BINDING OF PHYTIC ACID TO GLYCININ[1]

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ABSTRACT

The binding of phytic acid, the hexa*ortho*phosphate ester of *myo*-inositol, to glycinin, the major globulin of the soybean, was investigated over a broad range of pH. Above the isoelectric point of glycinin, pH 4.9, no binding could be detected by gel filtration through Sephadex G-75 at pH values of 6, 8, and 10. Between pH 5.0 and 2.5, binding that resulted in insoluble complexes occurred. The extent of binding was found to increase with decreasing pH, from a value of zero at the isoelectric point to a maximum value of 424 equivalents of phytate per mole glycinin dimer (360,000 daltons). The latter value is in good agreement with the 411 cationic groups calculated from prior knowledge of the 12 amino-terminal groups and the experimentally determined number of basic amino acid residues of the glycinin dimer. At pH 2.5, glycinin is fully dissociated into 2S subunits; at pH 4.5 – 5.0, the protein is presumably fully associated in the 11S form. At these pH values, the binding sites were judged to be fully accessible on the surface and to reflect the net positive charge of the protein. However, between pH 4.0 and 3.0, where glycinin exists in partially dissociated states, some of the binding sites were found to be hindered. Clearly, the state of quaternary structure is a determinant of the binding, and electrostatic interaction of carboxylate groups with the cationic binding sites is proposed to explain this hindrance. Calcium ions promoted dissociation of phytate-glycinin complexes at pH 3, as measured by dissolution of the insoluble complexes and by gel filtration through Sephadex G-75. The dissociation can be explained in terms of competition between Ca^{2+} and the cationic sites of the protein for the phosphate groups of phytate. At pH 3.0, a 105-fold equivalent excess of calcium with respect to the protein cationic groups was necessary to completely dissociate the complex.

In addition to other polyphosphate compounds, phytic acid, the hexa*ortho*phosphate ester of *myo*-inositol, has been shown to bind strongly to proteins below their isoelectric points (1). The binding is electrostatic in nature and involves the anionic phosphate groups of phytate and the cationic groups of proteins. Indeed, phytic acid has been employed as a reagent for the determination of arginyl, lysyl, and histidyl residues in proteins in the pH range of 3.0 to 2.5 (2). The complexes are generally insoluble below the isoelectric point of the protein, but dissolve at or below pH 2.0.

Because of their natural association within the protein bodies of the soybean (3), a detailed study of phytate binding to the isolated and purified soybean globulins is of interest. Glycinin, the 11S component, is the major globulin of the soybean, accounting for approximately 30% of the total protein in the seed (4). At acid pH, where phytate binding to glycinin occurs, the protein dissociates into subunits (5). Accordingly, we have examined the binding in light of the changes in quaternary structure, measured by ultracentrifugation, that occur at acid pH. In a mechanistic study of phytate binding in the pH range of 5 to 2, employing several proteins that do not have quaternary structure, two distinct classes of binding sites were found (6): the first class did not follow the law of mass action,

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i.e., the association constant was very large and could not be measured, whereas the second class had association constants in the range of $10^6 - 10^4$. We have found similar behavior in terms of phytate binding to glycinin between pH 4.0 and 3.0 and will refer to the first class of binding sites as primary and the second class as secondary. In addition, the dissociating effect of Ca^{2+} on glycinin-phytate complexes at pH 3.0 has been studied.

MATERIALS AND METHODS

Sodium Phytate

Crude sodium phytate was prepared (7) from commercial grade calcium phytate (Mann Research Lab, Inc.). The crude sodium salt was purified by ion-exchange chromatography (8) employing Baker CGa-541 resin (Cl form), 200–400 mesh, instead of the Dowex 1 × 2 originally used by the authors. Phosphorus analysis (9) was used to follow elution of the inositol phosphates from the column. The purified phytate was recovered from the appropriate effluent fractions by precipitation as the barium salt at pH 5. For characterization, the methyl ester was prepared from the barium salt by diazomethane methylation of the free phytic acid (10). The pure ester myo-inositol-1, 2, 3, 4, 5, 6-hexakis (dimethyl phosphate) was crystallized from ethyl acetate as colorless cubes mp 250° C (Köfler). Calculated for $C_{18}H_{42}O_{24}P_6$: C, 26.1; H, 5.1; P, 22.4; OCH_3, 44.9. Found: C, 26.14; H, 5.11; P, 22.16; OCH_3, 44.67.

For use in the binding studies, sodium phytate was prepared by conversion of the barium salt to the free acid with Dowex 50×8, followed by adjustment to the appropriate pH with aqueous NaOH.

Glycinin

Glycinin was purified (11) from hexane-defatted, unheated soybean meal (Nutrisoy 7B flakes) obtained from the Archer-Daniels-Midland Company. Essentially, the method involves cryoprecipitation of glycinin from the extract and subsequent purification by gel filtration through Sephadex G-200 (Pharmacia, Inc.).

Deionized water and commercial grade reagent chemicals were used throughout. A Radiometer TTT-1c pH meter was used for pH measurements, and absorption and turbidity measurements were made using a Zeiss PM Q II spectrophotometer.

Amino Acid Analysis

Amino acid analysis was performed to determine the total number of basic amino acid residues (potential phytate binding sites) in glycinin. A Beckman Model 120 C analyzer equipped with a Beckman Model 125 integrator was employed. Hydrolysis was performed in vacuum with 6*N* HCl at 110° C for 24 and 72 hr in triplicate; norleucine and α-amino-β-guanidinopropionic acid served as internal standards (12).

Dissociation at Acid pH

The extent of glycinin dissociation from the intact 11S component into 7S and 2S components was determined at 20° C and 60,000 rpm using a Beckman Model

L-2-65B preparative ultracentrifuge equipped with Schlieren optics. Conditions identical to those of the binding studies were used so the binding and dissociative behavior could be compared. Measurements were made after a 2-hr exposure of 1.5% glycinin solutions between pH 4.0 and 2.5 in water acidulated with HCl. Measurements at pH 4.5 and 5.0 were not possible, due to insolubility of the protein. A double-sector cell was used to permit solvent baseline correction. The values of sedimentation coefficients of each species determined under the above conditions are approximate because of the low ionic strength and thus are not reported. However, the values enabled identification of the 11S, 7S, and 2S components. The proportion of each component with respect to the total protein was estimated from the Schlieren patterns by weighing cut-out tracings of the patterns projected onto paper.

Binding Measurements Outside the Region of pH 5.0–2.5

At pH values of 10, 8, 6, and 2, where soluble mixtures of sodium phytate and glycinin existed, binding was investigated by gel filtration of 5-ml samples

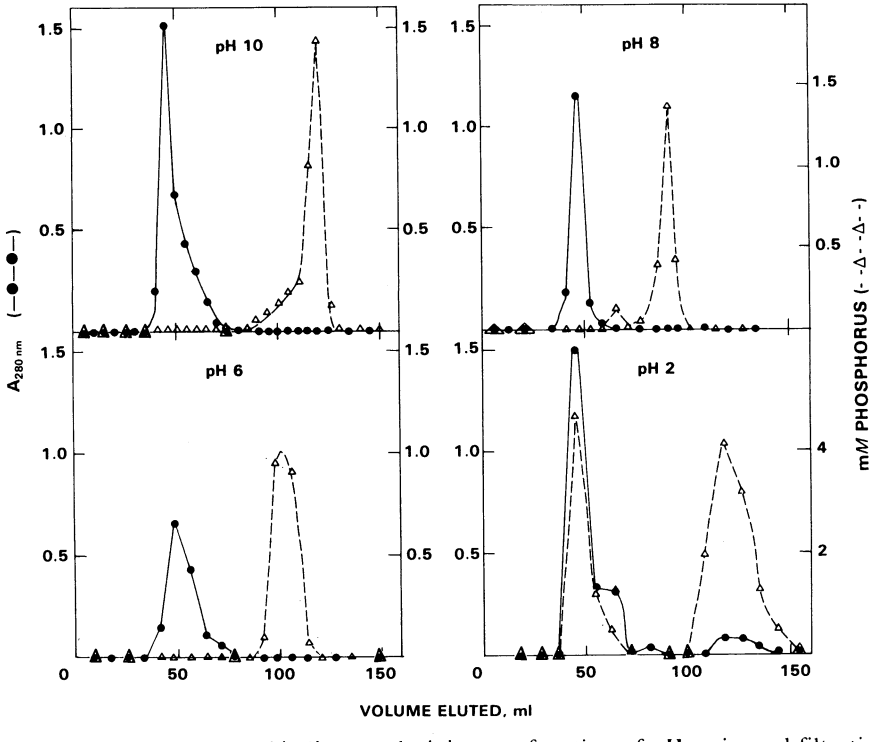


Fig. 1. Survey of phytate binding to glycinin as a function of pH, using gel filtration through Sephadex G-75. pH values for each permeation denoted within each pattern; left-hand ordinate (—●—●—) is $A_{280\ nm}$ (glycinin concentration); right-hand ordinate (- -Δ- - -Δ- -) is m*M* phosphorus (phytate concentration); column size, 1.6 × 85 cm; flow rate, 20 ml/hr; 5-ml samples applied. For details concerning solvents used for elution and amount of glycinin and phytate applied to columns, consult **Materials and Methods** section.

through 1.6 × 85-cm columns of Sephadex G-75 (Pharmacia, Inc.) at a flow rate of 20 ml per hr. Solvents used were: pH 10, water made alkaline with NaOH; pH 8, 0.05*M* tris-HCl buffer; pH 6, 0.05*M* 2(N-morpholino) ethane sulfonate · HCl buffer; pH 2, water acidulated with HCl. At pH 10 and 2, 40 mg glycinin and sodium phytate (164 μmoles P) were applied to the column; at pH 8.0 and 6.0, 20 mg glycinin and sodium phytate (14.3 μmoles P) were applied.

Glycinin was measured by absorbance at 280 nm and phytate detected by phosphorus analysis (9).

Binding Measurements between pH 2.5 and 5.0

Measurements were made at 25°C in water adjusted to the proper pH with 1*N* solutions of HCl and NaOH. Phosphorus analysis (9) was used to determine phytate concentration, and glycinin concentration was measured at neutral pH using $E^{1\%}_{280\text{nm, 1 cm}}$ of 9.2 (4). Stock solutions of sodium phytate at a concentration of 0.1716*M* as phosphorus, and glycinin solutions ranging from 0.02% (0.56 μ*M*) to 0.10% (2.8 μ*M*) were used for the binding studies.

Under the conditions of measurement, the glycinin-phytate complexes were totally insoluble between pH 2.5 and 5.0. Thirty minutes after the time of formation, the complexes were removed by centrifugation at 10,000 × *g* for 20 min at 25°C, and analysis of the supernatant fraction for phosphorus enabled calculation of bound phytate.

To present the results directly as number of sites occupied by ligand, binding results have been expressed as the ratio of moles phosphorus bound per mole glycinin; therefore, the binding in terms of phytate is one-sixth of the expressed values. A molecular weight of 360,000 for the glycinin dimer (13) was adopted to express the binding as well as to calculate the total number of potential binding sites from the amino acid composition.

The total number of binding sites was determined by adding sodium phytate to a 1.21 μ*M* glycinin solution at pH 2.5 to afford a phosphorus concentration of 846.2 μ*M*, and the binding was measured as previously described.

The number of binding sites available as a function of pH was determined by addition of sodium phytate to a 1.33 μ*M* glycinin solution at pH 6.1 to afford a phosphorus concentration of 833.0 μ*M*. Aliquots of the resulting solution were acidified to pH values between 5.0 and 2.5 and binding was measured as previously described.

The effect of phytate concentration on the binding at pH 3.0 was studied by adding increasing amounts of sodium phytate to a 1.11 μ*M* glycinin solution at pH 3.0 until the phosphorus concentration became 552 μ*M*. After each addition of phytate, the suspension was stirred 30 min before removing an aliquot. After centrifugation as previously described, the concentrations of glycinin and phytate were determined in the supernatant fractions which allowed the moles phosphorus bound per mole glycinin to be determined in the insoluble complex. At pH 3.0, an $E^{1\%}_{280\text{ nm, 1 cm}}$ of 7.89 was used to measure glycinin concentration.

Titration of Primary Binding Sites by Turbidimetry

Primary sites are defined as those fully accessible to the ligand and responsible for the quantitative precipitation of the protein when all such sites are occupied at a given pH. These sites were determined by measuring the increase in turbidity at 600 nm following addition of sodium phytate to glycinin solutions. After each

addition, the suspension was stirred 1 min prior to measurement. Adsorbance at 600 nm was plotted against phytate concentration, expressed as μM P, to provide typical titration curves, sigmoidal in shape. Estimation of the inflection point afforded the phytate concentration at which the complex precipitated; the ratio of moles P/mole glycinin at this point was considered to represent the number of sites in the protein participating in primary binding. To perform experiments as a function of pH, aliquots of a 1.11 μM glycinin solution at pH 6.1 were adjusted to pH values between 5.0 and 2.5 and titrated with sodium phytate.

TABLE I
Basic Amino Acid Content and Binding Sites of Glycinin at pH 2.5

Amino Acid	This Lab	Literature		
		Ref. (13)	Ref. (14)	Ref. (17)
Lys	138	141	172	119
His	67	60	59	60
Arg	194	186	181	162
Total basic residues	399	387	412	341
Total binding sites:				
Expected[a]	411	399	424	353
Found		424		

[a]Includes the 12 amino-terminal groups of the glycinin dimer (18).

TABLE II
Two-Stage Binding at pH 3

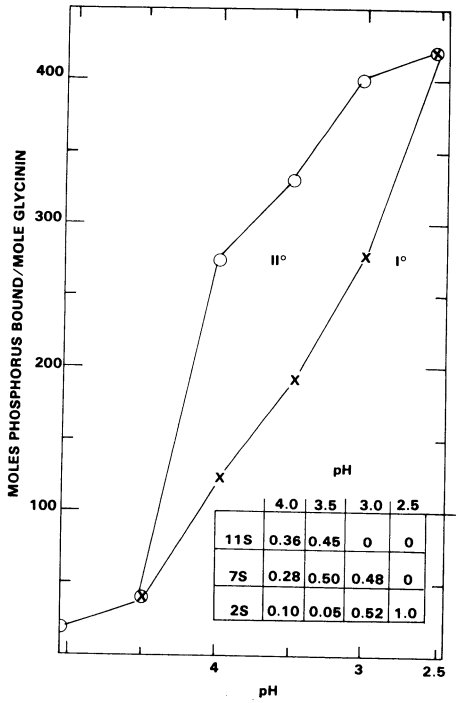
Total Phytate Added[a]	Phytate Concentration in Supernatant	Glycinin Concentration in Supernatant	Occupied Sites in Precipitated Complex[b]
Primary binding: Precipitation of complex			
0	0	1.11	0
57.2	47.0	1.07	255
114.4	88.5	0.972	186
171.6	106.0	0.847	249
228.8	122.0	0.708	265
286.0	106.0	0.506	298
343.2	0.0	0.000	309
Secondary binding: Exclusive binding to insoluble complex			
393.6[c]	3.0	0.000	352 (43)
448.1[c]	26.0	0.000	380 (71)
501.5[c]	52.0	0.000	406 (96)
552.1	100.0	0.000	407 (98)

[a]Concentrations are expressed as μM; phytate concentration expressed as μM phosphorus.
[b]Values calculated as the ratio of concentration of phytate to glycinin removed from solution; values in parentheses indicate number of secondary binding sites occupied.
[c]The association constant (4×10^5) for secondary binding was estimated from these rows of data, assuming a total of 98 secondary binding sites (last row). The value of 407 total occupied sites agrees well with the value of 400 at pH 3 shown in Fig. 2.

Effect of Ca^{2+} on Phytate-Glycinin Complex at pH 3.0

Turbidimetric titration with sodium phytate, as previously described, was performed on 1.11 μM glycinin solutions at pH 3.0 containing $CaCl_2$ at concentrations between 0 and 20 mM.

A glycinin-phytate complex was prepared at acid pH in order to demonstrate the effect of Ca^{2+} on its dissociation by gel filtration. To 20 mg glycinin dissolved in 30 ml water at pH 3.0 was added 1.25 ml 4.58 mM sodium phytate, resulting in a ratio of 640 moles P/mole protein. After 30 min, the precipitate which contained all the protein was isolated by centrifugation at 10,000 × g at 25° C for 10 min, washed thrice with 3.0 ml portions of water acidulated to pH 3.0 with HCl, and redispersed in 10 ml of the wash solvent. Ten milliliters of 0.24 M $CaCl_2$ was added, causing the complex to dissolve, and 5 ml of the solution was subjected to gel filtration through Sephadex G-75 using the conditions and techniques previously described, except that water acidulated to pH 3.0 with HCl was used for prior equilibration of the column and elution.



pH	4.0	3.5	3.0	2.5
11S	0.36	0.45	0	0
7S	0.28	0.50	0.48	0
2S	0.10	0.05	0.52	1.0

Fig. 2. Correlation of phytate binding with glycinin dissociation into subunits as a function of pH. At a given pH, the maximum number of occupied sites is shown by open circles, and the number of primary sites determined by turbidimetric titration is denoted by crosses; I° and II° denote regions of primary and secondary binding, respectively, as defined in the text. The inserted table in the lower right corner shows the proportion of glycinin that exists as 11S, 7S, and 2S components at pH values from 4.0 to 2.5 in the absence of phytate.

RESULTS AND DISCUSSION

pH Region of Binding

Coelution of glycinin and phytate during gel filtration through Sephadex G-75 did not occur at pH 10, 8, and 6, as shown in Fig. 1. Therefore, above the isoelectric point of glycinin which is 4.9 (14), no binding occurred. On the other hand, at pH 2, where the protein possesses a maximum positive net charge, binding in the form of a soluble complex was demonstrated by gel filtration. These experiments demonstrate that the net charge on the protein is the major determinant of phytate binding. At pH 10, some cationic groups reside in the protein and, under the conditions of low ionic strength used in this work, glycinin is known to be partially dissociated (15). At pH 8.0 and 6.0, an increased number of protonated basic residues are present with respect to pH 10 and the molecule is fully associated as the 11S component. Buried binding sites, electrostatic repulsion, and intramolecular salt bridges between the cationic binding sites and carboxylate groups are all possible reasons why phytate binding does not occur above the isoelectric point. The latter two appear most plausible, since conformational studies suggest that the hydrophilic side-chain residues are located primarily on the surface of glycinin (16).

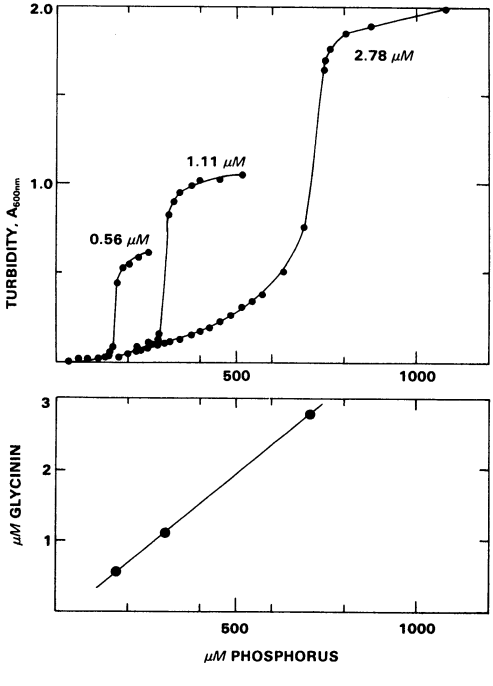


Fig. 3. Turbidimetric titration of glycinin by sodium phytate at pH 3.0. Upper graph: titration curves at various glycinin concentrations; values noted at each curve. Lower graph: relationship between midpoints of titration curves expressed in terms of μM phosphorus (sodium phytate) and glycinin concentration.

Equivalence of Phytate Binding Sites and Basic Residues

Table I indicates that agreement between the total number of phytate binding sites measured at pH 2.5 is, within experimental error, equal to the number of positively charged groups per mole of glycinin. The binding was found to be maximal at pH 2.5, where the net positive charge of the protein is greatest and higher orders of protein structure which hinder the binding have been largely destroyed. These results are in agreement with earlier studies (2) where phytic acid was used to determine the lysine, histidine, and arginine content of proteins.

In addition to the protein used in this work, Table I also presents the total number of basic residues and total potential binding sites calculated from the amino acid composition of three different preparations of the glycinin dimer adopting a molecular weight of 360,000. As can be seen in Table I, agreement between three of the four analyses in terms of total sites is good, ranging between 400 and 425, even though variation of the lysine and arginine content among the preparations is pronounced. Roughly 12.7% of the residues in glycinin contain potential phytate binding sites. Thus, the glycinin used in this study has a total content of basic amino acid residues similar to that of two of the three preparations.

Evidence for Two Major Classes of Binding Sites

At pH 3.0, the effect of phytate concentration on glycinin solubility and on binding was studied in detail. The results, presented in Table II, provide strong evidence for the existence of two major classes of binding sites. As the concentration of ligand increases, the protein concentration decreases to zero. At this point the phytate concentration is also zero, indicating the association constant at this stage of the binding to be extremely large. Further increases in phytate concentration had no effect on the solubility of the complex, but resulted in additional binding, up to a value of 407 moles P/mole glycinin, a value that agrees well with the maximal number of sites (400) determined at pH 3 (Fig. 2). Throughout the first stage of binding, up to the point of quantitative protein precipitation, primary binding is taking place, involving sites that are freely accessible on the protein surface. Thus, precipitation is a consequence of formation of an isoelectric phytate-glycinin complex, having a very large association constant. On the other hand, secondary binding, defined as that binding which occurs to sites hindered by steric inaccessibility or ionic interactions with carboxylate groups, is weaker than primary binding. This contention is supported by the data in Table II, where it is seen that not all the added phytate was firmly bound to the insoluble complex. An association constant of about 4×10^5 was calculated from the appropriate data, a value in accord with those found for phytate binding to both ovalbumin (19) and human serum albumin (20). Thus, at pH 3.0, 309 (76%) of the sites in glycinin were considered to be unhindered (primary), whereas the remaining 98 of the total sites were hindered (secondary).

At pH 3.0, the turbidimetric titration of glycinin solutions, varying in concentration between 0.56 μM (0.02%) and 2.78 μM (0.10%) with sodium phytate is shown in Fig. 3. The midpoint of the sigmoid represented the phase of transition of primary binding. As can be seen, the concentration of phytate corresponding to the midpoint was a linear function of the protein concentration and was equivalent to 280 moles P/mole protein, a value 91% of that found for

primary binding at this pH (309 sites, Table II).

Binding between pH 2.5 and 5.0

On the assumption that turbidimetric titration is a measure of the primary binding sites below the isoelectric point, experiments were performed between pH 4.5 and 2.5, and the results are shown in Fig. 4. By plotting midpoints of the titration curves as a function of pH, a linear relationship was observed between pH 4.5 and 3.0. Extrapolation to zero phosphorus concentration gave an intercept of pH 4.8, indicating that no phytate binding should occur at or above the isoelectric point where the net charge of the protein is zero or negative, respectively. These observations are consistent with and complementary to those obtained by gel filtration, and suggest that the precipitation of glycinin by phytic acid results from binding of the ligand to the cationic basic groups accessible on the protein surface, thus forming an isoelectric complex which is insoluble above pH 2.

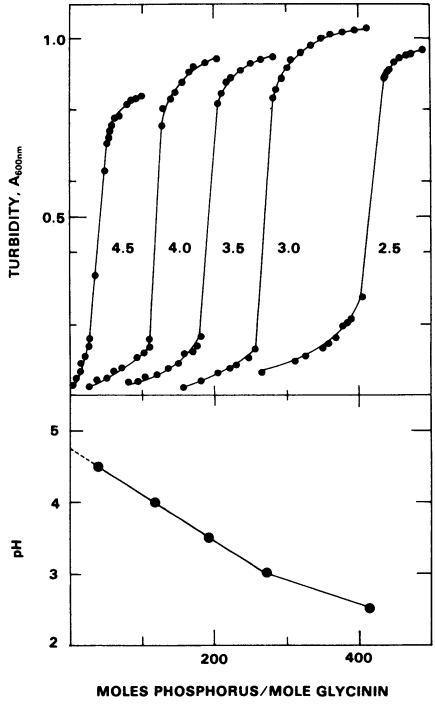


Fig. 4. Turbidimetric titration of glycinin by sodium phytate at pH values between 4.5 and 2.5. Glycinin concentration was 0.04% (1.11 μM). Abscissa for both graphs is sodium phytate concentration expressed as moles phosphorus/mole glycinin. Upper graph: turbidimetric titration curves at various pH values; pH of titration shown at the right of each curve. Lower graph: plot of midpoint of each titration, representing primary binding, and expressed as moles phosphorus/mole glycinin, against pH. Dashed line denotes extrapolation to a pH value of zero binding.

Shown in Fig. 2 are the maximum number of sites available for binding in the pH range 2.5 to 5.0, measured in the presence of a 1.5-fold excess of ligand, as compared with the number of primary sites found turbidimetrically at the same pH. The inset of the Figure indicates the extent of glycinin dissociation measured under conditions comparable to the binding experiments, in the absence of phytate.

The data indicate that secondary binding, evidenced by the departure of the two curves, occurred in the pH region in which glycinin was partially dissociated. Earlier studies on other proteins (6, 19) have shown clearly that higher orders of protein structure are a determinant of phytate binding. The net charge and the number of accessible binding sites are dependent upon tertiary structure and, in the case of glycinin, also upon quaternary structure. Secondary binding, therefore, appears to reflect higher orders of protein structure still extant in

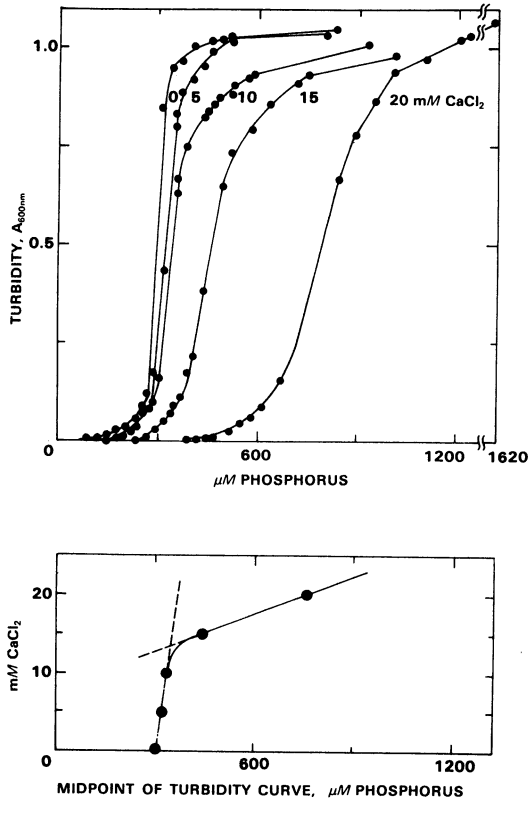


Fig. 5. Ca^{2+} inhibition on formation of phytate-glycinin complex at pH 3.0. Upper graph: glycinin solutions (0.04%:1.11 μM) at pH 3.0 containing increasing concentrations of $CaCl_2$ (values shown at the upper right of each curve) were titrated with sodium phytate, concentration expressed as μM phosphorus. Lower graph: relationship between $CaCl_2$ concentration and midpoint of turbidity curve; the intersection of the dashed lines gives the Ca^{2+} concentration at the onset of the binding inhibition.

partially dissociated glycinin, and represents sites impaired either by location in the interior of the molecule or by internal salt linkages with carboxylate groups. The role of carboxylate groups as a determinant of secondary binding is supported by the observation that the greatest extent of this binding occurs at pH 4, a value close to the pK_a values of side-chain carboxyl groups. Known interactions between carboxylate and cationic groups in human serum albumin (21) were proposed to explain the abnormal phytate binding observed at pH 4.1 and 3.3 with respect to that observed at pH 2.5 (20). In the case of glycinin, one cannot distinguish between the role of carboxylate groups in maintaining quaternary structure (5) and in solely determining binding behavior by electrostatic interactions (if indeed these functions are distinct).

The number of available binding sites at pH values where glycinin is presumably fully associated or fully dissociated can all be characterized as primary, and thus are believed to be fully accessible on the surface of the protein at pH 4.5 or the 2S subunits at pH 2.5.

Dissociation of Phytate-Glycinin Complexes by Ca^{2+} at Acid pH

Above the isoelectric point of soybean globulins, Ca^{2+} mediates phytate binding (22). In contrast, Ca^{2+} was found to decrease the stability and formation of phytate-glycinin complexes below the isoelectric point of glycinin. Gel filtration through Sephadex G-75 of a phytate-glycinin complex solubilized in the presence of a 110-fold equivalent excess of calcium ion with respect to the cationic protein groups indicated that 96% of the phytate had been dissociated from the protein at pH 3.0.

In a complementary experiment, the calcium concentration required to inhibit formation of insoluble phytate-glycinin complexes was determined at pH 3.0 by means of turbidimetric titration. Figure 5 indicates that the precipitation of glycinin by phytate was not significantly affected below a calcium concentration of 14 m*M*. Under the conditions of the experiment, this Ca^{2+} concentration corresponded to a 100-fold equivalent excess with respect to the basic residues of the protein. Since the same proportion of Ca^{2+} was required to dissociate the complex at pH 3.0, as demonstrated by gel filtration, dissolution of the complex by Ca^{2+} is evidently a result of dissociation. This effect of Ca^{2+} is ion-specific and not related to ionic strength; no dissociation, as measured turbidimetrically, occurred at NaCl concentrations of an equivalent ionic strength.

A similar inhibition of phytate binding to serum and almond albumin by Ca^{2+}, as well as by Zn^{2+} and Mg^{2+} at acid pH has been explained on the basis of chelation of the metal ion with a carboxylate and cationic group of the protein (6). In our opinion, direct competition between Ca^{2+} (or other multivalent cations) and basic residues of proteins for the phosphate groups of phytate appears to be a straightforward and logical explanation for the dissociation. This effect has been used successfully to prepare phytate-free soy protein isolates employing ultrafiltration at pH 3.0 in the presence of Ca^{2+} (23).

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