THE UNIVERSITY OF MANITOBA

THE INTERACTION OF PHYTIC ACID WITH LEGUME PROTEINS FROM *Vicia faba* (minor)

BY

RONALD W. MARTENS

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Abstract

The binding mechanism of phytic acid to fababean (Vicia faba var.Diana) protein and the subsequent control of binding were studied. In the literature it is hypothesized that a ternary structure composed of negatively charged protein, negatively charged phytic acid (pK 1.8) and a divalent cation may be functioning when protein isolates are prepared. Protein was concentrated by both physical and chemical methods with the resultant protein, phytic acid and mineral levels being determined in order to explore such a theory.

Protein was recovered by pin milling fababean seeds and air classifying the resultant flour (29.9 % protein) to obtain a 67.0% protein concentrate. When the protein was processed by this physical method, the phytic acid level increased from 1.57% in the flour to 4.10% in the protein concentrate. When the flour was subjected to chemical recovery techniques, protein isolates prepared by the isoelectric precipitation method (73.3% protein) contained 3.0% phytic acid. When the protein micellization method was used, the isolates (89.6% protein) contained 0.39% phytic acid and when the salt solubilization/acid precipitation method was the isolation procedure, the isolates (78.3% protein) contained 1.32% phytic acid. Additionally, protein isolates were prepared from flour by the isoelectric precipitation technique over a pH range from 7.0 to 12.0. By this method, 44-56% of the native phytic acid, 58-76% of the protein and

15-35 % of the native calcium were retained, depending upon the pH of solubilization.

Using highly purified reactants (protein, phytic acid and calcium chloride), different binary and ternary systems were studied. In each study system the resultant turbidity was monitored at 600 nm. and the soluble protein was measured at 279 nm. Of the three possible binary systems, only the calcium chloride and phytic acid combination resulted in a turbid state and this could be removed by filtration. When all three components were allowed to react, two types of turbidity were observed. One, typical of the binary complex was removed readily by filtration, while the second arising from the ternary complex could not be removed by this technique. In the ternary system, all other concentrations being equal, when the calcium chloride levels were altered, the level of turbidity and the amount of insoluble protein were related directly to the change in calcium levels. When different protein isolates were prepared in the presence of ethylenediaminetetracetate (EDTA), each fraction produced during the isoelectric precipitation method was monitored for phytic acid. It was found that as the EDTA levels increased, the amount of phytic acid in the isolate decreased, while that in the supernatant increased. These data suggest that blocking the ternary complex, which appears to be operating here, is a potentially useful approach in the preparation of protein isolates low in phytic acid.

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Introduction

During the maturation of a seed, myoinositol reacts with phosphoric acid to form myoinositol 1, 2, 3, 4, 5, 6 hexakis (dihydrogen phosphate) which is commonly known as phytic acid. Various levels of phytic acid have been found in cereal, legume and oilseed crops. Phytic acid, since it can be hydrolized, has the ability to bind cations and the resultant complex, due to its strength, cannot be digested in the human gastrointestinal tract (Omosaiye et al.,1979). Therefore, it is obvious that the very existence of a mineral in a foodstuff is not an adequate indicator of its availability to meet nutritional needs.

The study of phytic acid in seeds has shown that the compound interacts with proteins (O'Dell et al., 1976) and this interaction becomes important when considering the preparation of proteinaceous materials. It is thought (Okubo et al.,1976) that the most probable mechanism of protein and phytic acid binding is by electrostatic interaction since phytic acid has a pK of 1.8 and many plant proteins have acidic pK values. Since most protein isolate processing conditions are carried out under alkaline pH, the phytic acid and protein will probably both have a net negative charge. This being the case, it is hypothesized that a ternary complex may result where a multivalent cation bridges between the two negatively charged species (Omosaiye et al.,1979). It is the intent of this research to explore the

possible development of a ternary complex between fababean protein, a multivalent cation and phytic acid.

Literature Review

Occurrence

Phytate, the mixed calcium and magnesium salt of phytic acid, was first employed to describe the major organic phosphate component of seeds. Initially it was considered to be an intermediate in the fixation of carbon dioxide by chlorophyll. Although this concept has since been abandoned, the name has been retained and applied to similar materials from soil and from the blood of birds and turtles (Johnson and Tate, 1969).

Phytate, the salt of phytic acid has many and varied sources. It has not been found in bananas, celery, citrus fruit, lettuce, onions or prunes while only traces have been found in apples, broccoli, carrots and green beans. Moderate amounts of phytate are found in artichokes, figs, potatoes and strawberries while the greatest amount is in cereals, legumes and nuts (Harland and Harland, 1980).

The phytate found in seeds is located in various regions throughout the kernel. The endosperm of wheat and rice kernels were reported by Erdman and Forbes (1977) to be almost devoid of phytate while the germ and outer layers had quite high levels of the compound. Williams (1970) found that in wheat, Triticum vulgare cv., phytic acid was largely concentrated in the testa and pericarp fractions of the kernel. In the rice, the phytic acid region was found by autoradiographic techniques and electron microprobe x-ray analysis to be concentrated in the aleurone layer and scu-

tellum. It was proposed that the scutellum cells are the biosynthetic site of phytic acid in rice(Ogawa et al.,1979). The phytate of corn exists mainly in the germ (88%) unlike that of the soybean phytate (O'dell et al.,1972). In contrast to many other oilseeds and cereals, soybean phytate appears to be located ubiquitously throughout the seed. Peanut, cottonseed and sunflower phytate are concentrated within crystalloid type structures and since soybeans do not possess such bodies phytate is distributed throughout the kernel.

The amount of phytate which occurs naturally within cereals and oilseeds (Table 1) ranges from less than 1% in oats, corn, rice and barley to more than 5% in defatted sesame meal (Cheryan, 1980). In general, it has been found that cereal flours, in which the bran has been removed, contain less phytic acid than when the bran is present (Singh and Reddy , 1977; Cheryan, 1980).

Phosphorus, an important mineral nutritionally, has been found in most cereals and legumes as phytic acid. In the majority of leguminous seeds, phytate phosphorus accounts for approximately 80% of the total phosphorus (Reddy and Salunkhe, 1981) and in oilseed soybean meals 70-80% of the phosphorus is bound in the phytate complex (Saio et al.,1967). Rice phytate contains 80% of the total phosphorus in the seed (Ogawa et al.,1979) while wheat has 88% of its total phosphorus in the phytic acid form (Williams, 1970).

Table 1

Typical Phytic Acid Contents(% dry basis) of some cereals and oilseeds

Sample	Phytic Acid(%)
corn	0.89
wheat, soft	1.13
rice, brown	0.89
soybeans	1.40
peanut meal, defatted	1.70
sesame meal, defatted	5.18
rapeseed meal, defatted	3.69
cottonseed flour	
glandless	4.80
lima beans	2.52
navy beans	1.78
barley	0.99
oats	0.77
coconuts	2.38

Phytate, found in the raw product, will usually carry over into the finished product. Table 2 (Harland and Harland, 1980) lists the amount of phytic acid found in grains and the amount of phytic acid concentrated in breads made from some of those grains. Some breads in the list have added wheat flour.

Miller et al., (1980a) investigated the effect of the environment upon the concentration of phytic acid in four oat cultivators. The environment, rain and soil type, had a significant effect upon the levels of phytic acid in the oat cultivars.

Phytate in animal tissue has been researched and has been found to play a physiological role. In the animal, phytate is a constituent of the phospholipids in lipoproteins and membranes. In some animal species the compound is a required vitamin (Erdman and Forbes, 1977) while in others it is an allosteric regulator of oxygen release from hemoglobin (Chang and Schwimmer, 1977).

Phytate in plants is the principal storage form of phosphorus (Nahapetian and Bassiri, 1975). Erdman and Forbes (1977) reported that in potatoes, phosphorus is starch bound during the early stages but as the potato matures the phosphorus appears as phytic acid. It is thought that this occurs in order to prevent excessively high levels of inorganic phosphorus. In soybeans, phytic acid is believed to play an inhibitory role by restricting the availability of zinc to aflatoxin producing mold.

Table 2

Phytate(% dry weight) in grain and breads

Source	Phytic Acid (%)
Grains:	
wheat bran	3.70
soybeans	2.58
wild rice	2.20
barley	1.19
wheat flour	0.96
oats	0.77
corn	0.12
Breads:	
cornbread	1.36
whole wheat	0.56
rye	0.41
pumpernickel	0.16
raisin	0.09
french	0.03
white	0.03

As can be seen, phytic acid occurs to some degree in most foods that humans consume. Its presence in the seed has a positive role but as shall be seen later, its presence in food has a negative nutritional affect.

Structure

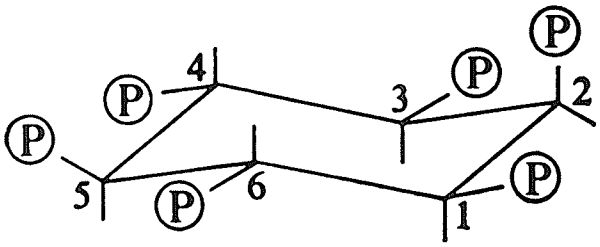
In the literature a controversy has developed over the detailed type of structure of phytic acid. Points at issue involve the many isomeric forms of the phosphate groups within the compound as well as cross linkages between phosphate groups which may occur within the molecule. The chemical structure is questioned therefore on the basis of multiple isomeric forms of hydroxyl groups (Erdman and Forbes, 1977). Two structures have been proposed as being the correct conformation of phytic acid (Fig. 1). The Anderson structure is a symmetrical hexaorthophosphate while the Neuberg structure is asymmetrical (Johnson and Tate, 1969).

Johnson and Tate (1969) studied the structure of phytic acid by the use of crystallization studies. The findings supported the Anderson structure (18 hydrogens) which has six acid hydrogens less than the Neuberg structure (24 hydrogens). The careful potentiometric titration results of Hoff-Jorgenson show that there are six strongly dissociated protons at pH less than 3.5 and that there are six weakly dissociated protons (pK 4.6-10). These results are in accord with the Anderson structure (Johnson and Tate, 1969). Brown et al., (1961), supporting the Neuberg structure, found that six of the eighteen acidic hydrogen mol-

Figure 1. Two proposed phytic acid structures:
1. Symmetrical hexaorthophosphate structure proposed by Anderson.
2. Asymmetrical hydrated tripyrophosphate structure proposed by Neuberg.

(Johnson and Tate, 1969)

Anderson (1)

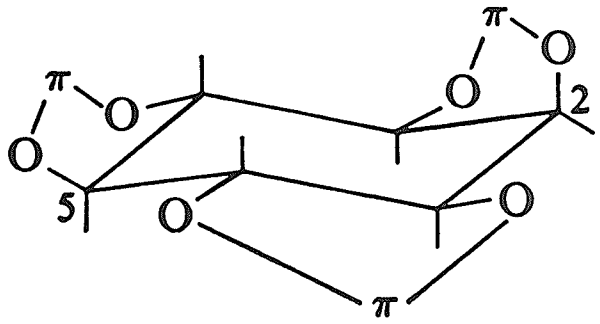


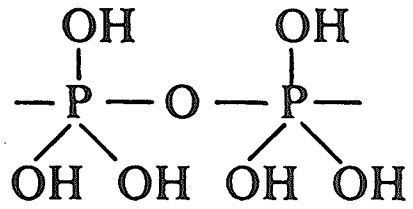
1

Ⓟ = OPO_3H_2

$\pm 3H_2O$

Neuberg (2)



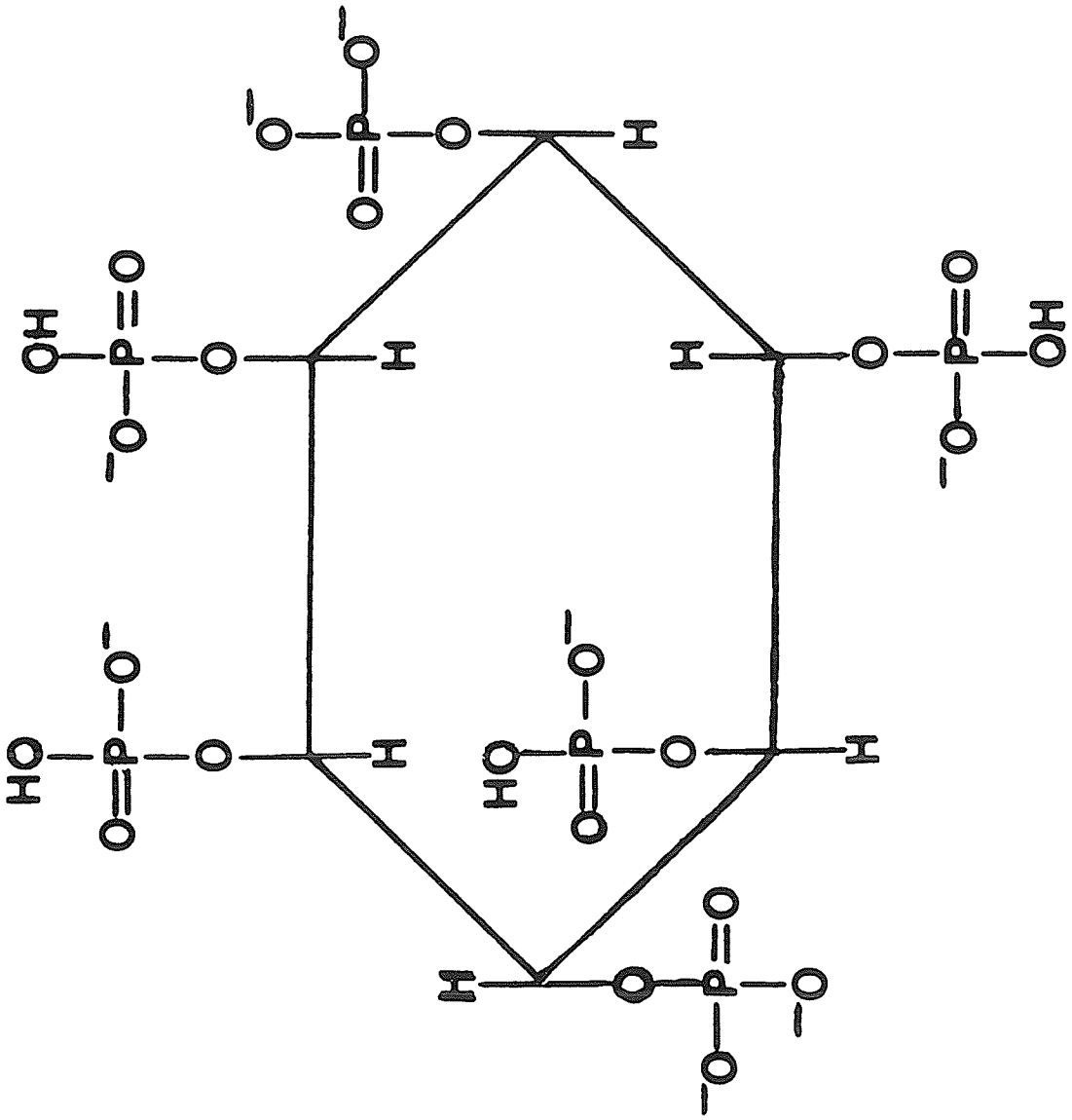
2 $\pi =$ 

ecules were not titratable in a basic solution. This analysis, which involved the titration of a phytic acid solution with a solution containing an excess of metal ions supported the 18 titratable hydrogen structure. Brown et al., suggests that the findings like those of Johnson and Tate of 12 titratable hydrogens is logical but incomplete since six protons are not titratable in water. Under ordinary conditions, normal salts are not readily formed and erroneous conclusions could be drawn on the basis of the number of moles of metal ion contained per mole of phytate.

The controversy over the configuration of phytic acid has gone on for many years, but now it is generally accepted that the Anderson structure is most likely correct (Cheryan, 1980). The suggestion by Brown et al., that there may be two phytic acids where the Anderson structure is simply the degradation product of the Neuberg structure, may still be valid. Assuming the Anderson structure to be correct, the proper name for phytic acid is myoinositol 1, 2, 3, 4, 5, 6 - hexakis (dihydrogen phosphate). Barre et al.,(1954,cited in Cheryan,1980) showed that of the 12 replaceable hydrogen protons, six are strongly removed at pH 1.8, two are weakly acidic at pH 6.3, while the remaining four are feebly dissociated at pH 9.7. In most food systems therefore, phytic acid will have a strong negative charge (Fig. 2) which will allow it to bind cationic species.

In food systems, although 80-90% of the phosphorus is bound in the phytic acid form, 10-20% is non-phytate phos-

Figure 2. Myoinositol 1,2,3,4,5,6 Hexakis (Dihydrogen Phosphate). Possible structure of phytic acid at neutrality based on the Anderson model. (Cheryan, 1980)



phorus. In rapeseed some of the phosphorus may be part of the lipid, carbohydrate or nucleic acid compounds. Nucleic acid of wheat flour constitutes the major portion, of the total non-phytic acid content of wheat (Gillberg and Tornell, 1976).

Nutritional Aspects of Phytic Acid

The nutritional evaluation of a foodstuff must not only take into consideration the concentration of a nutrient but it must also determine the biological availability of the nutrient to the consumer (O'dell and deBoland, 1972). As mentioned previously, phytic acid has a net negative charge so that it has the ability to bind minerals electrostatically. As far back as 1925 Mellanby attributed rickets in dogs to phytic acid binding of minerals in cereals (Erdman and Forbes, 1977).

It is apparent from the literature that cereal sources of minerals permit less efficient absorption of minerals compared to those from animal sources. When rats were fed egg white or casein as the protein source they required 12 ppm zinc in the diet as compared to 18 ppm when soybean was the protein source. Calculations showed that the efficiency of absorption of animal nutrients was 84% compared to 44% from soybeans (O'dell, 1969). In another study (Anon, 1979) immature rats were fed diets based on texturized vegetable protein (TVP) and egg albumen containing 14.5 or 100 ppm zinc. The lower level of zinc satisfied the requirement when egg albumen but not TVP was the source of protein. It

is clear that minerals are less available nutritionally when a predominantly plant diet is consumed and the main cause appears to be phytic acid.

Monogastric mammals lack or have low phytase activity in the intestinal tract and therefore cleavage of minerals complexed with phytic acid is limited. Clinical deficiencies of zinc, calcium and iron have been reported in segments of the Iranian population who consume up to 75% of their energy as unleavened whole wheat bread (Erdman and Forbes, 1977). Nahaptien and Bassiri (1975) speculated that the high phytate content of wheat may explain the high incidence of calcium and zinc deficiencies reported in mid-eastern countries where unleavened bread from whole wheat flour constitutes the major portion of the diet. In other studies (McCance, 1943) the absorption of iron has not been as efficient as is desired due to the presence of phytic acid in the diet of humans, however Morris and Ellis (1976) suggest that the monoferric phytate is possibly better absorbed than saturated ferric phytate.

A second cause for the decrease in absorption of minerals, besides the lack of phytase, has been proposed. When salts of phytic acid are formed they tend to decrease the solubility of the phytic acid and since the body absorbs soluble components only, the minerals become biologically unavailable (deBoland et al.,1975; Harland and Harland, 1980). Some researchers have found that calcium decreases the availability of zinc to the intestinal absorption site.

In rats, Oberleas et al., (1976) found that if excess calcium and phytic acid were present, zinc deficiencies occurred. There appears to be a three way interaction between zinc, calcium and phytic acid whereby zinc is complexed with phytic acid to form an insoluble compound. This finding is supported by O'dell (1969) who found that in the absence of phytate excess calcium had no detrimental effect.

Reddy and Salunkhe (1981), Atwal et al.,(1980) and deBoland et al., (1975) state that phytate has detrimental affects on rats because of its ability to bind calcium, magnesium, zinc, iron, copper and manganese. Zinc however, seems to be the mineral that is most selectively bound by phytic acid (Tolas, et al.,1976, Jones,1979) and becomes unavailable nutritionally because of the low solubility of the resulting salt (O'dell and deBoland, 1976). DeRham and Jost (1979) found this to occur when the zinc concentration was more than 1%. Harland and Harland (1980) found that the ratio of phytate to zinc may be a valuable method for the determination of zinc availability because in rats if the ratio were more than 10, a zinc deficiency occurred. The ratio for humans has not been determined as yet but in mid-east countries when zinc deficiencies occur, the molar ratio is greater than 20.

Kratzer et al.,(1958), Oberleas et al.,(1966) and O'dell (1969) found that when ethylenediaminetetracetate (EDTA) was included in the diet of poults and rats, growth was stimulated even in the presence of phytate. It has been

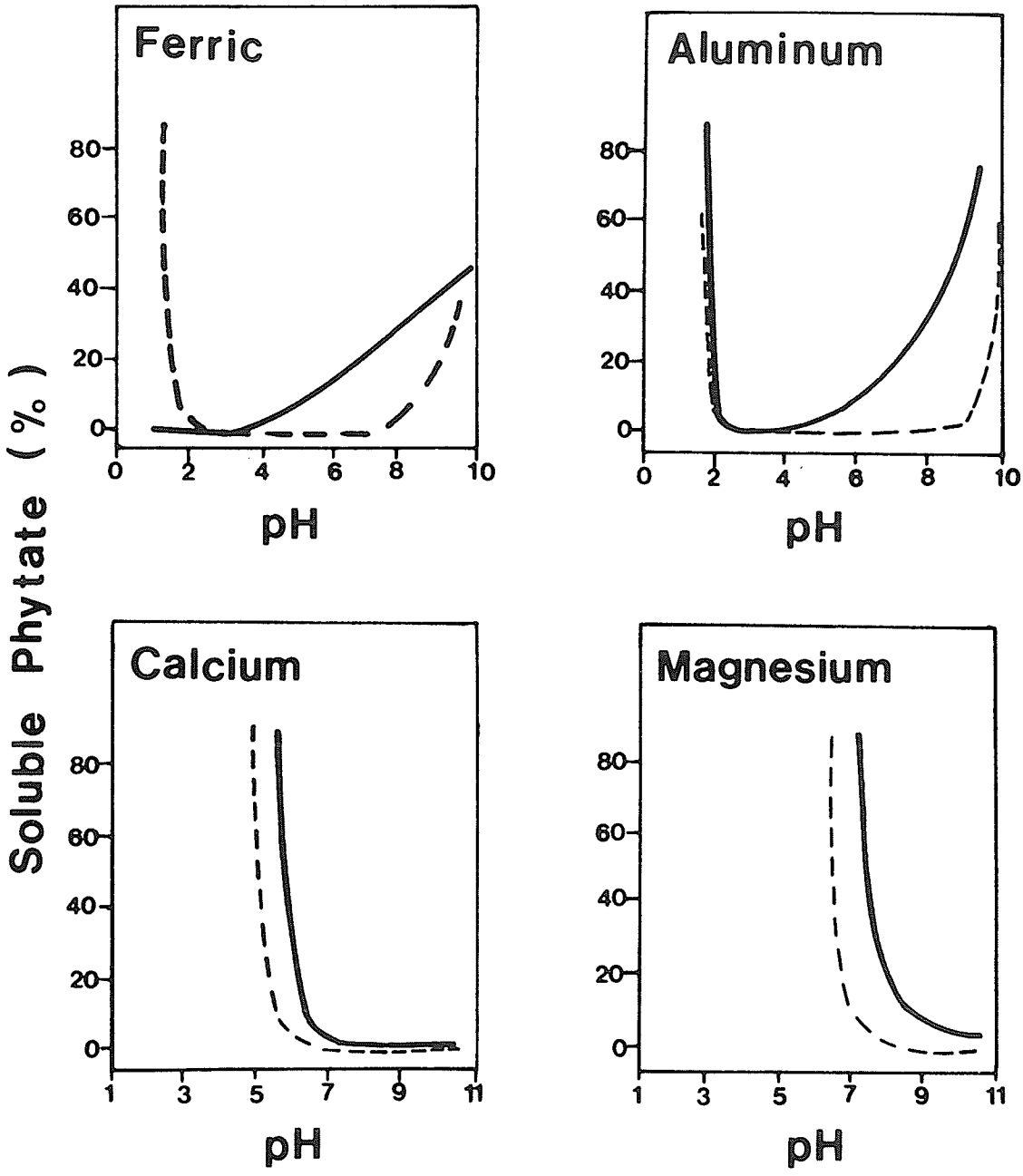
suggested that EDTA successfully competes with phytic acid for zinc and the resulting complex is either absorbed or the zinc is exchanged at the absorption site.

Solubility

One of the proposed mechanisms to explain the unavailability of minerals to the absorptive sites of the gut is the insoluble metal-phytic acid compound. Phytate in the native state is found to be quite soluble in some seeds; 99% of bean phytate, 90% of corn germ phytate and 60% of soybean flake phytate is extractable by water. When the amount of nitrogen removed by water was found to be only 40% in soybean flakes, O'dell and deBoland (1976) concluded that phytate was not bound to protein. In the native state phytic acid is in the soluble sodium or potassium salt form but if subjected to processing by heat, changes in pH and/or ionic strength the monovalent cation appears to be replaced by a divalent ion and thus becomes insoluble (Cheryan, 1980). The extractability of phytate from a biological system containing protein is dependent upon its chemical environment, i.e. the type of cations, ionic strength, nature of the proteins and pH of the solvent (Omosaiye and Cheryan, 1979).

Reference, however, must be made to the solubility of phytate in the absence of protein in order to compare its solubility in the presence of protein. In the absence of protein the tetra-, hexa- and dodeca-hydrogenated forms of phytic acid are soluble in water (de Rham and Jost, 1979). The calcium and magnesium salts of phytic acid in an aqueous

Figure 3. Solubility of various metal-phytate complexes at various pH levels. Full line indicates an equivilant ratio of metal to phytate phosphorus of 1.0. The broken line indicates an equivilant ratio of 3.75.
(Cheryan, 1980)


Ferric
Aluminum
Calcium
Magnesium
Soluble Phytate (%)
pH
pH
pH
pH
0
20
40
60
80
0 2 4 6 8 10
1 3 5 7 9 11

solution tend to be soluble at low pH and insoluble at high pH values (Fig. 3). A rapid decline in calcium phytate solubility occurred between pH 5.5 and 6.0, while magnesium phytate solubility decreased between 7.2 and 8.0. In contrast, aluminum phytate showed a higher solubility at extremes of pH and a minimum at pH 3.5. Ferric phytate was completely insoluble at low pH values (1.0-3.5) and slowly increased in solubility until it was 50% soluble at pH 10.0. At any pH level, if there were an increase in magnesium, calcium or aluminum, when their respective salts of phytic acid were being studied, the solubility decreased. Ferric phytate only showed this characteristic above pH 2.0 and below pH 2.0 ferric phytate solubility increased with increasing amounts of the ferric ion (Cheryan, 1980).

In the presence of proteins, the solubility of phytate salts changed. Gillberg and Tornell (1976) found that rapeseed meals have sufficient amounts of magnesium and calcium to permit the complete conversion of soluble phytate salts into insoluble salts at pH levels between 4 and 8. Between pH 8 and 10 the solubility of the complex began to increase concomittantly with protein so that these authors deduced that binding between protein and phytate was occurring. Above pH 10.0, the number of positive groups on the protein decreased because lysine has a pK of 10.53 reducing the number of binding sites which in turn caused a decrease in phytic acid binding. Omosaiye and Cheryan (1979), when examining the solubility of soybean phytate, found phytate to be

insoluble above pH 10.3 while deRham and Jost (1979) found the value to be pH 11.3. DeRham and Jost attributed the discrepancy to experimental differences such as the type of soybean, calcium level and temperature.

As well as protein affecting the solubility of phytate, phytate complexation with protein yields a less soluble protein (O'dell and de Boland, 1976). Bernardin (1978) found that potassium chloride, bromate and phytate all caused aggregation of wheat protein A-Gliadin with phytate having the greatest effect. These findings support Fontaine et al.,(1946) who added 500 mg of sodium phytate to a dialyzed peanut meal suspension at pH 1.4 and found that 20% of the total nitrogen was soluble as compared to 80% when only 135 mg of sodium phytate were used.

Binding Mechanism

In an aqueous system, where a positively charged metal is introduced, electrons in the solvated water molecules are displaced towards the metal and loss of hydrogen ions from the water molecules occurs more readily, thus forming metal hydroxides. When a hydroxyl group attaches to the complex, the positive charge on the metal is reduced by one. When metal ions, surrounded by water, and hydroxyl groups interact, polymers may be formed where the hydroxyl ions act as bridges between the metal ions (Fig.4). If the metal ions are trivalent, polymerization will occur when one hydroxyl group substitutes for one water in the first hydration shell of the ion. The charge on the ion will be reduced

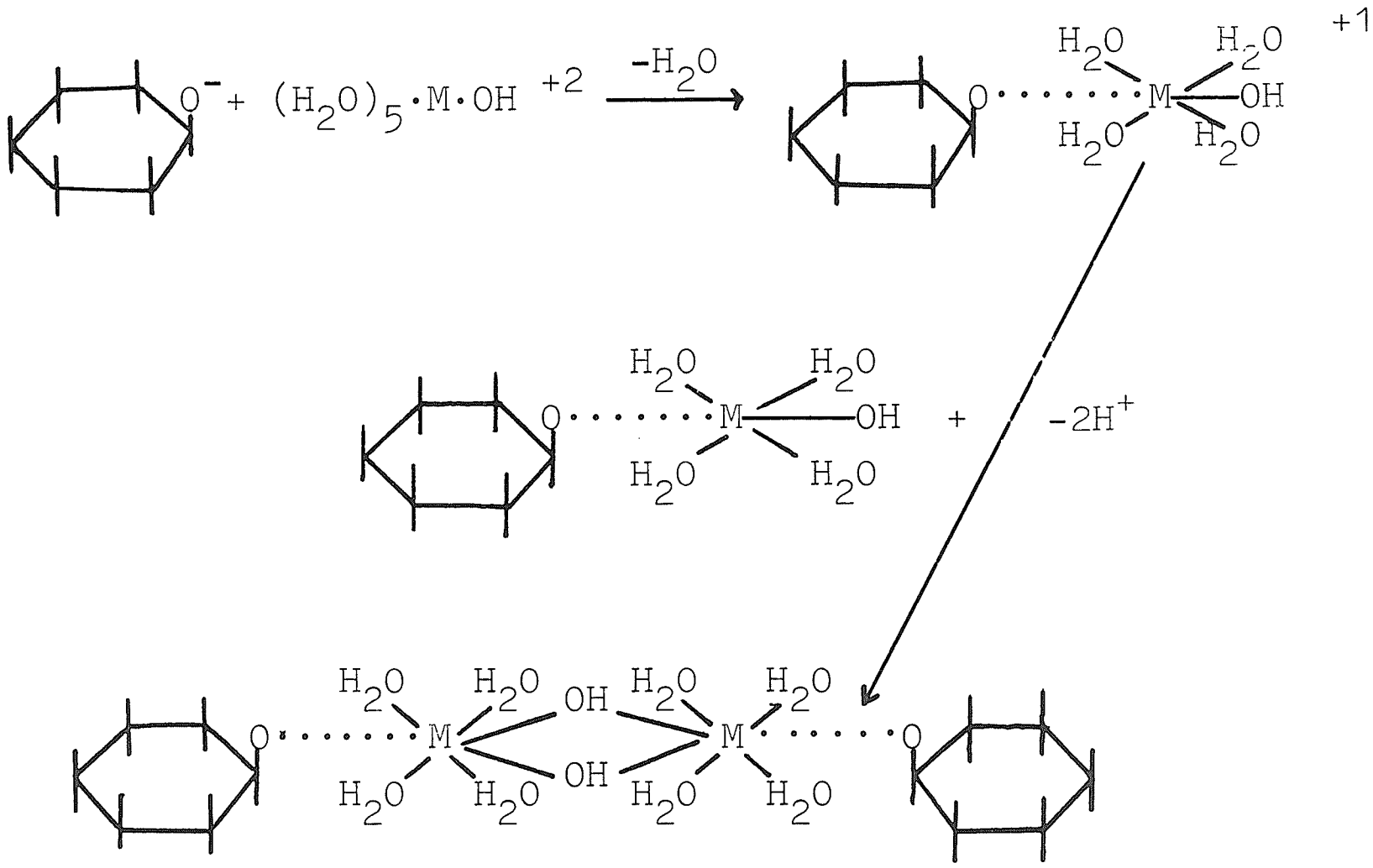
Figure 4. Binding of two hydrated metals by hydroxyl bridging.
(Chaberek and Martell, 1959)


HO
OH
M
HO
OH
+
H2O
OH
M
HO
OH2
OH
OH
OH
M
M
OH
OH
OH
+ H2O

from +3 to +2 and polymerization would occur according to figure 5.

When phytic acid is introduced into such a system, phytic acid with a pK of 1.8, would be negatively charged at most pH values. When phytic acid comes into contact with a positively charged species it would bind electrostatically. When two such complexes i.e. cation-phytic acid meet, they would be expected to polymerize as indicated (Fig.5). Since calcium is one of the most common metals attached to phytic acid, it shall be used as an example. Calcium exists as a divalent cation so that when the hydroxyl group attaches to the hydrated metal, the charge on calcium would be reduced by one. If the monovalent cation species were stable, the polymerization process would continue as in Figure 5. Monovalent calcium is not stable however, so that it becomes an intermediate between the divalent species and the neutral species which precipitates from solution. It is apparent, since calcium does not precipitate under alkaline conditions, that a water molecule is not substituted by a hydroxyl group and that calcium binds to phytic acid in the divalent form resulting in a univalent cation metal ligand complex. Polymerization not occurring, the metal ligand complex would seek out electron donors such as protein in order to fulfill its need of an electron. Metals such as calcium, magnesium, iron and zinc therefore have the potential to act as salt bridges between phytic acid and negatively charged proteins (Fig. 6). Phytic acid can therefore bind to protein

Figure 5. Possible complexation scheme of metal phytates. (Adapted from Chaberek and Martell, 1959)


O⁻ + (H2O)5·M·OH +2
-H2O
+1
H2O
H2O
O
M
OH
H2O
H2O
+
-2H+
OH
OH

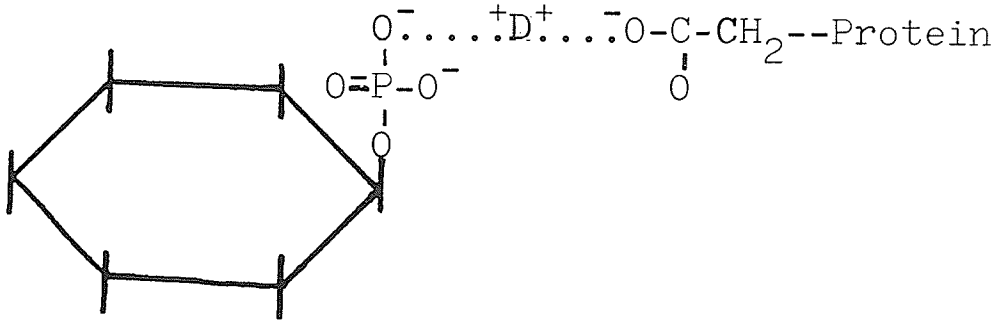
by salt bridges or it may bind solely to monovalent or divalent cations (Fig. 7).

Does experimentation support this theory of binding? Omosaiye and Cheryan (1979) using ultrafiltration found that phytic acid was bound to protein via two very different mechanisms depending upon the pH range of the experiment. Below the isoelectric point (pI) of the protein and above the pK of phytic acid (pH 1.8) a direct phytic acid - protein electrostatic interaction was one of the proposed mechanisms. At pH values above the isoelectric point of the protein the binding of negatively charged phytic acid and negatively charged protein was attributed to the bridging ability of multivalent cations as calcium (Fig. 8). Fontaine et al., (1946) extracted phytic acid and protein from peanut meal and found that both precipitated together. At pH levels below the pI of the protein, the mechanism of binding was described as a direct interaction of a positively charged protein and negatively charged phytic acid.

Reddy and Salunkhe (1981) dialysed black gram albumins above and below their isoelectric points. Of the phytates, 45% remained in the retentate at acidic pH and the ratio of phytate to protein was high. This finding was attributed to a direct electrostatic protein-phytic acid interaction. Above the isoelectric points, 69% of the phytic acid was retained during dialysis; it was suggested that this was the result of divalent cations mediating the binding of phytic acid and protein.

Figure 6. Possible bridging effect of a divalent cation (D^{++}) between protein and phytic acid (ternary complex).
(Cheryan, 1980)

Figure 7. Possible structure of phytic acid bound to a monovalent cation (M^{+}) or a divalent cation (D^{++})


O⁻....⁺D⁺...⁻O-C-CH2--Protein
O=P-O⁻
O
O

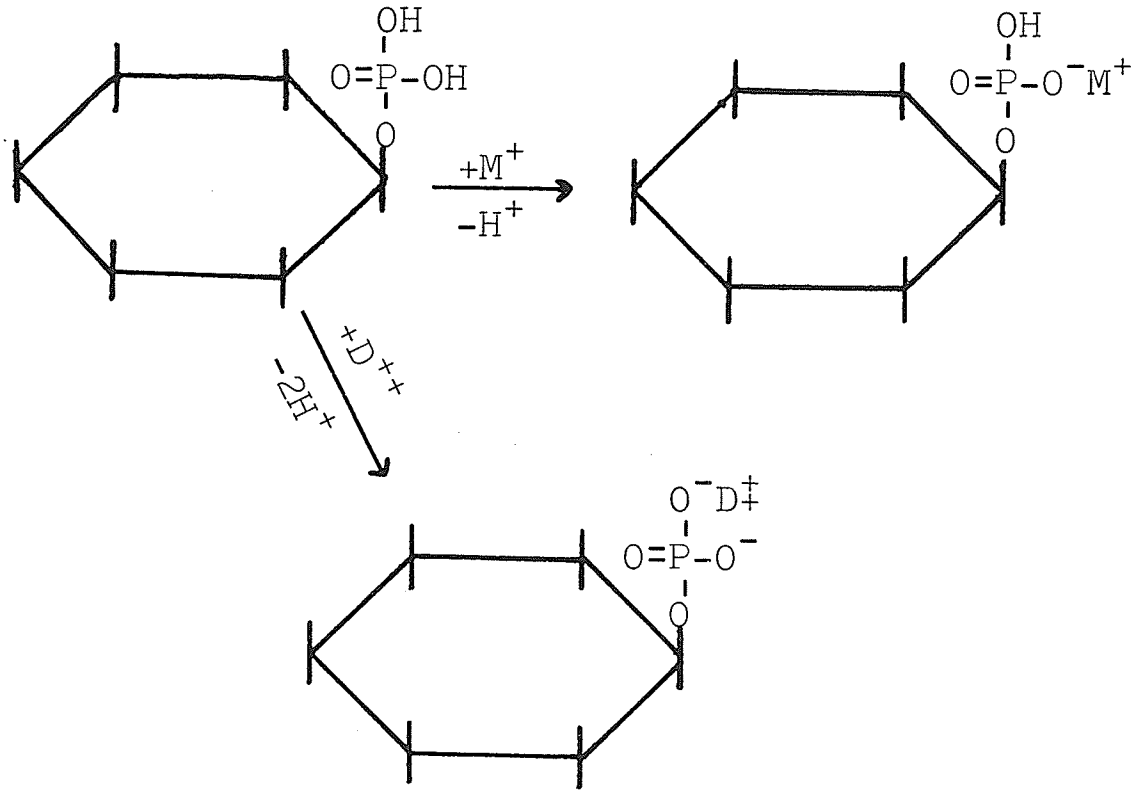

OH
O=P-OH
O
+M⁺
-H⁺
OH
O=P-O⁻M⁺
O
+D⁺⁺
-2H⁺
O⁻D‡
O=P-O⁻
O

Figure 8. Binary-ternary equilibrium reactions:
a) Binary equilibrium reaction.
b) Ternary equilibrium reaction.

$$\text{CATION} + \text{PHYTIC ACID} \longleftrightarrow (\text{CATION-PHYTIC ACID}) \quad \text{(a)}$$

$$\text{PROTEIN} + \text{CATION} + \text{PHYTIC ACID} \longleftrightarrow (\text{PROTEIN-CATION-PHYTIC ACID}) \quad \text{(b)}$$

Other studies have not produced such clear results. One such study by O'dell and deBoland (1976) compared the extraction of protein and the extraction of phytate from various sources. With soybean flakes, the phytic acid was firmly bound to the protein while in sesame and corn germ very little phytic acid was recovered with the protein. An attempt was made to solve the mystery by examining the amino acid content of each protein but suitable answers could not be found. It was concluded that a general statement as to the chemical environment or native state of phytate in plant seeds cannot be made. Cheryan (1980) stated that more work needs to be done in the area of the binding mechanism, especially above the pI of protein.

Phytase

Phytase (myoinositol hexaphosphate phosphohydrolase EC 3.1.3.8) hydrolyzes phytic acid to inositol and phosphoric acid (Lolas and Markakis, 1977) as measured by an increase in organic phosphorus. The enzyme is found in plant tissues but it is not active unless the seed is germinating (Chang et al.,(1977). Singh and Sedeh (1979) when working with triticale and Lolas and Markakis (1977) when investigating navy beans did not find any correlation between the occurrence of phytase and other components such as phytic acid, phosphorylase, glycerophosphatase, pyrophosphatase, calcium, magnesium, zinc and iron.

The enzyme is found in the gut of ruminants because of the kinds of microorganisms prevalent there. Since the

enzyme has the ability to degrade phytic acid to phosphorus and other minerals, all become available for digestion (Miller et al.,1980). In the monogastric mammal the presence of phytase is less clear (Wang et al., 1980). If it is present in man, Lolas and Markakis (1977) postulate, it may be difficult to observe its activity due to various inhibitors found in the diet.

Microorganisms used for the production of food products exhibit phytase activity. Shieh and Ware (1963) surveyed more than 2000 cultures of yeast, fungi and bacteria and found extracellular phytase activity to be mainly among the molds. Some 28 of 82 Aspergillus species, 1 of 58 Penicillium species and 1 of 37 Mucor species demonstrated activity with Aspergillis niger being the most active. Other microbes like Rhizopus, Neurospora, yeasts and bacteria were tested and found not to produce extracellular phytase activity. Many of these microorganisms however demonstrated intracellular activity. This finding is of significance to Oriental food fermentation since molds which produce high levels of intracellular phytase are not removed from the substrate (Wang, et al.,1980).

Phytase,like other enzymes, has characteristics peculiar to itself. The activation energy of plant phytase ranges from 8500 cal/mole(Chang et al.,1977) for mung beans to 41,500 cal/mole for wheat (Lolas and Markakis, 1977). Depending upon the plant source, there are different optimum pH and temperature values for each enzyme. In general, the

optimum pH ranges from pH 4.5 (Yamada et al.,1968) to pH 7.0 (Lolas and Markakis, 1977). The optimum temperature range is between 50° and 60°C (Lolas and Markakis, 1977, Chang, 1977). The relatively high optimum temperatures are of practical significance since appreciable activity would be expected during heat related food processes (Chang et al.,1977). Minerals like calcium in navy beans, magnesium and calcium in wheat and dwarf beans demonstrate an activation of plant phytase (Lolas and Markakis, 1977) and calcium activated phytase from Bacillus subtilis (Powar et al.,1967).

Phytase, activated by germination (Lolas and Markakis, 1977), is inhibited by many factors. Chang et al.,(1977) found that the substrate level in beans affects phytase activity so that 1.5 mM decreased activity by 35% and Lolas and Markakis (1977) found 10 mM of phytate to virtually halt activity in navy beans. When the inorganic phosphorus level reached 0.32 mM Chang et al.,(1977) found the activity to decrease to 30% and when inorganic phosphorus levels were 0.64 mM, activity decreased by 50%. Salts like zinc, iron, copper and mercury at 10^{-3} M exerted an inhibiting affect on phytase (Lolas and Markakis, 1977).

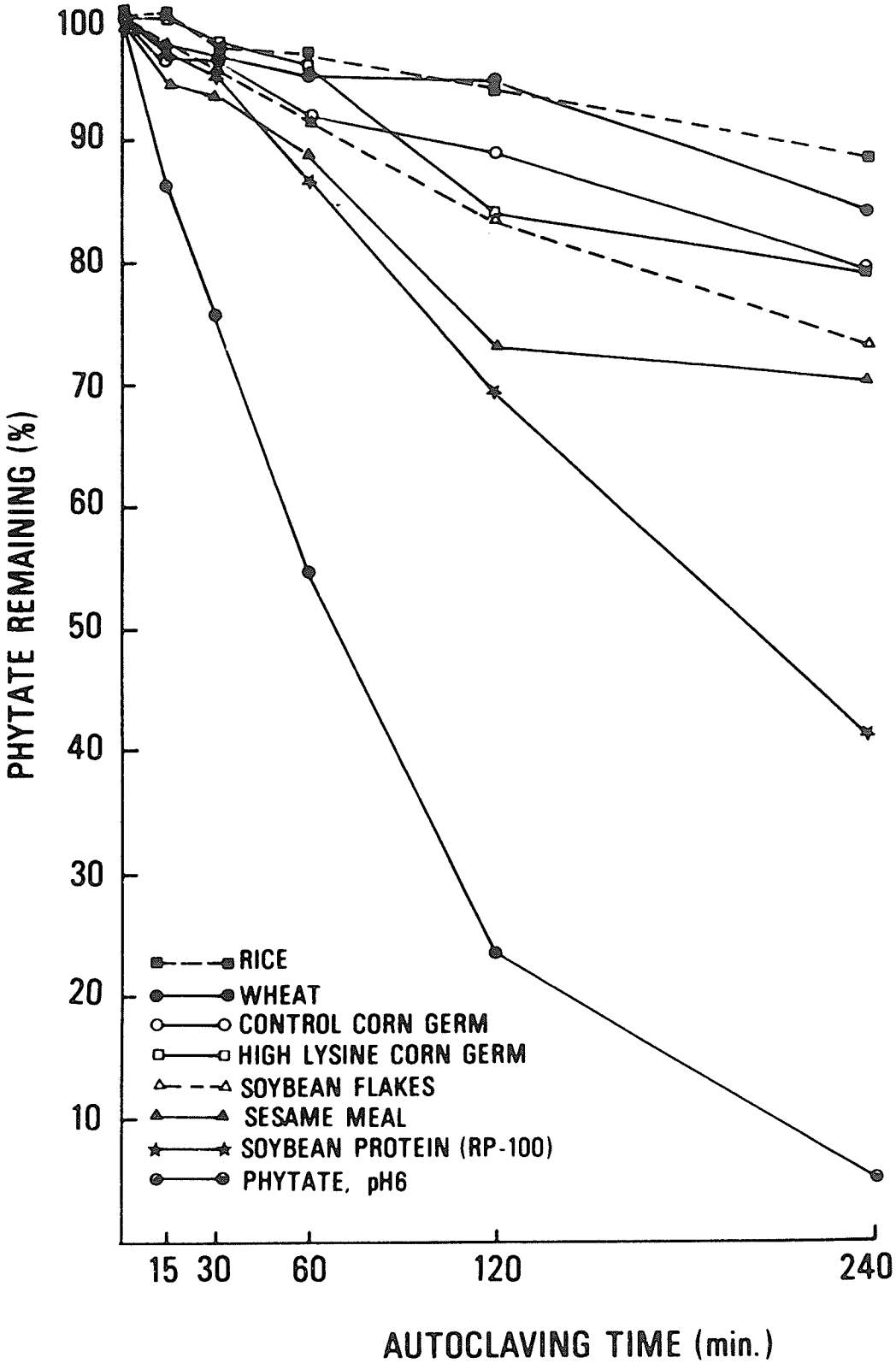
Reduction of Phytate

The reduction of phytate in a final food product has been the objective of many studies. This is an understandable goal considering the nutritional significance of phytic acid. Many methods have been used to accomplish the reduc-

tion of phytate. Since cereals have a very localized phytic acid concentration, in the bran and germ (Erdman and Forbes, 1977) and very little in the endosperm (O'dell and de Boland, 1977), it is logical to remove the bran and germ by physical milling practices. Harland and Harland (1980) found that there was 0.64% phytate in whole wheat flour but if the bran and germ were removed the level was lowered to 0.03%.

During the production of many products heat treatment is one of the steps. DeBoland et al.,(1975) compared the amount of remaining phytate in different products after they were autoclaved at 115°C (Fig. 9). Autoclaving aqueous inositol hexaphosphate at pH 6.0 resulted in an 80% loss of phytate in 2 hours while isolated soybean protein lost 70% after the same time. Rice, wheat, corn germ, soybean flakes and sesame meal did not lose nearly as much (less than 25%) of their phytate under the same conditions. Other investigators concur, autoclaving soybean protein increased the availability of zinc to turkey poults, pigs (O'dell,1969) and chicken poults (Kratzer et al.,1958). Lolas and Markakis (1977) reported that navy beans in water did not show any phytic acid destruction at 100°C for 75 minutes. It is speculated that the reason for the great differences that are observed is that of the phytate chemical environment (dO'dell,1969 DeBoland et al.,1975, Tangendjaja et al.,1981) which affects the degree of stabilization of phytic acid by binding mechanisms involving components of the environment.

Figure 9. Effect of heat upon phytate retention. Various sources of phytate heated at 115°C. (DeBoland *et al.*, 1975)


100
90
80
70
60
50
40
30
20
10
PHYTATE REMAINING (%)
RICE
WHEAT
CONTROL CORN GERM
HIGH LYSINE CORN GERM
SOYBEAN FLAKES
SESAME MEAL
SOYBEAN PROTEIN (RP-100)
PHYTATE, pH6
15 30 60 120 240
AUTOCLAVING TIME (min.)

Microorganisms, as discussed in the phytase section, do exhibit phytase activity. Tangendjaja et al.,(1981) reported that after 24 hours at 55°C and pH 5.1 added phytase reduced phytic acid content in rice bran by more than 70%. Preece et al.,(1960) found that during the malting of barley, phytase activity increased eighty fold over the period from the seed to green malt stage. Sudermadji and Markakis (1977) investigated the making of tempeh by soybean fermentation and reported that phytate was reduced by one third as measured by increased inorganic phosphorus. Harland and Harland (1980) demonstrated that during bread making, fermentation reduces phytate from 0.78 to 0.21% in rye bread under normal conditions. If the amount of yeast were doubled or the fermentation time were extended,phytate would virtually be reduced. Tangkongchitr et al., (1981) reported a 23% reduction of phytate in whole wheat bread where 17% occurred during fermentation.

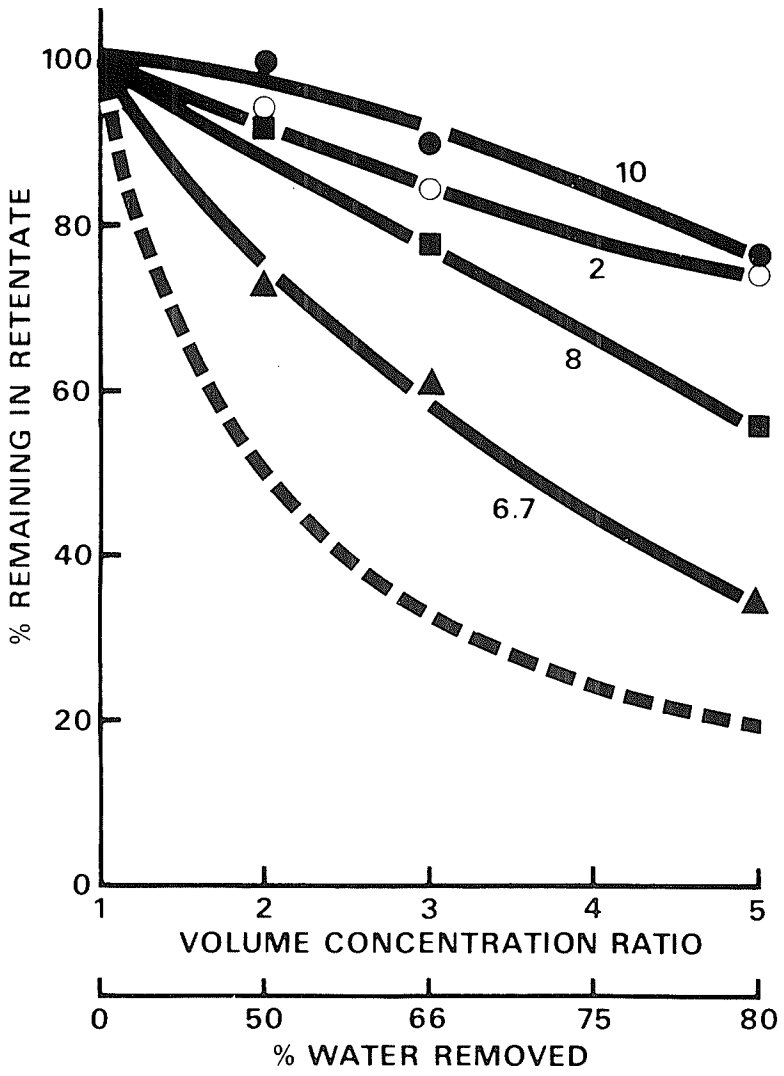
Water as an extractor has been investigated as a method for the removal of phytate. O'dell and deBoland (1976) found that 90% of corn germ phytate, 60% of soybean flake phytate, and none of the sesame meal phytate were extractable by water. DeBoland et al., (1975) concluded that water does not offer a practical method of removing phytate from foodstuffs except in cases like corn products where water is added and then removed during the wet milling process.

Separation of phytate from protein isolates is dependent upon the pH of the solution. Okubu et al.,(1975) found

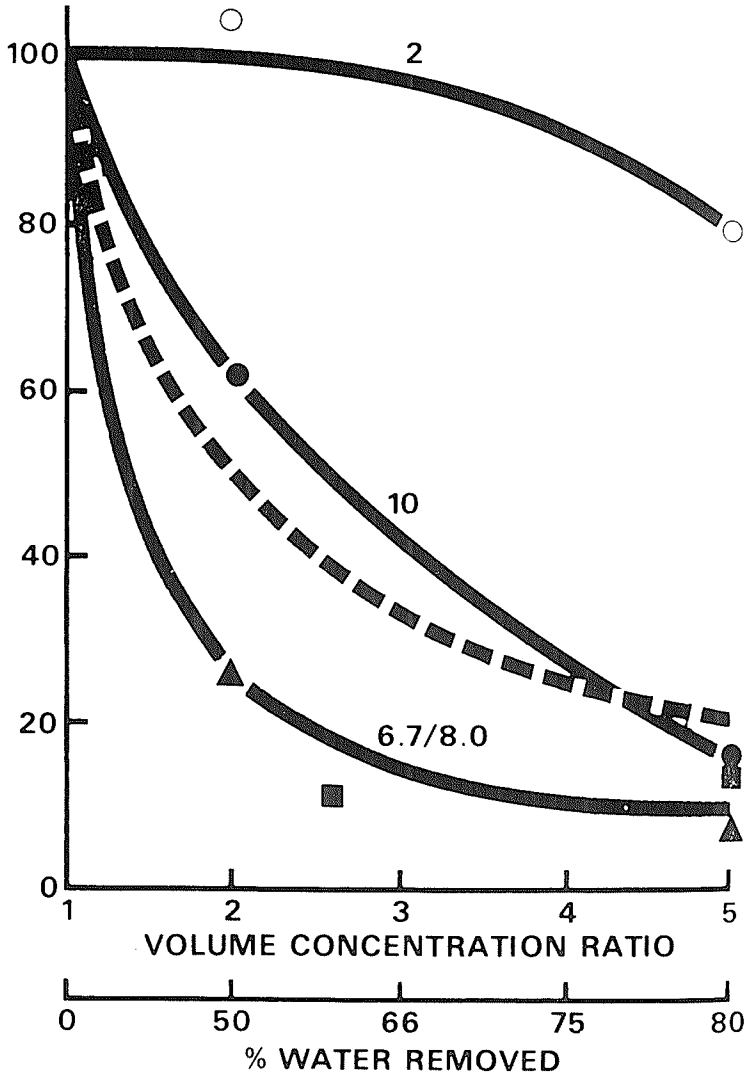
that near the isoelectric point of soybean globulins 95% of the phytic acid could be removed but much less was removed at other pH values. Omosaiye and Cheryan (1979) also found that during ultrafiltration, the most dominant factor that affected phytate removal was the pH (Fig. 10). Using soybean protein Omosaiye and Cheryan (1979) demonstrated that when one run was completed at various pH levels (Fig. 10a) the minimum amount of phytic acid that remained in the retentate occurred at pH 6.7. The maximum amount of phytic acid was retained at the two extreme pH levels of 2 and 10 and this was attributed to its binding with protein. When re-ultrafiltration was completed at the same pH levels (Fig. 10b), 80-92% of the phytate was removed from pH 6.7, 8 and 10. The increase in removal was attributed to the loss of cations which caused a reversal of the ternary complex of Figure 8, so that more phytate was in the binary state and could pass through the membrane. It has been found that if EDTA (Okubu et al.,1975), 8.5% sodium chloride or saturated ammonium sulfate (deRham and Jost, 1979) are added to the solution before ultrafiltration, disruption of the protein-phytic acid complex occurs resulting in decreased phytic acid retention.

Some factors however hinder the removal of phytate during food processes. During breadmaking Ranhottra (1973) discovered that an increased level of inorganic phosphorus will trigger the rephosphorylation of partially hydrolyzed phytic acid. Tangkongchitr et al., (1981) demonstrated that

Figure 10. Removal of phytic acid from soybean water extracts by ultrafiltration (a) and reultrafiltration (b). Variable is processing pH. Broken line indicates the theoretical or ideal behavior.
(Omosaiye and Cheryan, 1979)


% REMAINING IN RETENTATE
100
80
60
40
20
0
10
2
8
6.7
1
2
3
4
5
VOLUME CONCENTRATION RATIO
0
50
66
75
80
% WATER REMOVED

(a)


100
80
60
40
20
0
2
10
6.7/8.0
1
2
3
4
5
VOLUME CONCENTRATION RATIO
0
50
66
75
80
% WATER REMOVED

(b)

calcium acetate, magnesium acetate, monocalcium phosphate, sodium bicarbonate and ferrous sulfate protect phytate from fermentation hydrolysis. Ford et al., (1978), when making lipid protein concentrate, found that if calcium chloride levels were low (zero) and the pH was 5.5, then 90% of the phytate could be removed.

Methods of Analysis

Many different methods for measuring the levels of phytic acid have been developed which either measure inorganic phosphorus and relate it to phytic acid (Penny, 1976; Harland and Oberleas, 1977) or measure phytate directly (Latta and Eskin, 1980). The more recent methods involve two steps, extraction of phytate by a solvent and then precipitation of the phytic acid with controlled amounts of ferric ions (Cheryan, 1980). The history of the various methods will not be discussed here, only a few more recent analytical methods will be mentioned.

Extractability of phytate depends upon its association with other compounds within the food material, upon the pH, the type of solvent, the ionic strength and the endogenous iron (Reddy and Salunkhe, 1981). Kumar et al., (1978) found that 0.5 N hydrochloric acid (HCl) extracted the maximum amount of phytin phosphorus from cooked and uncooked peas as compared to water. Reddy and Salunkhe (1981) found that 3% trichloroacetic acid (TCA) was more complete and yielded higher phytate compared to other black gram extracts like 2% HCl, 2% hypochloric acid, 0.05M Tris (hydroxy methyl) amino

methane (TRIS) buffer and distilled water. Chang et al.,(1977) found in California small white beans at 60%, 3% TCA extracted more phytate than 0.5 N HCl or water. Therefore, it appears that 3% TCA is a good extraction medium.

The traditional methods of determination of phytate are based upon a method developed in 1914 by Heubner and Stadler (O'Neil et al., 1980). Many methods have been developed since then to make the task of phytate determination more accurate and more time efficient. To mention only a few, Maresse et al., (1961) compared volumetric, gravimetric and ion exchange techniques for the accuracy of phytic acid measurement. It was reported that ion exchange was the most accurate technique since it makes the distinction between the hexa- and ortho-inositol polyphosphate. Putnins and Yamada (1975) developed a colorimetric method for the determination of inorganic pyrophosphate by a manual or automated method and O'Neil et al., (1980) developed a nuclear magnetic resonance spectrometry method for phytate determination. Ellis et al., (1977) found that they could improve phytate detemination by incubating the ferric ion precipitate with 0.5 M HCl and thereby reduced to zero the interference of inorganic phosphorus. Harland and Oberleas (1977) developed a technique which involved phytate extraction with 1.2% HCl, ion exchange of the extract, digestion of the eluted phytate with concentrated nitric acid and concentrated sulfuric acid and colorimetrically measured phytate. The method is hard to use since the digestion step requires

careful monitoring in order to minimize loss due to incomplete or excessive digestion (Latta and Eskin, 1980; Tangkongchitr et al.,1981). Latta and Eskin (1980) developed a simple and more direct procedure for measuring phytate based on the reaction of the column eluant and a ferric chloride, sulfosalicylic acid solution. The Harland and Oberleas method and the Latta and Eskin method were both tried in this research but the latter method proved to be as accurate and much easier to use than the former method.

Materials and Methods

A. Starting Material

Whole fababeans (Vicia faba var., Diana, Ackerperle and Aladin) were all obtained from the Plant Science department at the Univerisity of Manitoba. Whole fababean flour (Vicia faba L. var.,minor, Diana) from the 1978 crop year was prepared at the Glenlea Research Station by an Alpine pin mill. From the same flour, prepared by pin milling, a protein concentrate was produced by air classification. The protein concentrate was further purified by gel filtration on Sephacryl S-300 using a K26/100 column (Pharmacia). The protein was eluted with 0.5N sodium chloride with 0.02% sodium azide incorporated as a microbial growth inhibitor.

Phytic acid was obtained from the Sigma Chemical Company of St. Louis, Missouri. The acid, lot 118c-0067, was extracted from corn and shipped as the sodium salt, it was 97% pure, contained 15% moisture by weight and had 12 sodium/mole. A calcium phytate, purchased from the Sigma Chemical Company was obtained from the Faculty of Human Ecology at The University of Manitoba.

Ion exchange resin, Amberlite 1R-120S, analytical grade, 200-400 mesh, chloride form was purchased from Bio-Rad. Laboratories of Richmond, CA. Disodium (Ethylenedinitrilo) tetraacetate (EDTA) from J.T. Baker Chemical Co. of Phillisburg, N.J., L-ascorbic acid from Aldrich Chemical Co. of Milwaukee, Wis., sulfuric acid and ammonium molybdate from Fisher Scientific Co. Fairlawn, N.J. and nitric acid

from McArthur Chemical Co. Montreal, P.Q. were purchased in the reagent grade form.

B. Methods of Analyses

1. Phytic Acid Analysis:

a) Preparation of phytic acid for standards: adopted from Angyal and Russell (Latta and Eskin, 1980).

i) place 70.0 ml of Amberlite 1R-120S in a beaker and wash three times with 100-150 ml of 5.0%. w/v HCl (114.0 ml conc. HCl/1000 ml).

ii) rinse the resin repeatedly with deionized water until neutral (ca. five rinses with 150 ml/rinse).

iii) add 300.0 mg of calcium phytate and 50.0 ml of deionized water and stir until the calcium phytate has dissolved.

iv) filter the supernatant through Whatman No. 1 filter paper into a crucible.

v) add 50.0 ml of deionized water to the resin, stir and filter into the same crucible.

vi) repeat the above rinsing procedure twice and filter into the same crucible.

vii) freeze and freeze dry.

viii) dilute the freeze dried material to 50.0 ml and store in the refrigerator.

ix) in triplicate, pipette 1.0 ml of phytic acid solution into a 100 ml volumetric and dilute to volume. Also prepare a blank in order to assess the acids for phytic acid.

x) Inorganic phosphorus determination performed by using 2.0 ml of the triplicate solutions for the color reaction of Harland and Oberleas (1977) in sec. B.1.b. The calculation is: Abs. sample/Abs. standard x 200 = ug Inorganic phosphorus/ml where the concentration of the standard is 2.0 ug/ml.

xi) Total phosphorus is determined by taking 2.0-5.0 ml aliquots of the triplicate solutions for digestion according to the Harland and Oberleas method (1977) sec. B. 1.b. After digestion, dilute to 25.0 ml and use 2.0 ml for the color test. The calculation is:

Total P(ug/ml) = Absorbance of sample/Absorbance of standard x conc. of standard x 500 where conc. of standard is 2 ug/ml.

xii) Organic phosphorus is found by: Total P (ug/ml) - Inorganic P (ug/ml).

xiii) Phytic acid (mg/ml) = Organic P (ug/ml) x 660/186 x 1/1000 The level of phytic acid is expected to be ca. 2.8 mg/ml.

b) Phosphorous determination (Harland and Oberleas, 1977 and Chen et al.,1956).

i) Reagents:

a) Phosphorous standard: Weigh out 0.0439 gm potassium phosphate (136.09g/mole) and dilute to 1.0 litre yielding a 10 ug/ml solution. Dilute the 10.0 ug P/ml to 2.0 ug/ml.

b) 2.5% Ammonium molybdate: dissolve 2.5 g of ammonium molybdate in distilled water and dilute to 100.0 ml.

c) 10.0% Ascorbic acid: dissolve 10.0 g of ascorbic acid in distilled water and dilute to 100.0 ml. Store in the refrigerator and use cold; this remains stable for up to seven weeks.

d) 2 N Sulfuric Acid: slowly and with mixing add 56.0 ml. of reagent grade sulfuric acid to distilled water and dilute to 1.0 l.

e) The color reagent is produced by addition of one volume of 2.5% ammonium molybdate and one volume of 10% ascorbic acid to three volumes of 2N sulfuric acid; this reagent is prepared fresh each day.

ii) Digestion:

a) pipet 5.0 ml of diluted column solvent into a 30.0 ml microkjeldahl flask (column eluant from B.l.a.ix. or from B.l.c.i.)

b) add 3 or 4 glass beads

c) add 3.0 ml of conc. nitric acid

d) add 0.5 ml of conc. sulfuric acid

e) rinse down the sides of flasks with approximately 10.0 ml of distilled water.

f) place flasks on a preheated digestion rack and observe until they begin to boil.

g) digest until dark brown fumes appear and then subside

h) remove flasks and cool for 5 minutes, then add 10-15 ml distilled water and swirl to mix.

i) place in a boiling water bath for 20 minutes.

j) allow flasks to cool

k) quantitatively transfer to a 25 ml volumetric, rinsing the flasks several times with distilled water and bring to volume.

iii) Color reaction:

a) pipet two ml of blank (water), standard or sample into 15.0 ml test tubes.

b) add two ml of the color reagent (B.l.b.i.e.)

c) tubes can be left overnight to develop color or may be mixed (using parafilm to close) and heated for 1.75 hours at 37°C

d) samples may be read in a spectrophotometer at 820 nm using the blank to zero the instrument.

iv) Calculating sample results:

Abs. sample = Abs. organic P
Abs. organic P = Abs. Total P - Abs. Inorganic P/5
%Phytic Acid = (Abs. sample/Abs. std) x conc. std x 2.22

c) Phytic Acid Determination (Latta and Eskin, 1980)

i) Sample extraction and column chromatography:

a) Weigh 5.00 g of sample into a 150 ml beaker and place on a magnetic stirrer.

b) While stirring add 100.0 ml of 2.4% HCl (54 ml conc. HCl/ 1000 ml).

c) mix for 1.0 hour.

d) centrifuge a portion of the sample at 20,000 x g in 50 ml tubes for 10.0 minutes.

e) remove 5-10 ml of clear supernatant and store in a

vial in the refrigerator.

f) use a column approximately 0.7cm x 27 cm containing a piece of glass wool at the bottom and 0.5 g of 200-400 mesh AGl- x 8 chloride anion exchange resin.

g) prepare the column with 15.0 ml of 5% w/v HCl.

h) rinse with 20.0 ml deionized water

i) dilute sample (e) 1/25 using a 25 ml volumetric flask and pipet 10.0 ml onto the column. When the phytic acid is less than 1.0%, dilute 1/5 instead of 1/25.

j) after the sample has passed through the column add 1.5 ml of 0.1 M NaCl.

k) then after (j) has passed through the column discard the eluant and place 25 ml volumetric flasks under the columns.

l) add 15.0 ml of 0.7 M NaCl to the column and collect the eluant.

m) fill the volumetric flasks to 25 ml and pour into large test tubes.

n) phytic acid can be assayed by determining the phosphorus (B.1.b.) or by reacting with the Wade reagent (B.1.c.ii).

ii) Color test using the Wade Reagent:

a) the reagent is prepared by combining 0.15 g hydrated ferric chloride and 1.50 g sulphosalicylic acid in water and diluting to 500 ml.

b) phytic acid standards are prepared to contain 10, 20, 30, 40 ug/ml from the 200 ug/ml stock solution. The standards must be stored in the refrigerator and brought to room temperatures prior to use.

c) pipet 3.0 ml of blank (water), standards and samples into 15 ml conical centrifuge tubes.

d) add exactly 1.0 ml of Wade Reagent

e) mix on a vortex type mixer

f) centrifuge for 10.0 minutes to precipitate phytic acid (5,000 x g)

g) pour supernatant into a cuvette and read the absorbance at 500 nm on a colorimeter using water to zero the instrument.

iii) Calculations:

a) absorbance readings for samples and standards are subtracted from the absorbance reading for the blank to obtain a final reading.

b) final readings for the standards are plotted against concentration to give a standard curve.

c) final readings for the samples are applied to the standard curve to determine the concentration.

d) the answer obtained is divided by eight to obtain an answer in percent phytic acid in the original sample.

e) moisture determinations should be done on the samples and the percent phytic acid reported on the dry weight basis.

f) if the dilution is 1/5 instead of 1/25, divide the resulting phytic acid by five.

2. Protein Determination:

The protein content of some samples was determined by the microkjeldahl method (AOAC, 1975a). Other samples used the method of Lowry et al., (1951) for the protein determination where bovine serum albumin was the standard protein.

3. Other assays:

Moisture determinations were performed by the Official Methods of Analysis (AOAC,1975b). All weighing was performed on Mettler digital display balances; models H6T,PL200 and HL 32 were used. All pH readings were done on the Radiometer pH meter 26. All absorbance readings were taken on the Bausch and Lomb spectronic 710 which has both tungsten and deuterium lamps.

C. Survey of phytic acid content in fababean varieties

Whole fababeans (var. Diana, Ackerperle and Aladin) were milled in a Chemical Rubber Company Micromill for three minutes. The moisture and phytic acid (Harland and Oberleas, 1977) analyses were performed. The hulls of the whole bean were removed in order to examine the cotyledons for phytic acid (Harland and Oberleas, 1977) and moisture. The hulls were removed by hand and then the cotyledons were milled in the same way as the whole bean. The phytic acid content of the hull of each variety was then determined by difference.

D. Methods of Isolating Protein

1) Isoelectric precipitation method: Prepared by adding 60.0 g of flour to 300.0 ml of distilled water at room temperature. The system was stirred by stir bar and Corning stir plate until the flour was evenly dispersed throughout. The pH was then increased to the desired level of solubilization by addition of 1.0 N NaOH and stirred for 30 minutes. The flour solution was then centrifuged at 8,000 xg for 10 minutes on the Sorvall RC2-B Centrifuge. The supernatant was decanted, adjusted to pH 4.5 with 1.0 N HCl, stirred for 5 minutes, centrifuged at 4,000 xg, the precipitate was collected and freeze dried.

2) 0.3 M NaCl/pH 4.5: Prepared by adding 60.0 g of flour to 300.0 ml of distilled water at room temperature and stirred until the flour was evenly dispersed. Added 5.265 g of NaCl (0.3 M) and stirred the the system for 30 minutes. Centrifuged at 8,000 xg for 10 minutes, decanted the supernatant, adjusted to pH 4.5 with 1.0 N HCl and stirred for 5 minutes. Centrifuged at 4,000 x g, collected and freeze dried the precipitate.

3) Protein Micellization Method (Murray et al.,, 1981): Prepare by adding 60.0 g of flour to 300.0 ml of distilled water at room temperature and stirred until evenly dispersed. Added 5.265 g of NaCl (0.3 M) and stirred the system for 30 minutes. Centrifuged at 8,000 xg for 15 minutes, decanted the supernatant and added cold tap water at a ratio of 1:3 (supernatant: water). Allowed the system to sit for 0.5 hours so that the protein could precipitate. Centrifuged at

4,000 xg for 15 minutes, collected and freeze dried the precipitate.

All isolates were examined by the Latta and Eskin (1980) method for phytic acid and all were analyzed for protein by the microkjeldhal method.

E. Addition of phytic acid to flour slurries

Protein isolates were prepared at pH 8.0 by the isoelectric precipitation method. Sodium phytate, commercially produced, was added to the distilled water after the flour. The sodium phytate was added at the levels of 1.12 g and 2.99 g and allowed to react during a 30 minute stirring period.

F. Effect of pH of solubilization upon the absorption of calcium by fababean flour

a) Calibration curve: A calibration curve was produced by measuring the relative millivolt (m.v.) values produced by free calcium. Calcium solutions were prepared from the Orion Research calcium stock solution. The Ionalyzer Model 701-A by Orion Research Incorporated, Cambridge MA, and the calcium electrode Model 93-20 from Orion Inc. were employed to measure the free calcium. Calcium (10^{-3} M) was arbitrarily set at +20.0 m.v. and the standard curve was produced covering the range of 10^{-4} M to 10^{-1} M calcium. All experiments using the electrode were referred to this graph for free calcium values.(Appendix 1).

b) Monitoring the effect of pH upon the binding of calcium by fababean flour: 20% flour solutions were prepared and adjusted to the desired pH levels of 8.0, 9.0, 10.0 and 12.0. Increments of 2.0 ml of 1.0 M calcium chloride were added to a maximum of 44 ml. Each aliquot of calcium was allowed to equilibrate before the next one was added. From the graphs produced, calculations were made(Appendix 1) to determine the amount of calcium that was bound. The amount that was bound was presented as percent bound.

G. Scatchard plots - kinetic considerations

Protein isolate (5.0 g) prepared by the protein micellization method (obtained from General Foods Ltd., Cobourg, Ontario) was dispersed in 23.2 ml of water. The pH was adjusted to 8.0 with 1.0 N sodium hydroxide and to this was added 1.0 ml of 4 M potassium chloride, obtained from Orion Research Inc. Calcium chloride (25.0 ml) at varying concentrations ($1.0x10^{-2}$,$2.0x10^{-2}$,$4.0x10^{-2}$,$5.0x10^{-3}$M) adjusted to pH8.00:.05 was added. The calcium electrode which was connected to the ion analyzer which in turn was connected to a Honeywell Electrik 19 recorder, was placed in the protein solution before the calcium was added. The system was enveloped with a plastic bag and contained a beaker of water in order to minimize sample evaporation. The system was allowed to run until equilibrium was reached (Appendix 2).

H. Effect of pH upon the binding of minerals and phytic acid to protein

Isolates prepared at solubilization pH values of 7.0, 8.0, 8.7, 9.4, 10.1, 10.5, 11.2 and 12.0 by isoelectric precipitation were tested for phytic acid (Latta and Eskin, 1980) and protein levels by the microkjeldahl method (AOAC, 1975a). The mineral content of each isolate was determined by the Soil Survey laboratory (Univerisity of Manitoba) using a wet digestion technique employing nitric and perchloric acids at a ratio of 2:1 for ashing. The solution remaining was filtered through Whatman No. 42 filter paper and brought to 25 ml in volumetric flasks. The samples were

then run on a Perkin-Elmer 560 atomic absorption spectrophotometer to monitor for various minerals in the samples. All samples were done in duplicate.

I. Turbimetric Studies:

A purified protein obtained by gel filtration on Sephacryl S-300 at a concentration of 1.5 mg/ ml (Lowry et al.,1951) was diluted by equal volume of 0.5N sodium chloride (NaCl) and 0.02% sodium azide (NaN_3). Before any experiments were pursued, all equipment was acid washed and rinsed with deionized water.

a) Binary systems:

i) A phytic acid and protein reaction was carried by mixing 9.7 ml of the 0.75 mg/ml protein with 0.1 ml of the NaCl/ NaN_3 solution and 0.2 ml of the 12.5 mg/ml phytic acid. These were mixed on a vortex mixer, allowed to stand for 15 minutes, turbidity at 600 nm was measured before and after filtration through Whatman No. 4 filter paper. The solution was then centrifuged at 15,000 xg and the protein absorbance was determined at 279 nm.

ii) 0.1 ml of 1.0 M calcium chloride was added to 0.2 ml of 12.5 mg/ml phytic acid and 9.7 ml of the NaCl/NaN_3 solution. The same analytical procedure was carried out as in case (i).

iii) 0.1 ml of 1.0 M calcium chloride ($CaCl_2$) 9.7 ml of the 0.7 mg/ml protein solution and 0.2 ml of the NaCl/NaN_3 were mixed and the same analytical procedure as in case (i) was carried out.

b) Ternary system: 9.7 ml of 0.75 mg/ml protein, 0.2 ml of 12.5 mg/ml phytic acid and lastly 0.1 ml of $CaCl_2$ at various levels so as to produce final concentrations of 4.0×10^{-2}, 1.0×10^{-2}, 1.0×10^{-3}, 1.0×10^{-4} M were added. The system was mixed and allowed to stand for 15 minutes. The turbidity was read before and after filtration on Whatman No. 4 filter paper at 600 nm. The solution was then centrifuged at 15,000 xg and the supernatant was monitored for protein at 279 nm.

J. Disodium (Ethylenedinitrilo) tetracetate (EDTA) Addition Studies:

Isoelectric precipitation isolates were prepared at pH 8.0, before anything was introduced into the water, EDTA was added. Isolates were prepared from extraction systems containing different amounts of EDTA (2.5, 5.0, 10.0, 15.0, and 20.0 g/system). Each fraction produced during the procedure was retained, weighed and monitored for phytic acid (Latta and Eskin, 1980) and protein by the microkjeldhal method (AOAC.,1975a).

Results and Discussion

The objective of this work, as stated initially, was to explore the possibility of a ternary complex as a mechanism of binding between phytic acid and protein. With this goal in mind, the following studies were undertaken.

A) Phytic acid in fababean varieties:

The amount of phytic acid in three fababean varieties is given in Table 3. Considering the varieties together, it may be concluded that the majority of phytic acid is found within the cotyledon while much less is present in the hull fraction of the seed. This being the case, it would be of limited usefulness to remove the hull as a method for significant reduction of phytic acid. It would however, be of value as one step in a method of removing phytic acid from fababean.

B) Phytic acid in protein preparations:

Protein was concentrated by a number of different methods and the results of these findings are given in Tables 4 and 5. It is determined from the results of Table 4 that when protein is concentrated by a physical method, phytic acid is also concentrated. Flour, made from ground whole fababeans (var. Diana, 1978), has a protein content of 30.03% and a phytic acid level of 1.61%. When the same fababean flour is air classified, the phytic acid level is increased to 4.23% while the protein level is concentrated to 66.65%. Griffiths and Thomas (1981) reported that air

Table 3 Phytic Acid Levels in Fababean Varieties

Variety	Year	Phytic acid (%)a		
		Whole	Cotyledon	Hull(b)
Diana	1979	0.95:.04	0.84:.05	0.11:.06
Diana	1978	0.73:.02	0.61:.05	0.13:.05
Ackerperle	1978	1.25:.02	1.20:.10	0.10:.10
Aladin	1977	1.45:.03	1.40:.10	0.10:.10

a. Analysis by Harland and Oberleas (1977) dry basis

b. Phytic Acid level found by difference

(Details of calculations are in Appendix 3).

Table 4 Phytic Acid and Protein Levels in Whole Fababean Flour and Protein Concentrate

	Phytic Acid (%)a	Protein (%)b
Flour	1.57:.01	29.9:.1
Concentrate	4.1:.2	67.0:.6

a. Analysis by Latta and Eskin (1980) dry basis

b. N x 5.7

(Details of calculations are in Appendix 3).

classification also concentrated phytic acid with protein to levels greater than 4% in fababeans. Since phytic acid and protein are intensified 2.6 and 2.2 fold respectively, some thought as to the reason is warranted. Phytic acid, during the air classification procedure is concentrated either because the method separates it from much of the starch in a similar way to protein or phytic acid is bound to protein in the native state and thereby is concentrated with the protein.

Since the literature and the physical type of protein concentration suggest a relationship between protein and phytic acid, it was decided to pursue protein recovery further. Various methods, namely isoelectric precipitation, protein micellization and salt solubilization with acid precipitation were undertaken and the isolates obtained by these processes are compared in Table 5.

When proteins are concentrated by the isoelectric precipitation (IEP) method, phytic acid tends to concentrate with the protein to a greater extent than it does with the other methods. This conventional method of protein isolation involves the manipulation of the various chargeable groups on the protein and thus by electrostatic repulsion, protein is solubilized. As Omosaiye and Cheryan (1979) found, the amount of phytic acid which is recovered with the protein is greatly dependent upon the pH of the system. Above the isoelectric point, usually at an alkaline pH, protein is negatively charged overall and since phytic acid is negatively

Table 5 Phytic Acid Levels in Isolates Prepared by Three Different Methods

Method of Preparation	Phytic Acid (%)a	Protein (%)b
Isoelectric Precipitation pH 8.0/pH 4.5	3.0:.1	73.3:.3
Protein Micellization	0.39:.05	89:1
Salt Solubilization/ acid precipitation	1.32:.05	78:2

a. Latta and Eskin (1980)

b. N x 5.7

charged, a ternary complex is quite conceivable. After centrifugation of the flour at pH 8.0, the solution is acidified to pH 4.5 where the salt bridge is quite weak (Omosaiye and Cheryan, 1979). In the isolate however, phytic acid was found. Upon examination of the fababean protein amino acids, glutamic and aspartic acids make up 32% of the protein. Glutamic acid, pK -COOH is 4.28 and aspartic acid pK B-COOH is 3.65; these acids, at pH 4.5, are still negatively charged resulting in a great potential for indirect (ternary) binding to occur.

The 0.3 M (1.2%) sodium chloride/pH 4.5 isolation method reduces the amount of phytic acid that is attached to the protein. The protein is solubilized at the natural pH of flour and water (pH 6.2:.1) by the salting-in effect of sodium chloride. Since the highest (alkaline) pH of the system is much less than the IEP method, fewer groups are negatively charged and therefore fewer groups are available for binding. DeRham and Jost (1979) found that if 8.5% sodium chloride were present in the solution, disruption of the binding occurred.

Since only 0.3 M sodium chloride was used during the salting-in process, the ionic strength of the sodium chloride did not play a part in reducing the amount of phytic acid bound to the protein. The phytic acid which was not bound to the protein was probably in the binary form, either bound to divalent or monovalent cations. If in the divalent salt form, phytate would precipitate from solution during

the first centrifugation step while the sodium phytate would remain soluble. When the pH was decreased to 4.5, the same reasoning as in the IEP method would be appropriate to explain the results. The sodium phytate however would remain soluble and not precipitate with the protein.

The protein micellization method of isolation uses the salting-in phenomenon for solubilizing the protein but a different method is used for precipitating out the protein. The hydrophobic nature of protein is taken advantage of in order to avoid the extremes of acid and alkali which tend to denature the protein structure and thus alter its functionality. The same reasoning as in the previous method is applicable in the protein micellization method of isolation. The remaining solution, the supernatant, of the first centrifugation step is diluted with water and thereby maintains the pH at the natural level of water. When the supernatant is diluted, the protein precipitates due to apparent hydrophobic interactions. When a protein molecule does not possess sufficient polar residues on its surface to allow the hydrophobic amino acids to be buried, some are exposed. In a salted-in condition, addition of water allows the proteins to be less salted-in and forces hydrophobic groups to coalesce. When the coalesced structures (micelles) become so large that the solution cannot support them, the protein precipitates. The micellization process is due to protein - protein interactions with the potential to exclude nonproteinaceous material such as phytic acid (Murray et al.,

1981). As evidenced in Table 5 the amount of phytic acid is substantially reduced by the protein micellization method as compared to the other two methods but the details of the actual exclusion phenomenon are not known.

C) Addition of phytic acid to flour slurries:

Generally, food protein isolates are prepared industrially via the isoelectric precipitation method. Since this is the case, the research to be carried out throughout this project will deal mainly with the IEP method.

As the literature has pointed out and the studies to this point have indicated, phytic acid and protein interact in some manner. In the way of further preliminary work it was decided to investigate whether or not the protein could have more phytic acid associated with it. In order to determine this point, additional phytic acid was added to flour slurries and protein isolates were prepared. At pH 8.0, the pH of solubilization, it was found that the level of phytic acid could be raised from 3.02% without any additional phytic acid to 4.4% and 7.0% if only 5 and 10 times the amount of native phytic acid were added to the slurry, respectively. The reason being that the reaction of Figure 8b could be driven further toward the ternary complex. The protein therefore is not saturated with phytic acid but simply is at equilibrium with that phytic acid which is probably in the binary salt form. Clearly, these proteins have the ability to bind considerable phytic acid.

D) Effect of the pH of solubilization upon the absorption of calcium by fababean flour:

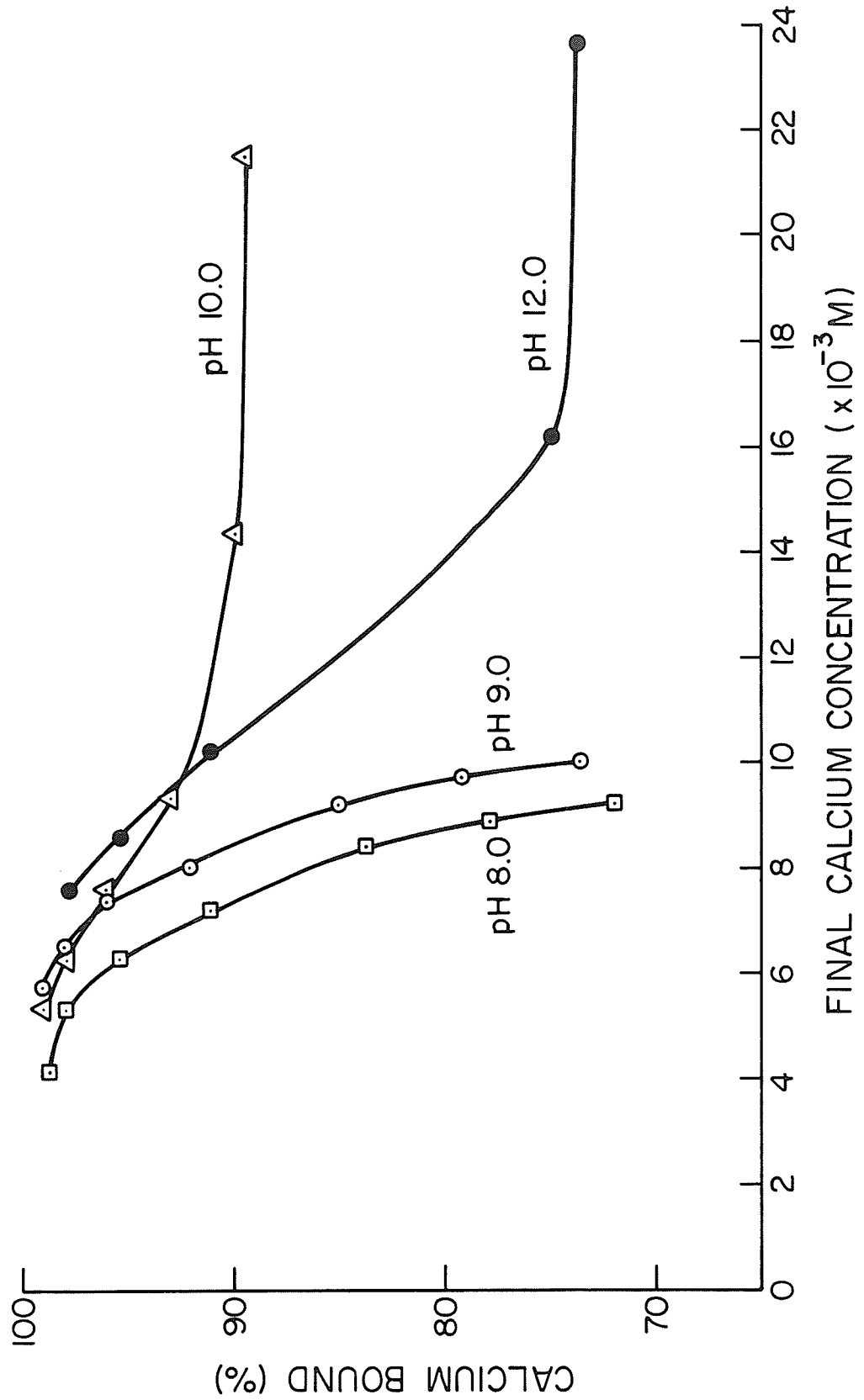
As has been shown in the previous section, the protein is not saturated with phytic acid but probably the amount of phytic acid bound depends upon the equilibria of all reactions occurring within the system. Since involved in the ternary complex is a divalent cation along with the protein and phytic acid, it was decided to explore the effect of pH upon the ability of the protein to bind the cation. Flour slurries were prepared at pH 8, 9, 10 and 12 and the amount of calcium that could be bound in each slurry was studied. As the pH of the slurry was altered (Fig. 11) there was a large change in the amount of calcium that could be bound. Due to the limitations of the experiment equilibriums were not obtainable for the pH 8 and 9 slurries but the data available indicate that at pH 8, less calcium was bound than at pH 9. The slurry at pH 10, at equilibrium, bound about 90% of the calcium added to the slurry which may be explained by degradation of the protein. If protein denaturation were the only determining factor of binding, the pH 12 system would have been expected to bind more than 90% of the calcium instead of the 74% that was observed. Cheryan (1980) explains this phenomenon by the following equation:

(Protein - divalent cation - phytic acid) + sodium

Protein - sodium + divalent cation - phytic acid.

The reaction proceeds to the right since at extreme alkali conditions, pH 12,there are many sodium molecules present.

Figure 11. Effect of pH upon the ability of a fababean flour slurry to bind calcium.


CALCIUM BOUND (%)
100
90
80
70
0
2
4
6
8
10
12
14
16
18
20
22
24
FINAL CALCIUM CONCENTRATION (x 10⁻³ M)
pH 10.0
pH 12.0
pH 9.0
pH 8.0

Instead of the divalent cation binding between phytic acid and protein the sodium replaces some of them, and thus reduces the ability of the protein to bind multivalent cations such as calcium.

E) Scatchard plots- kinetic considerations:

Appurao and Narasinga Rao (cited in Cheryan, 1980) have researched the binding of various cations to soy protein fractions by Scatchard plots. It was found that the most probable binding sites of those cations were the imidazole groups of histidine because good correlations existed between the moles of cation bound and the number of histidine residues for the pure protein fraction. In the presence of phytic acid however, abnormal Scatchard plots and much larger bound ratios were obtained suggesting that calcium was bound to phytic acid as well as protein.

Scatchard plots were developed by Scatchard (1949) in order to determine the classical first association constant and the number of binding sites on proteins. Using the law of mass action the following equation was proposed:

$$v/c = k\,(n - v)$$

where v = moles of ligand bound per mole of protein
c = concentration of free ligand at equilibrium
k = first association constant
n = number of binding sites

When v/c is plotted against v, a straight line develops if k is a constant value. The value of the intercept of the v/c axis yields the product kn and the intercept of the x axis is n. Curvature may indicate different intrinsic constants but it is not necessary to straighten the line if the corre-

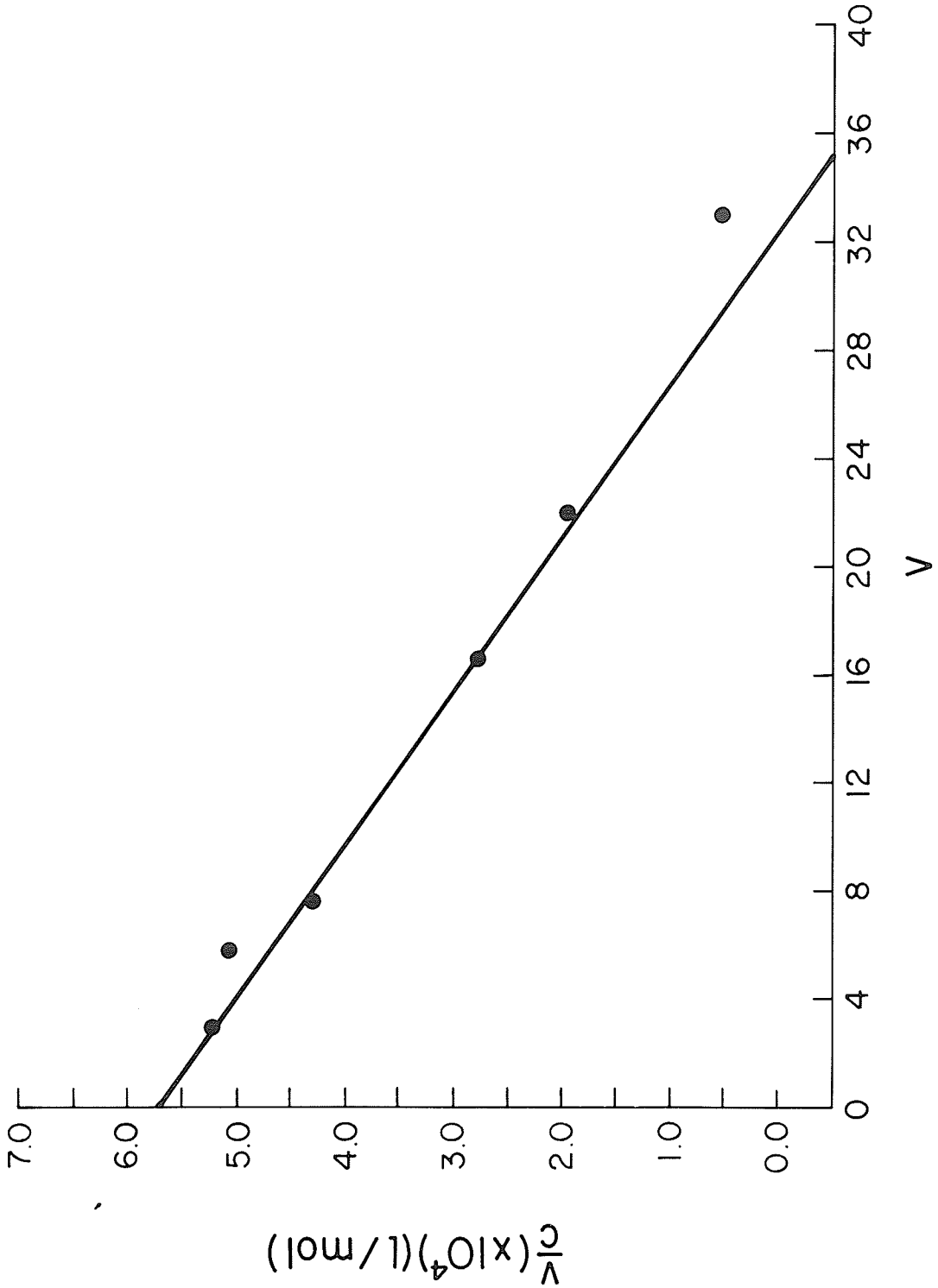
lation is good enough to determine the intercepts (Scatchard, 1949).

Protein solutions were prepared by adding isolate (via the protein micellization method) to water, adjusted to pH 8.0:.1, and to each solution different levels of calcium chloride were added. The result, a Scatchard plot is given in figure 12. The plot, as discussed, gives the number of binding sites on the protein to be 35 per molecule and the first association constant to be 1.62×10^{-3}. This indicates that the reaction tends toward the products since k is quite large. In such a mixed system, (not a pure protein) the value of k is for all reactions occurring within the system as opposed to the reaction occurring between calcium and a pure protein. Future work should involve only a purified protein and calcium; n and k should be obtained and the change in values monitored when phytic acid is added to such a system.

F) Effect of pH upon the binding of minerals and phytic acid to protein

As has been postulated and to a limited extent explored by various researchers (Cheryan,1980), the ternary complex formation studies were continued. Protein isolates were prepared at various pH levels of solubilization ranging from pH 7 to 12 so as to monitor the level of phytic acid and minerals recovered in each isolate. The objective of this study was to look for any trends which may occur as the pH of solubilization was altered.

Figure 12. Scatchard plot produced by the addition of calcium chloride (pH 8.0) to a protein isolate in solution at pH 8.0.


$\frac{V}{C}(\times 10^4)(L/mol)$
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
0
4
8
12
16
20
24
28
32
36
40
V

Each isolate was examined for the amount of phytic acid (Fig. 13) and the amount of calcium, magnesium and zinc (Fig. 14). In this series of isolates, each isolate had a phytic acid level between 2.5% and 3.5%, depending upon the pH of solubilization. Calcium, when compared to magnesium and zinc (Fig. 14), shows the greatest response to a change in the pH of solubilization. The level of magnesium and zinc in the isolates did not appear to be altered significantly as the pH of solubilization changed, unlike the response for calcium. In general, calcium is sequestered less preferentially than magnesium (Hole, 1980); this means that calcium is much more responsive to the differences of pH of solubilization than other minerals. The level of calcium within the isolates increased steadily until a high alkaline pH is reached where a subsequent decrease is noted.

Since during the protein isolate formation some of the components found within the starting material were concentrated, it is of value to determine the amount of protein, calcium and phytic acid recovered in each isolate. Calcium was the mineral chosen to be monitored throughout these experiments since it demonstrated the greatest response to pH change. Since calcium was so responsive to the change in pH it was hoped that it would demonstrate most readily any possible interactions.

Figure 15 demonstrates the amount of protein, calcium and phytic acid recovered within each isolate. The amount of protein recovered within each isolate increased, as

Figure 13. Phytic acid levels in protein isolates prepared by the isoelectric precipitation method where the pH of solubilization was altered over a range of 7.0 to 12.0.

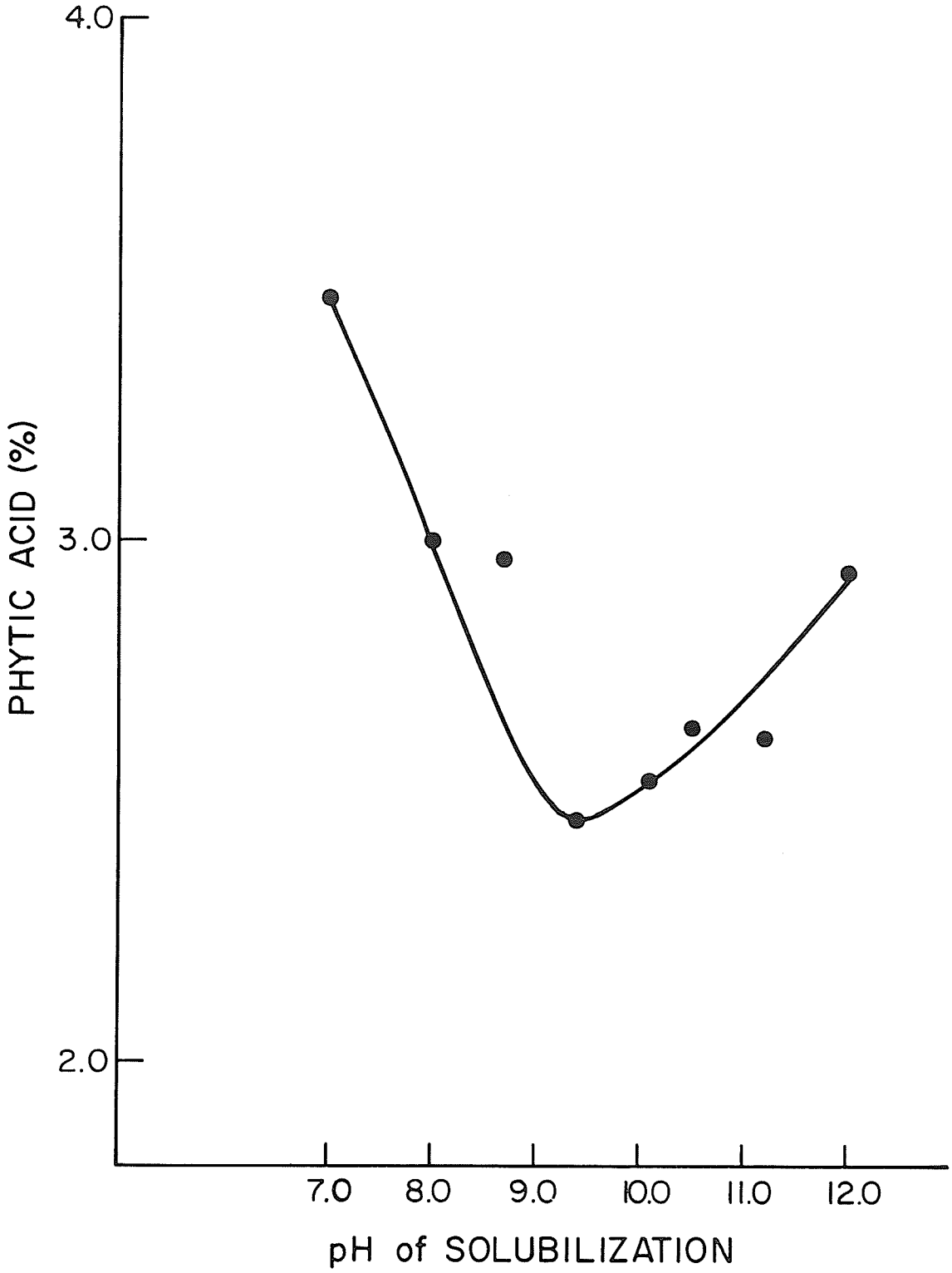

4.0
3.0
2.0
PHYTIC ACID (%)
7.0
8.0
9.0
10.0
11.0
12.0
pH of SOLUBILIZATION

Figure 14. Mineral levels in protein isolates prepared by the isoelectric precipitation method where the pH of solubilization was altered.

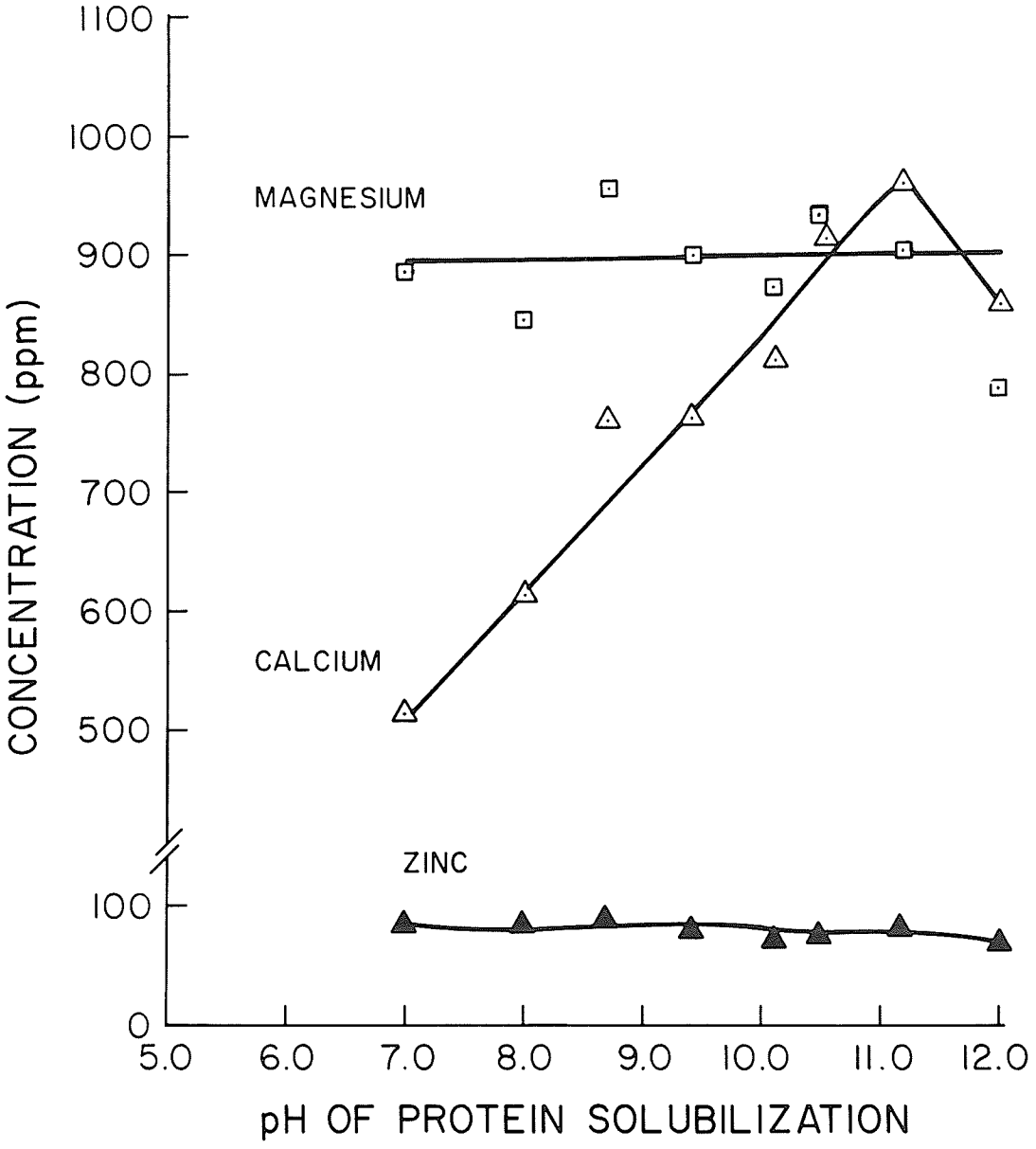
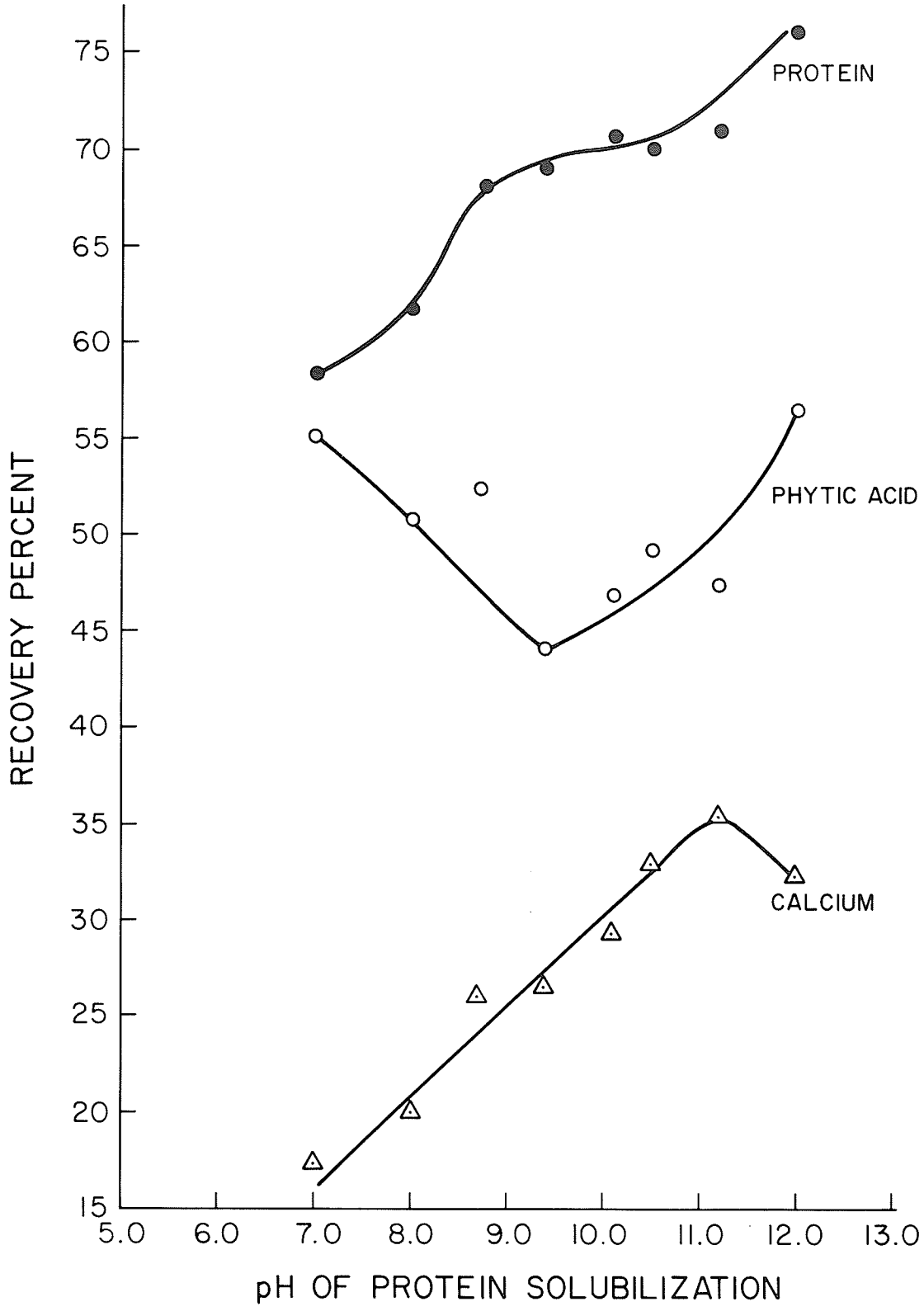

1100
1000
900
800
700
600
500
100
0
CONCENTRATION (ppm)
MAGNESIUM
CALCIUM
ZINC
5.0
6.0
7.0
8.0
9.0
10.0
11.0
12.0
pH OF PROTEIN SOLUBILIZATION

Figure 15. The effect of the pH of solubilization upon the recovery of protein, phytic acid and calcium in protein isolares.


RECOVERY PERCENT
75
70
65
60
55
50
45
40
35
30
25
20
15
PROTEIN
PHYTIC ACID
CALCIUM
5.0
6.0
7.0
8.0
9.0
10.0
11.0
12.0
13.0
pH OF PROTEIN SOLUBILIZATION

expected, since as the pH increases the protein becomes more negatively charged, resulting in a greater solubilization of the protein. Calcium, in the isolates, demonstrates a similar trend to that of protein until extreme alkali conditions (pH ¨ 11.0) were reached where upon it decreased. The reasoning for this decrease may be similar to that given by Cheryan (1980) where the sodium, because of its high ionic strength, displaced the calcium.

Phytic acid, if it responded ideally, would rise in a similar fashion to calcium and thus decrease when calcium decreased, since in the proposed ternary complex the mineral is thought to mediate the binding of protein and phytic acid. It would be expected that since the protein was being denatured with increasing levels of alkali, phytic acid would tend to be bound more and more until it reached extremes of alkali where the sodium would tend to disrupt the binding due to high ionic strength. Little work is known on phytic acid binding to fababeans. Since fababean storage proteins have two main species (P and Q) with molecular weights 340,000 and 140,000 d respectively, (Murray et al.,1981) and similar rates of migration on Sephacryl S-300, probably due to the horseshoe shaped structure of Q, some of the irregularity of these results may be due to the protein anomalies. As the results are quite different from those of other researchers working with different protein sources, one can only speculate at this point. Possibly there are two different types of binding mechanisms occurring where one

and then the other has more effect during the experiment. Due to the characteristics of the fababean protein, much different results are seen than in the case of soybean protein.

In the case of the fababean protein some conclusions may be drawn (Fig. 15). Of the native phytic acid 44-56% was retained, 58-76% of the protein was recovered and 15-35% of the calcium was retained in each isolate depending upon the pH of solubilization.

G) Turbidimetric studies:

The protein containing preparations used to this point were quite impure and contained many components making the results in the previous section difficult to interpret with regards to a ternary binding mechanism. It was decided therefore to use a purer system. While doing preliminary work it was found that protein, phytic acid and calcium precipatated only under certain conditions and combination of components, therefore turbidimetric studies were pursued involving the three key components.

A purified preparation of fababean storage protein, 1.5 mg/ml, was obtained by gel filtration on Sephacryl S-300, reagent grade calcium and sodium phytate were obtained from commercial producers. Binary and ternary systems were studied by allowing two or three of the components to be brought together in solution. The precipitation formed by various reactions was approximated by measuring the turbidity at 600 nm and the soluble protein was measured at 279

nm. A summary of the findings of both the binary and ternary combinations is found in Table 6.

The only precipitation which occurred between any of the binary systems was the reaction of calcium and phytic acid at pH 6.6. Most probably, the reaction was due to an electrostatic interaction between the two components where calcium replaced the sodium on the phytate molecule. It may be recalled from the literature review, that calcium phytate precipitates from solution at pH 6.6. Upon filtration of the turbid solution, simply through Whatman No. 4 filter paper, the complex could easily be removed as evidenced by the resultant absorbance.

When the three components were mixed together, calcium being the last to be added, the solution became cloudy immediately. Only a portion of this cloudiness could be removed by filtration which suggests that two types of precipitate were present. One precipitate, the binary complex of calcium and phytic acid, was shown to be removable by filter paper so that the other precipitate can only be explained as a ternary complex. After filtration and reading the absorbance at 600 nm, the solution was centrifuged at 15,000 x g. and the protein absorbance measured at 279 nm. It was reasoned that if protein became involved in the complex and if calcium and phytic acid together caused a protein precipitate, then the protein concentration should decrease as the calcium level increased. The protein concentration decrease should be observed by a decrease in protein absorbance in

Table 6 Turbidity formation in different reactant systems, all at pH 6.6

Binary System	A 600 (Before filt.)	A 600 (After filt.)	A 279
Calcium + Protein	0.001:.000	0.001:.000	0.354:.002
Phytic Acid + Protein	0.000:.000	0.004:.002	0.378:.001
Calcium + Phytic Acid	0.12:.03	0.004:.002	0.07:.01
Ternary System			
Calcium + Protein + Phytic Acid	0.30:.04	0.24:.01	0.30:.02

Reactant Concentrations:

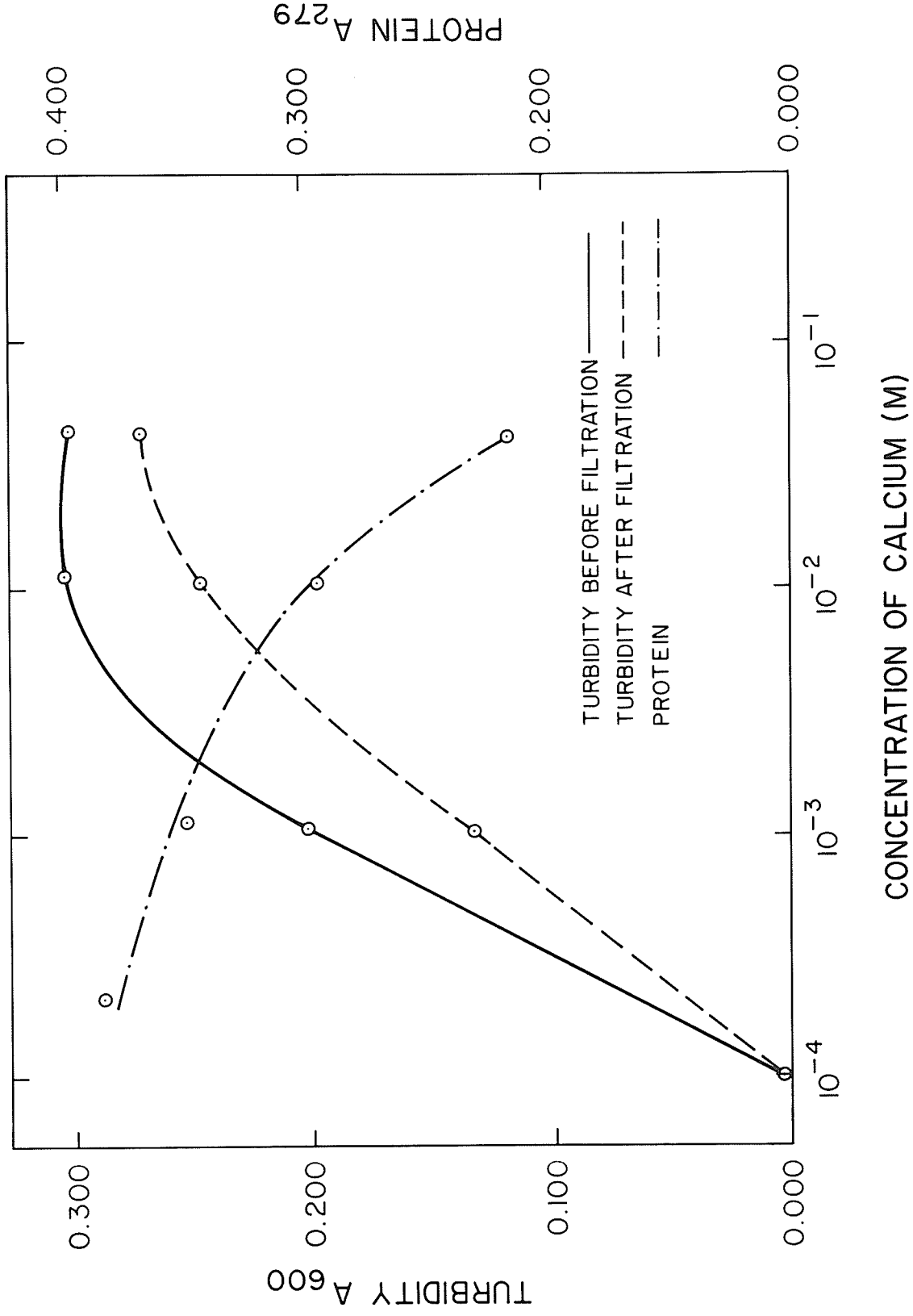
Calcium Chloride	10^{-2}M
Phytic Acid	250 ug/ml
Protein	250 ug/ml

accordance with Beer's law.

As the calcium concentration was increased (Fig. 16), all other reactant concentrations being equal, the turbidity before and after filtration increased while the soluble protein concentration decreased. Since the only binary complex to cause a precipitation was calcium phytate, the second precipitate must be due to the reaction between all three components. It can be concluded therefore that calcium is needed for the protein-phytic acid complex since the equilibrium can be shifted toward the ternary complex by simple addition of calcium to a system where calcium is a limiting factor. A second observation is in order, that is, at pH 6.6 the solubility of protein decreases and precipitates from solution when the three components interact.

These turbidimetric experiments support and extend work by Fontaine et al.,(1946) and Gillberg and Tornell (1976). Fontaine et al. (1946) found that phytate clouded protein solutions of peanut, soybean and cottonseed which were in some cases impossible to clarify by centrifugation; upon standing a flocculent precipitate occurred. A similar observation was made in this current work. Fontaine et al. (1946) found that the precipitate was high in phosphorus and stated that it probably consisted of phytin and some adsorbed proteins. The Gillberg and Tornell research involving rapeseed meal found turbidity to occur at pH less than 3.5 and between pH 5 and 11. The turbidity of the extracts was attributed to finely divided insoluble phytic acid

Figure 16. Turbidity changes in ternary systems with increasing calcium levels. Note the decrease in protein absorbance with increasing calcium.


TURBIDITY A_{600}
0.300
0.200
0.100
0.000
PROTEIN A_{279}
0.400
0.300
0.200
0.000
10^{-4}
10^{-3}
10^{-2}
10^{-1}
CONCENTRATION OF CALCIUM (M)
TURBIDITY BEFORE FILTRATION
TURBIDITY AFTER FILTRATION
PROTEIN

derivatives. The work done here demonstrates the usefulness of the filtration step in removing the binary type turbidity. There did not appear to be any difficulty in removing the turbid material by the filtration and the centrifugation steps and hence the spectrophotometric measurements in the ultraviolet region were not influenced.

H) Competitive Effects of EDTA

Since the ternary complex is a proposed mechanism in the literature and somewhat substantiated by the work reported here, a reduction in the concentration of divalent cations implies the breakdown or inhibition of the complex. As demonstrated by Omosaiye and Cheryan (1979) at alkaline pH, only a small reduction of phytate was observed in the first stage of ultrafiltration which represents mainly free phytic acid and a soluble complex of cation plus phytate. Upon dilution of the retentate to the original volume and repeating the ultrafiltration it was found that phytate was reduced. They explained this by observing that at the end of the first stage of ultrafiltration over one half of the salts were removed. Hence, when the retentate was rediluted the cation concentration was less, causing the reactions in Figure 8b to shift to the left resulting in a possible ternary complex reduction. It was therefore decided that since EDTA binds cations preferentially over phytic acid, that a similar reduction in the ternary complex should be observable.

During the preparation of isolates, three fractions

were produced namely the starch containing first precipitate, the supernatant and the isolate (fig. 17). At each stage of the procedure various fractions of the flour are obtained and usually all but the isolate are discarded. Since this is the case it was of interest to monitor the amount of phytic acid in each fraction as isolates were prepared at pH 8.0 with varying levels of EDTA added. The levels of EDTA added were chosen to demonstrate the effectiveness of EDTA upon the reduction of phytic acid levels in the isolate . EDTA effectively reduced the amount of phytic acid in the isolates (Fig.18) and the figure also points to the fraction where most of the phytic acid accumulated. The supernatant became that fraction which tended to accumulate the phytic acid, which did not bind to the protein isolate fraction. The level of phytic acid in the first precipitate remained constant. This suggests that the phytic acid level in the first precipitation step does not change much when EDTA is added to the system. It does demonstrate however, that when the pH is lowered to 4.5, that the majority of phytic acid is present in a soluble form.

It appears that the phytic acid not associated with protein does not become insoluble in the presence of EDTA. The EDTA, a stronger chelator than phytic acid, binds many of the divalent cations so that the ternary complex is partially blocked (Okubu *et al.*, 1975) rendering much of the phytic acid free of the protein. Since EDTA is added as the disodium salt and EDTA binds divalent cations preferen-

Figure 17. Isoelectric precipitation technique. Note the levels of phytic acid recovered in each fraction. Prepared at pH 8.0/ pH 4.5.

FABABEAN FLOUR (20% w/v)

PRECIPITATE
(phytic acid
recovery of
23.74%)

adjust pH to 8.0
stir 30 min.
centrifuge 8,000 x G

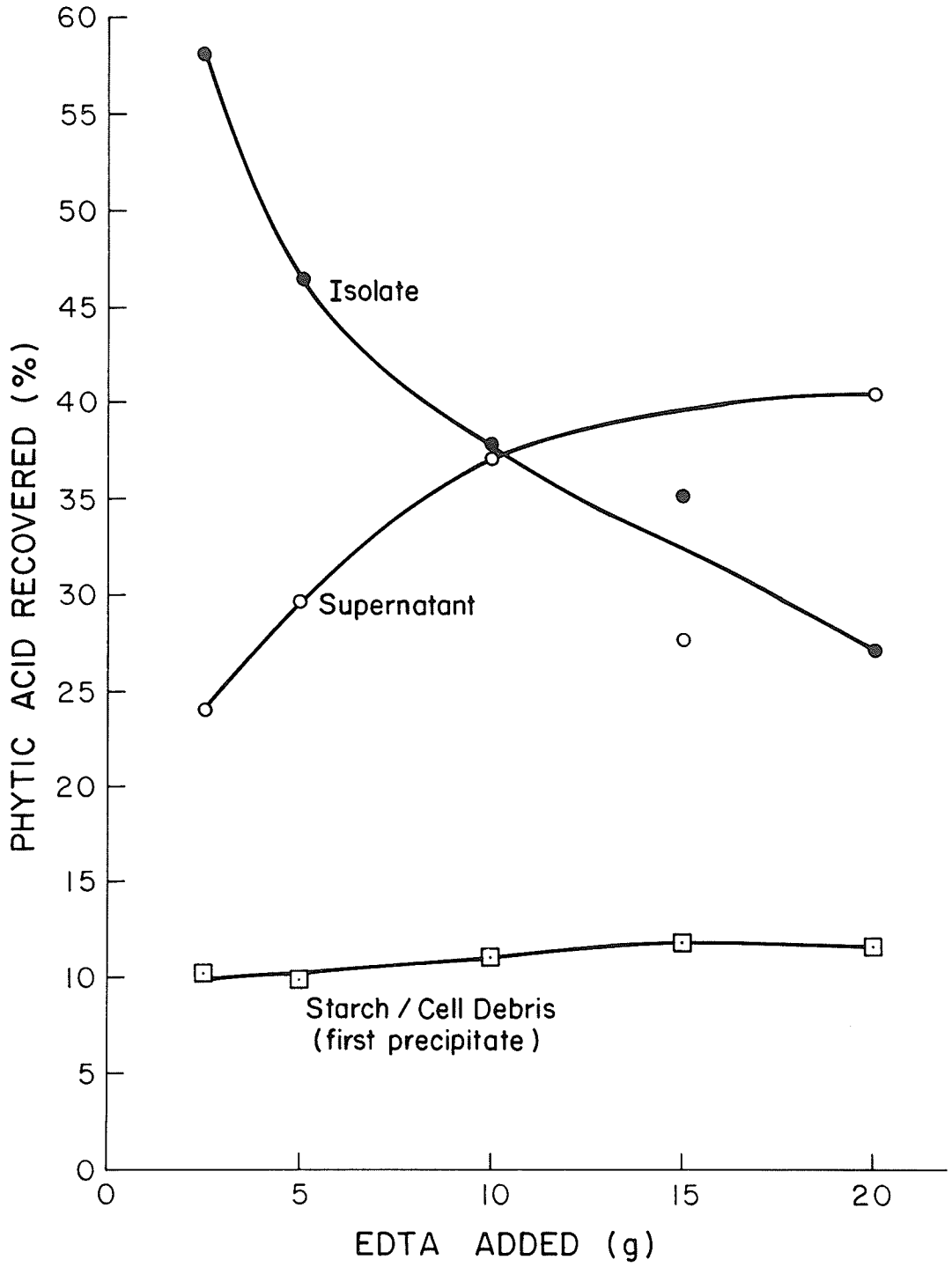
SUPERNATANT

SUPERNATANT
(phytic acid
recovery of
17.8%)

adjust to pH 4.5
stir 5 min.
centrifuge 4,000 x g

ISOLATE
(phytic acid
recovery of 50.56%)

Figure 18. Effect of EDTA addition upon the phytic acid levels in each fraction of the isoelectric precipitation method.


PHYTIC ACID RECOVERED (%)
60
55
50
45
40
35
30
25
20
15
10
5
0
Isolate
Supernatant
Starch / Cell Debris
(first precipitate)
0
5
10
15
20
EDTA ADDED (g)

tially to monovalent cations, it is speculated that sodium ions will be free in solution until phytic acid, which also is free in solution, chelates them. When this happens phytate remains in solution since the sodium phytate is soluble at alkaline pH. Sodium hydroxide, used to produce alkaline conditions, will also donate ions to the free phytic acid molecules to produce soluble phytate. It can be seen by the foregoing rationale therefore, that protein would have less phytic acid bound to it due to the presence of EDTA and possibly the majority of free phytate could remain soluble due to the formation of soluble phytate and be collected in the supernatant.

Phytic acid in the final isolate may be reduced when isolates are prepared in the presence of EDTA (Fig.18). Initially, in control extractions without any EDTA, about one half (50.56%; Fig. 17) of the total phytic acid in the starting material was present in the final isolate. When EDTA was added, the amount of phytate recovered in the isolate was reduced to one quarter (28.5%; Fig.18) of the phytic acid in the starting material. Further refinement of this approach to reducing phytic acid in the final isolate by competitively binding calcium should be possible.

Conclusions and Recommendations

The work carried out here has been made much easier because of the Latta and Eskin (1980) method. The method of Harland and Oberleas (1977) is as accurate as the Latta and Eskin method but it is much more difficult to obtain the phytic acid value with the latter method.

Phytic acid, the antinutritional factor of so many plant products, has been shown throughout this work to definitely concentrate with protein when isolates are prepared. Even when physical methods such as air classification or chemical methods such as isoelectric precipitation were used to produce isolates, phytic acid was concentrated. It appears that the best method for preparing isolates and avoiding phytic acid carry over is the protein micellization method,this method provides pure isolates which are low in phytic acid and still highly native.

When using the conventional approach to protein isolation, the isoelectric precipitation method, the peculiar fababean protein characteristics may affect the amount of phytic acid recovered. When working with fababeans one cannot assume that the same type of technology used on soybeans will achieve the same affect on fababeans as far as reducing the amount of phytic acid in the final isolate is concerned.

It is clear however, that calcium and possibly to a some extent other divalent cations are essential to promote the binding of phytic acid to protein. It has been shown by the turbidimetric studies and the EDTA addition studies that

phytic acid is bound to the protein and may be reduced in the final isolates by restricting the amount of divalent cation available for bridging protein and phytic acid.

The work presented here has generated some questions which if answered would aid in understanding the binding mechanism and in establishing systems for protein isolation which would exclude a large portion of native phytic acid. Since little work has been done on phytic acid binding to fababean protein it may be helpful to study the characteristics of fababean phytate and phytase. Information such as the solubility of fababean phytate, the optimum temperature and pH would aid in the quest for more general information.

It would be of value to pursue further experiments dealing with the binding mechanism. In order to explain the phytic acid recovery trend when the pH of solubilization is altered, it may be useful to examine the results in light of the amino acid constituents of the protein keeping in mind the number of exposed and buried reactive sites at each pH of solubilization.

Many more facts could be found by use of the Scatchard plots, as already pointed out in the results and discussion (section E), work with a pure system could be undertaken since such a system would allow more control. Many combinations could be set up so as to determine n and k values. For example at constant alkaline pH, the protein concentration could be held constant and varying levels of calcium could be added and then protein and calcium could be held constant

and varying levels of phytic acid added to it. Many combinations may be set up but when all three components are present two types of reactions would occur so that the calculation of n and k would need to reflect this consideration.

When pursuing the turbidimetric studies, it would be informative to add EDTA to the systems and observe the results. Hopefully when EDTA would be added, less turbid conditions would exist so that more protein would be soluble. This would occur due to the fact that the EDTA would bind the calcium and result in less calcium phytate,less ternary complex and less turbidity.

In summary, it is believed that the binding mechanisms involved at alkaline conditions, particularly those pH levels used in the food industry (pH 7.0-10.0), are the binary and the ternary complexes involving calcium-phytate as well as protein-calcium-phytate. These two complexes operate in equilibrium.

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Appendix 1

A sample calculation of the amount of calcium that is bound to flour as a function of the amount of calcium which is added. The following calculation is for flour initially at pH 8.0. From the graph of calcium chloride (Fig 1-1) added versus relative millivolt select six y-axis values and the corresponding level of calcium added. Convert the amount of calcium added to mole values. From the standard curve (Fig.1-2), determine the amount of free calcium present at each millivolt (m.v.) reading. The difference between the amount that is added and the amount that is free is the amount that is bound.

At pH 8.0, m.v. reading +10.0, .0054 moles of calcium chloride had been added.

0.0054 litres (l) of 1.0 M calcium chloride equals 0.0054 moles. From the standard curve, 0.00041 M yields a 10.0 m.v. reading.

$$0.00041\ M = X\ \text{moles}/0.3\ l$$
$$X\ \text{moles} = 0.00012\ \text{moles}$$

Therefore 0.0054-0.00012=0.0053 moles of the calcium was bound. OR (0.0053/0.0054)x100=98.1% of the added calcium was bound.

Figure 1-1. Conductivity of calcium in flour solutions.

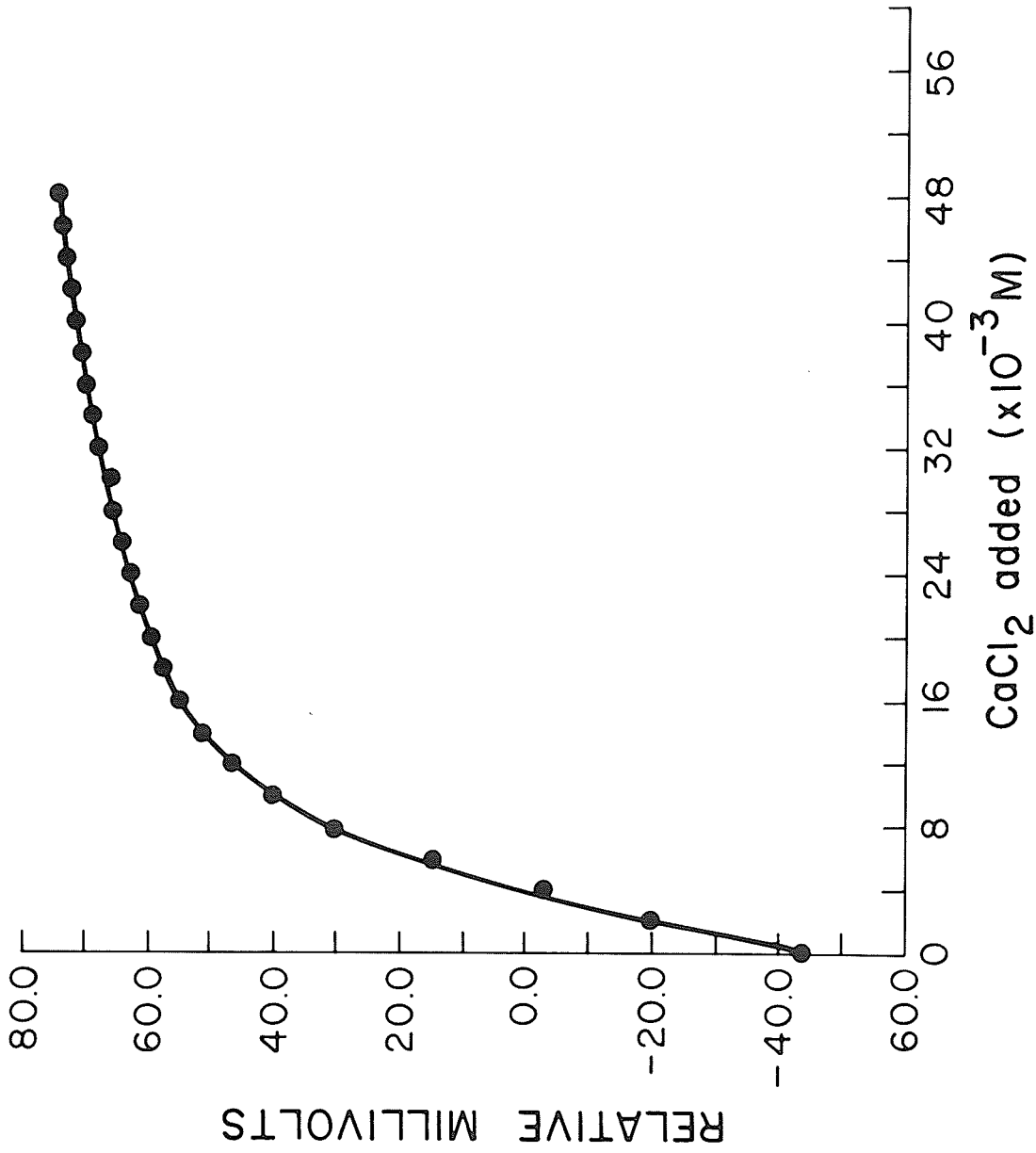
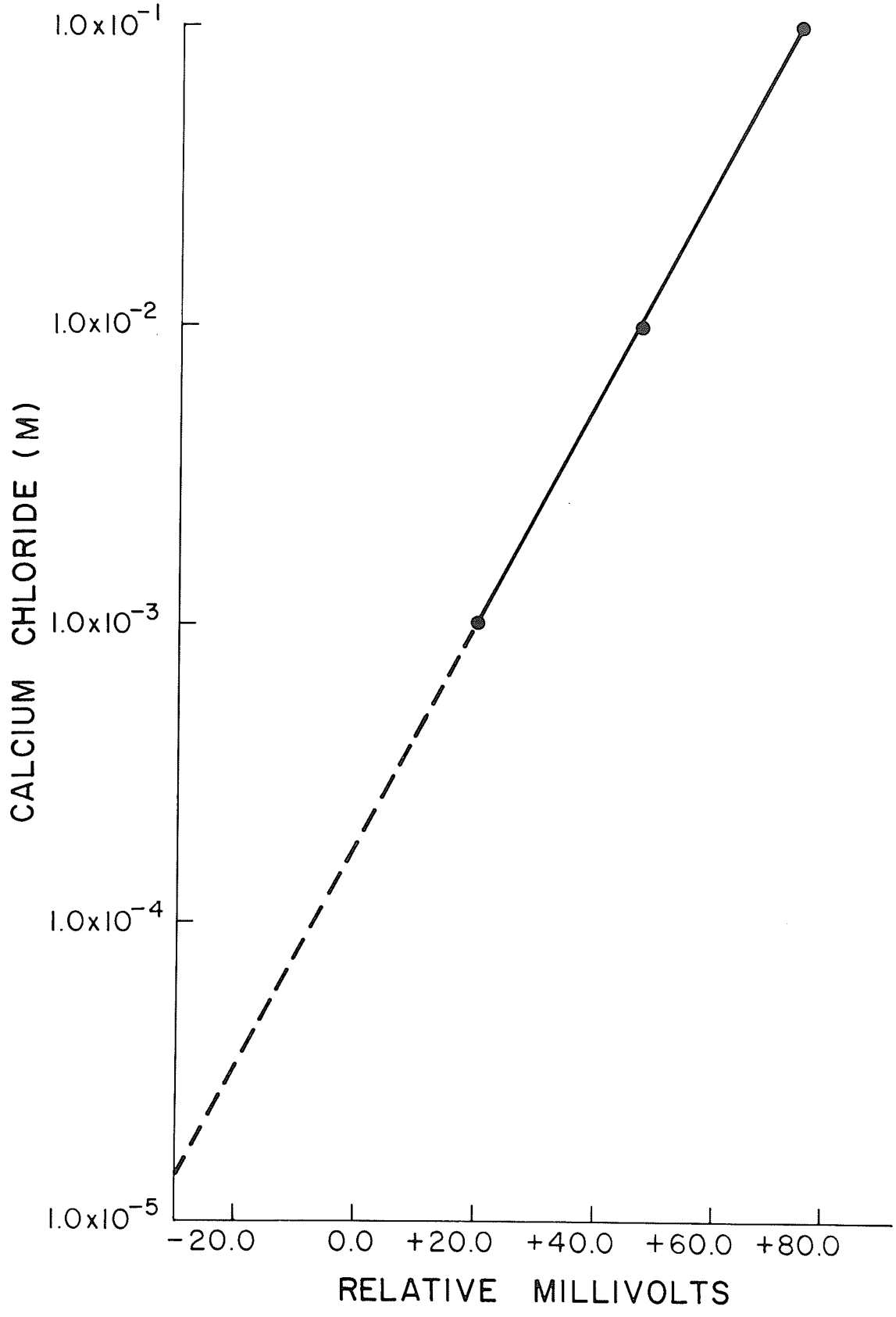

RELATIVE MILLIVOLTS
80.0
60.0
40.0
20.0
0.0
-20.0
-40.0
60.0
0
8
16
24
32
40
48
56
$CaCl_2$ added ($x10^{-3}$M)

Figure 1-2. Standard curve of the calcium ion electrode. 1.0×10^{-3} M calcium arbitrarily set at +20.0 mv.


1.0x10⁻¹
1.0x10⁻²
1.0x10⁻³
1.0x10⁻⁴
1.0x10⁻⁵
CALCIUM CHLORIDE (M)
-20.0
0.0
+20.0
+40.0
+60.0
+80.0
RELATIVE MILLIVOLTS

Appendix 2

The Scatchard plot was calculated as follows: When 0.04g of calcium was added to the protein using the standard curve of appendix 1 and the same type of calculation for the amount of free calcium and the amount of bound calcium, calculate v,c,v/c.

v=moles of bound ligand/moles of protein
v=(0.0276g/40.0g/mole)/(5.00g/240,000g/mole)
v=33.1 (no units)

The molecular weight (m.w.) of protein (240,000) is obtained from Appendix 7. Since fraction Q has m.w. 140,000 and P is 340,000 m.w.,the proportion of each not being known, the average of the two species was the molecular weight used for these calculations.

c=concentration of free ligand at equilibrium
c=(0.0124g free calcium/40.0g/mole)/(0.05 l)
c=0.0062 M

Therefore v/c=33.1/0.0062 mole/l
v/c=5,340.0 l/mole

The Hewlett Packard 9100 calculator using a program involving the least squares method for the determination of the slope was used to calculate values for the slope, y-intercept and the x-intercept for figure 12.

The slope of the graph is $(-1.62 \pm .09)\times 10^{3}$

The y-intercept is $(5.7 \pm .2)\times 10^{4}$
The x-intercept is $(3.5 \pm .1)\times 10^{1}$

Since the x-intercept (v)=n, $n=(3.5 \pm .1)\times 10^{1}$ The y-intercept (v/c)=kn. Therefore $k=(1.63 \pm .07)\times 10^{3}$. The value of the k uncertainty term is found by the Shoemaker and Garland,1974 (p.51) method

uncertainty=

$$1.63\times10^{3}\times[(.2\times10^{4}/5.7\times10^{4})^{2}+(.1\times10^{1}/3.5\times10^{1})^{2}]^{5}$$

uncertainty=0.07×10^{3}

Appendix 3

Sample calculation of uncertainty in the phytic acid level based on the dry weight.

Data: 1979 Whole fababean

Phytic Acid level (wet basis) $0.83 \pm .04\%$

Water content $14 \pm 1\%$

Calculation: $0.83 \pm .04 + [(14 \pm 1) \times (0.83 \pm .04)]$

$(0.83 \pm .04) + \text{ç}0.12 \pm .12[(1/14)^2 + (0.04/0.83)^2]^5$

$0.83 \pm .04 + .12 \pm .01$

$0.95 \pm [(.04)^2 + (.01)^2]^5$

uncertainty=$0.95 \pm .04$

Uncertainty determined by Shoemaker and Garland,1974 (p.51).

Appendix 4

Phytic acid level in protein prepared at various levels: Statistical analysis by Orthogonal polynomial reqression goodness of fit test.

Analysis done by the BMDP packaged program 5R, (pg. 444).

Polynomial Degree	D.F.	S.S.	M.S.	F.
0	3	2.99	0.998	22.52**
1	2	1.62	0.810	18.26**
2	1	0.09	0.087	1.96
Residual	28	1.24	0.044	

** significant at 1% level

A significant F-test indicates that a higher degree of polynomial should be considered.

Degree 0 is a constant

Degree 0, 1 is a linear relationship

Degree 0, 1, 2 is a quadratic relationship

In this case the relationship is quadratic.

Appendix 5

Statistical analysis of the level of calcium, zinc, protein and phytic acid recovery in isolates prepared at different pH of solubilization levels.

Analysis done by Orthogonal polynomial regression goodness of fit test.

Analysis done by the BMDP packaged program 5R (p. 444).

a) Calcium recovery:

Polynomial Degree	D.F.	S.S.	M.S.	F.
0	3	598.82	199.61	44.73**
1	2	44.11	22.05	4.94*
2	1	9.15	9.15	2.05
Residual	12	53.55	4.46	

b) Zinc recovery:

Polynomial Degree	D.F.	S.S.	M.S.	F.
0	3	198.50	66.17	2.98
1	2	5.85	2.92	0.13
2	1	0.99	0.99	0.04
Residual	12	226.36	22.20	

c) Magnesium recovery:

Polynomial Degree	D.F.	S.S.	M.S.	F.
0	3	22,357.85	7452.62	2.03
1	2	18,861.08	9430.54	2.57
2	1	4,752.60	4752.60	1.30
Residual	12	44,017.15	3668.10	

d) Phytic Acid recovery:

Polynomial Degree	D.F.	S.S.	M.S.	F.
0	3	381.37	127.12	9.11**
1	2	371.23	185.12	13.31**
2	1	49.99	49.99	3.58
Residual	28	390.60	13.95	

Appendix 5(Cont'd.)

e) Protein recovery:

Polynomial Degree	D.F.	S.S.	M.S.	F.
0	3	265.49	88.50	35.14**
1	2	21.07	10.54	4.18*
2	1	9.28	9.28	3.69
Residual	11	27.70	2.52	

When the F-test statistic is significant, a higher polynomial degree should be considered. When the polynomial degree 0, the relationship between x and y values is a constant. When degree, 1, the relationship is linear and when degree 2, the relationship is quadratic. The zinc recovery and the magnesium recovery F-test statistics indicate that the amount removed in each isolate is constant while the other F-test statistics indicate quadratic relationships between the percent recovered and the pH of solubilization.

** - significant at the 1% level
* - significant at the 5% level

Appendix 6

Summary of biochemical data characterizing the two associated species found in aqueous salt extracts of fababeans

	Species Q	Species P
Total molecuar weight	140,000 d	340,000 d
Molecular shape[1]	horseshoe	spherical
Maximum diameter	132 A	185 A
Dissociation constant for TNS[2] binding	8.85×10^{-6}	1.13×10^{-4}
Denaturation temperature[3]	93°C	107°C
Number of subunit species	2	3
Subunit molecular weights	54,300[4] 86,100[5]	54,300[4] 34,700 16,200

[1]By electron microscopy of the protein stained with either uranyl acetate or phosphotugstic acid.

[2]p toluidinyl naphthalene sulfonate (constants calculated from fluorescence data).

[3]By DSC in 0.5 M NaCl.

[4]Spectroscopic studies indicate units may not be identical.

[5]Only subunit with detectable intrachain disulfide bond.

(Murray, et al.,1981)

Appendix 7

Statistical analysis of the recovery of phytic acid in the different isoelectric precipitation fractions as affected by the addition of EDTA.

Analysis by the Orthogonal polynomial regression goodness of fit test, BMDP packaged program 5R (p. 444).

a) First precipitate:

Polynomial Degree	D.F.	S.S.	M.S.	F.
0	3	407.84	135.95	5.28*
1	2	60.43	30.22	1.17
2	1	47.52	47.52	1.84
Residual	16	412.16	25.76	

b) Supernatant:

Polynomial Degree	D.F.	S.S.	M.S.	F.
0	3	556.54	185.51	11.27*
1	2	2.10	1.05	0.06
2	1	1.99	1.99	0.12
Residual	16	263.45	16.47	

c) Isolate:

Polynomial Degree	D.F.	S.S.	M.S.	F.
0	3	721.52	240.51	51.04*
1	2	48.46	24.23	5.14*
2	1	6.06	6.05	1.28
Residual	14	65.96	4.71	

The analysis indicate that the relationship between phytic acid recovery and EDTA in the isolate is linear in the first precipitate and in the supernatant, while it is quadratic in the final isolate.

* - significant at the 5% level