

Phytic Acid and Metal Availability: A Study of Ca and Cu Binding

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ABSTRACT

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Two *in vitro* methods for the assay of the chemical form of elements were tested in solutions containing phytic acid. In the first method, we used the decrease in α -amylase activity to register the chelation of calcium by phytic acid. In the second method, the binding of copper by phytic acid was measured directly by electron paramagnetic resonance spectroscopy. In the

calcium- α -amylase system, phytic acid showed an effect on the same order of magnitude as ethylenediamine-NNN'N'-tetraacetic acid. The electron paramagnetic resonance measurements showed that at stomach pH, more copper was bound to amino acids than to phytic acid.

Nutritional researchers have become increasingly interested in the bioavailability of elements. One factor that regulates bioavailability is the presence of organic compounds with chelating properties.

Bioavailability is measured by *in vivo* methods. Rats, pigs, and even humans have been the subjects of research to determine the amount of elements they can utilize from different types of foodstuffs (Frölich and Lysö 1983, Morris and Ellis 1980, Sandström 1980). *In vivo* methods are expensive, however, and therefore not suitable for control purposes.

Our food is a complicated chemical mixture. Thus, the first step in developing new methods for analyzing it is to test the methods in simpler systems. The main objective of the present study was to test two *in vitro* methods for evaluating the amount of available elements in the presence of chelating compounds. Phytic acid was used as a test substance because its chelating ability is believed to be responsible for the decreased uptake of trace elements from food with a high content of whole grain flour (Reinhold et al 1976).

We measured the binding of calcium to phytic acid by the decrease in α -amylase activity in solutions containing this enzyme and phytic acid. In addition, the binding of copper to phytic acid was measured directly by electron paramagnetic resonance (EPR) at typical stomach pH values. We added other chelators to study the effects of these compounds upon the binding of metals to phytic acid.

Available Ca as Regulator of α -Amylase Activity

The stabilization of α -amylases by Ca ions is a well-known phenomenon. As early as 1908, the use of Ca salts to obtain optimal α -amylase activity in mashing was patented (Wallerstein 1908). Fischer and Haselback (1951) isolated the enzyme from malt and found that inactivation was induced by substances such as ethylenediamine-NNN'N'-tetraacetic acid (EDTA), which bound Ca^{2+} ions.

The nature of this inactivation is not clear. Calcium may either be necessary for enzymatic activity or only to stabilize the structure of the enzyme, thereby protecting it against proteolytic attack, but this is a matter of speculation. For our purposes, however, it is sufficient to know that an inhibition occurs.

There are two contradictory reports concerning the action of phytic acid upon α -amylase. Cawley and Mitchell (1968) used a penetrometer method and concluded that phytic acid did suppress the α -amylase activity by chelating the Ca necessary for enzymatic activity. Our measurements support this view.

On the other hand, Sharma et al (1978) concluded that the inhibition they observed when the α -amylase activity was measured with a soluble starch was not due to chelation of Ca by phytic acid, but to a reaction between phytic acid and the protein moiety of the enzyme.

EPR as an Analytical Tool

Electron paramagnetic resonance (EPR) spectroscopy may be used to detect metal ions containing unpaired electrons such as Cu^{2+} , Mn^{2+} , Fe^{3+} , and Cr^{3+} . The detectability of these ions and the characteristics of their EPR spectra depend on the number of unpaired electrons, the molecular structure, and the molecular motion. Unfortunately, Zn^{2+} , one of the most interesting ions nutritionally does not contain unpaired electrons and is unobservable by EPR. We have chosen, therefore, to study the effect of Cu^{2+} . Because this ion is somewhat more firmly bound than zinc (Vohra et al 1965), its behavior can be used to predict the maximum amount of zinc that will react with phytic acid.

MATERIALS AND METHODS

α -Amylase

Some of the microbial α -amylases contain Ca, which is so firmly bound that inhibition by chelating agents is impossible (Vallee et al 1959). According to Fischer and Stein (1960), inhibition may occur in malt amylases, and malted barley was therefore chosen as an enzyme source. Brewing malt contains 400–500 ppm calcium (*unpublished data*). To obtain an unstable enzyme, no further calcium was supplied in the isolation steps.

A modification of the procedures published by Schwimmer and Balls (1948) and Fischer and Haselback (1951) was used. Malt (1 kg) was homogenized in water and kept at 70°C for 15 min. After cooling, the solution was filtered through 60 g of diatomaceous earth (Celite). The filter cake was washed with distilled water to a total filtrate column of 2 L. $(\text{NH}_4)_2\text{SO}_4$ (700 g) was added, and the pH was adjusted to 6.0 with NH_3 . After 2 hr at room temperature, the solution was filtered through a folded filter (Schleicher and Schüll filter, 597½). The filter was washed with five times the filtrate volume of a solution containing 230 g of $(\text{NH}_4)_2\text{SO}_4/\text{L}$, pH 6.0. The enzyme was stored in small portions (for one thawing only) at -20°C.

Parallel experiments were done with a preparation of commercial malt enzyme (Sigma type V-A).

Phytic Acid

Materials from two suppliers were used. Phytic acid, sodium salt (Sigma P 5756, Sigma Chemical Company, St. Louis, MO), listed by the producer as 41.3% H_2O and 12 Na/mol, was used. Drying at 105°C in the laboratory yielded a slightly lower water content. Phytic acid (Fluka 80180, Fluka AG, Chemische Fabrik, Buchs SG, Switzerland), which also was used, was listed by the producer as 40% (w/v) in aqueous solution, $\text{C}_6\text{H}_{18}\text{O}_{24}\text{P}_6$.

There is disagreement about whether the actual phytic acid formula is $\text{C}_6\text{H}_{24}\text{O}_{27}\text{P}_6$ or $\text{C}_6\text{H}_{18}\text{O}_{24}\text{P}_6$ (Brown et al 1961). We used a rounded-off molecular weight of 1,000 (combining the uncertainties of the water content and the formula) when the effect of phytic acid was compared to the other chelators.

The element content of the two phytic acid preparations was analyzed by atomic absorption spectroscopy. The results are shown

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in Table I. The phytic acid preparation with the higher element content was used in the first step of the investigation, concerning the effect of increasing Cu-levels upon the amount of bound copper. The Na-phytate was used in the second part of the investigation.

Copper was supplied in the form of CuSO_4 in water.

Inhibition Studies

Phytic acid was compared to the synthetic chelators EDTA and N' -(2-hydroxyethyl) ethylenediamine- NNN' -triacetic acid (HEEDTA). Because the three compounds contain different numbers of dissociable protons, equal amounts by weight rather than equimolar amounts were used.

The following were added to four 100-ml volumetric flasks: 80 ml of ethanolamine, 0.02M, adjusted to pH 5.5 with malic acid; 400 mg EDTA (disodium dihydrat), Titriplex III, Merck 8418, E. Merck, Darmstadt, Germany; 400 mg HEEDTA (trisodium dihydrat), BDH 28678, BDH Chemicals Ltd., Poole, England; and 400 mg Na-phytate in 60 ml H_2O , pH 5.5.

Five hundred milligrams of the enzyme preparation was added to each flask. The flasks were filled to volume with distilled water. At 2-hr intervals, samples were taken from each flask for the determination of α -amylase activity.

Determination of α -Amylase Activity. α -Amylase activity was measured by means of an insoluble, dyed starch. The substrate was prepared according to a modification of the method described by Rinderknecht et al (1967). The colored starch was suspended in an organic buffer consisting of 0.12M ethanolamine, malic acid, and 0.01% calciumacetate.

Enzymatic activity was registered as the increase in soluble starch. Blanks consisting of chelators with no enzymes added did not cause any increase in the amount of soluble starch.

EPR Measurements. The measurements were made in the following way. First, the EPR spectrum of the pure copper was detected. Copper, dissolved in water, forms complexes of the type $\text{Cu}(\text{H}_2\text{O})_6^{2+}$ for $\text{pH} < 7$ (Kragten 1978), and the EPR spectrum is characterized by a broad, slightly unsymmetrical signal at $g \approx 2.20$ (Fig. 1). Second, the spectrum of a mixture of copper and the chelator was observed. Upon adding the chelator, the intensity of the $\text{Cu}(\text{H}_2\text{O})_6^{2+}$ signal decreased, and a new spectrum with a different shape appeared because of the formation of Cu-organic complexes. The shapes of the spectra from these different complexes depended upon the chelator, the concentrations, and the pH (Fig. 1b-f). The decrease in the $\text{Cu}(\text{H}_2\text{O})_6^{2+}$ EPR signal intensity was used to calculate the concentration of Cu-water and Cu-organic complexes as a function of Cu/organic compound ratio and pH.

The intensities of the EPR signals were obtained by fitting the observed line shape with a weighted sum of the line shapes for the complexes that were present. The weighting factors were equal to the fraction of copper in the respective complexes.

Three series of measurements were made with a total copper concentration of 10, 5, or 1 mmol/L respectively. All measurements except one were made with a concentration of 10 mmol/L phytic acid.

Both the phytic acid solutions and the metal solutions were pH-adjusted before mixing so that different pH values would be in the final mixtures. Adjusting the pH of phytic acid was a slow process, requiring at least 24 hr of reaction time. A faster pH

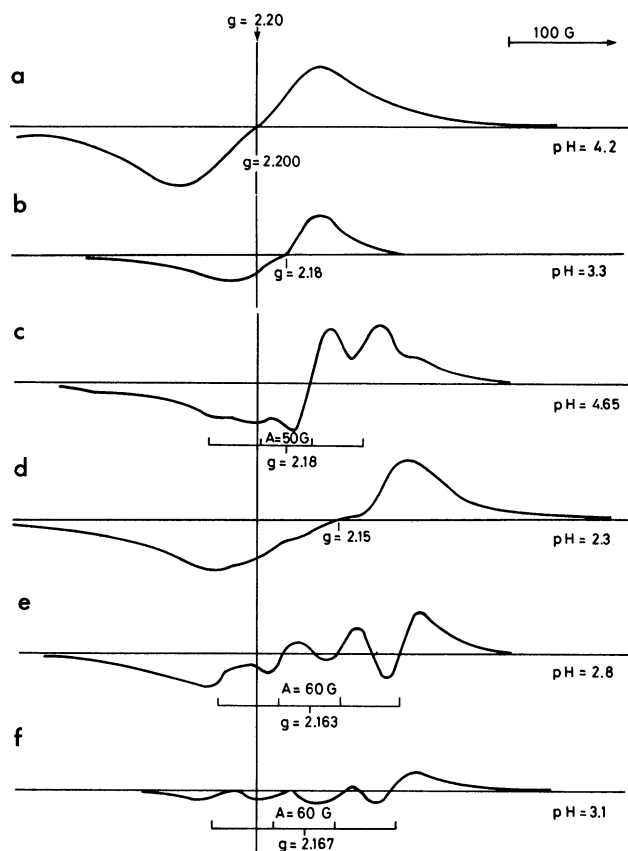


Fig. 1. Electron paramagnetic resonance spectra of different Cu^{2+} complexes in aqueous solution. a, $\text{Cu}(\text{H}_2\text{O})_6^{2+}$; b, Cu:Na-phytate; c, Cu:phytate; d, Cu:glycine; e, Cu:histidine; and f, Cu:EDTA. In b-f the signal from $\text{Cu}(\text{H}_2\text{O})_6^{2+}$, which is also present in these aqueous solutions, was subtracted.

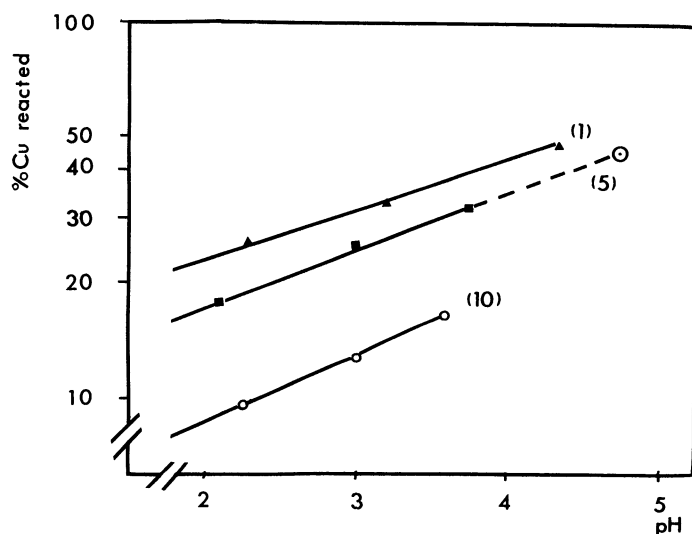


Fig. 2. Copper reacted (percent, log-scale) as a function of pH for varying total copper concentrations: \blacktriangle , 1 mM copper; \bullet , 5 mM copper; \blacksquare , 10 mM copper; and \circ , tenfold increase in phytic acid concentration.

TABLE I
Metal Content of Commercial Phytic Acid

| Preparation | Element | | | | | | | |
|-----------------------------|---------|---------|--------|----------------------|----------------------|----------------------|----------------------|----------------------|
| | Ca (mg) | Mg (mg) | K (mg) | Cu (μg) | Fe (μg) | Mn (μg) | Sr (μg) | Zn (μg) |
| Sodium phytate ^a | 0.435 | 0.030 | 0.0595 | 2.0 | 6.5 | 0.5 | <0.25 | 1.0 |
| Phytic acid ^b | 0.720 | 0.024 | 0.084 | 1.27 | 15 | 1.08 | 2.42 | 4.8 |

^a Per gram.

^b Per milliliter.

decrease occurred when the metal solution was added to the phytic acid. The pH values in Fig. 2 were recorded immediately after the two solutions were mixed.

RESULTS AND DISCUSSION

α -Amylase and Calcium

The isolated enzyme was stable over a very narrow pH range. pH 5.5 was found to be optimal and was therefore used in the inhibition experiments.

The inactivation of the enzyme as a function of time when HEEDTA, EDTA, or Na-phytate was added is shown in Fig. 3.

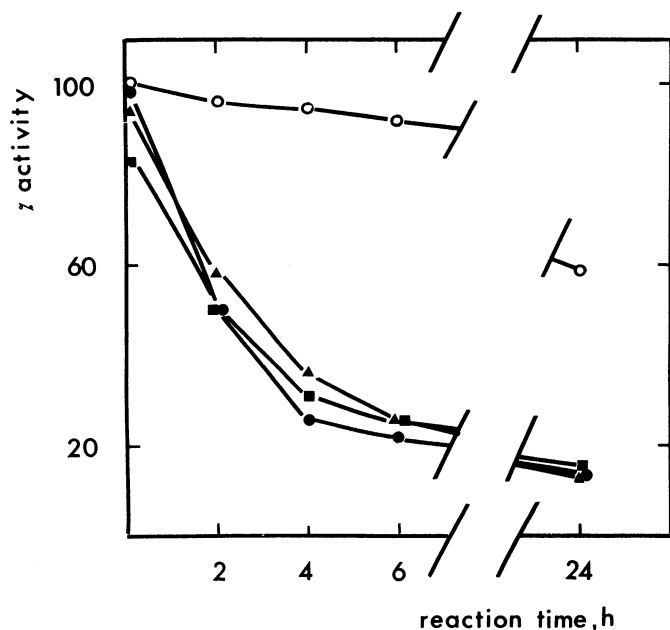


Fig. 3. Percent α -amylase activity as a function of time for the untreated enzyme (o) and mixtures of enzyme and chelator. The initial value for the untreated enzyme is assumed to be 100%. Chelators used were \bullet , EDTA; \blacktriangle , HEEDTA; and \blacksquare , phytic acid. pH = 5.5.

TABLE II
Concentration (mM of Free Cu ($\text{Cu}(\text{H}_2\text{O})_6^{2+}$) and Different Cu-Complexes in a Solution of 10 mM Cu and 10 mM A^a

| Chelator | $\text{Cu}(\text{H}_2\text{O})_6^{2+}$ | | Cu:A | | pH After Mixing |
|------------|--|--------------------|-------------------------|--------------------|-----------------|
| | Experiment ^b | Calc. ^c | Experiment ^b | Calc. ^c | |
| ... | 10.0 | 10.0 | 0.0 | 0.0 | 4.3 |
| Na-phytate | 8.6 | ... | 1.4 | ... | 3.3 |
| Glycine | 7.1 | 7.6 | 2.9 | 2.4 | 3.1 |
| Histidine | 6.2 | 6.0 | 3.8 | 4.0 | 2.8 |
| EDTA | 0.0 | 0.0 | 10.0 | 10.0 | 2.3 |

^a A = chelator.

^b Plus or minus 10%.

^c With the computer program Comics (Perrin and Syce 1967). No stability constants available for the Cu-Na-phytate complex.

TABLE III
Concentration (mM of Free Cu ($\text{Cu}(\text{H}_2\text{O})_6^{2+}$) and Different Cu Complexes in Solutions of 10 mM Cu, 10 mM phytate, and 10 mM A^a

| Chelator | $\text{Cu}(\text{H}_2\text{O})_6^{2+}$ | Cu:A | Cu:Na-Phytate | pH After Mixing |
|------------|--|-------------------------|-------------------------|-----------------|
| | Experiment ^b | Experiment ^b | Experiment ^b | |
| Na-phytate | 7.6 | 1.2 | 1.2 | 3.75 |
| Glycine | 7.4 | 1.5 | 1.2 | 3.35 |
| Histidine | 5.8 | 3.4 | 0.8 | 3.0 |
| EDTA | 0.0 | 10.0 | 0.0 | 2.65 |

^a A = chelator.

^b Plus or minus 10%.

After 24 hr, the activity of the untreated enzyme had decreased to 59% of the original activity. For the solutions containing chelators, however, the activity was only 13–14% of the initial activity of the untreated enzyme.

Equal amounts, by weight, of the three chelators were used. On a molar basis, this equaled $2.11 \cdot 10^{-3}$ mole HEEDTA, $2.15 \cdot 10^{-3}$ mole EDTA, and $4.7 \cdot 10^{-4}$ mole phytic acid per gram of crude α -amylase.

According to Brown et al (1961), the dominant phytic acid species at pH 5.5 is $\text{H}_{16}\text{Phy}^{-8}$, whereas the synthetic binders are present mainly as the divalent ion. As shown in Fig. 3, the activity decreased at the same rate for the three chelators, but less phytic acid than EDTA or HEEDTA (on a molar basis) was needed to produce a decrease. Thus, phytic acid was more effective than expected from the pK value of 3.94 at pH 7.4 reported by Gosselin and Coghlan (1953).

The inhibition experiment was repeated with the commercial malt enzyme preparation, which for some reason lacked the normal temperature stability of malt α -amylases. A decrease in pH to 4.0 was necessary for inhibition. Similar results were obtained as a function of time, although in this case EDTA gave a higher degree of inhibition than HEEDTA. With this enzyme, phytic acid started at the same point as HEEDTA, but after 24 hr it showed the same degree of inhibition as EDTA.

The similarity between EDTA and Na-phytate in the decrease of enzymatic activity as a function of time indicated that Na-phytate also reduces α -amylase activity through calcium binding.

Cu Binding to Phytic Acid

We plotted the percentage of copper reacting with phytic acid as a function of the pH of the mixture (Fig. 2). These copper ions give an EPR-detectable signal (Fig. 1c). No insoluble complexes were formed. For each concentration of copper, a logarithmic dependency is obtained between the pH and the percentage of copper reacted. A sample with a tenfold increase in phytic acid concentration falls on the same line for a fixed total copper concentration, as demonstrated by the highest measured value of the 5 mmol Cu/L line. We cannot explain this rather unusual stoichiometry, but conclude that it seems to be more important that a certain amount of copper ions are kept in an unreacted state than it is to fill the places available for binding at the chelator. Thus, only two factors determine the amount of free copper: the pH of the solution and the total amount of copper.

Using Na-phytate instead of phytic acid yielded in general a higher amount of reacted copper.

Exchange of Cu from Phytic Acid to Other Chelators

We have demonstrated that changes in the amount of copper bound by phytic acid may be measured by EPR. Our next problem is to determine how firmly this copper is bound, or to what extent it may be removed by other compounds with chelating ability. Both EDTA (O'Dell et al 1969) and histidine (Nielsen et al 1966) increase the bioavailability of zinc in diets containing phytic acid. Therefore, phytic acid was mixed with the amino acids glycine and histidine and with EDTA.

The investigation was made in two steps. First, the shape of the spectra and the amount of free copper were determined when EDTA, histidine, or glycine were used as chelators instead of phytic acid. The results are given in Table II. Typical spectra are shown in Fig. 1d–f. EPR spectra of Cu-amino acid complexes have previously been published by several investigators (Falk et al 1967, Malmstrom and Vännegård 1960).

If the binding constants for a chelator are known, the distribution between free and bound elements in a mixture of the chelator with various elements may be calculated. Available computer programs facilitate this type of calculation. In the present case, binding constants were found for EDTA and the amino acids (Sillen and Martell 1964), and the concentrations of bound copper were calculated with the computer program Comics (Perrin and Syce 1967). As shown in Table II, the calculated amounts of free and chelated copper agree with the measured concentrations.

In the second step of the investigation, the tabulated amounts of

chelated element were used as a basis for determining the changes in free copper concentration when Na-phytate or one of the above-mentioned chelators was added to a solution of copper and Na-phytate. After pH adjustment and mixing, the spectra of these solutions were recorded, and the concentrations of different Cu-complexes were calculated from the spectra. The results are given in Table III. The Na-phytate preparation used in this case was less contaminated with elements than the phytic acid. More normal stoichiometries were obtained: comparison of Tables II and III shows that an increased Na-phytate concentration gives an increased amount of bound copper.

As shown in Tables II and III, the amount of free copper is zero when EDTA is present and is highest when Na-phytate is used as the only chelator. It is interesting to observe from Table III the extent to which copper originally bound to phytic acid may be removed by the addition of amino acids. Na-phytate (20 mM) binds 2.4 mmol Cu. If 10 M of the Na-phytate is substituted with glycine, only a small decrease in the concentration of free copper is observed, and the glycine does not alter the average number of copper ions bound to each phytate molecule. This shows that Na-phytate and glycine have approximately equal binding strengths for copper at the experimental conditions used. Histidine, on the other hand, is able to bind more Cu ions. A significant number of these are taken from the Cu-phytate complexes (Cu: Na-Phy decreases from 1.2 to 0.8).

Thus, amino acids can reduce the amount of copper bound to phytic acid at a typical stomach pH. Zinc, which is of higher nutritional interest than copper, is not an EPR-detectable ion. We know, however, that both amino acids and phytic acid bind copper more strongly than zinc. We therefore expect the substitution between chelators to occur for zinc as well, but with a higher amount of free metal ions.

CONCLUSION

It is well known that phytic acid binds calcium. The measurements upon the α -amylase show that for the tested concentrations and pH, sodium-phytate on a molar basis is a slightly more effective calcium binder than HEEDTA and EDTA.

The measurements also show that in the malt α -amylase, the calcium binding is so loose that the enzyme may be used for the study of organic molecules with calcium binding ability.

With the aid of EPR, the distribution of certain elements between free and bound conditions may be registered for phytic acid as well as for other chelators. Phytic acid from different sources may show different behavior. It is therefore important to know the total composition of the system under investigation. An interaction between copper and other elements is suspected.

The concentrations used in this work were increased relative to the typical concentrations found in food so that they could be detected analytically. By studying such simple systems, information about the chemical reactions in our more complex food may be obtained. One such piece of information from this study is the fact that amino acids are able to partly reduce the amount of copper bound to the phytic acid.

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