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Iron(III)–Phosphoprotein Chelates: Stoichiometric Equilibrium Constant for Interaction of Iron(III) and Phosphorylserine Residues of Phosvitin and Casein[†]

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Appendix: Equilibrium Equations for Iron(III) Chelates of (Ethylenedinitrilo)tetraacetate, Nitrilotriacetate, Citrate, and Phosphoprotein *O*-Phosphorylserines

Jack Hegenauer

ABSTRACT: Estimates of the strength of iron binding to model phosphoproteins were obtained from equilibrium dialysis experiments. Iron-free phosvitin (chicken and frog) or α_{s1}-casein (cow) was dialyzed against the iron(III) chelates of nitrilotriacetate (NTA), (ethylenedinitrilo)tetraacetate (EDTA), or citrate. Protein-bound metal was measured at equilibrium; competition of chelator and phosphoprotein for iron(III) was determined by reference to comprehensive equilibrium equations presented in the Appendix. Analysis of the iron-binding data for phosvitin suggested that clusters of di-*O*-phosphorylserine residues (SerP·SerP) were the most probable iron-binding sites. A stoichiometric equilibrium constant of $10^{18.0}$ was calculated for the formation of the Fe^{3+}(SerP·SerP) chelate. When compared on the basis of phosphate content, casein bound iron more weakly than phosvitin. However, if the stoichiometric equilibrium constant for the formation of the casein Fe^{3+}(SerP·SerP) chelate ($10^{17.5}$) was adjusted to account for the fact that a smaller percentage of casein phosphoserines occurs in di-*O*-phosphorylserine clusters, the affinity of casein and phosvitin for iron was very similar. A theoretical comparison showed that the "strengths" of the ferric chelates can be ranked: EDTA > phosphoprotein di-*O*-phosphorylserine > citrate > NTA.

The binding of metal by phosphoproteins has great physiological and nutritional relevance. For example, the calcium–casein micelles of milk are important sources of calcium and phosphorus for children and adults, and the iron complex of phosvitin, the principal phosphoglycoprotein of egg yolk (Shainkin & Perlmann, 1971), is a rich source of dietary iron for many people, as well as an essential reservoir of this metal for embryonic development in the eggs of lower vertebrates (Clark, 1974). Physical–chemical studies of the iron–phosvitin complex have recently outlined the coordination requirements of bound iron(III). The structural model of the iron(III)–phosvitin complex proposed by Webb et al. (1973) and by Gray (1975) explains the high probability of iron binding by adjacent phosphorylserine residues, as well as the strong iron–iron interactions observed by measurements of magnetic susceptibility. We are now studying the ligand-exchange behavior of casein and phosvitin to provide biochemical explanations for observations that milk and egg yolk may inhibit the gastrointestinal absorption of dietary iron (Carmichael et al., 1975). A related aim of some nutritional importance is to explain and predict the large proportion of natural and supplemental iron that is found associated with the casein micelles of milk (Loh & Kaldor, 1973). A complete analysis of these questions requires an estimate of the strength of iron binding by phosphoproteins.

To determine optimal conditions for saturating phosphoproteins with iron(III) for physical–chemical studies, we considered four methods for the presentation of iron: (1) presentation of iron(II) under oxidizing conditions (Taborsky, 1963); (2) addition of simple iron(III) salts; (3) direct addition of iron(III) chelates to apoproteins (Webb et al., 1973); and (4) presentation of iron(III) chelates by equilibrium dialysis under conditions similar to those used by Aasa et al. (1963)

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and by Aisen et al. (1978) to determine the strength of binding of iron(III) to transferrin. The first of these possibilities was avoided because of evidence that autoxidation of iron(II) may render protein-bound phosphate labile (Grant & Taborsky, 1966). The second was eliminated because of the impossibility of maintaining a reasonable concentration of iron(III) at neutral pH without hydrolysis and polymerization of ferric oxyhydroxide (Spiro & Saltman, 1969). The third method creates some problems with the removal of unbound iron(III) chelate; in our experience, the use of gel filtration, for example, may perturb the iron-protein/iron chelate equilibrium by fractionating excess chelator. Preliminary attempts with phosvitin to implement the fourth possibility through equilibrium dialysis were successful, but it became evident that interactions of complex systems of ligands competing for iron(III) could not be interpreted without reference to a comprehensive equilibrium formulation. These equations, presented in the Appendix, have permitted the rational analysis of data obtained from dialyzing phosvitin and casein against iron(III) chelates and permits us for the first time to estimate the strength and stoichiometry of the binding of iron to phosphoproteins in general.

Materials and Methods

Preparation of Phosphoproteins

Chicken Phosvitin. Phosvitin was extracted by a novel method from defatted yolk granules (phosvitin-lipovitellin complex) and purified by ion-exchange chromatography. Commercial egg yolk powder (about 1 kg; General Biochemicals, Chagrin Falls, OH) was blended with 0.2 M NaCl and diluted with 35 L of detergent solution (0.5% Triton X-100-0.04 M NaCl-0.02 M NaH_2PO_4; unbuffered pH = 5.2). After standing overnight, the supernatant was siphoned off and the settled yolk granules were resuspended in another 35 L of detergent solution. Washing was repeated 6 times (at room temperature). The defatted yolk granules were recovered by low-speed centrifugation, suspended in about 8 L of 1 M KCl, stirred for 8 h, and centrifuged at 10000*g* for 20 min; the supernatant containing phosvitin dissociated from the lipovitellin complex was set aside. The residue was washed twice with another 8 L of 1 M KCl, the supernatants were combined, and the solution was filtered through a pad of diatomaceous earth to remove particulates. Phosvitin was isoelectrically precipitated from the filtrate by dropwise addition of 6 N HCl to pH 1.5. After standing overnight in the cold, the phosvitin formed an opaque, colloidal gel, which was recovered by centrifugation at 10000*g* for 20 min. The gel was washed twice by suspension-centrifugation in about 2 L of 0.01 N HCl to remove some residual salt and detergent, suspended in an equal volume of water, and redissolved by adding a minimal quantity of a saturated solution of sodium acetate. The phosvitin solution was then dialyzed twice against daily 10-volume changes of 0.05 M disodium (ethylenedinitrilo)tetraacetate (EDTA) (unadjusted pH = 4.55), dialyzed exhaustively against water, filtered to remove debris, and lyophilized to give 9-10 g of phosvitin (monosodium salt) from each kilogram of powdered egg yolk.

As a final purification procedure, the crude phosvitin was chromatographed on DEAE-cellulose as described by Wallace et al. (1966) using a 4 × 60 cm column of adsorbent (Whatman "DE-52") for 3 g of phosvitin. The column was equilibrated and the sample was dissolved in 50 mM Tris-HCl-5 mM EDTA, pH 8.0. The sample was washed into the column with enough Tris-EDTA buffer to remove unadsorbed metal-EDTA complexes, basic protein, and Triton X-100 (monitored by $A_{260\mathrm{nm}}$). The column was eluted with 2 L of a linear gradient (Hegenauer et al., 1965) of NaCl (0-0.5 M in Tris-EDTA buffer). Phosvitin was eluted last in the gradient and was well separated from a small amount of lipovitellin. The relative quantities of phosvitin and contaminating proteins were not readily deduced from the elution diagram because of the low molar absorptivity of phosvitin at 280 nm (Wallace, 1963). Phosvitin was recovered by acidifying the pooled eluate with HCl to pH 1.5, allowing the suspension to stand overnight in the cold, and centrifuging. The phosvitin gel was dissolved in dilute sodium acetate, dialyzed thoroughly against distilled water, and lyophilized. About 80% of the applied protein was recovered in the phosvitin fraction. The chicken apophosvitin gave a single band on polyacrylamide electrophoresis (Hegenauer et al., 1977). Elemental composition (Hegenauer et al., 1979a) of the deionized protein was 12.52% N, 10.66% P, and <0.0025% Fe; this agrees well with Wallace et al. (1966).

Frog Phosvitin. Ovaries were dissected from mature female leopard frogs (*Rana pipiens*), rinsed in 0.1 M NaCl, and frozen at −18 °C for as long as 3 years before use. Ovaries (2 kg) were thawed and blended in the cold with 4 L of 0.2 M NaCl. The thick black suspension was centrifuged at 10000*g* for 20 min, and the supernatant was discarded. The sticky black residue, containing yolk granules and adsorbed pigment, was washed twice with 4-L portions of 0.2 M NaCl by blending and centrifugation. The phosvitin was extracted with three 4-L portions of 1 M KCl, precipitated isoelectrically at pH 1.5, dialyzed, and lyophilized as described above for the chicken protein. About 6-8 g of crude phosvitin (monosodium salt) was obtained from each 2 kg of ovary. The frog phosvitin was further purified by chromatography on DEAE-cellulose as described above. Because the frog protein emerged earlier in the gradient relative to the chicken protein, the frog phosvitin was not well separated from lipovitellin and had to be rechromatographed. The frog apophosvitin gave a single band on polyacrylamide electrophoresis (Hegenauer et al., 1977). Elemental composition (Hegenauer et al., 1979a) of the deionized protein was 16.65% N, 10.55% P, and <0.0005% Fe; this agrees well with Wallace et al. (1966).

Cow Casein. α_{s1}-Casein was prepared from whole isoelectric casein as the fraction insoluble between 6.6 and 4.63 M urea (Hipp et al., 1952). The crude product (8 g) was further purified by chromatography on a 5 × 77 cm column of DEAE-cellulose (Whatman "DE-52") equilibrated with urea-bisulfite buffer (5 M urea-5 mM $NaHSO_3$-5 mM Na_2SO_3, pH 6.78) and eluted with 8 L of a linear gradient (Hegenauer et al., 1965) of 0-0.4 M NaCl in urea-bisulfite buffer. All buffers were kept under a nitrogen atmosphere to retard oxidation of bisulfite, and all procedures were conducted at room temperature. α-Casein was the last component to emerge from the column. The eluate was pooled, dialyzed exhaustively against 0.05 M EDTA and water, and lyophilized. The protein was as pure as that obtained by the related chromatographic method of Thompson (1966) and was homogeneous on starch-gel electrophoresis (El-Negoumy, 1966). The α_{s1}-casein B was the principal genetic variant, but small amounts of the A and C components were detected (Thompson, 1971).

Equilibrium Dialysis

Chelator Solutions. Stock solutions of trisodium citrate and Na_2EDTA were prepared by reference to the formula weights. Because of the uncertain purity and hydration state of disodium nitrilotriacetate (NTA; Sigma Chemical Co., St. Louis, MO), its concentration was estimated by nitrogen analysis, after wet-ashing with H_2SO_4–H_2O_2, by the colorimetric procedure of Koch & McMeekin (1924).

Iron(III) Chelates. General precautions for preparing ferric chelates without hydrolysis of iron have been described (Hegenauer et al., 1979c). In these experiments, ferric chloride (freshly prepared in 0.1 N HCl) was diluted in water to about one-fifth of the final desired volume. Solutions of chelator, buffer salts, and compensating electrolyte (NaCl) were each added slowly with good stirring; dilute NaOH or HCl (0.01 N) was added to achieve the desired pH before diluting to the final volume. Solutions were used within 24 h. In preparing radioiron chelates for dialysis against casein, $^{59}FeCl_3$ ("carrier-free", New England Nuclear, Boston, MA) in 0.1 N HCl was equilibrated with the ferric chloride solution for 2 h before adding chelator; the specific activity of ^{59}Fe(III) chelates was ~7 Ci/mol. Acidic solutions of NTA were buffered with 0.1–0.2 M acetate ($pK' = 4.756$); acidic solutions of citrate and EDTA were self-buffered. Alkaline solutions of NTA and citrate were buffered with 0.01 M Tris ($pK' = 8.01$) for phosvitin dialysis or with 0.01 M Hepes ($pK' = 7.55$) for casein dialysis. Ionic strength was brought to $I = 0.1$ with NaCl after the contribution of all ionized (uncomplexed) species of NTA or citrate (−1, −2, −3), EDTA (−1, −2, −3, −4), acetate (−1), Tris (+1), or Hepes (−1) with monovalent counterions was calculated. Final iron concentration of each chelate solution was determined by solution absorption spectrophotometry of the iron(II)–tris(bathophenanthroline disulfonate) complex after wet-ashing with $HClO_4$ (Avol et al., 1973). Final ligand concentrations were calculated from the concentrations of the stock solutions.

Phosvitin. Protein solutions were deionized (Dintzis, 1952), titrated, and equilibrated by dialysis with the buffer–electrolyte system used to prepare the corresponding iron chelate. Initial phosphate concentration of each apophosvitin solution was determined by solution absorption spectrophotometry of molybdenum blue after wet-ashing with $HClO_4$ (Allen, 1940). Equilibrium dialysis was carried out essentially as described by Hughes & Klotz (1956). Protein (up to 5 mL) was enclosed in boiled no. 8 Visking regenerated cellulose tubing and placed in a 250-mL screw-cap flask containing 100 mL of the iron chelate solution and 0.2 mL of toluene. The flask was suspended in a water bath at 25 ± 0.2 °C and agitated continuously by gentle reciprocal shaking. Equilibrium was established by 100 h for NTA and citrate and by 200 h for EDTA (Aasa et al., 1963; Aisen & Leibman, 1968); dialysis was allowed to proceed for 5 and 10 days, respectively. Duplicate (NTA and citrate) or quadruplicate (EDTA) experiments were performed at each metal/chelator ratio. Iron content of the diffusate was determined by solution absorption spectrophotometry (Avol et al., 1973) on duplicate samples withdrawn at the beginning of dialysis (before introducing protein) and at its conclusion; protein-bound metal was calculated as the difference after applying volume corrections. Nonspecific binding of iron(III) to the dialysis tubing (Hughes & Klotz, 1956) was negligible. The experimental design did not permit direct analysis of metal in the retentate compartment, but we assumed that Donnan equilibrium did not affect the distribution of the iron(III) chelate. Final pH was measured at 25 °C with a Beckman 39501 ceramic junction combination electrode.

Casein. Protein solutions were dialyzed against 0.1 M sodium perchlorate and then water to remove potentially contaminating EDTA (Price & Gibson, 1972). After titration to the desired pH, solutions were dialyzed against the buffer–electrolyte system of the corresponding iron chelate. Initial phosphate concentration of each solution was determined by the method described for phosvitin. Equilibrium dialysis was performed in plastic cell compartments separated by a single thickness of no. 20 Visking tubing, as described by Malmstrom (1953); the interior of the cells was coated lightly with silicon grease to prevent adsorption of iron to the acrylic surface. Two milliliters of protein solution and 2 mL of iron chelate were pipetted into the cell on opposite sides of the membrane. Filling holes were sealed with tape, and the cells were enclosed in plastic wrap to prevent evaporative water loss; cells were then agitated on a reciprocal shaker (1 excursion/s) for 5 days at 10 ± 0.5 °C. Five cells were loaded for each iron/chelator ratio. Duplicate samples (500 μL) were withdrawn from each cell compartment, and ^{59}Fe was measured in a Nuclear-Chicago γ well counter [2-in. NaI(Tl) crystal] with the spectrometer calibrated to give a window of 1.10 ± 0.011 MeV; at least 5×10^4 counts were accumulated for each sample. Since ^{59}Fe is assumed to be proportional to $^{55.847}$Fe, protein-bound metal in each cell was calculated from the relationship (^{59}Fe in retentate − ^{59}Fe in diffusate)/(^{59}Fe in retentate + ^{59}Fe in diffusate). We observed no change in the relative volumes of the protein and chelator compartments during dialysis, and no volume corrections were applied. Final pH was measured at 22 °C with a Radiometer GK2302C ceramic junction combination electrode.

Stability of Iron(III) Chelates. Numerous control experiments were performed to ensure that iron solutions remained soluble and, most importantly, diffusable throughout the dialysis period. In a few experiments with phosvitin (noted under Results), NTA concentration (determined by nitrogen analysis) was lower than anticipated, so that the Fe/NTA molar ratio was >1; iron was therefore initially polymeric and undiffusable. No precipitation of ferric oxyhydroxide was observed, however, and there was reason to believe that this iron had depolymerized and equilibrated by the end of the dialysis period. Diffusability of iron was confirmed (1) by placing iron solution and electrolyte in the diffusate and retentate compartments, respectively, and seeing if iron concentration was equal in both compartments at the end of dialysis or (2) by seeing if polymeric iron was retained on an Amicon PM-10 ultrafilter.

Metal-Ligand Equilibria and Calculations

Iron(III)–EDTA. The equations and association constants for the iron(III)–EDTA equilibrium [$I = 0.1$ (KCl), 20 °C] are those of Schwarzenbach & Heller (1951a). Equations for computing the equilibrium concentration of iron(III) are derived in the Appendix. An abbreviated derivation has been presented by Aasa et al. (1963).

Iron(III)–NTA. The equations and association constants for the iron(III)–NTA equilibrium are those given by Schwarzenbach & Heller (1951b) for the hydroxo and ternary monomeric complexes [$I = 0.1$ (KCl), 20 °C] and by Gustafson & Martell (1963) for the dimeric complex [$I = 1.0$ (KCl), 25 °C]. Equations for computing the equilibrium concentration of iron(III) are derived in the Appendix; these equations have not been explicitly formulated elsewhere.

Iron(III)–Citrate. The equations and association constants of Warner & Weber (1953) for the iron(III)–citrate equilibrium [$I = 0.1$ (NaCl), 30 °C] have been used. We have also taken into account the formation of the ferric dicitrate complex described by Spiro et al. (1967). Like Aisen et al. (1978), we have not included the formation of the ferric citrate dimer reported by Timberlake (1964); inclusion of this equilibrium led to the calculation of impossibly low iron(III) concentrations that were not consistent with experience. The formulation and constants derived by Hamm et al. (1954) for the iron(III)–citrate equilibrium [$I = 1.0$, 20 °C] are not considered here but lead to a similar expression for [Fe(III)]. Aisen et al. (1978) have recently presented a different treatment of the Warner–Weber/Spiro equilibria.

Iron(III)–Phosvitin and Iron(III)–Casein. We assumed that phosphate groups of phosphorylserine residues constitute the metal-binding sites of phosvitin (Taborsky, 1963; Grant & Taborsky, 1966; Donella et al., 1976) and casein (Ho & Waugh, 1965). Formulation of equilibrium equations for the interaction, however, requires additional definition of the physical structure of the metal chelate. No assumptions about the stoichiometry of the metal–phosphoprotein complex are required, but we have been guided by the common observation that one iron atom is bound for every two phosphate groups when phosvitin is fully saturated.

Nature of the Ligand. Many conceivable metal-binding ligands may be defined by permutations and combinations of phosphorylserine residues. We focussed our attention principally on two ligands: (1) the monodentate mono-*O*-phosphorylserine ligand SerP and (2) the bidentate di-*O*-phosphorylserine ligand SerP·SerP (composed of two adjacent phosphorylserine residues). We can generalize these two ligands to consider their interaction with a variety of di- and trivalent metal ions and at the same time satisfy possible restrictions about 1Fe/2P stoichiometry. Consideration of only mono- and bidentate ligands also leads to simpler equilibria and simpler algebra (see Appendix). We are aware of no evidence for cooperativity of metal binding to phosphoproteins which would suggest that two or more metal ions were bound more readily than one. Consideration of the stereochemistry of multiple sequences of phosphorylserine residues suggests that a maximum of two phosphorylserines satisfies the coordination requirements of iron(III) and rules out the likelihood that more than two phosphorylserines are needed to bind a single iron(III) ion. There is thus no reason to suspect that metal binding may involve phosphorylserine ligands more complex than those considered here. As our analysis will show (see Results), the iron-binding data for two dissimilar phosphoproteins, phosvitin and casein, can be rationalized fully on the basis of their phosphate content, so it seems unnecessary at this time to consider other potential ligands of a weaker nature (carboxyl, thiol, amine).

Nature of the Chelate. We restricted our attention to chelates of only one iron atom with mono- or bidentate phosphorylserine ligands. The three possibilities, which are illustrated diagrammatically in Figure 1, are (1) a mononuclear chelate composed of one iron atom and one monodentate ligand, Fe(SerP), (2) a binuclear chelate composed of one iron atom and two monodentate ligands, Fe(SerP)$_2$, and (3) a mononuclear chelate composed of one iron atom and one bidentate ligand, Fe(SerP·SerP). Although chelates 2 and 3 lead to equivalent 1:2 stoichiometry of metal–phosphorylserine, the formulation for the two equilibria is quite different. The monodentate binuclear chelate 2 must be considered the result of the interaction of two *independent* phosphorylserine residues, whereas the bidentate mononuclear chelate 3 must be formed from two *linked* (adjacent) phosphorylserine residues.

Generalized equilibrium equations for the interaction of protons and metal ions, including iron(III), with mono- or di-*O*-phosphorylserine residues of a phosphoprotein are derived in the Appendix. Accepted constants for the dissociation of protons from phosphorylserine residues in phosphoproteins were not available, so they have been estimated from available titration data of phosvitin (Mecham & Olcott, 1949) and a casein phosphopeptide (Österberg, 1960, 1961). These estimates are rationalized in the Appendix.

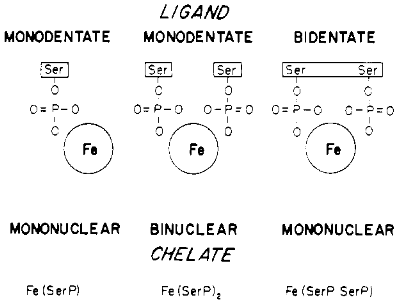

FIGURE 1: Nomenclature and diagrammatic representation of the interaction of an ion of iron with one or two *O*-phosphorylserine residues (Ser) of a phosphoprotein. Indications of valence have been omitted from the diagram. Denticity describes the number of metal coordinating groups of a ligand. Nuclearity defines the stoichiometry of the complex in terms of the number of ligands (of any complexity) coordinated to one iron atom.

Calculation of Fe(III)–Phosphoprotein Association Constants. In a system of Fe(III), phosphoprotein, and competing ligand, the free ionic iron concentration, [Fe^{3+}], in equilibrium with the iron–phosphorylserine complex is equal to [Fe^{3+}] in equilibrium with the iron–ligand complex. In our dialysis experiments, we estimated the amount of iron associated with the diffusable ligand (the concentrations of free iron and all iron–ligand complexes) by determining the iron concentration in the diffusate compartment; iron associated with the phosphoprotein (the concentration of iron–phosphorylserine complex) was calculated as the difference between the total iron in the retentate compartment and diffusable iron. Concentrations of monodentate and bidentate phosphorylserine ligands were set equal to [P]$_t$ and [P]$_t$/2, respectively, by determining the phosphate concentration of the phosphoprotein solutions. The very small free iron concentration, [Fe^{3+}], was calculated for the particular iron–ligand system with the help of the equilibrium equations given in the Appendix. This quantity was then introduced into the iron–phosphoprotein equilibrium equations to yield the stoichiometric association constant for the interaction.

Results

Strength of Iron(III) Binding to Phosvitin

Primary data for equilibrium dialysis experiments with phosvitin are given in Table I. So that we might derive the greatest possible generality from the results, we designed experiments to include a wide range of chelators, pH, ligand/metal ratios, and potential ternary ligands (e.g., acetate). The scarcity of frog phosvitin did not permit a more extensive analysis, so most of the experiments used chicken phosvitin. In a few experiments (B.1, C.1, E.1, G.1, L.1, Table I), the initial iron concentration (the sum of metal concentrations in both dialysis compartments at equilibrium) exceeded the chelator (NTA) concentration, so that a small proportion of the iron introduced in the diffusate was polymeric and therefore inaccessible to the protein at the start of dialysis. The final chelator/iron ratio in the diffusate compartment was, however, greater than 1, so polynuclear iron was likely to have depolymerized during the dialysis period. With these exceptions, instability (i.e., polymerization) of iron(III) chelates was not observed in experimental or control incubations. Polymerization of 1:1 Fe–NTA chelates within a short time of preparation has been noted by other workers (Webb et al., 1973); but in our opinion such metastability must have been due in part to the method of analysis since iron(III) interacts strongly with many gel filtration matrices, which are also

Table I: Primary Data and Stoichiometric Equilibrium Constants for the Binding of Iron(III) to Chicken and Frog Phosvitins with NTA, EDTA, or Citrate as the Competing Ligand[a]

		equilibrium concn ($M \times 10^4$)								log K[c]		
					iron(III)[b]							
expt	competing ligand	ligand	phosvitin phosphate	acetate	phosvitin (bound)	chelator (free)	protein Fe/P	pH	pFe	mononuclear monodentate Fe(SerP)	mononuclear bidentate Fe(SerP·SerP)	binuclear monodentate $Fe(SerP)_2$
A.1	NTA	9.499	9.787	96.618	4.488	0.481	0.459	4.64	12.92	14.65	17.57	21.37
A.2	NTA	14.248	9.787	96.618	4.342	0.621	0.444	4.64	13.04	14.75	17.55	21.21
A.3	NTA	18.997	9.787	96.618	4.225	0.715	0.432	4.64	13.16	14.85	17.57	21.15
A.4	NTA	21.846	9.787	96.618	4.173	0.777	0.426	4.64	13.21	14.89	17.58	21.13
A.5	NTA	28.495	9.787	96.618	4.010	0.953	0.410	4.64	13.30	14.94	17.56	21.02
B.1	NTA	4.750	9.636	193.237	4.728	0.282	0.491	4.75	13.03	14.72	18.14	22.60
B.2	NTA	9.499	9.636	193.237	4.635	0.400	0.481	4.75	13.25	14.92	18.04	22.19
B.3	NTA	14.248	9.636	193.237	4.496	0.514	0.467	4.75	13.37	15.01	17.91	21.81
B.4	NTA	18.997	9.636	193.237	4.391	0.644	0.456	4.75	13.45	15.07	17.85	21.63
B.5	NTA	23.749	9.636	193.237	4.246	0.777	0.441	4.75	13.51	15.10	17.77	21.41
C.1	NTA	4.773	10.018	116.505	5.134	0.520	0.512	4.65	12.52	14.34		
C.2	NTA	9.545	10.018	116.505	5.048	0.668	0.504	4.65	12.79	14.60		
C.3	NTA	14.317	10.018	116.505	5.014	0.772	0.500	4.65	12.97	14.77		
C.4	NTA	19.090	10.018	116.505	4.816	0.946	0.481	4.65	13.06	14.82	18.04	22.16
C.5	NTA	23.864	10.018	116.505	4.678	1.076	0.467	4.65	13.14	14.88	17.88	21.77
D.1	EDTA	5.116	18.961	0	0.520	4.407	0.027	3.69	15.24	16.44	19.50	21.95
E.1	NTA	4.727	12.196	0	2.865	2.154	0.235	8.00	18.56	18.06	18.52	21.42
E.2	NTA	9.453	12.196	0	1.906	3.192	0.156	8.00	18.80	18.08	18.47	21.25
E.3	NTA	14.179	12.196	0	1.245	3.884	0.102	8.00	18.95	18.02	18.37	21.09
E.4	NTA	18.906	12.196	0	0.845	4.235	0.069	8.00	19.09	17.98	18.31	21.00
E.5	NTA	23.635	12.196	0	0.527	4.554	0.043	8.00	19.20	17.87	18.19	20.85
F.1	NTA	4.750	11.632	0	3.645	1.086	0.313	7.40	17.59	17.29	17.86	20.97
F.2	NTA	9.499	11.632	0	3.429	1.450	0.295	7.40	17.86	17.53	18.07	21.13
F.3	NTA	14.248	11.632	0	3.233	1.621	0.278	7.40	18.07	17.70	18.21	21.24
F.4	NTA	18.997	11.632	0	2.913	1.929	0.250	7.40	18.16	17.73	18.22	21.19
F.5	NTA	23.749	11.632	0	2.728	2.175	0.235	7.40	18.25	17.79	18.25	21.20
G.1	NTA	4.682	18.961	0	2.875	1.978	0.152	7.95	18.50	17.76	18.15	20.74
G.2	NTA	9.363	18.961	0	2.186	2.735	0.115	7.95	18.77	17.90	18.26	20.81
G.3	NTA	14.044	18.961	0	1.472	3.424	0.078	7.95	18.90	17.84	18.18	20.69
G.4	NTA	18.726	18.961	0	1.066	3.837	0.056	7.95	19.02	17.81	18.14	20.63
G.5	NTA	23.410	18.961	0	0.684	4.231	0.036	7.95	19.12	17.70	18.02	20.49
H.1	EDTA	4.671	37.748	0	0.749	3.692	0.020	3.19	14.36	15.92	19.48	21.62
K.1*	NTA	9.453	9.922	96.154	4.557	0.415	0.459	4.62	12.95	14.70	17.65	21.45
K.2*	NTA	18.906	9.922	96.154	4.299	0.651	0.433	4.62	13.16	14.87	17.62	21.20
K.3*	NTA	28.358	9.922	96.154	4.120	0.864	0.415	4.62	13.30	14.98	17.64	21.12
L.1*	NTA	4.682	12.285	0	2.909	1.999	0.237	7.87	18.28	17.79	18.25	21.16
L.2*	NTA	9.363	12.285	0	2.075	2.862	0.169	7.87	18.54	17.86	18.26	21.07
L.3*	NTA	14.044	12.285	0	1.436	3.506	0.117	7.87	18.68	17.82	18.19	20.93
M.1	citrate	20.662	13.864	0	4.660	0.589	0.336	4.04	12.57	14.68	17.69	20.73
M.2	citrate	25.827	13.864	0	4.933	0.337	0.356	4.01	12.84	15.01	18.09	21.19
M.3	citrate	41.323	13.864	0	4.830	0.493	0.348	4.03	13.02	15.16	18.20	21.28
N.1	citrate	15.496	13.301	0	3.704	1.557	0.278	7.19	17.58	17.24	17.77	20.75
N.2	citrate	25.827	13.301	0	3.425	1.892	0.257	7.15	17.69	17.31	17.81	20.76
N.3	citrate	36.158	13.301	0	3.138	2.138	0.236	7.20	17.84	17.40	17.88	20.79
										16.29 ± 1.45	17.99 ± 0.28	21.22 ± 0.45

[a] Association constants for the binding of Fe(III) to the mono-*O*-phosphorylserine (monodentate) or di-*O*-phosphorylserine (bidentate) ligands have been computed from mutually exclusive equilibrium relationships (see Appendix) using the total concentrations of ligand, phosphate, acetate, metal, protein-bound metal, and hydrogen ion and are merely different ways of rationalizing the observed Fe(III)–phosvitin equilibrium. Constants were not calculated when the experimental results were inconsistent with the equilibrium formulation (i.e., when the Fe/P ratio exceeded 0.50). Experiments with frog phosvitin have been marked with an asterisk. [b] Dialysis compartments. [c] Units = M^{-n} (see Appendix).

capable of fractionating iron and chelator. In nearly all of the experiments reported here, an excess of chelator was present. In our experience, excess EDTA, NTA, or citrate prevents polymerization of iron(III) and maintains diffusability through conventional dialysis membranes. We observed a phosvitin-bound Fe/P molar ratio of slightly greater than 0.5 in only one set of experiments (C, Table I), so that certain further computations were not possible. This nearly perfect relationship between iron and phosphate groups at saturation is perhaps the best evidence for a 1:2 iron–phosphorylserine chelate.

Association Constants. Table I gives stoichiometric equilibrium constants computed for the modes of iron binding illustrated in Figure 1. Although the experiments mentioned above (B.1, C.1, E.1, G.1, L.1, Table I) were flawed by the presence of polymeric iron in the diffusate compartment at the start of dialysis, equilibrium constants computed for these data were indistinguishable from other groups and were therefore not excluded from the calculation of the averaged constants. The averages of these constants (certain omissions are discussed below) yield association constants of $10^{18.0}$ and $10^{21.2}$ for the formation of the Fe(SerP·SerP) and $Fe(SerP)_2$ complexes, respectively. We emphasize that these constants represent mutually exclusive equilibria, and not simultaneous transitions between the various mono- and di-*O*-phosphorylserine complexes.

Statistical Analysis. The statistical distribution of data values (Figure 2) yields much information about which of the three sets of equilibrium formulations derived in the Appendix best represents the experimental data (Dixon & Massey, 1969). Association constants computed for the mononuclear monodentate chelate, Fe(SerP), were not normally distributed, but fell into two distinct groups of values depending on the experimental pH (Figure 2); the standard deviation of this bimodal distribution was obviously very large. Experimentally derived formation constants for mononuclear bidentate Fe(SerP·SerP) and binuclear monodentate $Fe(SerP)_2$ complexes were approximately normally distributed, as shown by the straight lines obtained with a normal probability graph (Figure 2). Inspection of Table I shows that association constants derived for these two interactions were not markedly affected by the experimental pH or by the nature of the competing chelator, so the results do not appear to be biased by experimental design. Constants obtained with EDTA as the competing ligand (experiments D and H, Table I) lay conspicuously outside the normal distribution for the Fe(SerP·SerP) equilibrium but not that for the $Fe(SerP)_2$ equilibrium (Figure 2) and were therefore not used for computing the mean of the Fe(SerP·SerP) association constants. With this exception, data obtained with different chelators could not be distinguished by statistical methods, nor did we observe a significant difference between data for frog and chicken phosvitins. The standard deviation of values representing the Fe(SerP·SerP) complex was smaller than that for the $Fe(SerP)_2$ complex, as shown in Table I and by comparison of the slopes of the normal probability lines in Figure 2. When the standard deviations of these two distributions are compared with respect to their magnitude as a percentage of the mean values ("coefficient of variation"), the data for the Fe(SerP·SerP) formation constant showed the narrower distribution. By statistical criteria, then, the experimental data are best fitted by equilibrium equations for the formation of a mononuclear bidentate Fe(SerP·SerP) complex.

Error Analysis. We recognize the problem of propagation of constant errors in evaluating experimental data involving a large number of independent variables. Systematic errors usually do not appear as statistical fluctuations in the values and therefore cannot be eliminated by merely repeating the measurements. We have subjected the phosvitin data to error analysis (Crumpler & Yoe, 1940) and have determined the probable error of computing the logarithm of the association constant for each experiment. The results of this analysis are of academic interest only and are not presented here. In general, the systematic error is of the same order of magnitude as the sampling error (i.e., standard deviation). A complete description of assumptions, error values for experimental variables and for experimentally derived constants, and methods for performing the necessary partial differentiation and numerical analysis are given by Hegenauer (1970).

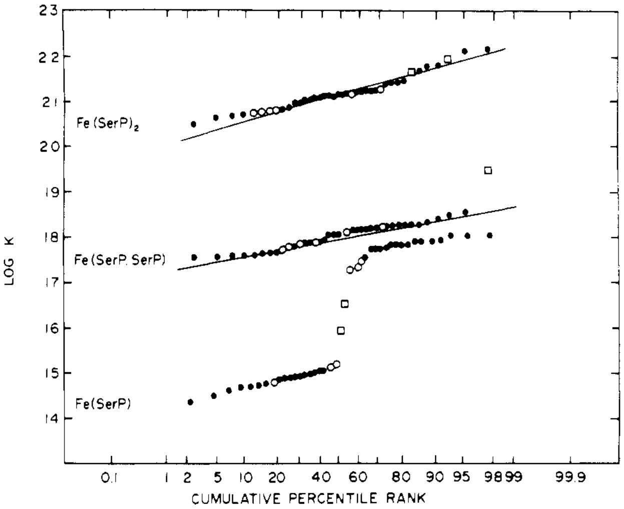

FIGURE 2: Normal probability plots for association constants (computed from data in Table I) for three iron(III)–O-phosphorylserine chelates. The linearity of such plots is a subjective measure of the "normality" of the data. The mean of each set of constants is defined approximately by the point at the 50th percentile. The steeper the slope of the probability plot, the greater the standard deviation. According to convention, the datum point representing the 100th percentile has been omitted. Experiments with different chelators are denoted by different symbols: NTA (●); citrate (○); and EDTA (□).

Without making further assumptions about the physical or steric likelihood of particular iron(III)–phosvitin interactions, we may conclude that (1) the data are not consistent with the equilibrium relationship involving a monodentate ligand and a mononuclear chelate, Fe(SerP), (2) the data are represented best by a 1:2 iron(III)–phosphorylserine complex, and (3) the particular equilibrium formulation used to arrive at a 1:2 chelate is of less importance than the final stoichiometry of the complex. The equilibrium formulation chosen to represent the interaction, however, determines the magnitude of the equilibrium constant, since $K_{Fe(SerP)_2}$ is proportional to the second power, and $K_{Fe(SerP \cdot SerP)}$ to only 2 times the first power, of the phosphorylserine concentration (see Appendix).

Strength of Iron(III) Binding to Casein

Primary data for a smaller number of equilibrium dialysis experiments with casein phosphoprotein are given in Table II. Averages of the stoichiometric equilibrium constants computed for the two most probable modes of iron binding are $10^{17.5}$ and $10^{20.5}$ for the formation of the Fe(SerP·SerP) and $Fe(SerP)_2$ complexes, respectively. Constants calculated for the iron–phosvitin interaction appear to be about $10^{0.5}$ larger than those for the corresponding iron–casein interaction. Equilibrium dialysis of casein solutions was carried out at 10 °C to inhibit microbial growth; equilibrium constants for the Fe(III)-NTA and Fe(III)-citrate competing equilibrium have, of course, been determined at different temperatures (see Metal-Ligand Equilibria and Calculations). We could not perform experiments at low pH because casein began to precipitate isoelectrically at pH <5.5. Experiments with casein were designed differently from those with phosvitin because they were carried out later in a different laboratory using vastly simpler apparatus and analytical methods. We recognize the difficulty in comparing the two sets of experiments, which were also performed at different temperatures, but we will show under Discussion that apparent numerical differences in the association constants for the two proteins may be reconciled on the basis of phosphorylserine sequences.

Discussion

Strength of Iron(III) Binding to Phosvitin. The affinity of phosvitin for Ca^{2+} and Mg^{2+} (Grizzuti & Perlmann, 1973), for Fe^{2+} (Taborsky, 1963), and for Fe^{3+} (Webb et al., 1973) has been widely recognized and discussed in the context of the protein structure of this interesting polyelectrolyte. The best-studied phosvitin, the phosphoglycoprotein from chicken egg yolk, contains 136 phosphoamino acids, including one phosphothreonine (Shainkin & Perlmann, 1971). About 56 mol % of the amino acids are phosphorylated. Frog and fish phosvitins are very similar to chicken phosvitin in physical-chemical properties and amino acid composition (Wallace, 1963; Wallace et al., 1966; Mano & Lipmann, 1966), so we may justifiably include them in our general discussion of metal-binding proteins.

At pH 6.5, phosvitin can bind at least one Ca^{2+} or Mg^{2+} to each dianionic phosphoamino acid (Grizzuti & Perlmann, 1973). When phosvitin is fully saturated with iron, however, a ratio of 1Fe/2P atoms has typically been observed (Webb et al., 1973; Taborsky, 1963). This nearly perfect stoichiometry may be attributed to the participation of adjacent phosphorylserine residues in sequestering this metal. Phosvitin has exceptional affinity for iron even at low pH (Greengard et al., 1964). This property has been explained partly as a "chelate effect" of clustered phosphorylserine sequences in the protein (Donella et al., 1976).

"Runs", or contiguous sequences, of phosphorylserine residues have now been identified in phosvitin (Williams & Sanger, 1959; Belitz, 1964; Shainkin & Perlmann, 1971; Clark, 1973), α_{s1}-casein (Mercier et al., 1971), β-casein (Manson & Annan, 1971; Ribadeau-Dumas et al., 1972), and brain phosphoproteins (Heald, 1961). Residue multiples up to $(SerP)_6$ have been identified in acid hydrolysates of phosvitin (Williams & Sanger, 1959), and a $(SerP)_8$ block has been positively identified in a pronase glycopeptide of phosvitin (Shainkin & Perlmann, 1971). Belitz (1964) has examined a tryptic peptide comprising a third of the phosvitin molecule and has identified phosphorylserine residues lying in groups of $(SerP)_3$ or $(SerP)_6$ interspersed among more basic amino acids. Of the 42 SerP residues contained in this peptide, 6 are in triplet groups rather than sextets; thus, only 2 out of the 42 SerP residues are not contained in even multiplets of $(SerP)_n$. If this peptide is typical of the remaining molecule, we may forecast that only 1/21, or 4.8%, of the SerP residues in phosvitin could not participate in the chelation of iron(III) by a di-*O*-phosphorylserine binding site. We might therefore expect an error of ~5% in the association constant determined for the Fe(SerP·SerP) bidentate complex since our calculations were based on the assumption that [SerP·SerP] equalled half the total phosphate concentration. This error would have to be included among the systematic (constant) errors en-

Table II: Primary Data and Stoichiometric Equilibrium Constants for the Binding of Iron(III) to Bovine α_{s1}-Casein with NTA or Citrate as the Competing Ligand[a]

		equilibrium concn (M × 10^4)								log K[c]		
					iron(III)[b]							
expt	competing ligand	ligand	α-casein phosphate	acetate	α-casein (bound)	chelator (free)	protein Fe/P	pH	pFe	mononuclear monodentate Fe(SerP)	mononuclear bidentate Fe(SerP·SerP)	binuclear monodentate $Fe(SerP)_2$
A.1	NTA	7.906	8.225	100.000	1.789	0.212	0.218	5.79	15.39	15.57	16.88	19.78
A.2	NTA	15.811	8.225	100.000	1.698	0.302	0.206	5.78	15.64	15.80	16.91	19.99
A.3	NTA	23.717	8.225	100.000	1.628	0.372	0.198	5.80	15.86	15.98	17.06	20.13
B.1	NTA	7.906	8.155	0	0.547	1.453	0.067	7.66	18.34	17.22	17.56	20.43
B.2	NTA	15.811	8.155	0	0.473	1.527	0.058	7.66	18.75	17.57	17.90	20.76
B.3	NTA	23.717	8.155	0	0.397	1.603	0.049	7.67	19.01	17.75	18.07	20.93
C.1	citrate	8.000	8.225	0	1.187	0.813	0.144	5.88	16.01	15.90	16.87	19.88
C.2	citrate	16.000	8.225	0	1.232	0.768	0.150	5.83	16.28	16.23	17.25	20.26
C.3	citrate	24.000	8.225	0	1.110	0.891	0.135	6.51	17.30	16.76	17.28	20.33
D.1	citrate	8.000	8.155	0	1.465	0.535	0.180	7.67	18.27	17.63	18.04	21.05
D.2	citrate	16.000	8.155	0	1.102	0.898	0.135	7.69	18.37	17.59	17.96	20.91
D.3	citrate	24.000	8.155	0	1.032	0.968	0.127	7.68	18.51	17.70	18.07	21.00
										16.81 ± 0.86	17.47 ± 0.52	20.45 ± 0.46

[a] Association constants for the formation of the Fe(III)-phosphoserine chelates have been computed from mutually exclusive equilibrium relationships as described in Table I. [b] Dialysis compartments. [c] Units = M^{-n} (see Appendix).

countered in deriving an association constant for Fe(SerP·SerP) but not necessarily for other interactions in which the SerP monodentate moiety is the putative ligand. Because of analytical uncertainties, it is doubtful that a 5% discrepancy in matching iron atoms with $(SerP)_2$ doublets would be observed as a significant deviation from 1Fe/2P stoichiometry.

Our data, together with the equations derived in the Appendix, have allowed us to compute a stoichiometric association constant for the interaction of iron(III) with phosvitin phosphorylserine residues. Knowledge of the magnitude of this stoichiometric constant permits us completely to describe or predict the concentrations of all species at equilibrium in a system of iron(III), phosvitin, and competing ligand like EDTA, NTA, or citrate at any pH. Equations for calculating free (ionic) iron concentration in solutions of iron(III) chelates have been based on accepted equilibria for known transitions. To treat the data in this way, we assume that iron and competing ligands are in equilibrium in a single phase. This condition was obviously not met initially in some experiments with the ~1:1 Fe–NTA chelate, which precipitated ferric oxyhydroxide in the diffusate compartment. Time of dialysis was probably long enough in these cases to permit complete depolymerization. Since the association constants determined with these data were not significantly different from others obtained with Fe(III)–NTA, equilibrium conditions were probably attained. Polynuclear chelates of iron(III)–NTA have been recognized (Gustafson & Martell, 1963), but since the kinetics are not known the equilibria have not been formulated. We cannot easily answer questions about how certain variables, constants, and experimental conditions might influence the magnitude of the association constants calculated here. When analytical errors can be estimated or when literature constants are presented with error limits, the computational error (usually small) can be determined (Hegenauer, 1970). Determining the consequences of changing certain parameters in order to identify the most sensitive or critical experimental variables is best handled by computer simulation techniques, which were beyond the scope of this investigation. Our studies have taken some liberties with experimental or analytical temperatures (e.g., performing casein dialysis at 10 °C to preserve protein stability) without a complete understanding of the effects of temperature on the iron–ligand equilibria. A study of the literature on the determination of association and dissociation constants used in this paper will disclose a similar lack of standardization of experimental conditions ($I = 0.1$ or 1.0; temperature = 20, 25, or 30 °C; electrolyte = NaCl or KCl). We can guess that the combined effects of these "errors" are perhaps less than the analytical error, because experiments with NTA and citrate generally gave constants of the same magnitude and because there was only a small difference in the association constants for the iron–phosvitin and iron–casein chelates when the data (obtained at different temperatures) were normalized to di-*O*-phosphorylserine concentration. The magnitude of the iron–phosphoprotein association constants depends, of course, on the nature and number of phosphorylserine ligands interacting with the iron (Table I). These association constants will also be influenced by uncertainties in the magnitude of the acid dissociation constants for the phosphorylserine residues, which we have estimated from literature data (see Appendix). It these acid dissociation constants are revised in the future, the new values may be used to update the iron(III)–phosphorylserine association constants simply by multiplying them by a factor (see Appendix) composed of appropriate "A's": $A_{\text{old}}/A_{\text{new}}$.

For lack of specific information, we have not considered the formation of ternary complexes of the iron(III)–phosphorylserines with other ligands (hydroxyl ion, buffer salts, or chelators). The protein–iron–ligand ternary complexes of transferrin are of course well-known (Aisen & Brown, 1977). We have obtained some preliminary evidence for the formation of spectroscopically distinct phosphoprotein–iron(III)–oxyanion complexes with phosphate, phosphite, and arsenate (J. Hegenauer and P. Saltman, unpublished experiments), but such anions were not present in the experiments reported here. We have not looked at the formation of hydroxo or aquo ternary complexes, although these are likely to be present, especially at alkaline pH, if four charged oxygens from two phosphorylserines are unable to satisfy the coordination requirements of octahedral iron(III). It should be noted, however, that ternary or mixed complexes would be less probable if bound iron(III) is tetrahedrally coordinated (Webb et al., 1973). We have already mentioned that association constants for the $Fe(SerP)_2$ and Fe(SerP·SerP) chelates could not be distinguished by experimental pH or competing ligand (Table I), so that our disregard of potential ternary complexes probably has a relatively small effect on the magnitude of the calculated equilibrium constant.

The "strength" of a metal chelate complex is best measured by the concentration of free (uncomplexed) metal ion in equilibrium with an excess of chelator. Because the principal association constant for metal and ligand is calculated with reference to the fully dissociated (deprotonated) ligand, the free metal concentration will depend greatly on pH, as well as on the formation of ternary (e.g., hydroxo) complexes by the chelate. It is therefore inappropriate to judge the strength or affinity of a metal–ligand complex solely by numerical comparison of the respective association constants. We may, however, display this competition between protons and metal by plotting free metal concentration as a function of pH. Figure 3 presents a graphic comparison of pFe computed at different pH values for 1:2 iron(III) chelates of the ligands considered in this study. Free iron concentration in a 1:2 mixture of iron(III) and phosvitin di-*O*-phosphorylserine ligands reaches a minimum plateau beyond pH 7 because of complete deprotonation of the dianionic phosphorylserine; a similar curve was generated for the interaction leading to the formation of the $Fe(SerP)_2$ complex using the appropriate association constant; the pFe vs. pH relationship is thus not affected so much by the magnitude of the association constant as by the equilibrium formulation. Minima are not observed with EDTA, NTA, or citrate because of incomplete dissociation at pH <10 and/or formation of hydroxo or dimeric complexes which compete for free iron. Between pH 4 and 7, chelator "strength" can be ranked: EDTA > phosvitin (i.e., di-*O*-phosphorylserine ligand) > citrate > NTA. Inspection of Figure 3 also suggests strategies for preparing iron(III)–phosvitin by competitive chelation. Seeking conditions which would maximize the difference in free iron concentration between phosvitin and competing ligand, we might choose to prepare iron(III)–phosvitin by dialyzing apophosvitin against 1:2 iron(III)–NTA at pH 5–6 in order to ensure a favorable transfer of iron. As Figure 3 suggests, dialysis of iron(III)–phosvitin against excess EDTA at pH 4–8 would favor the loss of iron from the phosphoprotein.

Strength of Iron(III) Binding to Casein. Divalent cations like Ca^{2+} are of obvious importance to the structure and stability of the α_{s1}-κ-casein micelles in milk. IR absorption studies of the interaction of Ca^{2+} and Mg^{2+} with α_{s1}-casein have pointed out the involvement of protein phosphate and

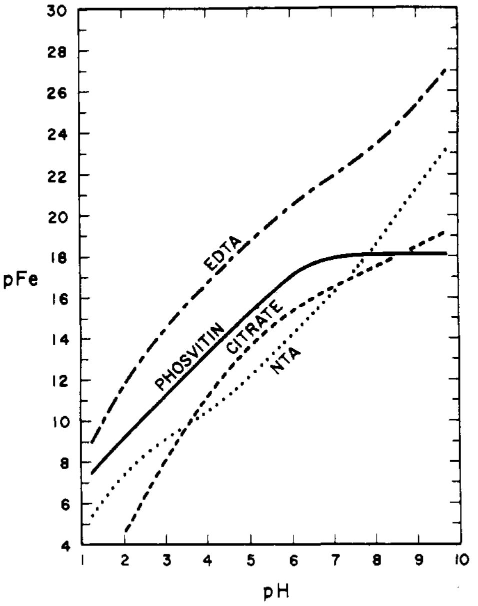

FIGURE 3: Free iron(III) concentration (expressed as –log [Fe^{3+}]) computed as a function of pH in solutions of iron and chelator (chelator/iron ratio = 2). The iron(III)–ligand equilibria (see Appendix) were solved for the following parameters: iron(III) concentration, 0.5 mM; ligand concentration (EDTA, NTA, citrate, or phosphoprotein bidentate di-*O*-phosphorylserine), 1 mM; and association constant for the iron(III)–di-*O*-phosphorylserine complex, $10^{18.0}$ M^{-1}.

carboxylate groups. Ono et al. (1976) identified nine high-affinity Ca^{2+} binding sites in equilibrium dialysis studies with α_{s1}-casein; carboxylate groups contribute additional low-affinity sites for binding Ca^{2+} at high concentration. The number of high-affinity sites (nine) corresponds closely to the total number of phosphorylserine residues (eight) shown to be present in α_{s1}-casein by sequence studies (Mercier et al., 1971). In both α_{s1}- and β-casein, four of the phosphorylserine residues are concentrated within a cluster in which three adjacent phosphorylserine residues are separated from a fourth by one neutral amino acid residue. The remaining phosphorylserines are not paired. Only 25% of the phosphorylserine residues in α_{s1}-casein are thus grouped in doublets, compared to 95% predicted for phosvitin. Fewer even-numbered multiplets would imply fewer di-*O*-phosphorylserine ligands as "high-affinity" sites for iron binding, so our assumption that [SerP·SerP] equals half the total phosphate concentration is certainly invalid for this protein. As a result, we would expect the stoichiometric association constant for the formation of Fe(SerP·SerP) to be lower for casein than for phosvitin. Our data, in fact, reflect this difference. Comparison of Tables I and II shows that the averaged association constant, $K_{Fe(SerP·SerP)}$, for α_{s1}-casein ($10^{17.5}$) is slightly smaller than that for phosvitin ($10^{18.0}$). If we instead assume that the concentration of "true" doublets [SerP·SerP] equals 0.25 × $[P]_t/2$, the same association constant for α_{s1}-casein is increased by approximately log 4, so that $10^{17.5} \times 10^{0.6} = 10^{18.1}$. If only these "high-affinity" di-*O*-phosphorylserine binding sites are considered, the iron-binding strength of α_{s1}-casein compares favorably to that of phosvitin. This simple treatment has, of course, ignored the contribution of the monodentate SerP singlets to the overall binding affinity, as well as other factors related to experimental conditions described above.

Effects of Protein Structure on Metal Binding. Binding of trivalent metal ions such as Fe^{3+} (Webb et al., 1973) and Al^{3+} (Hegenauer et al., 1977) reduces electrostatic repulsion between phosphate groups of phosvitin at neutral pH and induces a conformational change toward the β structure (Taborsky, 1963; Webb et al., 1973). The same shift from unordered polyelectrolyte to ordered β structure occurs upon binding of protons at low pH (Taborsky, 1968). Casein probably does not display similar polyelectrolyte behavior because of its much smaller phosphate concentration. Interesting "cooperativity" effects could be produced if the transition to a more ordered structure made metal-binding sites more or less accessible to additional metal ions. We have no evidence for such cooperativity or pH dependence of binding. Although the extent of iron binding by phosvitin (Fe/P molar ratio) generally increased with decreasing pH (Table I), this was not caused by a conformational change; rather, phosphorylserines bound iron more strongly than did competing NTA or citrate at lower pH (Figure 3). The association constant computed for the monodentate mononuclear chelate Fe(SerP) appeared to increase with decreasing pH (Table I), but this was undoubtedly an artifact of the equilibrium formulation, which obviously did not fit the data very well (Figure 2). It is significant that the numerical constants for the formation of the Fe(SerP·SerP) and $Fe(SerP)_2$ chelates were not influenced by experimental pH. Thus, data obtained at different pH values could be consolidated to calculate a single average association constant, and this constant could be used to predict iron–phosvitin concentration throughout a wide range of pH.

Table III: Comparative Binding Strength of Mg, Ca, and Fe to Three Conceivable Combinations of Phosvitin Phosphorylserines[a]

metal ion	log K^b M(SerP)	M(SerP·SerP)	$M(SerP)_2$	ref
Mg(II)	4.1	4.8	5.7	Grizzuti & Perlmann (1973)
Ca(II)	5.2	5.2	5.5	Grizzuti & Perlmann (1973)
Fe(III)	16.3	18.0	21.2	this paper

[a] Association constants for the Ca(II) and Mg(II) phosphorylserine chelates were computed by using the primary data of Grizzuti & Perlmann (1973) and the generalized ligand/generalized metal formulations presented in the Appendix. Values presented for the Mg(II) and Ca(II) chelates are the means of 9 and 10 sets of data, respectively. Constants presented for the Fe(III) chelates were taken from Table I. [b] Units = M^{-n} (see Appendix).

Relative Affinity of Mg(II), Ca(II), and Fe(III) for Phosphoproteins. Using Grizzuti & Perlmann's (1973) equilibrium dialysis data for binding of Mg(II) and Ca(II) to phosvitin with M_r 40 000 and 11.9% P, we may estimate the stoichiometric association constant for the interaction with mono- and bidentate ligands with the help of the generalized equilibrium equations derived in the Appendix for any metal, M^{m+}, and any ligand, $((SerP)_d)_n$. Table III presents these estimates alongside the constants for the iron(III)–phosvitin chelates derived in this paper. Considerations of stoichiometry as well as of experimental evidence (Grizzuti & Perlmann, 1973) dictate that we should consider Mg(SerP) and Ca(SerP) to be the most probable complexes formed in this interaction, just as the Fe(SerP·SerP) complex is sterically the most likely complex of trivalent iron. The very large increment in binding affinity between di- and trivalent ions suggests that iron(III) would displace Ca(II) from a phosphoprotein complex. We have, in fact, observed a very rapid donation of chelated iron(III) to the calcium casein micelle in fluid milk, which contains a high concentration of free calcium ion (Carmichael et al., 1975).

Biological Implications of Metal–Phosphoprotein Chelates. Our interest in iron–phosphoprotein complexes developed from an attempt to elucidate their role in iron nutrition. Complexation by casein has been invoked to explain the inhibition of iron absorption by whole milk (see Discussion in Carmichael et al., 1975). On the other hand, the small amount of native iron in milk occurs largely in the form of a casein complex, which we have shown to be a highly bioavailable form of dietary iron (Carmichael et al., 1975). Because of better understanding of iron–phosphoprotein equilibria and ligand-exchange reactions, it is now possible to use Fe(III) and Cu(II) chelates as trace element supplements for fluid milk without promoting lipid peroxidation, which causes instability and off-flavor in dairy products (Hegenauer et al., 1979a,b). It is thus important to quantitate the binding affinity of biologically important phosphoproteins in order to predict the ligand-exchange behavior of iron at different places in its gastrointestinal absorption and to understand the mechanisms of inhibition or facilitation of iron absorption.

In many species, phosvitin is a major storage form of iron for the development of the fertilized oocyte; mobilization of this metal during development is thought to occur via chelation by intra- or extracellular transferrin (Osaki et al., 1975). Appreciation of the relative affinities of iron(III) for phosvitin (see Discussion) and for transferrin (Aisen et al., 1978) suggests explanations for the data of Osaki et al. (1975), who have shown transfer of iron between iron–phosvitin and apotransferrin in vitro. Since phosvitin-bound iron may be in direct equilibrium with stronger chelators like transferrin, a larger portion of developmental storage iron evidently exists in a readily mobilizable form that does not require the reducing or chelating intermediates postulated for the mobilization of ferritin iron (Harrison et al., 1974). It remains to be determined whether ligand exchange of iron between phosvitin and transferrin can occur at a biologically significant rate by direct macromolecular contact without small molecular intermediates.

The task of elucidating the mode and strength of binding of divalent cations to phosvitin should be facilitated by experiments similar to those of Grizzuti & Perlmann (1973), which are interpreted according to the equilibrium formulations derived in the Appendix for generalized metals and phosphorylserine ligands. Such information will be essential to understand how metal-binding affects the metabolism of several classes of tissue phosphoproteins, including the mineralization behavior of bone and dentin phosphoproteins (Nawrot et al., 1976; Cohen-Solal & Glimcher, 1977).

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Appendix: Equilibrium Equations for Iron(III) Chelates of (Ethylenedinitrilo)tetraacetate, Nitrilotriacetate, Citrate, and Phosphoprotein *O*-Phosphorylserines

Text and equations contained in this Appendix were composed with the Burroughs B6700 CANDE text-editing system through the University of California at San Diego Computing Center.

List of Symbols

[ ]	molar concentration of the indicated species
[ ]t	total molar concentration of all indicated species
$K_{H\ldots}$	dissociation constant for hydrogen ion
$K_{Fe\ldots}$	association constant for iron
$K_{M\ldots}$	association constant for any metal (M)
X	ligand (EDTA, NTA)
X(OH)	ligand with protonated hydroxyl (citrate)
SerP	*O*-phosphorylserine ligand (monodentate)
SerP·SerP	di-*O*-phosphorylserine ligand (bidentate)
A	reciprocal of the proportion of unoccupied proton sites on the ligand
B	reciprocal of the proportion of total metal bound as the simple chelate (MX)
d	denticity of ligand; number of coordinating groups comprising the ligand
i, j	subscripts in operations involving summation
m^+	valence of metal ion
n	nuclearity of chelate; number of ligands (of any complexity) interacting with a single metal ion
Σ	summation function

(*1*) (*Ethylenedinitrilo*)*tetraacetate* (*EDTA*). (*A*) *H*(*I*)–*EDTA Equilibria* (*Schwarzenbach & Ackermann, 1947*)

$$H_4X \leftrightarrow H_3X^- + H^+$$

$$K_{H_4X} = \frac{[H_3X^-][H^+]}{[H_4X]} = 10^{-1.996} \qquad (1.1)$$

$$H_3X^- \leftrightarrow H_2X^{2-} + H^+$$

$$K_{H_3X} = \frac{[H_2X^{2-}][H^+]}{[H_3X^-]} = 10^{-2.672} \qquad (1.2)$$

$$H_2X^{2-} \leftrightarrow HX^{3-} + H^+$$

$$K_{H_2X} = \frac{[HX^{3-}][H^+]}{[H_2X^{2-}]} = 10^{-6.161} \qquad (1.3)$$

$$HX^{3-} \leftrightarrow X^{4-} + H^+$$

$$K_{HX} = \frac{[X^{4-}][H^+]}{[HX^{3-}]} = 10^{-10.262} \qquad (1.4)$$

(*B*) *Fe*(*III*)–*EDTA Equilibria* (*Schwarzenbach & Heller, 1951a*)

$$X^{4-} + Fe^{3+} \leftrightarrow FeX^-$$

$$K_{FeX} = \frac{[FeX^-]}{[X^{4-}][Fe^{3+}]} = 10^{25.1} \qquad (1.5)$$

$$FeX^- + OH^- \leftrightarrow FeX(OH)^{2-}$$

$$K_{FeX(OH)} = \frac{[FeX(OH)^{2-}]}{[FeX^-][OH^-]} = 10^{6.45} \qquad (1.6)$$

$$FeX(OH)^{2-} + OH^- \leftrightarrow FeX(OH)_2^{3-}$$

$$K_{FeX(OH)_2} = \frac{[FeX(OH)_2^{3-}]}{[FeX(OH)^{2-}][OH^-]} = 10^{4.53} \qquad (1.7)$$

No acetate complexes are formed (Schwarzenbach & Heller, 1951a). The presence of binuclear hydroxo species has not been considered here, but may be significant only in high concentrations of Fe(III)–EDTA (Gustafson & Martell, 1963).

(*C*) *Free Iron Concentration.* We wish to solve eq 1.1–1.7 simultaneously for [Fe^{3+}], so that we may express ionic iron concentration as a function of all congruous association and dissociation constants and of the total concentrations of iron, ligand, and hydrogen or hydroxyl ion. The stoichiometric equations are

$$[Fe]_t = [Fe^{3+}] + \sum_{i=0}^{2}[FeX(OH)_i]$$

or

$$[Fe]_t = [Fe^{3+}] + B[FeX] \qquad (1.8)$$

where

$$B = 1 + K_{FeX(OH)}[OH^-] + K_{FeX(OH)}K_{FeX(OH)_2}[OH^-]^2$$

and

$$[X]_t = \sum_{i=0}^{2}[FeX(OH)_i] + \sum_{j=0}^{4}[H_jX]$$

or

$$[X]_t = B[FeX^-] + A[X^{4-}] \qquad (1.9)$$

where

$$A = 1 + K_{HX}^{-1}[H^+] + K_{HX}^{-1}K_{H_2X}^{-1}[H^+]^2 + K_{HX}^{-1}K_{H_2X}^{-1}K_{H_3X}^{-1}[H^+]^3 + K_{HX}^{-1}K_{H_2X}^{-1}K_{H_3X}^{-1}K_{H_4X}^{-1}[H^+]^4$$

Subtracting eq 1.8 from 1.9 and solving for $[X^{4-}]$,

$$[X^{4-}] = ([X]_t - [Fe]_t + [Fe^{3+}])/A$$

Solving eq 1.5 for $[FeX^-]$ and substituting into eq 1.8,

$$[Fe]_t = [Fe^{3+}] + K_{FeX}[X^{4-}][Fe^{3+}]B \tag{1.10}$$

Substituting $[X^{4-}]$ into eq 1.10 and solving for $[Fe^{3+}]$,

$$[Fe]_t = [Fe^{3+}] + K_{FeX}[Fe^{3+}]\{([X]_t - [Fe]_t + [Fe^{3+}])/A\}B$$

or

$$[Fe^{3+}]^2\{(K_{FeX}B)/A\} + [Fe^{3+}]^1\{1 + (K_{FeX}B/A)([X]_t - [Fe]_t)\} + [Fe^{3+}]^0(-[Fe]_t) = 0 \tag{1.11}$$

The desired root is the maximum positive value provided by eq 1.11, solved with the aid of the quadratic equation.

(*2*) *Nitrilotriacetate* (*NTA*). (*A*) *H(I)–NTA Equilibria* (*Schwarzenbach et al., 1949*)

$$H_3X \leftrightarrow H_2X^- + H^+$$

$$K_{H_3X} = \frac{[H_2X^-][H^+]}{[H_3X]} = 10^{-1.89} \tag{2.1}$$

$$H_2X^- \leftrightarrow HX^{2-} + H^+$$

$$K_{H_2X} = \frac{[HX^{2-}][H^+]}{[H_2X^-]} = 10^{-2.49} \tag{2.2}$$

$$HX^{2-} \leftrightarrow X^{3-} + H^+$$

$$K_{HX} = \frac{[X^{3-}][H^+]}{[HX^{2-}]} = 10^{-9.73} \tag{2.3}$$

(*B*) *Fe(III)–NTA Equilibria* (*Schwarzenbach & Heller, 1951b*; *Gustafson & Martell, 1963*)

$$X^{3-} + Fe^{3+} \leftrightarrow FeX$$

$$K_{FeX} = \frac{[FeX]}{[X^{3-}][Fe^{3+}]} = 10^{15.87} \tag{2.4}$$

$$X^{3-} + FeX \leftrightarrow FeX_2^{3-}$$

$$K_{FeX_2} = \frac{[FeX_2^{3-}]}{[X^{3-}][FeX]} = 10^{8.45} \tag{2.5}$$

$$FeX(OH)^- + H^+ \leftrightarrow FeX$$

$$K_{FeX(OH)} = \frac{[FeX]}{[FeX(OH)^-][H^+]} = 10^{4.08} \tag{2.6}$$

$$FeX(OH)_2^{2-} + H^+ \leftrightarrow FeX(OH)^-$$

$$K_{FeX(OH)_2} = \frac{[FeX(OH)^-]}{[FeX(OH)_2^{2-}][H^+]} = 10^{7.77} \tag{2.7}$$

$$FeX + Ac^- \leftrightarrow FeXAc^-$$

$$K_{FeXAc} = \frac{[FeXAc^-]}{[FeX][Ac^-]} = 10^{2.3} \tag{2.8}$$

$$2FeX \leftrightarrow (FeX(OH)^{2-})_2 + 2H^+$$

$$K_{(FeX(OH))_2} = \frac{[(FeX(OH)^{2-})_2][H^+]^2}{[FeX]^2} = 10^{-6.0} \tag{2.9}$$

(*C*) *Free Iron Concentration.* We wish to solve eq 2.1–2.9 simultaneously for $[Fe^{3+}]$, so that we may express ionic iron concentration as a function of all congruous association and dissociation constants and of the total concentrations of iron, ligand, acetate, and hydrogen ion. The stoichiometric equations are

$$[Fe]_t = [Fe^{3+}] + \sum_{i=0}^{2}[FeX(OH)_i] + [FeX_2] + [FeXAc] + 2[(FeX(OH))_2] \tag{2.10}$$

or

$$[Fe]_t = [Fe^{3+}] + [FeX](B + K_{FeX_2}[X^{3-}] + K_{FeXAc}[Ac^-] + 2K_{(FeX(OH))_2}[FeX][H^+]^{-2}) \tag{2.11}$$

where

$$B = 1 + K_{FeX(OH)}{}^{-1}[H^+]^{-1} + K_{FeX(OH)}{}^{-1}K_{FeX(OH)_2}{}^{-1}[H^+]^{-2}$$

and

$$[X]_t = \sum_{i=0}^{2}[FeX(OH)_i] + 2[FeX_2] + [FeXAc] + 2[(FeX(OH))_2] + \sum_{j=0}^{3}[H_jX] \tag{2.12}$$

Subtracting eq 2.10 from 2.12,

$$[X]_t - [Fe]_t = [FeX_2] + \sum_{j=0}^{3}[H_jX] - [Fe^{3+}] \tag{2.13}$$

Solving eq 2.5 for $[FeX_2]$ and substituting into eq 2.13,

$$[X]_t - [Fe]_t + [Fe^{3+}] = K_{FeX_2}[FeX][X^{3-}] + A[X^{3-}] \tag{2.14}$$

where

$$A = 1 + K_{HX}{}^{-1}[H^+] + K_{HX}{}^{-1}K_{H_2X}{}^{-1}[H^+]^2 + K_{HX}{}^{-1}K_{H_2X}{}^{-1}K_{H_3X}{}^{-1}[H^+]^3$$

Solving eq 2.4 for [FeX] and substituting into eq 2.14, we obtain a polynomial in $[X^{3-}]$.

$$[X^{3-}]^2(K_{FeX_2}K_{FeX}[Fe^{3+}]) + [X^{3-}]^1A + [X^{3-}]^0\{-([X]_t - [Fe]_t + [Fe^{3+}])\} = 0 \tag{2.15}$$

If a value for $[Fe^{3+}]$ can be supplied, the desired root is the maximum positive value provided by eq 2.15, solved with the aid of the quadratic equation. $[Fe^{3+}]$ is initially indeterminate but may be given an estimated value (e.g., $[Fe]_t$) to begin a recursive procedure to improve the values of both $[X^{3-}]$ and $[Fe^{3+}]$.

Again solving eq 2.4 for [FeX] and substituting into eq 2.11, we obtain a polynomial in $[Fe^{3+}]$.

$$[Fe^{3+}]^2(2K_{FeX}{}^2K_{(FeX(OH))_2}[X^{3-}]^2[H^+]^{-2}) + [Fe^{3+}]^1\{1 + K_{FeX}[X^{3-}](B + K_{FeX_2}[X^{3-}] + K_{FeXAc}[Ac^-])\} + [Fe^{3+}]^0(-[Fe]_t) = 0 \tag{2.16}$$

The maximum positive value for $[Fe^{3+}]$ may be extracted from eq 2.16 with the quadratic formula if an approximate value of $[X^{3-}]$ is first obtained by eq 2.15. The new value of $[Fe^{3+}]$ can then be substituted into eq 2.16 to improve the approximation of $[X^{3-}]$, and so on. When applied recursively, successive approximations of $[Fe^{3+}]$ will converge on a stable value within 5 iterations.

(*3*) *Citrate.* (*A*) *H(I)–Citrate Equilibria* (*Warner & Weber, 1953*)

$$H_3X(OH) \leftrightarrow H_2X(OH)^- + H^+$$

$$K_{H_3X(OH)} = \frac{[H_2X(OH)^-][H^+]}{[H_3X(OH)]} = 10^{-2.94} \tag{3.1}$$

$$\mathrm{H_2X(OH)^- \leftrightarrow HX(OH)^{2-} + H^+}$$

$$K_{\mathrm{H_2X(OH)}} = \frac{[\mathrm{HX(OH)^{2-}}][\mathrm{H^+}]}{[\mathrm{H_2X(OH)^-}]} = 10^{-4.44} \qquad (3.2)$$

$$\mathrm{HX(OH)^{2-} \leftrightarrow X(OH)^{3-} + H^+}$$

$$K_{\mathrm{HX(OH)}} = \frac{[\mathrm{X(OH)^{3-}}][\mathrm{H^+}]}{[\mathrm{HX(OH)^{2-}}]} = 10^{-5.82} \qquad (3.3)$$

(*B*) *Fe(III)-Citrate Equilibria* (*Warner & Weber*, *1953*; *Spiro et al.*, *1967*)

$$\mathrm{H_3X(OH) + Fe^{3+} \leftrightarrow FeX(OH) + 3H^+}$$

$$K_{\mathrm{FeX(OH)}} = \frac{[\mathrm{FeX(OH)}][\mathrm{H^+}]^3}{[\mathrm{H_3X(OH)}][\mathrm{Fe^{3+}}]} = 10^{-1.82} \qquad (3.4)$$

$$\mathrm{X(OH)^{3-} + Fe^{3+} \leftrightarrow FeXO^- + H^+}$$

$$K_{\mathrm{FeXO}} = \frac{[\mathrm{FeXO^-}][\mathrm{H^+}]}{[\mathrm{X(OH)^{3-}}][\mathrm{Fe^{3+}}]} = 10^{9.46} \qquad (3.5)$$

Since an explicit constant for the formation of the ferric dicitrate complex is not available, we have followed the suggestion of Aisen et al. (1978) and have calculated this equilibrium constant from the original spectrophotometric titration data of Spiro et al. (1967). The near-UV absorbances of ferric monocitrate and ferric dicitrate are about equal upon the addition of two or more base equivalents per iron when the total concentrations of iron and citrate are 1 and 31 mM, respectively. From their data, the hydrogen ion concentration at which no further change in the relative concentrations of the monocitrate and dicitrate complexes is observed corresponds to pH 9.5. With these parameters, we obtain a value of $10^{-7.98}$ for this association constant. Aisen et al. (1978) considered that the concentrations of ferric monocitrate and ferric dicitrate were equal at pH 7.7 and thus obtained a value of $10^{-6.17}$ for this constant. This is not consistent with the original data (Figures 1 and 2) of Spiro et al. (1967) which clearly show that pH 7.7 corresponds to the addition of only about 1.5OH/Fe and that the concentration of the monocitrate complex is not minimized until 2OH/Fe (=pH 9.5) have been added. Since omission of the dicitrate equilibrium has relatively little effect on the calculated pFe, the difference between our estimate for this constant and that of Aisen et al. (1978) is likely to be insignificant.

$$\mathrm{FeXO^- + X(OH)^{3-} \leftrightarrow Fe(XO)_2{}^{5-} + H^+}$$

$$K_{\mathrm{Fe(XO)_2}} = \frac{[\mathrm{Fe(XO)_2{}^{5-}}][\mathrm{H^+}]}{[\mathrm{FeXO^-}][\mathrm{X(OH)^{3-}}]} = 10^{-7.98} \qquad (3.6)$$

(*C*) *Free Iron Concentration.* We wish to solve eq 3.1–3.6 simultaneously for $[\mathrm{Fe^{3+}}]$, so that we may express ionic iron concentration as a function of all congruous association and dissociation constants and of the total concentrations of iron, ligand, and hydrogen ion. The stoichiometric equations are

$$[\mathrm{Fe}]_t = [\mathrm{Fe^{3+}}] + [\mathrm{FeXO^-}] + [\mathrm{FeX(OH)}] + [\mathrm{Fe(XO)_2{}^{5-}}] \qquad (3.7)$$

$$[\mathrm{X}]_t = [\mathrm{FeXO^-}] + [\mathrm{FeX(OH)}] + 2[\mathrm{Fe(XO)_2{}^{5-}}] + \sum_{i=0}^{3}[\mathrm{H}_i\mathrm{X(OH)}] \qquad (3.8)$$

Subtracting eq 3.7 from 3.8,

$$[\mathrm{X}]_t - [\mathrm{Fe}]_t = [\mathrm{Fe(XO)_2{}^{5-}}] + A[\mathrm{X(OH)^{3-}}] - [\mathrm{Fe^{3+}}] \qquad (3.9)$$

where

$$A = 1 + K_{\mathrm{HX(OH)}}{}^{-1}[\mathrm{H^+}] + K_{\mathrm{HX(OH)}}{}^{-1}K_{\mathrm{H_2X(OH)}}{}^{-1}[\mathrm{H^+}]^2 + K_{\mathrm{HX(OH)}}{}^{-1}K_{\mathrm{H_2X(OH)}}{}^{-1}K_{\mathrm{H_3X(OH)}}{}^{-1}[\mathrm{H^+}]^3$$

Solving eq 3.5 and 3.6 simultaneously for $[\mathrm{Fe(XO)_2}]$ and substituting into eq 3.9, we obtain a polynomial in $[\mathrm{X(OH)^{3-}}]$.

$$[\mathrm{X(OH)^{3-}}]^2(K_{\mathrm{Fe(XO)_2}}K_{\mathrm{FeXO}}[\mathrm{Fe^{3+}}][\mathrm{H^+}]^{-2}) + [\mathrm{X(OH)^{3-}}]^1A + [\mathrm{X(OH)^{3-}}]^0\{-([\mathrm{X}]_t - [\mathrm{Fe}]_t + [\mathrm{Fe^{3+}}])\} = 0 \qquad (3.10)$$

Since eq 3.10 has the standard quadratic form, the maximum positive root may be obtained from the quadratic formula if a value for $[\mathrm{Fe^{3+}}]$ can be supplied as discussed in the previous section.

Solving eq 3.4–3.6 in terms of $[\mathrm{X(OH)^{3-}}]$ and substituting into eq 3.7,

$$[\mathrm{Fe}]_t = [\mathrm{Fe^{3+}}] + K_{\mathrm{FeXO}}[\mathrm{X(OH)^{3-}}][\mathrm{Fe^{3+}}][\mathrm{H^+}]^{-1} + K_{\mathrm{FeX(OH)}}[\mathrm{Fe^{3+}}][\mathrm{X(OH)^{3-}}]K_{\mathrm{HX(OH)}}{}^{-1}K_{\mathrm{H_2X(OH)}}{}^{-1}K_{\mathrm{H_3X(OH)}}{}^{-1} + K_{\mathrm{Fe(XO)_2}}K_{\mathrm{FeXO}}[\mathrm{X(OH)^{3-}}]^2[\mathrm{Fe^{3+}}][\mathrm{H^+}]^{-2}$$

or

$$[\mathrm{Fe^{3+}}] = [\mathrm{Fe}]_t/(1 + K_{\mathrm{FeXO}}[\mathrm{X(OH)^{3-}}][\mathrm{H^+}]^{-1} + K_{\mathrm{FeX(OH)}}[\mathrm{X(OH)^{3-}}]K_{\mathrm{HX(OH)}}{}^{-1}K_{\mathrm{H_2X(OH)}}{}^{-1}K_{\mathrm{H_3X(OH)}}{}^{-1} + K_{\mathrm{Fe(XO)_2}}K_{\mathrm{FeXO}}[\mathrm{X(OH)^{3-}}]^2[\mathrm{H^+}]^{-2}) \qquad (3.11)$$

The approximate value of $[\mathrm{Fe^{3+}}]$ obtained with eq 3.11 can then be substituted into eq 3.10 to improve the approximation of $[\mathrm{X(OH)^{3-}}]$, and so on. When applied recursively, successive approximations of $[\mathrm{Fe^{3+}}]$ will converge on a stable value within 5 iterations.

(*4*) *Phosphoprotein O-Phosphorylserine.* (*A*) *Estimation of Dissociation Constants.* (*a*) *Data of Mecham & Olcott* (*1949*). Taborsky (1968) has estimated values of $\mathrm{p}K_1'$ and $\mathrm{p}K_2'$ of the phosvitin phosphate groups to be 2.5 and 7.2, respectively, using the potentiometric titration curve of acid-washed phosvitin (in water solution) reported by Mecham & Olcott (1949). Mano & Lipmann (1966) reported values of approximately 3 and 7 for $\mathrm{p}K_1'$ and $\mathrm{p}K_2'$ of the phosphorylserine residues in a salmon egg phosphoprotein (water titration). In making these estimates, the relatively minor contribution of other acidic groups to the acid dissociation equilibria in the two pH regions was ignored, and no correction was made for self-neutralization by amino groups at the start of the titration.

Our estimates of acid dissociations should, however, be consistent with other equilibrium constants derived under "standard" conditions, namely, $I = 0.1$ at 20 °C. The values of $\mathrm{p}K_1'$ and $\mathrm{p}K_2'$ should decrease with increasing ionic strength because of a weakening of the electrostatic attraction between negatively charged protein and protons. A shift in $\mathrm{p}K_1'$ and $\mathrm{p}K_2'$ is, in fact, evident from the titration curve of acid-washed phosvitin in 1.0 M NaCl (Mecham & Olcott, 1949). Taborsky (1968) has presented evidence based on optical rotatory dispersion measurements of phosvitin for such a shift in the $\mathrm{p}K_1'$ transition with increasing ionic strength.

Using the titration curve of Mecham & Olcott (1949) for acid-washed phosvitin in 1.0 M NaCl, we estimated the midpoint of the second titration region, corresponding to $\mathrm{p}K_2'$, to be ~6.3; a similar graphic estimate of the $\mathrm{p}K_1'$ was not attempted because the shape of the curve in this region did not indicate a midpoint and was undoubtedly complicated by the titration of carboxyl groups and by partial neutralization of phosphates by amino groups at the isoionic point.

(*b*) *Data of Österberg* (*1961*). A somewhat different estimate of pK_1' and pK_2' was made by using the data of Österberg (1961) obtained from potentiometric titrations of a phosphopeptide (derived fom bovine α-casein) in 0.15 M KCl at 25 °C. The amino acid sequence of this particular phosphopeptide was not determined, but its composition has been given by Österberg (1960) as $\mathrm{HOOC\text{-}Lys(Asp_3,Thr,Ser_6,Glu_{10},Pro,Gly,Ala_2,Val_2,Met_2,Ilu_4,Lys)Asp\text{-}NH_2,[(NH_3)_3,(PO_4H_3)_7]}$. Its titration behavior was characterized by a "crowded" distribution of groups with the same charge and suggested the existence of very acidic sequences in the peptide attributable to phosphorylserine residues.

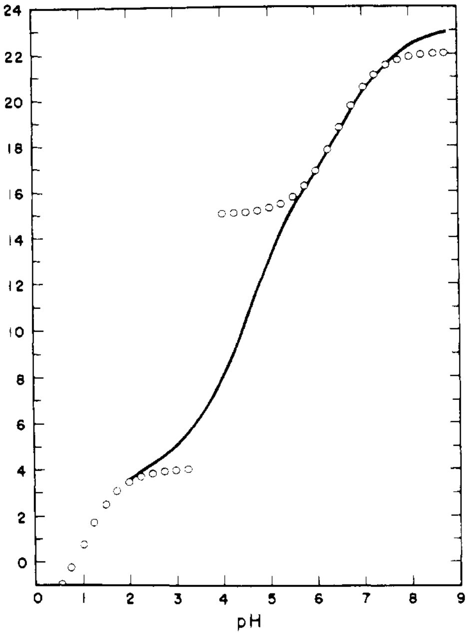

FIGURE A1: Österberg's (1961) titration curve of a casein phosphopeptide (solid line) showing presumed titration regions (○) attributable to the first and second acidic dissociations of the *O*-phosphorylserine residues. The dissociations of the isolated phosphates in the phosphopeptide were calculated by using *apparent* constants derived from Österberg's data.

Österberg (1961) has analyzed his titration data in the form of intrinsic ionization constants and electrostatic parameters, using the relationship (cf. Edsall & Wyman, 1958), $\mathrm{pH} - \log (A_i/\{1 - A_i\}) = pK_i - 0.868W'\bar{Z}$. The intrinsic ionization constants for pK_1 and pK_2 were reported as 0.9 and 5.3, respectively, and the electrostatic parameter, W', was given as 0.071 for both dissociations.

We may convert Österberg's (1961) intrinsic constants into *apparent* dissociation constants if $\bar{Z}$ (the average net charge on the phosphopeptide) is known, since, at the midpoint of the appropriate dissociation, $\mathrm{pH} = pK'$ and $\log (A_i/\{1 - A_i\}) = 0$, so that $pK' = pK_i - 0.868W'\bar{Z}$. From Österberg's titration curve, $\bar{Z} = -4$ when $7\mathrm{PO_3H^-} + 3\mathrm{NH_3^+}$ have been titrated; therefore, the midpoint of the first phosphate transition is represented by $\bar{Z} = -0.5$ when $3.5\mathrm{PO_3H^-} + 3\mathrm{NH_3^+}$ groups have been titrated. Similarly, $\bar{Z} = -22$ when $7\mathrm{PO_3^{2-}} + 11\mathrm{COO^-} + 3\mathrm{NH_3^+}$ groups have been titrated; the midpoint of the second phosphate transition is represented by $\bar{Z} = -18.5$ when $3.5\mathrm{PO_3H^-} + 3.5\mathrm{PO_3^{2-}} + 11\mathrm{COO^-} + 3\mathrm{NH_3^+}$ groups have been titrated. Calculated values of pK_1' and pK_2' are 0.93 and 6.44, respectively.

These calculated apparent dissociation constants were fitted to Österberg's (1961) titration curve by first calculating $\bar{h}$ (the number of moles of hydrogen ion dissociated) as a function of pH for $n = 7$ isolated phosphate groups by the relationship $\mathrm{pH} = pK_{1,2}' + \log (\bar{h}/\{n - \bar{h}\})$ and then calculating the average net charge ($\bar{Z}$) on the phosphopeptide by $\bar{Z} = \bar{h} + 3\mathrm{NH_3^+}$ for the first transition and $\bar{Z} = \bar{h} + 11\mathrm{COO^-} + 3\mathrm{NH_3^+}$ for the second transition. The results of this fitting are shown in Figure A1 and indicate that the calculated apparent dissociation constants predict rather well the shape of the titration curve for the appropriate pH regions. In addition, the calculated pK_2' (6.44) agrees reasonably with that determined graphically (6.3) from the titration curve of Mecham & Olcott (1949) for phosvitin in 1.0 M NaCl.

Table AI

n	chelate	d	ligand	Fe(III) complex
1	mononuclear	1	monodentate	Fe(SerP)
1	mononuclear	2	bidentate	Fe(SerP·SerP)
2	binuclear	1	monodentate	$\mathrm{Fe(SerP)_2}$
2		2		not considered

(*B*) *H(I)–Phosphorylserine Equilibria.* (*a*) *Monodentate Ligand* (*SerP*)

$$\mathrm{SerPH_2} \leftrightarrow \mathrm{SerPH^-} + \mathrm{H^+}$$

$$K_{\mathrm{SerPH}} = \frac{[\mathrm{SerPH^-}][\mathrm{H^+}]}{[\mathrm{SerPH_2}]} = 10^{-0.93} \tag{4.1}$$

$$\mathrm{SerPH^-} \leftrightarrow \mathrm{SerP^{2-}} + \mathrm{H^+}$$

$$K_{\mathrm{SerP}} = \frac{[\mathrm{SerP^{2-}}][\mathrm{H^+}]}{[\mathrm{SerPH^-}]} = 10^{-6.44} \tag{4.2}$$

(*b*) *Bidentate Ligand* (*SerP·SerP*)

$$\mathrm{SerPH_2{\cdot}SerPH_2} \leftrightarrow \mathrm{SerPH^-{\cdot}SerPH_2} + \mathrm{H^+}$$

$$K_{\mathrm{SerPH\cdot SerPH_2}} = \frac{[\mathrm{SerPH^-{\cdot}SerPH_2}][\mathrm{H^+}]}{[\mathrm{SerPH_2{\cdot}SerPH_2}]} = 10^{-0.93} \tag{4.3}$$

$$\mathrm{SerPH^-{\cdot}SerPH_2} \leftrightarrow \mathrm{SerPH^-{\cdot}SerPH^-} + \mathrm{H^+}$$

$$K_{\mathrm{SerPH\cdot SerPH}} = \frac{[\mathrm{SerPH^-{\cdot}SerPH^-}][\mathrm{H^+}]}{[\mathrm{SerPH^-{\cdot}SerPH_2}]} = 10^{-0.93} \tag{4.4}$$

$$\mathrm{SerPH^-{\cdot}SerPH^-} \leftrightarrow \mathrm{SerP^{2-}{\cdot}SerPH^-} + \mathrm{H^+}$$

$$K_{\mathrm{SerP\cdot SerPH}} = \frac{[\mathrm{SerP^{2-}{\cdot}SerPH^-}][\mathrm{H^+}]}{[\mathrm{SerPH^-{\cdot}SerPH^-}]} = 10^{-6.44} \tag{4.5}$$

$$\mathrm{SerP^{2-}{\cdot}SerPH^-} \leftrightarrow \mathrm{SerP^{2-}{\cdot}SerP^{2-}} + \mathrm{H^+}$$

$$K_{\mathrm{SerP\cdot SerP}} = \frac{[\mathrm{SerP^{2-}{\cdot}SerP^{2-}}][\mathrm{H^+}]}{[\mathrm{SerP^{2-}{\cdot}SerPH^-}]} = 10^{-6.44} \tag{4.6}$$

(*C*) *Metal–Phosphorylserine Equilibria.* (*a*) *Monodentate Ligand* (*SerP*)

$$n\mathrm{SerP^{2-}} + \mathrm{M}^{m+} \leftrightarrow \mathrm{M(SerP)}_n^{(2n-m)-}$$

$$K_{\mathrm{M(SerP)}_n} = \frac{[\mathrm{M(SerP)}_n^{(2n-m)-}]}{[\mathrm{SerP^{2-}}]^n[\mathrm{M}^{m+}]} \tag{4.7}$$

(*b*) *Bidentate Ligand* (*SerP·SerP*)

$$n\mathrm{SerP^{2-}{\cdot}SerP^{2-}} + \mathrm{M}^{m+} \leftrightarrow \mathrm{M(SerP{\cdot}SerP)}_n^{(4n-m)-}$$

$$K_{\mathrm{M(SerP\cdot SerP)}_n} = \frac{[\mathrm{M(SerP{\cdot}SerP)}_n^{(4n-m)-}]}{[\mathrm{SerP^{2-}{\cdot}SerP^{2-}}]^n[\mathrm{M}^{m+}]} \tag{4.8}$$

(*c*) *Generalized Ligand* (($SerP)_d$)

$$n(\mathrm{SerP^{2-}})_d + \mathrm{M}^{m+} \leftrightarrow \mathrm{M((SerP)}_d)_n^{(2dn-m)-}$$

$$K_{\mathrm{M((SerP)}_d)_n} = \frac{[\mathrm{M((SerP)}_d)_n^{(2dn-m)-}]}{[(\mathrm{SerP^{2-}})_d]^n[\mathrm{M}^{m+}]} \tag{4.9}$$

where $m = +3$ for Fe(III) and the metal chelates are defined as in Table AI.

(*D*) *Association Constant for Metal–Phosphorylserine Complexes.* We may solve the generalized relationship (eq 4.9) for the association constant in terms of the total concentrations of metal–phosphorylserine complex, phosphorylserine, hydrogen ion, and free metal ion. Phosphorylserine concentration is estimated by phosphate assay, so that $[(\mathrm{SerP})]_\mathrm{t} = [\mathrm{P}]_\mathrm{t}$ and $[(\mathrm{SerP})_2]_\mathrm{t} = [\mathrm{P}]_\mathrm{t}/2$. The concentration of metal–phosphorylserine complex is set equal to the protein-bound metal concentration measured in the retentate compartment. At equilibrium, the free metal concentration in the metal–phosphoprotein system is by definition equal to that in the competing metal–chelator system. Thus, for the special case of iron, the free iron concentration $[\mathrm{Fe}^{3+}]$ is first obtained by solving the equilibrium equations for the iron(III) chelates of EDTA (eq 1.11), NTA (eq 2.16), or citrate (eq 3.11). The stoichiometric equations are

$$[\mathrm{M}]_\mathrm{t} = [\mathrm{M}^{m+}] + [\mathrm{M}((\mathrm{SerP})_d)_n{}^{(2dn-m)-}] \qquad (4.10)$$

and

$$[(\mathrm{SerP})_d]_\mathrm{t} = \sum_{i=1}^{d}\sum_{j=0}^{2}[(\mathrm{SerPH}_j)_i] + n[\mathrm{M}((\mathrm{SerP})_d)_n{}^{(2dn-m)-}] = A_d[(\mathrm{SerP}^{2-})_d] + n[\mathrm{M}((\mathrm{SerP})_d)_n{}^{(2dn-m)-}] \qquad (4.11)$$

where, for $d = 1$ and 2,

$$A_1 = 1 + [\mathrm{H}^+]K_{\mathrm{SerP}}{}^{-1} + [\mathrm{H}^+]^2K_{\mathrm{SerPH}}{}^{-1}K_{\mathrm{SerP}}{}^{-1}$$

and

$$A_2 = 1 + [\mathrm{H}^+]K_{\mathrm{SerP\cdot SerP}}{}^{-1} + [\mathrm{H}^+]^2K_{\mathrm{SerP\cdot SerP}}{}^{-1}K_{\mathrm{SerP\cdot SerPH}}{}^{-1} + [\mathrm{H}^+]^3K_{\mathrm{SerP\cdot SerP}}{}^{-1}K_{\mathrm{SerP\cdot SerPH}}{}^{-1}K_{\mathrm{SerPH\cdot SerPH}}{}^{-1} + [\mathrm{H}^+]^4K_{\mathrm{SerP\cdot SerP}}{}^{-1}K_{\mathrm{SerP\cdot SerPH}}{}^{-1}K_{\mathrm{SerPH\cdot SerPH}}{}^{-1}K_{\mathrm{SerPH\cdot SerPH_2}}{}^{-1}$$

Solving eq 4.11 for the uncomplexed phosphorylserine concentration, $[(\mathrm{SerP}^{2-})_d]$, and substituting into eq 4.9,

$$K_{\mathrm{M((SerP)}_d)_n} = [\mathrm{M}((\mathrm{SerP})_d)_n{}^{(2dn-m)-}]A_d{}^n \times \{[(\mathrm{SerP})_d]_\mathrm{t} - n[\mathrm{M}((\mathrm{SerP})_d)_n{}^{(2dn-m)-}]\}^{-n}[\mathrm{M}^{m+}]^{-1} \qquad (4.12)$$

(*E*) *Free Metal Concentration.* We may also wish to determine $[\mathrm{M}^{m+}]$ in an isolated system of metal and phosphoprotein if the association constant is known. An exact solution in terms only of total concentrations of metal, phosphorylserine, and hydrogen ion is feasible but algebraically cumbersome if we generalize the equations for $n = 1$ or 2. A computationally simpler approach is to solve eq 4.12 for $[\mathrm{M}^{m+}]$, using a value for $[\mathrm{M}((\mathrm{SerP})_d)_n]$ first provided by eq 4.10. Calculations can be started by setting $[\mathrm{M}]_\mathrm{t}$ equal to $[\mathrm{M}((\mathrm{SerP})_d)_n]$ and then refining the solution of $[\mathrm{M}^{m+}]$ by 5–10 iterations, as described for eq 2.16 and 3.11.

(*5*) *Programming Considerations.* The equations derived above were implemented for machine calculation in PL/1 programming language. In several places considerable precision can be lost during subtraction of one small number from another while calculating the discriminant of the quadratic equation. Capability for computing with increased precision (e.g., "float (16) decimal" in PL/1 or "double precision" in Fortran) is desirable to prevent loss of significance. When using smaller (desktop) computers, it may be necessary to avoid exact solutions for $[\mathrm{Fe}^{3+}]$ via the quadratic equation and instead calculate by successive approximations and convergence.

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