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Phosphoprotein Particles: Calcium and Inorganic Phosphate Binding Structures†

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ABSTRACT: Phosphoprotein particles were isolated in their native state from the physiological fluid of the estuarine clam *Rangia cuneata*, and the characteristics of the mineral ion–protein complex which constitutes the native particle were investigated by using mineral ion binding and mineral ion exchange techniques. The particles are aspartic acid rich, highly phosphorylated proteins containing calcium, magnesium, and inorganic phosphate ions and covalently cross-linked via histidinoalanine residues. Twenty-nine percent of the amino acid residues are phosphorylated. In their native state, the particles contain a protected pool of calcium and inorganic phosphate ions and an exchangeable pool of calcium and magnesium ions. The Ca/PO_4 ratio in the protected pool is about 2.5. The number of binding sites for the protected mineral is unknown, but on the average, the native particles contain about 0.2 inorganic phosphate ion per organic phosphate residue. There is 1.0 exchangeable metal ion binding site per organic phosphate residue, and there is probably a phosphoserine residue at each site. These sites bind calcium with an apparent binding constant (K_{Ca}) of 4×10^3 M^{-1} at 50% saturation under physiological conditions, and K_{Ca}/K_{Mg} is about 1.6. In vivo, about 85% of the exchangeable sites are occupied. The total number of calcium ion binding sites (N) in the phosphoprotein particles is related to the number of organic phosphate residues (P_o) and the number of bound inorganic phosphate ions (P_i) by the equation $N = P_o + 2.5P_i$. The phosphoprotein particles probably serve as both the transporter and reserve source of calcium ions for shell development.

Aspartic acid rich, highly phosphorylated proteins are associated with two very different mineralized tissue structures. Phosphophoryn is a component of vertebrate tooth dentin (Veis & Schlueter, 1964; Dimuzio & Veis, 1978a) where it is concentrated at the mineralization front (Weinstock & Leblond, 1973). Phosphoprotein particles are components of the extrapallial fluid and innermost shell lamella of some species of bivalve mollusks, i.e., clams (Marsh & Sass, 1983). Both phosphophoryn and phosphoprotein particles are postulated intermediates in the mineralization process (Veis, 1978; Marsh & Sass, 1983), largely on the basis of their localization at mineralizing fronts and their ability to sequester large quantities of calcium ions (Lee et al., 1977; Zanetti et al., 1981; Marsh & Sass, 1983).

The invertebrate shell and vertebrate dentin are mineralogically very different; the former is a calcium carbonate (aragonite or calcite) tissue, and the latter is a calcium phosphate (hydroxyapatite) tissue. The associated phosphoproteins have some interesting similarities and differences. Both phosphoprotein particles and phosphophoryn are rich in aspartic acid and phosphoserine residues (Lee et al., 1977; Lyaruu et al., 1982; Termine et al., 1980; Marsh & Sass, 1983). On the other hand, histidine accounts for about 34% of the particle residues but for only about 1% of the phosphophoryn residues. The molecular weight of rat phosphophoryn is about 38 000 (Jontell, 1982); in this study, the molecular weight of the phosphoprotein particles is estimated to be about 54 million. The particles are composed of phosphoprotein monomers of an undetermined size covalently

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cross-linked through histidinoalanine residues (Sass & Marsh, 1983).

Veis has postulated that phosphophoryn, secreted by the odontoblasts, combines with calcium ions at the mineralization front and nucleates hydroxyapatite mineral (Veis, 1978; Dimuzio & Veis, 1978b). However, Zanetti et al. (1981) concluded that phosphophoryn was incapable of selectively extracting calcium ions from tissue fluid in order to form mineral, on the basis of the strong competition of magnesium and sodium ions for the calcium binding sites in vitro. To what extent phosphophoryn is in fact charged with calcium ions or interacts with dentin calcium phosphate crystallites in situ is unknown, because the mineral and protein are necessarily dissociated during protein isolation. However, techniques are available for the isolation of the phosphoprotein particles in their native state from the extrapallial fluid of the estuarine clam *Rangia cuneata* (Marsh & Sass, 1983). The experiments summarized in this paper describe (1) the mineral ion–protein association of the native phosphoprotein particles as it occurs in vivo in the physiological fluid of *Rangia cuneata* and (2) the in vitro mineral binding characteristics of particles previously demineralized with either ethylenediaminetetraacetic acid (EDTA)[1] or dilute acid. Mineral binding to native particles and in vitro to previously demineralized particles is very different, and the mineral ion–protein complex which constitutes the native particle could not have been predicted from in vitro studies on demineralized particles alone.

The long-range goal of this project and related projects from other laboratories is to precisely define the function of the aspartic acid rich, highly phosphorylated proteins in the mineralization process. The occurrence of these interesting proteins in the very different shell and dentin systems makes it possible to investigate aspects of the problem in one system which may not be easily or directly approached in the other.

Materials and Methods

Preparation of Native, Demineralized, and Partially Dephosphorylated Phosphoprotein Particles. Native phosphoprotein particles were isolated from the extrapallial and total fluids of *Rangia cuneata* which had been adapted to either fresh water or 50% seawater as described previously (Marsh & Sass, 1983). Briefly, the fluid was centrifuged at 600*g* to remove cells and filtered successively through 5.0- and 0.45-μm filters to remove smaller particulate material. Then the particles were sedimented from the fluid by centrifugation at 100000*g* for 30 min. The particles were washed by dissolving in water and sedimenting as before.

The native particles were demineralized by mixing an aqueous solution of the particles with a solution of 50 mM EDTA buffered with 50 mM Tris, pH 8.3, in 50 mM NaCl. A 5-fold excess of EDTA over metal ions was used. The demineralized particles were isolated by sedimentation at 100000*g* for 30 min and then washed with 50 mM NaCl and water.

The demineralized particles were partially dephosphorylated by treatment with potato acid phosphatase as described by Rhodes et al. (1959); 0.8 mL of phosphatase (60 units/mL) was added to 35 mL of demineralized particles (5.0 μmol of organic phosphate/mL) and dialyzed overnight against 600 mL of 0.1 M sodium acetate, pH 5.3 at 37 °C. Only 25% of the organic phosphate was liberated, and addition of more enzyme did not liberate a significant amount of additional phosphate. The partially dephosphorylated particles were isolated by sedimentation at 100000*g* for 30 min. The pellet was dissolved in water and the pH adjusted to 8.3 with 1 N NaOH and resedimented.

Electron Microscopy. A 3-μL drop of an aqueous solution of the native particles (0.8 μmol of organic phosphate/mL) was placed on a Formvar-coated electron microscope grid and allowed to stand for 1 min. The bulk of the solution was withdrawn with a strip of filter paper, and the grid was allowed to dry and then positively stained for 5 min with 0.4% lead citrate in 0.1 N NaOH. The grids were carbon coated on both sides in a Denton evaporator; then a drop of solution containing latex spheres 0.109 μm in diameter was placed on the grids for 1 min and withdrawn as described above. The particles were visualized and photographed in a Philips 200 electron microscope operated at 80 kV.

Partial Specific Volume. The apparent partial specific volume was determined by weighing an aqueous solution of the native particles (60 μmol of organic phosphate/mL) in a calibrated 1.0-mL pycnometer. A measured aliquot of the solution was lyophilized and dried to constant weight in a vacuum desiccator over P_2O_5. The apparent partial specific volume, ϕ, was calculated with the equation $\phi = (1/W_p)[(1/\rho_s) - (1 - W_p)/\rho_w]$ where W_p is the weight fraction of the particles, ρ_s is the density of the solution, and ρ_w is the density of water at 24 °C.

Analytical Methods. Calcium, magnesium, and organic and inorganic phosphate were measured as previously described (Marsh & Sass, 1983). Sodium was determined by atomic emission spectroscopy in a 0.2% KCl solution. The concentration of free ions in the extrapallial or total fluid was determined by measuring their concentration in the 100000*g* supernatant fraction after sedimenting the phosphoprotein particles. Aliquots of solutions containing radioactive isotopes were dissolved in Aquasol-2 (New England Nuclear, Boston, MA) and counted with a Packard PLD liquid scintillation counter.

Mineral Ion Exchange in Phosphoprotein Particles. Four identical solutions were prepared containing native particles (1.0 mM organic phosphate, pH 8.3) and free Ca, Mg, Na, and PO_4 ions. One solution was unlabeled, and the other three were labeled with 0.5 μCi/mL of either ^{45}Ca, ^{28}Mg, or $^{33}PO_4$. ^{28}Mg was obtained from the Brookhaven National Laboratory. The solutions were allowed to stand at 4 °C, and at intervals, the particles were sedimented from 8-mL aliquots of each solution. Isotopically labeled solutions and their supernatant fluids were counted to determine total and free counts. The unlabeled solution and its supernatant fluid were assayed for calcium, magnesium, and inorganic phosphate to determine total and free ions. Bound ions and counts were calculated by difference. The unexchanged calcium ($[Ca]_u$) bound to the particles was calculated as follows: $[Ca]_u = [Ca]_b - (SA_b/SA_f)[Ca]_b$, where $[Ca]_b$ is the number of calcium ions bound to the particles per inorganic phosphate residue and SA_b and SA_f are the specific activities of the bound and the free calcium, respectively. The unexchanged magnesium and inorganic phosphate were calculated in the same manner.

Two solutions were prepared containing demineralized particles (1.0 mM organic phosphate, pH 8.3), 1.5 mM $^{40}CaCl_2$, and 1 μCi/mL $^{45}CaCl_2$. In the first solution, the calcium isotopes were mixed first, and then the particles were added. The concentration of bound calcium was determined by the ratio of the bound to the total counts multiplied by the total calcium concentration. In the second solution, the particles were first equilibrated with $^{40}CaCl_2$, and then $^{45}CaCl_2$

[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

was added. The concentration of unexchanged calcium was determined as before by using the concentration of bound calcium determined with the first solution. The experiment was repeated in the presence of 50 mM NaCl.

Titration of Native Particles with Acid and Base. Eight-milliliter aliquots of the native particles (1.0 mM organic phosphate, pH 8.3) dissolved in 50 mM NaCl were titrated with 1 N HCl to give a final pH of 7.5–3.0. The particles were sedimented at 100000*g* for 30 min, and the supernatant fluid was assayed for liberated calcium, magnesium, and inorganic phosphate ions. Bound mineral was calculated as the difference between total and liberated mineral. Additional aliquots were readjusted to pH 8.3 with 1 N NaOH after acidification, and bound and liberated ions were determined as before.

Titration of Native Particles with EDTA. Solutions were prepared which contained native particles (1.0 mM organic phosphate, pH 8.3), NaCl (1.25 mM), Tris (50 mM, pH 8.3), and EDTA, pH 8.3 (0.15–1.5 mM). EDTA was added last. An identical set of solutions was prepared except that $CaCl_2$ was added 15 min after the addition of EDTA and at a concentration equivalent to EDTA. Calcium, magnesium, and inorganic phosphate bound to the particles were determined as described above.

Titration of Demineralized and Partially Dephosphorylated Particles with Calcium. Solutions were prepared containing demineralized particles (1.0 mM organic phosphate, pH 8.3) and 10 μCi of $^{45}CaCl_2$/mL (0.09–1.8 mM). Aliquots of each solution were removed, and the particles were sedimented from the remainder at 100000*g* for 30 min. Each solution and its supernatant fluid were counted to determine the total and free calcium concentration. The concentration of bound calcium was calculated by difference. Partially dephosphorylated particles were titrated in the same manner at the same particle concentration; therefore, the organic phosphate concentration was only 0.75 mM. The demineralized particles were also titrated in solutions containing 50 mM NaCl.

Other Procedures. When either native or demineralized particles were equilibrated with a large excess of mineral ions, the particles were sedimented from solution by centrifugation at 100000*g* for 30 min and washed with an equal volume of water, and then the bound mineral ion was measured directly on the isolated particles. The free mineral ion concentration was measured in the 100000*g* supernatant fraction.

Results

General Properties. The phosphoprotein particles are components of both the extrapallial fluid and the hemolymph (Marsh & Sass, 1983). In this study, the ionic composition of the extrapallial fluid and the total fluids (a mixture of the hemolymph and extrapallial fluid) was similar with respect to calcium, magnesium, sodium, and inorganic phosphate ions; likewise, the distribution of mineral ions bound to particles derived from both the extrapallial fluid and the total fluids was also similar. Therefore, in the experiments described here, the phosphoprotein particles were isolated from the total fluids which will be referred to as the physiological fluid. Throughout the isolation procedure, the particles are in equilibrium with the soluble components of the physiological fluid except for the final water wash. Therefore, the isolated particles are in a native state; i.e., the mineral ion–protein association in the isolated particles is qualitatively, quantitatively, and structurally similar to the mineral ion–protein association in vivo.

When dissolved in water or 50 mM NaCl, the native particles have a pH of 8.3. The particles are water soluble by the following criteria: they are completely miscible with water and neither aggregate nor settle from solution. However, since the molecular weight is very large, the particles are quantitatively sedimented when centrifuged at 100000*g* for 30 min.

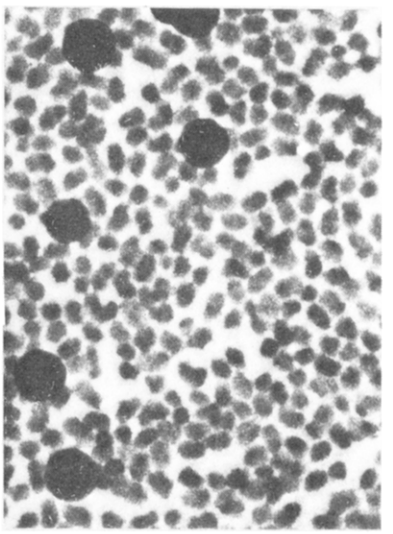

FIGURE 1: Electron micrograph of native phosphoprotein particles stained with lead. Large structures are latex spheres with an average diameter of 0.109 μm.

An electron micrograph of the native particles stained with lead is shown in Figure 1. The particles vary in appearance from roughly elliptical structures (40 × 57 nm) to roughly circular structures 40 nm in diameter. A prolate ellipsoid of axial dimensions 40 × 40 × 57 nm is a three-dimensional approximation of the particle structure and corresponds to a particle volume of 28.7×10^6 mL/mol. Generally, the densest particles observed have circular profiles, consistent with the particle being viewed down its long axis. The particle profiles are neither truly circular or elliptical; instead, they have a rather angular contour. The prolate ellipsoid structure is used only to obtain an approximate volume. Demineralized particles prepared for electron microscopy in the same manner are indistinguishable from the native particles.

The apparent partial specific volume of the native particles is 0.531 mL/g, corresponding to an apparent density of 1.88 g/mL. The particle solutions may be far from ideal, so the apparent density may be too large. However, the particles appear very compact in the electron microscope. On the basis of the particle volume and the apparent density, the molecular weight of the particles is about 54 million.

The amino acid composition of the phosphoprotein particles determined in previous studies (Marsh & Sass, 1983; Sass & Marsh, 1983) is presented in Table I. Ninety percent of the amino acid residues are phosphoserine (29%), aspartic acid (22%), histidine (34%), and lysine (4.6%). Another 6% of the amino acid residues are involved in covalent cross-linkages via the histidinoalanine residues. In order to show clearly the similarities and differences between the phosphoprotein particles and bovine phosphophoryn, the amino acid composition of the latter, taken from Lee et al. (1977), is included in Table I along with the amino acid composition of the particles exclusive of the histidine and cross-linking residues. When the cross-links and histidine residues of the particles are ignored, the compositions of the two phosphoproteins are very similar, phosphoserine and aspartic acid accounting for about 80% of the residues in both. In the particles, histidinoalanine may cross-link domains with a high aspartic acid and phosphoserine content—perhaps compositionally and functionally similar to phosphophoryn—to domains which are essentially polyhistidine (Sass & Marsh, 1983).

Table I: Amino Acid Composition of Phosphoprotein Particles and Phosphophoryn[a]

residue	phosphoprotein particles: native	phosphoprotein particles: exclusive of histidine and cross-links	phosphophoryn[b]
aspartic acid	21.5	36.0	37.6
phosphoserine	28.8	48.2	41.8
histidine	34.3		1.0
N^{τ}-histidinoalanine	4.5[c]		
N^{π}-histidinoalanine	1.5[c]		
lysine	4.6	7.7	4.9
threonine	0.6	1.0	1.4
glutamic acid	1.2	2.0	3.7
glycine	1.1	1.9	4.4
alanine	0.4	0.7	1.3
valine	0.7	1.1	0.6
arginine	0.9	1.4	0.7
tyrosine			0.4
phenylalanine			0.5
leucine			0.8
half-cystine			0.4
isoleucine			0.4
methionine			0.2

[a] Units are mole percent. [b] From Lee et al. (1977). [c] On the basis of leucine equivalents.

Table II: Free and Particle-Bound Mineral Ions in the Physiological Fluid of *Rangia cuneata* Maintained in Fresh Water and 50% Seawater

aquarium	ion	free concn (mM)	bound to particles (mol/mol of organic PO_4)
fresh water	Ca	4.67	1.20
	Mg	1.01	0.113
	PO_4	0.124	0.182
50% seawater	Ca	5.90	0.528
	Mg	23.03	0.627
	PO_4	0.296	0.134

Mineral Ion Binding. *Rangia cuneata* is an osmoregulating species when maintained in a fresh water environment. Under these conditions, the sodium ion concentration of the physiological fluid is about 30 mM; the free calcium concentration varies from 4 to 10 mM and the free magnesium concentration from 1 to 2 mM. The physiological fluid is isoosmotic with the environmental water when specimens are maintained in 50% seawater aquariums. Here the sodium ion concentration is about 250 mM, and the free calcium and magnesium concentrations are about 6 and 23 mM, respectively, in both the aquarium water and the physiological fluid. The distribution of mineral ions bound to the native particles is affected by the mineral ion distribution in the physiological fluid (Table II). All minerals bound to the particles are expressed as the number of ions bound per organic phosphate residue. In this study, particles contained 1.3–1.45 metal ions (calcium plus magnesium) and 0.17–0.21 inorganic phosphate ion per organic residue when isolated from fresh water specimens and 1.2–1.3 metal ions and 0.13–0.17 inorganic phosphate ion per organic phosphate residue when isolated from 50% seawater specimens. Native particles derived from fresh water specimens are referred to as low Mg particles, because magnesium is only about 10% of the total bound metal ions. Particles derived from 50% seawater specimens are referred to as high Mg particles since over 50% of the metal ions are magnesium. Particles demineralized after isolation are sodium salts.

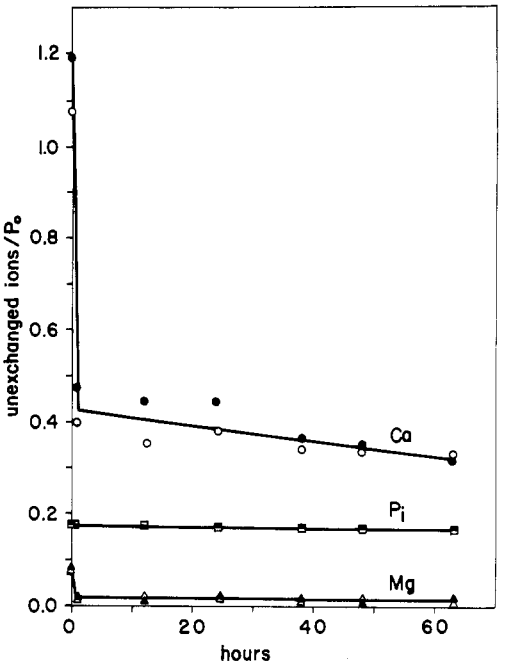

FIGURE 2: Equilibration of the low Mg particles with free ^{45}Ca (●, ○), ^{28}Mg (▲, △), and $^{33}PO_4$ (■, □) ions in 3 mM NaCl (●, ▲, ■) and in 50 mM NaCl (○, △, □). Particle and free ion concentrations are given in the text. Data are plotted as the number of unexchanged ions bound to the particles per organic phosphate residue (P_o) as a function of time in hours. P_i is inorganic phosphate.

The binding and exchange experiments were performed under as nearly physiological conditions as possible. Particle solutions of 1.0 mM in organic phosphate were utilized since the physiological fluid contains particles at a concentration of 1.0–3.0 mM with respect to organic phosphate. The pH of the isolated native particles when dissolved in water or 50 mM NaCl is 8.1–8.3, and the pH of the physiological fluid is similar. Generally, it was unnecessary to include buffer in the exchange and binding experiments, since the pH of the particle solutions remained constant over the experimental period. However, the particle solutions were buffered with Tris at pH 8.3 during titration with EDTA, because chelation of alkaline earth metals by EDTA generates hydrogen ions (Garvin, 1964).

The mineral ions contained in the native phosphoprotein particles are distributed into two distinct pools, i.e., a pool which is exchangeable with free ions in the medium and a pool which is not. This was demonstrated by equilibration of low Mg particles at a concentration of 1.0 mM with respect to organic phosphate with a solution containing 1.0 mM $CaCl_2$, 0.1 mM $MgCl_2$, 0.04 mM inorganic phosphate, and either 3 or 50 mM NaCl. The free mineral ions were labeled with either ^{45}Ca, ^{28}Mg, or $^{33}PO_4$ ions, and the exchange of unlabeled bound mineral ions with free labeled mineral ions was followed over a number of hours. The data are given in Figure 2 and are expressed as the number of unexchanged mineral ions bound to the particles per organic phosphate residue as a function of time. Two distinct calcium pools are apparent. One pool exchanges completely with free calcium ions within 1 h. The other pool (approximately 35% of the total calcium) is protected; it exchanges very slowly or not at all.

The rate of exchange of calcium from the protected pool cannot be determined, since there is a slow net loss of mineral from the particles over extended periods in dilute solution (Figure 3). The slow decrease in unexchanged calcium ions with time is due principally to net loss of calcium from the

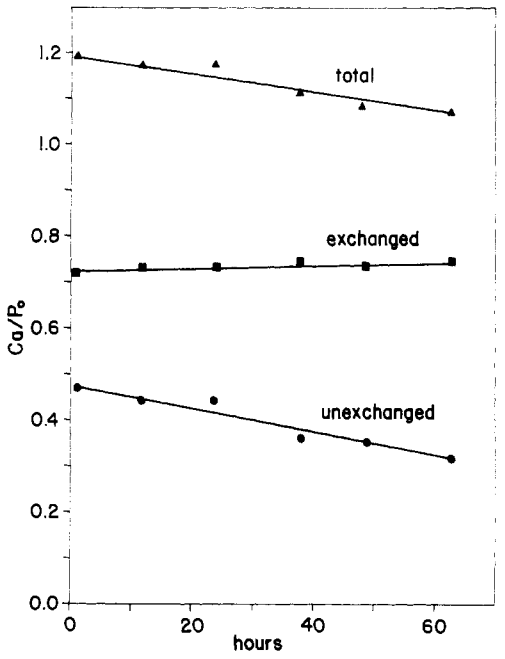

FIGURE 3: Equilibration of low Mg particles with free ^{45}Ca ions in a 3 mM NaCl solution. Particle and free ion concentrations are given in the text. Data are plotted as the total (▲), exchanged (■), and unexchanged (●) calcium ions bound to the particles per organic phosphate (P_o) as a function of time in hours.

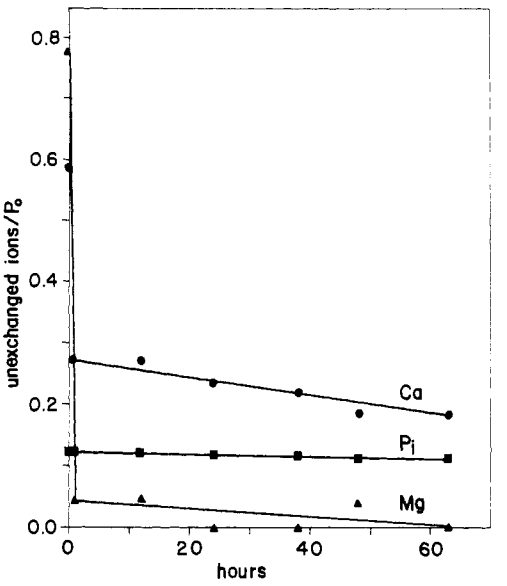

FIGURE 4: Equilibration of high Mg native particles with free ^{45}Ca (●), ^{28}Mg (▲), and $^{33}PO_4$ (■) ions in 8 mM NaCl. Particle and free ion concentrations are given in the text. Data are plotted as the number of unexchanged ions bound to the particles per organic phosphate residue (P_o) as a function of time in hours. P_i is inorganic phosphate.

particles and only minimally to exchange of unlabeled calcium for ^{45}Ca. However, native particles can be stored as concentrated aqueous solutions (40–60 mM organic phosphate) at 4 °C for up to 1 week without significant loss of mineral. The exchange experiments are thus initiated immediately upon dilution of the particles, and the number of unexchanged mineral ions in the protected pool is determined by extrapolating the amount of slowing exchanging mineral to zero time.

Approximately 35% of the calcium in low Mg particles is protected. These particles contain little magnesium, most of which is exchangeable with free ^{28}Mg ions (Figure 2). In contrast, essentially all of the inorganic phosphate is protected, exchanging either very slowly or not at all with free $^{33}PO_4$ ions. The $Ca/Mg/PO_4$ ratio in the protected pool is 2.5/0.1/1.0.

The number of mineral ions in the protected mineral pool is similar when measured in either 3 or 50 mM NaCl solutions (Figure 2). However, the total calcium bound in the presence of 50 mM NaCl is decreased due to competition by sodium ions for the exchangeable binding sites (Figure 2, zero time point). When the measurements are made in 50 mM NaCl, the ionic strength is similar to that of the physiological fluid in fresh water animals (about 50 mM), but the ratio of divalent cations to sodium ions is about 10-fold less than in the physiological fluid. When measurements are made in 3 mM NaCl, the ionic strength of the solution is much lower than the physiological fluid, but the ratio of divalent cations to sodium ions is comparable to the in vivo ratio. These two sets of conditions bracket the physiological milieu of the particles, and it can be concluded that in vivo the mineral ions of the phosphoprotein particles are also distributed between exchangeable and protected pools.

The distribution of mineral ions in the protected pool of high Mg particles was determined as described above for low Mg particles. Here, however, the particles were equilibrated with a solution containing 0.8 mM Mg, 0.2 mM Ca, 0.04 mM PO_4, and 8 mM Na ions. This is approximately the same relative concentration of the free ions in the physiological fluid from which the particles were derived. The data are given in Figure 4 and show the number of unexchanged mineral ions bound to the particles per organic phosphate residue as a function of time. In the high Mg particles, approximately 50% of the calcium ions are protected; essentially all of the inorganic phosphate is protected, but only about 5% of the magnesium ions are protected. The $Ca/Mg/PO_4$ ratio in the protected pool is 2.3/0.3/1.0. Although only 30% of the exchangeable metal ions in the high Mg particles are calcium, about 90% of the metal ions in the protected pool are calcium, which indicates that the protected binding sites have a high degree of specificity for calcium ions.

Table III: Mineral Ion Distribution in Native Phosphoprotein Particles Dialyzed against $CaCl_2$, $MgCl_2$, and NaCl[a]

dialysis solution	Ca	Mg	PO_4
none	1.28	0.15	0.185
50 mM $CaCl_2$	1.22	0.02	0.170
50 mM $MgCl_2$	0.02	0.96	0.005
50 mM NaCl	0	0	0

[a] Units are ions bound per organic phosphate residue.

The phosphoprotein particles will not sequester calcium phosphate unless a portion of the exchangeable binding sites is occupied with calcium ions. This was demonstrated by exhaustive dialysis of low Mg native particles against 50 mM $MgCl_2$ or 50 mM NaCl to saturate the exchangeable sites with magnesium or sodium ions. When the exchangeable sites were saturated with magnesium or sodium ions, the protected calcium phosphate pool was lost (Table III). When the particles were dialyzed in a similar manner against 50 mM $CaCl_2$, little if any of the inorganic phosphate was lost.

Demineralization of the native phosphoprotein particles is only partially reversible; i.e., exchangeable ions will rebind to demineralized particles, but the protected pool is irreversibly lost. This is demonstrated in the following experiments with low Mg particles. First, duplicate solutions of the particles were titrated with EDTA. Calcium ions were added to one set of solutions after EDTA and in an amount equivalent to the EDTA concentration (Figure 5). When titrated with EDTA alone, both metal and inorganic phosphate ions were removed from the particles. After readdition of calcium ions,

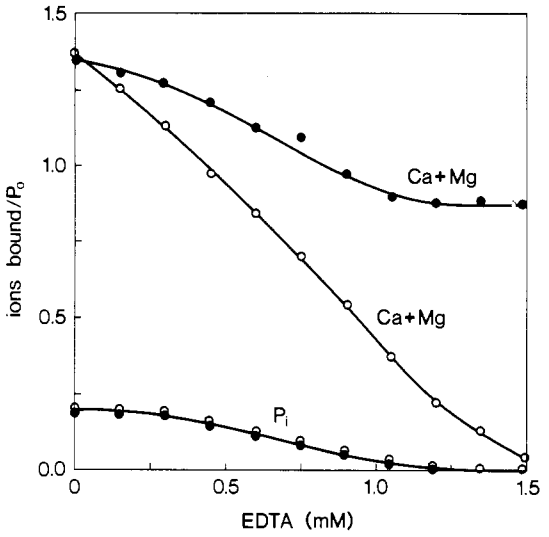

FIGURE 5: Titration of the native particles (1.0 mM organic phosphate) with EDTA in 50 mM Tris, pH 8.3. The number of mineral ions bound to the particles per organic phosphate residue (P_o) is shown as a function of EDTA added. (○) Titration with EDTA alone; (●) titration with EDTA followed by addition of an equivalent amount of $CaCl_2$. P_i is inorganic phosphate.

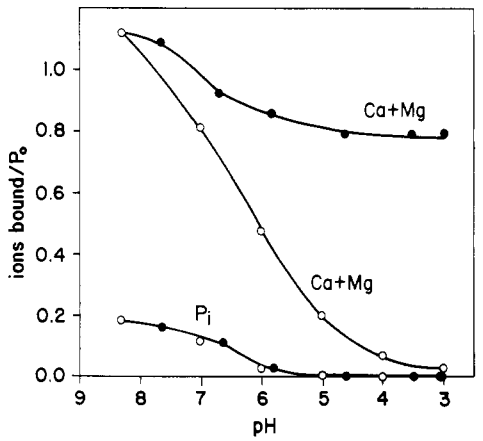

FIGURE 6: Number of calcium, magnesium, and inorganic phosphate (P_i) ions bound to native particles per organic phosphate residue (P_o) as a function of pH. (○) The native particles in 50 mM NaCl (1.0 mM organic phosphate, pH 8.3) were titrated with 1 N HCl to the pH on the horizontal axis. (●) Identical particle solutions titrated with 1 N HCl to the pH on the horizontal axis and then readjusted to pH 8.3 by addition of 1 N NaOH.

a portion of the lost metal ions would rebind to the particles (approximately 65%), but any loss of inorganic phosphate was completely irreversible.

In a similar manner, titration of the native low Mg particles dissolved in 50 mM NaCl, pH 8.3, with HCl simultaneously removes both metal and inorganic phosphate ions as the pH is lowered to 3.0 (Figure 6). If the pH of the particle solution is returned to 8.3 with NaOH after each interval in the acid titration, about 70% of the metal ions rebind. However, any loss of inorganic phosphate is completely irreversible even when the pH is lowered to only 7.5.

Once the particles have been demineralized in vitro, they cannot be returned to a native state even when reequilibrated with the physiological fluid from which they were derived. This is demonstrated by the data in Table IV which give the number of mineral ions in the native phosphoprotein particles and in EDTA demineralized particles reequilibrated at a concentration of 1.0 mM with respect to organic phosphate with the 100000g supernatant fraction of the physiological fluid from which the native particles were just isolated. After

Table IV: Mineral Ions Bound to Native Particles and to Demineralized Particles Reequilibrated with Physiological Fluid

	physiological[a] fluid (mM)	phosphoprotein particles[b] I[c]	II[d]	I minus II
Ca	4.83	1.179	0.769	0.410
Mg	1.04	0.116	0.115	0.001
PO_4	0.127	0.171	0	0.171
Ca/PO_4				2.40

[a] Free ion concentration in the physiological fluid. [b] Units are ions bound per organic phosphate residue. [c] Native particles isolated from the physiological fluid. [d] Demineralized particles reequilibrated with 100000g supernatant fraction of the physiological fluid.

Table V: Calculation of K_{Ca}/K_{Mg}[a] for Demineralized Particles Equilibrated with Physiological and Synthetic Fluid

fluid	C_{Mg}/C_{Ca}	ν_{Ca}/ν_{Mg}	K_{Ca}/K_{Mg}
physiological[b]	0.243	6.69	1.57
synthetic	0.250	6.15	1.54

[a] As defined in eq 3. [b] 100000g supernatant fraction of the physiological fluid.

demineralization and reequilibration with the physiological fluid, the net loss of bound mineral is equivalent to 35% of the calcium and all of the inorganic phosphate originally present in the native particles (Table IV, I minus II). The Ca/PO_4 ratio of the lost mineral is 2.4, which corresponds to the Ca/PO_4 ratio of 2.5 in the protected mineral pool of low Mg particles, as determined above by ^{45}Ca and $^{33}PO_4$ exchange. Thus, the mineral which does not rebind to demineralized particles represents the protected mineral pool in the native particles. When demineralized particles are equilibrated with $CaCl_2$ in water or 50 mM NaCl, all calcium bound to the particles is exchangeable with free $^{45}CaCl_2$.

The purpose of the remainder of the experiments summarized here is to determine the number of exchangeable binding sites in the phosphoprotein particles and to estimate the apparent binding constants for calcium and magnesium ions at these sites in vivo.

First, it must be established that the calcium and magnesium ions of the physiological fluid which are not bound to the particles behave as free ions. When two substances, Ca and Mg, compete for the same binding sites, ν_{Ca} and ν_{Mg} (the number of calcium and magnesium ions bound per organic phosphate residue) are related to the free ion concentrations C_{Ca} and C_{Mg} as shown in eq 1 and 2 (Tanford, 1961).

$$\frac{\nu_{Ca}}{n - \nu_{Ca} - \nu_{Mg}} = K_{Ca}C_{Ca} \quad (1)$$

$$\frac{\nu_{Mg}}{n - \nu_{Ca} - \nu_{Mg}} = K_{Mg}C_{Mg} \quad (2)$$

n is the number of binding sites per organic phosphate residue. Dividing eq 1 by eq 2 and rearranging give

$$K_{Ca}/K_{Mg} = (\nu_{Ca}/\nu_{Mg})(C_{Mg}/C_{Ca}) \quad (3)$$

Unless the binding sites are ideal (i.e., identical and independent), K_{Ca} and K_{Mg}, the apparent binding constants, will be a function of sites occupied. The demineralized phosphoprotein particles (which bind only exchangeable mineral ions) were equilibrated at a concentration of 1.0 mM with respect to organic phosphate with (1) the 100000g supernatant fraction of the physiological fluid and (2) a synthetic solution containing a similar calcium, magnesium, and sodium ion concentration, i.e., 4.5 mM $CaCl_2$, 1.0 mM $MgCl_2$, and 30 mM

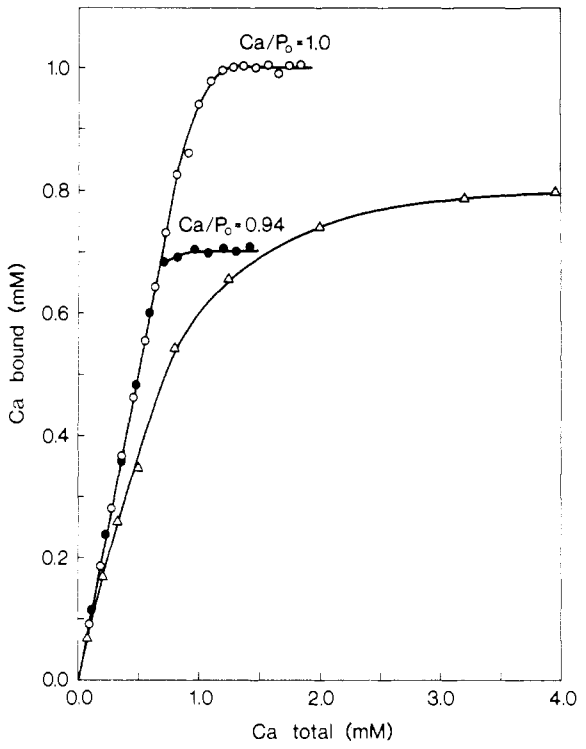

FIGURE 7: Titration of demineralized phosphoprotein particles (1.0 mM organic phosphate, pH 8.3) with $^{45}CaCl_2$ in a water solution (○) and in 50 mM NaCl (△). Titration of 25% dephosphorylated particles (0.75 mM organic phosphate, pH 8.3) in water (●). The concentration of bound calcium is shown as a function of the total calcium concentration. P_o is organic phosphate.

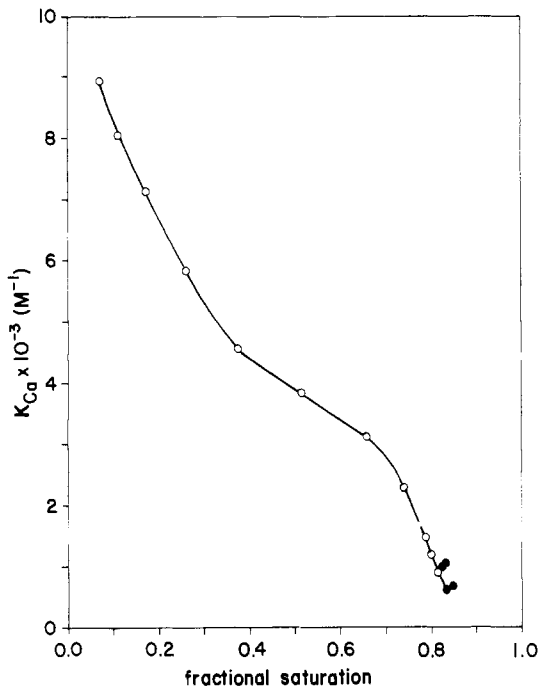

FIGURE 8: Apparent calcium binding constant (K_{Ca}) for demineralized particles as a function of the fractional saturation (ν_{Ca}/n) in 50 mM NaCl (○). K_{Ca} for the exchangeable binding sites on low Mg native particles in equilibrium with physiological fluid as a function of the fractional saturation, $\nu_{Ca}/(n - \nu_{Mg})$, assuming $n = 1.0$ (●).

NaCl. The calculated ratio K_{Ca}/K_{Mg} was 1.57 in the physiological fluid and 1.54 in the synthetic solution (Table V). Since the ratio of the apparent binding constants is similar under both sets of conditions and since the mineral ions in the synthetic solution are free, they must also be effectively free in the physiological fluid.

To determine the maximum number of exchangeable metal ion binding sites, demineralized particles were titrated with $^{45}CaCl_2$ at pH 8.3 in aqueous solutions free of any competing cations (Figure 7). Under these conditions, the demineralized particles saturate sharply and bind a maximum of 1.0 calcium ion per organic phosphate residue. Treatment of demineralized particles with acid phosphatase liberates 25% of the organic phosphate. Calcium binding to completely phosphorylated and partially dephosphorylated particles is compared in Figure 7. The former bind 1.0 and the latter 0.95 calcium ion per organic residue. The numbers are not significantly different, so it can be inferred that there is probably one organic phosphate residue at each exchangeable binding site.

When demineralized particles are titrated with $^{45}CaCl_2$ under approximately physiological conditions in 50 mM NaCl, the affinity for calcium is reduced, and the particles do not saturate sharply (Figure 7). The binding sites are nonideal in both water and 50 mM NaCl solutions, since Scatchard plots relating free and bound calcium ions are not linear (not shown). An apparent binding constant can be calculated at any degree of saturation with eq 1 by setting ν_{Mg} equal to 0 (eq 4). Figure 8 shows the apparent binding constant in 50

$$K_{Ca} = \frac{\nu_{Ca}}{(n - \nu_{Ca})C_{Ca}} \quad (4)$$

mM NaCl as a function of the fraction of binding sites occupied (ν_{Ca}/n where $n = 1.0$). The curve is triphasic, consisting of three nearly linear domains. The apparent binding constant at 50% saturation is only 4×10^3 M^{-1} in 50 mM NaCl. In the absence of NaCl (i.e., low ionic strength), the binding constant is 3×10^6 M^{-1} at 50% saturation.

It is now possible to estimate the apparent binding constants K_{Ca} and K_{Mg} for the exchangeable binding sites on the native phosphoprotein particles in vivo by assuming that the native particles also contain one exchangeable metal binding site for each organic phosphate residue. Since the protected mineral pool is not in equilibrium with the free ions, ν_{Ca} and ν_{Mg} in eq 1 and 2 represent the number of exchangeable calcium and magnesium ions bound to the particles per organic phosphate residue. ν_{Ca} and ν_{Mg} can be calculated by assuming that all inorganic phosphate associated with the particles is protected. For each inorganic phosphate ion, there are 2.5 protected calcium ions and 0.1 protected magnesium ion in the low Mg particles as determined above. Therefore

$$\nu_{Ca} = \nu_{Ca}' - 2.5\nu_P' \quad (5)$$

$$\nu_{Mg} = \nu_{Mg}' - 0.1\nu_P' \quad (6)$$

where ν_{Ca}', ν_{Mg}', and ν_P' are the total number of calcium, magnesium, and inorganic phosphate ions bound to the particles per organic phosphate residue. K_{Ca}, calculated with eq 1, for the native phosphoprotein particles in equilibrium with the physiological fluid ranged from 0.6×10^3 to 1.05×10^3 M^{-1} in four different preparations (Table VI). To compare the apparent calcium binding constants of native particles in vivo and demineralized particles in vitro, K_{Ca} (in vivo) was included in the plot in Figure 8 (closed circles). In vivo, the fractional saturation with calcium ions, ν_{Ca}/n (abscissa, Figure 8), is replaced by $\nu_{Ca}/(n - \nu_{Mg})$ since the effective number of calcium binding sites is decreased in the presence of magnesium ions. K_{Ca} for the exchangeable sites on native particles in vivo agrees well with K_{Ca} for demineralized particles in vitro at comparable saturations. K_{Ca}/K_{Mg} for the exchangeable sites on the native particles in vivo varied from 1.50 to 1.69 (Table VI), consistent with a value of 1.57 obtained with demineralized particles above. Therefore, it can be concluded that the

Table VI: Calculation of K_{Ca} and K_{Mg} for Exchangeable Binding Sites on Native Phosphoprotein Particles in Vivo[a]

C_{Ca} (mM)	C_{Mg} (mM)	n[b]	ν_{Ca}	ν_{Mg}	$K_{Ca} \times 10^{-3}$ (M^{-1})	$K_{Mg} \times 10^{-3}$ (M^{-1})	K_{Ca}/K_{Mg}	$(\nu_{Ca} + \nu_{Mg})/n$
4.67	1.01	1.0	0.745	0.095	1.00	0.59	1.69	0.84
4.83	1.04	1.0	0.753	0.099	1.05	0.64	1.64	0.85
8.21	1.17	1.0	0.785	0.075	0.68	0.46	1.50	0.86
8.38	1.23	1.0	0.773	0.073	0.60	0.39	1.56	0.85

[a] Calculated with eq 1, 2, 5, and 6. [b] n is assumed to be 1.0.

native particles in vivo also have one exchangeable metal ion binding site for each organic phosphate residue, as was assumed, and that the apparent K_{Ca} is also about 4×10^3 M^{-1} at 50% saturation. In vivo, about 85% of these exchangeable sites are occupied [$(\nu_{Ca} + \nu_{Mg})/n$, Table VI].

Discussion

Native Phosphoprotein Particle. On the basis of the experimental findings presented in this paper, the following conclusion may be drawn concerning the mineral ion–protein association in the native phosphoprotein particles in equilibrium with the physiological fluid. The particles are high molecular weight (about 54 million) covalently cross-linked structures which contain an exchangeable pool of calcium and magnesium ions and a protected pool of predominantly calcium and inorganic phosphate ions. The Ca/PO_4 ratio in the protected pool is about 2.5. The number of binding sites for the protected mineral is unknown, but on the average, the particles contain about 0.2 inorganic phosphate ion per organic phosphate residue. There is 1.0 exchangeable metal ion binding site for each organic phosphate residue. At 50% saturation, the apparent K_{Ca} for these sites is 4×10^3 M^{-1}. K_{Ca} is only about 1.6-fold greater than K_{Mg}. In vivo, about 85% of the exchangeable sites are saturated.

The drawing in Figure 9 summarizes what is known of the mineral–protein complex which constitutes the native phosphoprotein particle. The protected calcium and inorganic phosphate ions are displayed inside of a closed loop to indicate that they exist within a protected domain and are inaccessible for exchange with free ions in the medium. It has not been established with certainty that the protected calcium and inorganic phosphate occupy the same domain as opposed to two distinct domains, one containing protected calcium ions and the other protected inorganic phosphate. However, since the presence of inorganic phosphate in the particles is dependent upon the presence of calcium, it is probable that inorganic phosphate binds to the protein through protected calcium ions. The inorganic phosphate ions in Figure 9 are depicted in a triply charged state, but the actual state of ionization is completely unknown since the protected domain ions are not in equilibrium with the hydrogen ions in the medium. The number of protected domains per particle and the number of inorganic phosphate ions per domain are unknown. Figure 9 represents the smallest number (two) possible to account for a whole number of calcium ions (five). Outside the protected domain are the exchangeable metal ion binding sites which in vivo are occupied by calcium and magnesium. Each site probably contains an organic phosphate (phosphoserine) residue which is doubly charged, consistent with a pK_a of 6.8 reported by Lee et al. (1977) for the phosphoserine residues in dentin phosphophoryn. Nothing is known concerning the participation of other amino acid residues at the exchangeable binding sites. Likewise, there is no information concerning the amino acid residues in the protected domain, except for the probable absence of phosphoserine, since all of these residues can be accounted for at the exchangeable binding sites.

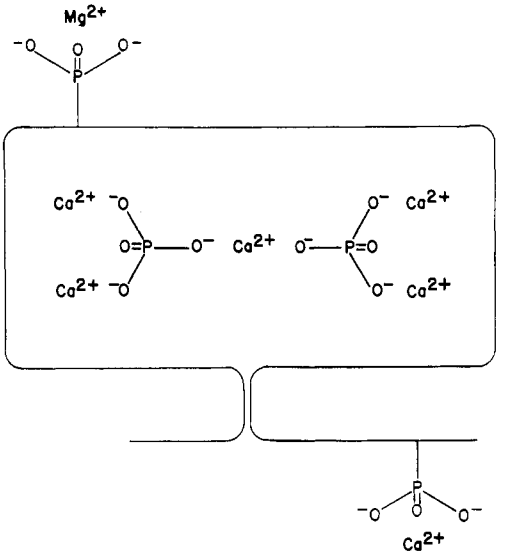

FIGURE 9: Drawing depicting a protected calcium–inorganic phosphate binding site (inside the loop) and exchangeable metal ion binding sites (outside the loop) within the native phosphoprotein particle. See the text.

The total number of calcium binding sites (N) on the phosphoprotein particle can be summarized by the equation $N = P_o + 2.5P_i$ where P_o is the number of organic phosphate residues and P_i is the number of bound inorganic phosphate ions. P_o is constant, but P_i is variable.

Demineralized Phosphoprotein Particle. Demineralization of the native particles, either by lowering the pH or by treatment with EDTA, irreversibly denatures the particles; i.e., the protected mineral pool cannot be reinserted in demineralized particles even under physiological conditions. Therefore, it is apparent that preformed phosphoprotein particles are not mineralized in the hemolymph or extrapallial fluid in vivo. Phosphoprotein monomers may be cross-linked into particles and charged with a protected mineral pool intracellularly in a completely different ionic environment before being discharged into the extracellular fluid, which influences only the distribution of exchangeable mineral ions. Alternatively, the phosphoprotein may be secreted as monomers which in turn may sequester a protected mineral pool of predominantly calcium and inorganic phosphate ions even in the presence of a large excess of magnesium; the mineralized monomers may then cross-link extracellulary into particles.

Comparison of Phosphoprotein Particles and Phosphophoryn. The phosphoprotein particles and phosphophoryn are highly phosphorylated, aspartic acid rich proteins which sequester mineral ions and are concentrated at mineralization fronts (i.e., invertebrate shell and vertebrate dentin). It is highly desirable, therefore, to make comparisons between these two proteins, not only for the purpose of determining if they are functionally similar but also as an aid in determining precisely what their function is with respect to extracellular mineralization. At low ionic strength, calcium binding constants of 1.1×10^7 M^{-1} at pH 8.2 for rat dentin phosphophoryn

(Zanetti et al., 1981) and 3.6×10^4 M^{-1} at pH 6.5–7.0 for bovine dentin phosphophoryn (Lee et al., 1977) have been reported. The apparent calcium binding constant for the exchangeable sites on the phosphoprotein particles at low ionic strength is 3×10^6 M^{-1} at pH 8.3 and 50% saturation, comparable to the binding constant reported for rat phosphophoryn. The disaggreement between the binding constants for rat and bovine phosphophoryn is probably due to the difference in pH at which the measurements were made. In bovine phosphophoryn, the number of calcium binding sites is a function of the protein concentration. At higher concentrations, the number of sites is roughly equivalent to the number of organic phosphate residues, comparable to the number of exchangeable sites per organic phosphate residue in the phosphoprotein particles. Since the number of binding sites in phosphophoryn was determined by equilibrium measurements employing a calcium ion specific electrode, it can be concluded that these binding sites are all exchangeable.

A comparison between native phosphoprotein particles and native phosphophoryn cannot be made, because the latter is necessarily isolated under demineralizing conditions. Whether or not phosphophoryn in vivo acquires a protected mineral pool before it is incorporated into the dentin matrix at the mineralization front is unknown. Zanetti et al. (1981) concluded that in vivo phosphophoryn was incapable of selectively extracting calcium ions from tissue fluids in order to form mineral, on the basis of the strong competition of magnesium and sodium ions for the calcium binding sites in vitro. However, this conclusion is no longer justifiable, because phosphophoryn, like the phosphoprotein particles, may in vivo acquire a protected mineral pool which is specific for calcium phosphate and is not in equilibrium with the extracellular fluid. Veis (1978) postulated that phosphophoryn binds to collagen at the dentin mineralization front and nucleates and catalyzes the deposition of hydroxyapatite (calcium phosphate) crystals. If native phosphophoryn is similar to native phosphoprotein particles, the role of phosphophoryn can be extended to include the actual transport of mineral ions to the calcification front of dentin.

Phosphoprotein Particles and Shell Mineralization. The phosphoprotein particles are components of the hemolymph, extrapallial fluid, and innermost shell lamella of some species of bivalve mollusks including *Rangia cuneata* (Marsh & Sass, 1983). The physiological significance of the particles in the hemolymph is not considered here.

The following observations demonstrate a physiological role for the phosphoprotein particles in shell formation. The extrapallial fluid bathes the inner shell surface dorsal to the pallial attachment and separates the innermost shell lamella from the soft tissues of the animal. The phosphoprotein particles, which occur at a maximal concentration of 3.0 mM with respect to organic phosphate in the extrapallial fluid, are concentrated about 200-fold in the innermost shell lamella where they occupy about 20% of the volume (Marsh & Sass, 1983). Since the lamella particles are necessarily derived from the fluid, they initially contain an exchangeable pool of calcium and magnesium ions and a protected pool of calcium phosphate. Once incorporated into the lamella, however, the particles are altered; i.e., they develop a less compact morphology. The altered morphology cannot be attributed solely to demineralization, because native and demineralized particles are ultrastructurally indistinguishable. The altered particles are not dephosphorylated (Marsh & Sass, 1983). Thus, the decreased density of the lamella particles is probably due to a reduced molecular weight or state of aggregation produced by either a proteolytic enzyme or an uncoupling agent. The phosphoprotein particles are not incorporated into the mature, mineralized shell layers.

Since phosphoprotein particles are concentrated and altered in the innermost shell lamella but not incorporated into the mature shell, the particles probably serve as both the transporter and reserve source of calcium ions for shell development. At this time, there is no information to demonstrate how the protected calcium phosphate in the particles is utilized to generate a mature shell layer of crystalline calcium carbonate devoid of phosphoprotein. If the protected mineral in the particles were calcium carbonate, or the shell were calcium phosphate, it would be reasonable to suggest that altered particles release protected mineral ions resulting in an extreme local elevation of free ions and the spontaneous precipitation of a solid mineral phase. However, if this mechanism operates in the shell system, there must also be a physiological process for converting solid calcium phosphate derived from the particles to crystalline calcium carbonate. The presence or absence of such a process has not been established but is currently under investigation.

Registry No. Calcium, 7440-70-2; magnesium, 7439-95-4; phosphate, 14265-44-2.

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