This is to certify that the

dissertation entitled

YEAST PHYTASE AND WHEAT INOSITOL PHOSPHATES

presented by

NARSIMHA REDDY NAYINI

has been accepted towards fulfillment
of the requirements for

PH.D. degree in FOOD SCIENCE & HUMAN NUTRITION

Pericles Markakis
Major professor

Date 11-11-83

YEAST PHYTASE AND WHEAT INOSITOL PHOSPHATES

By

Narsimha Reddy Nayini

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Food Science
and Human Nutrition

1983

ABSTRACT

YEAST PHYTASE AND WHEAT INOSITOL PHOSPHATES

By

Narsimha Reddy Nayini

Wheat contains phytic acid, myoinositol 1, 2, 3, 4, 5, 6-hexakis (di-hydrogen phosphate), which may bind minerals and reduce their bioavailability. Enzymes present in bread yeast and plant tissues can hydrolyze phytic acid to phosphoric acid and inositol through a series of intermediate inositol phosphates.

The objectives of this research were to study (a) the properties of yeast phytase (b) the effect of milling extraction rate and bread fermentation time on the entire spectrum of inositol phosphates present in wheat flour and bread and (c) the metal-binding properties of the wheat inositol phosphates.

The yeast phytase was extracted with 2% $CaCl_2$, purified by ammonium sulfate fractionation and DEAE-cellulose chromatography. The phytase showed an optimum pH of 4.6 and optimum temperature of $45^{o}C$ with phytic acid as substrate. The enzyme activity is increased by 1 mM of Fe^{2+} and decreased by chelating agents. The yeast phytase had K_m =

0.21 mM with phytate as substrate. The enzyme shows broad specificity and hydrolyzes several phosphomonoesters besides phytic acid and other inositol phosphates. It is a non-specific phosphomonoesterase characterized by potent pyrophosphatase activity.

Hexa- (IP_6), penta- (IP_5), tetra- (IP_4), tri- (IP_3), di- (IP_2), mono-phosphate (IP_1) along with inorganic phosphate (P_i) were found in all flours and breads studied. Inositol hexaphosphate, inorganic phosphate and total phosphorus increased as the extraction rate increased from 70% to 90% to 100%. When the doughs were subjected to various fermentation times, from 0 to 120 min, a decrease in IP_6 and an increase in P_i were observed. There were always, however, intermediate inositol phosphates which did not follow any trend in their quantitative changes, but an overall phosphate balance could be obtained only by considering their presence.

Commercial white bread contained more P_i, IP_6 and total phosphorus than expected, assuming a 70-75%-extraction, because of added phosphates which probably slowed down the hydrolysis of IP_6. The distribution of inositol phosphates in commercial whole wheat bread was 17.4% IP_6, 20.0% IP_5, 13.0% IP_4, 12.0% IP_3, 6.5% IP_2, 5.5% IP_1 and 25.6% P_i.

All six wheat inositol phosphates and phosphoric acid showed the ability to precipitate the minerals Ca, Cu, Fe, and Zn at pH's 4, 5 and 6, except IP_1 and phosphoric acid

which did not precipitate zinc at pH 4. With a few exceptions, the phosphorus to calcium atom ratio was 1 : 1 in the inositol phosphates isolated from wheat. Iron and copper showed a decrease in the ratio of phosphorus to metal as the pH rose from 4 to 5. With a rise in pH, zinc showed an increase in the P : Zn ratio in IP_2 and IP_3, no change in IP_5 and a decrease in IP_4 and IP_6.

ACKNOWLEDGMENTS

The author is deeply indebted to his major professor Pericles Markakis for his assistance in conducting this study, encouragement and patience throughout the course of this work.

Appreciation is also expressed to professors Robert J. Brunner, Jerry N. Cash, Wanda L. Chenoweth from the department of Food Science and Human Nutrition and David R. Dilley of the department of Horticulture for their critical evaluation of this manuscript.

The author is most greatful to his brother Dr. Krishna R. Nayini and sister in-law Dr. Rama R. Nayini for their encouragement throughout the graduate program.

TABLE OF CONTENTS

LIST OF TABLES

Table | Page

LIST OF FIGURES

INTRODUCTION

Phytic acid has become the subject of active research for sometime because of its influence in both functional and nutritional properties of foods. A great number of studies have been conducted to understand its biochemistry and physiological role in plants and animals. Phytates, which represent a complex class of naturally occurring compounds, are the main source of phosphorus in many seeds.

Extensive use of cereals and legumes is a definite hope for reducing the hunger and malnutrition of the world. As the world pupulation increases so increases the demand for protein. Plant proteins, however, are frequently associated wiht metabolic inhibitors and phytic acid is one of them. Phytic acid affects solubility and other characterestics of protein. Phytic acid also chelates important minerals (Zn, Fe, Ca, Cu) and reduces their bioavailability.

Wheat, as all cereal grains, contain phytic acid. During the bread fermentation, enzymes present in the yeast hydrolyse phytic acid to inositol phosphates poorer in phosphorus than phytic acid and finally to inositol and phosphoric acid. Although extensive work has been done on phytic acid and its metal-binding properties, little is known about the properties of the yeast phytase, distribution

of intermediate inositol phosphates in flour and bread and their chelating characteristics.

The objectives of this research were to study (a) the purification and properties of yeast phytase (b) the effect of extraction rate on the wheat flour (c) the effect of fermentation time on the inositol phosphates present in bread and (d) the mineral-binding properties of the wheat inositol phosphates and phosphoric acid.

LITERATURE REVIEW

Phytase enzyme

Phytase (myo-inositol hexaphosphate phosphohydrolase, E.C.3.1.3.8) is the enzyme capable of hydrolyzing myo-inositol hexakis-dihydrogen-phosphate to yield inositol and free orthophosphate via inositol penta- to monophosphates as intermediary products. Phytase was the first enzyme known to liberate inorganic phosphate from organic phosphorus compounds (Suzuki et al., 1907) and as such has widespread distribution in plant and animal tissues, in many species of fungi and in certain bacteria (Cosgrove, 1966).

Though most of the dry seeds contain phytate, the presence of it is not necessarily associated with phytase activity. There has been reported no phytase activity in oats (McCance and Widdowson, 1944) and mung beans (Mandal and Biswas, 1970), moderate phytase activity in barley (Preece and Grav, 1962) and high activity in wheat (Peers, 1953). The distribution and quantity of phytase are not in proportion to phytic acid content in seeds and not correlated with glycerophosphate and pyrophosphate activities in plant tissues (Courtois and Perez, 1948 a; Saio, 1964). During sprouting, all seeds possess phytase activity which increases with the progress in germination and is accompanied by an increase in the inorganic phosphate and decrease in the

phytate content of seed (Courtois and Perez, 1948 b; Peers, 1953; Mayer, 1958; Mandal and Biswas, 1970).

Phytase activity was shown to be present in germinated pulses but not in ungerminated pulses, while phosphatase activity was found present in both germinated and ungerminated pulses. However, germinated pulses showed greater phosphatase activity (Belavady and Banerjee, 1953). Courtois and his collaborators have carried out extensive work on the occurrence, specificity and mechanism of phytase (Fleury and Courtois, 1945; Fleury and Courtois, 1947; courtois, 1947a; courtois, 1947b; courtois and Joseph. 1947; Courtois, 1948; Courtois and Perez, 1948a, 1948b; Courtois and Joseph, 1948; Courtois and Perez, 1949; Courtois, 1951; Barre et al., 1956). They observed that phytase behaves as a distinct enzyme different from the majority of other phosphomonoesterases. They noticed that phytase hydrolyzed inositol hexaphosphate whereas glycerophosphatase preparations were inactive towards inositol hexaphosphate, but could hydrolyze lower phosphate esters of inositol (Courtois, 1945). Later they found that the phytase from wheat bran was active on both phytic acid and glycerophosphate whereas a common phosphatase associated with the phytase was inactive on phytic acid but active on glycerophosphate (Fleury and Courtois, 1947). They applied to the wheat bran the customary techniques of separation and purification of enzymes but could not single out any evidence for the existence of a

phytase that hydrolyzes specifically only the phytic acid (Courtois, 1947a). They concluded that wheat bran and mustard seed, two of the materials with which they worked most of the time, contain two distinct enzymes: a common phosphomonoesterase capable of hydrolyzing α-glycerophosphate but not phytic acid and a phytophosphatase (phytase) capable of hydrolyzing both substrates (Courtois, 1947b; Courtois and Joseph, 1947).

Gibbins and Norris (1963) distinguished two enzymes in Dwarf french bean, the one being active towards phenyl phosphate but not towards phytate, and the other was active towards both phytate and phenyl phosphate. The first was an acid phosphatase and the second a phytase. Attempts to purify the phytase enzyme have been proved quite tedious. Nagai and Funahashi (1962) purified the wheat bran phytase more than 1500 times. The purified preparation was not a phytate specific phosphatase, but had all the characteristics of a nonspecific acid phosphomonoesterase with broad substrate specificity to various phosphomonoesters at pH 5.0. Their preparation had a potent pyrophosphate activity which is characteristic of plant nonspecific acid phosphomonoesterase.

Peers (1953) found the phytase enzyme to be more dispersed throughout the wheat kernel than its substrate, phytate, yet the enzyme was found primarily in the aleurone (39.5%), endosperm (34.1%) and scutellum (15.3%). He also

reported that enzyme activity was higher in hard wheats than soft but the variation in activity among the species was not large. Both enzyme and substrate found in the endosperm have been associated with protein bodies (Morton and Raison, 1963).

The action of phytase on phytates is by steps as it is shown below (Sloane-Stanley, 1961):

$$\underset{(IP_6)}{\text{myoinositol hexaphosphate}} + H_2O \longrightarrow \underset{(IP_5)}{\text{myoinositol pentaphosphate}} + \underset{(P_i)}{H_3PO_4}$$

followed by

$$IP_5 + H_2O \longrightarrow IP_4 + P_i, \text{ etc.}$$

The stepwise reaction has been proved by Mihailovic and co-workers (1965) who found that wheat phytate was completely decomposed within seven days of germination. Using paper chromatography, they examined extracts of wheat made at various stages of germination where they observed the formation of intermediate penta-, tetra-, tri-, di-, and monophosphates of myoinositol. In ripe wheat grain before germination, only inositol hexaphosphate was present. The stepwise hydrolysis is in agreement with the results of in vitro studies of the action of phytase preparations

on phytic acid. With the use of paper chromatography, Preece and co-workers (1960) found hexa-, tetra-, and tri- phosphates in barley. Malt contained all the above esters plus the diphosphate, the presence of which suggests that degradation occurs. The failure to detect di- and mono-esters in barley is attributed to limitations of the method, or, if they are present, their amounts must be very small. de Boland et al (1975) found only hexa-phosphate in the mature seeds of corn, wheat, rice, soy-beans and sesame. Glass and Geddes (1959) found an increased level of inorganic phosphorus along with lower phytate levels in wheat stored under elevated temperature and moisture conditions.

Chemistry of phytic acid

The presence of a Ca-Mg salt of an organic phosphate in the aleurone layer of wheat endosperm was first reported by Pfeffer (1872). Winterstein (1897) later showed that a similar substance phytic acid extracted from the seeds of Indian mustard (*Sinapsis nigra*) gave myo-inositol and orthophosphoric acid after hydrolysis with hydrochloric acid. Later Michel-Durand (1939) stated phytic acid to be as ubiquitous in the plant kingdom as starch.

The chemical designation of phytic acid is myo-inositol 1, 2, 3, 4, 5, 6-hexakis (dihydrogen phosphate). The name "phytic acid" has been used interchangeably in the lit-

erature with the term "phytin" which more correctly refers to the mixed Ca and Mg salt of the acid. Phytic acid and its isomers are unique in nature for being the only biologically produced molecules containing six phosphate groups on adjacent carbon atoms.

The structure of phytic acid has been controversial for some time. The more recent work of Johnson and Tate (1969) indicates that cereal grain phytic acid has the myo-inositol hexaorthophosphate structure suggested by Anderson(1914).

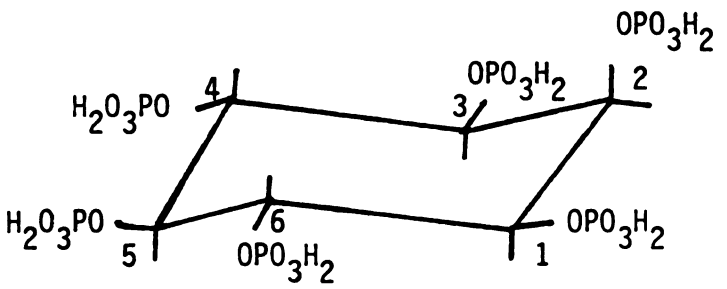


Figure 1.--Anderson's structure

Biochemistry of phytic acid

Phytates are found in a wide variety of foods as was demonstrated in an early study of Averill and King (1926), who reported a wide range of phytate levels as influenced by variety and byproduct of numerous cereals and nuts.

According to Earle and Milner (1938), phosphorus compounds found in seeds may be classified into four groups: phytates, phosphotides, nucleic compounds, and inorganic phosphorus compounds. Phytic acid is the principal form of phosphorus in many seeds; 60-90% of all the phosphorus in some seeds is present as phytic acid (Barre, 1956).

Occurence

Utilizing scanning electron microscopy, Pomeranz (1973) observed that in the case of barley, phytate is in the form of potassium and magnesium salts instead of the calcium-magnesium complex normally thought to be present in most other cereals. Phytic acid levels in 18 varieties of barley were found to range from 0.97 to 1.08% dry weight (Lolas et al., 1976). Makower (1969) reported that mature dry pinto beans contained approximately 1% phytic acid whereas immature beans contained about 0.13%. In addition, low levels of phytic acid were found in the pod at all stages of maturity. Walker (1974) reported that embryo development to maturity in phaseolus vulgaris requires approximately 36 days. Approximately 90% of the phytic acid was found between days 24 and 30. During germination 90% of the phytic acid was lost by day 10. Lolas and Markakis (1975) measured the phytic acid content of 50 cultivated varieties of P.vulgaris grown over a 2-year period and found a range of 0.54-1.58% of dry basis.

In addition, they also noted that 99% of the total phytic acid was in a water soluble form.

Anderson (1914c) was among the first to identify phytate in corn. Later, DeTurk et al (1933) followed phytate levels in corn from pollination to maturity and observed that phytate was not present in the leaves, stems, tassels, or cobs of the plant and that phytate began to increase in the kernels approximately 3 weeks after pollination and increased to maturity. O'Dell et al (1972b) demonstrated that in corn approximately 90% of the phytate is concentrated in the germ portion as compared to the endosperm and hull portions. Engle and Guinn (1959), in working with germinating cottonseed, noted the dephosphorylation of phytate resulted in the accumulation of inorganic phosphorus. Wozenski and Woodburn (1975) measured the phytic acid level in four food-grade cottonseed products and found significantly higher phytate levels in products of glandless seeds than in products of glanded ones.

Ashton and Williams (1958) found that phytate phosphorus is gradually broken down into inorganic phosphorus during germination of oats and that no phytate phosphorus was present after 2 weeks of germination. During panicle emergence and up to the milk ripe stage, they found no phytate in maturing oats; however, at maturity approximately 60% of the phosphorus was in the form of phytate. Asada and Kasai (1962) reported that during the early stage of

rice ripening a major portion of the myo-inositol was in the free state but at the end of ripening period most of the myo-inositol was in the phosphate ester form which represented approximately 80% of the total phosphorus in the product. Free myo-inositol and myo-inositol phosphate was found in the grains, leaves and stems, and roots of rice, but the low levels in the latter two portions compared to the grain level indicated that biosynthesis occured in the grains themselves from sugars. Kennedy and Schelstraete (1975) reported that phytic acid was primarily found in the outer layers of rice grain. Specifically, 2% of the outside kernel was found to contain 23 times more phytic acid than the intact kernel, and removal of the outer 13% of the kernel resulted in an endosperm that contained no detectable phytic acid.

Phytates in soy appear to be unique in that although associated with protein bodies, they appear to have no specific site of localization (Tombs, 1967). Lolas et al., (1976) have reported that the phytic acid content of 15 soybean varieties ranged from 1.0 to 1.47% on dry weight, representing between 51.4 and 57.1% of the total phosphorus. de Boland et al., (1975) reported on the phytic acid content of several commercially available soy products. Soy meal had a level of 1.42%, flakes, 1.52% and isolate, 1.52%.

Jennings and Morton (1963b), reported that the

initiation of rapid phytic acid synthesis in wheat could be correlated to the time of restriction of supply of water to the endosperm during maturation. Williams (1970) also investigated the effect of water stress on phytic acid formation in maturing wheat and found similar results. O'Dell et al., (1972b) found a level of 0.32% phytate in the whole kernel of wheat with approximately 87% of it being associated with the aleurone layer, 13% in the germ, 2% in the endosperm, and none in the hull portion. Morris and Ellis (1976) have reported that most of the phytate associated with wheat bran is in the form of mono-ferric phytate, which in turn is probably bound to cationic sites of proteins or other cellular components. In evaluating 38 wheat varieties, Lolas et al., (1976) found a phytic acid range of 0.62-1.35% (dry weight) in whole kernels, whereas the bran portion had phytic acid levels ranging from 4.59 to 5.52%, demonstrating that foods containing added wheat bran could have very high phytate levels. Figure (2) shows the longitudinal section of wheat kernel.

Physiological Roles

Several physiological roles have been suggested for phytic acid in plants. Phytic acid has been generally regarded as the chief storage form of both phosphorus and inositol in almost all seeds. Hall and Hodges (1966) attempted to obtain an overall description of phosphorus

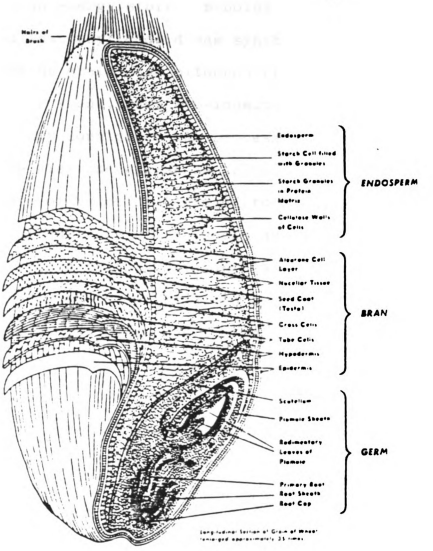


Figure 2.--Longitudinal section of wheat kernel (Baking science and technology)

metabolism associated with the germination of oats. Their results confirmed that phytic acid represents the primary storage form of phosphorus, about 53% of the total phosphorus. Biswas and Biswas (1965) proposed that phytic acid represents an energy store. Sobolev and Rodionova (1966) reported that phytic acid was synthesised by a mixture of aleurone grains and mitochondria isolated from ripening sunflower seeds, when myo-inositol and succinate were present. They considered the process of phytin formation by the aleurone grains as an important link in the general chain of reactions leading to quenching of the physiological activity of the seeds during ripening. They further proposed that the biosynthesis of phytates is not associated with the utilization of hexose phosphate or glycolysis products but is due to the stepwise phosphorylation of inositol. Williams (1970) presented evidence that phytic acid serves only as a source of phosphorus and cations for the germinating seed. He suggests that the synthesis of strongly chelating phytic acid exerts an effect on the cellular metabolism by combining with multivalent cations. He further states that these multivalent cations play a significant role in the control of many cellular processes, particularly those involving phosphotransferases on which energy metabolism depends.

Asada and co-workers (1968) found that phytate

contains over 80% of the total phosphorus of mature rice grain and the turnover of phytate phosphorus is practically nil in the resting grain. From that they concluded that phytate is the final product of phosphorus metabolism in the ripening process. Samotus (1965) suggested that formation of phytic acid in seeds and tubers is a means of preventing the accumulation of excessively high levels of inorganic phosphate. He proposed the following mechanism of phosphorus distribution in potato tuber. Inorganic phosphorus penetrates into the tuber during plant growth; a part of this phosphorus is engaged in metabolic transformations and the remainder is bound in the form of phytin and phosphostarch. Support of the above claim is provided by the observations of Asada and Kasai (1959) who found an enhanced accumulation of phytic acid, relative to other phosphorus compounds in rice grains, upon increased applications of phosphorus fertilizer to the rice plant.

Protein-phytate complexes

Insoluble complexes are formed between protein and polyphosphates. When polyphosphates, such as phytic acid are added to protein solution below the pH of their isoelectric point, precipitation occurs, and the extent of the reaction is controlled by the pH of the system. Phytic acid forms salt-like linkages with basic groups on the protein molecule such as those of arginine, lysine

and histadine units (Cosgrove, 1966). Myers and Iacobucci (1974) suggest that charged carboxyl groups are a major factor in explaining the binding behavior of phytate to glycinin between pH 3.0 and 4.0. Calcium ion has been shown to have an effect on phytate binding to glycinin. Okubo and co-workers (1974) assume that calcium ion mediates phytate binding to glycinin above the isoelectric point, and both soluble and insoluble complexes can be formed. They give as a possible explanation of the binding that calcium ion acts as a bridge between the carboxyl groups of the protein and the phosphate groups of phytate.

Okubo and co-workers (1976), working with glycinin, a major globulin of the soybean, showed no binding above the isoelectric point of pH 4.9. The extent of binding was found to increase with decreasing pH, from a value of zero at the isoelectric point to a maximum value of 424 equivalents of phytate per mole glycinin dimer (360,000 daltons). They also showed that calcium ions promoted dissociation of phytate-glycinin complexes at pH 3 and they contributed this to the competition between Ca^{2+} and the cationic sites of the protein for the phosphate groups of phytate. At pH 3.0, a 105 fold equivalent excess of calcium with respect to the protein cationic groups was necessary to completely dissociate the complex. Saio and co-workers (1967) studied the effects of protein-calcium-phytic acid relationships on the solubility characteristics

of soybean meal protein and found that the combinations among proteins, calcium and phytic acid are very labile in the alkaline range above pH 8.0, especially by heating. The same workers (1968) also found that phytic acid affects the binding of calcium by a cold insoluble protein fraction of soybean meal. Elimination of phytic acid from soybean meal extracts is considered an essential preliminary step to the study of the individual soybean protein (Smith and Rackis, 1957).

Wang (1971) described changes in the isoelectric focusing behavior of soybean whey protein caused by the addition or elimination of phytate which influences the net charges of proteins. It has been shown that phytic acid exhibits an inhibitory effect on the peptic digestion of ovalbumin and elastin (Barre, 1956). This effect is related to its property to form insoluble combinations with proteins, below their isoelectric point, in an acid medium, and in a range of pH which corresponds precisely to the optimum for the action of pepsin.

Destruction of phytic acid during bread making

Phytic acid is hydrolyzed during the breadmaking process. Consumption of whole wheat breads that have been made with little or no fermentation has caused considerable concern because of their high phytate levels (Reinhold 1971, 1972). Reinhold (1971) suggested that

yeast might contribute in decreasing the phytic acid content of leavened bread. Yeast or sourdough fermentation of dough has been shown to lower phytate levels by one-third to half (Reinhold, 1972). Earlier, Mellanby (1944) had demonstrated that fermentation time, temperature, pH, and humidity could all significantly influence phytase activity. Reinhold (1975) reported that phytate loss due to the action of phytase was rapid in fermented bread made from 75-90% extraction wheat but was slow for 95-100% extraction products. de Lang et al (1961) reported that phytate is completely hydrolyzed in low extraction flours.

Harland and Harland (1980) showed a significant reduction in phytate contents by increasing the amount of yeast and time of fermentation in rye, white and whole wheat breads. In contrast, Tangkongchitr et al (1981b) did not find a significant loss of phytate with higher yeast levels. Ranhotra (1972) reported 20 times more phytic acid in wheat protein concentrate than wheat flour, and thus the inclusion of wheat protein concentrate into bakery items can present phytate-related problems inspite of the fact that a high level of phytase activity is associated with it. Addition of calcium has shown to inhibit phytate hydrolysis in bread making (Ranhotra, 1972), and thus additives high in calcium content, such as certain whey products, can diminish phytase activity (Ranhotra, 1973). Ranhotra et al (1974) found little

phytase activity in a number of commercially available soy products. They also showed that the addition of 10% soy protein concentrate to the bread formulation resulted in phytate hydrolysis in excess of 80%, in contrast to 22% observed in a whey-soy blend product, probably due to high residual level of calcium in the latter blend. Knorr et al., (1981) found a reduction of upto 1/8 and 1/2 of the initial phytate concentration, upon addition of commercial phytase and phosphatase respectively. They also reported that storage of whole wheat bread for up to 96 hrs at room temperature further reduced phytate phosphorus.

Nutritional aspects of inositol phosphates

Ruminants are able to utilize phytates. Ellis and Tillman (1961) investigated the availability of phosphorus in wheat bran fed to sheep. They found appreciable amounts of phytin to have been digested. Rumen micro organisms show high phytase activity and evidence exists that the hydrolysis of phytates is due to the phytase of these organisms and is not dependent on phytases present in the feed (Raun et al., 1956).

Non-ruminants do not seem to be able to utilize phytates although phytase has been shown to be present in the intestinal mucosa of rats (Pileggi, 1959) and in human faeces, the latter being possibly bacterial in orgin (Courtois and Perez, 1949). The ability of man to hydrolyse

phytates remains a controversial subject, though some hydrolysis in the digestive tract occurs probably due to microbial phytases or non-enzymatic cleavage. Evidence has been presented recently that man probably possesses phytase (Bitar and Reinhold, 1972), but the lack of phytate cleavage may be caused by inhibitors of phytate hydrolysis present in foods such as bread (Reinhold et al., 1973). Phytases can only act on phytates in solution and the extent to which phytates are hydrolyzed depends largely on their solubility. This in turn depends on the ions with which they are associated and on the level of calcium in the diet.

Zinc deficiency was first recognized by Prasad et al., (1963) in Egyptian boys whose diets consisted mainly of bread and beans. These patients, who were characterized by dwarfism and hypogonadism, showed a response to zinc supplementation. Erdman (1979) reported that the greatest impact of phytic acid relative to human nutrition is its reduction of zinc bioavailability. Likuski and Forbes, (1964, 1965) showed an inverse relationship between the level of phytic acid in the diet and zinc bioavailability. Forbes and Parker (1977) demonstrated that zinc added to rat diets in the form of whole fat soy flour was significantly less utilized than zinc added as zinc carbonate to an egg white diet. In the case of soy-fortified wheat bread, Ranhotra et al (1978) found that the bioavailability

of zinc was not affected because most of the bound zinc was liberated due to the action of phytase on phytate during fermentation.

Momcilovi and Shah (1976b) studying the bioavailability of zinc to rats from several infant formulas and breakfast cereals concluded that the infant cereal was the poorest source of zinc. Reinhold et al., (1973a,b), utilizing human subjects found a positive correlation between the phytate levels and unavailability of zinc. The interaction of zinc, calcium and phytic acid has also been investigated by using the pig (Oberleas et al., 1962) and rat (Oberleas et al., 1966) as models and found that high levels of calcium in conjunction with phytate decreased zinc bioavailability.

Iron represents the other nutritionally significant mineral that has been associated with phytate binding. Although there is little doubt that the consumption of a diet containing added phytate (Na) lowers the iron balances in human subjects (Turnbull et al., 1962), the effect of phytate naturally present in foods has been reported to have no effect or only slightly depressing effect on the utilization of iron by rats (Ranhotra et al., 1974). This has been questioned by Davis and Nightingale (1975) who reported a significant effect. Morris and Ellis (1976) isolated monoferric phytate from wheat bran which they found to be water sobuble and of

high biological value to the rat. They also postulated that the monoferric phytate in bran was bound to calcium sites of proteins with utilization being through solubilization by an ion-exchange type mechanism instead of through phytate hydrolysis.

Definite evidence that the presence of phytates in diets cause a reduction in copper availability has been obtained by Davis and Nightingale (1975) in studies with rats. Davis et al., (1962) had earlier reported that diets containing an isolated soy bean protein reduced the availability of copper in chickens. However, and in view of the high phytate content of soybean meal (Common, 1940) and the ability of soybean protein to complex with phytates, it would seem likely that phytates are involved in copper unavailability.

Anticalcifying and rachitogenic properties were attributed to consumption of cereals by Bruce and Callow, 1934; Harris and Bunker, 1935; Cruickshank et al., 1945; Walker, 1951. In the case of dogs Mellanby (1949) demonstrated that phytate addition to their diets reduced calcium absorption and subsequently induced rickets. In contrast, Walker et al., (1948) reported that human diets high in phytates improved the retention of dietary calcium and magnesium. However, in a later study, Reinhold et al (1973b) could not confirm human adaptation to high-phytate diets.

Forbes (1964) reported that in rats, dietary calcium depressed weight gain, feed intake and femur zinc concentration, especially in the presence of soy protein. Likuski and Forbes (1965) showed that dietary calcium and phytic acid also decreased magnesium absorption. Van Den Berg et al., (1972) studying the influence of phytic acid and its derivatives on inhibiting calcification in the rat found that although phytate itself was inert, phytic acid and its hydrolysates were potent inhibitors of calcification.

In the case of wheat breads, Reinhold et al (1975) contended that fiber and not phytate is primarily responsible for poor calcium absorption. They reported that the ability to bind calcium is a function of fiber concentration.

Evans and Pierce (1981) isolated a calcium-phytate complex, the chemical composition of which indicates penta substituted calcium phytate, despite rather widely varying P/Ca ratios in the reaction mixture, Earlier, Hoff-Jorgensen had previously (1944) reported penta-calcium phytate. Evans and Pierce (1982) studying the simultaneous interaction of several metal ions with phytic acid could isolate only amorphous powders with non-stiochiometric atomic ratios, however, Hay (1942) reported a hexa-calcium phytate salt in the corn.

MATERIALS AND METHODS

Enzyme extraction

The crude enzyme solution was prepared according to (Lolas and Markakis, 1977) with some modifications, from baker's yeast cake. The yeast cells were hydrated with distilled water for 30 min, broken by Polytron (Brinkmann Instruments, Inc., Westbury, New York) and extracted with a 10:1 ratio of 2% $CaCl_2$ to yeast. The enzyme solution was centrifuged at (2000 g) for 30 min at 2^{o} C. The clear supernatant solution had a pH of 5.3.

Ammonium sulfate fractionation

Sufficient solid ammonium sulfate was added to the crude enzyme solution (Dixon, 1953) with continuous mechanical stirring to make it 30% saturated. The enzyme solution was kept for 30 min at 2^{o}C and centrifuged (2000 g) for 20 to 30 min at 2^{o}C. The residue was discarded and the supernatant solution was made 80% $(NH_4)_2SO_4$ saturated followed by the same treatment as above. The fraction precipitating between 30% and 80% saturation contained all the phytase activity. This was dissolved in a small volume of 0.01 M tris-maleate buffer pH 6.5 and dialysed for about 48 hours in the same buffer in a

cold room ($2^{o}C$).

Assay procedure

Phytase activity was assayed by measuring the rate of increase in inorganic phosphorus, liberated by the action of phytase, using the ascorbic acid method (Watanabe and Olsen, 1965). The reactions were carried out in small glass-stoppered test tubes in a 45 $\pm$ $1^{o}C$ water bath. The typical reaction mixture had a total volume of 1.2 ml and contained 0.2 ml of 0.6 M acetate buffer, pH 4.6; 0.15 ml of 8 mM sodium phytate (SIGMA Chemical Co; St. Louis, MO) previously adjusted to pH 4.6 (with 1 N HCl), 0.2 ml enzyme solution and water to 1.2 ml. Final concentration of buffer and phytate were 0.1 M and 1 mM, respectively; and incubation time usually 30 mins. After incubation, samples were withdrawn from the digest, deproteinized by adding 0.8 ml of 10% TCA, centrifuged in small 2 ml conical centrifuge tubes and orthophosphate determination was carried out on the supernatant according to the method described below under the title "Determination of inorganic phosphorus by the ascorbic acid method". The activity values were corrected from a control which contained boiled enzyme.

Enzyme activity was expressed in international units, one unit being the activity which results in the liberation of 1 μmole of inorganic phosphorus per minute ---

(recommended by the Commission on Enzymes of the International Union of Biochemistry) (Whitaker, 1972).

Determination of inorganic phosphorus by the ascorbic acid method

The steps of the method are as follows:

1. Prepare reagent A.

Dissolve 12 g of ammonium molybdate in 250 ml deionized H_2O. In 100 ml of deionized H_2O dissolve 0.2908 g of antimony potassium tartrate. Add both of the dissolved reagents to 1 liter of 5 N H_2SO_4, mix throughly, make to 2 liters and store in a brown glass bottle in the refrigerator.

2. Prepare reagent B.

Dissolve 0.264 g ascorbic acid in 50 ml of reagent A and mix. This reagent does not keep more than 12 hours and must be prepared before analysis.

3. Pipette aliquots containing 0.01 to 0.015 μmole of orthophosphate into 5 ml volumetric flasks.

4. Add deionized H_2O to make the volume to 4 ml, and then add 0.8 ml reagent B.

5. Make to volume with deionized H_2O and mix. The color is stable in 10 min and is measured at 700 nm.

6. A standard curve was prepared using a standard phosphorus (predried KH_2PO_4) solution in the same manner as above against a blank containing 4.2 ml H_2O and 0.8 ml

of reagent B. The following linear regression equation was used for estimating the P_i content $A_{700} = 0.003 + 3.204 \times C$. The correlation coefficient corresponding to this equation was ($r = 0.989$).

Effect of pH on enzyme activity

Standard assay procedures were used to determine reaction rates over the pH range 3.6 to 6.0. Acetate buffers were used except for pH 6.0 where a tris-maleate buffer was used. The buffers had a final concentration of 0.1 M in the assay mixture. The results were expressed as percent of activity at pH 4.6 and plotted against pH.

Effect of incubation temperature on reaction rate

The reaction rates were determined at temperatures from 40°C to 60°C at 5 degree intervals using standard assay procedures. The inorganic phosphorus liberated was measured after 30 min of incubation.

Effect of substrate concentration on reaction rate

The effect of phytic acid concentration (final concentrations up to 10 mM) on activity was tested by measuring initial reaction velocities. The Michaelis constant and maximum velocity values were calculated by plotting 1/(initial velocity) against 1/(substrate

concentration) (Lineweaver and Burk, 1934). For the determination of K_m, the initial velocities of reaction were measured over the range 0.05 to 8.0 mM phytate.

Effect of various metal ions on phytase activity

Ca^{2+} ($CaSO_4.2H_2O$); Cd^{2+} ($CdCl_2.2\frac{1}{2}H_2O$); Co^{2+} ($CoCl_2.6H_2O$); Cu^{2+} ($CuSO_4.5H_2O$); Fe^{2+} ($FeSO_4.7H_2O$); Hg^{2+} ($HgCl_2$); Mg^{2+} ($Mg(NO_3)_2.6H_2O$); Mn^{2+} ($MnSO_4.H_2O$); Zn^{2+} ($ZnSO_4.7H_2O$) ions at final concentrations of 10^{-5}M, 10^{-4}M and 10^{-3}M were investigated to determine their effect on enzymatic activity. These ions were incorporated into the assay by the addition of each separately and the results were checked against those of controls containing no metal ion.

Effect of chelating agents on phytase activity

Standard assays were performed which contained ethylenediamine tetra-acetic acid (EDTA), sodium oxalate, sodium potassium tartrate and sodium citrate at final concentrations of 10^{-6}M, 10^{-4}M, and 10^{-2}M. The results were compared with those of controls containing no chelating agent.

Separation of phytase from phosphatase

Diethlaminoethyl (DEAE) cellulose (20g) was treated according to the procedure described by Whitaker (1972). It was packed in a glass column (3.0 cm x 50 cm) to a height of about 27 cm. The column was equilibrated with 0.01 M tris-HCl pH 7.4 buffer, until the pH of the effluent was identical with that of the applied buffer. The enzyme solution was dialysed against the same buffer for 24 hours and about 30 ml of enzyme solution, having a protein content of 3.3 mg/ml, was charged gently at the top of the column. The concentrations of NaCl solution in 0.01 M tris-HCl buffer, used for elution of the protein from the column, were 0.15 and 0.40 M (stepwise method). The 0.4 M NaCl concentration was applied after all the phosphatase enzyme had been eluted. The chromatographic procedure was carried out at room temperature. The collected fractions (10 ml) each were assayed for protein content, phosphatase and phytase activity. The assay for the phosphatase activity was carried out under the conditions as that of the phytase enzyme.

For the determination of protein the spectrophotometric method of Warburg and Christian (1942) was used and the protein content calculated by the Kalckar (1947) formula:

1.45 x (absorbance at 280 nm) − 0.74 x (absorbance at 260 nm) = mg protein/ml

Substrate specificity

The DEAE-cellulose separated phytase, after dialysis, was used to determine its activity against 5'- adenylic acid (AMP), α and β- glycerophosphate, sodium pyrophosphate, phenyl phosphate, inositol pentaphosphate, inositol tetraphosphate, inositol triphosphate, inositol diphosphate, inositol monophosphate and phytic acid. The intermediate inositol phosphates were obtained from the wheat extracts. The activities of the above substrates were compared with that of phytic acid. Standard assay procedures were used with the only difference that all the substrates, except phytic acid and the intermediate inositol phosphates, were added in the assay mixture at a final concentration of 10 mM.

Preparation of bread

Two experiments involving bread were conducted. Variable milling extraction rate was the factor studied in the first one, and variable fermentation time was studied in the second experiment.

A. Soft Red Wheat (SRW) flours of 70%, 80%, 90% and 100% extractions were obtained from Soft Wheat Quality Laboratory, Wooster, Ohio. The breads were made according to AACC-10-10 procedure (1962). The formulation of test pan bread were: flour: 100 g ; yeast: 3.0 g; salt: 1.5 g; sugar: 5.0 g; and water: 70 ml. The yeast was first

hydrated in a mixing bowl for 5 min and then sugar, salt and flour were added to it. The ingredients were first blended at low speed for 1 min and then the dough was mixed at high speed for 7 min in a Hobart mixer. The doughs were fermented for 120 min at 30°C and 85% R.H. The dough were given a 10 min bench rest after fermentation at room temperature. The loaves were then molded, panned and later proofed for 40 min at 30°C and 85% R.H. The proofed doughs were baked at 218°C for 20 mins. The breads were air dried, ground and stored in a dessicator for later use. Inositol phosphate analysis was conducted on the flours as well as on the breads made from them.

B. The wheat flours (WF) and whole wheat flours (WWF) that were used in the fermentation experiment were obtained from a local market. All the breads were made according to AACC-10-10 procedure and with same formulation of test pan bread as used in extraction experiment, except the doughs were fermented for 30, 60, 90 and 120 min at 30°C and 85% R.H. The 0 min fermentation dough was immediately used for separation of inositol phosphates. The breads were later air dried and stored in a dessicator for later use.

Commercial whole wheat and white breads obtained from local market were also analysed for the entire spectrum of phosphates.

Separation of inositol phosphates

Separation of inositol phosphates was done according to the method of Saio (1964). The inositol phosphates were extracted from 1 g of wheat flour or bread sample with 10 ml of 3% TCA. After centrifugation at 12,000 x G, 1 ml of the extract was chromatographed on a Dowex 1 x 8 (200-400 mesh, Cl^- form, 1.1 x 11 cm) column. The extract was eluted with 600 ml 0 - 1.0 N HCl linear gradient at a flow rate of 2 ml per minute. Eluant was collected in 120 tubes (5 ml per tube) using a fraction collector (Rinco Instruments Co., Greenville, ILL).

Phosphorus determination of chromatographic fractions

The solution in each fraction was evaporated to dryness at 40°C by blowing air onto the surface of the solution through a manifold (Figure 3).

1 ml of 70% perchloric acid was added to each fraction containing dry residue. The tubes were heated for 45 min to release the phosphorus from the inositol phosphates. The phosphorus content of each fraction was then determined colorimetrically according to Allen's method (1940) with slight modifications. The method was based on the formation of phosphomolybdic acid which was reduced to an intense blue complex. Reagents used in phosphorus determination were as follows:

Perchloric acid: a 70% solution

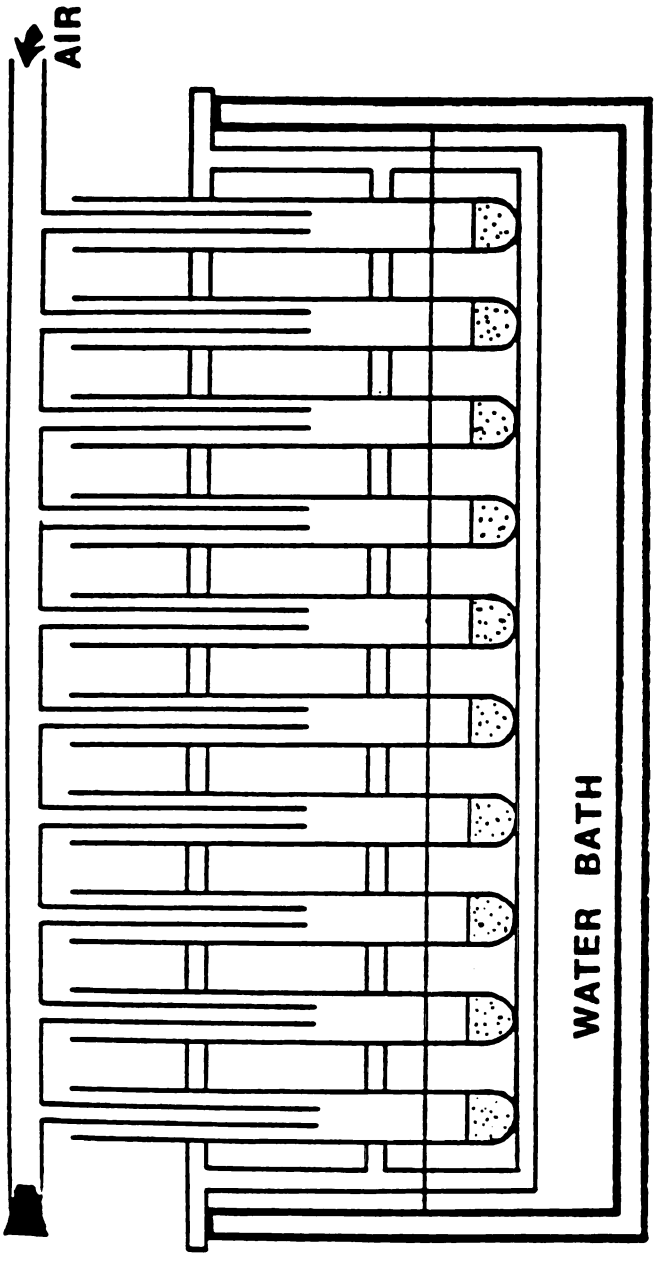


Figure 3.--Apparatus used for evaporating liquid in tubes.

Amidol reagent prepared by dissolving 2 g 2,4-diaminophenol dihydrochloride and 40 g sodium bisulfite in distilled water and diluted to 200 ml. The solution was kept in a brown bottle and discarded after 1 week.
Ammonium molybdate solution: an 8.3% solution.
Standard phosphorus solution: a 50 μg P per ml solution was prepared by dissolving 0.2197 g KH_2PO_4 (dried at 105°C) and diluted to 1 L.

After hydrolysis with perchloric acid, 1 ml amidol reagent and 1 ml of ammonium molybdate solution were added to each fraction, and the volume was adjusted to 10 ml with water. The solution was mixed using a Vortex mixer, and after 5-30 minutes the absorbance was measured at 675 nm with a Beckman DU Model 2400 Spectrophotometer (Beckman Instruments, Inc., Fullerton, CA.).

A standard curve was previously prepared using a series of standard solution containing 0 to 70 μg P per 10 ml.

The following linear relationship was obtained:

$$A_{675} = 0.0124\ C + 0.0021$$
$$(r = 1.000)$$

where C was the concentration of P in μg per 10 ml.

The amount of phosphorus per tube was plotted against the tube number to illustrate the separation of inositol phosphates. Identification of the chromatogram was done by combining new fractions of each separated peak. These

solutions were dried, and hydrolyzed with 6 N HCl in ampules at 110°C for 48 hours. This was done to liberate phosphoric acid from inositol phosphate without destroying the inositol moiety. The solution containing free inositol and inorganic phosphorus was subjected to inositol determination (Agranoff et al., 1958) as described by Saio (1964) and to phosphorus determination (Allen, 1940).

Inositol determination

In the inositol determination, 2 ml sample containing approximately 0.01 to 0.5 μmole of inositol, 2 ml of 1 M acetate buffer (pH 4.7), and 0.4 ml of 0.01 M sodium periodate were mixed, and the absorbance at 260 nm was immediately read. The absorbance was read again after 30 minutes at room temperature and after 16 hours in water bath at 45°C. The difference in the absorbance before and after heating at 45°C was due to the oxidation of inositol.

A standard curve was previously prepared using a series of standard solution of 0 to 250 μM. The following relationship was obtained:

$$-\Delta A_{260} = 0.0024\ C + 0.00667$$

$$(r = 0.999)$$

where C was the concentration of inositol in μM.

Inositol phosphates collection

After identification of the chromatogram, the test tube fractions representing a particular inosiotl phosphate were collected, pooled and stored under refrigeration. Similar separations were repeated till sufficient amounts of inositol phosphate pooled fraction was concentrated at $40^{o}C$ under vacuum. 0.1 ml of the concentrate was analysed for phosphorus according to Allen's method (1940). The inositol phosphates were reported as phosphorus content. The following were the concentrations obtained: IP_1 = $5.01 \times 10^{-3}M$; IP_2 = $8.6 \times 10^{-3}M$; IP_3 = $1.5 \times 10^{-3}M$; IP_4 = $7.5 \times 10^{-3}M$; IP_5 = $5.9 \times 10^{-3}M$; IP_6 = $5.0 \times 10^{-3}M$. The phosphoric acid (H_3PO_4) concentration was $5.0 \times 1.0^{-3}M$.

Precipitation of inositol phosphate-metal complex

3 ml of each inositol phosphate solution was transferred in a 100 ml centrifuge tube, to which a 5 ml solution containing 5000 ug of metal was added. The pH was adjusted using 5% Na_2CO_3 and 5% HCl. The centrifuge tubes were adjusted to pH 4, 5 and 6. In addition to inositol phosphates, phosphoric acid was also used in the metal complexation experiment. All the tubes were made in duplicate and were later allowed to set overnight at room temperature.

The tubes were centrifuged at 10,000 x G for 15 min. The supernatant was discarded carefully and the precipitate

was dried at room temperature. All the precipitate from a single centrifuge tube was transferred into a Kjeldahl flask and wet digested using 20 ml HCl and 5 ml H_2SO_4 for 2 hrs. The contents were transferred to a 100 ml volumetric flask and brought to volume. This solution was used in the analysis of metal and phosphorus. The metals used in this research were calcium as calcium chloride, copper as copper sulfate, iron as ferrous sulfate, and zinc as zinc sulfate.

Analysis of calcium

Calcium was determined by using IL 951 Atomic Absorption spectrophotometer according to the manufacturer's guide. The absorption was read at 422.7 nm using 320 µm slit width. To depress interferences due to silicon, aluminium, phosphates and sulfates, 19 ml of 10,000 ppm strontium as strontium chloride was added to 1 ml of sample.

A standard curve was previously prepared using a series of standard solution containing 0 to 80 µg of Ca per ml. The following linear relationship was obtained;

$$A_{422.7} = 2.004\ C + 0.098$$

$$(r = 0.999)$$

Where C was the concentration of calcium in µg.

Analysis of copper

The copper was determined by using the Zincon method (1954). Reagents used for the copper determination were as follows:

Buffer pH 5.2: A Clark and Lubs buffer of pH 5.2 was made using 50 ml M/5 potassium biphthalate and 29.95 ml M/5 sodium hydroxide diluted to 200 ml.

Zincon solution: 0.130 g of finely powdered 2-carboxy-2-hydroxy-5-sulfoformazyl benzine (Zincon) was added to 2 ml of 1 M sodium hydroxide and diluted to 100 ml. This gives a concentration of 0.002 M based on a purity of 68% for the reagent. These solutions were deep red in color and were stable for about one week.

A 1 ml aliquot of sample was transferred to 50 ml volumetric flask and brought to approximately a neutral solution by adding sodium bicarbonate. A 10 ml aliquot of the buffer, was added to it and shaken vigorously. Later, a 3 ml of Zincon solution was added and was brought to volume using deionized water. The contents in the flask were mixed thoroughly and measured for the absorbance at 600 nm with a Beckman DU model 2400 Spectrophotometer against a reagent blank.

A standard curve was previously prepared using a series of standard solution containing 0 to 2.0 µg of Cu per ml. The following linear relationship was obtained:

$$A_{600} = 0.0072\ C + 0.0025$$

$$(r = 0.995)$$

where C is the concentration of Cu in μg per ml.

Analysis of iron

The iron content was determined according to AOAC (1975). The reagents used for iron determination were as follows:

Orthophenanthroline solution: a 0.1% solution.

Hydroxylamine hydrochloride solution: a 10% $H_2NOH.HCL$ solution.

Acetate buffer solution: prepared by dissolving 8.3 g anhydrous sodium acetate and 12 ml of acetic acid which was then diluted to 100 ml.

One half ml of sample containing iron was transferred into a 25 ml volumetric flask.

One ml of hydroxylamine hydrochloride was added, the flask was rotated and allowed to stand a few minutes. Subsequently, 10 ml of acetate buffer and 1 ml of orthophenanthroline solution were added. The contents were adjusted to volume, and the absorbance was measured at 510 nm with a Beckman DU Model 2400 Spectrophotometer against a reagent blank.

A standard curve was previously prepared using a series of standard solution containing 0-125 μg Fe per ml. The following linear relationship was obtained:

$$A_{510} = 0.0072\ C + 0.0238$$

$$(r = 0.996)$$

where C was the concentration of Fe in μg per ml.

Analysis of zinc

The zinc was determined by using Zincon method (1954). The reagents used for the zinc were as follows: Zincon solution: 0.130 g of finely powdered 2-carboxy-2-hydroxy-5-sulfoformazyl benzine (Zincon) was added to 2 ml of 1 M sodium-hydroxide and diluted to 100 ml. This gives a concentration of 0.002 M based on a purity of 68% for the reagent. These solutions were deep red in color and were stable for about one week. Buffer pH 9.0: A Clark and Lubs buffer, pH 9.0, was made using 50 ml M/5 H_3BO_3, M/5 KCl and 21.30 ml of M/5 NaOH diluted to 200 ml.

A 1 ml aliquot of the sample was transferred to 50 ml volumetric flask in a neutral solution. A 10 ml of the buffer, and 3.0 ml of the zincon reagent solution were added. The volume was brought to the mark with deionised water, mixed throughly, and measured for the absorbance at 620 nm with Beckman DU Model 2400 Spectrophotometer against the reagent blank.

A standard curve was previously prepared using a series of standard solution containing 0 to 2.4 μg of Zn per ml.

The following linear relationship was obtained:

$$A_{620} = 0.3759\ C - 0.0055$$

$$(r = 0.999)$$

where C was the concentration of Zn in μg per ml.

RESULTS AND DISCUSSIONS

Phytase extraction

Several salts (NaCl, $CaCl_2$, KCl), water, buffers (usually 0.2 M acetate pH 5.3 and 0.1 M tris-maleate pH 6.5) were tested in an effort to extract all the phytase from yeast cells. Among all the extractants tested $CaCl_2$ at the 2% level was considered as the ideal extractant resulting in a clear supernatant having a constant pH of 5.0.

Effect of pH and incubation temperature on enzyme activity

The pH optimum for the yeast phytase was found to be 4.6 (Figure 4) with relatively rapid diminution in activity on either side of this optimum. Various figures have been reported in the literature as the optimum pH of phytase activity; 5.15 as the optimum of wheat flour phytase (Peers, 1953); 5.0 for wheat bran phytase (Nagai and Funahashi, 1962); 5.2 for the Dwarf bean phytase (Gibbins and Norris, 1963); 5.6 for the corn phytase (Chang, 1967); 7.5 for the phytase of germinating mung beans (Mandal et al., 1972); 5.3 for navy bean phytase (Lolas and Markakis, 1977) and two pH peaks (5 and 7) were observed in the phytase activity of germinating

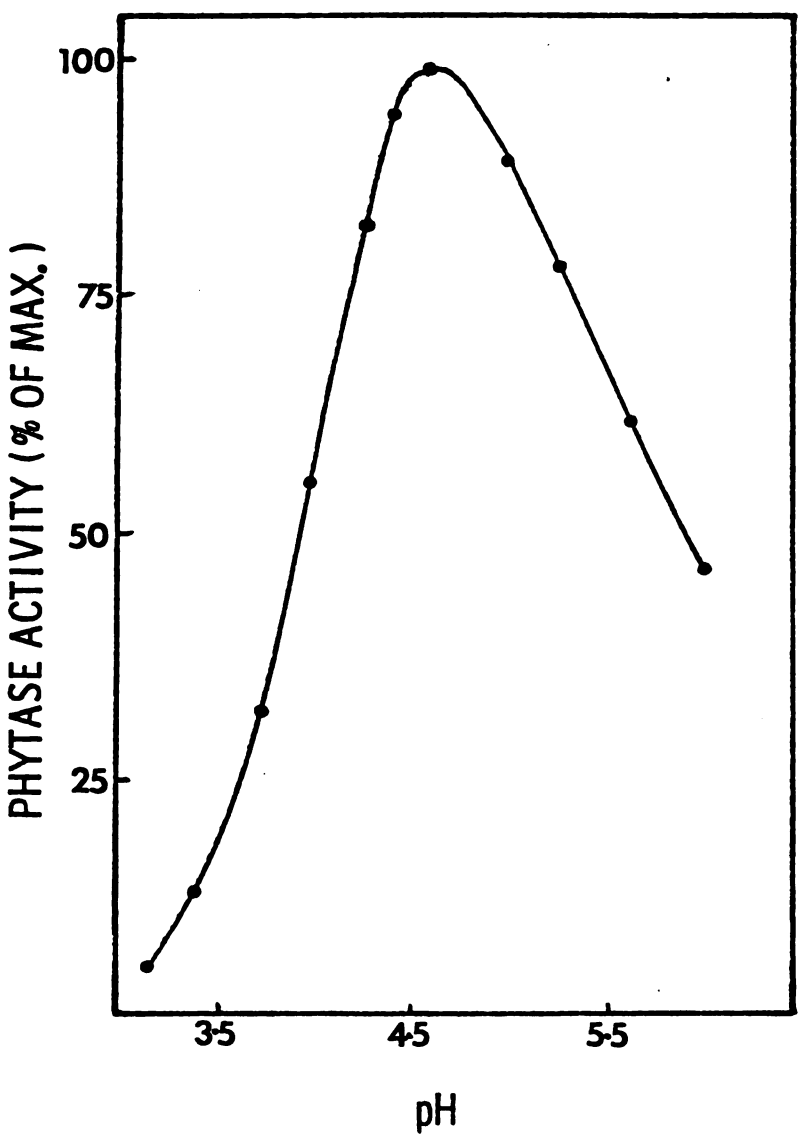


Figure 4.--Effect of pH on yeast phytase activity. Phytase was incubated at 45°C with 1 mM sodium phytate in the presence of 0.1 M acetate buffer except for pH 6.0 (0.1 M tris-maleate buffer).

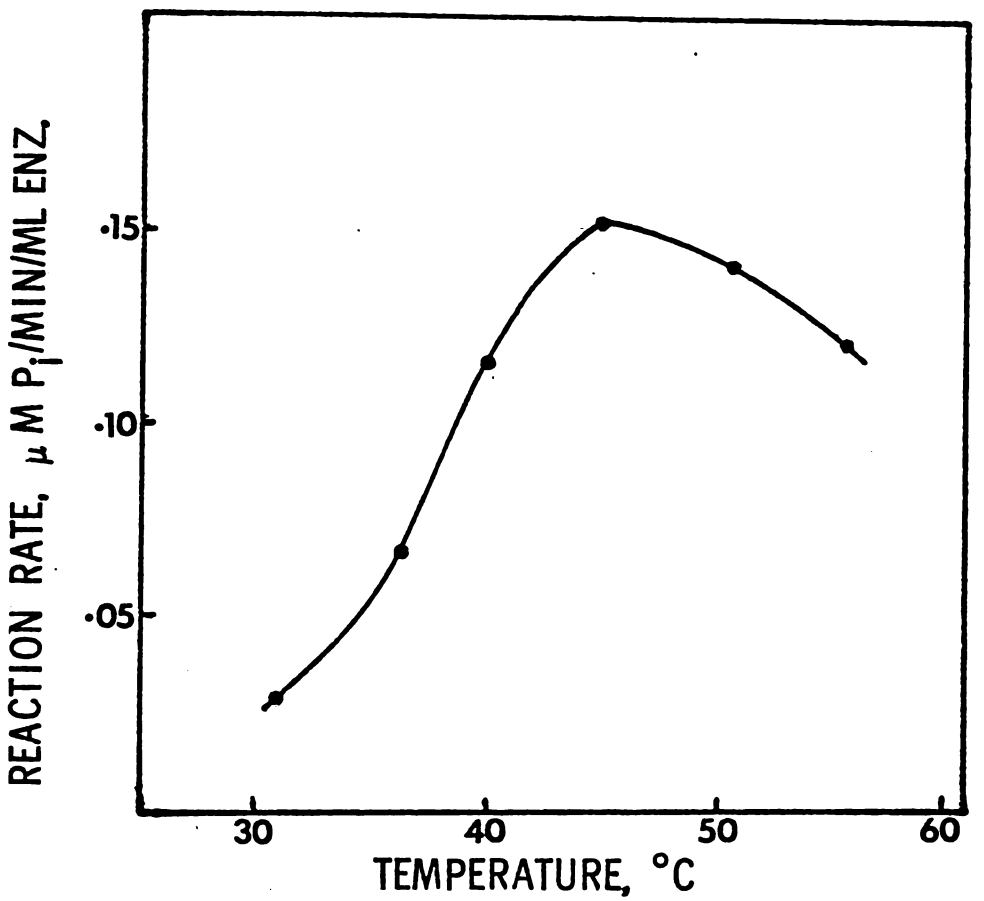


Figure 5.--Effect of temperature on yeast phytase activity. Incubation time. 30 min: buffer, 0.1 M acetate pH 4.6: substrate, sodium phytate, 1 mM.

lettuce seeds (Mayer, 1958).

The optimum temperature of activity for yeast phytase is about 45°C (Figure 5) which is measured as the rate of formation of orthophosphate against the temperature of incubation for a period of 30 minutes. Peers (1953) obtained a value of 55°C for the optimum temperature of wheat phytase. Mandal and co-workers (1972) found 57°C as optimum for the germinating mung bean phytase while Lolas and Markakis (1977) found 50°C as the optimum for navy bean phytase.

Effect of substrate concentrations on activity

The initial velocity of the reaction, in terms of umoles of orthophosphate liberated/min per ml of enzyme, was calculated and plotted against substrate concentration (Figure 6). Phytase is shown to be inhibited by concentrations of substrate higher than 1 mM, and practically stopped the reaction over 10 mM. A similar finding has been reported for the Dwarf bean phytase (Gibbins and Norris, 1963) and for the Navy bean phytase (Lolas and Markakis, 1977). Gibbins and Norris (1963) attribute the inhibition of phytase by high substrate concentrations to be indicative of a two-point attachment of the phytate to the enzyme.

From a plot of 1/v against 1/s (v = velocity as

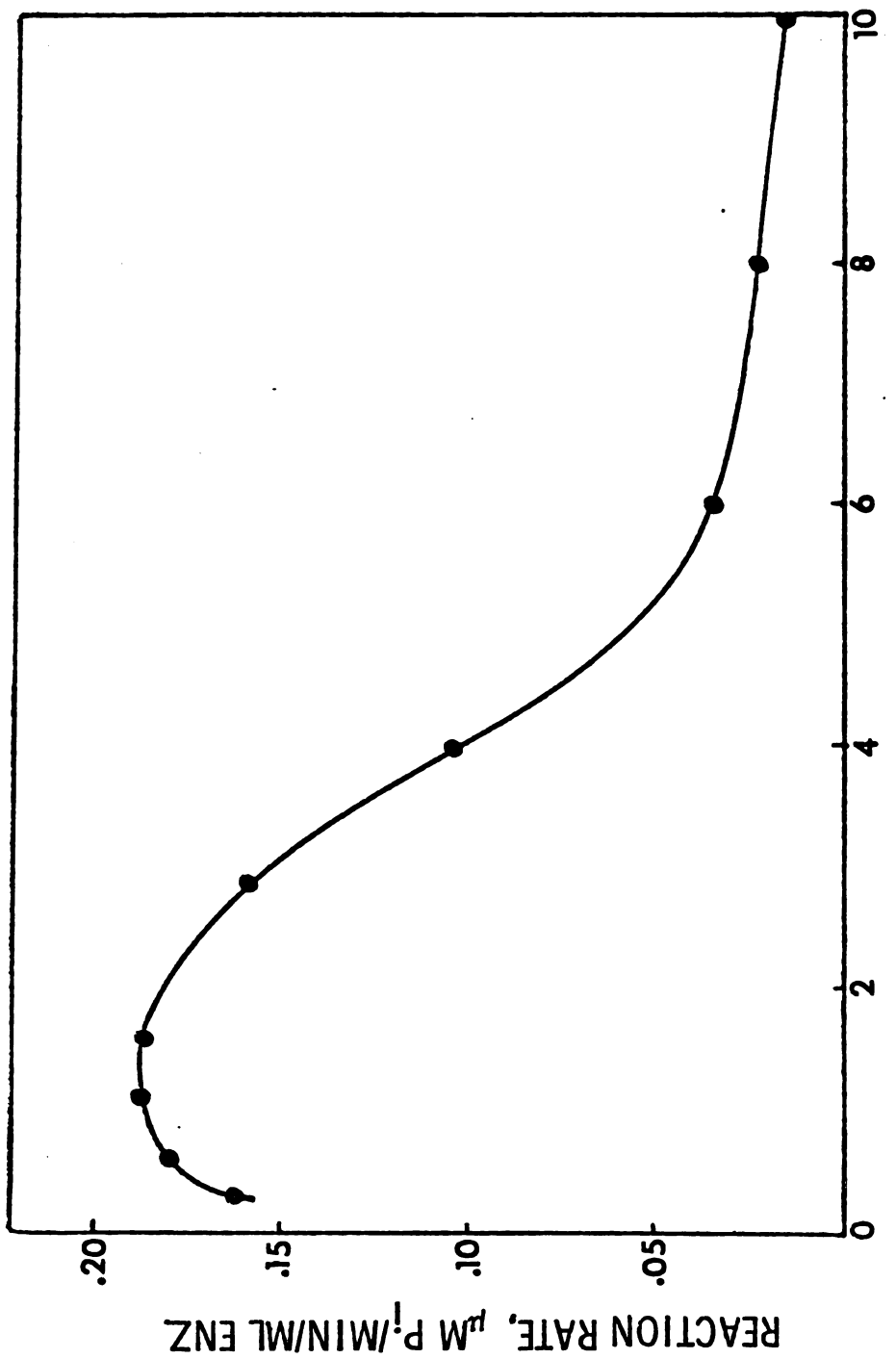


Figure 6.--Effect of substrate concentration on the yeast phytase activity. The enzyme was incubated at 50°C in 0.1 M acetate buffer, pH 5.3, with the indicated concentrations of sodium phytate.

enzyme units/ml enzyme, s = substrate concentration as molarity) the Michaelis constant K_m was found to be 0.21 mM and the V_{max} 0.005 umoles/min per ml enzyme (Figure 7). Other Michaelis constants for phytase reported in literature are 0.091 mM for corn phytase (Chang, 1967); 0.33 mM for wheat phytase (Peers, 1953); 0.57 mM for wheat bran phytase (Nagai and Funahashi, 1962); 0.15 mM for the Dwarf bean phytase (Gibbins and Norris, 1963); 0.65 mM for the mung bean phytase (Mandal et al., 1972); and 0.018 mM for the Navy bean phytase (Lolas and Markakis, 1977).

Effect of metal ions on phytase activity

As shown in Table 1, none of the metallic ions tested including magnesium and calcium had any effect upon phytase activity with the exception of cadmium, mangnese, mercuric ions that have inhibitory effects at final concentration of 10^{-3}M and higher. This inhibitory effect of metallic ions may be due to strong affinity to phytic acid itself and resulting in some competition between the metallic ions and enzyme for the substrate. Obviously, heavy metallic ions can have an adverse effect on the enzyme protein.

Peers (1953) reported activation of wheat phytase by magnesium and calcium ions. Gibbins and Norris (1963)

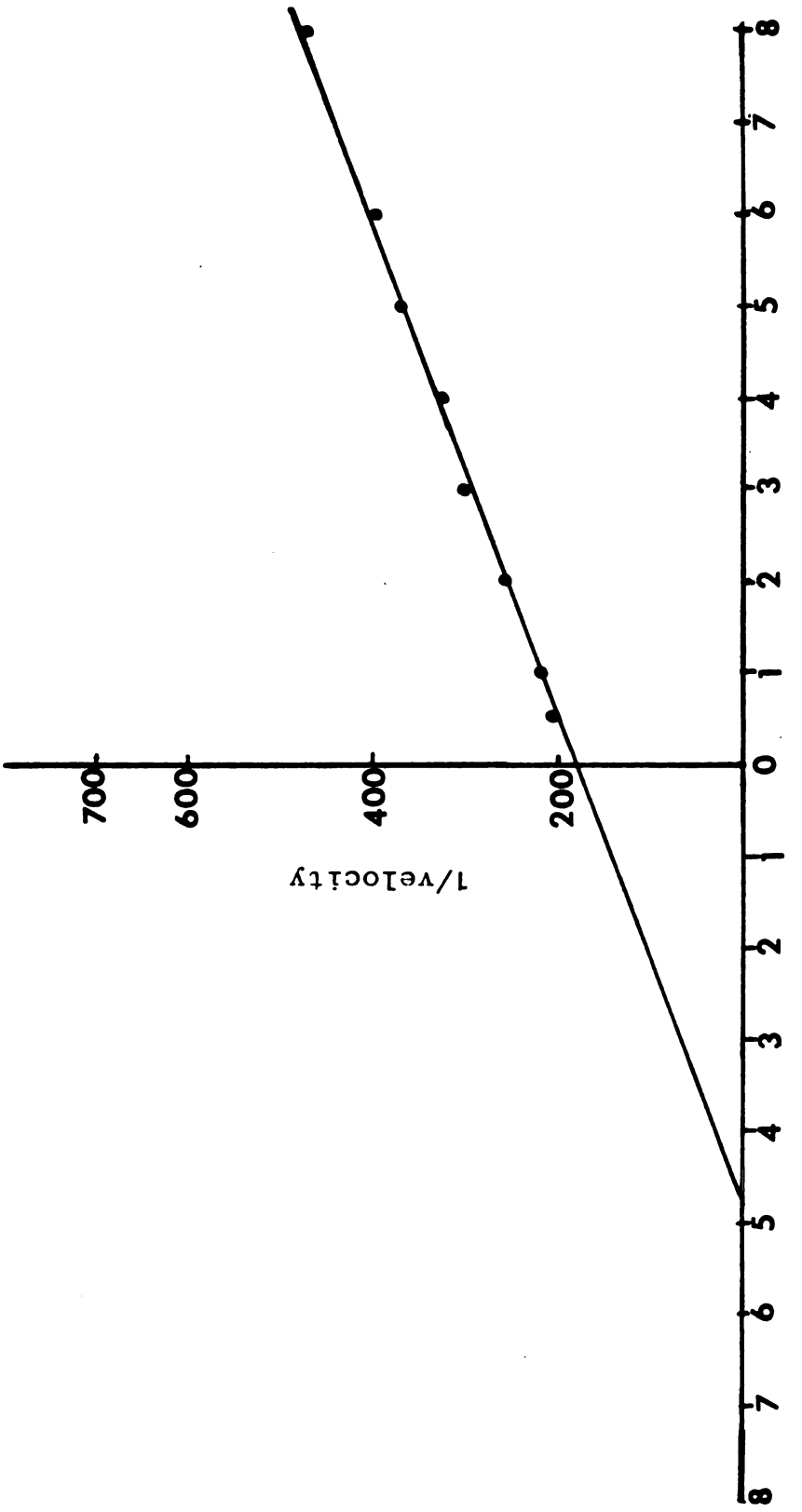


Figure 7.--Lineweaver-Burk plot of yeast phytase with socium phytate as substrate. (velocity expressed in μmoles/min per ml enzyme).

Table 1.--Effect of metal ions on the yeast phytase acitvity.

Metal ions	Relative Activity@		
	10^{-5} M	10^{-4} M	10^{-3} M
$CaSO_4$	99%	100%	106%
$CdCl_2$	102	79	72
$CoCl_2$	101	101	94
$CuSO_4$	95	116	115++
$FeSO_4$	105	125	130++
$HgCl_2$	100	80++	66++
$Mg(NO_3)_2$	94	91	97
$MnSO_4$	109	90	87++
$ZnSO_4$	103	103	97++

@The activity without added metal ions was taken as 100%.
++Precipitation was observed.

also observed a small increase in the activity of the Dwarf bean phytase by the same metals whereas no activation was observed in the bran wheat phytase (Nagai and Funahashi, 1962). Lolas and Markakis (1977) showed an increase of 35% in activity of Navy bean phytase by cobalt in the enzyme mixture. The yeast phytase, in the present study, showed an increase in activity by about 30% as a result of the addition of 10^{-3}M Fe^{2+} in the enzyme mixture. There is no data in literature on the

effect of Fe^{2+} on phytase activity. However, a sample of wheat phytase (SIGMA Chemical Co., St. Louis, Missouri) when assayed did not show any effect in the presence of 10^{-3}M Fe^{2+}.

Effect of chelating agents on yeast phytase activity

As shown in Table 2, all the chelating agents tested showed a decrease in the phytase activity. In the presence of EDTA, even at the low concentrations of 10^{-6}M, the activity of yeast phytase decreased by 72%. This behaviour of the yeast phytase may be due to the fact yeast phytase is activated by Fe^{2+}, and the presence of chelating agents in the reaction mixture may result in non-availability of Fe^{2+} for the activation of the enzyme.

Table 2.--Effect of chelating agents on yeast phytase activity.

Chelating Agent	Relative Activity@		
	10^{-6} M	10^{-4} M	10^{-2} M
Citrate	100%	68%	50%
Oxalate	80	64	47
Tartrate	93	90	39
EDTA	72	66	40

@The activity without added chelating agent was taken as 100%.

Separation of phytase from phosphatase

Figure 8 illustrates the fractionation of a crude enzyme extract from yeast phytase on a DEAE column. The first enzyme emerging from the column is a phosphatase that hydrolyzes ∝-glycerophosphate or phenyl phosphate but not phytic acid. The second enzyme is the phytase which utilizes all the above substrates. As a further proof of the different nature of enzymes is the fact that the phytase fraction,but not the phosphatase fraction showed an increase in the activity on phytate by about 30% in the presence of 10^{-3}M Fe^{2+}. Thus it is evident that a clear distinction can be drawn between the two enzymes.

Substrate specificity of yeast phytase

The yeast phytase purified by DEAE-cellulose chromatography, had a broad sutstrate specificity, catalyzing the hydrolysis of all the phosphomonoesters tested Table 3.

Yeast phytase hydrolyzes all the substrates tested and is characterized by a potent pyrophosphate activity. Among the substrates used, pyrophosphate was the most preferred substrate while inositol pentaphosphate was the least preferred substrate. The enzyme was most active on inositol monophosphate, followed by inositol triphosphate, inositol diphosphate, inositol hexaphosphate, inositol

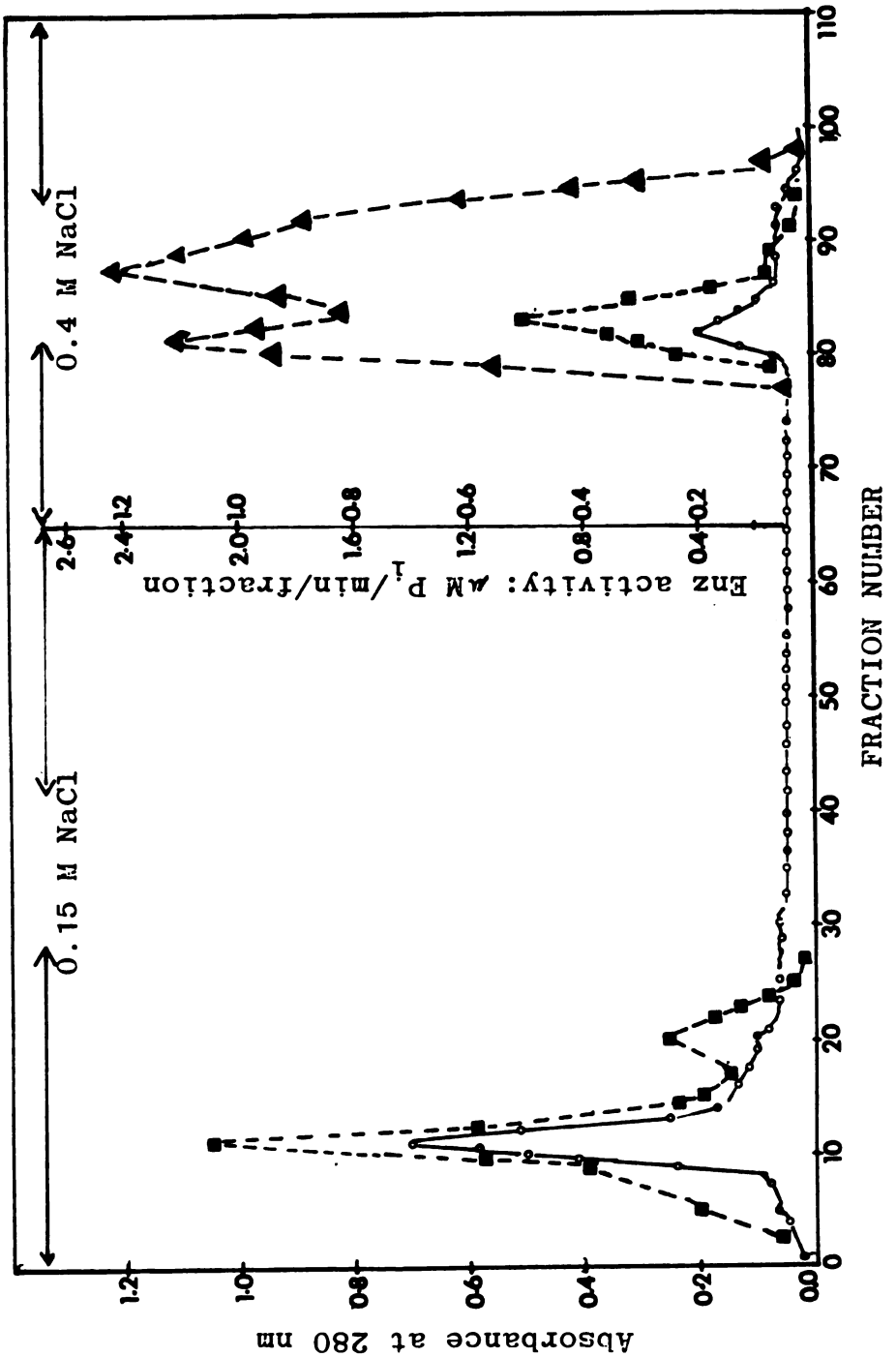


Figure 8.--DEAE-cellulose chromatography of yeast extract. Protein was eluted from column with NaCl solution in 0.01 M TRIS-HCl buffer, pH 7.4. , absorbance at 280 nm: , phosphatase activity. , phytase activity. The left scale in the center is for phosphatase and the roght for phytase activity.

Table 3.--Substrate specificity of purified yeast phytase.

Substrate	Activity (units/ml enzyme)@	Relative Activity
Phytic acid	0.011	1.0
Inositol pentaphosphate	0.0042	0.39
Inositol tetraphosphate	0.0107	0.97
Inositol triphosphate	0.0319	2.87
Inositol diphosphate	0.0176	1.59
Inositol monophosphate	0.4590	41.37
5'-Adenylic acid (AMP)	0.0281	2.53
α-Glycerophosphate	0.0228	2.06
β-Glycerophosphate	0.0286	2.58
Phenyl phosphate	0.4420	39.90
Pyrophosphate	4.1220	371.35

@Unit = One unit is the activity which results in the liberation of 1 μmole of inorganic phosphorus per minute.
ml of enzyme corresponds to 0.15 g of yeast (dry basis).

tetraphosphate, and inositol pentaphosphate among the inositol phosphates tested.

As yeast phytase can hydrolyse all the intermediate inositol -phosphates, though at different rates, this may be the reason for the absence of any particular pattern in their presence in bread samples.

Effect of extraction rates on the inositol phosphates in wheat flours and breads

The inositol phosphates from flours and bread were separated on Dowex 1 x 8 (Cl^-form) with 0 to 1.0 N HCl linear gradient elution. The peaks appearing in the chromatogram were identified by calculating the molecular ratio of phosphorus to inositol (Table 4).

Table 4.--Ratio of phosphorus to inositol in fractions obtained from Dowex 1 x 8 (Cl^-) chromatography[@]

Fraction #	phos-phorus (µg)	Ino-sitol (µg)	µmole P/ µmole inositol		Inositol phosphates identified[b]
			Observed	Theory	
4 - 10	124.2	ND[c]	—	—	P_i
13 - 18	38.5	225.0	1.00	1	IP_1
23 - 31	101.6	303.6	1.94	2	IP_2
34 - 48	201.7	423.3	2.76	3	IP_3
52 - 59	150.6	224.8	3.91	4	IP_4
63 - 79	210.2	257.9	4.78	5	IP_5
82 - 107	418.7	416.2	5.83	6	IP_6

@ Wheat flour bread fermented for 2 hours.

b Inositol mono- (IP_1), di- (IP_2), tri- (IP_3), tetra- (IP_4), penta- (IP_5), and hexaphosphate (IP_6).

c Not detected.

The chromatographic separation of the six inositol phosphates, i.e. monophosphate (IP_1), diphosphate (IP_2),

triphosphate (IP_3), tetraphosphate (IP_4), pentaphosphate (IP_5), and hexaphosphate (IP_6), plus inorganic phosphate (P_i), is illustrated in Figure 9. A more quantitative account of these fractions in various extraction flours and breads is given in Table 5. The Table 5 also shows that IP_6 to be the dominant inositol phosphate in all of the flours. The percentage of IP_6 increased with increasing extraction rates. The percent of total P present as P of IP_6 was 27%, 33%, 38% and 51.7% for 70%-extraction (E_{70}), 80%-extraction (E_{80}), 90%-extraction (E_{90}) and 100%-extraction (E_{100}) wheat flours, respectively. This is due to the higher concentration of IP_6 (phytic acid) in the outer layers of the wheat kernels (Hay, 1942); more of these layers are present in higher extraction flours. Fermentation reduced the phytate contents by about 70% in both 70%-extraction breads (EB70) and 80%-extraction breads (EB_{80}), 67% in 90%-extraction bread (EB_{90}), but only 58% in 100%-extraction breads (EB_{100}). This is in accordance with de Lange et al (1961) who observed only 40 - 50% reduction of phytate in whole wheat bread. The slower rate of disappearence of phytate in EB_{100} may be attributed to the sluggish action of the yeast phytase due to the presence of inhibitors. Calcium, which is present in large quantities in higher extraction

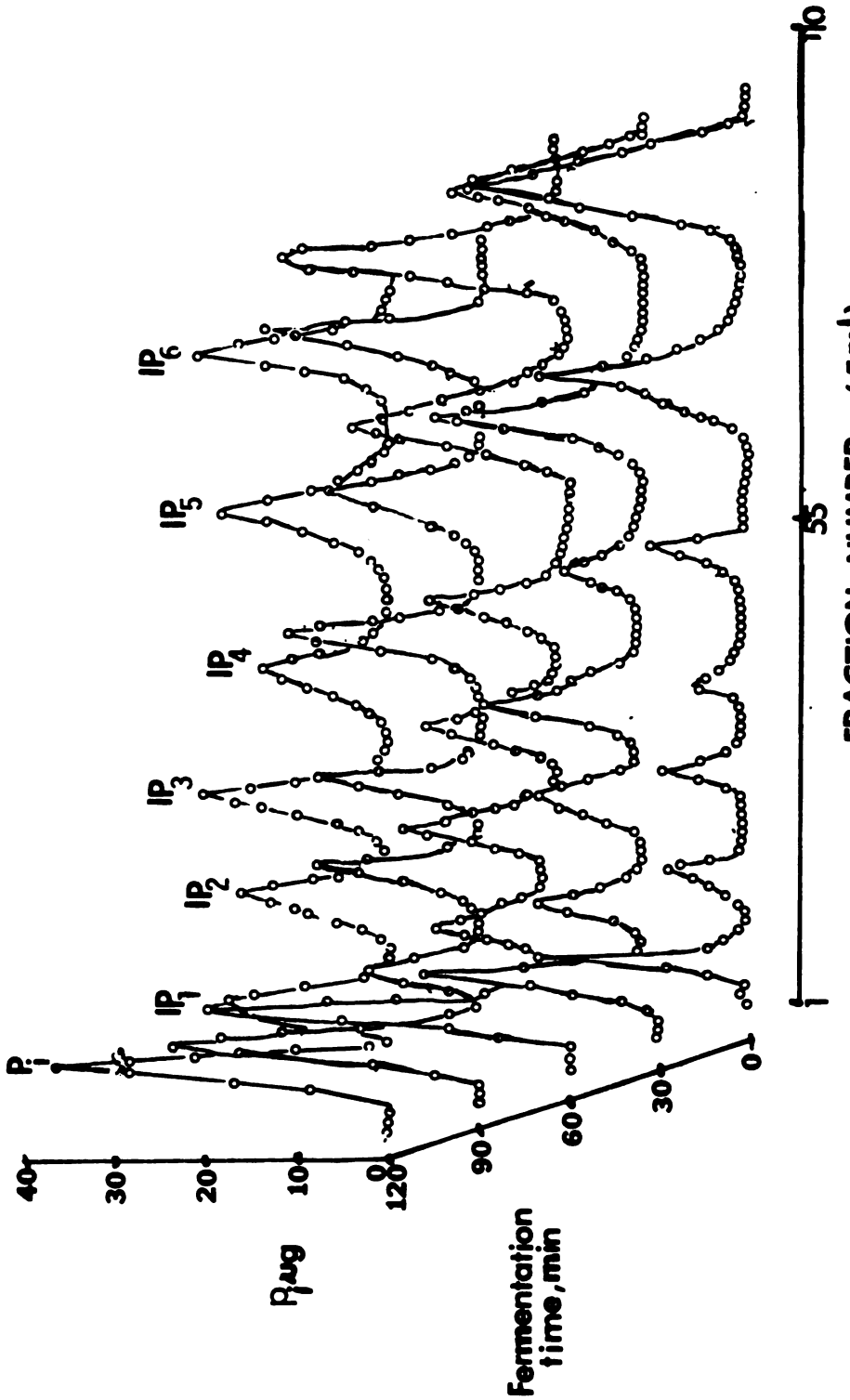


Figure 9.--Elution pattern of inositol phosphates on Dowex 1x8 (Cl^-) column.

Table 5.--Concentration of inositol phosphates and inorganic phosphate in flours of varying extraction rate and breads made from them (mg of P present[b] in each fraction[@] per 100 g of flour or bread, dry basis).

Extraction rate	Product	P_i	IP_1	IP_2	IP_3	IP_4	IP_5	IP_6	sum of P
70%	Flour	17	10	11	12	26	22	36	134
	Bread	29	5	28	41	6	15	11	135
80%	Flour	23	7	14	33	14	55	72	218
	Bread	71	23	17	31	25	30	21	218
90%	Flour	27	20	23	50	34	34	115	304
	Bread	94	22	29	39	40	44	38	306
100%	Flour	31	17	17	40	36	69	222	432
	Bread	126	34	35	38	25	81	94	433

@ P_i = inorganic phosphate; IP_1 to IP_6 = inositol phosphates possessing 1 to 6 phosphate groups per inositol residue, respectively.

b All the breads were fermented for 2 hr.

flours has been reported to possess inhibitory effect on phytase (Ranhotra, 1972). In addition, high extraction flours may contain more phytate in a protein-bound state and this protein-bound phytate might not be acessible to the enzymatic activity (Ranhotra, 1972; Fontaine et al., 1942). Finally, the substrate inhibition due to higher concentrations of phytate on the enzymatic activity of phytase, might have been resulted in slower destruction of phytate in EB_{100}.

As the phytate content decreased, there was a concurrent increase in inorganic phosphorus. After 2 hours of fermentation, the percent of total phosphorus represented by phytic acid P in bread ranged from 7.9%, 8.6%, 12.2%, and 21.7% in EB_{70}, EB_{80}, EB_{90} and EB_{100}, respectively. After taking intermediate inositol phosphates into consideration, it was possible to balance the phosphate content of the various extraction flours. Except for the IP_5 content, which showed a steady increase with extraction rate, all other intermediate inositol phosphate contents fluctuated in both flours and breads.

Effect of fermentation time on inositol phosphates in wheat flours and breads

The elution of inositol phosphates was satisfactory and was identical to the elution pattern Figure 9. A more quantitative account of these fractions in both 70%-extraction bread (WB) and whole wheat (WWB) prepared after fermentation of varying duration is given in Table 6.

As shown in Figure 9 the separation of inositol phosphates and P_i is satisfactory. The breads made of either flour, WWB and WB, contained all six types of inositol phosphates before being subjected to the panary fermentation. Ferrel, 1978, reported only phytic acid to be present in wheat flour. He chromatographed the

wheat flours immediately after milling, whereas in our work, the flours were obtained from the local market, and some hydrolysis of the phytic acid by, a phytase native to the wheat had occurred in the time intervening after milling.

Table (6) shows that IP_6 is the dominant inositol phosphate in both the unfermented breads analysed, although WWB contains more than twice the amount of IP_6 present in WB. Lolas et al (1976) found 62 - 80% of the total phosphorus to be in the phytate form in whole wheat. As the fermentation progressed, phytic acid was dephosphorylated to other inositol phosphate forms (inositol mono-, di-, tri-, tetra-, and penta- phosphate) and inorganic phosphate. A scheme of dephosphorylation of phytic acid by phytase was suggested by Tomlinson and Ballou (1962) (Figure 10). The remaining inositol phosphates collectively contain almost as much P as IP_6 in WWB, and 1.7 times as much as in WB. Fermentation reduced the phytate content with the fastest decrease occurring during the first 30 minutes. The rate of phytate degradation was more in WWB than in WB and this may be due to the presence of more phytate and the enzyme phytase in WWB than in WB flours. Peers (1953) found the phytase primarily in the aleurone, endosperm and scutellum. Breads prepared after 120 min fermentation sustained a 72% to 77% loss of IP_6. In the literature,

Table 6.--Wheat flour bread contents in inositol phosphates as a function of fermentation time (mg of P present in each of the fractions/ 100 g of bread, dry basis).

Fermentation time, min	Type of bread	P_i @	IP_1	IP_2	IP_3	IP_4	IP_5	IP_6	Total phosphorus
0	WWB[b]	45	36	37	52	98	191	398	857
	WB	40	62	29	94	33	63	192	513
30	WWB	95	68	116	127	89	157	206	858
	WB	57	32	102	75	39	92	116	514
60	WWB	110	88	104	100	112	175	175	862
	WB	64	43	71	98	51	83	101	511
90	WWB	131	79	122	160	90	139	133	854
	WB	77	50	43	71	92	114	73	520
120	WWB	157	112	125	112	128	127	92	860
	WB	110	56	48	108	40	101	54	517

@ P_i = inorganic phosphorus; IP_1 to IP_6 = inositol phosphates containing 1 to 6 phosphates per inositol residue.

b WWB = whole wheat bread; WB = 70%-extraction wheat bread.

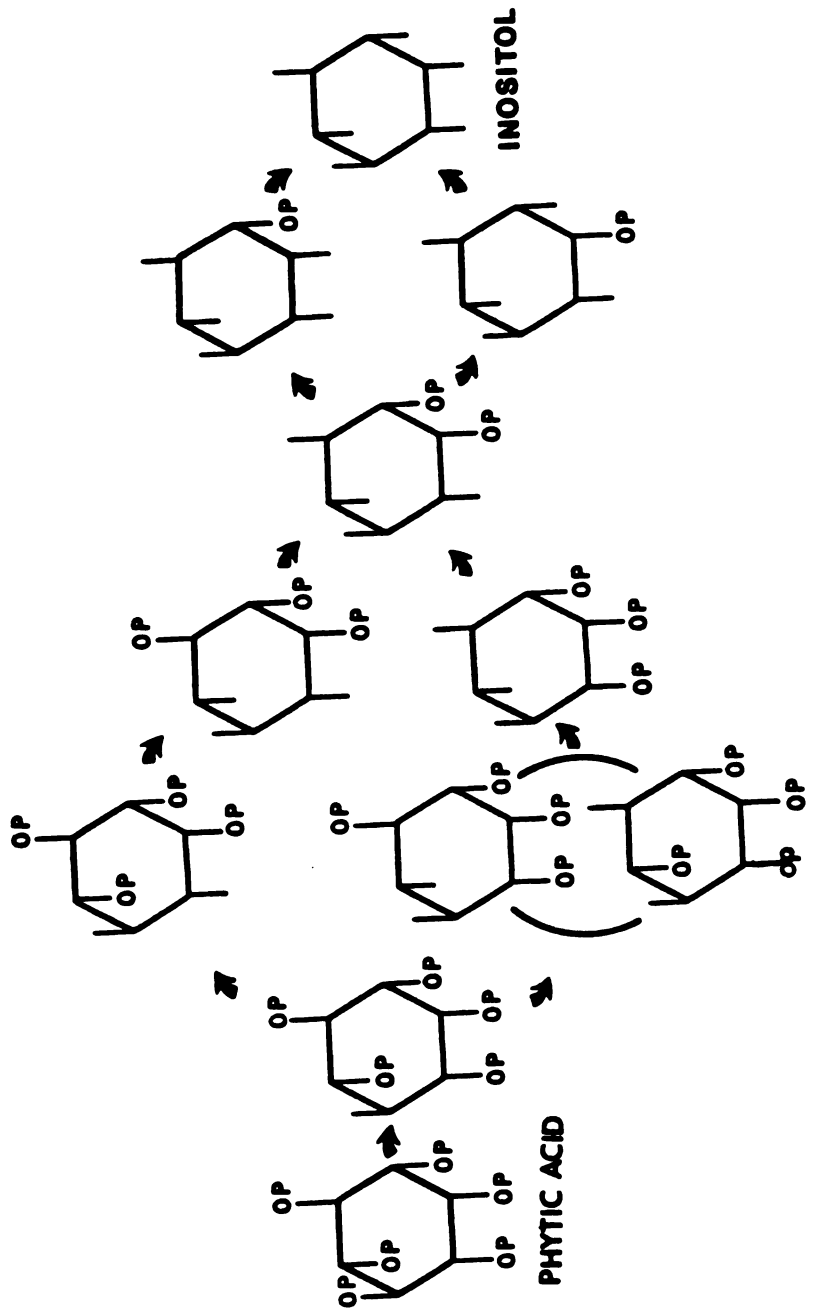


Figure 10.--Scheme of dephosphorylation of phytic acid by phytase. (Tomlinson and Ballou)

the figures pertaining to phytate destruction in bread-making vary greatly: from 100% for white bread (Pringle and Moran, 1942), to 40 - 50% for whole wheat bread (deLange et al., 1961), to 13% for village flat breads made in Iran (Reinhold, 1972). As the phytate content decreased, the inoraganic phosphate content increased (Figures 11 & 12). The largest increase in P_i in WWB occurred during the last 30 minutes of fermentation. This may be due to the activity of wheat phytase in the earlier stages of fermentation, as is present in large amounts in whole wheat flour and yeast phytase activity in the later stages of fermentation. But not even after 120 minutes of fermentation can the loss of IP_6 be accounted for by the P_i rise. Only when the P content of the intermediate inositol phosphates are taken into consideration can a acceptable P balance be achieved. Tongkongchitr et al., (1981) could account for almost all of the phytate P loss by the increase in inorganic P, but this occurred after 8 hours of fermentation by which time perhaps almost all of the intermediate inositol phosphates were dephosphorylated. Such prolonged fermentation time would lead to lowering of loaf quality and a decrease of the nutrient content of bread (Reinhold, 1975). The intermediate inositol phosphates content of breads fluctuated with fermentation time, which is probably due to several dephosphorylation reactions

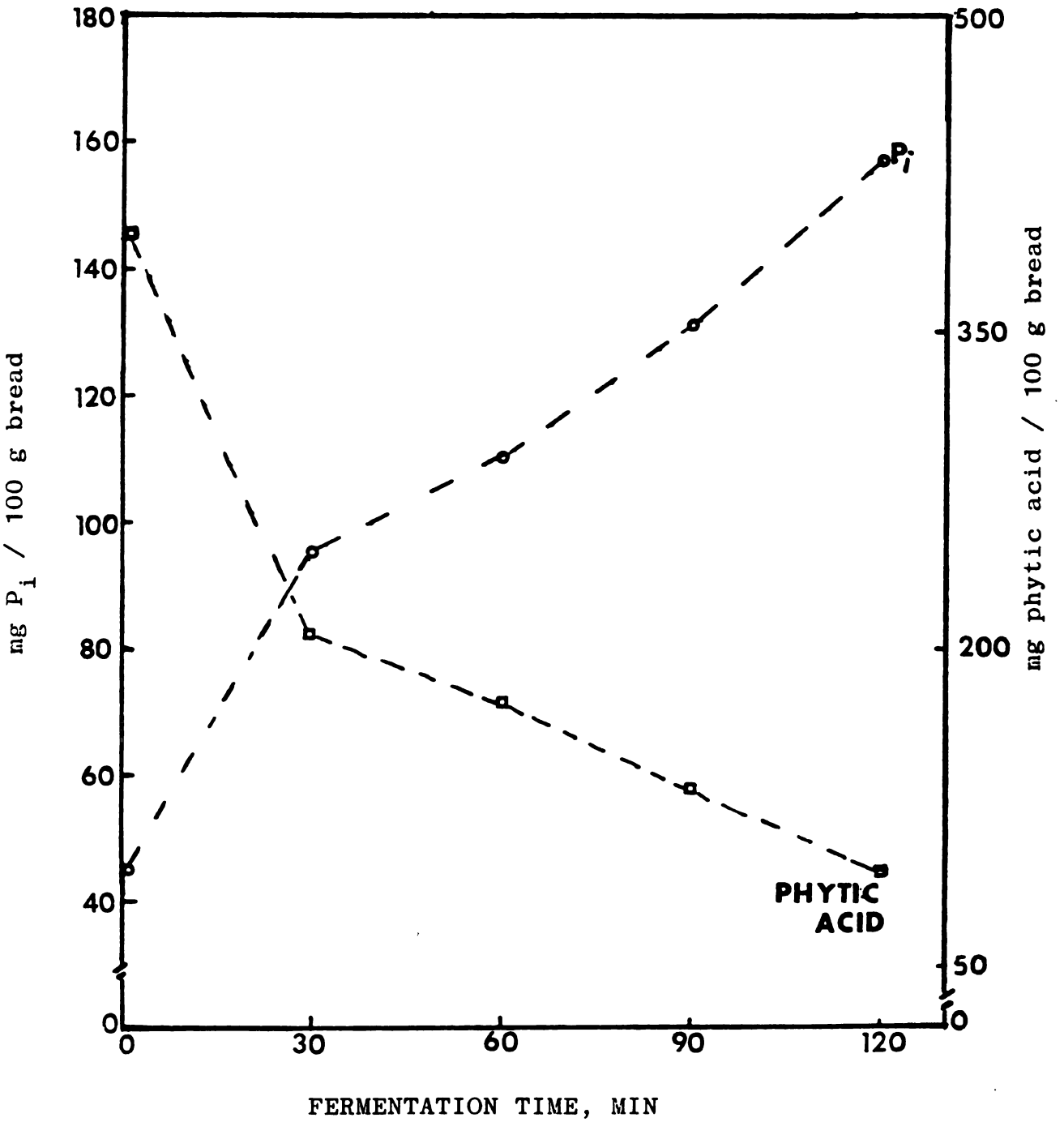


Figure 11.--Effect of fermentation time on the phytic acid and P_i content of whole wheat bread.

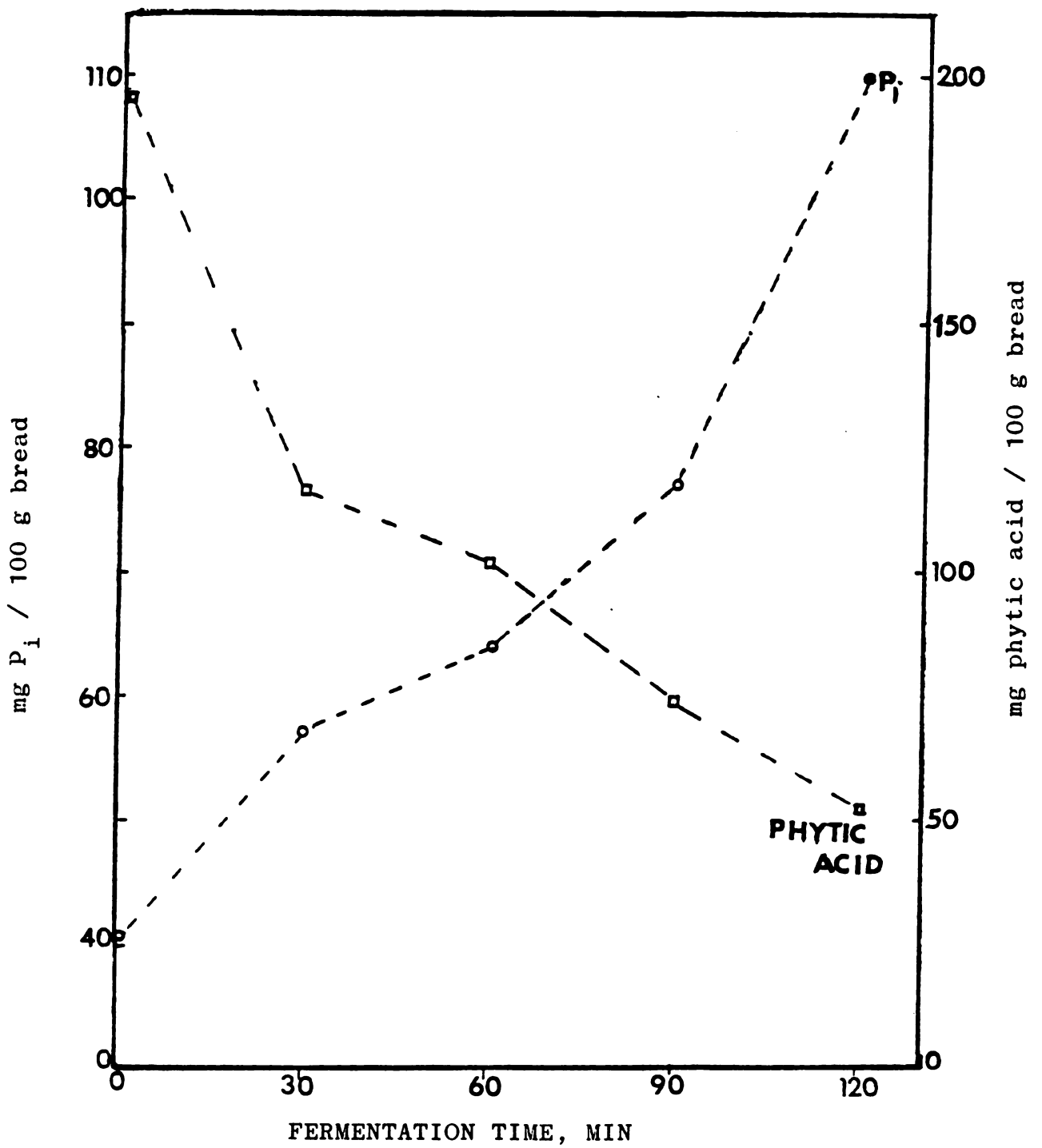


Figure 12.--Effect of fermentation time on the phytic acid and P_i content of wheat bread.

occurring simultaneously.

Inositol phosphates in commercial breads

The inositol phosphate contents of commercial breads are given in Table 7. The white bread shows more P_i, IP_6 and total phosphorus than expected, assuming a 70 - 75% extraction. The excessive amounts of P_i and total phosphorus must be due to the added mono-calcium and di-calcium phosphates listed on the label of the white bread. The rather large quantity of phytic acid may be attributed to the inhibition of the yeast phytase by the added calcium in the form of calcium propionate, mono-calcium phosphate, calcium sulfate and di-calcium phosphate; such inhibition was previously observed by Ranhotra (1972). The whole wheat breads showed only 17.4% of phosphorus in the phytate form, and other inositol phosphates did not show any pattern due to continuous phosphorylation and dephosphorylation reactions. The whole wheat breads did not contain any added calcium or phosphates according to the label.

INOSITOL PHOSPHATE-METAL BINDING

Inositol monophosphate-metal complexes: IP_1-metal complexes were formed at all the pH's studied except at pH 4 with zinc (Table 8). IP_1-Ca precipitate showed phosphorus to

Table 7.--Concentration of inositol phosphates and inorganic phosphates in the commercial white and whole wheat breads (mg of P present in each fraction@ per 100 g of bread, dry basis).

Product	P_i	P_1	P_2	P_3	P_4	P_5	P_6	sum of P
Commercial White Bread	100	3	12	40	11	61	92	319
Commercial Whole Wheat Bread	120	26	30	55	60	94	81	466

@ P_i = inorganic phosphate; IP_1 to IP_6 = inositol phosphates possessing 1 to 6 phosphate groups per inositol residue, respectively.

White Bread Ingredients: Flour, Corn syrup, Whey, Yeast, Soybean oil, Salt, Sodium stearate, Non-fat milk, Corn flour, Calcium propionate, Monoglyceride, Monocalcium phosphate, Calcium sulfate, Dicalcium phosphate, Barley malt, Ammonium sulfate, Fungal enzymes, Potassium bromate, Niacin, Ferrous sulfate, Azo di carbonate, Potassium iodate, Thiamin hydrochloride, and Riboflavin.

Whole Wheat Bread Ingredients: Ground wheat, Unbleached enriched flour (wheat flour), Malted barley, Niacin, Iron, Thiamin mono-nitrate, Riboflavin, Corn syrup, Vegetable shortening, Salt, Yeast, Vinegar.

Table 8.--Percentages of metal and phosphorus precipitated from Inositol monophosphate solution at pH's 4,5 and 6.

Metal	pH	% metal	% phosphorus	P/metal in precipitate
Ca (as $CaCl_2$)	4	30.01	23.25	1.0
	5	31.28	28.52	1.17
	6	33.68	31.07	1.00
Cu (as $CuSO_4$)	4	9.62	14.08	2.99
	5	62.04	30.27	1.00
	6	95.61	8.40	@
Fe (as $FeSO_4$)	4	50.16	13.92	0.500
	5	77.10	14.26	0.333
	6	93.22	6.55	@
Zn (as $ZnSO_4$)	4	—	—	—
	5	35.50	8.41	0.500
	6	38.58	4.40	@

@ These precipitates contain both metal hydroxide and inositol monophosphate-metal complex.

— No precipitate formed.

calcium atom ratio of 1 : 1 at pH's 4 and 6, while at pH 5 a ratio of 6 : 5 was observed. The IP_1-Cu precipitate at pH 4 had a phosphorus to copper atom ratio of 3 : 1; at pH 5 the ratio was 1 : 1. The IP_1-Fe precipitate analysis showed a phosphorus to iron atom ratio of 1 : 2 at pH 4 and 1 : 3 at pH 5. The IP_1-Zn precipitate displayed a phosphorus to zinc atom ratio of 1 : 2 at pH 5.

As the pH increased there was an increase in the percent of metal precipitated. The influence of pH was more pronounced in the copper and iron precipitation than in the calcium precipitation. Increasing the pH from 4 to 5 resulted in a large change in the atom ratio of phosphorus to copper: from 3 : 1 to 1 : 1. For a similar pH increase the atom ratio of P : Fe changed from 1 : 2 to 1 : 3. Calcium did not show any change in the atom ratio of 1 : 1 with phosphorus as the pH increased.

Inositol diphosphate-metal complexes: IP_2-metal complexes were formed at all pH levels (Table 9). The IP_2-Ca complex analysis showed a simple atom ratio of phosphorus to calcium of 1 : 1 at pH's 4, 5 and 6. The IP_2-Cu precipitate showed a phosphorus to copper atom ratio of 2 : 1 at pH 4 and 1 : 1 at pH 5. The IP_2-Fe precipitate, at pH 4 had a phosphorus to iron atom ratio of 1 : 1, at pH 5 the ratio was 1 : 2. The IP_2-Zn complex displayed a phosphorus to zinc atom ratio of 1 : 2 at pH 4 and 1 : 1 at pH 5.

Table 9.--Percentages of metal and phosphorus precipitated from Inositol diphosphate solution at pH's 4,5 and 6.

Metal	pH	% metal	% phosphorus	P/metal in precipitated
Ca (as $CaCl_2$)	4	29.01	22.47	1.00
	5	31.50	24.41	1.00
	6	32.62	25.26	1.00
Cu (as $CuSO_4$)	4	20.98	20.47	2.00
	5	72.24	35.24	1.00
	6	90.82	12.52	@
Fe (as $FeSO_4$)	4	49.88	27.68	1.00
	5	81.54	22.63	0.50
	6	97.51	9.53	@
Zn (as $ZnSO_4$)	4	17.23	4.08	0.50
	5	34.72	16.45	1.00
	6	49.54	4.52	@

@ These precipitates contain metal hydroxide and inositol diphosphate-metal complex.

All the metals showed an increase in the percent of metal precipitated with an increase in pH. The increase in IP_2-metal complex precipitation was larger in copper, iron and zinc than in calcium. With an increase in pH from 4 to 5, the atom ratio of P : Cu decreased from 2 : 1 to 1 : 1. For a similar increase in pH, the P : Fe atom ratio changed from 1 : 1 to 1 : 2, while the zinc precipitation there was an increase in the P : Zn atom ratio from 1 : 2 to 1 : 1. There was no change in the atom ratios of IP_2-Ca complexes with a change in pH.

Inositol triphosphate-metal complexes: IP_3-metal complexes were precipitated at all pH's (Table 10). IP_3-Ca complex formed a simple phosphorus to calcium atom ratio of 1 : 1 at all the pH's studied. The IP_3-Cu precipitate displayed a phosphorus to copper atom ratio of 2 : 1 at pH 4, and 1 : 1 at pH 5. The IP_3-Fe precipitate analysis showed a phosphorus to iron atom ratio of 1 : 1 at pH 4 and 1 : 3 at pH 5. The IP_3-Zn complex showed a phosphorus to zinc atom ratio of 1 : 2 at pH 4 and 1 : 1 at pH 5.

The effect of pH on the IP_3-metal complexes was significant in copper, iron and zinc, while calcium showed only a slight increase. All the metals showed an increase in percent metal precipitated with an increase in pH. Increasing the pH from 4 to 5 resulted in decrease in the atom ratio of phosphorus to copper from 2 : 1 to 1 : 1.

Table 10.--Percentages of metal and phosphorus precipitated from Inositol triphosphate solution at pH's 4, 5 and 6.

Metal	pH	% metal	% phosphorus	p/metal in precipitated
Ca (as $CaCl_2$)	4	25.12	19.47	1.00
	5	30.36	23.53	1.00
	6	32.28	25.02	1.00
Cu (as $CuSO_4$)	4	15.74	15.36	2.00
	5	70.51	34.39	1.00
	6	99.71	25.31	@
Fe (as $FeSO_4$)	4	58.78	32.63	1.00
	5	94.61	35.00	0.66
	6	99.91	20.04	@
Zn (as $ZnSO_4$)	4	9.46	2.24	0.500
	5	42.91	20.34	1.00
	6	50.20	21.06	@

@ These precipitates contain metal hydroxide and inositol triphosphate-metal complex.

For a similar pH increase the atom ratio of P : Fe in the IP_3-Fe precipitate decreased from 1 : 1 to 1 : 1.5, while the IP_3-Zn precipitate showed an increase in the atom ratio phosphorus to zinc from 1 : 2 to 1 : 1. Calcium did not show any change in the atom ratio of 1 : 1 between phosphorus and calcium with an increase in pH.

Inositol tetraphosphate-metal complexes: IP_4-metal complexes were precipitated at all pH's studied (Table 11). The IP_4-Ca complex showed simple atom ratio of phosphorus to calcium of 1 : 1 at the pH's 4, 5 and 6. The IP_4-Cu precipitate analysis showed a phosphorus to copper atom ratio of 3 : 2 at pH 4, and 1 : 1 at pH 5. The IP_4-Fe complex had a phosphorus to iron atom ratio of 1 : 1 at pH 4, and 1 : 3 at pH 5, while in the IP_4-Zn complex the P : Zn ratio was 1 : 1 at pH 4 and 6 : 5 at pH 5.

As the pH increased there was an increase in the percent of metal precipitated. The influence of pH was greater in the copper, iron and zinc precipitation than in the calcium precipitation. Calcium did not show any difference in the atom ratio of 1 : 1 with phosphorus precipitates as pH increased. The phosphorus to copper atom ratio decreased from 1 : 0.68 to 1 : 1 with an increase in pH from 4 to 5. For a similar pH increase the atom ratio of phosphorus to iron changed from 1 : 1 to 1 : 1.5, while P : Zn atom ratio changed from 1 : 1 to

Table 11.--Percentages of metal and phosphorus precipitated from Inositol tetraphosphate solution at pH's 4, 5 and 6.

Metal	pH	% metal	% phosphorus	P/metal in precipitate
Ca (as $CaCl_2$)	4	21.92	16.98	1.00
	5	27.16	21.05	1.00
	6	46.42	35.98	1.00
Cu (as $CuSO_4$)	4	35.84	25.34	1.48
	5	53.24	25.97	1.00
	6	99.06	11.64	@
Fe (as $FeSO_4$)	4	57.66	32.10	1.00
	5	75.10	27.75	0.66
	6	85.18	14.24	@
Zn (as $ZnSO_4$)	4	12.62	5.98	1.00
	5	61.33	24.43	0.84
	6	63.32	20.54	@

@ These precipitates contain metal hydroxide and inositol tetraphosphate-metal complex.

1 : 1.19.

Inositol pentaphosphate-metal complexes: The IP_5-metal complexes were precipitated at all pH's (Table 12). The IP_5-Ca complex upon analysis showed a phosphorus to calcium atom ratio of 1 : 1 at pH 4 and 6 : 5 at pH's 5 and 6. The IP_5Cu and IP_5-Zn precipitates both displayed the phosphorus to metal atom ratio of 1 : 1 at pH's 4 and 5. The IP_5-Fe precipitates showed phosphorus to iron atom ratios of 1 : 1 at pH 4, and 1 : 2 at pH 5.

Increasing the pH resulted in an increase in the percent of metal precipitated. The influence was more pronounced in calcium, copper and zinc precipitation than in iron precipitation. Calcium showed an increase in the atom ratio of P : Ca from 1 : 1 to 6 : 5 with an increase in the pH from 4 to 5 or 6. Increasing the pH from 4 to 5 did not change the atom ratio of both P : Cu and P : Zn from 1 : 1. For a similar increase in pH, the IP_5-Fe precipitate showed a decrease in the P : Fe atom ratio from 1 : 1 to 1 : 2.

Inositol hexaphosphate-metal complexes: Insoluble IP_6-metal complexes were formed at all of the pH's studied (Table 13). The IP_6Ca precipitate showed a phosphorus to calcium atom ratio of 6 : 5 at pH's 4, 5 and 6. Similar observations reported by Evans and Pierce (1981). Although a hexa-calcium phytate salt has been reported by Hay (1942), in a similar

Table 12.--Percentages of metal and phosphorus precipitated from Inositol pentaphosphate solution at pH's 4, 5 and 6.

Metal	pH	% metal	% phosphorus	P/metal in precipitate
Ca (as $CaCl_2$)	4	26.46	20.51	1.00
	5	47.76	44.06	1.19
	6	53.11	48.89	1.19
Cu (as $CuSO_4$)	4	52.55	25.64	1.00
	5	78.06	38.08	1.00
	6	98.90	10.15	@
Fe (as $FeSO_4$)	4	63.76	35.39	1.00
	5	68.76	19.08	0.50
	6	97.70	29.28	@
Zn (as $ZnSO_4$)	4	48.01	22.76	1.00
	5	62.64	29.70	1.00
	6	70.03	7.95	@

@ These precipitates contain metal hydroxide and inositol pentaphosphate-metal complex.

Table 13.--Percentages of metal and phosphorus precipitated from Inositol hexaphosphate solution at pH's 4, 5 and 6.

Metal	pH	% metal	% phosphorus	P/metal in precipitate
Ca (as $CaCl_2$)	4	28.10	25.77	1.18
	5	31.46	29.16	1.19
	6	39.44	36.82	1.19
Cu (as $CuSO_4$)	4	78.94	38.51	1.00
	5	92.64	45.18	1.00
	6	99.90	21.22	@
Fe (as $FeSO_4$)	4	75.16	41.72	1.00
	5	75.44	41.87	1.00
	6	82.10	33.39	@
Zn (as $ZnSO_4$)	4	43.08	20.43	1.00
	5	71.96	17.06	0.50
	6	77.96	31.96	@

@ These precipitates contain metal hydroxide and inositol hexaphosphate-metal complex.

study Hoff-Jorgensen (1944) reported only the penta-calcium phytate. The IP_6-Cu and IP_6-Fe precipitate observed a phosphorus to metal ratio of 1 : 1 at pH's 4 and 5. The IP_6-Zn complex showed a P : Zn atom ratio of 1 : 1 at pH 4 and 1 : 2 at pH 5.

As the pH increased there was an increase in the percent of metal precipitated. The influence was more pronounced in copper and zinc. The calcium did not show any change in the atom ratio of P : Ca with an increase in pH, and was 6 : 5. Similar atom ratios were observed by Evans and Pierce (1981) over a pH range of 5 to 6. Increasing the pH from 4 to 5 did not change the atom ratio of P : Cu and P : Fe, which stayed at 1 : 1. Evans and Pierce (1982) showed phosphorus to copper atom ratio of 6 : 5 at pH 6, but copper starts forming hydroxides at pH 6 (Britton, 1925), thus the differences in the atom ratio may be due to hydroxide formation. The IP_6-Zn precipitate observed an decrease in the atom ratio of P : Zn from 1 : 1 to 1 : 2 with a pH increase from 4 to 5.

Phosphoric acid-metal complexes: The phosphoric acid-metal complexes were observed at all pH's studied except at pH 4 with zinc (Table 14). The H_3PO_4-Ca precipitate showed a phosphorus to calcium atom ratio of 1 : 1 at pH's 4, 5 and 6. The H_3PO_4-Cu complex analysis showed a phosphorus to copper atom ratio of 3 : 1 at pH 4 and 1 : 1 at pH 5.

Table 14.--Percentages of metal and phosphorus precipitated from phosphoric acid solution at pH's 4, 5 and 6.

Metal	pH	% metal	% phosphorus	P/metal in precipitate
Ca (as $CaCl_2$)	4	29.12	22.57	1.00
	5	32.96	25.54	1.00
	6	34.91	27.05	1.00
Cu (as $CuSO_4$)	4	9.04	13.23	2.98
	5	59.02	28.78	1.00
	6	99.71	6.78	@
Fe (as $FeSO_4$)	4	47.12	19.80	0.75
	5	72.66	20.16	0.50
	6	79.98	10.01	@
Zn (as $ZnSO_4$)	4	—	—	—
	5	8.38	1.98	0.50
	6	43.38	11.08	@

@ These precipitates contain metal hydroxide and phosphoric acid-metal complex.

— No precipitate formed.

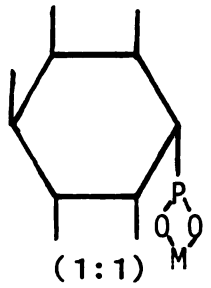
The H_3PO_4-Fe precipitate observed a phosphorus to iron atom ratio of 3 : 4 at pH 4 and 1 : 2 at pH 5. The H_3PO_4-Zn complex isolated at pH 5 showed a atom ratio of 1 : 2.

As the pH increased there was an increase in the percent of metal precipitated. The influence of pH was larger in copper and iron precipitation than in calcium and zinc precipitation. Increasing the pH from 4 to 6 did not change the atom ratio of the calcium precipitate, which stayed at 1 : 1. The H_3PO_4-Cu precipitate showed a decrease in the atom ratio of phosphorus to copper from 3 : 1 to 1 : 1 with an increase in pH from 4 to 5. The H_3PO_4-Fe precipitate also showed a decrease in the atom ratio of P : Fe, which changed from 1 : 1.32 to 1 : 2 with pH increase from 4 to 5.

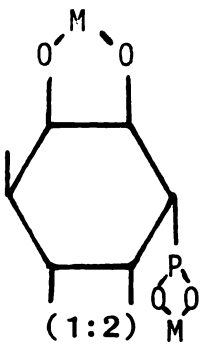
From the inositol phosphate-metal complex ratios, the possible structures that could be written are given in pages 80 to 83.

POSSIBLE STRUCTURES OF INOSITOL PHOSPHATE-METAL COMPLEXES

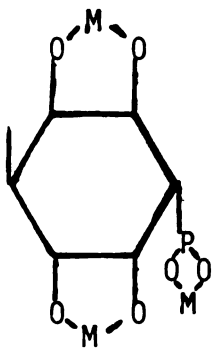
INOSITOL MONOPHOSPHATE-METAL COMPLEX



(1:1)

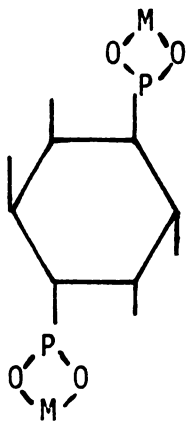


(1:2)

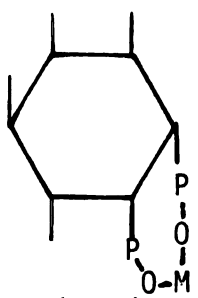


(1:3)

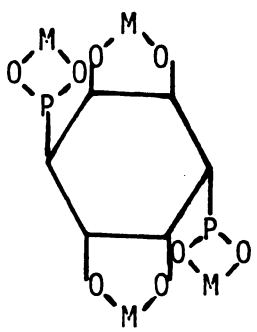
INOSITOL DIPHOSPHATE-METAL COMPLEX



(1:1)

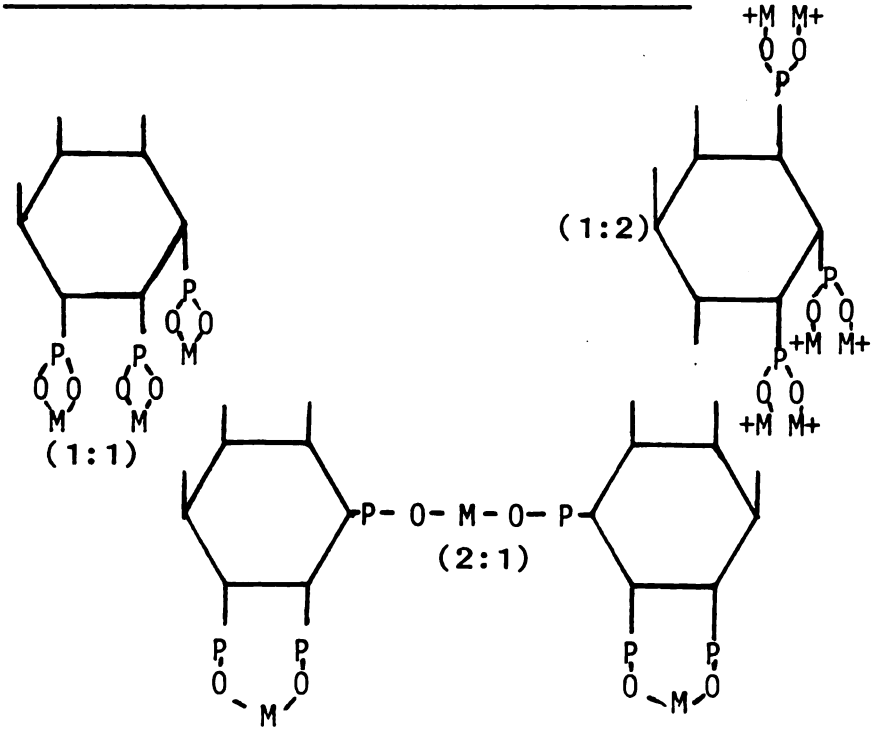


(2:1)

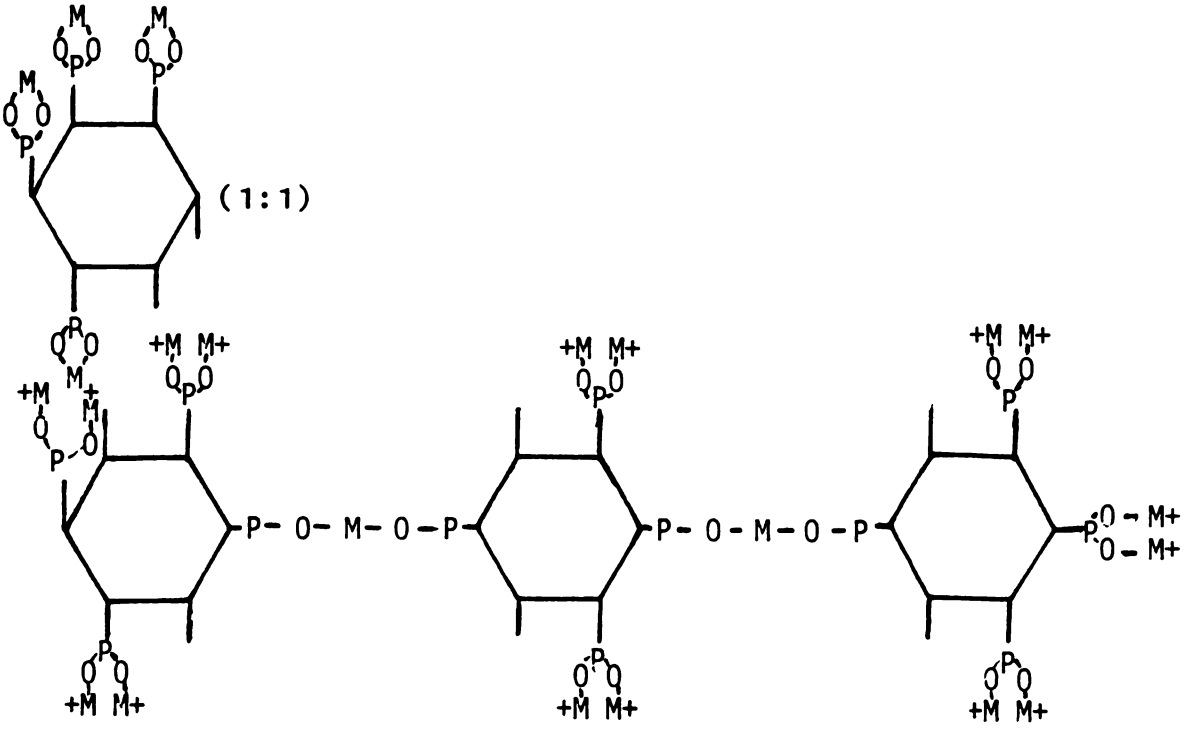


(1:2)

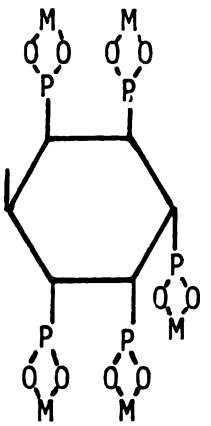
INOSITOL TRIPHOSPHATE-METAL COMPLEX



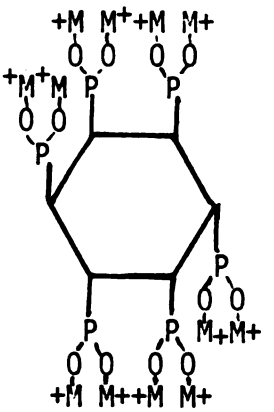
INOSITOL TETRAPHOSPHATE-METAL COMPLEX



INOSITOL PENTAPHOSPHATE-METAL COMPLEX

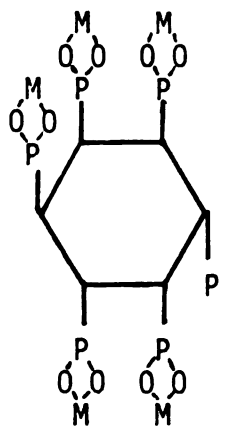


(1:1)

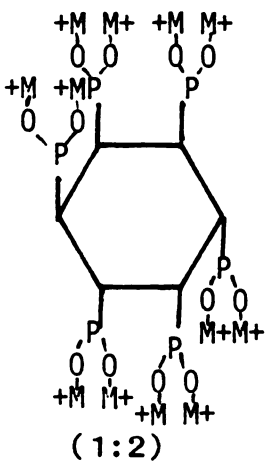


(1:2)

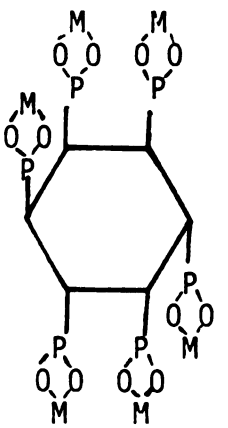
INOSITOL HEXAPHOSPHATE-METAL COMPLEX



(6:5)

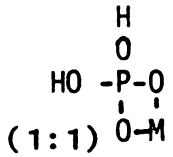


(1:2)

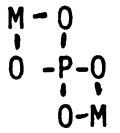


(1:1)

PHOSPHORIC ACID-METAL COMPLEX

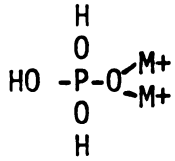


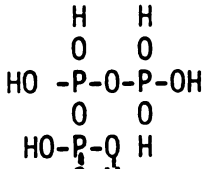
(1:1)



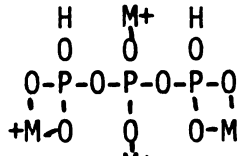
OR

(1:2)





(3:1)



(3:4)

CONCLUSIONS

The yeast phytase had an optimum pH of 4.6 and optimum temperature of 45°C with acetate buffer and phytic acid as substrate. The Michaelis constant with phytate as substrate was 0.21 mM. The phytase could be separated from an associated phosphatase by DEAE-cellulose chromatography. The purified phytase shows a broad specificity being able to hydrolyze a number of phosphomonoesters besides phytic acid and other inositol phosphates and can be characterized as a nonspecific phosphomonoesterase with phytase and potent pyrophosphatase activity. This enzyme is inhibited by high concentrations of phytic acid. The enzyme activity is increased by 1 mM of Fe^{2+} and decreased by chelating agents.

Inositol hexa-, penta-, tetra-, tri-, di-, and monophosphates along with inorganic phosphate were present in both whole wheat flour (WWF) and wheat flour (WF). IP_6 was the dominant inositol phosphate in unfermented doughs prepared with either WWF of WF, although the WWF dough contained more than twice the amount of IP_6 present in WF dough. The remaining inositol phosphates collectively contained almost as much P as IP_6 in WWF dough, and 1.7 times as much as in WF doughs. Fermentation reduced the phytate content with the fastest decrease occurring during

the first 30 min. The rate of phytate degradation was greater in WWF dough than in WF dough. The content of intermediate inositol phosphates fluctuated with fermentation time and it was only after considering their phosphorus content that an overall phosphorus balance could be achieved.

The 70%, 80%, 90% and 100%-extraction flours obtained from soft red winter wheat flours contained all of the six inositol phosphates, plus inorganic phosphate. The amounts of P_i, IP_6 and total phosphorus increased with increasing extraction rate. The percent of IP_6 phosphorus was 27%, 33%, 38% and 52% of all phosphates in the 70%-extraction, 80%-extraction, 90%-extraction, and 100%-extraction wheat flours, respectively. Dough fermentation for 2 hours reduced the phytate contents by about 70% in both 70%-extraction bread (EB70) and 80%-extraction breads (EB80), 67% in 90%-extraction bread (EB90) but only 58% in 100%-extraction bread (EB100). As the phytate content decreased there was a concurrent increase in orthophosphate. After 2 hours of fermentation, the phytic acid contents in bread was already reduced to 7.9% to, 8.6% , 12.2% and 21.7% of total phosphate in EB70, EB80, EB90 and EB100 respectively. A phosphate balance could be achieved only after taking intermediate inositol phosphates into account.

Two commercial breads were also analysed for the entire spectrum of phosphates. The white bread contained

more P_i, IP_6, and total phosphorus than expected, assuming a 70-75%-extraction. The excessive amount of P_i and total phosphorus were due to added phosphorus. The high amounts of phytic acid may be due to the inhibition of phytase by the added calcium.

All six wheat inositol phosphates and phosphoric acid showed the ability to precipitate the minerals Ca, Cu, Fe and Zn at pH levels 4, 5 and 6, except IP_1 and phosphoric acid, which did not precipitate zinc at pH 4. Calcium formed a simple atom ratio of 1 : 1 with all the inositol phosphates with following exceptions: with IP_1 at pH 5, IP_5 at pH's 5 and 6, and IP_6 at pH's 4, 5, and 6. In these the ratio was 6 : 5. Copper showed a decrease in the atom ratio of P : Cu with an increase in pH from 4 to 5: IP_1, 3 : 1 to 1 : 1; IP_2 and IP_3, 2 : 1 to 1 : 1; IP_4, 1.5 : 1 to 1 : 1; phosphoric acid, 3 : 1 to 1 : 1. Both IP_5 and IP_6 did not show any changes in the atom ratio of 1 : 1, with an increase in pH. Thus copper formed a simple atom ratio of 1 : 1 at pH 5 with all inositol phosphates and phosphoric acid. Iron displayed a decrease in the atom ratio of P : Fe with an increase in pH from 4 to 5: IP_1, 1 : 2 to 1 : 3; IP_2 and IP_5, 1 : 1 to 1 : 2; IP_3 and IP_4, 1 : 1 to 1 : 1.5; phosphoric acid, 1 : 1.33 to 1 : 2. No change in atom ratio of 1 : 1 was observed in IP_6-Fe complex for a similar rise in pH. Zinc showed an increase in molar ratio of 1 : 2 to 1 : 1 with an increase in pH

from 4 to 5 in both IP_2 and IP_3. For a similar change in pH there was a decrease in atom ratios: IP_4, 1 : 1 to 1 : 1.19 and IP_6, 1 : 1 to 1 : 2; while IP_5 did not show any change in atom ratio of 1 : 1.

LITERATURE CITED

BIBLIOGRAPHY

AACC. 1962. "Approved Methods" 7th ed. American Association of Cereal Chemists, St. Paul, Minn.

Agranoff, B. W., Bradley, and R. O. Brady. 1958. The enzymatic synthesis of inositol phosphatide. J. Biol. Chem. 233:1077.

Allen, R. J. L. 1940. The estimation of phosphorus. Biochem J. 34:858.

Anderson, R. J. 1914. Concerning the organic phosphoric acid of cottonseed meal. J. Biol. Chem. 17:141.

Anderson, R. J. 1914c. Concerning phytin in corn. J. Biol. Chem. 17:165.

Asada, K. and Z. Kasai. 1959. Formation of phytin and its role in the ripening process of rice plant. Mem. Res. Inst. Food Sci., Kyoto Univ. 18:32.

Asada, K. and Z. Kasai. 1962. Formation of myo-inositol and phytin in ripening rice grains. Plant Cell Physiol. 3:397.

Asada, K., K. Tanaka, and Z, Kasai. 1968. Phosphorylation of myo-inositol in riepning grains of rice and wheat. Incorporation of phosphate-32P and myo-inositol-3H into myo-inositol phosphates. Plant Cell Physiol. 9:185.

Ashton, W. M. and P. C. Williams. 1958. The phosphorus compounds oats. I. The content of phytate phosphorus. J. Sci. Food Agric. 9:505.

Association of Official Analytical Chemists. 1975. Official methods of analysis. Washington, D. C.

Averill, H. P. and C. G. King. 1926. The phytin content of foodstuffs. J. Am. Chem. Soc. 48:724.

Barre, R., Courtois, J. E and Wormser, G. 1956. Action de quelques cations divalents sur diverses preparations glycerophosphatasiques et phytasiques du son de ble. Bull. Soc. Chim. Biol. 38:387

Barre, R. 1956. Influence de l'acide phytique sur 'la digestion pepsique de differentes protein. Ann. Pharm. Fr. 14:182.

Belavady, B. and Banerjee, S. 1953. Studies on the effect of germination on the phosphorus values of some common Indian pulses. Food Res. 18:223.

Britton, H. T. S. 1925. Electrometirc studies of the precipitation of hydroxides. J. Chem. Soc. 127:2110.

Biswas, S. and B. B. Biswas. 1965. Enzymatic synthesis of guanosine diphosphate. Biochim. Biophys. Acta. 108:710.

Bitar, K. and J. G. Reinhold. 1972. Phytase and alkaline phosphatase activities in intestinal mucosa of rat, chicken, calf and man. Biochim. Biophys. Acta. 268:442.

Chang, C. W. 1967. Study of phytase and flouride effects in germinating corn seeds. Cereal Chem. 44:129.

Common, R. H. 1939. Phytic acid and mineral metabolism in poultry. Nature, 143:379.

Cosgrove, D. J. 1966. The chemistry and biochemistry of inositol polyphosphates. Rev. Pure Appl. Chem. 16:209

Courtois, J. 1947a. Recherches sur la phytase. III.-Essais de separation de l'activite glycerophosphatasique et l'activie phytasique du son de ble. Biochim. Biophys. Acta. 1:270.

Courtois, J. 1947b. Recherches sur la phytase. V.-Etude preliminaire de l'hydrolyse des inositohexaphosphates et glycerophosphates par les graines de Moutarde blanche. Bull. Soc. Chim. biol., Paris, 29:944.

Courtois, J. and Joseph, G. 1947. Recherches sur la phytase. VI-Action de diverses preparations phosphatasiques sur quelques esters phosphoriques de l'inositol. Bull. Soc. Chim. biol., Paris, 29:951.

Courtois, J. 1948. Recherches sur la phytase. VII.-Action de preparations phosphatasiques purifiees de moutarde-blanche sur divers esters phosphoriques. Bull. Soc. Chim. biol., Paris, 30:37.

Courtois, J. and C. Perez. 1948a. Recherches sur la phytase. VIII. Teneur de inositophosphates et activite phytasique de diverses graines. Bull. Soc. Chim. Biol. 30:195.

Courtois, J. and C, Perez. 1948b. Recherches sur la phytase. XI.-Exxais en vue d obtenir des matieres premieres dont l activite phytasique initiate a ete articiellment accrue. Bull. Soc. Chim. Biol. 30:631

Courtois, J. and C. Perez. 1949. Presence dune phytase acide dans les feces humaines. Bull. Soc. Chim. Biol. 31:1373.

Courtois, J. 1951. Les esters phosphorique l'inositol. Bull. Soc. Chim. biol., Paris, 33:1075.

Davis, N. T. and R. Nightingale. 1975. The effect of phytate on intestinal absorption and secretion of zinc and whole body retention of zinc, copper, iron and manganese in rats. Br. J. Nutr. 34:243.

Davis, P. N., L. C. Norris, and F. H. Kratzer. 1962. Interference of soybean proteins with the utilization of trace minerals. J. Nutr. 77:217.

de Boland, A. R., G. B. Garner, and B. L. O Dell. 1975. Identification and properties of "phytate" in cereal grains and oilseed products. J. Agric. Food Chem. 23:1186.

de Lange, D. J., C. P. Joubert, and S. F. M. du Preez. 1961. The determination of phytic acid and factors which influence its hydrolysis in bread. Proc. Nutr. Soc. South Afric. 2:69.

DeTurk, E. E., J. R. Holbert, and B. W. Hawk. 1933. Chemical transformations of phosphorus in the growing corn plant, with results on two first-generation crosses. J. Agric. Res. (Washington, D.C) 46:121.

Dixon, M. 1953. A nomogram for ammonium sulfate solutions. Biochem. J. 54:457.

Earle, F. R. and R. T. Milner. 1938. The occurrence of phosphorus in soybeans. Oil Soap. 15:41.

Ellis, L. C. and A. D. Tillman. 1961. Utilization of phytin phosphorus in wheat bran by sheep. J. Animal Sci. 20:606.

Erdman, J. W. 1979. Oilseed phytates: nutritional implication. J. Am. Oil Chemists Soc. 56:736.

Ergle, D. R. and G. Guinn. 1959. Phosphorus compounds of the cotton embryo's and their changes during germination. Plant Physiol. 34:476.

Evans, W. V. and A. G. Pierce. 1981. Calcium-phytate complex formation studies. J. Am. Oil Chem. 58:850.

Fleury, P. and J. Courtois. 1947. Recherches sur la phytase. II.-Cinetiques comparees de l'hydrolyse du glycerophosphate et de l'inositohexaphosphate par le son de ble. Biochim. Biophys. Acta. 1:256.

Forbes, R. M. and H. M. Parker. 1977. Biological availability of zinc in and as influenced by whole fat soy flour in rat diets. Nutr. Repts. Intern. 15:681.

Glass, R. L. and W. F. Geddes. 1959. Grain storage studies. XXVII.-The inorganic phosphorus content of deteriorating wheat. Cereal Chem. 36:186

Gibbins, L. N. and F. W. Norris. 1963. Phytase and acid phosphatase in the Dwarf bean, Phaseolus vulgaris. Biochem. J. 86:67.

Hall, J. R. and T. K. Hodges. 1966. Phosphorus metabolism of germinating oat seeds. Plant Physiol. 41:1459.

Harland, B. F. and J. Harland. 1980. Fermentation reduction of phytate in rye, white and whole wheat breads. Cereal Chem. 57:226.

Hay, J. G. 1942. The distribution of phytic acid in wheat and a preliminary study of some of the calcium salts of this acid. Cereal Chem. 19:326.

Hoff-Jorgensen, E. K. 1944. Investigations on the solubility of calcium phytate. K. Dan. Vidensk. Selsk. Math. Fysisk. Medd. 21:NR 7.

Jennings, A. C. and K. K. Morton. 1963b. Changes in nucleic acids and other phosphorus containing compounds in developing wheat grains. Aug. J. Biol. Sci. 16:332.

Johnson, L. F. and M. E. Tate. 1969. Structure of phytic acid. Can. J. Chem. 47:63.

Kalckar, H. M. 1947. Differential spectrophotometry of purine compunds by means of specific enzymes. II.-Studies of the enzymes of purine metabolism. J. Biol. Chem. 167:461.

Kennedy, B. M. and M. Schelstraete. 1975. Chemical, physical and nutritional properties of high protein flours and residual kernal from the over milling of uncoated milled rice. III. Iron, calcium, Magnesium, Phosphorus Sodium, Potassium, and Phytic acid. Cereal Chem. 52:173.

Knorr, D., T. Watkins, and B. Carlson. 1981. Enzymatic reduction of phytate in whole wheat breads. J. Food. Sci. 46:1866.

Likiski, H. J. A. and F. M. Forbes. 1965. IV. Effects of calcium and phytic acid on the utilization of dietary zinc. J. Nutr. 85:230.

Lineweaver, H. and D. Burk. 1934. The determination of enzyme dissociation constants. J. Am. Chem. Soc. 56:658.

Lolas, G. M. and P. Markakis. 1975. Phytic acid and other phosphorus compounds of beans. J. Agric. Food Chem. 23:13.

Lolas, G. M., N. Palamidis. and P. Markakis. 1976. The phytic acidtotal phosphorus relationship in barley, oats, soybeans, and wheat. Cereal Chem. 53:867.

Makower, R. U. 1969. Changes in phytic acid and acid-soluble phosphorus in maturing pinto beans. J. Sci. Fd. Agric. 20:82.

Mandal, N. C. and B. B. Biswas. 1970. Metabolism of inositol phosphates. I-Phytase synthesis during germination in cotyledons of mung beans (Phaseolus aureus). Plant Physiol. 45:4.

Mandal, N. C. Burman, S. and B. B. Biswas. 1972. Isolation, purification and characterization of phytase from germinating mung beans. Phytochemistry. 11:495.

Mayer, A. M. 1958. The breakdown of phytin and phytase activity in germinating lettuce seeds. Enzymologia. 19:1.

McCance, R. A. and E. M. Widdowson. 1944. Activity of the phytase in different cereals and its resistance to dry heat. Nature (London). 153:650.

Mellanby, E. 1944. Phytic acid and phytase in cereals. Nature (London). 154:394.

Michel-Durand, E. 1939. Le phosphore des vegetaux (Presses Univ-de France, Paris).

Mihailovic, M. L., M. Antic, and D. Hadzijev. 1965. Chemical investigation of wheat. 8 — Dynamics of various forms of phosphorus in wheat during its ontogenesis. The extent and mechanism of phytic acid decomposition in germinating wheat grain. Plant and Soil. 23:117.

Momcilovic, B. and B. G. Shah. 1976b. Bioavailability of zinc in infant foods. Nutr. Rep. Int. 14:717.

Morris, E. R. and R. Ellis. 1976. Isolation of monoferric phytate from wheat bran and its biological value as an iron source to the rat. J. Nutr. 106:753.

Morton, R. K. and J. K. Raison. 1963. A complex ontracellular unit for incorporation of amino acids into storage protein utilizing adenosine triphosphate generated from phytate from phytate. Nature (London). 200:429.

Myers, D. V. and G. A. Iacobucci. 1974. Binding of phytic acid to glycinin. Cereal Science Today. 19:401.

Nagai, Y. and S. Funahashi. 1962. Phytase (myoinositol hexaphosphate phosphohydrolase) from wheat bran. Part I.-Purification and substrate specificity. Agr. Biol. Chem. 26:794.

Oberleas, D., M. E. Muhrer. and B. L. O Dell. 1962. Effects of phytic acid on zinc availability and parakeratosis in swine. J. Anim. Sci. 21:57.

Oberleas, D., M. E. Muhrer. and B. L. O Dell. 1966. Dietary metal-complexing agents and zinc availability in the rat. J. Nutr. 90:56.

O'Dell, B. L., A. R. de Boland, S. R. Koirtyohann. 1972. Distribution of phytate and nutritionally important elements among the morphological components of cereal grains. J. Agr. Food Chem. 20:718.

Okubo, K., G. A. Iacobucci, and D. V. Myers. 1974. Effects of Ca^{+2} on phytate binding to glycinin. Cereal Science Today. 19:401.

Okubo. K., D. V. Myers, and G. A. Iacobucci. 1976. Binding of phytic acid to glycinin. Cereal Chem. 53:513.

Peers, F. G. 1953. The phytase of wheat. Biochem. J. 53:102.

Pfeffer, E. 1872. Pringsheim jahrbucker fur wiss. Bot. 8:429, 475, cited by Courtois, J. 1951. Les esters phosphoriques de linositol. Bull. Soc. Chim. Biol., Paris. 33:1075.

Pileggi, V. J. 1959. Distribution of phytase in the rat. Arch. Biochem. Biophys. 80:1

Pomaranz, Y. 1973. Structure and mineral composition of cereal aleurone cells as shown by scanning electron microscopy. Cereal Chem. 50:353.

Prasad, A. S., A. Miale., Z. Farid., H. H. Sanstead., A. R. Schubert, and W. J. Darby. 1963. Biochemical Studies on Dwarfism, Hypogonadism and Anemia. Arch. Internal. Med. 111:407.

Preece, I. A. and H. J. Grav. 1962. Studies on phytin. II.-Preliminary study of some barley phosphotases. J. Inst. Brew. 68:66.

Preece, I. A., H. J. Grav, and A. T. Wadham. 1960. Studies on phytin. I.-The inositol phosphatases. J. Inst. Brew. 66:487.

Ranhotra, G. S. 1972. Hydrolysis during breadmaking of phytic acid in wheat protein concentrate. J. Food Sci. 37:12.

Ranhotra, G. S. 1973. Factors affecting hydrolysis during breadmaking of phytic acid in wheat protein concentrate. Cereal Chem. 50:353.

Ranhotra, G. S., C. Lee, and J. A. Gelroth. 1978. Bioavailability zinc in soy-fortified wheat bread. Nutr. Rep. Int. 18:487.

Ranhotra, G. S., R. J. Loewe, and L. V. Puyat. 1974. Phytic acid in soy and its hydrolysis during breadmaking. J. Food Sci. 39:1023.

Raun, A., E. Chang, and W. Burroughs. 1956. Phytate phosphorus hydrolysis and availability to rumen microorganisims. J. Agric. Food Chem. 4:869.

Reinhold, J. G. 1971. High phytate content of rural iranian bread: a possible cause of human zinc deficiency. Am. J. Clin. Nutr. 24:1204.

Reinhold, J. G. 1972. Phytate concentrations of leavened and unleavened iranian breads. J. Food Nutr. 1:187.

Reinhold, J. G., F. Ismail-Beigi, and B. Faradji. 1975. Fibre vs phytate as determinant of the availability of calcium, zinc and iron of breadstuffs. Nutr. Rept. Int. 12:75.

Reinhold, J. G., H. Hedayati., A. Lahimgarzadeh, and K. Nasr. 1973. Zinc, calcium, phosphorus, and nitrogen balances of iranian villagers following a change from phytate-rich to phytate poor diets. Ecol. Food Nutr. 2:157.

Reinhold, J. G., A. Lahimgarzadeh., N. Khosrow, and H. Hedayati. 1973. Effects of purified phytate and phytate-rich bread upon metabolism of zinc, calcium, phosphorus, and nitrogen in man. Lancet. 1:283.

Rush, R. M. and J. H. Yoe. 1954. Colorimetric determination of zinc and copper with 2-carboxy-2-hydroxy-5-sulfoformazyl benzine. Anal Chem. 26:1345.

Saio, K. 1964. The change in inositol phosphates during the ripening of rice grains. Plant and Cell physiol. 5:393.

Saio, K., E. Koyama, and T. Watanabe. 1967. Protein-calcium-phytic acid relationships in soybeans. Part I.-Effects of calcium and phosphorus on solubility characteristics of soybean meal protein. Agric. Biol. Chem. 31:1195.

Saio, K., E. Koyama, and T. Watanabe. 1968. Protein-calcium-phytic acid relationships in soybean. Part II.-Effects of phytic acid on combination of calcium with soybean meal protein. Agric. Biol. Chem. 32:448.

Samotus, B. 1965. Role of phytic acid in tuber. Nature (London) 206:1372.

Sloane-Stanley, G. H. 1961. Phytase, in Biochemists Handbook. Ed. C. Long. (E. and F. N. Spon, London).

Smith, A. K, and J. J. Rackis. 1957. Phytin elimination in soybean protein isolation. J. Amer. Chem. Soc. 79:633.

Sobolev, A. M, and M. A. Rodionova. 1966. Phytin synthesis by aleurone grains in ripening sunflower seeds. Sov. Plant Physiol. 13:958.

Suzuki, U., K. Yoshimura, and M. Takaishi. 1907. Ueber ein Enzym "Phytase" das "Anhydro-oxy-methylen diphosphorsaure" spaltet. Tokyo Imperial Univ Coll. Agric. Bull. 7:503.

Tangkongchitr, U., P. A. Sieb, and R. C. Hosney. 1981. Phytic acid. II. Its fate during breadmaking. Cereal Chem. 58:229.

Tombs, M. P. 1967. Protein bodies of the soybean. Plant Physiol. 42:797.

Turnbull, A., F. Cleton., C. A. Finch., L. Thompson, and J. Martin. 1962. Iron absorption. IV. The absorption of hemoglobin iron. J. Clin. Invest. 41:1897.

Walker, K. A. 1974. Changes in phytic acid and phytase during early development of Phaseolus vulgaris. Planta. 116:91.

Wang, L. C. 1971. Effect of phytate in isoelectric focusing of soybean whey proteins. Cereal Chem. 48:229.

Warburg, O. and W. Christian. 1942. Isolierung und Kristakisation des Garungsferments Enolase. Biochem. Z. 310:384.

Watanabe, F. S. and S. R. Olsen. 1965. Test of an ascorbic acid method for determining phosphorus in water and $NaHCO_3$ extracts from soil. Soil Sci. Soc. Am. Proc. 29:677.

Whitaker, J. R. 1972. Principles of enzymology for the Food Sciences. Marcel Dekker, Inc., N. Y.

Williams, S. G. 1970. The role of phytic acid in the wheat grain. Plant Physiol. 45:376.

Winterstein, E. 1897. Ueber einen phosphorhaltigen pflanzenbestandtheil welcher bei der spaltung liefert. Ber Dtsch. Chem. Ges. 30:2299.

Wozenski, J, and M. Woodburn. 1975. Phytic acid (Myoinositol hexa phosphate) and phytase activity in flour cotton seed protein products. Cereal Chem. 52:665.

APPENDIX 1

Table.--Recovery of phytic acid added to the control sample [a]

Sample	PA in control sample (mg)	Added PA[b] (mg)	Total PA (mg)	PA found (mg)	% Recovery
Control	12.05	–	–	–	–
Control + 10 mg NaPhy	12.05	5.24	17.57	16.57	95.84
Control + 20 mg NaPhy	12.05	10.47	22.52	21.37	94.89

a Average of three determinations.

b PA = phytic acid + 52.37% of standard NaPhy.