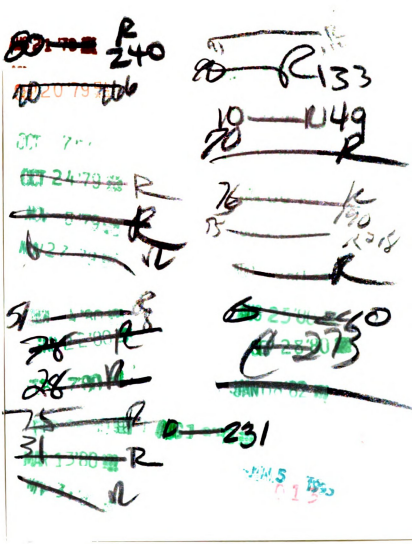

ABSTRACT

THE PHYTATE AND PHYTASE OF DRY BEANS
(PHASEOLUS VULGARIS)

By

George Menelaus Lolas

Beans, as many other seeds, are rich sources of phosphorus. A great amount of this phosphorus is bound in the form of phytic acid and considered to be unavailable by the animal organism. In addition, phytic acid influences the availability of several mineral elements by its chelating properties.

The objective of this study was to provide data on the phytic acid content of many varieties of beans and seek a possible relationship between the contents of beans in phytic acid and that in the other phosphorus compounds. Furthermore, the enzyme phytase from the Sanilac Navy bean variety was extracted and studied. Its effect on phytic acid during germination was also investigated.

The beans of this study contained on a dry weight basis 0.54 - 1.58% phytic acid, 0.259 - 0.556% total phosphorus, 0.021 - 0.044% inorganic phosphorus and 0.050 - 0.135% other than phytic acid organic phosphorus. The phytic acid phosphorus represented 53.6 - 81.6% of the total phosphorus with an average value of 69.3%. A high correlation coefficient of 0.9847 between total phosphorus and phytic acid provides the possibility of estimating phytic acid by determining

total phosphorus with great savings in time and effort. The observation was made that phytic acid is in a water-soluble form.

The phytase enzyme was extracted by 2% $CaCl_2$ (an extractant that eliminated the problem of contamination with the substrate phytic acid) and purified by ammonium sulfate fractionation and DEAE-cellulose chromatography. The enzyme showed an optimum pH of 5.3 with acetate buffer and phytic acid as substrate and an optimum temperature of 50°C. The Michaelis constant with phytate as substrate was 0.018 mM. The activation energy of hydrolysis of phytic acid was 11500 cal/mole and the inactivation energy of enzyme 55800 cal/mole. The enzyme shows a broad specificity being able to hydrolyze a number of phosphomonoesters besides phytic acid. It is a nonspecific phosphomonoesterase characterized by a potent pyrophosphatase activity. The enzyme increases in activity in the presence of 1 mM Co^{++}.

Germination of the bean seed is accompanied by a 7-fold increase in phytase activity with a parallel increase in the amount of orthophosphate and decrease in the amount of phytic acid.

THE PHYTATE AND PHYTASE OF DRY BEANS

(*PHASEOLUS VULGARIS*)

By

George Menelaus Lolas

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DEDICATION

To My Family and

Bosom Friend

Vasilis Kaloudis

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INTRODUCTION

A great number of studies are being conducted today in an effort to establish the physiological role of phytic acid and its enzyme phytase in plants and animals. Phytic acid is the main source of phosphorus in many seeds, and does not seem to be utilized by non-ruminant animals resulting in phosphorus deficiencies when the phosphorus of diet is primarily in the form of phytates. Furthermore, high-phytate intake interferes with the absorption of several mineral elements, such as Ca, Fe and Zn.

Hunger and malnutrition problems in the world could be lessened by more extensive use of food legumes. As the world population increases so increases the demand for protein. Plant proteins, however, are frequently associated with metabolic inhibitors and phytic acid is one of them. Solubility of the proteins is often required to obtain desired functional properties, as soluble ingredients are always easier to formulate into foods. Phytic acid, being associated with almost all plant proteins, affects their solubility and other characteristics. Phytic acid, because of its strong chelating properties, renders the biological value of plant proteins lower, than otherwise would be, by sequestering some trace minerals.

Phosphorus is an expensive nutrient and should not be wasted in the form of phytic acid. The only way to make the phytate phosphorus available is, probably, through the action of phytase contained

in foods and feedstuffs and possibly in intestine. A study of the enzyme and its characteristics are useful in contributing to the understanding of its function in plant growth and animal nutrition.

This research had a dual objective: (a) to study quantitatively the phytic acid of dry beans and its relationship to other constituents of beans, and (b) to study the enzyme(s) that hydrolyze phytic acid with a view of utilizing this enzymatic activity for reducing the phytic acid content of beans.

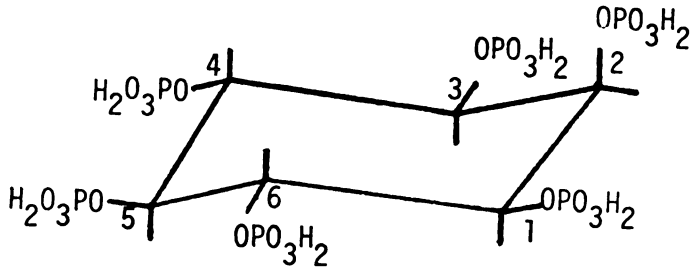
LITERATURE REVIEW

Chemistry of phytic acid

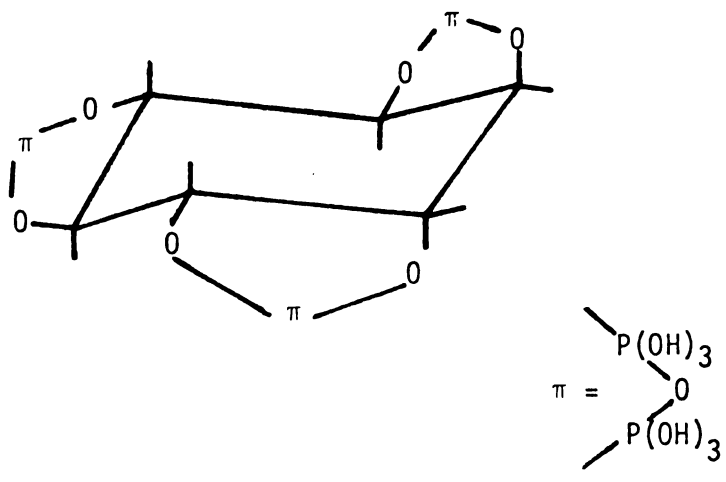
Pfeffer (1872) was the first who showed the presence of a Ca-Mg salt of an organic phosphate in the aleurone grains of wheat endosperm. Winterstein (1897) later showed that a similar substance extracted from the seeds of Indian mustard (Sinapsis nigra) gave myoinositol and orthophosphoric acid after hydrolysis with hydrochloric acid. It has been stated that phytic acid is as ubiquitous in the plant kingdom as starch (Michel-Durand, 1939).

The chemical designation of phytic acid is myo-inositol 1,2,3, 4,5,6-hexakis (dihydrogen phosphate) (IUPAC-IUB, 1968). The name "phytic acid" has been used interchangeably in the literature 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 sometime. The more recent NMR work of Johnson and Tate (1969) indicates that cereal grain phytic acid has the myoinositol hexaorthophosphate structure suggested by Anderson (1914) and not the myoinositol tripyrophosphate structure proposed by Neuberg (1908).



Anderson's structure



Neuberg's structure

Accurate methods for the estimation of phytic acid are desirable since many foods and animal feedstuffs contain phytic acid as an important source of phosphorus. Heubner and Stadler (1914) were the first to describe a method for the determination of phytic acid. The method is based on the titration of phytic acid with standard acidified ferric chloride solution with the formation of insoluble ferric phytate, in the presence of ammonium thiocyanate indicator. The

titration is carried out in 0.5 N HCl extracts of plant material. The method is not reliable as the end point, shown by the reddish brown ferric thiocyanate, is indefinite and is taken arbitrarily as the point at which the color persists for five minutes.

Other methods have generally relied on the marked insolubility of the ferric phytate in acid media. Inorganic phosphate is not precipitated under these conditions and it is believed that organic phosphates, other than phytic acid, remain in solution. Precipitation methods are divided into direct methods, in which the ferric phytate is removed and determined as phosphorus (Pons et al., 1953) or inositol (Cosgrove, 1966), and indirect methods in which an excess of ferric chloride is added to precipitate the phytate; the ferric iron left in solution is then determined by a standard colorimetric method, or the precipitate is purified and the bound iron is determined by a standard colorimetric method after decomposition of the ferric phytate salt (Makower, 1970; Wheeler and Ferrel, 1971). Different values for phytic acid have been obtained using different analytical methods (Marrese, 1961).

Determination of the iron depends upon a constant composition in the precipitate which has been reported to have an iron/phosphorus ratio of 4:6 (Earley, 1944; Wheeler and Ferrel, 1971). Anderson (1963) studied the effects of the ratio of iron to phosphorus and of acid concentration on the precipitation of phytic acid as ferric phytate. He concluded that the mole ratio of added Fe to total P is critical, soluble complexes can be formed in the presence of excess iron, precipitation is not quantitative at pH 1.0 in hydrochloric

acid, a commonly used extractant, and that precipitation is improved by heating.

Wheeler and Ferrel (1971) investigated the effectiveness of two extractants, trichloroacetic acid (TCA) and hydrochloric acid, in extracting phytic acid from wheat and wheat fractions. They found that TCA extracts contained more phytate phosphorus and were much cleaner than those obtained with HCl.

Biochemistry of phytic acid

Beans and other seeds are rich sources of phosphorus. According to Earle and Milner (1938), phosphorus compounds found in seeds may be classified into four groups: phytates, phosphatides, nucleic compounds, and inorganic phosphorus compounds. Phytic acid is the principal form of phosphorus in many seeds; 60-90% of all the phosphorus in these seeds is present as phytic acid (Barré, 1956).

Several physiological roles have been suggested for phytic acid in plants. Phytic acid has been generally regarded as the chief storage form of both phosphate and inositol in almost all seeds. Hall and Hodges (1966) attempted to obtain an over-all description of phosphorus metabolism associated with the germination of oats. Their results confirmed that phytic acid represents the primary storage form of phosphate in oat seeds (about 53% of the total phosphorus). That the phytic acid represents an energy store has been proposed by Biswas and Biswas (1965). Sobolev and Rodionova (1966) reported that phytic acid was synthesized by a mixture of aleurone grains and mitochondria isolated from ripening sunflower seeds when myoinositol

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. Recently, Williams (1970) presented evidence that phytic acid serves only as a source of phosphorus and cations for the germinating seed. He believes that synthesis of the strongly chelating phytic acid exerts an effect on the cellular metabolism by combining with multivalent cations which are known to play a significant part 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 can be considered a final product of phosphorus metabolism in the ripening process. Samotus (1965) claims that formation of phytic acid in seeds and tubers is a means of preventing the accumulation of excessively high levels of inorganic phosphate. He suggests 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) on enhanced accumulation of phytic acid, relative to other phosphorus compounds in rice grain, when increased applications of phosphorus fertilizer are made to the plant.

Insoluble complexes are formed between proteins and polyphosphates. When polyphosphates, such as phytic acid, are added to protein solutions at a pH below the isoelectric point, precipitation takes place, the extent of the reaction being controlled by the pH of the system. Phytic acid appears to form salt-like linkages with basic groups on the protein molecule such as those of arginine, lysine and histidine units (Cosgrove, 1966). Myers and Iacobucci (1974) believe 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 carboxylate groups of the protein and the phosphate groups of phytate. 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 protein, calcium and phytic acid are very labile in the alkaline range above pH 8.0, especially by heating. The same workers (1968) found, also, 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 proteins (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 (Barré, 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.

The formation of large molecular complexes between phytic acid and proteins had been observed very early, also, by Mattson (1946) during his studies on the cookability of yellow peas. He considered phytic acid to be powerfully attracted by the protein cations and link these together forming large molecular complexes which, having lost their charge, coagulate isoelectrically. To test his theory he dissolved a little legumin in 8% acetic acid and filtered to get a clear solution. A little sodium phytate was dissolved in another portion of the acetic acid solution. When the two solutions were mixed there was formed a copious, flocculent precipitate which was difficultly soluble in 1 N HCl, was readily dissolved with a slight excess of NaOH, but which was insoluble in concentrated acetic acid. He found the stability of the formed compound to be as great as that of ferric phytate, and concluded that other proteins must form protein-phytate complexes in acid media.

Phytic acid in nutrition

The animal nutritional importance of phytic acid lies in its ability to chelate several mineral elements, especially Ca, Mg, Fe, Zn, Mn, Cu, Mo, and Cr, and thereby reduce their availability in the intestinal tract (Oberleas, 1973; Rackis, 1974). Bruce and Callow (1934) found that phytic acid reduces the absorption of calcium and is responsible for the rachitogenic properties of certain cereals. Melanby (1949) induced rickets in puppies raised on a low calcium diet containing phytate, whereas controls raised on the same diet without added phytate did not become rachitic. He found that by increasing the phytate in the diet not only the phytate but also the Ca increases in the faeces. Its main anti-calcifying effect depends on the fact that it competes in the intestine with inorganic phosphate for Ca. In this way phytate limits the amount of Ca available for absorption under the influence of vitamin D. Taylor (1965) concludes that rickets occurring in animals fed on cereal diets low in Ca is due to a deficiency of Ca and with diets high in Ca to a deficiency of phosphorus.

Roberts and Yudkin (1960) caused Mg deficiency in albino rats fed purified diets containing sodium phytate in levels ranging from 1 to 10%. The rats showed signs ranging from diminished growth at the 1% level to severe illness and death at the 10% level. Addition of calcium or inositol did not improve the health of rats, hence eliminating calcium or inositol as possible reasons

for the illness. Addition of magnesium in the diet resulted in improving the appearance of the animals.

Sodium phytate, added in the diet of a group of adolescent boys, reduced the absorption of radioactive iron by 15 times indicating that added soluble phytates can interfere with iron absorption (Sharpe et al., 1950). Since the normal Indian diet, based on cereals and pulses, contains approximately 50% of the total phosphorus as phytin phosphorus, Apte and Venkatachalam (1962) assessed the safe level of iron intake to provide sufficient absorption in normal subjects consuming that typical diet.

O'Dell and Savage (1960) furnished evidence that phytate decreases zinc availability in chicks. They found that zinc in isolated soy protein is less available than that in casein and zinc in a casein-phytic acid complex, which contains an amount of phytic acid comparable to that found in isolated soy protein, is also less available than that in untreated casein. Maddaiah and co-workers (1964) found that the most stable complexes with phytic acid formed in the physiological pH ranges were those of zinc. This stability of the metal-phytic acid complexes studied was in the order $Zn^{2+} > Cu^{2+} > Co^{2+} > Mn^{2+} > Ca^{2+}$. By adding sodium phytate to a low-zinc chick diet, they depressed the growth rate and assumed that phytic acid is the culprit in rendering dietary zinc unavailable. Human zinc deficiency was studied by Reinhold and co-workers (1973). They found that consumption of phytate in quantities similar to those eaten regularly by adult villagers in Iran can result in disturbances of Zn, Ca, P and Fe metabolism.

In general, the zinc in animal products is more readily absorbed than that in plant products, particularly those that arise from plant seeds; and chelating agents, whether man-made or naturally occurring in foods, may compete with phytate to increase zinc availability (O'Dell, 1969). Oberleas and Prasad (1969), studying the effect of zinc on plant protein utilization, found that addition of zinc to plant-seed protein diets results in growth rates that are comparable to those achieved by the use of animal protein in similar amounts.

Extensive use, in recent years, of isolated soybean protein, which contains phytic acid, has been associated with a number of mineral deficiencies. Davis and co-workers (1962) studied the effect of isolated soybean protein on the availability of zinc, manganese, copper and iron with and without EDTA. They found that EDTA made the above metals more available and reduced the chick's requirement for these trace elements. EDTA, presumably, competed with phytic acid for these metals. Reid and co-workers (1956) reported significant growth increases in chicks and poults fed purified isolated soybean protein basal diets on supplementation with molybdenum. Finally, phytate significantly decreased chromium transport through the rat intestine (Nelson et al., 1973).

These results indicate that a compound such as phytate that complexes with such a broad spectrum of metals may produce a wide variety of deficiencies depending on which element first becomes limiting under specified dietary conditions. They further point out the fallacy of depending only on chemical concentrations to

specify levels of trace elements needed in a nutritionally adequate diet (Oberleas, 1973).

Phytic acid in food technology

Phytates might play some role in the cookability of leguminous seeds. In dried leguminous seeds, the deleterious effect of calcium and magnesium ions on the cooking quality is considered to be due to the cross linking of uronic acid groups of pectin. The result is a rigid structure between the middle lamellae and cell walls that makes thermal breakdown difficult and is believed to lead to a hard-cooking seed. Mattson (1946) showed that the cookability of peas was related to their phytic acid and calcium contents. He put forth the view that the pectin in the middle lamella layer formed insoluble calcium and magnesium pectates which were responsible for the poor cookability of some peas. He suggested that if sufficient phytic acid was present in the peas, it would form insoluble salts with calcium and magnesium ions and hence prevent these inorganic ions to crosslink the uronic acid groups of pectin. Crean and Haisman (1963) found that phytic acid in dried peas is wholly water soluble and assumed that it is present as an acid potassium salt. They concluded that the influence of phytate ions on texture is small and failed to support Mattson's (1946) hypothesis. The presence of free phytate ions cannot prevent the deterioration in texture caused by the calcium and magnesium ions, as the phytate is only one component in a complex equilibrium which must also include the pea proteins, the cell

wall uronic acids and the low-ester pectins in the middle lamellae. Rosenbaum and co-workers (1966) made a detailed study of the effects of the phytic acid contents of peas and calcium ions in the cooking water on the cookability of two different samples of peas with different cookability characteristics. Their experiments showed that there was a significant correlation between the cookability of individual peas (seed coats removed) and their phytic acid contents when the peas were cooked in distilled water. However, they did not find such a correlation between cookability and calcium content.

Biochemistry of phytase

Phytase (myoinositol hexaphosphate phosphohydrolase, E.C. 3.1.3.8) is the enzyme that catalyses the hydrolysis of phytic acid to inositol and free orthophosphate via inositol penta- to monophosphates as intermediary products.

The enzyme was named and described the first by Suzuki and co-workers (1907) who noticed that an impure preparation of phytase from rice and wheat bran had the ability to hydrolyze phytates to inorganic phosphate and inositol. Phytase has been one of the first enzymes known to liberate inorganic phosphate from organic phosphorus compounds. The enzyme is widely distributed 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 for 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 Pèrez, 1948a; Saio, 1964). During sprouting all the seeds possess phytase activity which increases with the progress of germination and is accompanied by an increase in the inorganic phosphate and decrease in the phytate content of seed (Courtois and Pèrez, 1948b; 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). Phytase present in the mature seeds appears to have little, if any, effect on the phytate in the dry or dormant seed. Glass and Geddes (1959) showed increased inorganic phosphorus and decreased phytate in wheat stored at increased moisture content and temperature. They attributed this to the phytase, which is activated under these conditions, and suggested that high levels of inorganic phosphorus can be used as an index of a sample stored under poor conditions.

Courtois and his collaborators have carried out extensive work on the occurrence, specificity and mechanism of phytase (Fleury and Courtois, 1937; Courtois, 1945; Fleury and Courtois,

1947; Courtois, 1947a; Courtois, 1947b; Courtois and Joseph, 1947; Courtois, 1948; Courtois and Pèrez, 1948a, 1948b; Courtois and Joseph, 1948; Courtois and Pèrez, 1949; Courtois, 1951; Barré 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 β-glycerophosphate but not phytic acid and a phytophosphatase (phytase) capable to hydrolyzing both substrates (Courtois, 1947b; Courtois and Joseph, 1947). In another experiment they found phytase to hydrolyze a number of phosphorus esters plus phytic acid whereas all the phosphatase preparations could hydrolyze all the phosphorus esters except phytic acid (Courtois and Joseph, 1947; Courtois, 1948).

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 phenylphosphate. 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 pyrophosphatase activity which is characteristic of plant nonspecific acid phosphomonoesterase.

In wheat, Peers (1953) found the phytase enzyme to be more dispersed throughout the wheat grain than its substrate, phytate, yet the enzyme is found primarily in the scutellum and the aleurone layers. 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. A 1500-fold purified phytase from wheat bran (Nagai and Funahashi, 1963) hydrolyzed all the myoinositol phosphates in a manner that removed the phosphate groups of them one by one. Relative initial rates of hydrolysis of these inositol phosphates were nearly the same and the activation energy of hydrolysis was about 11000 cal/mole for all these substrates. When mixed substrate was used the enzyme showed a preferential attack on the highest member of the phosphates present. From the mixed substrate rate test it was concluded that wheat bran phytase is a single enzyme.

Nutritional aspects of phytase

Ruminants are able to utilize the phytate of diet. 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 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 in the diet 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 origin (Courtois and Pèrez, 1949). Rapoport and co-workers (1941) failed to find phytase activity in the plasma and cells of a number of mammals which included man, rabbit, guinea pig, and calf, but they did report the presence of a magnesium-insensitive phytase in the plasma of the lower vertebrates goose, pigeon, turtle, and frog. It is maintained that possible absence of intestinal phytase in man and non-ruminant animals is responsible for the evidence of mineral-deficiency diseases when the diet is characterized by high phytate content. Nelson and co-workers (1971) have shown that phytic acid added in the diet of chick constitutes a less serious problem when the diet contains active phytase extracted from mold (Aspergillus ficuum NRRL 3135).

The ability of man to hydrolyze phytates remains a controversial subject, though some hydrolysis in the digestive tract

occurs probably due to microbial phytases or nonenzymatic 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 the food 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 Ca in the diet.

Supplementation of white wheat flour with wheat protein concentrate or soy protein preparations not only results in a substantial increase in protein quality and quantity but in phytic acid content as well. Ranhotra (1972; 1974) studied the hydrolysis during breadmaking of phytic acid in wheat protein concentrate and soy protein preparations. He observed the amount of phytic acid hydrolyzed during breadmaking to be decreased with increasing concentrations of wheat protein concentrate (WPC) until in all-WPC bread virtually none of the phytic acid initially present was hydrolyzed, despite the increase in phytase activity. Phytic acid increases linearly when increasing concentrations of WPC are added to wheat flour, but the phytase activity does not increase linearly and the pH of the breads baked from these blends increases progressively above the optimum for phytase activity (Ranhotra, 1973). He explained the progressive decrease in the rate of phytic acid hydrolyzed during breadmaking, as WPC increases in the blends, to

be caused by increased inhibition of phytase activity or rephosphorylation (by excessive accumulation of inorganic phosphorus) of partially hydrolyzed phytic acid, or both (Ranhotra, 1972).

MATERIALS AND METHODS

Analysis for phytic acid and other phosphorus compounds

All the beans analyzed were grown in Michigan and obtained from the Crop and Soil Science Department of Michigan State University. They represented fifty varieties and lines and included crops of three years, 1971, 1972 and 1973.

The beans were ground to a fine flour which was dried in a vacuum oven and then put into small glass containers and kept in a dessicator until the time of analysis. An electric grinder bearing a cooling system (Chemical Rubber Co., Cleveland, Ohio) was used for grinding the beans. The bean flour could pass an 80 mesh sieve. All the analyses described below were carried out in duplicate or triplicate. Solution percentages are reported as weight of solute per 100 ml of solution.

Total phosphorus determination

Total phosphorus was determined colorimetrically after digestion of the sample with perchloric acid according to Allen's method (1940). The method is a variant of the Fiske & Subbarow method (1925) which is based on the formation of phosphomolybdic acid and its subsequent reduction to a blue compound which can be measured colorimetrically. The method makes use of the following solutions:

Ammonium molybdate solution: An 8.3% solution of the A.R. salt in distilled water.

Perchloric acid: A 60% solution.

Amidol reagent: This is the reducing agent. It is composed of 2 g amidol (2,4-diaminophenol dihydrochloride) and 40 g sodium bisulphite dissolved in distilled water and diluted to 200 ml. The solution must be kept in a well-stoppered brown bottle and discarded after one week.

Hydrogen peroxide: A 30% solution.

Standard phosphate solutions: A stock solution of KH_2PO_4 (dried previously in the oven) is prepared containing 1 mg phosphorus per ml. Suitable standard solutions for the calibration of the spectrophotometer are obtained by dilution of the stock solution.

A standard curve was prepared (Figure 1) by using a series of standard solutions in 25 ml volumetric flasks in a range of concentrations of 0.00 to 0.16 mg P/25 ml. In each flask, containing the proper amount of phosphorus, 2 ml perchloric acid, 2 ml amidol reagent and 1 ml ammonium molybdate are added in that order. Water to 25 ml is added, the solution is mixed and the color is measured after an interval of 5-30 min with a Beckman DU spectrophotometer at 675 nm.

For the estimation of total phosphorus in bean flour a carefully weighed amount of the material, usually 20-30 mg of dried flour, is placed in a micro-Kjeldahl flask and a 2.2 ml perchloric acid is added. The flask is heated over a micro-burner until the contents have become colorless. Addition of a few drops of H_2O_2 to complete the combustion was not necessary in most cases. After cooling the flask, its contents are thoroughly rinsed into a

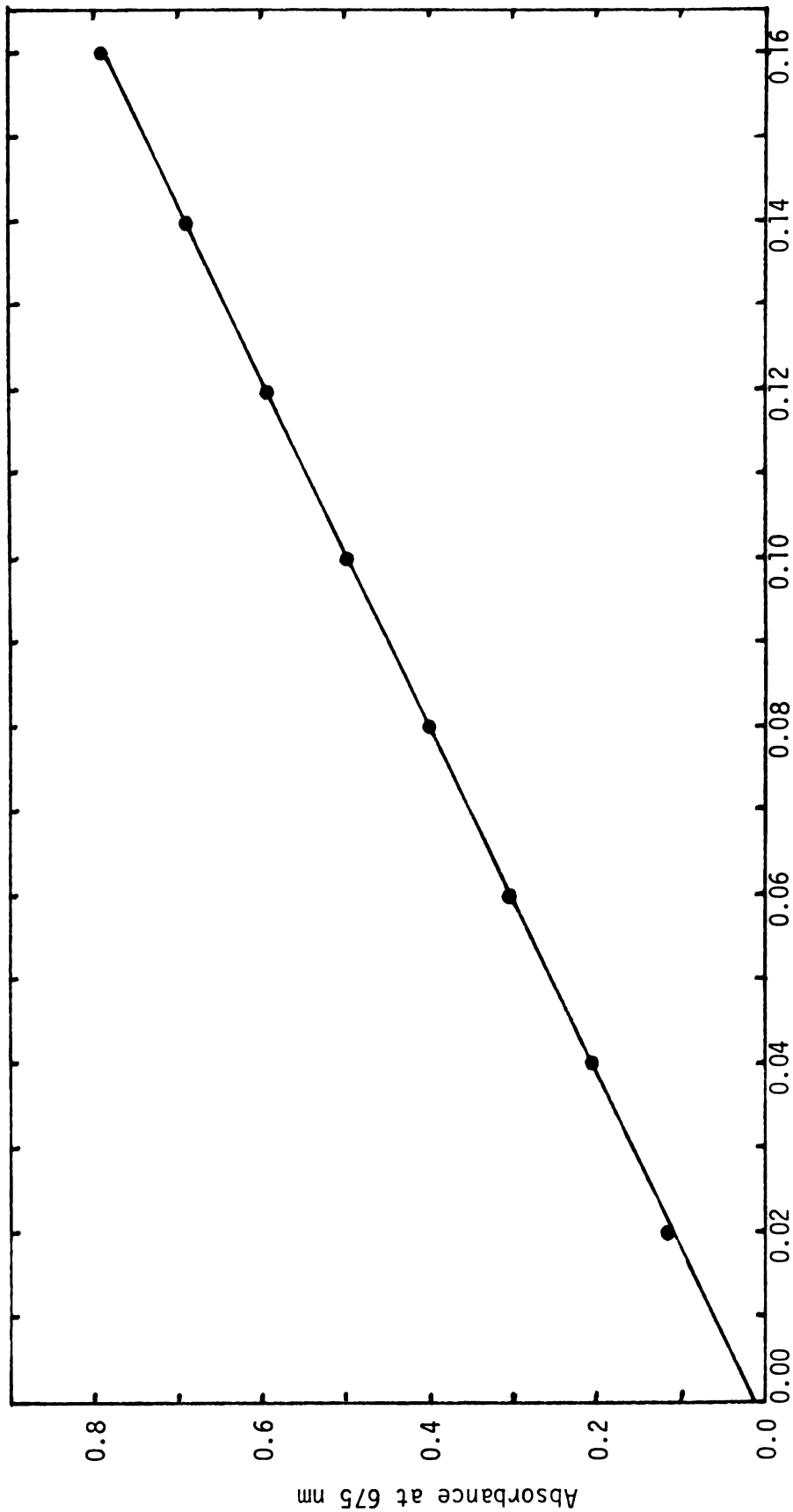


Figure 1.--Standard curve for the determination of total phosphorus. Linear regression equation: $A_{675} = 0.011 + 4.863 \times C$.

25 ml volumetric flask and the analysis is continued with the same manner as with the standards. The absorbance is independent on the amount of perchloric acid in the range of 1.0-2.4 ml perchloric acid/25 ml. To be sure that one is in this range, it is suggested, for better results, that the digestion of the sample be continued till the solution is almost all but a very small amount (less than 0.4 ml) evaporated.

Determination of phytic acid

For the determination of phytic acid a combination of two methods was used. The extraction and precipitation of phytic acid were performed according to the method of Wheeler and Ferrel (1971), whereas the iron of the precipitate was measured by Makower's method (1970). The steps of the method, modified in its details, are as follows:

1. Weigh 2 g finely ground flour (80 mesh) estimated to contain 15 to 35 mg phytate phosphorus into a 125 ml Erlenmeyer flask.

2. Extract with 40 ml 3% TCA (trichloroacetic acid) for 45 min with mechanical shaking (300-350 rpm).

3. Centrifuge the suspension at 20,000g.

4. Transfer a 10 ml aliquot of the supernatant into a glass 40 to 50 ml conical centrifuge tube.

5. Add 5 ml $FeCl_3$ solution (made to contain 2 mg ferric iron per ml in 3% TCA) to the aliquot by blowing rapidly from the pipet.

6. Heat the tube and contents in a boiling-water bath for 1 hr. If the supernatant is not clear after 30 min, add 1 or 2 drops of 3% sodium sulfate in 3% TCA and continue heating.

7. Centrifuge for 15 min at about 2000g and carefully decant clear supernatant.

8. Wash precipitate twice by dispersing well in 20 to 25 ml 3% TCA, heating in boiling-water bath 5 to 10 min, and centrifuging.

9. Repeat wash once with water.

10. Disperse the precipitate in 5 ml H_2O and add 5 ml 0.6 N NaOH.

11. Heat in boiling water for 45 min to coagulate $Fe(OH)_3$.

12. Centrifuge for 15 min at 2000g and decant carefully.

13. Wash precipitate with water, recentrifuge and decant.

14. Precipitate is dissolved in 5 ml 0.5 N HCl with heating in boiling water, usually 10 to 15 min.

15. Transfer to 100 ml volumetric flask and make to volume with 0.1 N HCl.

16. Analyze for iron. Transfer 1 to 2 ml of the above solution to a 25 ml volumetric flask, add 1 ml 10% hydroxylamine hydrochloride solution, rotate flask, and let stand a few minutes. Add 9.5 ml 2M sodium acetate solution and 1 ml 0.1% orthophenanthroline solution, dilute to volume and mix. Let stand at least 5 min and read absorbance in the spectrophotometer at 510 nm (AOAC, 1970). Compare results to a standard curve constructed by the use of standard solutions of $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ (Figure 2).

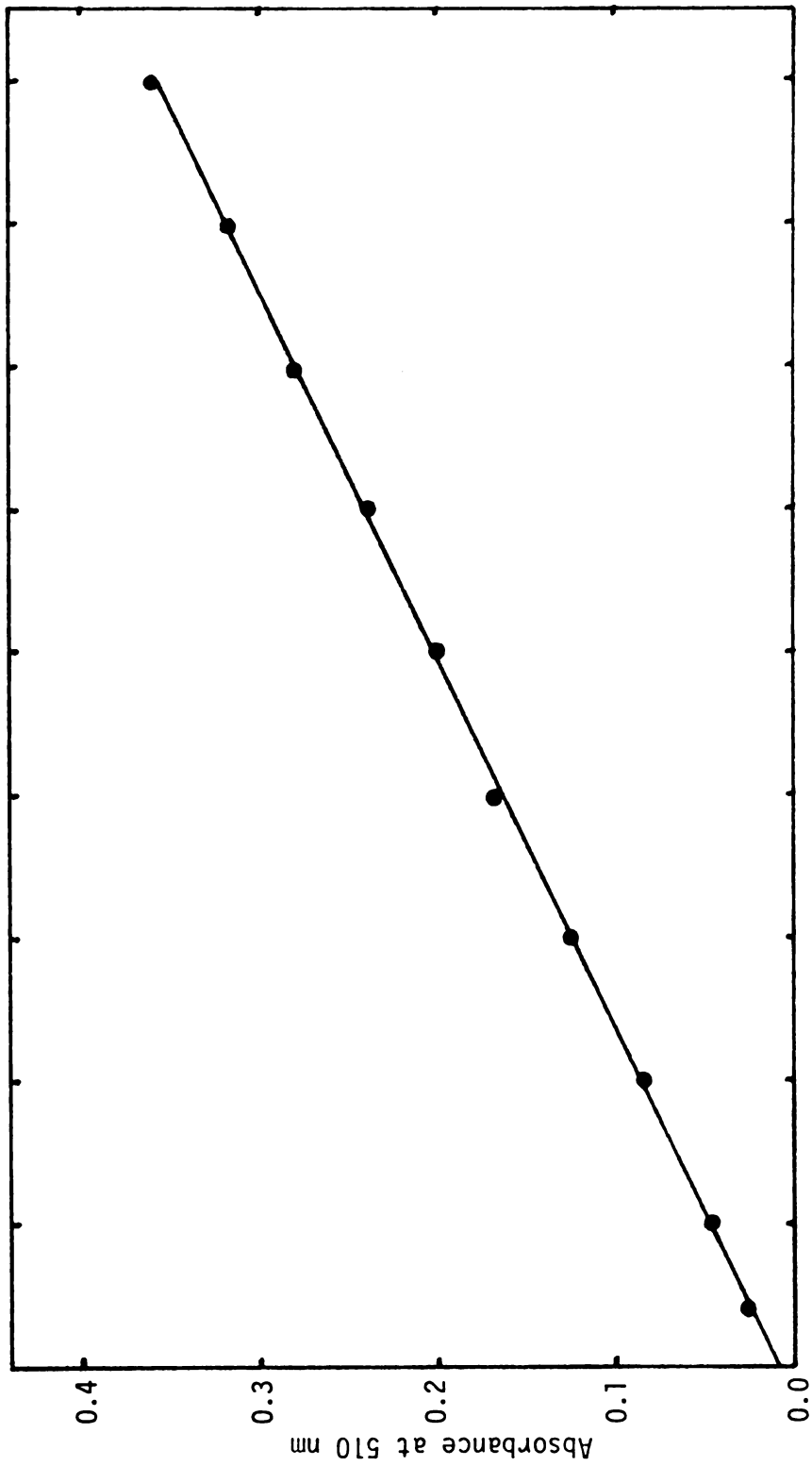


Figure 2.--Standard curve for the determination of iron. Linear regression equation: $A_{510} = 0.006 + 0.783 \times C$.

A 4:6 Fe/P atomic ratio was used to calculate the phytic acid content.

For the determination of phytic acid in germinating beans, the germinating beans were ground with 3-5 g of sea sand in a mortar in the presense of 3% TCA. The homogenate was made to volume with 3% TCA and shaken for 45 min with mechanical shaking (300-350 rpm). The analysis was continued starting from step #3.

Determination of inorganic phosphorus

The determination of inorganic phosphorus was based on the colorimetric method of Pons and Guthrie (1946). The method employs extraction of phosphomolybdic acid with isobutyl alcohol and subsequent reduction of it by stannous chloride. The method is as follows:

1. Weigh 1 g finely ground flour (80 mesh) into an 125 ml Erlenmeyer flask.

2. Extract with 0.75 N TCA for 1 hour with mechanical shaking (300-350 rpm).

3. Centrifuge the suspension at 20,000g.

4. Pipet a 2 ml aliquot into an 125 ml separatory funnel with a mark at 20 ml.

5. Add 5 ml of the molybdate-sulfuric acid reagent (prepared by dissolving 50 g of ammonium molybdate, $(NH_4)_6Mo_7O_{24}.4H_2O$, in 400 ml of 10 N sulfuric acid and 500 ml of water, making up to 1 liter, and storing in a brown bottle) and deionized water to the 20 ml mark.

6. Add 10 ml of isobutyl alcohol and shake for 2 minutes.

7. Discard the aqueous layer, and wash by shaking once with 10 ml of 1 N H_2SO_4.

8. Add 15 ml of dilute stannous chloride, shake for 1 min and then discard the aqueous layer. The dilute stannous chloride is prepared by diluting 1 ml of stock solution to 200 ml with 1 N H_2SO_4 just before use. The stock solution is composed of 10 g stannous chloride hexahydrate or 8.25 g of the dihydrate salt dissolved in 25 ml of concentrated HCl and stored in a glass-stoppered brown bottle.

9. Transfer the blue isobutyl alcohol layer to a 50 ml volumetric flask, washing the funnel with 95% ethyl alcohol, and make to volume with 95% ethyl alcohol.

10. Measure the absorbance of the blue solution in a spectrophotometer at 730 nm after 40 minutes against a blank containing all reagents.

11. Prepare a calibration curve (Figure 3) by pipetting known concentrations of inorganic phosphorus in the range 0.000 to 0.045 mg P, obtained by diluting stock phosphate solution, into 125 ml separatory funnels and developing the color exactly as outlined in the above procedure.

Estimation of residual phosphorus

Residual phosphorus representing nonphytic acid organic phosphorus was calculated by subtracting inorganic phosphorus and phytic acid phosphorus from total phosphorus.

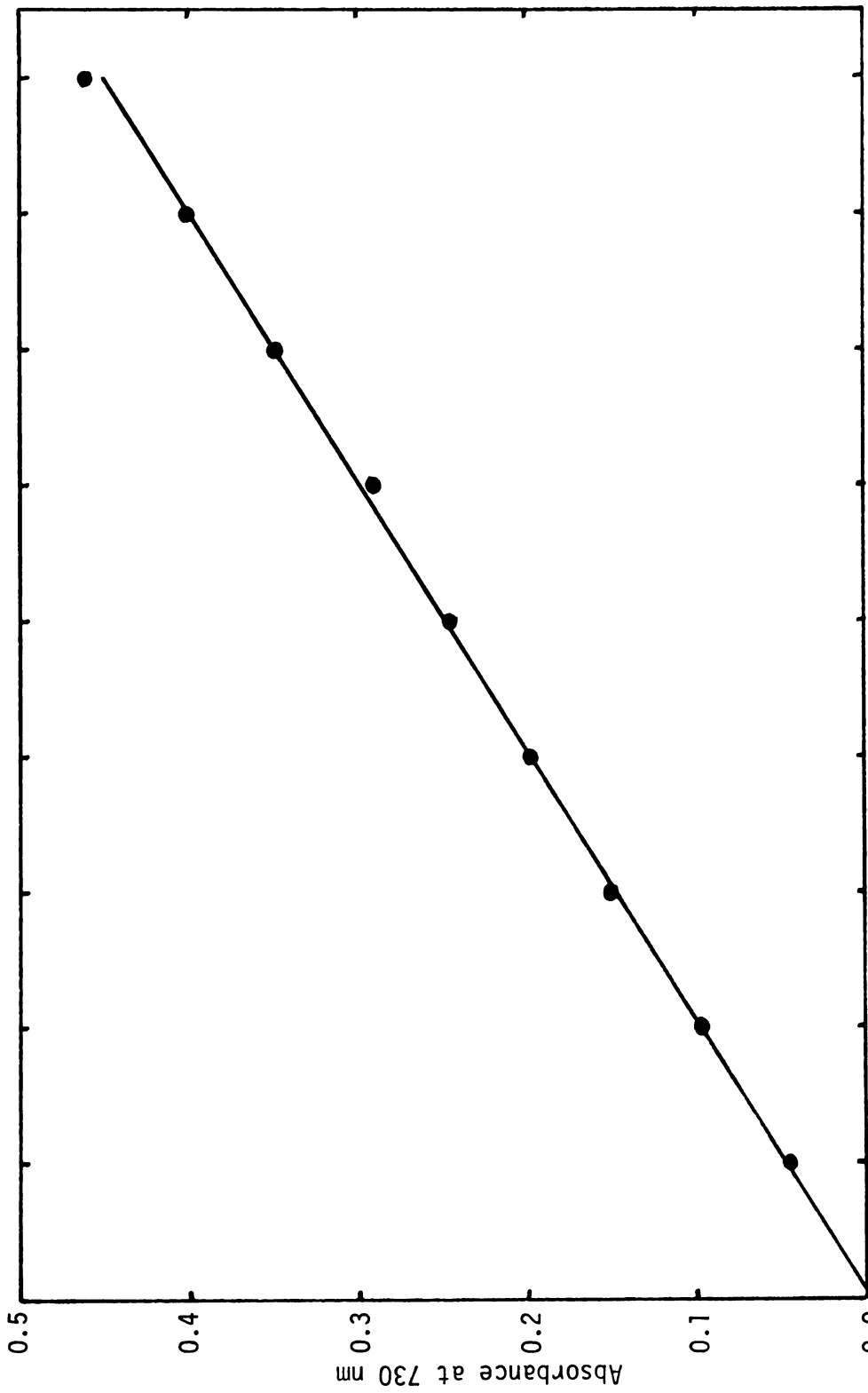


Figure 3.--Standard curve for the determination of inorganic phosphorus. Linear regression equation: $A_{730} = -0.008 + 10.260 \times C$.

Isolation of a protein-phytate complex

A protein-phytate complex was isolated from bean flour according to the method of Rackis and co-workers (1961). The method is as follows:

1. 100 g flour of freshly ground beans (80 mesh; Sanilac navy beans) was doubly extracted with deionized water in ratios of water to flour 10:1 and 5:1 under mechanical shaking at room temperature for 1 hour.

2. After centrifugation at 20,000g the supernatant was acidified to pH 4.4 with 1 N HCl and the precipitate was centrifuged out.

3. The received supernatant was adjusted to pH 8.0 with 1 N NaOH and let stand 1 hour at 0°C.

4. The precipitate formed is the protein-phytate complex. After centrifugation, the supernatant was discarded and the precipitate was lyophilized.

Nitrogen determination

The AOAC (1970) micro-Kjeldahl method was followed for the determination of the nitrogen content of beans, using mercuric oxide as a catalyst.

Enzyme extraction

The 1973 crop of Sanilac Navy Beans was used for all the experiments with the phytase enzyme.

The crude enzyme solution was prepared by extracting the bean flour with a 10:1 ratio of 2% $CaCl_2$ to flour, mechanical shaking

(300 rpm) for 30 min at room temperature and centrifuging (20000g) for 30 min at 2°C. The enzyme from the germinating beans was prepared by first homogenizing the tissue in a mortar with sea sand, mechanical shaking of the homogenate as above, filtering through cheese cloth and centrifuging it under the same conditions as the flour.

The clear supernatant in both cases had a pH of 5.0.

Ammonium sulfate fractionation

Sufficient solid ammonium sulfate was added to the crude enzyme solution (Dixon, 1953) with continuous mechanical stirring to make it 35% saturated, kept for 30 min at 2°C and centrifuged (20000g) for 20 to 30 min at 2°C. The residue was discarded and the supernatant was made 80% $(NH_4)_2SO_4$ saturated followed by the same treatment as above. The precipitate, the fraction precipitating between 35% 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°C).

This partially purified phytase was used as the enzyme source in most of these experiments. The enzyme obtained from the 6-8 days germinating beans possessed a much stronger activity and was used for the determination of the biochemical parameters of the bean phytase.

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 50 ± 1°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 5.3, 0.15 ml of 8 mM previously adjusted to pH 5.3 (with 1 N HCl) sodium phytate (SIGMA Chemical Co., St. Louis, Missouri), 0.2 ml enzyme solution and water to 1.2 ml. Final concentrations of buffer and phytate were 0.1 M and 1 mM , respectively; and incubation time usually 30 minutes. After incubation, samples were withdrawn from the digest, deproteinized by adding TCA to a final concentration of 0.7 N 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 or the enzyme without incubation (value at time zero).

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).

Deproteinization

The enzymatic reaction was stopped by the use of TCA. This has to be in the proper concentration to precipitate the maximum amount of protein and so give clear solutions for the subsequent colorimetric determination of inorganic P. To find the concentration of TCA at which the maximum amount of protein is precipitated (or at which the minimum amount of nitrogen remains in solution) this method was followed (Becker et al., 1940).

A 1-g bean flour sample was weighed into an 125 ml Erlenmeyer flask and extracted with 40 ml TCA solution of different concentrations ranging from 0.0015 to 4.0 N with mechanical shaking for 30 minutes. The suspension was centrifuged for 10 min at 2000g and nitrogen was determined in an aliquot of the supernatant by the Kjeldahl method (duplicate determinations from the extraction stage). The results were plotted as percent of total nitrogen extracted against the logarithms of TCA concentration.

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 thoroughly, make to 2 liters and store in a brown glass bottle in the refrigerator.

2. Prepare reagent B.

Dissolve 0.264 g of ascorbic acid in 50 ml of reagent A and mix. This reagent does not keep more than 24 hours and must be prepared the day of 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.

6. Calibrate the method (Figure 4) 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. Measure absorbance at 700 nm.

Effect of pH on enzyme activity

Standard assay procedures were used to determine reaction rates over the pH range of 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 5.3 and plotted against pH.

Effect of incubation temperature on reaction rate

The reaction rates were determined at temperatures from 35°C to 60°C at 5 degree intervals using standard assay procedures. The progress curves at the different temperatures were plotted

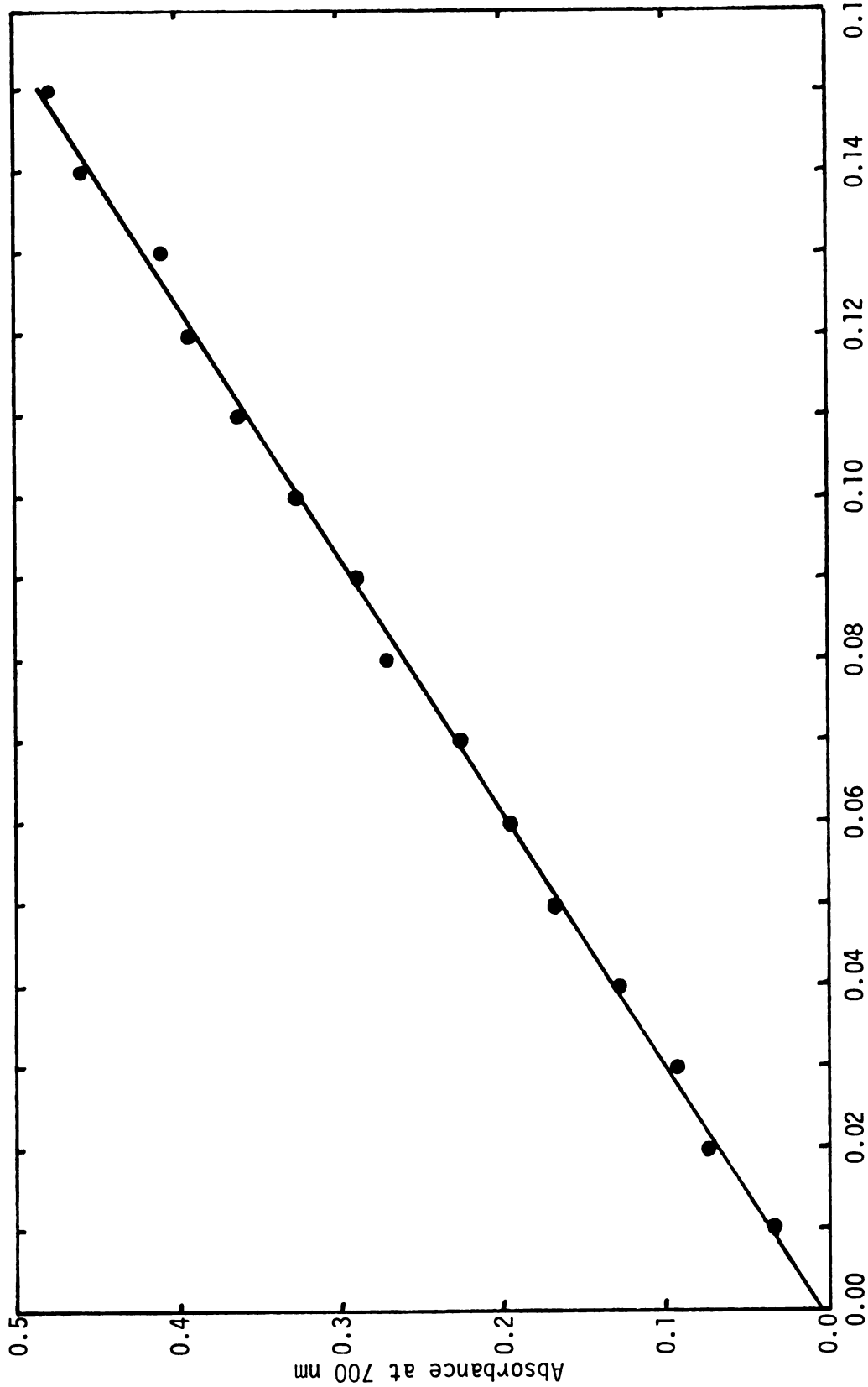


Figure 4.--Standard curve for the determination of inorganic phosphorus by the ascorbic acid method. Linear regression equation: $A_{700} = 0.003 + 3.204 \times C$.

against 30 min intervals. The rate of formation of liberated orthophosphate as a function of temperature was also plotted.

The activation energy of hydrolysis of phytic acid was calculated from the obtained data. The tangent on each curve represents the reaction rate k (μmole liberated Pi/min per ml enzyme). The slope of the curve lnk vs 1/T is equal to -Ea/R according to the Arrhenius equation

$$k = A \exp(-Ea/RT)$$

where Ea is the activation energy for the transformation of phytic acid to orthophosphate (Pi), T is the absolute temperature, A is a constant and R the gas constant (1.99 $cal \cdot deg^{-1} \cdot mole^{-1}$).

Thermal inactivation of phytase in solution

Standard assay mixtures not containing the substrate were kept for 10 min at different temperatures from 30°C to 80°C at 5 degree intervals in a water bath. After the 10 min incubation, they were put immediately in an ice-bath, the substrate was added and activity was measured according to the standard procedure. The results were compared to that of a control without heat treatment and plotted as percent remaining activity against the temperature of the 10 min heat treatment.

From the above results it was decided that 58°C and 68°C were suitable temperatures for observing the rate of inactivation. Standard assay mixtures not containing the substrate were heat-treated at 58°C and 68°C for varying periods of time, stored in an

ice-bath, substrate was added and activity determined with the usual way. The natural logarithm of the percent remaining activity was plotted against time. The tangents to the two curves represent the rate constants $k_{58°}$ and $k_{68°}$. The energy of inactivation is then obtained by substituting these values in the Arrhenius equation:

$$E_a = \frac{RT_1T_2}{(T_2 - T_1)} \ln \frac{k_2}{k_1}$$

Effect of enzyme concentration on reaction rate

Varying amounts of enzyme solution (0.1 to 0.8 ml) were used under standard procedures to test for linearity of response. The results were plotted as activity against concentration of enzyme.

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 were calculated 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 0.02 to 0.11 mM phytate at pH 5.3.

Effect of inhibitors on phytase activity

Enzyme inhibition was investigated by performing standard assays in the presence of varying concentrations of sodium fluoride, iodoacetamide and N-ethylmaleimide and the results, compared with those of controls, were plotted as percent of remaining activity against concentration of inhibitor.

Effect of reducing agents on phytase activity

The effect of cysteine, ascorbic acid, reduced glutathione and β-mercaptoethanol at four final levels of concentration of 10^{-4}M, 10^{-3}M, 10^{-2}M and 10^{-1}M in the assay mixture was determined under the established standard conditions and the results were compared with those of controls containing no reducing agent.

Effect of various metal ions on phytase activity

Zn^{2+}, Mn^{2+}, Mg^{2+}, Fe^{2+}, Fe^{3+}, Cu^{2+}, Ca^{2+}, Hg^{2+}, Cd^{2+}, Ni^{2+}, Co^{2+}, Al^{3+}, Ag^{+} 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.

The effect of Co^{2+} concentration on activity was plotted to establish the optimum concentration at which maximum activity exists.

Effect of chelating agents on phytase activity

Standard assays were performed which contained ethylenediamine tetra-acetic acid (EDTA), sodium potassium tartrate, sodium oxalate and sodium citrate at final concentrations of 10^{-6}M, 10^{-4}M and 10^{-2}M.

In another experiment the effect of 10^{-4} and 10^{-3}M (final concentrations) EDTA on activity was studied in an assay mixture containing 10^{-3}M Co^{2+} (final concentration).

Effect of NaCl concentration on phytase activity

Standard assays containing 0.1 to 0.6 M NaCl were performed to test the effect of NaCl on activity. The results were plotted as percent remaining activity against NaCl concentration.

Separation of phytase from phosphatase

Diethylaminoethyl (DEAE) cellulose (15-20 g) was treated according to the procedure described by Whitaker (1972). It was packed in a glass column (2.5 cm x 50 cm) to a height of about 25 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 7.8 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 0.4 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 (15 ml) were assayed for protein content, phosphatase and phytase activity. The assay for the phosphatase activity was carried out under the same 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 and phytic acid. 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 but phytic acid were added in the assay mixture at a final concentration of 10 mM.

Germination of beans

The dry seeds of beans were sterilized by dipping in 1% $Ca(OCl)_2$ for 5-10 min, rinsed with sterilized water and soaked for

about 12 hours in sterilized water at room temperature. After the seeds were fully soaked they were placed on a thick piece of filter paper in a perforated tray. Between seeds and filter paper a piece of cheese cloth (2 to 4 layers) was placed whose two ends were dipped in two trays containing sterilized water. The whole system was in a chamber the temperature of which was maintained at 25°C to 27°C. Germination was allowed to proceed in darkness.

RESULTS AND DISCUSSION

Analysis of phosphorus compounds

Expressed on a dry weight basis, the results of the bean analyses are shown in Table 1. The ranges are: for phytic acid, 0.54-1.58%; for total phosphorus, 0.259-0.556%; for inorganic phosphorus, 0.021-0.044%; and for other than phytic acid organic phosphorus, 0.050-0.135%. The phytic acid phosphorus represents 53.6-81.6% of the total phosphorus, with an average of 69.3%. So about 70% of total phosphorus is tied up in the form of phytic acid and this amount is unavailable to those people whose diet is primarily composed of dry beans.

The total phosphorus and the phytic acid content of beans were found to be related by the linear regression equation

$$Y = 0.141 + 0.273\ X$$

where Y is the percentage content in total phosphorus and X the percentage content in phytic acid. The correlation coefficient was 0.9847 and the standard error of estimate 0.017%. A graphic presentation of that relationship is shown in Figure 5. The correlation coefficient between phytic acid and inorganic phosphorus was 0.4550 and that between total phosphorus and inorganic phosphorus was 0.4956.

Table 1.--Content of beans in total P, phytic acid, inorganic P, organic P other than phytic P, and nitrogen.[a]

Varieties	Total P, % dry wt	PA, % dry wt	PA P as % of total P	Inorg P, % dry wt	Inorg P as % of total P	Org P other than PA P, % dry wt	% org P other than PA P, as % of total P	N, % dry wt
1971								
Black turtle soup	0.335	0.70	58.8	0.031	9.3	0.107	31.9	3.36
G.N., Nebraska #1	0.327	0.67	57.7	0.035	10.7	0.103	31.6	3.55
G.N., Tara	0.363	0.83	64.4	0.033	9.1	0.096	26.5	3.43
G.N., U.I. #59	0.333	0.68	57.5	0.034	10.2	0.108	32.3	3.53
G.N., U.I. #61	0.286	0.56	55.1	0.035	12.2	0.093	32.6	3.05
G.N., U.S. #1140	0.283	0.60	59.7	0.031	11.0	0.083	29.4	3.16
N.B., Gratiot	0.369	0.75	57.2	0.033	8.9	0.125	33.8	3.51
N.B., Michelite-62	0.365	0.78	60.2	0.034	9.3	0.111	30.5	3.68
Pinto, #111	0.310	0.64	58.1	0.037	11.9	0.093	29.9	3.71
Pinto, U.I. #114	0.305	0.61	56.3	0.036	11.8	0.097	31.9	3.57
Red Kote	0.373	0.71	53.6	0.038	10.2	0.135	36.2	4.14
R.M., Big Bend	0.308	0.61	55.8	0.035	11.4	0.101	32.9	3.93
R.M., U.I. #34	0.313	0.64	57.6	0.031	9.9	0.102	32.5	3.91
R.M., U.I. #36	0.270	0.54	56.3	0.039	14.4	0.079	29.3	3.43
S.W., U.I. #1	0.259	0.55	59.8	0.031	12.0	0.073	28.2	3.29
S.W., #59	0.340	0.75	62.1	0.021	6.2	0.108	31.7	3.66
1972								
N.B., Sanilac[b]	0.497	1.32	74.8	0.040	8.1	0.085	17.2	4.12
N.B., Seafarer	0.362	0.74	57.6	0.039	10.8	0.115	31.7	3.63
N.B., Seaway	0.448	1.21	76.1	0.034	7.6	0.073	16.4	3.69
1973								
Calif. R.K., #1104	0.497	1.20	68.0	0.041	8.3	0.118	23.8	3.93
G.N., U.I. #31	0.502	1.37	76.8	0.032	6.4	0.084	16.8	3.42
Jules	0.525	1.44	77.2	0.038	7.2	0.082	15.5	3.62
N.B., #20444	0.531	1.47	78.0	0.032	6.0	0.085	16.0	3.70
N.B., #20449	0.528	1.47	78.4	0.034	6.4	0.080	15.2	3.72
N.B., #20454	0.553	1.53	77.9	0.038	6.9	0.084	15.2	3.61
N.B., #20455	0.520	1.33	72.0	0.039	7.5	0.106	20.5	3.48
N.B., #20457	0.534	1.43	75.4	0.038	7.1	0.093	17.5	3.66
N.B., #20459	0.542	1.50	77.9	0.039	7.2	0.081	14.9	3.76
N.B., #20460	0.549	1.58	81.0	0.041	7.5	0.063	11.5	3.91
N.B., #20462	0.507	1.36	75.5	0.035	6.9	0.088	17.6	3.62
N.B., #20463	0.539	1.38	72.1	0.036	6.7	0.114	21.2	3.57
N.B., #20464	0.555	1.52	77.1	0.039	7.0	0.088	15.9	3.75
N.B., #20465	0.498	1.30	73.5	0.027	5.4	0.105	21.1	3.79
N.B., #20466	0.546	1.48	76.3	0.034	6.2	0.095	17.5	3.81
N.B., #20467	0.523	1.41	75.9	0.043	8.2	0.083	15.9	3.77
N.B., #20468	0.538	1.41	73.8	0.041	7.6	0.100	18.6	3.86
N.B., #20469	0.539	1.36	71.1	0.041	7.6	0.115	21.3	3.75
N.B., #20470	0.544	1.35	69.9	0.043	7.9	0.121	22.2	3.79
N.B., #20471	0.556	1.52	77.0	0.038	6.8	0.091	16.2	3.74
N.B., #20472	0.528	1.33	70.9	0.041	7.8	0.112	21.3	3.74
N.B., #20473	0.490	1.29	74.1	0.037	7.6	0.090	18.3	3.79
N.B., #20474	0.553	1.45	73.8	0.040	7.2	0.106	18.9	3.96
N.B., #20475	0.533	1.46	77.1	0.042	7.9	0.080	15.0	3.89
N.B., #20476	0.523	1.33	71.6	0.036	6.9	0.113	21.5	3.82
N.B., #20477	0.532	1.42	75.2	0.039	7.3	0.093	17.5	3.60
N.B., #20478	0.504	1.29	72.1	0.039	7.7	0.102	20.2	3.67
Oregon #58	0.476	1.36	80.5	0.038	8.0	0.055	11.6	3.83
R.M., Big Bend	0.429	1.10	72.2	0.035	8.2	0.084	19.6	3.15
Royal R.K.	0.509	1.30	71.9	0.044	8.6	0.099	19.5	3.65
Swedish Brown	0.483	1.40	81.6	0.039	8.1	0.050	10.3	3.27

[a] Abbreviations used are: P, phosphorus; PA, phytic acid; G.N., Great Northern; N.B., Navy Bean; R.M., Red Mexican; S.W., Small White; R.K., Red Kidney. [b] Twelve analyses in this variety for phytic acid gave a mean and standard deviation of 1.32 ± 0.01.

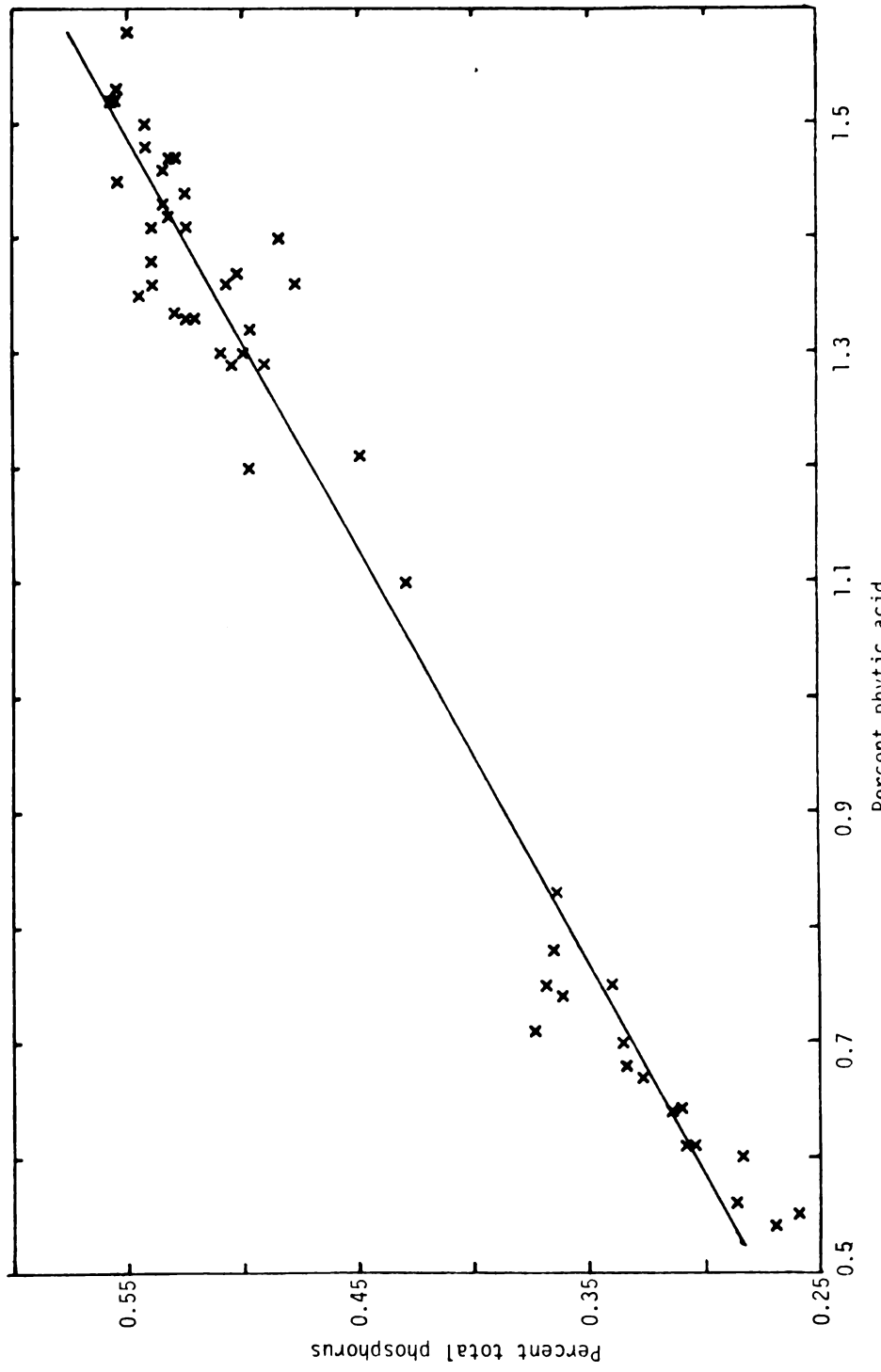


Figure 5.--Relationship between total P and phytic acid contents (dry basis) of beans. Linear regression equation: % Total P = 0.141 + 0.273 x (% phytic acid) ($r = 0.9847$).

On the basis of these data, it appears that one can estimate the phytic acid content of mature dry beans by determining total phosphorus. This is an advantage, especially when a great number of samples are to be analyzed. Phytic acid analysis is time consuming and tedious, whereas total phosphorus determination by the colorimetric method is much faster and easier.

It should be noted that a similar relationship has been found between total phosphorus and phytic acid contents of oats and barley with high correlation coefficients (Lolas et al., 1975).

The initial bean flour sample yielded 0.47% (dry weight basis) of protein-phytate complex, analysis of which showed that it is composed of 14.2% water, 2.7% nitrogen (or 0.3% of total nitrogen of flour), 12.9% total phosphorus (or 12.1% of total phosphorus of flour), and 0.024% inorganic phosphorus. A great amount of total phosphorus seems to be in the form of phytate. The limited amount of material did not allow a sound analysis for phytic acid.

Because of the formation of a protein-phytate complex, it was thought that a relation might exist between protein and phytic acid. A correlation coefficient of 0.3655 was obtained between protein and phytic acid contents indicating no close relation between these constituents of beans. This means that increase in the amount of protein in a variety by genetic crossings is not going to be accompanied necessarily by an increase in the amount of phytic acid.

The following experiment indicates that the phytic acid of beans is present in a soluble form rather than as insoluble phytin. Bean flour was extracted with water first at the 10:1 water/flour ratio for 1 hour under shaking, the slurry was centrifuged, and the sediment was extracted similarly a second time with a water/flour ratio of 5:1. After a second centrifugation, a residue was obtained which upon extraction with 3% TCA [the phytin solvent used in the Wheeler and Ferrel (1971) method] was found to contain approximately 0.4% of the total phytic acid. This agrees with Sandegren's (1948) contention that phytic acid exists principally as the water-soluble sodium-potassium salt in barley. It has been also reported by Crean and Haisman (1963) that the phytic acid in dried peas is wholly water-soluble, probably stored in the peas as a potassium hydrogen salt. Considering the metabolic function of phytic acid in the seed as a source of phosphorus (Williams, 1970) it seems likely that it should be stored in a relatively soluble form.

However soluble the phytic acid might be, it was found that it does not come out of the dry seed during soaking for 24 or 48 hours in distilled water at room temperature.

Cooking of beans for 75 minutes in distilled water at 100°C and subsequent analysis for phytic acid of the whole mixture did not reveal any destruction of phytic acid. This should be expected since phytic acid is hydrolyzed only under acidic conditions, the hydrolysis being accelerated by the application of heat. During cooking, an increase in the amount of inorganic

phosphorus was observed (about 35% of the initially existed), apparently from the hydrolysis of other labile phosphorus esters.

Phytase extraction

The use of $CaCl_2$ as an extractant of an enzyme might look peculiar. Extraction by water and buffers (usually 0.2 M acetate pH 5.3 and 0.1 M tris-maleate pH 6.5) of bean flour and subsequent ammonium sulfate fractionation could not avoid contamination of enzyme by the natural substrate (phytic acid) present in the flour. Such a problem was not observed when the beans were well germinated (more than 6 days). Other investigators have not reported a problem of similar nature, probably, because the majority of them worked with germinating seeds. Since phytic acid forms complexes with proteins, it is extracted and subsequently precipitated in a form which is bound to proteins and so a method should be found to break these complexes without destroying the enzyme. The method used at the beginning of this work was extraction of bean flour with water, precipitation of proteins at pH 4.4 and raise of pH of supernatant to pH 8.0 with NaOH after centrifugation (Rackis et al., 1961). After leaving it in cold for 1 hour the phytic acid precipitated and was centrifuged out. The supernatant was adjusted to pH 6.5 and fractionated with ammonium sulfate (fraction between 35% and 80%). The received enzyme was not contaminated with the substrate, especially when a saturated $Ca(OH)_2$ solution was used instead of NaOH.

The above method had a number of disadvantages. Some of the enzyme was precipitated at pH 4.4, use of $Ca(OH)_2$ increased

the volume of the solution considerably, some complications were observed during ammonium sulfate fractionation and the whole procedure was time consuming and tedious. Several salts were tested, then, in an effort to separate the substrate from the enzyme. Among the salts tested $CaCl_2$ at the 2% level was considered as the ideal extractant. The problem was eliminated and clear supernatants were obtained having a constant pH of 5.0. Apparently, $CaCl_2$ precipitates phytic acid and breaks any protein-phytate complexes. These complexes probably are used during the development of the plant as phytic acid breaks down to inositol and inorganic phosphorus and this is the reason why the problem was not observed in the germinating beans.

Deproteinization

Trichloroacetic acid in concentrations of 10% or 20% has been added to enzyme digests for terminating enzyme activity and removing protein. In the extraction of bean flour with various concentrations of TCA, the minimum amount of nitrogen was removed with the acid concentrations lying between 0.6 N and 0.8 N acid, 0.7 N acid being taken as the midpoint. Figure 6 shows the percent of total nitrogen extracted at various acid concentrations. Though there is no sharp, clear-cut line of demarcation between proteins and nitrogenous nonprotein compounds, TCA was used at a final concentration of 0.7 N to stop enzymatic activity and precipitate protein. At this concentration the minimum amount of nitrogen remains in solution or the maximum amount of protein has

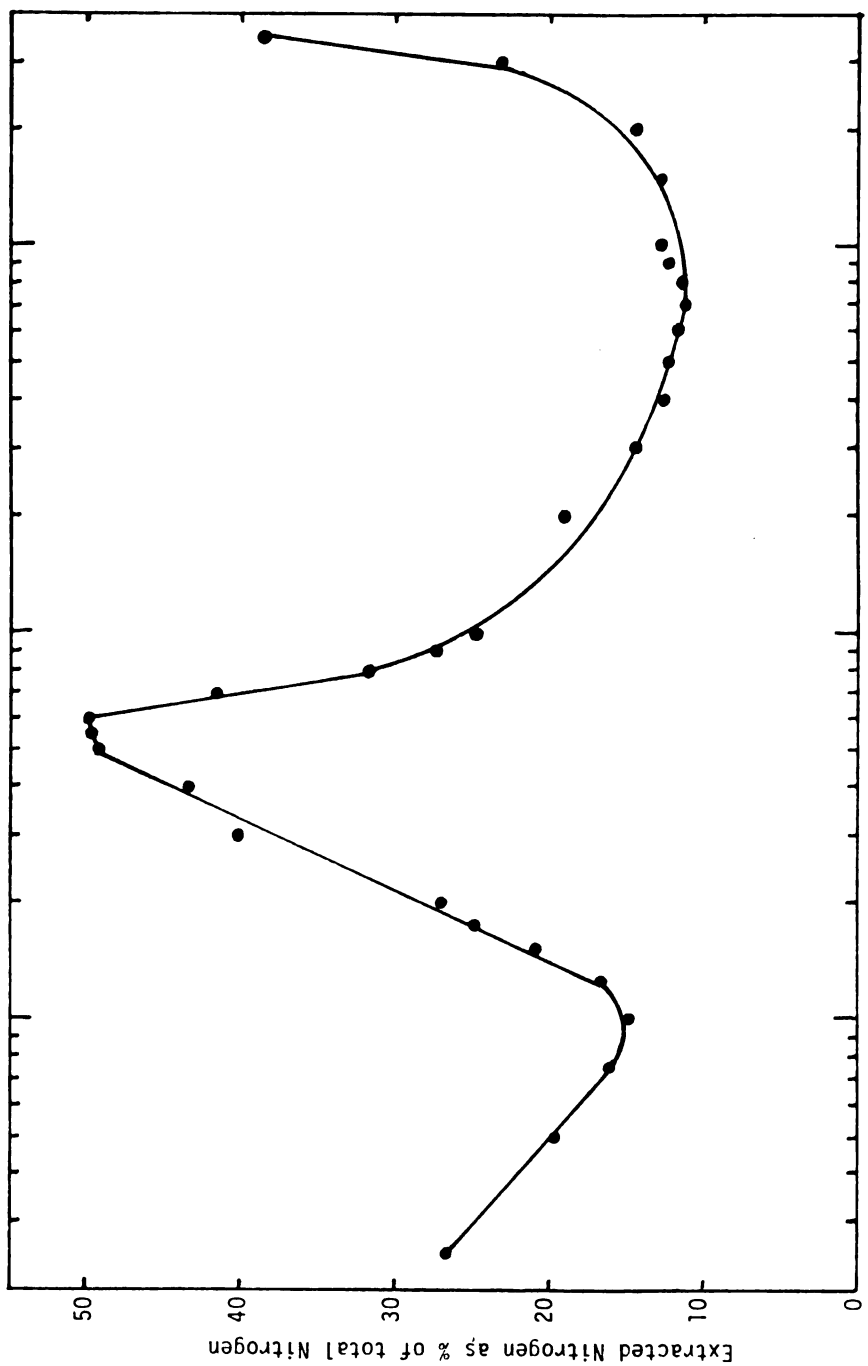


Figure 6.-- Nitrogen extracted from bean flour by different concentrations of TCA.

been precipitated. Thus protein, being removed, does not interfere in the subsequent colorimetric determination of orthophosphate.

Effect of pH and incubation temperature on enzyme activity

The pH optimum for the Navy bean phytase of mature and germinating seeds was found to be 5.3 (Figure 7) 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; 5.15 for the optimum pH 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) and two pH peaks (5 and 7) have been observed in germinating lettuce seeds (Mayer, 1958).

The progress curves at various temperatures have been plotted in Figure 8 and these show the optimum temperature of activity to be about 50°C. The progress curves at the different temperatures with the exception of that of 60°C indicate that the decomposition of phytate proceeded at a relatively constant rate during the first hour of incubation period. Peers (1953) obtained a value of 55°C for the optimum temperature of wheat phytase while Mandal and co-workers (1972) found 57°C as optimum for the germinating mung bean phytase. Figure 9 is a plot of the rate of formation of orthophosphate against the temperature of incubation for a period of 30 minutes.

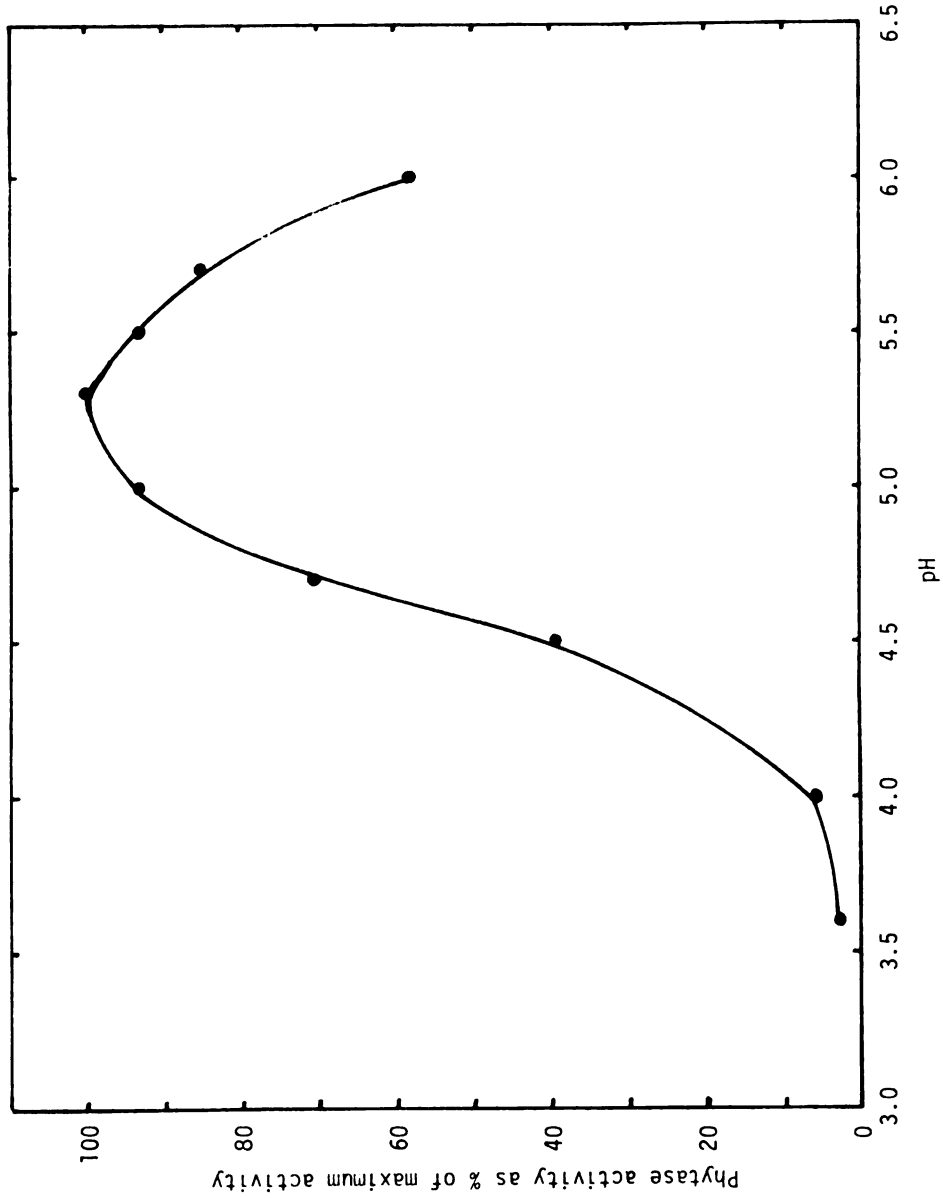


Figure 7.--Effect of pH on bean phytase activity. Phytase was incubated at 50°C with 1 mM sodium phytate in the presence of 0.1 M acetate buffers except for pH 6.0 (0.1 M tris-maleate buffer).

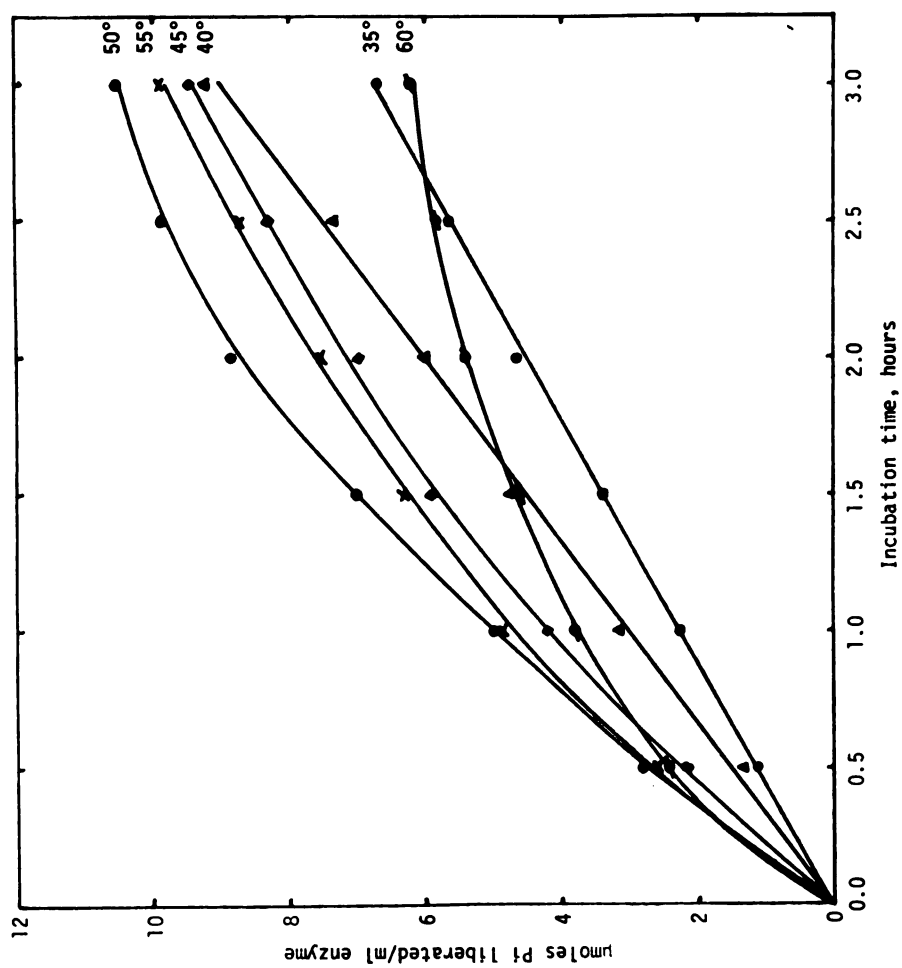


Figure 8.--P_i formation with time in a phytate-phytase system at various temperatures. Measured in 0.1 M acetate buffer, pH 5.3, and 1mM sodium phytate.

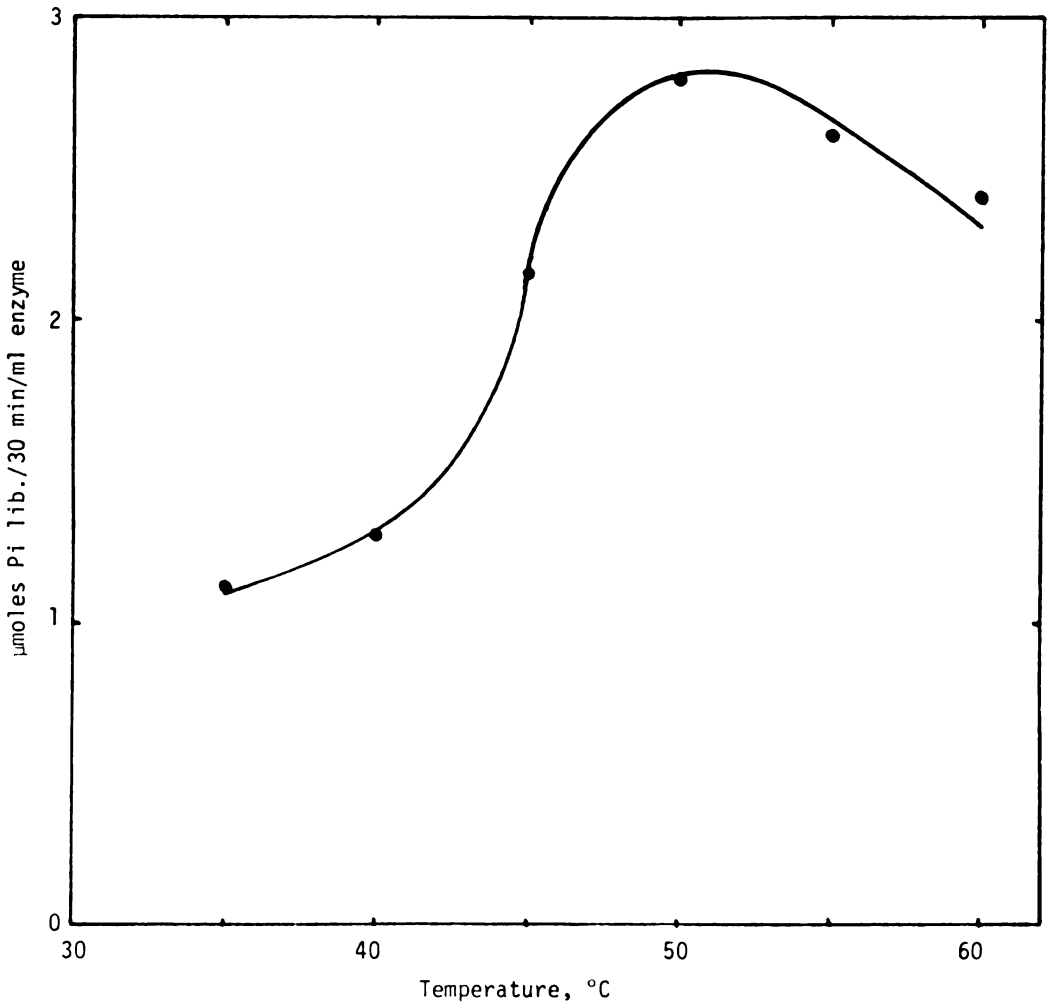


Figure 9.--Effect of temperature on bean phytase activity. Incubation time, 30 min; buffer, 0.1 M acetate pH 5.3; substrate, sodium phytate, 1 mM.

The activation energy of hydrolysis of phytic acid has been calculated from the data of Figure 8 by drawing the tangent (reaction rate constant) on each progress curve at time zero. Figure 10 is an Arrhenius plot of the natural logarithm of the reaction rate against the inverse of the absolute temperature. The slope of this curve gives the activation energy of hydrolysis for phytic acid which was found to be 11500 cal/mole. Nagai and Funahashi (1962) reported an energy of activation of about 12000 cal/mole for the wheat bran phytase, whereas Mandal and co-workers (1972) found 8500 cal/mole between 37°C and 57°C for the germinating mung bean phytase. In general, activation energies for transformation of reactants to products (catalysis) in enzyme-catalyzed reactions fall within the range of 6000 to 15000 cal/mole (Whitaker, 1972).

Thermal stability of phytase

As shown in Figure 11, assays with the preheated enzyme preparation at the various temperatures indicated that heating up to 50°C for 10 min did not depress the enzyme activity. However, at 65°C the activity was depressed to about 45% of the control value and at 80°C was practically completely inhibited. Peers (1953) in his study of the thermal inactivation of wheat phytase, reported that the preheated enzyme preparation of whole-meal (dry sample) at 80°C for 10 min did not lose any activity and that at 90°C there was about 40% of the enzyme activity of the control. However, he found that the enzyme in solution was stable up to 50°C for 10 min and the activity was completely inhibited at 70°C

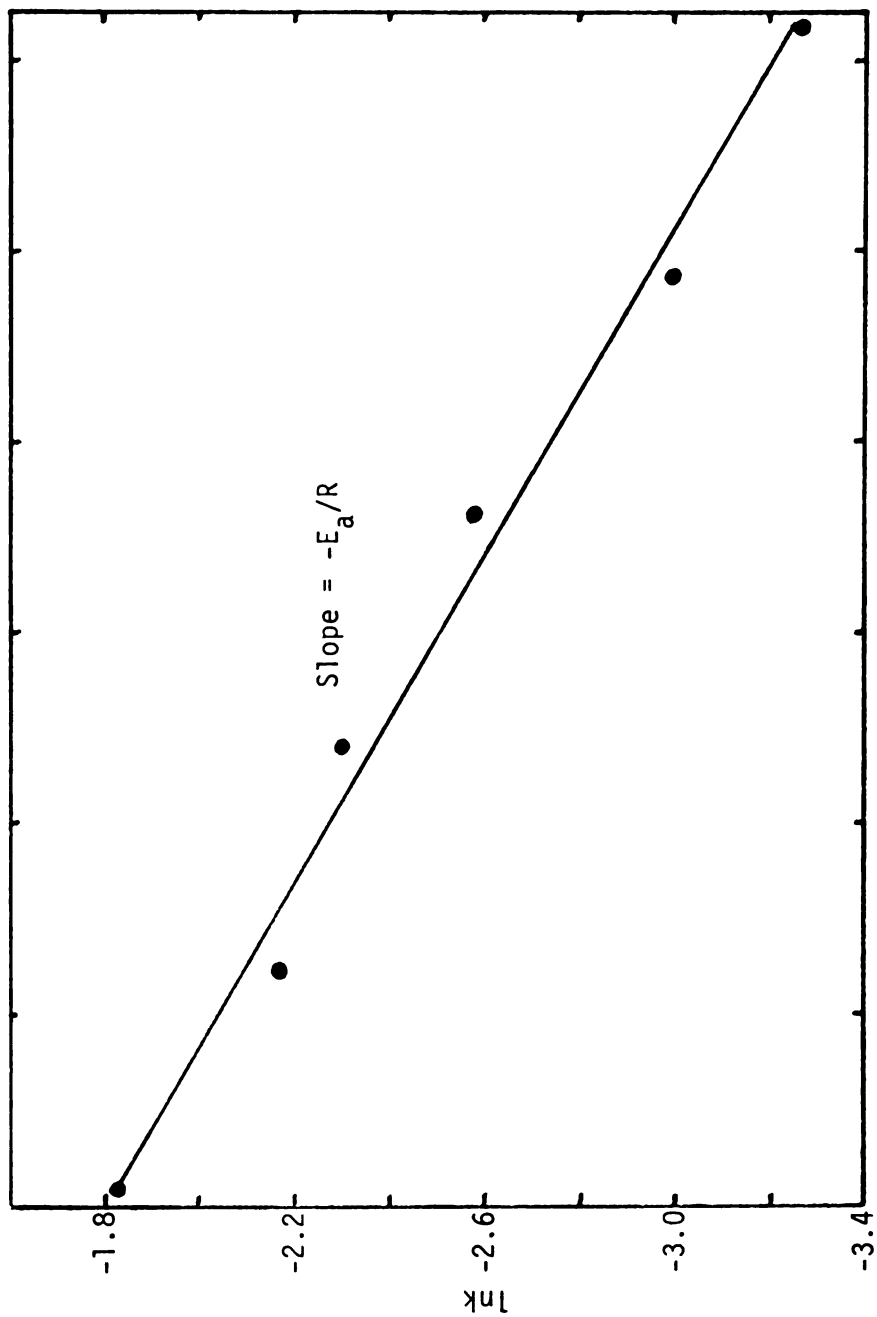


Figure 10.--Effect of temperature on the rate constant of the reaction in 0.1 M acetate buffer, pH 5.3, and 1 mM sodium phytate. Linear regression equation: $\ln k = 15.55 - 5.79 \times 10^3\ 1/T$.

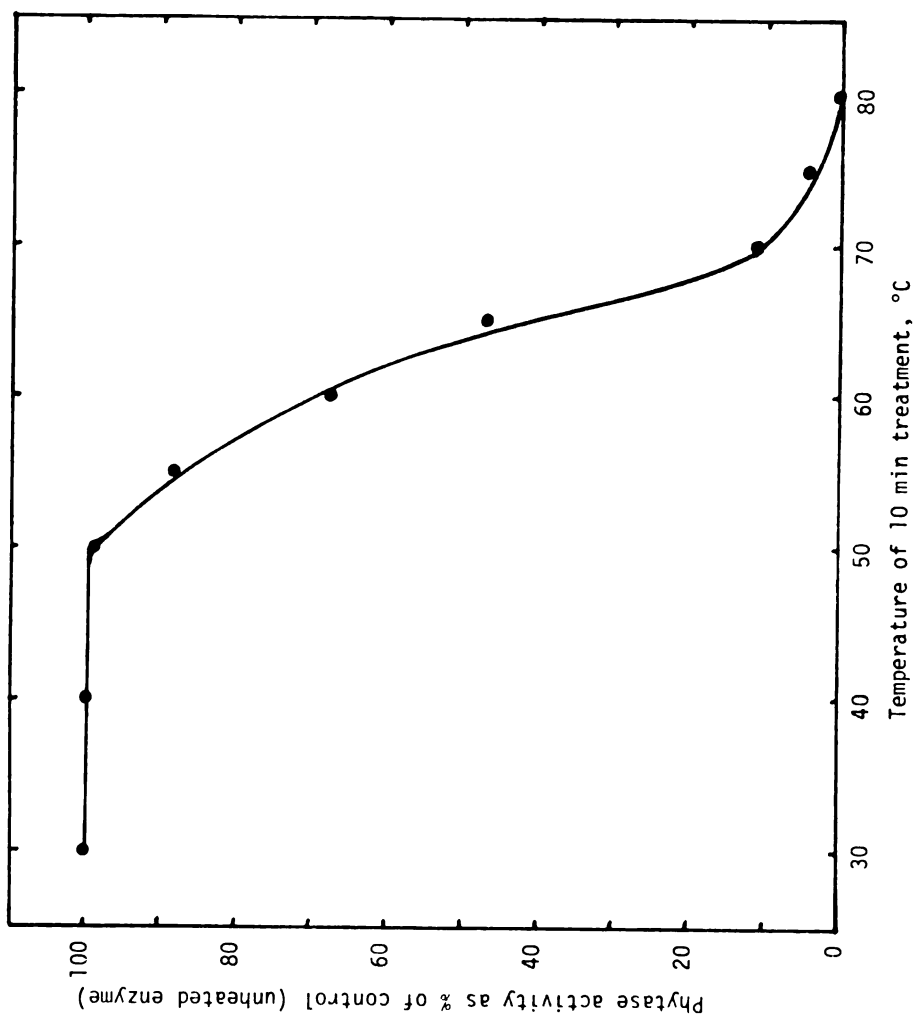


Figure 11.--Thermal inactivation of phytase. Aliquots of enzyme in 0.1 M acetate buffer, pH 5.3, were heated at various temperatures for 10 min. Activities were determined after adding the substrate at 50°C.

for 10 min. Corn phytase was found, also, to be stable during heat treatment up to 50°C for 10 min and at 80°C to have about 10% of the control activity. So, the resistance of phytase to heat treatment seems to vary, depending on the different sources of enzyme.

From the results of Figure 11, it was decided that 58°C and 68°C were suitable temperatures for observing the rate of inactivation. The results of these experiments are shown in Figure 12 where the natural logarithm of percentage activity remaining is plotted against time of heating. The curves are not linear, which indicates that the heat inactivation of phytase, under these conditions does not follow the unimolecular rate law which is frequently observed with other enzymes. The same was found to be true in the heat inactivation of wheat phytase (Peers, 1953).

By drawing the tangents to the two curves obtained in Figure 12 the pseudomonomolecular rate constants $k_{58°} = 0.014$ and $k_{68°} = 0.168$ are derived. By substituting these values in the Arrhenius equation

$$Ea = \frac{RT_1T_2}{(T_2 - T_1)} \ln \frac{k_2}{k_1}$$

the energy of inactivation was obtained. This was 55800 cal/mole. A value of 41600 cal/mole has been reported for the wheat phytase (Peers, 1953) between 55°C and 65°C. Activation energies for denaturation of enzymes usually fall within the range of 50000 to 150000 cal/mole (Whitaker, 1972).

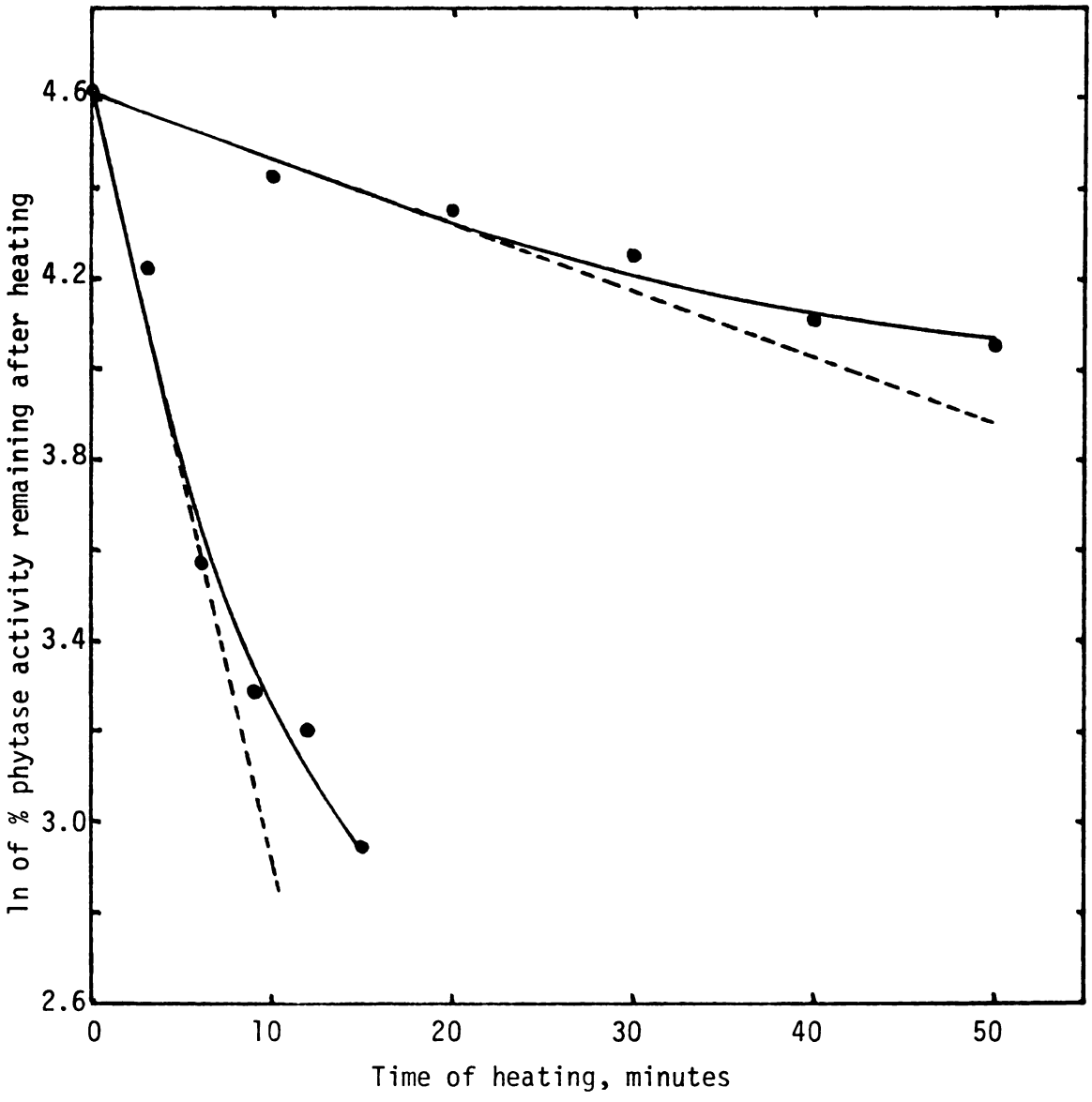


Figure 12.--Rate of denaturation of phytase at 58°C and 68°C. Buffered samples of phytase (0.1 M acetate, pH 5.3) were heated at 58°C and 68°C for varying periods, sodium phytate was added and the activity determined at 50°C.

The value of the temperature coefficient of inactivation $Q = k_{(t+10)}/k_t$ for the interval 58°C to 68°C is 12.

Mandal and co-workers (1972) found the mung bean phytase to be protected 80-85% when heated in the presence of phytate. They believe that the protection is mediated through the liberated inorganic phosphorus.

The experiments on the rate of thermal inactivation of the enzyme show that it is relatively stable and the high temperature for optimum activity is, presumably, partly a reflexion of this thermostability.

The enzyme was stable at 2°C for at least six weeks in 0.01 M tris-maleate buffer pH 6.5. The enzyme can, also, withstand freezing and subsequent thawing without great loss of activity, as it is shown by the following experiment. A small quantity of enzyme in 0.01 M tris-maleate buffer pH 6.5 was kept frozen for about one month. During this period the enzyme periodically was thawed at room temperature and frozen again for about 100 times. At the end of this period the activity was about 70% of that which initially existed. Lyophilization of an enzyme solution in 0.01 M tris-maleate buffer pH 6.5 and reconstitution of the dry matter did not have any significant effect on the activity of the enzyme.

Effect of enzyme and substrate concentrations on activity

Figure 13 shows that a linear relation exists between activity and enzyme concentration for the enzyme-concentration range used.

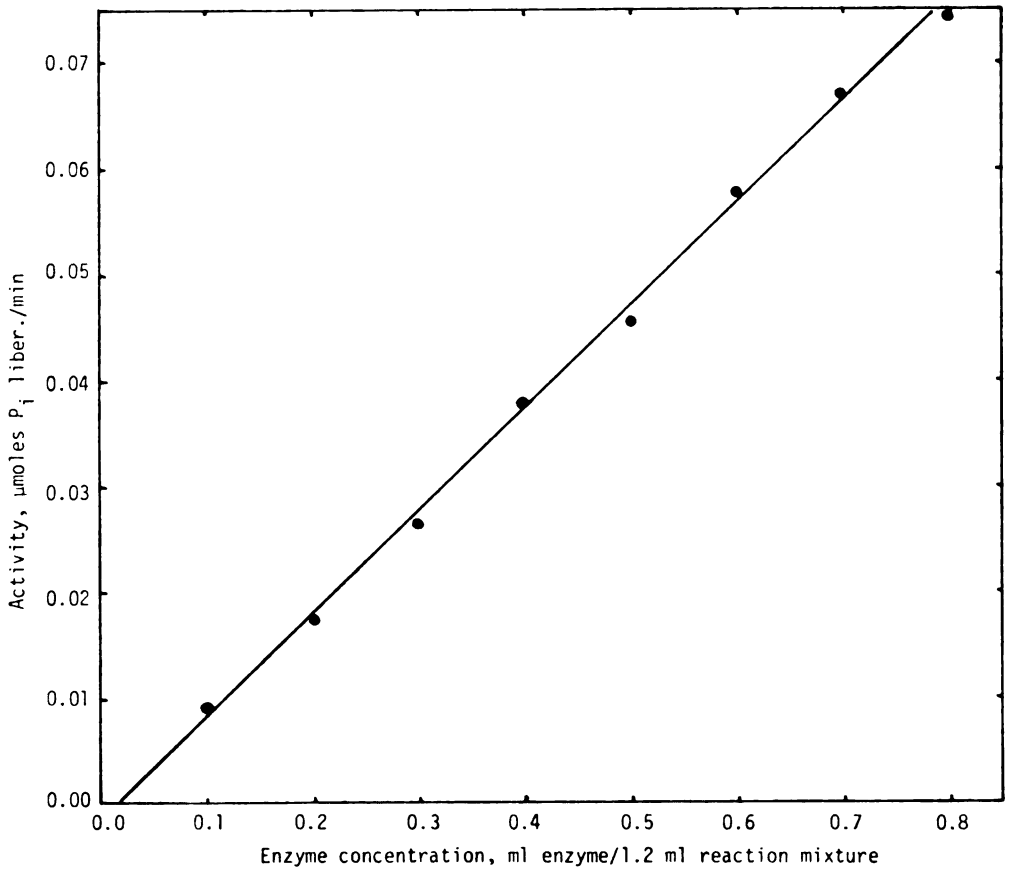


Figure 13.--Effect of enzyme concentration on phytase activity. Activities were determined in 0.1 M acetate buffer, pH 5.3, and 1mM sodium phytate at 50°C. Linear regression equation: Activity = -0.001 + 0.096 x (Enz. concentr.).

The initial velocity of the reaction, in terms of μmoles of orthophosphate liberated/min per ml of enzyme, was calculated and plotted against substrate concentration (Figure 14). Phytase is shown to be inhibited by concentrations of substrate higher than 1 to 2 mM, and practically stopped at substrate concentrations over 10 mM. A similar finding has been reported for the Dwarf bean phytase (Gibbins and Norris, 1963). Gibbins and Norris (1963) consider 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 enzyme units/ml enzyme, s = substrate concentration as molarity) the Michaelis constant K_m was found to be 0.018 mM and the V_{max} 0.2 μmoles/min per ml enzyme (Figure 15). 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 bran wheat phytase (Nagai and Funahashi, 1962), 0.15 mM for the Dwarf bean phytase (Gibbins and Norris, 1963) and 0.65 mM for the mung bean phytase (Mandal et al., 1972).

Inhibitors of phytase activity

The effect of fluoride, iodoacetamide and N-ethylmaleimide on phytase activity was studied. Figure 16 shows the effects of inhibitors on activity at the indicated final concentrations. The enzyme inhibition induced by fluoride was proportional to the various concentrations of sodium fluoride ranging from 0.0001 to 0.05 M.

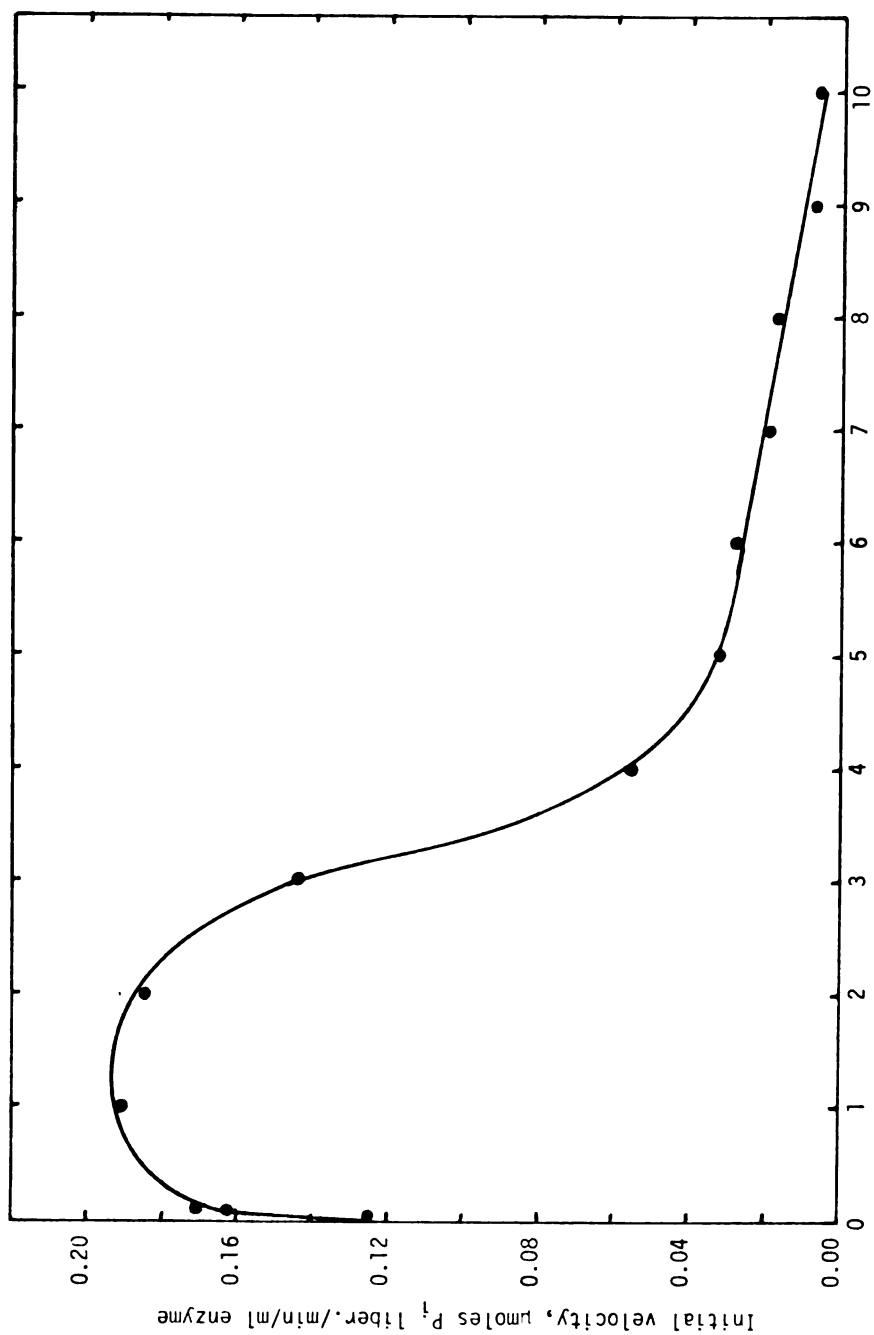


Figure 14.--Effect of substrate concentration on phytase activity. The phytase enzyme was incubated at 50°C in 0.1 M acetate buffer, pH 5.3, with the indicated concentrations of sodium phytate.

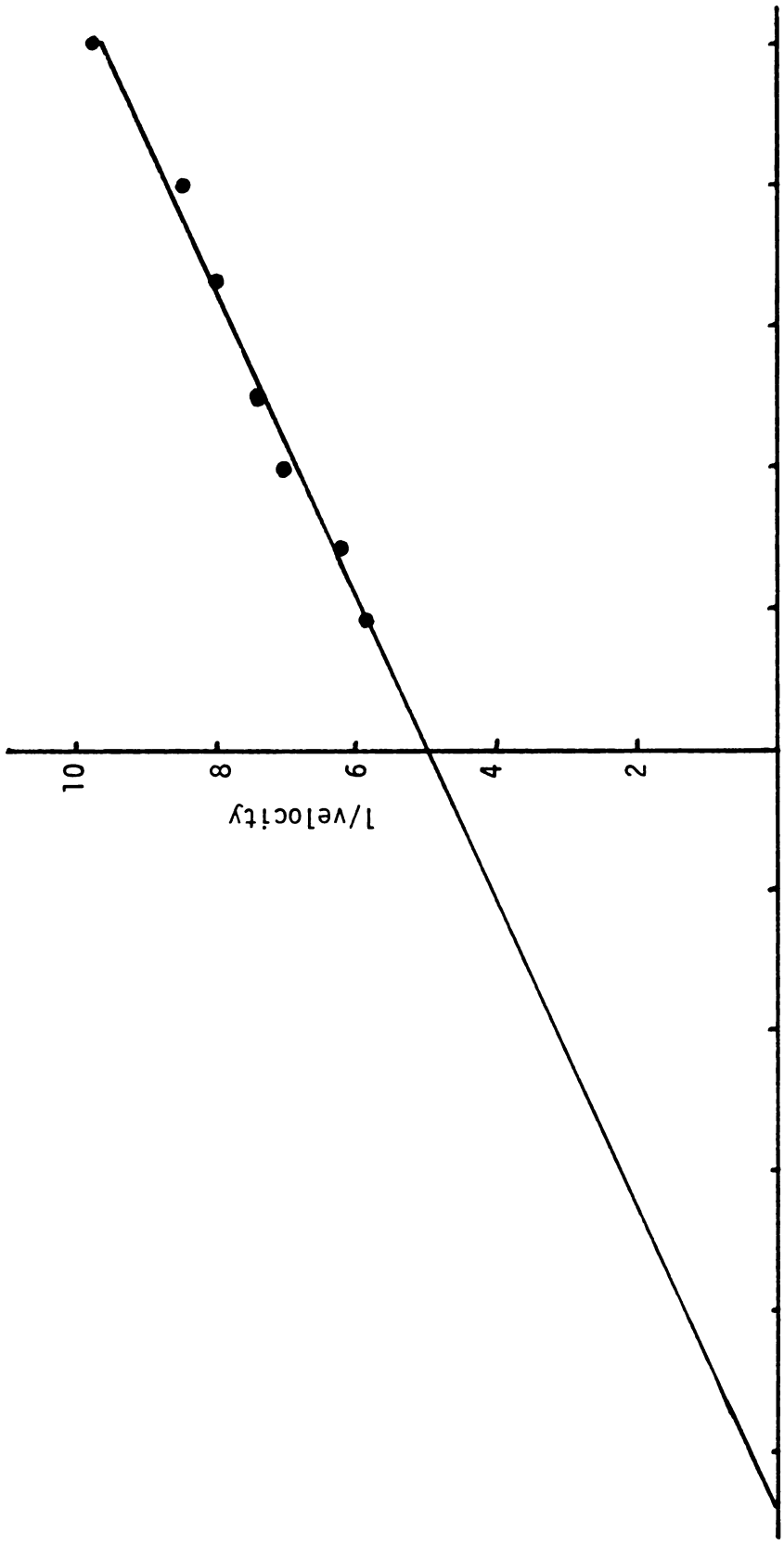


Figure 15.--Lineweaver-Burk plot for bean phytase with sodium phytate as substrate. Linear regression equation: $\frac{1}{V} = 4.999 + 0.092 \frac{1}{S}$.

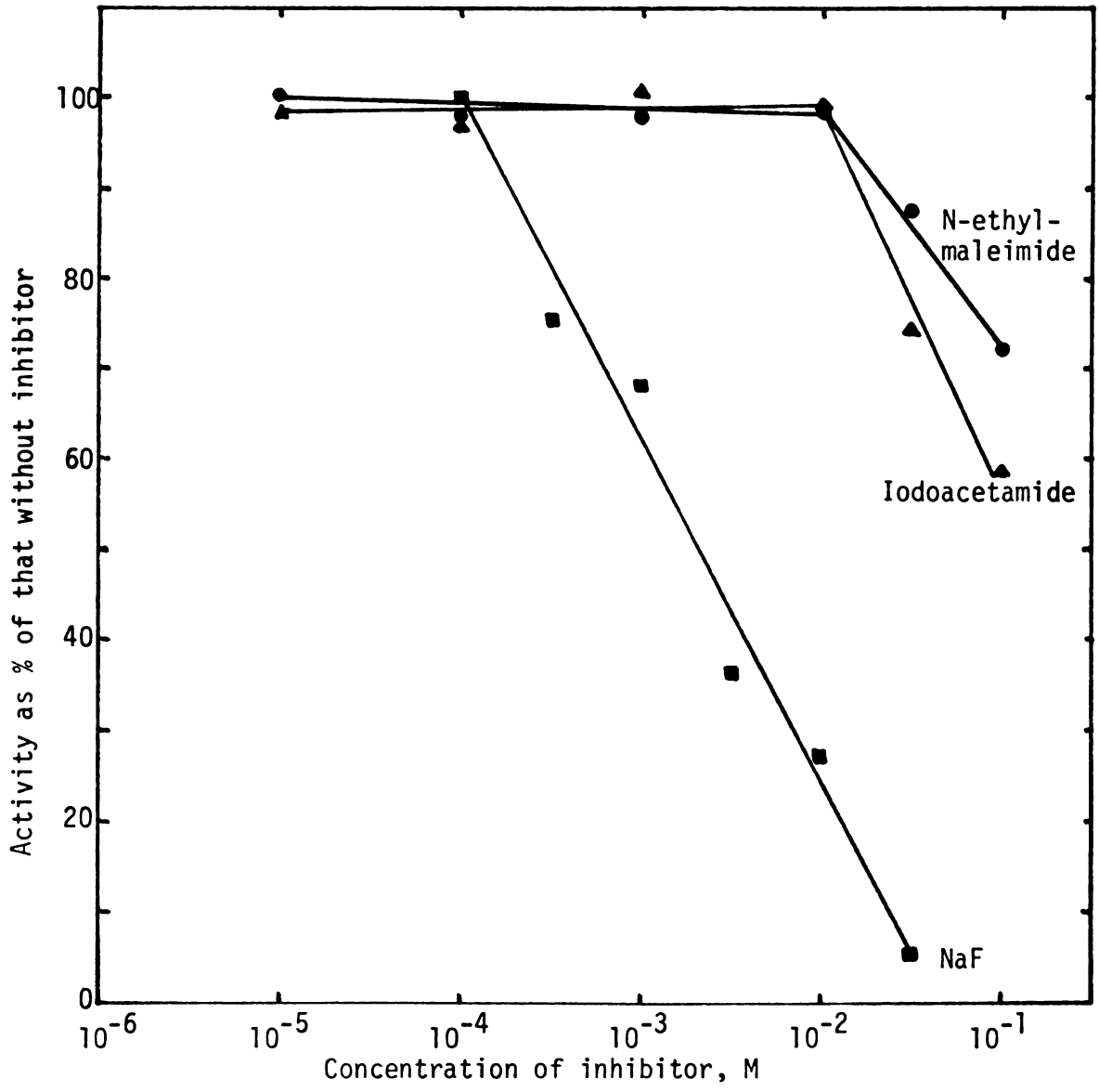


Figure 16.--Effect of inhibitors on phytase activity. Enzyme was incubated at 50°C and 0.1 M acetate buffer, pH 5.3, with 1mM sodium phytate and inhibitors at the indicated concentrations.

Iodoacetamide and N-ethylmaleimide did not have any effect on activity up to the level of 0.01 M. This means that the hydrolysis of phytic acid is not dependent on the presence of essential -SH groups at or near the active site. Alkylating reagents react with -SH groups irreversibly in such a manner that the reaction

$$\text{Enzyme-SH} + ICH_2CONH_2 \longrightarrow \text{Enzyme-S-}CH_2CONH_2 + HI$$

proceeds far to the right, with formation of a covalent derivative of the enzyme.

Sodium chloride at relatively high concentrations shows an inhibitory effect on phytase activity as it is shown in Figure 17.

Effect of reducing and chelating agents on phytase activity

As shown in Table 2, L-cysteine, ascorbic acid, reduced glutathione and β-mercaptoethanol did not have any effect on phytase activity even at a final concentration of 0.01 M.

Chelating agents such as tartrate, citrate, oxalate and EDTA (ethylenediamine tetraacetic acid) had no effect on the phytase activity tested, as shown in Table 3.

Effect of metal ions on phytase activity

As shown in Table 4, all the metallic ions tested including magnesium and calcium did not show any effect upon phytase activity with the exception of zinc, ferrous, ferric, cupric and mercuric ions that have inhibitory effects at final concentration of 10^{-3} M. It must be taken into consideration that these inhibitory metallic

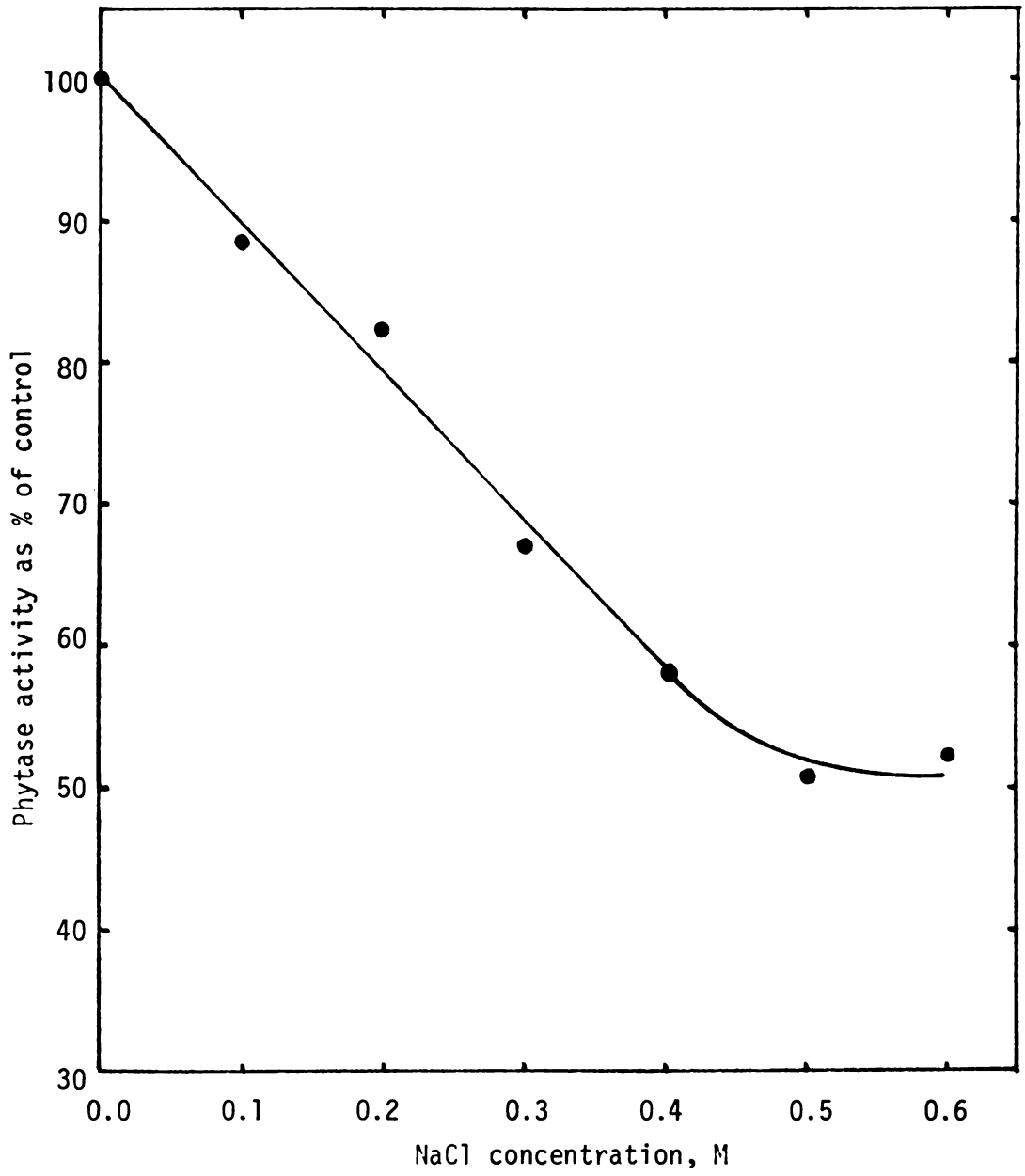


Figure 17.--Effect of NaCl on activity. Activity was measured with the indicated concentrations of NaCl in 0.1 M acetate buffer, pH 5.3, and 1 mM sodium phtate at 50°C.

Table 2.--Effect of reducing agents on phytase activity.

Reducing Agent	Relative Activity*			
	10^{-4} M	10^{-3} M	10^{-2} M	10^{-1} M
L-cysteine	107%	105%	109%	10%
Ascorbic acid	96	104	98	36
Glutathione (red.)	109	110	103	18
β-mercaptoethanol	94	96	104	76

*The activity without added reducing agents was taken as 100%.

Table 3.--Effect of chelating agents on phytase activity.

Reducing Agent	Relative Activity†		
	10^{-6} M	10^{-4} M	10^{-2}M
Tartrate	91%	97%	90%
Citrate	92	108	94
Oxalate	103	105	94
EDTA	100	109	111

†The activity without added chelating agents was taken as 100%.

Table 4.--Effect of metal ions on phytase activity.

Metal Ions	Relative Activity*		
	10^{-5} M	10^{-4} M	10^{-3} M
$ZnSO_4$	96%	83%	59%†
$MnSO_4$	101	106	110
$MgSO_4$	102	106	107
$FeSO_4$	93	76†	69†
$FeCl_3$	102	84†	63†
$CuSO_4$	105	93	44†
$CaSO_4$	102	101	111
$HgCl_2$	103†	54†	25†
$CdCl_2$	97	101	99
$NiCl_2$	105	107	103
$CoCl_2$	102	106	137
$AlCl_3$	99	98	98
$AgNO_3$	94	88†	90†

*The activity without added metal ions was taken as 100%.
†Precipitation was observed.

ions have a rather strong affinity to phytic acid itself and so there is some competition between the metallic ions and enzyme for the substrate. Obviously, heavy metallic ions such as cupric, silver and mercuric ions can have an adverse effect on the enzyme protein. Strange is the behavior of silver ion which does not show any inhibitory effect even at 10^{-3} M. An explanation might

be that since silver is monovalent it does not require much phytic acid for its precipitation, and being removed as a precipitate does not harm the enzyme which can act on the rest of the substrate. However, in wheat bran phytase silver ion at a higher concentration of 10^{-2} M depressed the activity to 66% of that which initially existed (Nagai and Funashashi, 1962).

Peers (1953) reported activation of wheat phytase by magnesium and calcium ions. Also, Gibbins and Norris (1963) 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). The Navy bean phytase, in the present study, showed an increase in activity by about 35% as a result of the addition of 10^{-3} M Co^{2+} in the enzyme mixture. There are no data in the literature on the effect of Co^{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 Co^{2+}. Figure 18 shows the optimum concentration of cobalt for maximum activity.

Separation of phytase from phosphatase

The fractionation of a crude enzyme extract from beans on a DEAE column is illustrated in Figure 19. The first enzyme emerging from the column is a phosphatase that hydrolyzes β-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

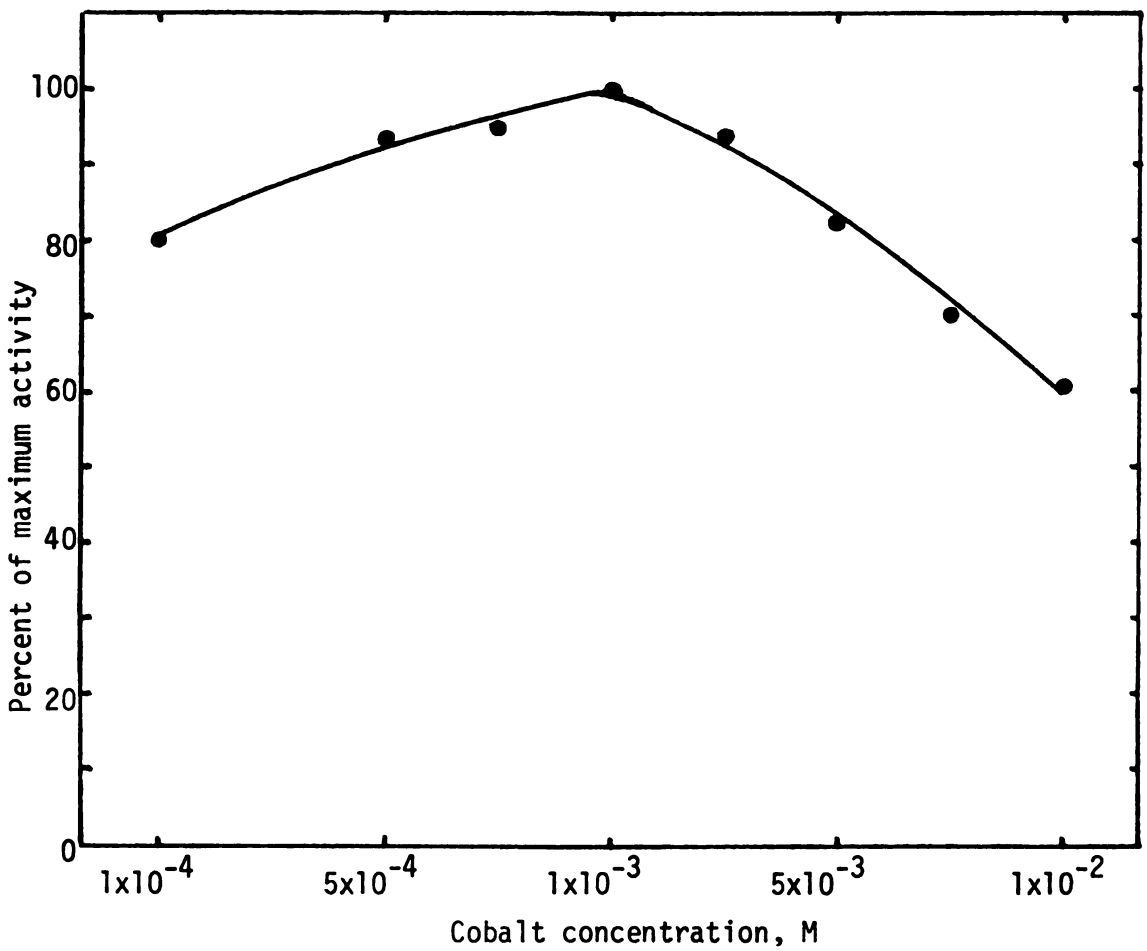


Figure 18.--Effect of Co^{2+} on phytase activity. The enzyme was incubated at 50°C in 0.1 M acetate buffer, pH 5.3, with 1 mM sodium phytate and Co^{2+} at indicated concentrations.

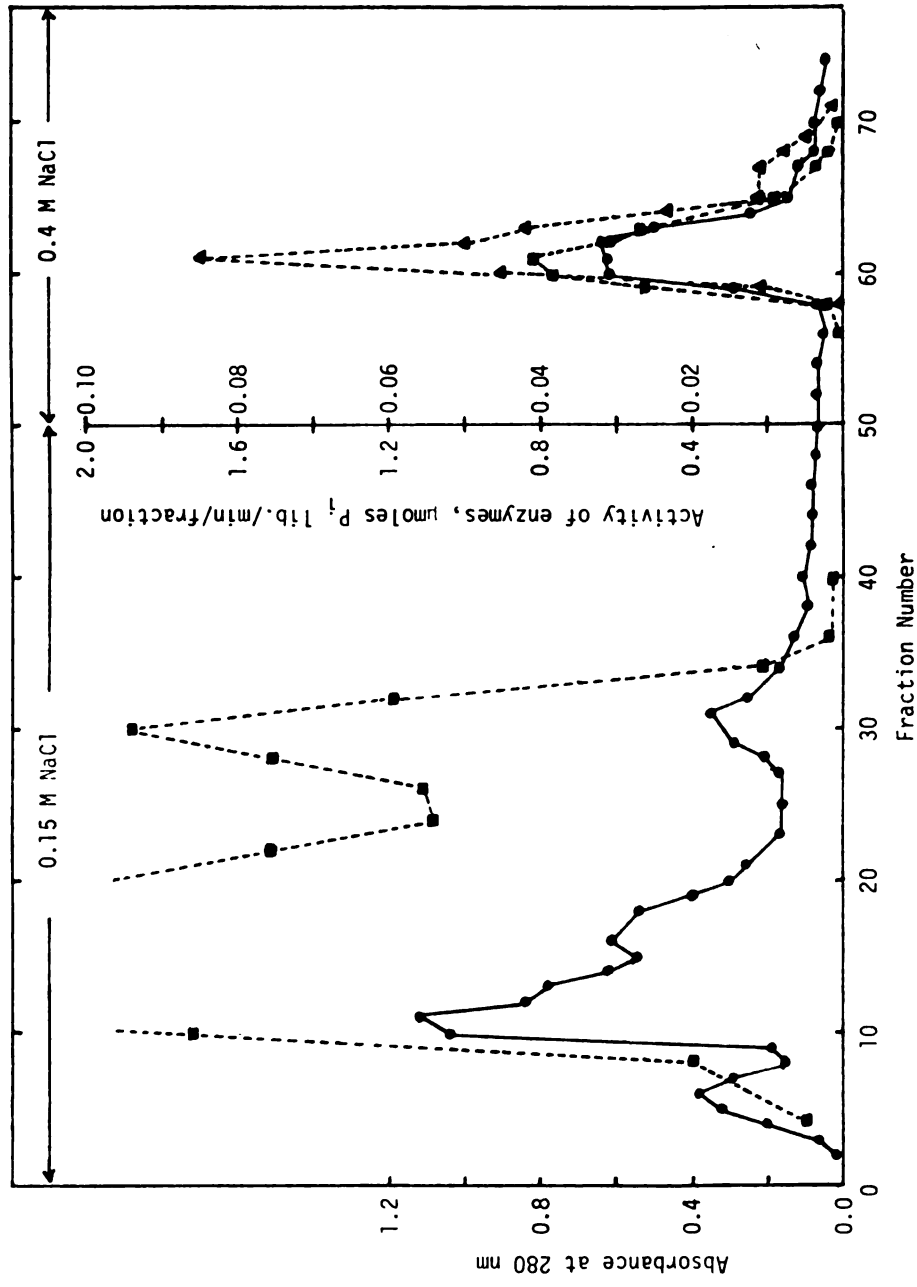


Figure 19.--DEAE-cellulose chromatography of bean extract. Protein was eluted from column with NaCl solutions in 0.01 M tris-HCl buffer, pH 7.4. ●, absorbance at 280 nm; ■, phosphatase activity; ▲, phytase activity. The left scale in the center, is for phosphatase and the right for phytase activity.

the fact that the phytase fraction, but not the phosphatase fraction, shows an increase in activity on β-glycerophosphate by about 35% in the presence of 10^{-3} M Co^{2+}. It is evident that a clear distinction can be drawn between the two enzymes.

Substrate specificity of phytase

As shown in Table 5, the phytase, purified by DEAE-cellulose chromatography, had a broad substrate specificity catalyzing the hydrolysis of all the phosphomonoesters tested.

Phytase hydrolyzes much easier and faster all the substrates tested except phytic acid and is characterized by a potent pyrophosphatase activity. The same broad specificity was shown by a 1500-fold purified preparation of wheat bran phytase (Nagai and Funahashi, 1962). Courtois and Pèrez (1948a) found in all the seeds they studied that phytase hydrolyzed pyrophosphate and

Table 5.--Substrate specificity of purified phytase preparation.

Substrate	Activity (units/ml enzyme)	Relative Activity
Phytic acid	0.003	1.0
5'-Adenylic acid (AMP)	0.029	9.7
β-Glycerophosphate	0.046	15.3
α-Glycerophosphate	0.057	19.0
Phenyl phosphate	0.185	61.7
Pyrophosphate	2.205	735.0

glycerophosphate more rapidly than phytic acid. The Navy bean phytase follows the same rule by being able to hydrolyze several phosphomonoesters in addition to phytic acid.

Phytase activity and breakdown of phytic acid during germination

Phytase activity increases during the first 6 to 8 days of germination (Figure 20). On the 6th day of growth this activity is about 7 times higher than that of the mature seed. A 6.5-fold has been reported for wheat on the 5th day of germination (Peers, 1953) and a 40-fold increase for corn on the 4th day of germination (Chang, 1967). No increase in activity was observed when beans were soaked in distilled water for 24 or 48 hours. Even during germination for 2 days no significant increase in activity was observed. An abrupt increase in activity was observed from the fourth to the sixth day of germination. The activity subsided after the tenth day.

The increase in phytase activity is accompanied by a decrease in phytic acid, which is used by the enzyme, and increase in the amount of orthophosphate (Figure 21). From Figures 20 and 21 it can be deduced that the first 2 days of the germination period represent a latent period during which neither significant increase in phytase activity and orthophosphate is observed nor diminution in the content of phytic acid. Phytic acid is broken down to liberate orthophosphate which can be used for synthesis of organic phosphate substances in the developing plant. All the

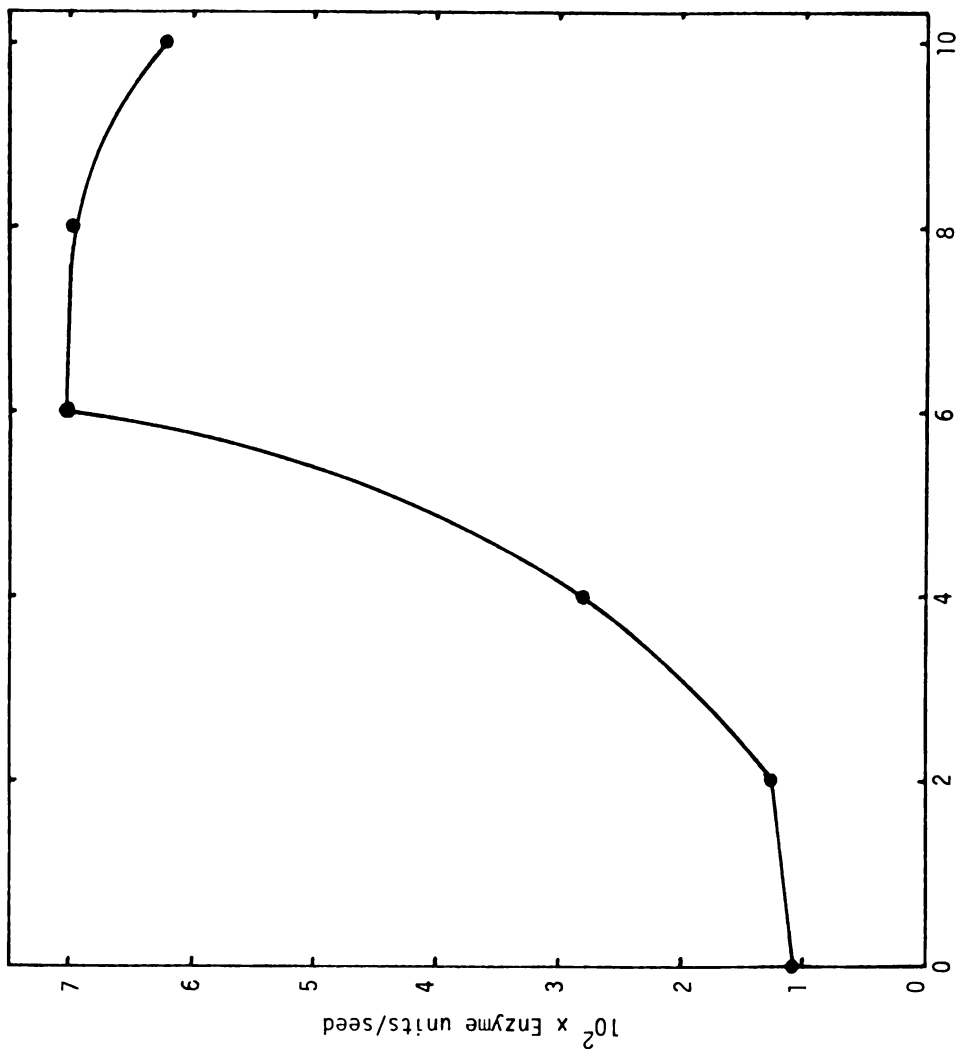


Figure 20.--Effect of germination on phytase activity.

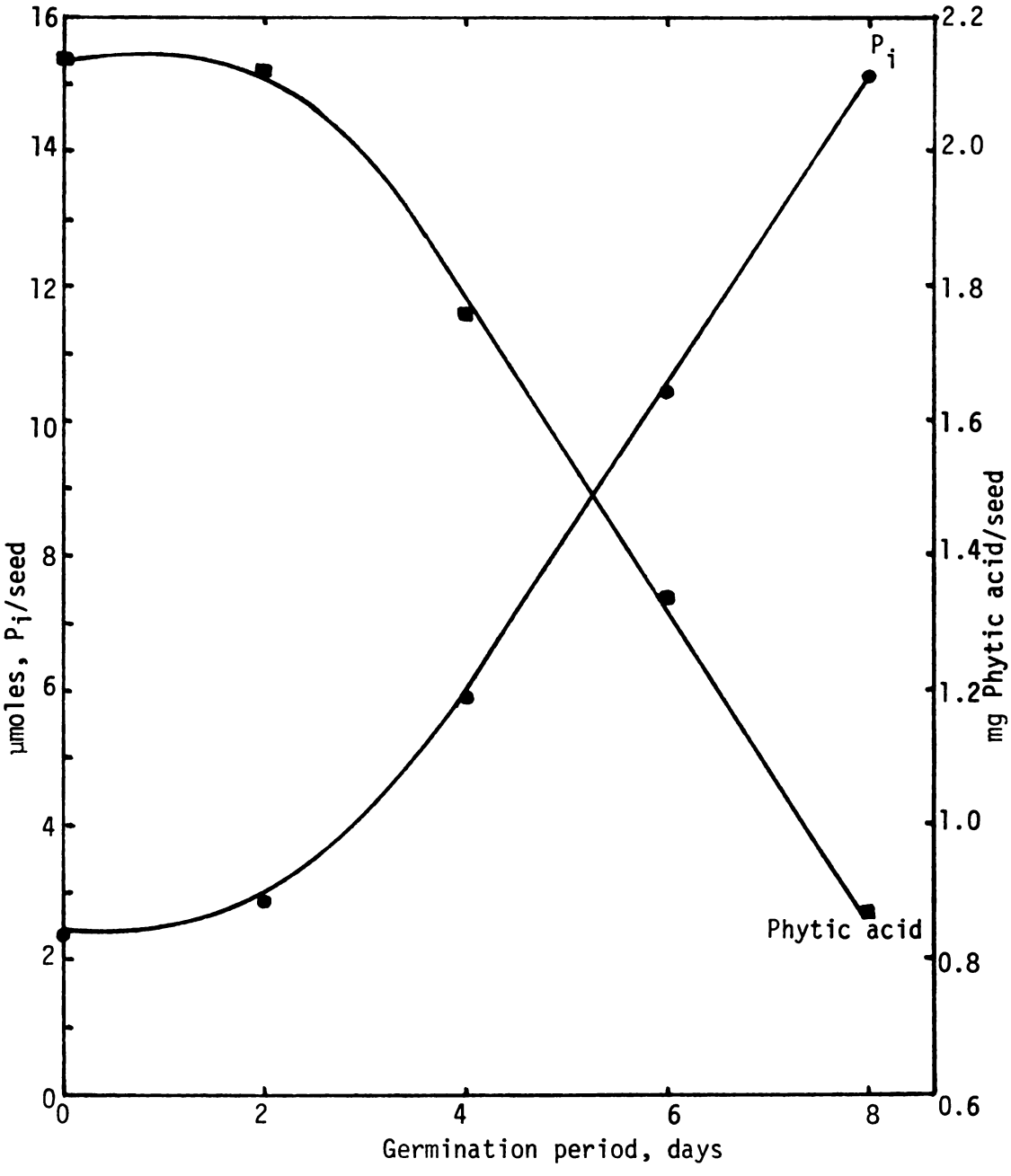


Figure 21.--Effect of germination on phytic acid and P_i content of germinating beans.

loss in phytic acid phosphorus was recovered as a gain in inorganic phosphorus in this experiment.

Sartirana and Bianchetti (1967) using ^{32}P inorganic phosphate indicated that the rate of phytin breakdown is controlled in vivo by the concentration of inorganic phosphate, through the inhibition of phytase activity.

The weak phytase activity in the dry and soaked bean seeds accompanied by the non-destructibility of phytic acid during cooking is an important factor in the bean diet since the animal organism must depend on the presence of intestinal phytase for breaking phytates and making available the inorganic phosphorus to the animal.

CONCLUSIONS

Fifty cultivated varieties and lines of mature dry beans (*Phaseolus vulgaris* L.) were analyzed for phytic acid, total phosphorus, inorganic phosphorus, and other than phytate organic phosphorus. The respective concentrations on a dry weight basis were 0.54-1.58%, 0.259-0.556%, 0.021-0.044%, and 0.050-0.135%. A correlation coefficient of 0.9847 was found between total phosphorus content and phytic acid content. The high correlation coefficient allows the estimation of phytic acid through the determination of total phosphorus by the linear regression equation

$$Y = 0.141 + 0.273\ X$$

where Y is the percentage content in total phosphorus and X the percentage content in phytic acid. A protein-phytate complex was also isolated. The observation was made that 99.6% of the total phytic acid was in a water-soluble form.

The Navy bean phytase had an optimum pH of 5.3 with 0.1 M acetate buffer and phytic acid as substrate and an optimum temperature of 50°C. The Michaelis constant with phytate as substrate was 0.018 mM. The activation energy of hydrolysis of phytic acid was 11500 cal/mole and the inactivation energy of enzyme 55800 cal/mole. 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 can be characterized as a nonspecific phosphomonoesterase with phytase and potent pyrophosphatase activity. This enzyme is inhibited by high concentrations of phytate. No effect on the activity was caused by the addition of Mg^{2+} or Ca^{2+}, metals that have been shown to activate phytase from other sources, but Co^{2+} increased activity by about 35%.

Soaking of beans did not affect the phytic acid content and the phytase activity of the beans. Cooking did not destroy phytic acid. Germination was followed by an increase in phytase activity and breakdown of phytic acid.

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