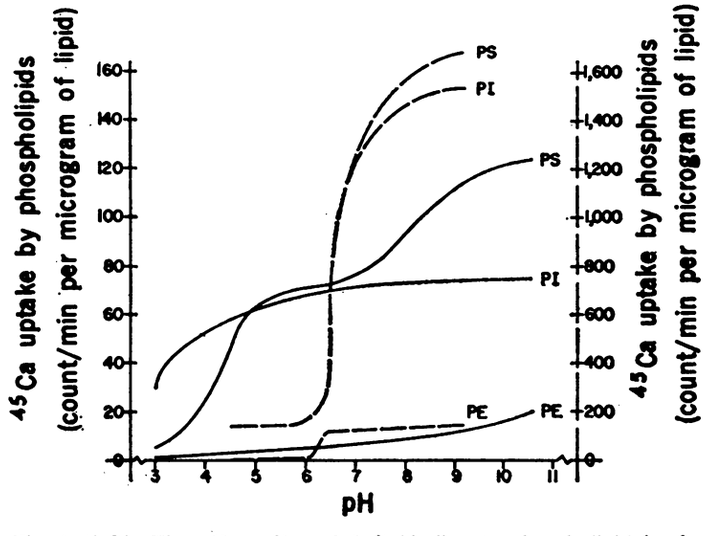
# Phospholipid–Calcium Phosphate Complex: Enhanced Calcium Migration in the Presence of Phosphate

Abstract. *In the presence of acidic phospholipids, inorganic phosphate greatly enhances the net migration of calcium ions from the aqueous phase to the organic phase, an effect that does not occur at less than the physiological* p*H. The calcium complex in the organic phase is shown by electron microscopy to consist of spherules, composed of stoichiometric amounts of calcium, inorganic phosphate, and phospholipid. The demonstration of complex formation between calcium phosphate and acidic phospholipids adds support to the concept that phospholipids are involved in biological mineralization.*

Data have been presented which indicate that phospholipids may be involved in calcification at sites of primary mineralization. This postulation was based on the results of lipid analysis of the epiphyseal plate of the bovine fetus, on the basis of histologic evidence that a sudanophilic staining material was associated with mineral in the matrix surrounding the cartilage cells at the site of primary calcification. Biochemical evidence indicated (i) that there was a progressive increase in the amount of lipids during endochondral calcification, (ii) that acidic phospholipids were progressively more difficult to remove as mineralization advanced through the epiphyseal plate, and (iii) that the metabolic pattern of phospholipids associated with the mineral phase was distinct from that of other phospholipids not so associated (*1*). Several investigators have shown evidence from electron microscopy for the involvement of membrane-bound, electron-opaque bodies in the earliest stages of mineralization in the epiphyseal plate (*2*). These studies led us, using an in vitro system and commercially purified phospholipids (*3*), to examine the factors affecting the ability of phospholipids to sequester $Ca^{2+}$. Although many investigations, with a variety of techniques, have been carried out on calcium-phospholipid binding (*4*), we centered our attention on the effects of inorganic phosphate ($P_i$) and $Mg^{2+}$, since these ions are most likely to affect the calcification process.

We used a modification of Feinstein's method (*5*). A mixture of chloroform, methanol, and water (2 : 1 : 2, by volume) was allowed to separate, the upper phase being used for the preparation of all aqueous solutions and the lower phase for the dissolution of the phospholipids. To a 10-ml screw-cap test tube were added the following: 1.5 ml of the lower phase containing 60 μg of lipid per milliliter, 0.9 ml of aqueous buffer solution containing the ion under examination, and 0.1 ml of aqueous $^{45}Ca$-labeled $CaCl_2$ to give a 1 m*M* solution of $Ca^{2+}$ with a radioactivity of approximately 0.13 μc per micromole. The system was immediately shaken vigorously for 15 seconds, centrifuged for 15 minutes at low speed, and allowed to stand overnight. The radioactivity of aliquots from both phases was determined with the use of a gas-flow counter (Nuclear-Chicago).

The *p*H profiles were established for $Ca^{2+}$ binding to phospholipids (Fig. 1, solid lines). The aqueous phase was buffered with a universal buffer (*6*). In general, these profiles were in agreement with those in the literature (*4*), even though the methods used were different. However, one exception was the profile for phosphatidylethanolamine (PE), which did not correspond to the values shown by Joos and Carr (*7*). At the physiological *p*H, one $Ca^{2+}$ complexed with two phosphatidylserine (PS) molecules or with slightly more than two molecules of phosphatidylinositol (PI). The approximate molecular weights of the lipids were calculated from data compiled by Ansell and Hawthorne (*8*). Phosphatidylcholine was tested in this system, but, as indicated by Dervichian (*9*), failed to bind $Ca^{2+}$ in the presence of 5 m*M* $Na^+$ in the universal buffer. The dashed lines (Fig. 1) demonstrate the changed *p*H profiles in the presence of phosphate buffer (*10*). The net migration of $Ca^{2+}$ from the aqueous to the organic phase increased markedly, as measured by the appearance of



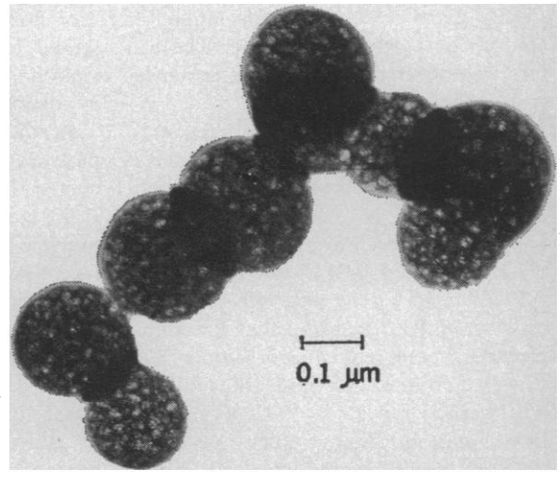




Fig. 1 (left). The *p*H profiles of $Ca^{2+}$ binding to phospholipid in the absence (solid lines, left ordinate scale) and in the presence (dashed lines, right ordinate scale) of $P_i$. The $Ca^{2+}$ and $P_i$ concentrations were both 1 m*M*, to give a molar ratio of $Ca^{2+}$ to $P_i$ of 1 in the aqueous phase for the experiments in which both $Ca^{2+}$ and $P_i$ were used (*6*, *10*). Fig. 2 (right). Electron micrograph of the complex from the organic phase after reaction of PS with a molar ratio of $Ca^{2+}$ to $P_i$ of 1 (1 m*M* $Ca^{2+}$, 1 m*M* $P_i$) in the aqueous phase (0.05*M* tris buffer, *p*H 7.5). The overall diameter of an individual spherule is approximately 175 to 280 Å.

$Ca^{2+}$ in the organic phase and the character of the changed *p*H profiles. The transition region of all three phospholipids for their enhanced binding of $Ca^{2+}$ occurred between *p*H 6 and 7, the *p*H at which calcium phosphate begins to precipitate.

Magnesium ions weakly inhibited the binding of $Ca^{2+}$ to PS, in the presence of 1 m*M* $Ca^{2+}$ in tris buffer (*11*). When a solution of $Mg^{2+}$ of the same molar concentration as that of the $Ca^{2+}$ solution was used, $Mg^{2+}$ decreased calcium-phospholipid binding by only 6 percent in the presence of 1 m*M* $P_i$ and by 18 percent in the absence of 1 m*M* $P_i$. At a tenfold higher concentration, $Mg^{2+}$ decreased calcium-phospholipid binding by 48 percent in the presence of 1 m*M* $P_i$ and by 59 percent in the absence of 1 m*M* $P_i$; at a 100-fold higher concentration, $Mg^{2+}$ almost abolished $Ca^{2+}$ binding. Hendrickson and Fullington (*12*), using an aqueous micellar system, found little difference in the stability constants of $Ca^{2+}$ and $Mg^{2+}$ complexes of PS. However, the results of the ion competition studies with our biphasic system show that $Mg^{2+}$ produces significant inhibition of the net $Ca^{2+}$ migration from the aqueous to the organic phase only when the ratio of $Mg^{2+}$ to $Ca^{2+}$ far exceeds that of serum, and that PS has a distinctly greater affinity for $Ca^{2+}$ than for $Mg^{2+}$. Bachra *et al.* (*13*) have shown that $Mg^{2+}$ inhibits the formation of hydroxyapatite and favors the formation of noncrystalline calcium phosphate. Our studies show, however, that the preferential binding of PS with $Ca^{2+}$ rather than with $Mg^{2+}$ is not strictly dependent on the formation of amorphous calcium phosphate. The effect was observed both in the presence and in the absence of $P_i$.

Sixteen samples of the isolated calcium phosphate–lipid complex have been analyzed. These were prepared from systems in which the organic phase contained PS and the aqueous phase contained 0.01*M* tris buffer (*p*H 7.5), 1 m*M* $Ca^{2+}$, and either 1 m*M* or 10 m*M* $P_i$. To ensure that the complex recovered from the organic phase was free from excess lipid, the complex was washed repeatedly with a mixture of ethanol and diethyl ether (3 : 1, by volume). For chemical analysis, dried samples of the complex were dissolved in concentrated formic acid and partitioned between the upper and lower phases of a mixture of chloroform, methanol, and 1*N* HCl (200 : 100 : 75, by volume) to separate the phospholipid (lower phase) from the $P_i$ and $Ca^{2+}$ (upper phase). The amount of $Ca^{2+}$ was measured by the method of Banerjee *et al.* (*14*), and $P_i$ and lipid phosphorus, as a measure of PS, by the method of Martin and Doty (*15*). The molar ratio for $Ca^{2+} : P_i : PS$ was found to be 12.12 ($\pm$ 0.31) : 6.74 ($\pm$ 0.17) : 1.0 (values in parentheses are standard errors).

In some experiments, the $Ca^{2+}$ concentration was increased from 1 m*M* to 3 m*M*, with the $P_i$ concentration being kept constant. No increase in the amount of $Ca^{2+}$ migration was observed in any of these cases.

The structural characteristics of an unstained and unfixed preparation of the calcium phosphate–phospholipid complex were examined by electron microscopy (Fig. 2). A sample of the organic phase, containing PS, $Ca^{2+}$, and $P_i$ was concentrated under a stream of nitrogen gas to remove excess chloroform. A drop of this concentrate was transferred to a 400-mesh Formvar and carbon-coated copper grid, blotted dry immediately, and examined. The units of the clusters appear to be discrete hollow spherules, 175 to 280 Å in overall diameter. From the data of Luzzati and Husson (*16*) for the dimensions of phospholipid micelles in aqueous systems, and the observed stoichiometry and dimensions of the microspheres, we postulate that both the center and the exterior surfaces of the complex are lined with PS molecules, with their polar ends attached to $Ca^{2+}$. X-ray diffraction studies have shown the complex to be noncrystalline. In this respect the complex is similar to the noncrystalline microspheres observed by Weber *et al.* (*17*), from in vitro preparations of calcium phosphate in aqueous media.

In the absence of lipid and $P_i$ no measurable migration of $Ca^{2+}$ occurred. In the absence of lipid but with $P_i$ in the aqueous phase, a small dense precipitate accumulated at the interface between phases, but no $Ca^{2+}$ appeared in the organic phase.

Because of this observation, the following question was asked: Were $Ca^{2+}$ and $P_i$ being transferred to the organic phase, in the presence of PS, as ions, or as a solid? The answer to this query necessitated the use of a different biphasic system, one in which the organic phase was the upper one. A benzene-ethanol-water (2 : 1 : 2, by volume) system was chosen. Where used, the $P_i$ concentration was 1 m*M*. The aqueous (lower) phase was used to make 0.05*M* tris buffer (*p*H 7.5), and the organic (upper) phase was used to dissolve the lipid. In the absence of lipid and with or without $P_i$, no $Ca^{2+}$ appeared in the organic phase. In the presence of PS, and with or without $P_i$, $^{45}Ca$ migration occurred in amounts comparable to those obtained with the chloroform-methanol-water system. In tubes lacking PS but containing $P_i$, a white interface material was present as a thin layer. Samples without $P_i$ or PS showed no interface precipitate.

The upper phase was carefully withdrawn from the tubes that contained no PS and replaced with a new organic phase containing PS. The mechanics of the reaction procedure were repeated, and the upper and lower phases were sampled for $^{45}Ca$. Calcium ions migrated from the lower to the upper phase under this condition, but there was no difference between samples containing $P_i$ and those without $P_i$. This amount of $Ca^{2+}$ transferred in the presence of $P_i$ was the same as that transferred in the absence of $P_i$ in other experiments. We conclude from this experiment that a solid containing $Ca^{2+}$ and $P_i$ is not formed prior to the enhanced movement of $Ca^{2+}$ into the organic phase. Rather, an ionic interaction between $Ca^{2+}$, $P_i$, and PS appears to be involved in this phenomenon. The solid precipitate of $Ca^{2+}$ and $P_i$ formed in the absence of PS is quite different from that formed during desiccation preparatory to chemical analysis or electron microscopy, which also contains the lipid.

These data illustrate the manner in which phosphate enhances the ability of acidic phospholipids to bind $Ca^{2+}$. They also provide a rational basis for the earlier observation that acidic phospholipids are bound with newly forming mineral during in vivo mineralization (*1*). Recent electron micrographs of endochondral calcification reveal that membrane-coated electron-opaque bodies are secreted from chondrocytes into the matrix, probably by budding off of the cytoplasmic processes (*18*). Matthews *et al.* (*19*) noted a large amount of labeling with $^{45}Ca$ in cytoplasmic processes during the early period following isotope injection. The labeled calcium was seen first in the endoplasmic reticulum, then in the Golgi apparatus, and later in the cytoplasmic processes. If calcium phosphate, complexed with phospholipid and other membrane components, is indeed extruded from the chondrocyte during calcification, as these studies suggest,

this would explain the remarkable buildup of phospholipid in the extracellular matrix of calcifying cartilage seen both histologically and by direct chemical analysis (*1*). In addition, Termine and Posner (*20*) demonstrated a relationship between zinc deficiency, which causes a decrease in bone cephalin and an increase in neutral lipid, and the presence of an increased amount of crystalline apatite in chick epiphyses, at the expense of amorphous calcium phosphate. This evidence suggests a correlation between the presence of phospholipids and the stabilization of amorphous calcium phosphate in epiphyseal tissue.

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## Butterfly Feeding on Lycopsid

Abstract. *Larvae of* Euptychia westwoodi *feed on* Selaginella, *a lycopsid. This is the first feeding record in the Satyrinae outside the monocotyledons and one of the few records of a butterfly feeding on other than a seed plant. Clues to possible evolutionary origins of this habit are found in the oviposition behavior of other* Euptychia *species.*

All the previously known plants fed on by larvae of the large nymphalid subfamily Satyrinae (perhaps 2000 species) are monocotyledons, primarily Gramineae and Cyperaceae, but occasionally Restionaceae, Zingiberaceae and Palmeae (*1*). It was therefore with considerable surprise that we observed a small *Euptychia* species, *E. westwoodi* Butler, ovipositing on *Selaginella horizontalis* (Presl) in the laboratory clearing of the Barro Colorado Island research station, Panama Canal Zone. Captured female *E. westwoodi* oviposited readily on *S. horizontalis* in the laboratory.

A careful search of the *S. horizontalis* mats which were thick on the slopes around the laboratory building revealed eggs and larvae which were reared in the laboratory on this plant. Larval density was approximately 0.5 larvae per square meter of *Selaginella* cover. A second species, *Selaginella articulata*, growing in the forest shade, was not attacked by any insect.

Our observations (*2*) of the reproductive behavior of other tropical *Euptychia* species provide clues to the origin of this choice of food plant. Of the six species observed in Trinidad, all are grass- or sedge-feeders as larvae. However, only three (*E. hesione*, *E. palladia*, *E. libye*) glue their eggs directly on the larval food plant. In contrast, *E. hermes* and *E. renata* test the food plant while alighting on it briefly, then fly a short distance and glue their eggs to dead leaves, bark, twigs, or other plants nearby. An alternative strategy, exemplified in Trinidad by *E. penelope*, is that of testing the food plant, then dropping an egg to the ground. These habits presumably evolved in response to patterns of egg predation. Chalcid predation is heavy in those species which oviposit directly on the food plant. This is also the case among nonsatyrines in Trinidad such as *Heliconius ethillus* and *Mechanitis polymnia*, which owing to the relative inaccessibility of the edible parts of their food plants do not have the egg-scattering options that are available to grass-feeders such as satyrines and skippers (Hesperioidea).

In the light of these observations, it is possible that the switch from feeding on monocots to feeding on *Selaginella* occurred as a consequence of habitual oviposition away from the larval food plant. This hypothesis is supported by recorded ovipositions on *S. horizontalis* in Trinidad by both *E. hermes* and *E. renata*, although larvae of these species will not eat this plant in the laboratory. The alternative hypothesis, that oviposition on *Selaginella* arose as a consequence of some chemical similarity between grasses and *Selaginella*, leading to frequent "mistakes" in oviposition, also receives support from an observed oviposition on *S. horizontalis* by *E. palladia*. This species normally lays on the leaves of grasses (*Paspulum convexum* and *Oplismenus hirtellus*).

These observations, five in number, of occasional oviposition on *Selaginella* by grass-feeding species that habitually lay eggs both on and off their food plants may suggest that this habit is not accidental. For example, it may be a safeguard against egg desiccation in the dry season. In any case, mutant or recombinant larvae which could mature on *Selaginella* would acquire several advantages. They would avoid both the long and energetically expensive trip to find appropriate food after hatching and exposure to a suite of predators operating in the litter. Furthermore, they would probably face low predator pressure on the *Selaginella*. In Trinidad, this plant was unmolested by insect herbivores aside from an occasional sawfly larva, whereas in Panama at least 90 percent of the observed damage could be ascribed to larvae of *E. westwoodi*. In