quality or in number of bands in the protein patterns. For the differentiation of yeasts at the species level, complementary and more sensitive methods, especially that of serological and enzymic, are necessary. A combination of isoelectric focusing with the enzymogram technique offers a relatively simple and efficient method in this connection. An application of the above technique on yeast enzymes will be published (Drawert and Bednář, 1982).

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Calcium Binding to Phytic Acid

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Phytic acid (*myo*-inositol hexaphosphate), a substance present in large amounts in most plant seeds, may, through chelation, suppress the absorption of important polycationic nutrilites such as Ca^{2+}, Zn^{2+}, and Fe^{3+}. I have, therefore, investigated the binding of Ca^{2+} to phytic acid and its dependence on temperature, pH, and ionic strength by Ca^{2+}-selective potentiometry. Scatchard plots showed downward curvature, indicating the existence of intrinsically different binding sites. Their affinities for Ca^{2+} increased sharply with pH, and between 5 and 40 °C the interactions displayed positive entropy and enthalpy changes. The study also showed the presence of two soluble Ca^{2+}–phytate species, Ca_1–phytate and Ca_2–phytate, whereas all other Ca^{2+}–phytate complexes precipitated even at low pH. The important nutritional consequences of this phenomenon are discussed.

The occurrence of phytic acid [*myo*-inositol 1,2,3,4,5,6-hexakis(dihydrogen phosphate)] as a mixed Ca^{2+}–Mg^{2+} salt in aleurone grains was first reported in 1872 (Pfeffer, 1872). It constitutes 1–6% by weight of most cereals, legumes, and oilseeds. It has been shown to interfere with the nutritional bioavailability of minerals by forming insoluble complexes with di- and trivalent cations (Maddaiah et al., 1964; Vohra et al., 1965). With a continually expanding global need for food protein, increasing concern arises over the presence of antinutritional agents such as phytic acid in plant-derived foods. Several excellent recent review articles discuss the occurrence, chemistry, and nutritional aspects of phytic acid (Erdman, 1979; O'Dell, 1979; Cheryan, 1980; Maga, 1982).

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Although the insolubility of metal salts of phytic acid is the proposed mechanism by which it contributes to nutrilite deficiency, there is a paucity of information on the interactions of phytic acid with cations in vitro. Several studies have demonstrated that most phytate–mineral complexes precipitate at intestinal pH (Maddaiah et al., 1964; Vohra et al., 1965; Oberleas, 1973; Evans and Pierce, 1981) and that an excess of one cation may potentiate the precipitation of another cation by phytate (Byrd and Matrone, 1965), presumably by coprecipitation. However, no quantitative study of these interactions has appeared yet, and furthermore no distinction is made in the literature between the formation of a cation–phytate complex and its precipitation. This lack of information is surprising in view of the nutritional relevance of this phenomenon and the existence of extensive compilations of association constants for various other metal–ligand complexes (Ringbom, 1963; Sillen and Martell, 1964, 1971; Martell and Smith, 1974; Smith and Martell, 1975).

An in-depth investigation of the association of phytic acid with cations is of prime nutritional and also physicobiochemical importance. The present paper describes a potentiometric study of the binding of Ca^{2+} to phytate and the effects of temperature, pH, and ionic strength. Ca^{2+} was chosen because of its biological relevance and because of the ease, accuracy, and sensitivity of Ca^{2+}-selective potentiometry. Due to the superb selectivity of the Ca^{2+} electrode, the binding of other cations in the presence of Ca^{2+} may be examined by this versatile and convenient method.

MATERIALS AND METHODS

Materials. Sodium phytate was purchased from Sigma Chemical Co. and converted to the free acid by treatment with Dowex 50 and Chelex 100 to assure the removal of any contaminating divalent cations. It was stored at −20 °C in small aliquots at a concentration of approximately 48.5 mM as determined by phosphate analysis on the wet-ashed phytate standard; potentiometric titration with $CaCl_2$ at pH 10.4 gave a concentration of 50.67 mM, which was used for all subsequent calculations due to the excellent precision and accuracy of titrimetry.

A standard $CaCl_2$ solution was prepared and its molarity was determined to be 1.005 ± 0.007 M by titration with disodium ethylenediaminetetraacetate (Na_2EDTA) in 100 mM 1,3-bis[tris(hydroxymethyl)aminomethyl]propane (Bis-Tris-propane), pH 9.4, at 20 °C using a calcium electrode. Dowex 50 and Chelex 100 were obtained from Bio-Rad Laboratories. All other chemicals were of reagent grade or better.

Potentiometric Measurements. Potentiometric measurements were made with a Radiometer Ca^{2+}-selective electrode, type F2112Ca, a Radiometer Ag/AgCl reference electrode, type K801, and an Orion digital millivolt meter, Model 701A. Temperature control of ±0.1 °C was maintained at the indicated temperatures by a refrigerated circulating bath (Forma Scientific, Model 2095), and the samples were stirred at a slow and constant rate. Under these conditions, the noise was ±0.1 mV or less, and the drift during a period of 1 h was 0.3 mV or less. A Nernstian slope of 102 ± 1% of theoretical was obtained between 5 and 40 °C.

The buffers used for titrations were (A) 50 mM acetic acid/triethanolamine (Ac/TEA), pH 4.8 (prepared by titrating 50 mM acetic acid with triethanolamine), (B) 50 mM 2-(*N*-morpholino)ethanesulfonic acid (Mes)/TEA, pH 6.0, (C) 50 mM 3-(*N*-morpholino)propanesulfonic acid (Mops)/TEA, pH 7.2, (D) 50 mM 3-[tris(hydroxymethyl)methyl]aminopropanesulfonic acid (Taps)/TEA, pH 8.4, (E) 50 mM Bis-Tris-propane/HCl, pH 9.4, and (F) 50 mM 3-(cyclohexylamino)-1-propanesulfonic acid (Caps)/triethylamine, pH 10.4. The ionic strength was increased, when called for, by adding tetramethylammonium chloride (TMAC); this compound is inert to both the Ca^{2+} electrode and phytic acid.

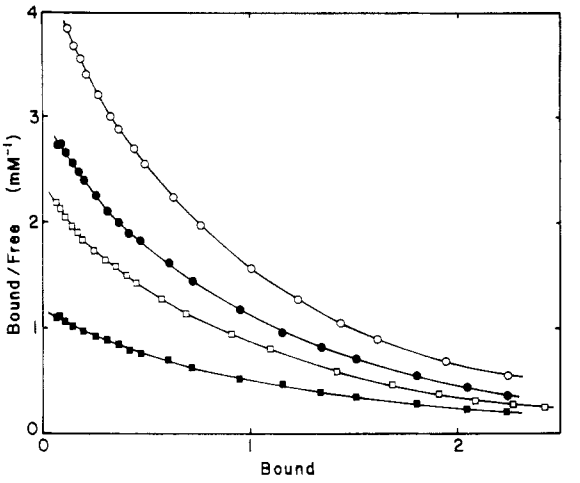

Figure 1. Scatchard plots of binding of Ca^{2+} to phytic acid. 3.04 mM phytic acid in 50 mM Ac/TEA, pH 4.8, was titrated with 1.005 M $CaCl_2$ at 5 (□), 20 (●), and 40 °C (○). The binding at 20 °C was corrected for electrostatic interactions by multiplying the concentrations of free Ca^{2+} by e^{-4wZ}, where w = 0.02 and Z = the average charge of phytate (■). The amount of bound Ca^{2+} is expressed in units of $[Ca^{2+}]_{bound}/[phytate]$.

Immediately before each titration, a semilogarithmic standard curve was prepared by adding increments of $CaCl_2$ to 100 mL of buffer under the respective conditions and measuring the potential. Then a phytate solution was titrated with $CaCl_2$ and millivolt readings were recorded. The concentration of free Ca^{2+} (free) was calculated from the standard curve at each point. The concentrations of total Ca^{2+} and phytate were corrected for dilution, and the average number of Ca^{2+} bound per phytate (bound) was calculated by subtracting free from the total Ca^{2+} concentration and dividing it by the phytate concentration.

The association constants and number of binding sites were determined by a standard Scatchard analysis (Scatchard, 1949; Scatchard et al., 1957). These initial values were refined by fitting them to the Adair equation by an iterative procedure (Adair, 1925). All association constants reported in this study have not been corrected for competitive H^+ equilibria, electrostatic interactions, or ionic strength.

RESULTS

The curvature of the Scatchard plots shown in Figure 1 clearly indicates the presence of more than one class of binding sites. The association constants for the different sites were calculated under the assumption that at pH 4.8 a maximum number of three Ca^{2+} ions bind to phytate. The existence of additional low-affinity sites, however, would have little bearing on the validity of my proposed model, since their influence on the value of K_1 would be insignificant. Computer-simulated binding isotherms generated from the Adair equation (eq 1)

$$B = \frac{K_1F + 2K_1K_2F^2 + 3K_1K_2K_3F^3}{1 + K_1F + K_1K_2F^2 + K_1K_2K_3F^3} \quad (1)$$

(where B = average number of Ca^{2+} bound per phytate, F = concentration of free Ca^{2+}, and K_1, K_2, and K_3 = calculated association constants) were in excellent agreement with the experimental binding isotherms (Figure 2).

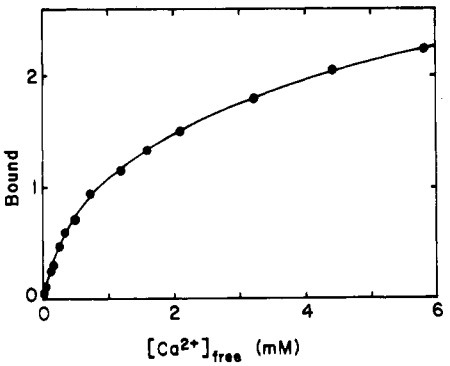

Figure 2. Binding isotherm of Ca^{2+} to phytic acid. The titration was performed at 20 °C under the same conditions as in Figure 1. Circles represent experimental data points, and the solid line represents the theoretical curve generated mathematically from the Adair equation by using the following parameters: K_1 = 2890 M^{-1}; K_2 = 340 M^{-1}; K_3 = 200 M^{-1}. The amount of bound Ca^{2+} is expressed in units of $[Ca^{2+}]_{bound}/[phytate]$.

Typical standard deviations for the association constants determined in separate experiments were 4% for K_1, 10% for K_2, and 20% for K_3.

To distinguish between the existence of three classes of intrinsically different sites or one class of three identical sites that bind Ca^{2+} with successively decreasing affinities due to electrostatic interactions, I plotted the left-hand side of eq 2 vs. 1.736Z:

$$\log \frac{B}{F(3-B)} = \log K_{intr} - 1.736wZ \qquad (2)$$

where B = average number of Ca^{2+} bound per phytate, F = concentration of free Ca^{2+}, Z = average charge of phytate, K_{intr} = intrinsic association constant, and w = electrostatic correction factor.

These Z plots showed curvature at all temperatures, which indicated the presence of at least two intrinsically different classes of binding sites. The electrostatic correction factors estimated from these plots were always 50–100 times smaller than the calculated ones. The effect of electrostatic correction on the Scatchard plots is demonstrated by the bottom line in Figure 1, which was obtained by multiplying the concentration of free Ca^{2+} by e^{-4wZ}, where Z is the average charge of phytate (=2B − 6) and w = 0.02 (as approximated from the above Z plots). Given the described circumstances, electrostatic correction of our data obviously fails to improve the analysis, and the calculation of apparent association constants is subject to far fewer uncertainties.

The Scatchard plots shown in Figure 1 never extended beyond an X value of 2.2–2.4 due to precipitation of the complex. This X value was independent of the phytate concentration over a 100-fold concentration range. These results suggest that Ca_1–phytate and Ca_2–phytate are two very soluble species, whereas the addition of a third Ca^{2+} ion to the complex results in precipitation even at very low concentration.

The apparent association constants for Ca^{2+} binding to phytate show a strong ionic strength dependence (Figure 3). The magnitude of the ionic strength effect is proportional to both the charges of the interacting species and the change in charge accompanying the binding, as predicted by the Güntelberg equation (Alberty and Daniels, 1978):

$$\log y_i = \frac{-z_i^2 A I^{1/2}}{1 + I^{1/2}} \qquad (3)$$

where y_i = activity coefficient of species i, z_i = charge of species i, A = Debye–Hückel constant (=0.52 at 20 °C), and I = molar ionic strength.

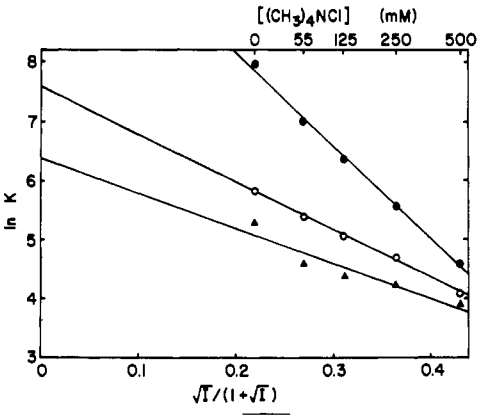

Figure 3. Ionic strength dependence of Ca^{2+} binding to phytic acid. Solutions of 50 mM Ac/TEA, pH 4.8, containing 3.04 mM phytic acid and increasing amounts of tetrametylammonium chloride were titrated with $CaCl_2$ at 20 °C. The natural log of the association constants K_1 (●), K_2 (○), and K_3 (▲) are plotted vs. $I^{1/2}/(1 + I^{1/2})$.

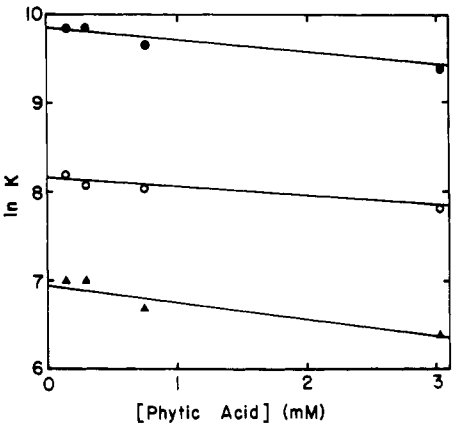

Figure 4. Effect of phytate concentration on association constants K_1 (●), K_2 (○), and K_3 (▲). The titrations were carried out in 50 mM Mes/TEA, pH 6.0, at 20 °C.

From the intercepts in Figure 3, the intrinsic association constants at 20 °C, pH 4.8, were calculated ($K_{0,1}$ = 81.1 mM^{-1}, $K_{0,2}$ = 2.0 mM^{-1}, $K_{0,3}$ = 0.59 mM^{-1}), which are markedly higher than the apparent association constants obtained in 50 mM Ac/TEA, pH 4.8, containing 3.04 mM phytic acid and no tetramethylammonium chloride (K_1 = 2.89 mM^{-1}, K_2 = 0.34 mM^{-1}, and K_3 = 0.20 mM^{-1}).* It should be noted that the term intrinsic may be a slight misnomer, since it implies correction for competitive H^+ equilibria and electrostatic interactions in addition to zero ionic strength.

The apparent association constants are also strongly influenced by the concentration of phytic acid (Figure 4); due to the high charge of phytate, only slight changes in concentration will alter the overall ionic strength of the solution substantially. The calculated ionic strength dependence observed in Figure 3 was significantly higher than that of Figure 4, for reasons not fully understood.

The affinity of phytic acid for Ca^{2+} increases with temperature as shown in Figure 5. The entropy changes for the formation of the three Ca^{2+}–phytate complexes are positive (26.0 cal mol^{-1} K^{-1} for K_1, 19 cal mol^{-1} K^{-1} for K_2, and 19 cal mol^{-1} K^{-1} for K_3), presumably as a result of the partial disruption of the oriented layer of water around the highly charged phytate ion. The enthalpy changes calculated from the slopes in Figure 5 were 2.98 kcal/mol

Table I. Effect of pH on Ca^{2+} Binding to Phytic Acid[a]

pH	K_1	K_2	K_3	K_4	K_5	K_6	Z^b
4.8	2.89	0.34	0.20	<0.2	<0.2	<0.2	−6.1
6.0	12.1	2.5	0.60	<0.6	<0.6	<0.6	−6.9
7.2	22.7	22.7	22.7	<22.7	<22.7	<22.7	−8.0
8.4	>1000	>1000	>1000	285	285	285	−8.9
9.4	>1000	>1000	>1000	516	516	516	−9.4
10.4	>1000	>1000	>1000	>1000	>1000	>1000	−10.5

[a] The values of the apparent association constants (mM^{-1}) were determined at 20 °C in 50 mM pH buffer containing 3.04 mM phytic acid (pH 4.8, 6.0, 7.2) and 0.15 mM phytic acid (pH 8.4, 9.4, 10.4). [b] The average charge of phytate (Z) in the absence of Ca^{2+} was calculated on the basis of the following acid dissociation constants: pK_1 for C-1, C-2, C-3, C-4, C-5, and C-6 = 1.5, 1.1, 1.5, 2.1, 1.7, and 2.1, respectively; pK_2 for C-1, C-2, C-3, C-4, C-5, and C-6 = 5.70, 6.85, 12.00, 10.00, 7.60, and 10.00, respectively (Costello et al., 1976).

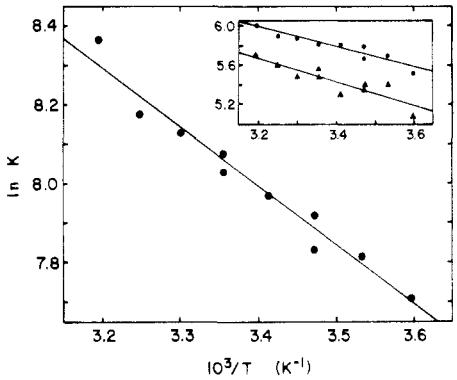

Figure 5. Temperature dependence of the first association constant K_1. Solutions of 3.04 mM phytic acid in 50 mM Ac/TEA, pH 4.8, were titrated with 1.005 M $CaCl_2$ at 5, 10, 15, 20, 25, 30, 35, and 40 °C. The inset shows the effect of temperature on K_2 (●) and K_3 (▲).

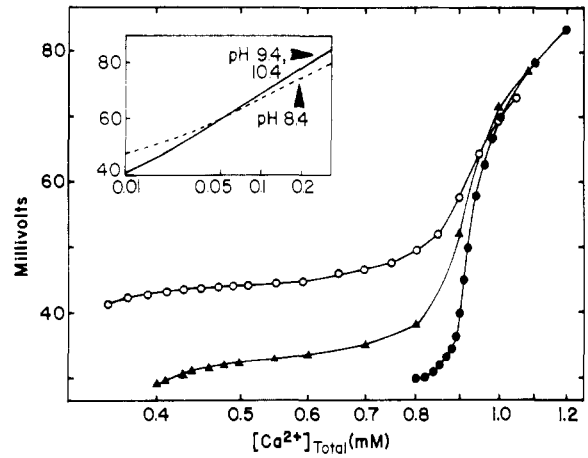

Figure 6. Titration of 0.15 mM phytate with 1.005 M $CaCl_2$ at pH 8.4 (○) pH 9.4 (▲), and pH 10.4 (●). The inset shows the calibration curves in the absence of phytic acid.

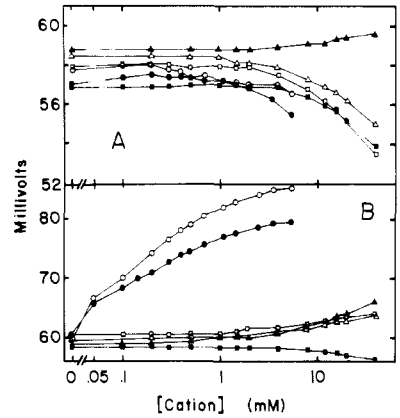

Figure 7. Competitive binding of Li^+ (▲), Na^+ (△), K^+ (□), Mg^{2+} (●), and Ba^{2+} (○) in the presence of 0.15 mM phytic acid and 0.945 mM $CaCl_2$ (panel B) and with no phytic acid in the presence of 0.104 mM $CaCl_2$ (panel A). All titrations were carried out in 50 mM Caps/triethylamine, pH 10.4, at 20 °C. The effects of TMAC on the electrode and on the binding of Ca^{2+} to phytic acid are represented by the solid squares (■).

(K_1), 2.0 kcal/mol (K_2), and 2.4 kcal/mol (K_3).

A change in pH from 4.8 to 10.4 caused a several thousand-fold increase in the association constants (Table I). At pH 4.8 and 6.0 the Scatchard plots looked very similar to those in Figure 1, with a maximum number of three binding sites. At pH 7.2 all the experimental points fell on a nearly straight plot between 0 and 1.2 bound and between 37 and 23 bound/free on the Scatchard plot, and the extrapolated intercept was three; however, the maximum number of sites could not be estimated. At pH 8.4 and 9.4 all the measurable bound/free values were lying between 3 and 6 on the abscissa in Figure 1, and at pH 10.4 they fell on a line between 5.95 and 6.00. Obviously such Scatchard plots are fraught with large uncertainties and merely indicate the lower limit of the association constants and the total number of binding sites. In the case of these high affinities of phytate for Ca^{2+}, however, the number of sites may be determined with far better accuracy from titration plots (Figure 6) by dividing the concentration of total Ca^{2+} at the equivalence point by the phytate concentration. The sharpness of the end point increases with pH, as predicted. The determination of the association constants at high pH would require either the use of high phytate concentrations whose Ca^{2+} salts precipitate above pH 5.0 or the measurement of very low Ca^{2+} concentrations which lie below the detection limit of our electrode.

Finally, I investigated the binding of Li^+, Na^+, K^+, Mg^{2+}, and Ba^{2+} to phytic acid by measuring the displacement of Ca^{2+} from Ca_6–phytate by increasing amounts of these competitive cations (Figure 7). A quantitative analysis of the data would be extremely difficult because of several interfering phenomena: (A) The addition of metal halides increases the ionic strength of the medium, which influences both the association constants between Ca^{2+} and phytate and the millivolt readings in the absence of phytate by lowering the activity coefficients for all ionic species according to eq 3. (B) Certain cations such as Li^+ interfere with Ca^{2+}-selective potentiometry at very high concentrations. (C) The solutions were not at equilibrium as indicated by their slight cloudiness. This would invalidate any thermodynamic treatment of the competitive binding results. Under all equilibrium conditions, however, available binding sites were not fully occupied by Ca^{2+}, and total binding of Mg^{2+} and Ba^{2+} by occupying free sites and displacing Ca^{2+} could not be evaluated.

Despite these shortcomings, several conclusions may be drawn from the results shown in Figure 7: (1) TMAC does

not bind to phytic acid or interfere with the Ca^{2+} electrode, two criteria necessary for a suitable ionic strength adjuster. (2) Both Mg^{2+} and Ba^{2+} bind to phytic acid very strongly. No other divalent cations were examined because of their strong interference with the Ca^{2+}-selective electrode. (3) The interactions of phytic acid with Li^+, Na^+, and K^+ are several orders of magnitude weaker than those with divalent cations. This is in excellent agreement with the valency rule of Schulze and Hardy for flocculation values of sols (Overbeek, 1952).

DISCUSSION

The results provided by the present study demonstrate that phytic acid exhibits a high affinity for Ca^{2+} over a wide pH range. The degree and tightness of binding depend on pH, temperature, ionic strength, and size and valency of the cation, in agreement with existing electrostatic theories. The interactions will also be influenced by the conformation of phytic acid, which exists in the chair form with five phosphate groups equatorially and one axially oriented in dilute solution (Johnson and Tate, 1969), in the inverted chair conformation when crystallized (Blank et al., 1971), and in the boat form at high ionic strength (Costello et al., 1976).

There exists a vast body of literature on the in vitro precipitation of cations by phytate and on its in vivo effects on the gastrointestinal absorption of minerals. These results are complemented by the present study that for the first time demonstrated (A) the chelation of Ca^{2+} by phytate in acidic medium, even at pH 2.0 (results not shown), and (B) the presence of two soluble complexes, Ca_1–phytate and Ca_2–phytate. The chelation of Ca^{2+} at low pH suggests that substantial amounts of cations will be bound to phytate in the stomach which may facilitate the precipitation of the complex during the subsequent passage through the intestine. Of even greater nutritional significance is the existence of two soluble Ca^{2+}–phytate species. Indeed, Morris and Ellis (1976) reported that monoferric phytate was a soluble and bioavailable chelate, raising the possibility that all metal–phytate complexes at a low metal to phytate ratio may be soluble at intestinal pH. This would have important nutritional consequences: Submarginal mineral fortification may be more harmful than beneficial depending on the total ionic composition of the diet, as the deleterious nutritional effects of phytic acid arise from the insolubility of its metal salts and not from their formation. Thus, optimal intestinal uptake of cations would occur at very low and at very high metal to phytate ratios. This hypothesis currently under investigation may explain some of the ambiguous and conflicting results in the literature on the in vivo effects of dietary phytate on zinc and iron absorption.

The methodology developed during this investigation will also be required to study the interactions of Ca^{2+} with *myo*-inositol mono-, di-, tri-, tetra-, and pentaphosphate esters, which are now under preparation in our laboratory. The discovery of a *myo*-inositol phosphate ester capable of forming very strong and soluble metal chelates would have far-reaching nutritional and medical consequences because of its numerous applications in both fields.

Registry No. Phytic acid, 83-86-3; Ca, 7440-70-2; Li, 7439-93-2; Na, 7440-23-5; K, 7440-09-7; Mg, 7439-95-4; Ba, 7440-39-3.

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