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CALCIFICATION OF LIPOSOMES AND RED CELL GHOSTS *IN VITRO* AND *IN VIVO*

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Abstract

To study the role of membranous phospholipids in calcification, liposomes made of phosphatidylserine and red blood cell ghosts (RBCG) prepared from rat and canine blood were incubated in minimal essential medium (MEM) with 2.5 mM (mmol/l) calcium and 1.3 mM phosphate, pH 7.4 (MEM-2.5), and homologous (rat) or autologous (canine) plasma filtrate (serum) at 37°C for up to 1 week. Calcification was determined by electron probe microanalysis, electron diffraction, and depletions of calcium and phosphate from MEM-2.5 and sera. Liposomes and rat-RBCG incubated in MEM-2.5 and serum calcified in a week, whereas isolated collagen and elastin did not. Liposomes and rat-RBCG implanted in rat peritoneal cavities calcified in 4 weeks. Calcification of both liposomes and RBCG under identical conditions suggests that phospholipids in membrane may play a role in calcification.

Canine RBCG incubated in MEM-2.5 and serum began to calcify on day one and grew heavier with further incubations. Calcification of RBCG in autologous serum indicates that calcification is prevented by normal red cells. The capacity to prevent calcification by red cells is apparently abolished by removal, during RBCG preparation, of the cytoplasmic content.

Key Words: Liposome, red cell ghosts, calcification, minimal essential medium, serum, peritoneal implantation.

Introduction

In view of the frequent occurrence of calcification in fat necrosis, the prospective role of lipids in calcification has long been entertained. Klots in 1905 (cited in Abraham, 1970) maintained that fatty acids released in necrotic tissues form salts with calcium which are gradually replaced by calcium phosphate. The first scientific clue for the lipids' role in calcification was presented by Irving (1958), who demonstrated a zone of sudanophilia along the front of calcification in developing teeth. It became gradually apparent that of the lipid components, phospholipids (PL) were primarily associated with this calcification (Irving and Wuthier, 1968). Because of their stronger affinity for calcium, acidic PL, especially phosphatidylserine (PS) have been suggested to be a promoter (**nucleator**) of calcium hydroxyapatite (**apatite**) formation (Boskey, 1989; Boyan *et al.*, 1989; Wuthier, 1988). The concept that PL play a role in calcification was supported by the discovery of matrix vesicles (**MV**) by electron microscopy (**EM**) of the epiphyseal cartilage (Anderson, 1967; Bonucci, 1967) and calcified human aortic valve (Kim and Huang, 1971). Numerous studies of both physiological and pathological calcifications have reiterated that MV are the primary site of calcification (Anderson, 1983, 1988; Kim, 1983a, b). Subsequent studies have also disclosed that lipids seen in calcified tissues mainly correspond to accumulated MV. However, the mechanism of MV calcification is far from being understood.

One of the theories for the mechanism of calcification has been that PS forms a complex with hydrophobic proteins (proteolipids) and/or with calcium and phosphate (P_i) (calcium-phospholipid-phosphate complex, **Ca-PL- P_i**) and these complexes induce calcification (Boskey and Posner, 1977; Ennever *et al.*, 1977). Proteolipids and **Ca-PL- P_i** have been isolated only from calcified tissues and they were able to cause apatite formation *in vitro* and *in vivo* (Boskey, 1989; Boyan *et al.*, 1989; Raggio *et al.*, 1986).

There has been another line of evidence that PL play a role in calcification. Liposomes made of PL have been shown to promote apatite formation (**nucleate**) in metastable solutions (Eanes *et al.*, 1984, 1987; Eanes

and Hailer, 1985; Kim, 1979, 1983a). These observations have suggested that PL in a membranous conformation may be able to nucleate apatite from a metastable solution by heterogenous nucleation (Kim, 1983b) or through selective transports of Ca^{2+} and P_i across the membrane simulating MV calcification (Skrtec and Eanes, 1992a, b).

In addition, spherular calcific deposits were frequently observed in large thrombi such as occur in aortic aneurysms. The spherules were proved to be calcified red cells by EM (Kim, unpublished data). Fossilized red cells were also frequently observed in human urinary stones (Kim, 1982). To further substantiate the nucleation of apatite by PL in membranes under physiological conditions and to compare the calcifiability of artificial and natural membranes, calcification of liposomes and red blood cell ghosts (RBCG) was attempted in *in vitro* and *in vivo*. Both liposomes and RBCG calcified in every experiment indicating that PL in membranes are likely to be a nucleator of apatite.

Materials and Methods

Phospholipids, ATP and other chemicals were purchased from SIGMA (St. Louis, MO) and were used without further purification. Culture media were purchased from GIBCO (Grand Island, NY). For RBCG, Fisher 344 inbred rats, 2 years of age, and Beagle dogs, 12 years of age, were used. Analytical grade reagents, acid washed glassware and sterile techniques were used throughout the study.

For the preparation of liposomes, $2 \mu\text{M}$ ($\mu\text{mol/l}$) PS, $1.5 \mu\text{M}$ cholesterol and $0.2 \mu\text{M}$ dicetyl phosphate were dissolved in 1.0 ml of chloroform. A thin lipid film was formed in a round bottom flask on a rotary evaporator. The film was suspended in 5 ml of Ca^{2+} , Mg^{2+} -free Hank's balanced salt solution (HBSS) by sonication using a Branson sonicator for 30 seconds. The suspension was centrifuged at 15,000 g for 10 minutes. The supernatant was removed with a pipette and the concentrated liposomes in 0.5 ml HBSS were suspended in minimal essential medium (MEM) or serum by using a Vortex.

Rat bloods obtained by aortic punctures were collected in heparinized centrifuge tubes (14.3 USP unit sodium heparin/ml). The blood was centrifuged at 15,000 g for 10 minutes at 4°C , and the supernatant plasma was filtered through nitrocellulose filters with a pore size of $0.2 \mu\text{m}$, pooled and stored at 4°C until use. The buffy coat was removed from the resultant pellet and the remaining red cells were used for RBCG preparation.

Red cell ghosts were prepared by the method of Steck and Kant (1974). Red cells were washed 3 times in phosphate buffered saline (150 mM NaCl, 5 mM sodium phosphate, pH 8.0). One ml of the packed red cells was rapidly suspended in 40 ml of 5 mM sodium phosphate, pH 8, containing 1 mM MgSO_4 . Lysed red cells were pelleted by centrifugation at 22,000 g for 10

minutes. After washing lysed cells twice in 5 mM sodium phosphate, the resultant ghosts were resealed by suspending in 40 ml phosphate buffered saline at 37°C for 40 minutes, and pelleted.

Collagen was extracted from rat tails by the method of Thomas and Tomita (1967). After removing the skin, the tails were cut into 1 cm pieces and decalcified in 10% ethylenediaminetetraacetic acid (EDTA) with daily changes of the solution until it became calcium free. Pieces were then placed in 3% Na_2HPO_4 to remove soluble proteins, 20 ml per piece, at 4°C with constant stirring for 6 days. The pieces were additionally treated with 15% KCl for 6 days to remove glycosaminoglycans (Thomas and Tomita, 1967). For isolation of elastin, rat aortae were cut into 1 cm pieces, decalcified as above, and digested in 95.5% formic acid at 45°C for 4 days with daily changes of the acid (Seligman *et al.*, 1975). Isolated collagen and elastin were dialyzed for 3 days against H_2O , vacuum dried, and stored at 4°C until use. Residual lipids were removed from isolated collagen and elastin by placing tissues in an excess amount of chloroform-methanol mixture (3/1:v/v) with stirring for 3 days and daily changes of the solvent. Portions of collagen and elastin were coated with PS by placing 10 one mm^3 cubes of collagen or elastin in 0.5 ml of chloroform in which $2 \mu\text{M}$ of PS was dissolved, and then the tissue was vacuum dried.

To determine the calcium concentration at which liposomes and RBCG calcify, the total concentration of calcium in MEM, pH 7.4, was adjusted to 1.8-2.8 mM in a 0.1 mM increment with CaCl_2 . The total P_i was adjusted to 1.3 mM with K_2HPO_4 and was left unchanged. Liposomes prepared in each flask, RBCG prepared from 1 ml of packed red cells and 5 pieces of 1mm^3 cubes of the lipid-free or PS-coated collagen and elastin were suspended in 10 ml of MEM or serum in 15 ml coned plastic tubes. The tubes were tightly screw capped and incubated at 37°C on a rotary drum for 7 days. One half of the harvested samples was filtered through nitrocellulose filters, 1 cm in diameter and $0.2 \mu\text{m}$ in pore size, rinsed with isotonic ammonium acetate, and vacuum dried.

The concentration of total calcium in the filtrate was measured by using a Calcette Autotitrator (Precision Systems, Inc., Sudbury, MA). Calcification of liposomes, RBCG demonstrable by electron probe microanalysis (EPM), and calcium depletion from MEM, began at 2.2 mM calcium and became consistent at 2.5 mM. Therefore, subsequent experiments in MEM were performed at 2.5 mM calcium.

The above mentioned amounts of liposomes, RBCG and elastin were placed in Millipore chambers (Millipore Corp., Bedford, MA). The sealed chambers were implanted in pairs, using elastin as controls, in rat peritoneal cavities and harvested in weeks 1, 2, 3 and 4. Because jelly-like deposits formed in the chambers after incubation, samples from the chambers were directly smeared on carbon planchets. Portions of the jellies were processed for transmission electron microscopy (TEM). The chambers into which host cells had entered

were discarded and the experiment was repeated.

For scanning electron microscopy (SEM) and EPM, nitrocellulose filters with filtered samples were glued onto carbon planchets with a carbon glue and sputter coated with carbon or gold. Samples were examined in an ETEC Autoscan SEM equipped with a KEVEX energy-dispersive X-ray detector and a Tracor-Northern 880 multichannel analyzer, or a JEOL 100CX analytic microscope coupled with a KEVEX detector and a Tracor-Northern 5500 multichannel analyzer. For examination in JEOL 100CX, carbon planchets with mounted samples were cut into pieces with a razor blade and a hammer to a size which would fit into the specimen holder. EPM was performed at 20 keV, a working distance of 10 mm and 45° tilt. For TEM and electron diffraction (ED), the remaining halves of the harvested MEM or serum were centrifuged at 10,000 g for 10 minutes. The resultant pellets were embedded in agar and cut into 1 mm cubes. The cubes were fixed in 4% glutaraldehyde in cacodylate buffer, pH 7.4 for 4 hours at 4°C, postfixed in 1% osmium tetroxide in s-collidine buffer for one hour, dehydrated in ethanol and propylene oxide, and embedded in Epon. Thin sections for ED were additionally carbon coated in an evaporator. ED was carried out at 80 keV, a camera length of 160 cm and a standardized exposure time of 60 seconds. The jellies obtained from Millipore chambers were processed for TEM without embedding in agar.

Since it is difficult to obtain large volumes of rat serum, the study was repeated with canine serum. Canine bloods were obtained by leg vein punctures from three dogs. Serum and right side out red cell ghosts (RORBCG) were prepared as described above for the rat RBCG preparation. Inside-out ghosts (IORBCG) were prepared by the method of Steck and Kant (1974). One ml pellet of lysed red cells was diluted in 40 ml of 0.5 mM sodium phosphate, pH 8, at 4°C. Pelleted membranes were suspended in 1 ml of 0.5 mM sodium phosphate and passed 5 times through a 27 gauge needle using a syringe. No attempt was made to remove unsealed ghosts or RORBCG from the preparation.

Canine red cell ghosts, both RORBCG and IORBCG, were incubated in 10 ml of autologous serum or MEM-2.5 as described above and were harvested on days 1, 2, 3 and 7. Samples for SEM, EPM, TEM and ED were prepared as described above. Ca^{2+} in the harvested sample filtrates was measured by using an Orion 901 Ion Analyzer (Orion Research, Cambridge, MA) coupled with a Ca^{2+} selective electrode (Radiometer America, Cleveland, OH). Phosphate was measured by the method of Itaya and Ui (1966). Five ml of a color reagent prepared by mixing 4.2% $(\text{NH}_4)_6\text{MoO}_{24}\cdot 4\text{H}_2\text{O}$ in 5N HCl and 0.2% Malachite Green (1:3/v:v) were added to 1 ml of each sample and the color developed was measured at 660 nm. The linearity was checked at each measurement with gradient concentrations of KH_2PO_4 . Measurements were made in duplicates. RORBCG incubation in MEM-2.5 was repeated with addition of 1 mM ATP in MEM.

Results

Liposomes and rodent RBCG incubated in rat-serum and MEM with calcium concentrations greater than 2.2 mM depleted calcium from the solutions and showed peaks of Ca and P by EPM in a week. Liposomes incubated in MEM-2.5 revealed solid spherular aggregates mixed with flat, collapsed lamellae by SEM (Fig. 1). Solid spherules displayed tall peaks of Ca and P by EPM, whereas flat lamellae did not (Fig. 2). TEM revealed needle shaped crystals deposited mainly on the outer surface of liposomes (Fig. 3a). Rat-RBCG incubated in MEM-2.5 showed calcific deposits on the inner surface of the membranes. The deposits in both liposomes and rat-RBCG gave a powder pattern of apatite by ED (Fig. 3b).

Liposomes and rat-RBCG incubated in serum for a week showed smaller spherules than those in MEM-2.5 and contained Ca and P by EPM. SEM appearance of liposomes and rat-RBCG incubated in serum was obscured by a coating of what appeared to be organic substances, giving a snow-covered appearance (Fig. 4). TEM of liposomes incubated in serum demonstrated smaller and discrete vesicles. Calcific deposits in both liposomes and rat-RBCG in serum were seen within their lumens. The needles within liposomes incubated in rat serum were relatively sparse. It appeared that electron lucent organic deposits had obscured some of the needles, which might account for the observation of sparse needles. In addition to needle shaped crystals, there frequently were granular particles in the lumen of liposomes and RBCG (Fig. 5 and 6). It was not possible to identify the granules by ED probably due to its limit in sensitivity. TEM also demonstrated fuzzy deposits of organic materials on the walls of liposomes and rat-RBCG.

Calcific deposits in rat-RBCG incubated in both MEM-2.5 and serum were seen mainly on the inner surfaces of RBCG (Fig. 6a). The deposits appeared to be adherent to the inner surfaces of the membranes; the deposits remained attached to the membrane in RBCG with ruptured membranes as well (Fig. 6b). Lipid-free collagen and elastin failed to calcify in serum or MEM with the highest concentration of calcium (2.8 mM), whereas PS coated collagen and elastin calcified in MEM-2.5.

Liposomes and RBCG incubated in the rat peritoneal cavities began to show peaks of Ca and P by EPM within 2 weeks. SEM and TEM morphologies were similar to the above mentioned serum incubated liposomes. However, needle shaped crystal deposition in the lumen was scanty to absent in rat-RBCG incubated in the peritoneal cavities. Instead, TEM of RBCG incubated in Millipore chambers for 3 to 4 weeks frequently demonstrated characteristic, thick walled vesicles in which needle shaped crystals were radially embedded (Fig. 7a). Although ED was unsuccessful, EPM of the spherules on both carbon planchet mounted jelly and the thick-walls identified by TEM yielded peaks of Ca and P (Fig. 7b).

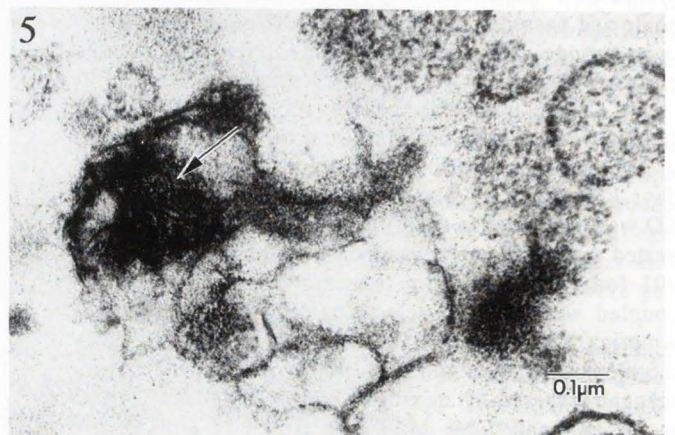
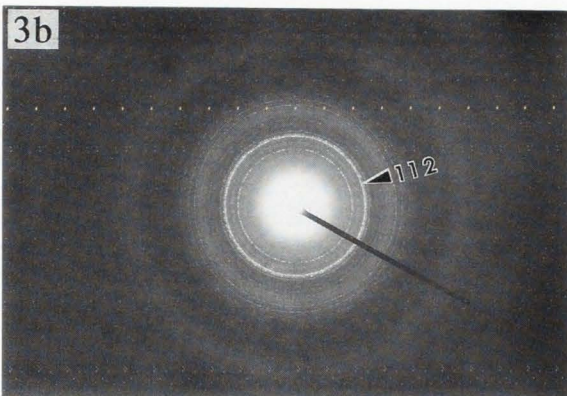
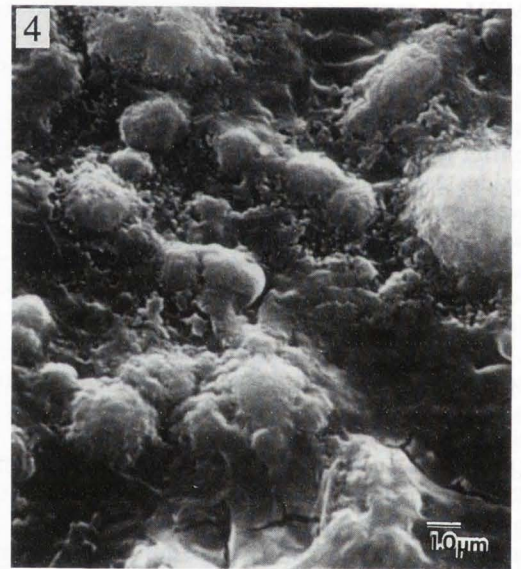
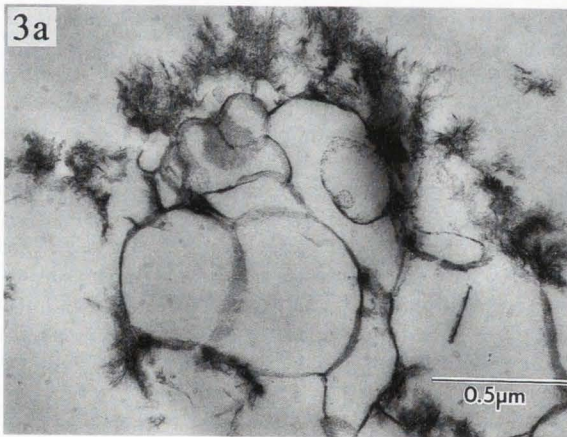
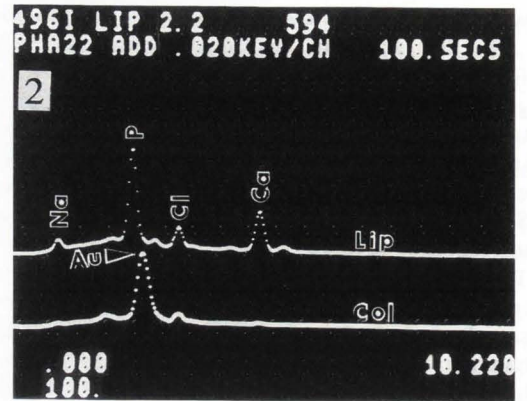
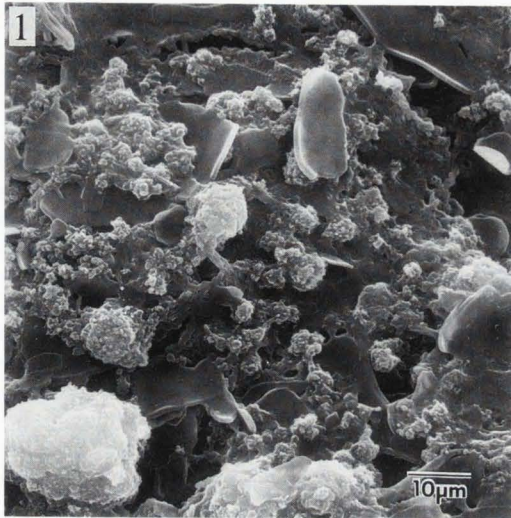


Figure 1. Scanning electron micrograph of liposomes incubated in MEM-2.5 for a week. Spherular, brighter aggregates of liposomes yielded tall peaks of Ca and P by EPM. Flat, collapsed lamellae did not contain Ca or P.

Figure 2. EPM of liposomes incubated in MEM with 2.2 mM calcium (upper spectrum) yielded peaks of Ca and P, whereas collagen incubated with 2.8 mM calcium for a week (lower spectrum) did not.

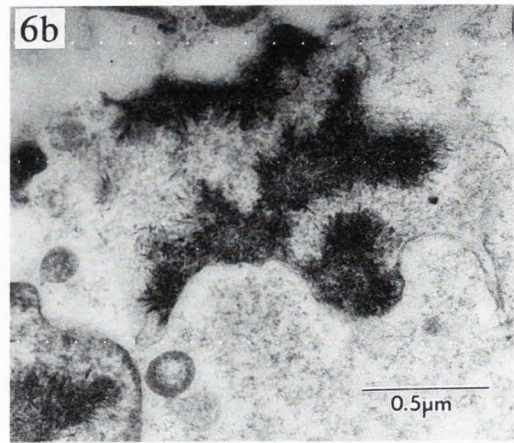
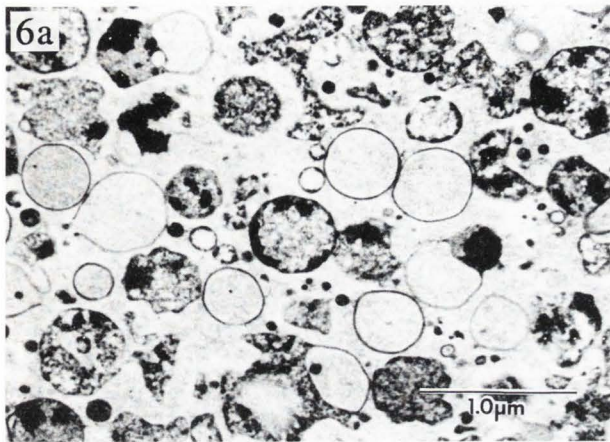


Figure 6. a (above). Transmission electron micrograph of rat-RBCG incubated in homologous, pooled serum for a week. Calcific deposits are seen on the inner surface of the membrane. RBCG frequently contain granular deposits. **b (above, right).** Needle shaped crystal deposits on the inner surface of a ruptured RBCG. Needles remain attached to the membrane.

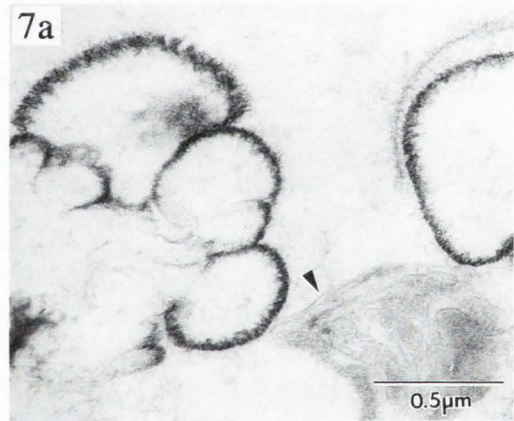
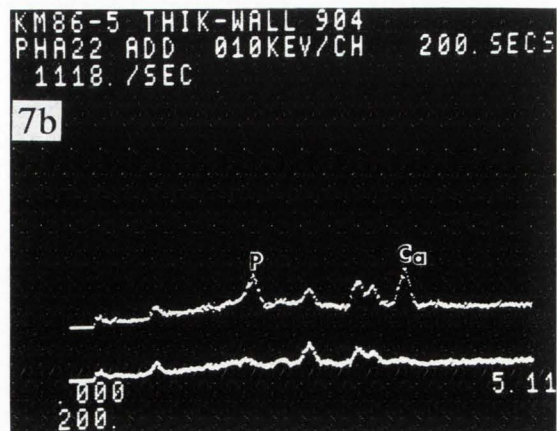


Figure 3 (facing page, bottom left). Transmission electron micrograph (3a) of liposomes incubated in MEM-2.5 for a week. Crisp needle shaped apatite crystals (ED pattern of needles, 3b) in the "hair on end" fashion are seen mainly on the outer surface of liposomes.

Figure 4 (facing page, right column). Scanning electron micrograph of rat-RBCG incubated in serum for a week. Spherular RBCG are covered by organic material. The larger and brighter spherules yielded taller peaks of Ca and P than smaller ones.

Figure 5 (facing page, right column). Transmission electron micrograph of liposomes incubated in rat serum for a week. Needle shaped crystals are seen mainly in the lumen (arrow). The needles appear obscured by what appears to be organic deposits. There are granular deposits in adjacent liposomes.



Liposomes incubated in millipore chambers did not form such thick-walled vesicles.

Canine RORBCG incubated in MEM-2.5 or autologous serum progressively depleted Ca^{2+} and P_i from the solutions beginning in day 1 (Fig. 8). SEM and TEM morphologies of canine RORBCG were similar to that of rat-RBCG. EPM detected small peaks of Ca and P on day 1 in both MEM-2.5 and serum incubation specimens and the peaks grew taller with longer incubations (Fig. 9). Needle shaped crystal deposits demonstrable by TEM began to appear after 3 days of incubation. Slight differences between RORBCG and IORBCG were noted in their rate of Ca^{2+} and P_i depletions from MEM-

Figure 7. a. Rat RBCG in a Millipore chamber implanted in the rat peritoneal cavity for 4 weeks. RBCG developed into thick-walled vesicles in which needle shaped crystals are radially embedded. Membranes of normal thickness are seen in the vicinity (arrowhead). **b.** EPM of the thick wall seen by TEM in a thin section. The lower spectrum is obtained from the adjacent background.

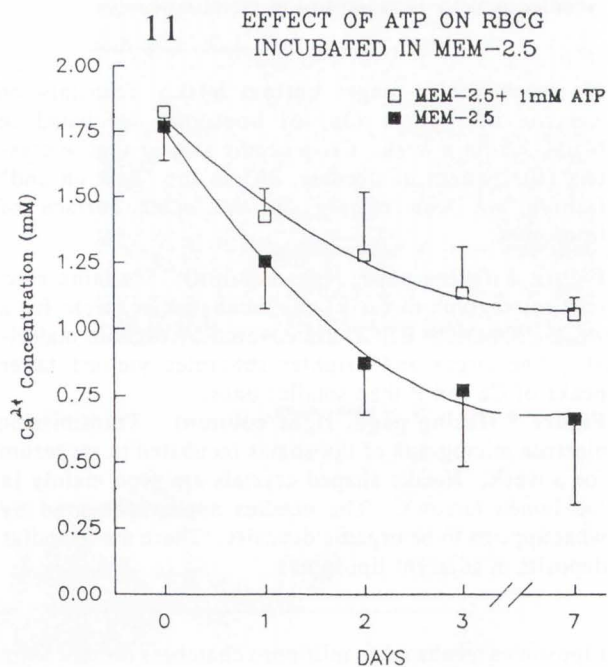
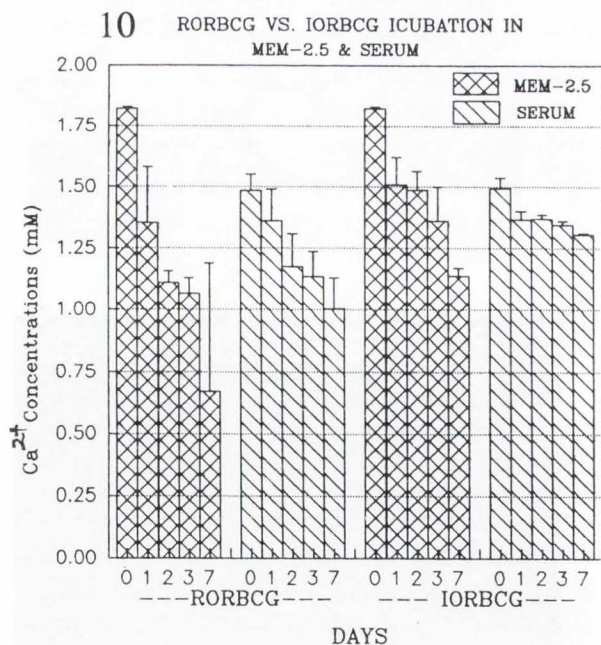
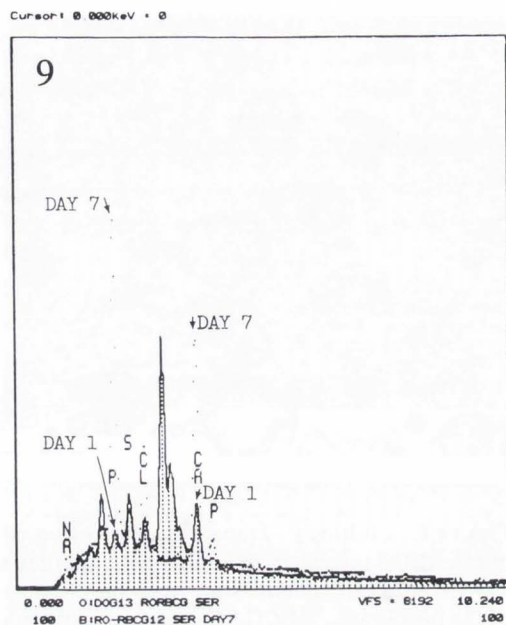
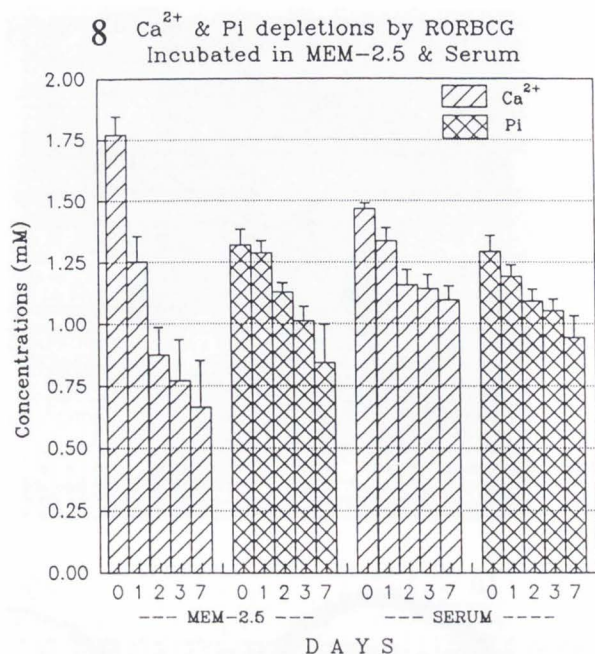


Figure 8. Depletions of Ca^{2+} and P_i from MEM-2.5 and serum by canine RORBCG. Note that Ca^{2+} concentration in MEM-2.5 at 0-time is higher than in serum. The depletions appear to have occurred at a faster rate in MEM-2.5 than in serum.

Figure 9. EPM of canine RORBCG incubated in autologous serum. Small peaks of Ca and P developed in day 1 (lower spectrum) and grew taller in day 7 (upper spectrum). Note little changes in other elements.

Figure 10. A comparison of Ca^{2+} depletions by canine RORBCG and IORBCG in MEM-2.5 and serum. Little difference was noted.

Figure 11. Canine RORBCG incubated in MEM-2.5 with (upper curve) and without 1 mM ATP (lower curve). The rate of Ca^{2+} depletion by RBCG was slower with added ATP.

2.5 or serum (Fig. 10). The purpose of IORBCG experiment was to see if calcific deposits occur on the outer surfaces (the original inner surfaces) of the ghosts. However, IORBCG incubated in both MEM-2.5 and serum formed aggregates of multilamellar curlicues. It was not possible to determine the loci of calcification by TEM. Addition of 1 mM ATP to MEM-2.5 incubation of RORBCG resulted in a decrease in the rate of Ca^{2+} depletion but showed little effect on the detectability of Ca and P by EPM (Fig. 11).

Discussion

Calcification of both liposomes and rat-RBCG in MEM-2.5, serum and millipore chambers incubated in the peritoneal cavities can be taken as definitive evidence that PL in membranous conformations are capable of promoting apatite formation under physiological conditions. Calcification of PS-coated collagen and elastin further substantiates the role of PS in calcification. The harsh treatment of collagen and elastin during their extractions and delipidizations can hardly be considered physiological. Nevertheless, the inability of these tissues to calcify under the conditions in which liposomes and RBCG calcified demonstrates that PL have an inherent ability to initiate apatite formation presumably by heterogeneous nucleation.

The deposition of needle shaped apatite in the "hair on end" fashion on the outer surface of the liposomes in MEM-2.5 suggests nucleation of apatite by heterogeneous nucleation. A delay of a few days in the appearance of calcific deposits detectable by TEM compared to EPM of serum incubated RORBCG suggests that early calcific deposits have been dissolved during the specimen preparation for TEM.

It is interesting that liposomes incubated in rat serum gave rise to intraliposomal calcification in contrast to those incubated in MEM-2.5. Ca^{2+} and P_i have apparently crossed the liposome membrane, whereas calcification inhibitors, e.g., proteins, are likely to have been blocked by the membrane. Certain PL have been shown to be ionophoric for calcium, and PS is very weakly so (Tyson *et al.*, 1976). Serum and the peritoneal fluid must have an ionophoric effect on the liposome membrane. For instance, insulin has been shown to cause transport of considerable amounts of Ca^{2+} and Mg^{2+} across liposome membranes made of phosphatidylcholine (PC) (Brimble and Ananthanarayanan, 1992). The paucity of the needles on the outer surface of serum incubated liposomes can be attributed to the presence of inhibitors of calcification in serum (Blumenthal, 1989; Nancollas and Zawacki, 1989; Garnett and Dieppe, 1990). Granular deposits within serum incubated liposomes may be a form of partly dissolved calcific deposit. Similar granular deposits were frequently observed in human dystrophic calcifications (Kim and Huang, 1971; Kim, 1978, 1983b).

Other investigators found that when P_i was trapped within liposomes and the liposomes were suspended

in a metastable solution without added ionophore, extraliposomal calcification took place (Skrtic and Eanes, 1992a). It was thought that the apatite formed within these liposomes penetrated through the membrane followed by their extraliposomal growth. When liposomes made with PS and without PS were compared, Ca^{2+} depletion from the solution was greater with liposomes without PS (Skrtic and Eanes, 1992b). These results were attributed to a PS-induced adherence of the outer liposome membrane to developing crystals thus restricting the availability of apatite (and perhaps PS) to the solution (Eanes and Hailer, 1987; Skrtic and Eanes, 1992b). In contrast, PC or other PL may be as effective as PS for the nucleation of apatite.

The difference between serum versus MEM-2.5 incubation of RBCG was not as conspicuous as in liposomes; in both, calcific deposits were seen on the inner surface of RBCG. A down-gradient influx of Ca^{2+} and P_i into sealed RBCG must have taken place to give rise to this calcification. Ca^{2+} entry into normal red cells has been shown to occur through Ca^{2+} -channels like in other cells (Engelman and Duhm, 1987). Red cells appear not to possess the $\text{Na}^+/\text{Ca}^{2+}$ -exchanger (Ferreira and Lew, 1977).

In contrast to eukaryotic cells in which P_i entry occurs via a Na^+/P_i cotransporter, P_i entry into red cells occurs through a Cl^-/P_i exchanger (Werle and Pederson, 1989). Some P_i entry through the exchanger of the RBCG membrane is likely to persist. Selective transport of Ca^{2+} and P_i and blockage of inhibitors by the RBCG membrane is an unlikely mechanism. Unsealed RBCG calcified as rapidly as sealed RORBCG (results not shown).

Phospholipids are asymmetrically distributed across the plasma membrane (Op den Kamp, 1979). PL asymmetry is maintained by ATP-dependent enzymes, flippases (Devaux, 1988). The presence of PS on the inner surface of MV, as seen in normal plasma membranes, was believed to be responsible for calcific deposits occurring on the inner surface of MV. However, it has recently become apparent that increased cytosolic Ca^{2+} and loss of ATP rapidly abolish the asymmetry of all major phospholipids in RBCG (Schrier *et al.*, 1992; Williamson *et al.*, 1992). Therefore, calcific deposits observed on the inner surface of RBCG can no longer be attributed to the presence of PS on the inner surface of the membrane. However, similar calcifiability of liposomes and RBCG under physiological conditions in this study supports the theory that PL are a likely candidate for an apatite nucleator. Recently, annexins, a group of proteins which glue cytoskeletal proteins to PL of the membrane, have been implicated as a potential mechanism for MV calcification (Genge *et al.*, 1992).

The nature of the thick-wall formation by RBCG incubated in the peritoneal cavities is obscure. Remarkably similar thick-walled vesicles in which apatite crystals were embedded were frequently observed in human dystrophic calcifications (Kim, 1976, 1983b) and decalcified urinary stones (Kim, 1983c). In view of the

thick-wall formation by RBCG but not by liposomes, it appears that intrinsic proteins of the cell membrane(s) are involved in the formation of the thick-wall. The mechanism of calcification induced by cell membranes is apparently not limited to the function of their ion transports or heterogeneous nucleation of apatite on their surfaces.

The most significant finding in this study is that RBCG calcified in the autologous serum in which they previously resided. This indicates that normal red cells are capable of preventing calcification in that same serum. Emptying the cell content evidently eliminates the mechanism of preventing calcification by normal red cells. Loss of Ca^{2+} -pump activity secondary to the removal of ATP is likely to be a major factor in RBCG calcification.

Calcium is mainly an extracellular ion and P_i an intracellular ion. Through the action of ATP-fueled Ca^{2+} -pumps, cytosolic Ca^{2+} ($[\text{Ca}^{2+}]_i$) is maintained at an extraordinarily low level (Carafoli, 1987). Probably due to the lack of $[\text{Ca}^{2+}]_i$ -buffering organelles, i.e., mitochondria and endoplasmic reticulum, $[\text{Ca}^{2+}]_i$ in normal red cells is higher than in other cells and is estimated to be 400-700 nM (Simons, 1982). Intracellular P_i ($[\text{P}_i]_i$) in red cells is estimated to be 1-10 mM (Bevington *et al.*, 1986). By compartmentalization of the two major ions needed for calcification across the plasma membrane, calcification is normally prevented (Kim, 1983b; Kretsinger, 1990). It is, therefore, tempting to speculate that the elimination of the barrier between the two major ions across the RBCG membrane has equilibrated $[\text{Ca}^{2+}]_i$ and $[\text{P}_i]_i$ with those in the serum and, in turn, resulted in calcification. The elimination of inhibitors, e.g., Mg^{2+} , ATP and pyrophosphate, which are normally present in the cytosol, is likely to have augmented RBCG calcification as well. The exposure of the inner surface of RBCG membrane, which has been adapted to an extraordinarily low $[\text{Ca}^{2+}]_i$, to serum concentrations of Ca^{2+} and P_i apparently is sufficient enough to bring about apatite nucleation.

Hydrolysis of ATP by ATPases of MV membrane has been postulated to increase P_i in MV and bring about calcification (Hsu, 1992; Kanabe *et al.*, 1983). Extracellular ATP has been shown to increase the permeability of the plasma membrane of susceptible cells (Gordon, 1986). The lack of a promotive effect by external ATP on RBCG indicates that RBCG do not respond to external ATP. The reason for a slight decrease in depleting Ca^{2+} and P_i by RBCG incubated in MEM-2.5 with added ATP needs further clarification. A condensed layer of Ca^{2+} forms on the outer surface of the plasma membrane by an electric attraction of Ca^{2+} by PL. Neutral PL as well as acidic PL attract Ca^{2+} (Seelig, 1990; Tatulian, 1987). The concentration of Ca^{2+} in the condensed layer is estimated to be two orders of magnitude greater than the surrounding solution and appears to increase exponentially in relation to Ca^{2+} concentration in the solution (Seelig, 1990). Calcium binding appears to occur on the inner surface of liposomes when they are

made permeable to Ca^{2+} by an ionophore, X537A (Eanes *et al.*, 1984).

It is likely that the layer of Ca^{2+} condensation is absent from the inner surfaces of normal plasma membranes due to an extraordinarily low $[\text{Ca}^{2+}]_i$ and PL asymmetry. In view of the reversal of PL asymmetry in RBCG with increased $[\text{Ca}^{2+}]_i$ and loss of ATP, the formation of a similar layer of Ca^{2+} condensation on the inner surface of RBCG is likely. There is evidence that Ca^{2+} binding to the inner surface of RBCG indeed occurs (Long and Mouat, 1971). Although PL are likely to be the structures of the membrane which nucleate apatite, the mechanism of the selective occurrence of calcification along the inner surface of RBCG remains to be elucidated.

Conclusions

This study presents evidence that the plasma membrane of red cells calcifies in the autologous serum from which the membrane has been isolated. The inner surface of the plasma membrane appears to have an inherent ability to nucleate apatite when it is exposed to serum concentrations of Ca^{2+} and P_i . Calcification is apparently prevented by compartmentalization of Ca^{2+} and P_i across the plasma membrane of normal cells by the action of ATP-driven ion pumps. Devitalization of red cells by removing cytosolic contents abolishes the capacity of the red cells to prevent calcification. Identification of the loci of calcification on the inner surface of the plasma membrane is likely to open a new avenue in the field of research on calcification. Loss of ATP and, in turn, of the Ca^{2+} -pump activity is likely to be a key mechanism in calcification.

In view of the documented scrambling of PL asymmetry resulting from increased $[\text{Ca}^{2+}]_i$ and nucleation of apatite by liposomes made of PC, the current concept that PS is primarily responsible for calcification occurring on the inner surfaces of MV membranes should be reevaluated. The theorized formation of a zone of Ca^{2+} condensation by electrostatic attraction on the inner surface of RBCG, resulting from the scrambling of PL asymmetry and Ca^{2+} influx, is likely to have a role in calcification.

Calcification of RBCG is similar to that of MV and dystrophic calcification in humans. In view of their ready accessibility, RBCG may serve as an ideal model with which to study the mechanism of calcification.

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Discussion with Reviewers

I.F. Miller: The low contrast areas within the crystal clusters seen in Figure 3a seem to reveal crystals with a flattened or platy aspect. Could the "needle-like" images in these clusters be platy crystal seen on edge on?

Author: Geological apatite and dental enamel apatite are known to be of plate shaped habit. Plate shaped crystals are occasionally seen in soft tissue calcification as well. It is possible that some of the needles in this observation may be side profiles of plates. However, well formed plates were not seen in this study.

I.F. Miller: Since the liposomes were prepared in the same Hank's medium, why should the liposomes be smaller in serum than in MEM-2.5? Also in serum, the crystals appear to be in more intimate contact with the liposomal membrane than in MEM. This closer association would indicate tighter crystal-membrane bonding.

Would the author comment on this and why it occurs?

Author: The larger size of liposomes in MEM-2.5 than in serum was mainly due to more frequent aggregation of liposomes in MEM-2.5 than in serum. As for the adherence of apatite crystals to the membranes, it appeared to be more conspicuous in MEM-2.5 than in serum. Crystals in Figure 3a probably obscure the liposome membrane to which they are attached. Loose crystals, if any, are likely to have been washed away during the sample preparation. More significant is that calcification in serum incubation took place within liposomes.

I.F. Miller: Despite the large errors in some of the measurements, the trend appears for RORBCG to deplete Ca²⁺ and P_i faster than IORBCG.

A.L. Boskey: It appears that RORBCG in MEM-2.5 depleted Ca at a rate much faster than IORBCG, and that rates in serum were slower than those in MEM.

Author: Apatite in a metastable solution proliferate autocatalytically. The rate of ion depletions observed in this study mainly reflects crystal proliferation and thus serves as a supplementary means to determine the occurrence of calcification. It was not meant to study the kinetics of crystal nucleation and growth. The slower depletion of Ca²⁺ and P_i from serum than in MEM-2.5 is attributable to the presence of inhibitors and a lower Ca²⁺ concentration in serum. Multilayering of IORBCG is likely to have limited free membrane surfaces exposed to the solution. A preliminary study suggests that the multilayering is Ca²⁺ and/or Mg²⁺ dependent. It is difficult to attach a quantitative significance to the difference between RORBCG and IORBCG at this stage. Despite the multilayering, IORBCG calcified.

I.F. Miller: Although the data appear to support a calcifying role for PL in the author's experimental solutions, the data do not necessarily imply a similar role *in vivo*, only that such a role is theoretically possible. Other factors such as non-collagenous protein/collagen associations may, in actuality, be more important in collagenous calcifications.

Author: I agree with the comment. However, calcification of liposomes and RBCG in serum raises a possibility that membranes may calcify *in vivo*. Collagen *in vivo* is complexed with proteoglycans and lipids, and perhaps other macromolecules. When these were removed, collagen did not calcify in MEM-2.5 and rat serum. Isolated proteoglycans have been shown to inhibit calcification.

I.F. Miller: Could the delay in depleting Ca²⁺ and P_i by RBCG in the presence of ATP have been due to the intrinsic inhibitory effect of ATP itself on calcification?

A.L. Boskey: ATP in solution inhibits hydroxyapatite growth and proliferation; this effect cannot be discarded.

Author: ATP has been shown to inhibit transformation of amorphous calcium phosphate to apatite in aqueous solutions and thus inhibit apatite formation. It is possible that such inhibition took place in this experiment. In

order to act upon intra-RBCG calcification, ATP must enter RBCG where calcification took place. To my knowledge, such entry of external ATP into the cell has not been documented. However, ATP evidently passes freely across the mitochondrial membranes. Possible effects of extracellular ATP to ion transports across the plasma membrane should also be considered.

Reviewer II: What is the role of membrane zeta potential on the calcification process?

Author: Determination of zeta potential of plasma membranes is one of the methods with which Ca^{2+} condensation on the membrane surface is measured. Recent advent of nuclear magnetic resonance (NMR) techniques further confirms the occurrence of Ca^{2+} condensation. To my knowledge, the role of membrane zeta potential on calcification has not been tested. As discussed in the text, neutral phospholipids, i.e., PC, are as effective as PS in attracting Ca^{2+} (Seelig, 1990; Tatulian, 1987) and inducing calcification (Skrtic and Eanes, 1992b). The zeta potential of crystal surfaces has been extensively studied for its role in crystal growth.

A.L. Boskey: The results of this study contradicts those of Eanes and others in that they conclude that free PS blocks hydroxyapatite formation. It is later concluded that other phospholipids may be equally important, a finding that is not in conflict with the literature.

Author: Calcification of MV which occurs predominantly in the lumen of MV has been attributed to the presence of PS on the inner surface of the membrane. This is the reason that PS was used in this study. The results show that PS-liposome can induce calcification under a near physiological condition. However, studies by Eanes' group indicate that PC may be more effective in promoting calcification in a metastable solution. We have made limited studies on liposomes made of several other PL including PC. Liposomes made of PC calcified in MEM-2.5 and rat serum. One of the problems in the studies of calcification is the lack of uniformity in the composition of metastable solutions used. Most laboratories use solutions of their own choice. This makes correlation of the results difficult. It is significant that liposomes calcified in serum in this study.

A.L. Boskey: What per cent of lipid in calcified tissues corresponds to extracellular MV? This can be calculated from Wuthier's early fractionation.

Author: It was not measured. As opposed to MV in the epiphyseal cartilage, membranous vesicles which calcify in vascular and other soft tissues are morphologically complex. Furthermore, adjacent cells usually demonstrate considerable structural changes which are likely to affect the profile of extracted lipids. The concept of MV may not apply directly to all calcifying vesicles.

A.L. Boskey: Liposome mediated calcification may occur by a different mechanism. The liposome serves as a "protected environment" in which Ca and P can accu-

mulate free from inhibitors in the surrounding matrix. Thus it is not that the liposome contains nucleators, but rather that it contains a high Ca^{2+} concentration. Can your observations be more easily explained in terms of this concept?

Author: Deposition of needle shaped apatite on the outer surface of liposomes in MEM-2.5 suggests that liposomal membranes serve as a substrate for heterogeneous nucleation of apatite. Calcification occurring within the lumen of liposomes in serum was a surprise finding. As for Ca^{2+} and P_i concentrations within liposomes and RBCG, an active accumulation of these ions against concentration gradients is theoretically inconceivable. For occasional observations of accumulation of a greater than expected amount of Ca^{2+} by MV or other membranous vesicles require a careful interpretation. The possibility of forming a condensed layer of Ca^{2+} on the inner surface of the membrane should be taken into account. An entry of excess amounts of Ca^{2+} has indeed been observed in ionophore-treated liposomes and RBCG (Eanes *et al.*, 1984; Long and Mouat, 1971). Such bound calcium is unlikely to raise the ionic Ca^{2+} concentration within liposomes and RBCG. There is no known mechanism by which membranous PL can increase ions against their concentration gradients. Energy dependent Ca^{2+} accumulation occurs in mitochondria and endoplasmic reticulum where the direction of Ca^{2+} -pumps is reversed. Plasma membranes of live cells pump Ca^{2+} outward. Concerted functions of the membranes maintain a very low $[\text{Ca}^{2+}]_i$ and a higher concentration of $[\text{P}_i]_i$ within the cell. Therefore, an equilibration of ions across the plasma membrane is possible only when cytosolic ATP is lost or ion pump activities are blocked. The depletions of Ca^{2+} and P_i in MEM-2.5 and serum is mainly attributable to continuous growth and proliferation of apatite. Since addition of liposomes to MEM-2.5 and serum causes calcification, membranous PL apparently can initiate apatite formation presumably by heterogeneous nucleation. The possible role of other components of the complex cell membranes in calcification remains to be determined.

A.L. Boskey: Why was ammonium acetate used to wash the materials?

Author: Washing samples with water tends to form droplets in which ions may concentrate and result in artificial deposits upon drying. Ammonium acetate is hydrophilic and volatile, therefore, thought to minimize artifactual deposits formed during sample drying. It is frequently used in sample preparations for EPM.

A.L. Boskey: Calcification induced by lipids, liposomes, or other materials is a function of concentration inside the membrane, phosphate concentration outside the membrane, as well as Ca concentration and pH. How did these vary in your experiments? Will that variation account for some of the observation?

Author: No Ca^{2+} was added to the inside of liposomes. This is the reason that calcification of liposomes in

MEM-2.5 occurred on their outer surfaces. When liposomes were filled with and suspended in MEM-2.5, intraliposomal calcification was frequently observed (results not shown). The major problem with *in vitro* experiments is that ion concentrations can not be maintained homeostatically as *in vivo*. A computer controlled steady state of ion concentrations by continuously supplementing consumed ions has been used in crystallization studies. However, the device is expensive and it is very difficult to control bacterial contamination in it. To compensate for the problem, a relatively large volume of MEM-2.5 and sera were used in this study. With regard to pH changes, apatite formation is known to acidify the solution. Some minimal pH changes in the later stage of calcification was noted in this study. When MEM-2.5 was additionally buffered with 20 mM HEPES, no significant difference in calcification of liposomes and RBCG was noted. The initial pH of the solution is most important since pH can affect nucleation and growth of apatite.

A.L. Boskey: The extent of mineral deposition should be discussed in terms of the Ca accumulated per mole of lipid, at least per volume, and not in terms of qualitative EM; quantitative chemical data can then be discussed.

Author: The surface area of liposomes rather than the amount of PL would be of a greater significance in liposome calcification. As discussed above, the size of liposomes can vary significantly and they frequently aggregate. For a meaningful quantitation of PL in relation to apatite, liposome titration would be in order. Dynamic physicochemical phenomena at the solid (liposome)-solution interphase involved in calcification is beyond the scope of this study. In the author's opinion, the deposition of needle shaped apatite on the surface of liposomes in the "hair on end" fashion observed by EM is more significant since it indicates apatite formation by heterogeneous nucleation. The purpose of this study was to see liposomes and RBCG calcify under near physiological conditions. The results show that they can.

A.L. Boskey: It is important to distinguish free PS and Ca-PS-P₁-proteolipid complexes (CPLX) when discussing their role in promoting calcification. PS can retard apatite growth due to its binding to mineral crystals, CPLX has a lower affinity and does not have such an inhibitory effect. The conformation and status of PL may be as important as their identity.

Author: MEM contains essential amino acids but no complex hydrophobic proteins, that are the component of proteolipids. Having an affinity for apatite, PS has been shown to inhibit apatite growth by coating the crystal surface, whereas CPLX promotes apatite growth [see Boskey AL, Dick BL (1991). The effect of phosphatidylserine on *in vitro* hydroxyapatite growth and proliferation. *Calcif. Tiss. Int.* **49**: 193-196]. Although no specific attempt was made, apatite crystals attached parallel to the liposomal surface was not seen in this study. PS may play an inhibitory role in earlier stages of apatite growth. Liposomal PS is unlikely to envelope vertically oriented apatite on the liposomal surface. The capacity of autocatalytic growth of apatite evidently exceeds any growth inhibition in MEM-2.5. Inhibition of apatite growth by PS is likely in the thick-walled vesicles. It will be interesting to know if the thick-wall contains CPLX and/or proteolipids.