Scanning Microscopy

Volume 9 | Number 4 Article 19

9-19-1995

Apoptosis and Calcification

Kookmin M. Kim
Louisiana State University Medical Center

Scanning Microscopy, Vol. 9, No. 4, 1995 (Pages 1137-1178) 0891-7035/95$5.00+.25
Scanning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA

APOPTOSIS AND CALCIFICATION

Kookmin M. Kim

Department of Pathology, V. A. Medical Center and LSU Medical Center,
510 Stoner Avenue, Shreveport, Louisiana 71101-4295, USA.

(Received for publication May 3, 1995 and in revised form September 19, 1995)

Abstract

Calcification in necrosis has long been known. Of the tissue components, the cells are most vulnerable. Nevertheless, little attention has been paid to the role of cell death in calcification. This review attempts to update the mechanism of calcification with an emphasis on the role of apoptosis in calcification. A brief review on the basic sciences relevant to calcification is followed by a discussion of abnormal Ca^{2+} and P_i homeostasis in cell injury and apoptosis. Concomitant increases in Ca^{2+} and P_i in blebs (and matrix vesicles) formed by apoptotic and/or necrotic cells are apparently the primary mechanism of calcification. In addition, membranous cellular degradation products (CDP) resulting from cell disintegration *in toto* frequently serve as the nidus of calcification. Published data on physiological calcification are compared with findings in various dystrophic calcinoses. This led to the conclusion that apoptosis most likely underlies the mechanism of both physiological and pathological calcifications. It is concluded that calcification is an important function of apoptosis. The mechanism of calcification by CDP and morphology of the resultant calcific deposits are complex.

Key Words: Cell death, Cellular degradation products, blebbing, matrix vesicles, calcium phosphate crystals, phospholipids, Ca^{2+} and P_i metabolism, cell aging, vascular calcinosis, neoplastic calcinosis, nephrocalcinosis, psammoma bodies, stones.

*Address for correspondence:
Kookmin M. Kim
Department of Pathology,
V. A. Medical Center,
510 Stoner Avenue,
Shreveport, Louisiana 71101-4295, USA.
Telephone number: 318-221-8411 ext. 6276
FAX number: 318-424-6093

Introduction

A variety of functions from development, growth, tissue remodeling, differentiation, clonal selection in the immune system, cell aging to oncogenesis has been ascribed to apoptosis. This review appends an additional function to apoptosis, i.e., calcification.

It has long been known that calcification follows tissue necrosis (Virchow, 1855). Of the tissue components, the most vulnerable are the cells. Nevertheless, little attention has been paid to the role of cell death in calcification. Recent advances on the mechanism of cell death indicate that the loss of ion regulation in cell death may play the primary role in calcification. Widespread occurrence of apoptosis may account for the common incidence of calcification in various tissues.

Cell death is currently classified under apoptosis and necrosis. Programmed cell death in developing vertebrates has been known for several decades. On the basis of morphological characteristics, Kerr *et al.* (1972) introduced the concept of apoptosis as naturally occurring cell death that plays an opposite role to mitosis in regulation of the cell population. Apoptotic cells are said to display cell shrinkage, nuclear chromatin condensation and blebbing of the plasma membrane, whereas necrotic cells show cell swelling (Arends and Wyllie, 1991). However, detailed observations revealed a considerable overlap in morphological changes between the two (Clarke, 1990).

Programmed cell death has been best characterized in a nematode, *Caenorhabditis elegans*, mediated primarily by ced-9 and other gene products. It subsequently became apparent that suppression of apoptosis in mammals is mediated by bcl-2, their equivalent of ced-9. These observations ignited an explosion of data in the field. In addition to the opposing effects of bcl-2 and p53, proto-oncogenes, especially c-myc, and cytokines, e.g., transforming growth factor beta (**TGF-β**), have been shown to be essential for apoptosis in certain types of cells (see, Stewart, 1994). Of the changes described in apoptosis, DNA fragmentation, that gives the laddering pattern on agar gel and peripheral chromatin condensation, are generally construed as the hallmark for apop-

tosis (Arends *et al.*, 1990; Arends and Wyllie, 1991). However, the reliance on these criteria resulted in a confusion as to what constitutes apoptosis (Farber, 1994). For instance, cell death due to a variety of cell injuries, i.e., radiation and chemotherapy to malignant neoplasms, are commonly included in apoptosis. Apoptosis does not occur through a uniform pathway (Evans, 1993). Furthermore, changes characteristic of apoptosis can occur without DNA fragmentation (Cohen *et al.*, 1992; Ucker *et al.*, 1992), in interphase nuclei incubated in a mitotic extract (Nakagawa *et al.*, 1989), anoxic injury to myocardiocytes (Tanaka *et al.*, 1994), okadaic acid treated hepatocytes (Boe *et al.*, 1991), normal kidneys (Enright *et al.*, 1994), and exposure of the cells to a low temperature (Liepins and Younghusband, 1985). The extent of these changes occurring in non-apoptotic conditions remains to be determined. The process of cell death is complex and likely to affect practically every aspect of cell functions. Although the distinction between apoptosis and programmed cell death has been proposed (Schwartz *et al.*, 1992), they are usually considered synonymous (Stewart, 1994). It will be quite some time before a clear concept of apoptosis is outlined. This review is not intended for an in depth discussion of apoptosis but for an establishment of the linkage between cell death, both apoptosis and necrosis, and calcification. Naturally occurring cell death without notable cause, as seen in the epiphyseal cartilage, aging vascular connective tissue and microscopic "tumor necroses", are considered apoptotic in this review.

Basic sciences relevant to calcification and calcium and phosphate homeostasis in relation to cell death are briefly reviewed. Published data on physiological calcification are compared with calcification resulting from apoptosis in human. This leads to the conclusion that apoptosis underlies the mechanism of both physiological and pathological calcifications.

It is also aimed to update the mechanism of calcification with an emphasis on the role of membrane related phenomena during cell death. For brevity, attempts were made to cite recent reviews instead of the original articles. For the potential role of extracellular matrix in calcification, readers are referred to other reviews, e.g., see, Boskey (1992).

Ion activity products and calcification

As ion concentrations increase in a solution, the incidence of collisions between solubilized ions, e.g., Ca^{2+} and phosphate ($\mathbf{P_i}$), increases. The ions form clusters of critical size, or nuclei. Addition or deletion of one more molecule to or from the nucleus will lead to crystallization or dissolution of the nucleus. Once the nucleus is formed, the subsequent growth of crystal occurs very rapidly (Boistelle, 1986; Nancollas, 1976). The nuclear size for each crystal varies with the degree of solute concentration; the greater the concentration, the smaller the sizes of the nuclei and the higher the nucleation frequency (Boistelle, 1986).

Nucleation of a crystal solely dependent upon saturation of solutes is said to be **homogenous**. Addition of solid particles to the solution catalyzes nucleation, causing **heterogenous nucleation**. The surface of the solid serves as a substrate for heterogeneous nucleation by reducing the size of the nucleus and the energetic barrier between the nucleus and the solution. Fewer molecules are required for a nucleus to reach the critical size on the substrate than the number needed in homogeneous nucleation. The closer the surface structure of the substrate is to structure of the crystal surface, the better substrate the solid will be (Boistelle, 1986). Thus, apatite is the best substrate for apatite nucleation. In view of the abundance of solids in tissues, homogenous nucleation is unlikely to take place *in vivo*. However, the ideal substrate for heterogeneous nucleation has yet to be identified.

Mammalian extracellular fluid (**ECF**) is metastable with respect to calcium hydroxyapatite (**apatite**). In isolated serum, apatite nucleation does not occur but addition of apatite seed to serum results in a rapid proliferation of apatite, reaching a new equilibrium between apatite and serum at 1/3 of the original concentrations of Ca^{2+} and P_i (Neuman and Neuman, 1958). This finding raises two critical issues; apatite nucleation *in vivo* occurs in a seemingly **undersaturated** solution and the formed apatite is maintained in an **oversaturated** solution. Through a delicate balance of the degree of supersaturation in ECF, it is evidently possible to induce calcification by certain local mechanism(s) and to confine the deposits to calcified tissues. As is the case with many biological phenomena, there is not yet a clear definition of calcification. With the current lack of knowledge, calcification usually refers to nucleation and growth of apatite. The maintenance of apatite in calcific deposits in disequilibrium with ECF has rarely been dealt with.

In addition to ion concentrations, many factors, i.e., temperature, pH, ionic strength, impurities, etc. affect crystal formation. A number of promoters or inhibitors of apatite nucleation are known to exist in tissue fluids. Nucleation, growth and proliferation of crystals are discontinuous processes and are affected by different sets of promoters and inhibitors (Termine, 1972). Crystal nucleation requires an induction time, which also depends on the degree of supersaturation (Nancollas, 1976). In general, newly formed crystals need to mature in order to yield recognizable X-ray diffraction patterns. This is one of the reasons that apatite in its early stage of formation is difficult to detect.

Table 1. Interplanar spacings (dA) of 3 different crystals determined by electron diffraction*.

hkl	dA[1] CO_3AP	dA Porous	hkl	dA[2] (OH)AP	dA Needles	hkl	dA[3] $Ca_3(PO_4)_2$	dA Solid
002	3.46	3.45	111	3.88	3.88	104	6.49	6.46
102	3.17	3.19	002	3.43	3.44	110	5.21	5.20
210	3.04	3.04	210	4.04	4.06	202	4.39	4.40
112	2.78	2.78	211	2.81	2.82	024	4.06	4.12
300	2.68	2.70	202	2.63	2.66	10$\underline{10}$	3.45	3.48
202	2.62	2.64	301	2.49	2.53	214	3.21	3.22
310	2.231	2.26	212	2.29	2.30	217	2.88	2.89
222	1.929	1.94	310	2.25	2.26	128	2.757	2.76
213	1.838	1.83	203	2.00	2.01	220	2.607	2.62
004	1.736	1.71	213	1.76	1.75	21$\underline{10}$	2.52	2.50

*Line intensities that were taken into account are not included.
[1]Calcium carbonate apatite, Joint Committee on Powder Diffraction; Standard data file, #19-272.
[2]Calcium Hydroxyapatite, the data file, #24-33.
[3]Whitlockite, the data file, #9-169.

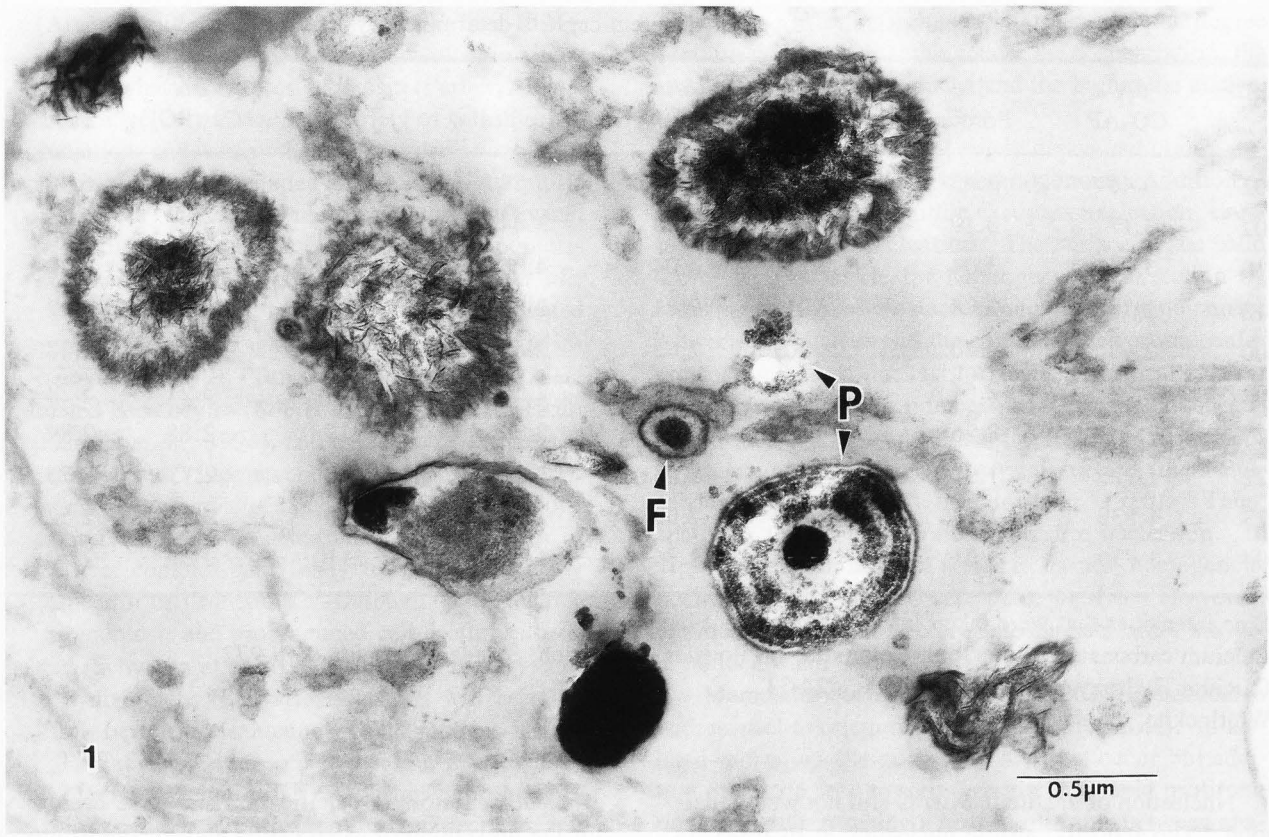
Nucleation of apatite *in vivo* is still not well understood. Theoretically, apatite nucleation in ECF occurs because of local increases in Ca^{2+} and P_i concentrations, the presence of an ideal substrate for heterogeneous nucleation, a local increase or decrease in promotors or inhibitors, or by a combination of these factors. Most theories of calcification fall into one of these categories.

Calcium phosphate crystals

Crystals are formed by the orderly packing of (atoms and) molecules within the minimum space. The stability of crystals is derived from the compact fitting of molecules which are brought together by electrostatic or van der Waals attractive force. The strength of the electrostatic or van der Waals force increases by a factor of 4 or 128, respectively, as the distance between molecules is reduced by one half (Fagan and Ward, 1992). Commonly noted misconceptions are that calcification results from chemical reactions between Ca^{2+} and P_i, and organic complexes of Ca^{2+} and/or P_i serve as ion donors for calcification. This is theoretically unlikely since the attractive forces of solubilized molecules are weaker than chemical bonds. It is, however, possible that certain molecules formed by chemical bonds may serve as building blocks for the formation of apatite. If an organic complex of Ca^{2+} or P_i accidentally fits into a developing crystal, it disturbs the order of arrangement of molecules within the growing crystal, and the complex will inhibit crystal growth. Polyphosphonates are efficient inhibitors of calcification and have been used for the prevention of calcification and for the treatment of Paget's disease. Polyphosphonates are believed to inhibit apatite formation by their aberrant fitting to the growing crystal (Blumenthal, 1989; Fleisch 1980). The strong affinity of Ca^{2+} for P_i suggests a better spatial fit between these two molecules. It is interesting that solubilized proteins inhibit calcification whereas the same proteins, when they are immobilized on the surface of solids, promote calcification (Nancollas and Zawacki, 1989).

Crystals in nature, especially biological ones, are seldom, if ever, ideal (Bloss, 1971). For instance, the Ca/P ratio of the skeletal apatite is consistently lower than the theoretical value of 1.67. The discrepancy is attributed to crystal defects and/or the coexistence of crystals of a lower Ca/P ratio. A variety of crystal defects such as deletions or substitutions of molecules in apatite has been described (Termine, 1972). Halogens frequently substitute for OH^- in apatite. Fluorine substitution for OH^- in apatite is believed to increase the stability of apatite in teeth. A considerable proportion of phosphate in skeletal apatite is replaced by carbonate, which is a known cause for a lower Ca/P ratio of calcific deposits (LeGeros, 1981).

In synthetic metastable solutions, **amorphous** (non-crystalline) calcium phosphate (**ACP**) with a stoichiometry of 3 Ca - 1.8 P_i tends to form first, followed by a spontaneous transformation to apatite. Inasmuch as the formation product of ACP is lower than apatite, it is an



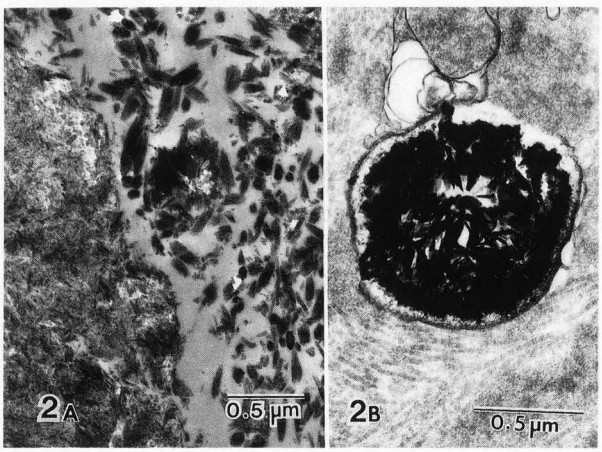


Figure 1. An area of calcific deposits in human aortic valve. In addition to needle shaped apatite, porous particles in concentric layering (P), fine fibrils (F) and solid deposits are seen. The deposits are seen either within membranous vesicles (CDP) or needles are radially embedded in thick-walled vesicles.

Figure 2. (**a**) Plate shaped apatite in a calcific aortic stenosis. The deposit is from an area of massive calcification. (**b**) A calcified CDP in a carotid atheromatous plaque. Triangular plate shaped crystals are seen in the center. Adjacent vesicles are either empty or contain granular matter.

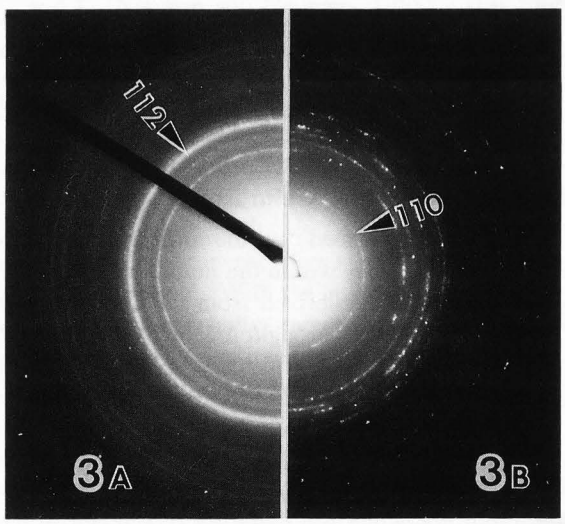


Figure 3. (**a**) Electron diffraction of porous particles yielded the pattern of carbonate apatite. (**b**) A solid deposit yielded the pattern of whitlockite.

---

attractive candidate to explain why calcification occurs in ECF (Eanes and Termine, 1983). ATP can inhibit calcification by restraining the transformation of ACP to apatite (Blumenthal, 1989). In addition to ACP, other calcium phosphates that have higher solubility products

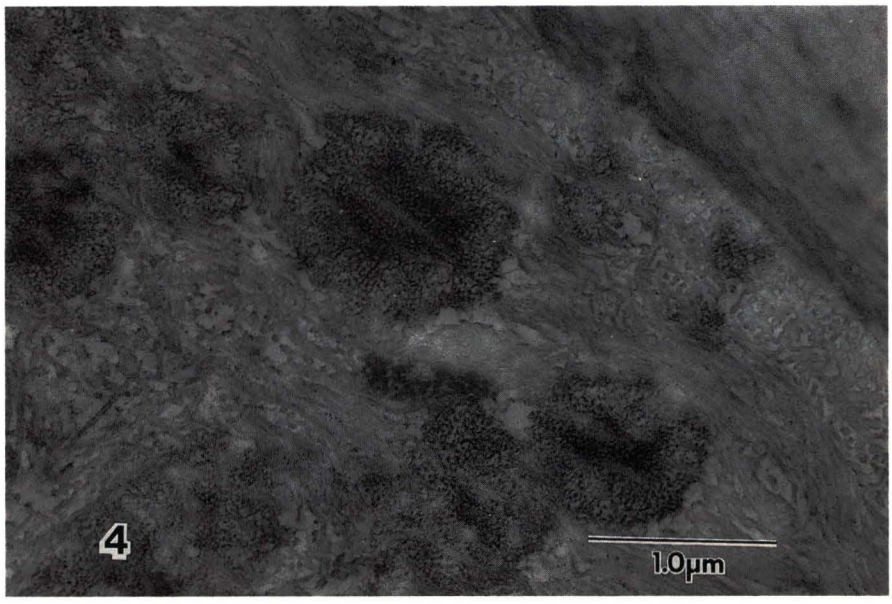


Figure 4. Spherular aggregates of radiating fibrils in paravertebral ligamentum flavum (yellow ligament). These deposits are shown to be associated with crystal ghosts or chondrocalcin.[4] A portion of elastic fiber (E) is seen in the right upper corner.

or lesser thermodynamic stabilities, i.e., octacalcium phosphate (**OCP**; Brown *et al.*, 1981), whitlockite (tricalcium phosphate) and brushite (dicalcium phosphate dihydrate, DCP) have been identified in experimental studies of calcification (Eanes and Termine, 1983). As in ACP, the lower stabilities of these calcium phosphates may help them serve as precursors to apatite formation in calcification. However, the precursor theory suffers because of the difficulty of demonstrating the occurrence of precursors *in vivo*. Furthermore, the lack of standardization in experimental conditions, especially of the solution composition, makes it difficult to correlate studies from different laboratories. It is noteworthy that isolated matrix vesicles have been shown to nucleate OCP in a synthetic ECF (Sauer and Wuthier, 1988). A lower pH favors the formation of DCP, which, as a result, is frequently seen in urinary stones (see, Kim, 1982). Non-apatitic calcium phosphates and ACP may account for the lower Ca/P ratios of calcific deposits *in vivo*, as well.

In fact, morphology of calcific deposits in tissues is complex (**Figs. 1 and 2**). Regardless of the tissues involved, the deposits are similar. In addition to needle-shaped apatite, plates, porous particles and solid deposits are frequently seen. The needles and plates yielded the electron diffraction pattern of hydroxyapatite, porous particles yielded the pattern of carbonate apatite, and solid deposits, of whitlockite (**Fig. 3**; **Table 1**). The nature of other morphologically distinct deposits remains to be determined. In certain cartilaginous tissues and in ligamentum flavum, calcific deposits at times assumed spherical aggregates formed by radiating fibrils (**Fig. 4**). However, calcified membranous vesicles of less than 500 nm were common in these tissues. The formation of radiating fibrils in cartilaginous tissues has been attributed to crystal ghosts (Bonucci *et al.*, 1987) or chondrocalcin (Poole and Rosenberg, 1986). In addition to calcium phosphates, a variety of other crystalline deposits occur in humans (Johnson, 1986).

A critical fact which is frequently overlooked in studies of calcification is that there is not yet a satisfactory means of detecting a nucleus of apatite *in vivo*. The so-called "initial stage of calcification" reported in the literature actually refers to an already advanced stage of calcification from a molecular point of view. Furthermore, sample preparations for the analyses of calcific deposits may have sufficient artifacts to mislead one in the interpretation of the results. Handling samples in aqueous solutions can dissolve early calcific deposits. Dehydration increases local ion concentrations and can induce additional deposits which did not exist in the original specimen. For instance, the processing of samples for electron microscopy (**EM**) can remove as much as 80% of calcium from tissues (Morgan, 1979). Cryopreparation of tissues applied to X-ray microanalysis eliminates much of these artifacts and has provided valuable information (Roomans, 1991). However, these conventional methods are hardly sensitive enough for the detection of crystal nuclei. This inability to detect apatite nuclei hinders much needed progress in the field of calcification. The accumulations of Ca^{2+} and P_i by isolated matrix vesicles or by other experiments are usually thought to precede apatite formation. Alternatively, the uptake of Ca^{2+} and P_i may result from the reduction of ion concentrations in matrix vesicles (MV) brought about by the growth of preformed apatite nuclei. The current interpretation of biological calcification is thus akin to that of archeology in fast motion.

Hopefully, a direct visualization of the early stages of crystal formation by advanced optical techniques and

the reconstruction of the three dimensional view of apatite formation with the aid of space filling models and computer programs, which include simulation of the *in vivo* environment, will help better elucidate the process of nucleation and growth of apatite. In order to be able to control undesirable calcinosis or to prevent bone loss, it is important to understand the multistep process of apatite nucleation and growth. This will allow us to pinpoint the critical step to which we might apply an efficient crystal poison or stabilizer and thus prevent undesirable calcification or calcium loss from bone.

Ca^{++} and P_i Homeostasis

Calcium and phosphate are ubiquitous in biological systems and have many vital functions. Both are absorbed solely through the gastrointestinal tract and excreted through the kidneys. Plasma concentrations of Ca^{2+} and P_i undergo circadian rhythmic changes; the Ca^{2+} x P_i product is highest early in the morning (Kemp *et al.*, 1992; Markowitz *et al.*, 1981). With the recent advent of fluorescent calcium probes, significant progress has been made in understanding calcium homeostasis.

Calcium is mainly an extracellular ion whereas P_i is mainly intracellular. This separation of ions is theorized to prevent calcification (Kim, 1983a, 1983b). Through evolution of P_i utilization for high energy phosphates, cells appear to have adapted to avoid calcification by separating the ions of strong affinity, Ca^{2+} and P_i, with the plasma membrane (Kretzinger, 1990). Disruption of the plasma membrane ion barrier in cell injury and death is likely to increase the local Ca^{2+} x P_i product sufficiently enough to bring about calcification (Kim, 1983a; Trump *et al.*, 1980).

Ca^{++} homeostasis

Cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) is maintained at 50-200 nM, and extracellular Ca^{2+} ($[Ca^{2+}]_o$), at 1.3 mM. The short-term maintenance of $[Ca^{2+}]_o$ is accomplished by parathormone (**PTH**) and calcitonin which act on plasma membrane receptors and ion channels of the target cells (Bonga and Pang, 1991). Any changes in $[Ca^{2+}]_o$ is promptly corrected by movements of Ca^{2+} into and out of the cells especially of bone and kidney. In parathyroid, kidney and other tissues, the cells are equipped with Ca^{2+}-sensing cell-surface receptors (Brown *et al.*, 1995). Within the cell, increased $[Ca^{2+}]_i$ is buffered by mitochondria and endo(sarco)-plasmic reticulum (**ER, SR**), maintaining $[Ca^{2+}]_i$ and $[Ca^{2+}]_o$ within physiological ranges. Nuclear membrane also participates in controlling $[Ca^{2+}]_i$ (Nicotera *et al.*, 1990). Long-term regulation of $[Ca^{2+}]_o$ is accomplished by the hormonal form of vitamin-D (1,25-$(OH)_2$-vitamin D_3, **VD_3**) by controlling the absorption and the excretion of Ca^{2+} and P_i.

The extraordinarily low $[Ca^{2+}]_i$ enables this ion to serve as a second messenger for a variety of agonist stimuli to plasma membrane receptors (Carafoli, 1987). The agonists, by binding to receptors, bring about transient increases in $[Ca^{2+}]_i$ following a cascade of reactions within the plasma membrane (Berridge, 1993). Increased $[Ca^{2+}]_i$ activates Ca^{2+}-pumps, which are located in the plasma, the ER and the mitochondrial inner membranes. The excess $[Ca^{2+}]_i$ is promptly returned to ground status by these pumps. Long-term maintenance of $[Ca^{2+}]_i$ is accomplished mainly by the Ca^{2+}, Mg^{2+}-ATPase (Carafoli, 1994), Na^+/Ca^{2+}-exchanger (Lingrel and Kuntzweiler, 1994) and Ca^{2+}-channels (Tsien, 1990) in the plasma membranes. Active cells usually devote some 25 to 50% of their energy to ion pumps especially the Na^+,K^+-ATPase (Lingrel and Kuntzweiler, 1994). Ca^{2+},Mg^{2+}-ATPase shares a small portion of the energy. Unlike Mg^{2+}, Ca^{2+} does not function directly as an enzymatic cofactor. The maintenance of net $[Ca^{2+}]_i$ thus results from a complex interplay of ion regulations by cell membranes. An excessive increase in $[Ca^{2+}]_i$ connotes malfunction of the transporters.

A sustained increase in $[Ca^{2+}]_i$ is one of the early events that precede cell death, both apoptosis and necrosis (Nicotera *et al.*, 1992; Trump and Berezesky, 1992). Overloaded $[Ca^{2+}]_i$ has profound effects on the structure and function of the cell by paralyzing the second messenger role and activating Ca^{2+}-dependent enzymes. Increased Ca^{2+} also collapses the proton-motive potential gradient across the mitochondrial inner membrane resulting in cessation of ATP synthesis (Malis and Bonventre, 1989).

Phosphate metabolism

Because of the difficulty in measuring P_i, relatively little is known about cellular P_i metabolism. Attempts are made to develop sensitive probes for P_i (Carbantchik and Gregor, 1992; Webb, 1992). With the recent advent of nuclear magnetic resonance (**NMR**) spectroscopy, some progress in the field has been made. Concentrations of cytoplasmic free P_i ($[P_i]_i$) measured by NMR range from 0.5 to 2 mM depending on the tissue. In perfused rat hearts, the mitochondrial P_i concentration was estimated to be 2.6 times that of $[P_i]_i$ (Garlick *et al.*, 1983). A large proportion of $[P_i]_i$ exists as organic phosphates, especially ATP. Cytosolic ATP is higher than 5 mM.

Being a constituent of ATP, DNA, phosphoproteins and phospholipids, P_i is essential for cellular energy metabolism, growth and replication. Of all anions, P_i is best suited for the diester linkages of DNA and energy bonds (Westheimer, 1987). In mammalian cells with a well developed Na^+/K^+-ATPase, P_i enters the cell

mainly through the Na^+/P_i cotransporter. P_i transport across the red cell membrane and the mitochondrial inner membrane occurs via Cl^-/HCO_3^--, and H^+/P_i-exchange, respectively (Wehrle and Pederson, 1989).

The steady state of $[P_i]_i$ apparently is maintained primarily by mitochondrial utilization of $[P_i]_i$ for the synthesis of ATP. Phosphorylation of proteins and lipids in the ER and their hydrolysis are likely to contribute to the $[P_i]_i$ steady state. The equilibrium between $H_2PO_4^-$ and HPO_4^{2-} serves as the primary buffer system in vertebrates for the maintenance of acid-base balance in ECF. Calcium phosphates constitute skeletal mineral in mammals, whereas calcium carbonates form in mollusks that are primarily buffered by carbonate.

P_i transport has been studied most extensively in kidney and intestine, where the whole body P_i homeostasis is regulated. The P_i resorption through the epithelial cells involves two separate processes: active uptake at the apical membrane and transport across the basolateral membrane. Little is known about the transport across the basolateral membrane. Parathyroid hormone is the major regulator of renal P_i reabsorption. The hormone brings about rapid changes in the plasma P_i concentration through its action on renal tubules. In the same way that serum PTH level is controlled by the plasma Ca^{2+}, renal P_i regulation depends on $[Ca^{2+}]_o$, as well. The mechanism of regulation of renal P_i handling by PTH does not yet appear clear (Quamme *et al.*, 1989). VD_3 stimulates renal and intestinal reabsorption of P_i but the mechanism is similarly unclear (Quamme and Shapiro, 1987). Na^+-dependent P_i uptake by cultured osteoblasts has been shown to be enhanced by insulin and fluoride (Kunkler *et al.*, 1991; Seltz *et al.*, 1991). Insulin-like growth factor-I (**IGF-I**) and PTH stimulate Na^+-P_i cotransport by epiphyseal chondrocytes (Montessuit *et al.*, 1994). IGF-I also stimulates renal tubular Na^+-P_i cotransport (Barac-Nieto and Spitzer, 1994).

The best studied of P_i transports in eukaryotes is the $H^+/H_2PO_4^-$ exchanger of the mitochondrial inner membrane (Wohlrab, 1986). The energy for P_i transport is derived from the proton gradient. In addition to its utilization for ATP synthesis, mitochondrial P_i cotransports respiratory substrate, i.e., malate and succinate, via P_i/dicarboxylate exchange. The exchanger is independent of H^+/P_i cotransport. The translocated dicarboxylate in the cytoplasm may participate in gluconeogenesis. There has been increasing evidence that several fold increases in $[P_i]_i$ occur in cell injury (*vide infra*).

Mitochondria

Because isolated mitochondria have a large capacity to accumulate Ca^{2+}, their role in calcification has been extensively studied (Trump *et al.*, 1980). Mitochondrial

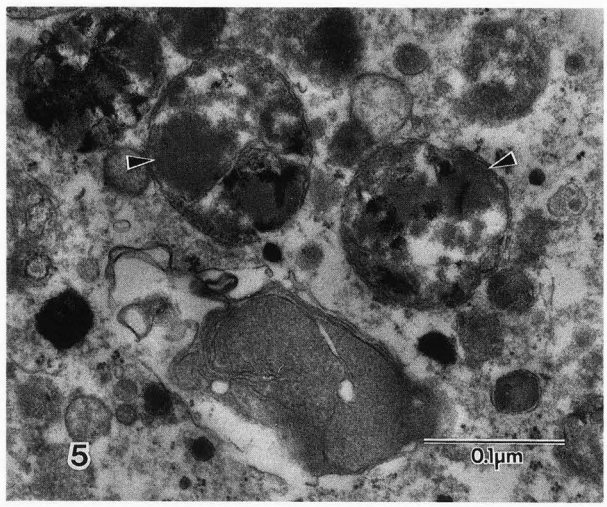


Figure 5. Canine acute tubular necrosis induced by bleeding. Calcific deposits are seen at flocculent densities (arrowhead) in mitochondria and other CDP in sloughed off proximal tubular epithelial cell.

---

calcification occurs commonly under experimental conditions. However, mitochondrial calcification *in vivo* is usually limited to massive tissue necrosis, e.g., myocardial infarction and acute renal tubular necrosis (see Kim, 1983a; **Fig. 5**). Contrary to the earlier belief, Ca^{2+} in mitochondrial matrix ($[Ca^{2+}]_m$) in unstimulated cells is probably lower than $[Ca^{2+}]_i$ (Somlyo *et al.*, 1985; Wendt-Gallitelli, 1986). In stimulated cells with $[Ca^{2+}]_i$ around 800 nM, $[Ca^{2+}]_m$ is estimated to be twice $[Ca^{2+}]_i$ (McCormack *et al.*, 1992). The Ca^{2+} uptake increases exponentially in relation to extramitochondrial Ca^{2+}. The maximum capacity of mitochondrial Ca^{2+} uptake is around 10 times that of their Ca^{2+} efflux. The large capacity to accumulate Ca^{2+} by mitochondria is apparently reserved for excessive increases in $[Ca^{2+}]_i$ in order to minimize the resultant cell damage (Gunter and Pfeifer, 1990; McCormack and Denton, 1993).

Mitoitochondrial Ca^{2+} uptake is catalyzed by a powerful electrophoretic Ca^{2+}-uniporter, which is driven by the proton-motive potential gradient of the mitochondrial inner membrane. The gradient is generated by outward proton pumping, either coupled to the electron transport system or ATP hydrolysis (Gunter *et al.*, 1994; McCormack and Denton, 1993). The principal Ca^{2+} efflux mechanism in normal mitochondria is an electro-neutral Na^+/Ca^{2+}-exchange, which is also driven by the proton gradient through the action of a highly active Na^+/H^+-exchanger (McCormack, 1985). There also appears to be a Na^+-independent egress of Ca^{2+} which is catalyzed by a direct $Ca^{2+}/2H^+$-exchange. High concentrations of $[Ca^{2+}]_m$ open pores on the mito-

chondrial inner membrane which are normally closed. Ca^{2+}-dependent opening of the pores is enhanced by oxidative stress and by P_i (Crompton *et al.*, 1988).

The primary function of $[Ca^{2+}]_m$ is to relay the second messenger role of $[Ca^{2+}]_i$, which is elicited by agonists binding to the plasma membrane receptors, to mitochondria (Denton and McCormack, 1990). Ca^{2+} is the only known second messenger capable of entering mitochondria. There it functions without binding to calmodulin. Rises in $[Ca^{2+}]_m$ will increase up to five-fold the activities of three key dehydrogenases, pyruvate, NAD^+-isocytrate and 2-oxyglutarate dehydrogenases. The increased $[Ca^{2+}]_i$ in agonist-stimulated cells that triggers energy consuming events also promotes mitochondrial ATP synthesis by stimulating these enzymes. $[Ca^{2+}]_m$ thus has an ability to balance energy production with the demand that is incurred by increased $[Ca^{2+}]_i$ (Gunter and Pfeifer, 1990; McCormack *et al.*, 1992). Increased $[Ca^{2+}]_m$ is also thought to enhance energy metabolism by inhibition of pyrophosphatase through mitochondrial swelling (Davidson and Halestrap, 1989) and stimulation of ATP-synthase (Futai *et al.*, 1989).

Abnormal increases in $[Ca^{2+}]_i$ have profound effects on mitochondrial functions. Ca^{2+} uptake by mitochondria proportionately reduces the potential gradient across the mitochondrial inner membrane (Gunther and Pfeiffer, 1990). A massive accumulation of $[Ca^{2+}]_m$ collapses the potential gradient and results in the loss of ATP (Malis and Bonventre, 1989). Pfeiffer *et al.* (1979) suggested that PLA_2 activation may cause permeability changes by opening pores in the mitochondrial inner membrane of injured cells. Mitochondrial dysfunction caused by increased $[Ca^{2+}]_i$ will increase $[P_i]_i$ by reversal of ATP/P_i and create a vicious circle in apoptosis and cell injury.

Mitochondria exposed to anoxia undergo cyclic changes of swelling and shrinkage, followed by the development of flocculent densities in the matrix. According to Trump *et al.* (1973), these densities are formed by lipolysis and signal the irreversibility of cell injury. Mitochondrial calcification usually occurs in association with these flocculent densities (Trump *et al.*, 1980; **Fig. 5**). Myocardial mitochondrial calcification has been described in Reye's syndrome and idiopathic cardiomyopathy (Hammar and Krous, 1977, Min and Wheeler, 1985). Mutilation and obscurity of mitochondria following disintegration of apoptotic cells *in toto*, which is common in various tissues with calcinosis (*vide infra*), may account for the relative sparsity of mitochondrial calcinosis.

In order to calcify, mitochondria must accumulate sufficient Ca^{2+} prior to the collapse of the membrane potential. Mitochondrial Ca^{2+} accumulation in cell injury can be attributed to residual substrates for energy production in mitochondria. Mitochondria can rapidly accumulate Ca^{2+} as long as ATP is available (Fiskum and Lehninger, 1982). In ischemic injury to myocardium, cells associated with greater changes in Na and K detected by X-ray microanalysis develop mitochondrial calcification (Buja *et al.*, 1985). In view of their calcium content, mitochondrial granules have been suspected of playing a role in calcification. However, only certain granules contain calcium and the granules tend to disappear in the early stages of cell injury (Buja *et al.*, 1985).

Mitochondria seem to have dual roles in calcification in cell death. In addition to accumulation of $[Ca^{2+}]_m$ and calcification, the collapse of proton gradient and the subsequent failure to synthesize ATP lead to an increase in $[P_i]_i$, that will participate in calcification. Of the known cell components, high energy phosphates, especially of ATP, are the most readily available source for $[P_i]_i$.

Endoplasmic reticulum

Despite its ability to accumulate Ca^{2+} and P_i and its role in controlling Ca^{2+} homeostasis (Meldolesi and Villa, 1993; Pozzan *et al.*, 1994), the potential role of the ER in calcification has hardly been touched upon. As opposed to the earlier view that mitochondria are the major intracellular Ca^{2+} storage site, the ER serves as the main Ca^{2+} storage organelle (Somlyo *et al.*, 1985). The ER contains Ca^{2+}-binding proteins, especially calsequestrin and calreticulin, that contribute to Ca^{2+}-storage (Milner *et al.*, 1992). Agonist binding to the plasma membrane results in breakdown of phosphoinositides by phospholipases C following a cascade of reactions in the membrane and liberates inositol triphosphate (**IP_3**; Berridge, 1993). Acting as a second messenger, IP_3 binds to the specific receptor on the ER membrane and triggers Ca^{2+} release from the ER and increases $[Ca^{2+}]_i$ to up to 1000 nM. A variety of cell functions, e.g., muscle contraction, cell division etc., has been ascribed to the transient increase in $[Ca^{2+}]_i$.

In non-excitable cells, an influx of Ca^{2+} through non-voltage dependent Ca^{2+}-channel also contributes to the increase in $[Ca^{2+}]_i$. In excitable cells, dramatic increases in Ca^{2+} occur through voltage gated channels. It has recently become apparent that the capacitative influx of Ca^{2+} is stimulated by depletion of intracellular Ca^{2+} stores (Clapham, 1995). Increased $[Ca^{2+}]_i$, by binding with calmodulin, stimulates nitric oxide synthase in the cytosol and emanates nitric oxide, another second messenger (Bredt and Snyder, 1994; Vallance and Collier, 1994). A report indicates that nitric oxide regulates refilling of the depleted intracellular Ca^{2+} store(s) by stimulating Ca^{2+} influx through the action of cGMP (Xu *et al.*, 1994b). Calcium uptake by the ER depends on a Ca^{2+}-ATPase (MacLennan *et al.*, 1992). The pump

is stimulated by calmodulin and is anion dependent; oxalate enhances the pump in experimental conditions.

Calcification in the ER seems to be rare and has been described in the skeletal muscle and nerve terminals (Bonucci and Sadun, 1972; McGraw *et al.*, 1980). Experimental precipitation of calcium phosphate has been described in the isolated ER (DeMeis *et al.*, 1974). In spite of its role in phosphoprotein and phospholipid synthesis, relatively little is known about P_i transport across the ER membrane. The sarcoplasmic reticulum of skeletal muscle contains a transporter for P_i which also carries oxalate and pyrophosphate (Carley and Rucker, 1982). External Ca^{2+}, Mg^{2+} and ATP are required for the uptake of P_i by the ER (Stefanova *et al.*, 1991).

Ca^{2+} and P_i in cell death

Sustained increase in $[Ca^{2+}]_i$ has been most extensively studied in cell injury and is believed to cause the majority of cell morbidity (Gunter and Pfeifer, 1990; Trump and Berezesky, 1992). The increase in $[Ca^{2+}]_i$ is attributable to the loss of ATP and the resultant decline in Ca^{2+}-pump activities of the membranes. The increased $[Ca^{2+}]_i$ activates many enzymes, i.e., phospholipase A_2 (**PLA_2**), neutral proteases, endonucleases and NO-synthase (Nicotera and Orrenius, 1992; Bredt and Snyder, 1994). Activation of endonucleases, especially DNAse-I, is believed to be responsible for DNA fragmentation in apoptosis. Experimental elevation of $[Ca^{2+}]_i$ has been shown to activate this enzyme and to fragment DNA (McConkey *et al.*, 1989). Neutral proteases (calpains) are thought to cause autolysis of dying cells (Fagan *et al.*, 1992). Nitric oxide inhibits glycolysis and DNA repair and can kill target cells, especially tumor cells (Bredt and Snyder, 1994; Vallance and Collier, 1994).

A variety of Ca^{2+}-binding proteins are involved in cytoskeletal organization (Bennet and Weed, 1986). Overloaded $[Ca^{2+}]_i$ disintegrates the cytoskeleton and is believed to cause blebbing of the plasma membrane (Smith *et al.*, 1991). Increases in Ca^{2+} in the early stages of cell injury is biphasic; an early transient $[Ca^{2+}]_i$ surge is followed by a prolonged and greater rise in $[Ca^{2+}]_i$. The transient increase is evidently due to an intracellular redistribution of Ca^{2+}, the second increase is due to an influx of $[Ca^{2+}]_o$ (Gasbarrini *et al.*, 1992). The mechanism of Ca^{2+} influx in cell injury is incompletely understood; it may not occur through Ca^{2+}-channels. The Na/Ca^{2+}-exchanger may contribute to the Ca^{2+} influx in ischemic and toxic injury to the cells (Tani, 1990).

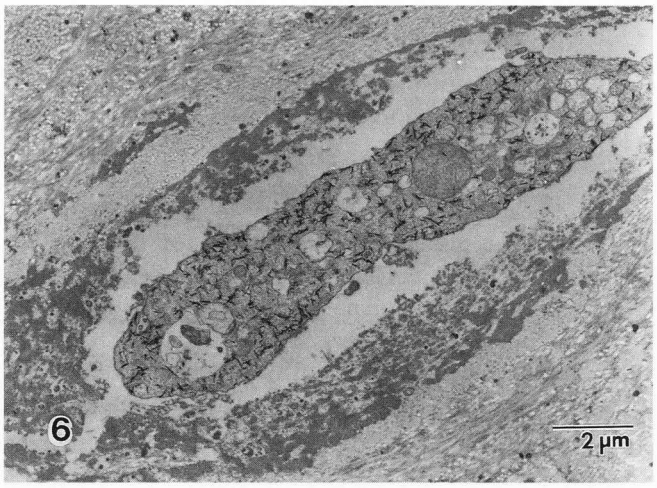
Activated PLA_2 eliminates phosphatidylcholine (**PC**) and produces eicosanoids, lysophospholipids and xanthine. Lysolipids are toxic to the cell (Orrenius *et al.*, 1991). Lysolipids associated with PLA_2 have also been detected in isolated MV (Wuthier, 1988). Xanthine production has been theorized to lead to lipoperoxidation and further damage to the membrane. Lipid peroxidation of the membrane, in addition to an accelerated degradation of phospholipids (**PL**), has been implicated in the mechanism of hypoxic injury to the cells (Dargel, 1992). The plasma membrane damaged by phospholipases may be involved in the increase in $[Ca^{2+}]_i$ by increasing the membrane permeability and make the injury irreversible (Farber, 1990).

Decreased utilization of $[P_i]_i$ and the resultant increase in $[P_i]_i$ in association with an increase in $[Ca^{2+}]_i$ has been known for some time in anoxic injury to renal tubules (Humes *et al.*, 1986). Anoxic injury to cultured hepatocytes causes within 2 hours a decrease of β-ATP by 85% associated with a 2- to 3-fold increase in $[P_i]_i$, and a 10-fold increase in the P_i/ATP ratio (Gasbarrini *et al.*, 1992). The reversal of ATP/P_i has been demonstrated by NMR in kidneys *in vivo* following various injuries (Hricak, 1989) and in anoxic injury to cultured myocardial cells (Humphrey and Garlick, 1991) and endothelial cells (Block *et al.*, 1989). Although $[P_i]_i$ derangements in apoptosis appear to be less well studied, an increase in P_i is likely since elevated $[Ca^{2+}]_i$ in apoptosis will cause a decrease in mitochondrial ATP synthesis. A similar reversal of ATP/$[P_i]_i$ was observed in cultured hypertrophic chondrocytes prior to matrix vesicle formation (Wuthier, 1993) and irradiated fibrosarcoma cells (Mahmood *et al.*, 1995).

Concomitant increases in $[Ca^{2+}]_i$ and $[P_i]_i$ in cell injury and in apoptosis provide an ideal milieu in which to nucleate apatite. Of all the theories on calcification, none had demonstrated such increases in Ca^{2+} and P_i, with the exception of MV, in a compartmentalized environment. Since ATP is known to inhibit calcification (Blumenthal, 1989), the decrease in cytosolic ATP may have an additional effect on calcification in cell injury and apoptosis. Occasionally, apatite deposition in the cytosol that can be attributed to increases in $[Ca^{2+}]_i$ and $[P_i]_i$ is seen in injured cells (**Fig. 6**).

Scavenging and calcification

Inasmuch as apoptosis is wide spread, the question arises whether every dying cell in the human body calcifies and if so why is calcification not more frequent? The answer apparently lies in the efficient scavenging system. Cells in general have a limited life span and are replaced by divisions of stem cells. Apoptotic cells of the surface epithelia are removed by desquamation and excretion. Calcification of such desquamated cells is very frequent. For instance, some 45% of adult females develop calcification in their breast and the incidence is increased to 56% in carcinoma (Thomas *et al.*, 1993).



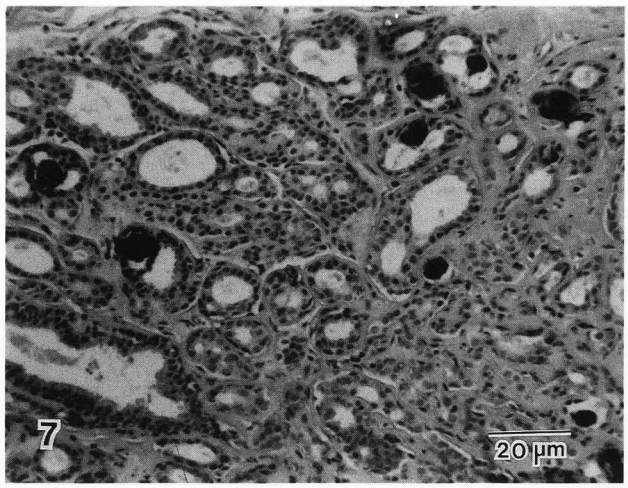


Figure 6 (at left). Needle shaped apatite deposited in the cytosol of a fibroblast in a rheumatic valvulitis. A massive calcification was present in the vicinity (calcific aortic stenosis).

Figure 7. A fibroadenoma of female breast. Calcific deposits are seen mainly in the duct lumina.

The calcification occurs mainly in the mammary duct lumina (Anastassiades *et al.*, 1984) in association with cell debris and degenerated cells (Stegner and Pape, 1972; **Fig. 7**). Calcification also commonly occurs in urinary casts that originate from desquamated tubular cells (*vide infra*).

In connective tissues where dead cells cannot escape to the outer environment, the cells are removed by phagocytes. Apoptotic cells in the tissues that are rich in capillaries have been shown to be promptly phagocytized by macrophages. Exposures of phosphatidylserine, of side-chain sugars due to loss of sialic acid from the sialoglycoprotein receptor and possibly of thrombospondin binding moiety on the outer surface of apoptotic cells are thought to be the factors which macrophages recognize (Savill *et al.*, 1993). Development of an aging antigen, especially of red cells, has also been shown to be a mechanism of scavenging obsolete cells (Kay, 1994). Macrophages have a large capacity to phagocytize apatite, as well (van der Meulen *et al.*, 1993). Tissues which commonly calcify, e.g., cartilage and vascular connective tissue, are avascular and, therefore, the accumulation of CDP tends to exceed the rate of scavenging. This results in the sequestration of CDP *in situ* (**Fig. 8**). In capillary-rich tissues, i.e., healing wounds, CDP are effectively scavenged by macrophages. No cell debris remains and calcification does not usually take place.

When rat aortic segments placed in Millipore chambers were incubated in the peritoneal cavity, a massive calcification occurred, whereas calcification was minimal in subcutaneously grafted aorta, where inflammatory cell infiltration was noted. Thus, because of lack of scavenging in the chambers, calcification could not be prevented (Kim, 1978).

Blebbing in cell injury and apoptosis

Blebbing of the plasma membrane is one of the means of exocytosis of macromolecules and is probably a widespread phenomenon. Megakaryocytes bud profusely to form platelets and other blood cells have been shown to bud plasma membrane vesicles. Platelets, in turn, bleb freely (Bode *et al.*, 1991). Virus escape the host cell via budding of the plasma membrane. Malignant tumor cells shed plasma membrane components at a higher rate than normal cells of the same type (Dainiak, 1991). The extent and the role of blebbing *in vivo* are largely unknown.

Blebbing in cell injury attained relatively early attention (Zollinger 1948). A variety of agents of cell injury has been shown to cause blebs (see, Phelps *et al.*, 1989). An alteration of the interaction between the plasma membrane and the cytoskeletal fibers is apparently involved in blebbing (Smith *et al.*, 1991). Asymmetry of phospholipids in the membrane may also contribute to blebbing (Farge and Devaux, 1992; Ferrel *et al.*, 1985). Temperature shifts from 0°C to 37°C of cultured cells induce blebbing which seems to be due to microtubule disruption (Liepins and Younghusband, 1985). Heating of cell cultures to 45°C also causes blebbing (Borrelli *et al.*, 1986). Thermal shock to cultured cells has been shown to increase $[Ca^{2+}]_i$ via Na^+/Ca^{2+}-exchange (Kiang *et al.*, 1992; Mikkelsen *et al.*, 1991). Blebs formed by the plasma membranes of injured cells seems to become more rigid indicating significant changes in the membrane structure (Florine-Casteel *et al.*, 1991).

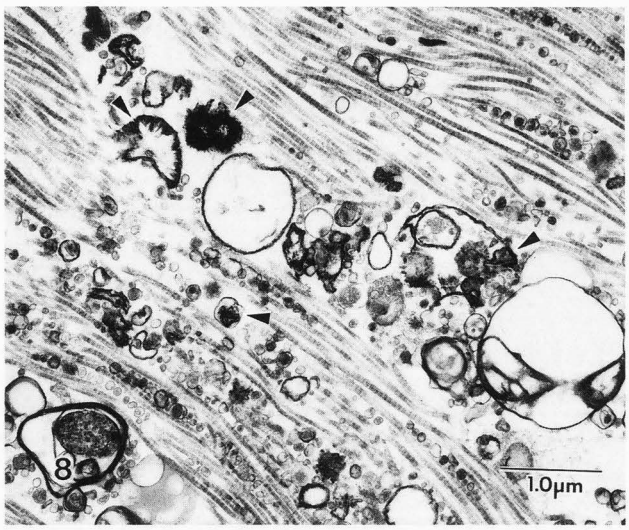
Figure 8 (at right top). Accumulation of CDP between collagen fibrils in canine aortic valve. Calcific deposits are seen in CDP (arrowhead). Collagen fibers are free of calcification. From Kim *et al.*, 1986, with the publisher's permission.

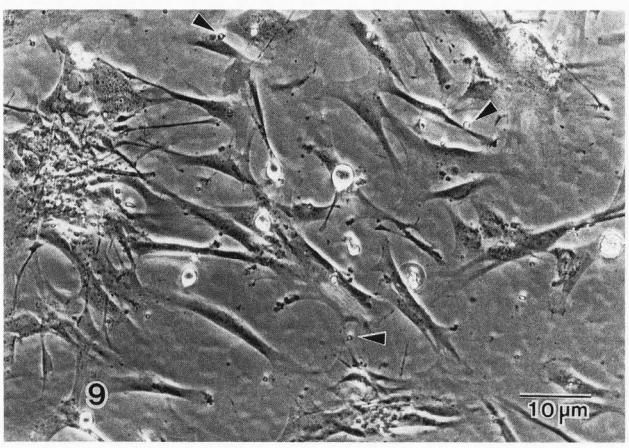
Figure 9 (at right middle). Human dermal fibroblasts in culture. After repeated population doublings, cells cease to divide and shed blebs (arrowhead). A few large blebs are artifacts that occurred during photography. When incubated in MEM-2.5 blebs calcified in a week, whereas live cells did not calcify in MEM-2.5.

The size of blebs vary widely depending on the type and the severity of injury (Trump *et al.*, 1980; Phelps *et al.*, 1989).

Hypoxic and toxic injuries to cultured cells concentrate a large amount of Ca^{2+} in protrusions of the plasma membrane prior to their blebbing (Lemaster *et al.*, 1987; Smith *et al.*, 1991). Blebbing could be construed as the cells' defense mechanism to eliminate overloaded $[Ca^{2+}]_i$ *en masse*. Direct measurements of P_i at the time of bleb formation other than MV formation by hypertrophic chondrocytes (Wuthier, 1993) appear to be not yet available. However, the high concentration of Ca^{2+} in the blebs must be accompanied by equivalent anions, i.e., P_i, the predominant intracellular anion that increases in cell injury. Blebs resulting from apoptosis and cell injury, as seen in MV formation, are likely to contain a high concentration of P_i as well and to provide an ideal environment in which calcification might develop. The observation of calcific deposits mainly in the membranous CDP in dystrophic calcifications supports this view (**Fig. 8**).

It should be noted that, in order to obtain homogeneous population of MV, cell debris (CDP) are eliminated by centrifugation prior to isolation of MV from the growth plate or cell cultures. The potential role of CDP in calcification thus has not been properly tested. Blebbing is common in cultured cells during cell division. Any subtle injury to cultured cells, e.g., nutritional deficiency or contamination, tends to cause blebbing. As cells grow old after repeated population doublings *in vitro*, they bleb profusely. When blebs formed by senescent canine valvular fibroblasts were collected and incubated in minimal essential medium in Earl's salt with the total calcium adjusted to 2.5 mM (**MEM-2.5**), they calcified in a week, whereas, live cells failed to calcify in the same solution (Kim, 1994). Similar results were obtained with human dermal fibroblasts (**Fig. 9**). Blebbing and the resultant calcification are similar to that of MV formed by chondrocytes in the epiphyseal cartilage. There is a remarkable similarity between the composition of isolated MV and injured cell plasma





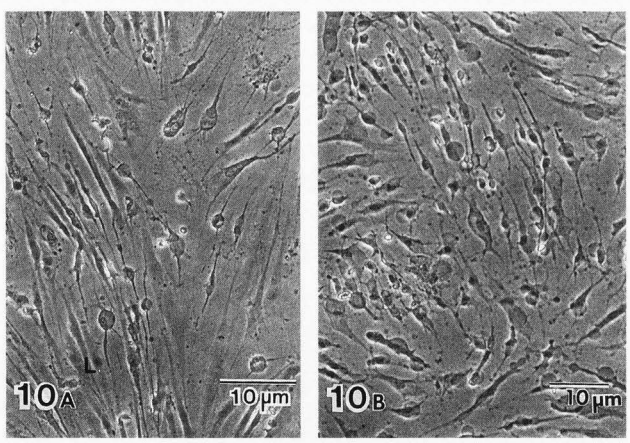


Figure 10. (**a**) Human dermal fibroblasts treated with 10 μM A23187 for 24 hours. Cells developed large sized blebs. (**b**) Same cells treated with 5 μM iodoacetate formed larger blebs. The size of blebs vary depending on the severity and type of cell injury.

membranes that give rise to blebs. In addition to elevated Ca^{2+} and P_i, MV also retain PLA_2 activity (Schwartz and Boyan, 1988). The PLA_2 activity may explain the depletion in PC and an increase in lysophospholipids in MV (Wuthier, 1988). In the face of the similar enzyme activities, MV formation may well be an epiphenomenon of impending apoptosis of hypertrophic chondrocytes (*vide infra*). Increased $[Ca^{2+}]_i$ by ionophoric treatment of hypertrophic chondrocytes leads to MV formation (Iannotti *et al.*, 1994). The ionophoric treatment has been known to cause blebbing in other cells (George *et al.*, 1982; Nicotera *et al.*, 1986; Trump *et al.*, 1980; **Fig. 10**). The formation of MV by hypertrophic chondrocytes may occur by the same mechanism as blebbing by apoptotic and necrotic cells. It is noteworthy that blebbing and cell death can occur by ATP depletion only without an increase in $[Ca^{2+}]_i$ (Jurkowitz-Alexander *et al.*, 1992).

Physiological Calcification

Calcification is divided into physiological and pathological. Extra-skeletal calcification is said to be pathological. Pathological calcifications are further classified into dystrophic and metastatic. Dystrophic calcification occurs in normocalcemic and normophosphatemic individuals. Metastatic calcification results from abnormal elevations of extracellular Ca^{2+} and/or P_i concentrations. However, the initial process of apatite nucleation in metastatic calcification is likely to be the same as in the dystrophic, differing mainly in the rate of apatite proliferation. Metastatic calcification is frequently extensive, and, therefore, may cause organ failures. The distinction in the fundamental mechanism between various types of calcification is not always clear and is arbitrary.

Although it has been held that the mechanisms of physiological and dystrophic calcification are distinctive from each other, detailed comparisons of the two are not available. The formation of the common final product, apatite, may imply that the fundamental mechanism of calcification in both may be similar, if not identical.

Calcification in bone

The most heavily calcified bone is the least suitable for the study of apatite nucleation. The abundance of collagen in bone has led us to believe that collagen may play a role in apatite nucleation (Glimcher, 1989). Although calcification in MV has been demonstrated in developing bone (Anderson, 1989), bone growth depends mainly on apatite seeds inherited from the epiphyseal cartilage. Since apatite has an inherent ability to grow autocatalytically in the metastable ECF, the mechanism of curbing the spread of calcification into the surrounding soft tissue is far more significant than the nucleation of apatite in bone. There is a continuous flow of extravasated fluid from capillaries towards the periphery of cortical bones through canaliculi and lacunae where osteocytes reside (Dillaman *et al.*, 1991).

Between calcified tissues and ECF, there must be a barrier which limits the spread of calcification. The barrier is apparently formed by live tissue since dystrophic calcification is limited to necrotic tissue. When a dead fetus remains unnoticed *in utero*, indiscriminate calcification of the entire fetus leads to the development of a lithopedion. When ATP synthesis was inhibited in organ cultured bones with iodoacetic acid, a hypermineralization resulted (Ramp, 1975; Scarpace and Neuman, 1976b). It is very interesting that a co-culture of fibroblasts inhibited calcification of osteoblasts that were cultured in the same dish (Ogiso *et al.*, 1991). This inhibition is suspected to be due to the secretion of prostaglandin E_2 by fibroblasts. Prostaglandins have been shown to have an inhibitory effect on bone growth and has been implicated to be one of the causes of osteoporosis (Raisz *et al.*, 1993). Cultured rat calvaria and cells from calvaria including fibroblasts have been shown to secret calcification inhibitors (Ohya *et al.*, 1988).

The barrier between the bone fluid and general ECF is apparently formed by a metabolically active, functional membrane (also called bone membrane; Ramp, 1975). In order to maintain this $[Ca^{2+}]$ differential, the bone membrane must be able to pump net Ca^{2+} and/or P_i out of the bone compartment through an energy consuming mechanism. Such a polarized mechanism within the bone membrane has yet to be identified. Furthermore, as opposed to the membranes of polarized epithelial cells, the bone membrane appears to be incomplete with open channels between the cells which allow free movements of fluid (Ramp, 1975). Theoretically, $[Ca^{2+}]$ in the bone fluid which faces the apatite mass must be near 1/3 of that of serum (Neuman and Neuman, 1958). The influx of Ca^{2+} and P_i across the bone membrane of organ cultured bone has been shown to be passive. When the periosteum and endosteum were removed from cultured calvaria, Ca^{2+} influx into the bone was increased (Scarpace and Neuman, 1976a, 1976b).

Ca^{2+} influx into bone cells is increased by PTH (Boden and Kaplan, 1990). Nutritional and humoral factors, such as VD_3 and PTH, might exert part of their effects on Ca^{2+} homeostasis by influencing the rate at which Ca^{2+} is pumped out of the bone.

Epiphyseal cartilage calcification

It is widely accepted that MV play the primary role in epiphyseal calcification (Anderson, 1989; Iannotti, 1990). Matrix vesicle calcification, therefore, is considered to be more or less synonymous with epiphyseal

or frequently with physiological calcification. Matrix vesicles were discovered in the epiphyseal cartilage (Anderson, 1967; Bonucci, 1967). They are present mainly in the matrix of the longitudinal septa of the proliferative and the hypertrophic zones. Calcification in similar membranous vesicles has been shown in a variety of pathological calcifications (Kim, 1983a, 1983b; Anderson, 1988). Thus, MV are widely accepted as an efficient inducer of both physiological and pathological calcifications. However, the mechanisms for the formation of MV and their calcification are still incompletely understood.

The representative views for the contemplated mechanism of MV calcification are: an active uptake (of Ca^{2+} and P_i), alkaline phosphatase, phospholipids, and apoptosis theories. A variety of intra-MV and extracellular Ca^{2+}-binding organic molecules have been implicated in potential mechanisms for calcification (Boskey, 1992). As pointed out earlier, Ca^{2+} or P_i bound to organic molecules can hardly be a promotive factor for calcification.

Active uptake theory

Isolated MV have been shown to contain exceptionally high concentrations of Ca^{2+} and P_i. The findings led to a theory that MV are extracellular organelles that are able to accumulate Ca^{2+} and P_i and thereby initiate calcification. This theory is based on the observation that isolated MV accumulate Ca^{2+} and P_i from a simulated cartilage fluid (Wuthier, 1988). However, a possibility that the uptakes may be the result of rather than the cause of apatite nucleation should be considered. Furthermore, hypertrophic chondrocytes already contain high $[Ca^{2+}]_i$ and $[P_i]_i$ especially in their cell processes prior to MV formation (Wuthier, 1993). The resultant MV contain a high concentration of Na^+, an extracellular cation, as well as increased Ca^{2+} and P_i. The increases indicate that MV are no longer able to actively transport ions. Since ATP-dependent Ca^{2+}-transporters across the plasma membrane are normally directed outward, an active uptake of Ca^{2+} by an energy dependent mechanism is unlikely. The increased Na^+ makes it unlikely that the Na^+-dependent P_i uptake exists in MV. Of the known Ca^{2+} transports, Na^+/Ca^{2+}-exchange is the only theoretically feasible mechanism for an active uptake of Ca^{2+} by MV. The elevated Na^+ in MV also indicates that the Na^+/K^+- and the Na^+/Ca^{2+}-exchangers are no longer functioning in MV. Na^+/Ca^{2+}-exchange has been demonstrated in osteoblasts (Krieger, 1991). The exchanger in chondrocytes or MV appears not to have been well studied. Existing concepts of ion transports do not support the active uptake theory.

A group of Ca^{2+} binding proteins, annexins (calpactins) glue cytoskeletal proteins to the membrane (Swairjo and Seaton, 1994). Annexins are one of the families of membrane associated Ca^{2+}-binding proteins that are widely distributed in mammalian cells and share the ability to bind phospholipids to cytoskeletons. The binding of calpactin to phospholipids greatly increases its affinity for Ca^{2+}. Recently, the possibility of annexin V playing a role in MV calcification by acting as a Ca^{2+}-channel has been raised (Genge *et al.*, 1992; Rojas *et al.*, 1992). Annexin V is known to be a slow Ca^{2+} channel (Berendes *et al.*, 1993). Functioning as a voltage gated Ca^{2+} channel, and with its collagen binding capacity, annexin V may stimulate Ca^{2+} uptake by MV (Kirsch and Wuthier, 1994). It is noteworthy that acidic PL can be ionophoric for Ca^{2+} (Tyson *et al.*, 1976). Certain hormones, e.g., insulin, are known to be ionophoric for Ca^{2+}, as well (Brimble and Ananthanarayanan, 1992). Interestingly enough, amyloid in Alzheimer's disease, when inserted into the membrane, can function as a Ca^{2+} channel (Arispe *et al.*, 1993).

Recently, Na^+-dependent P_i uptake was demonstrated in MV isolated from rachitic chicken growth plates. In MV isolated from normal chicken growth plate, the rate of P_i uptake was greater than that of rachitic MV but was not Na^+-dependent. The symporter apparently was unrelated to alkaline phosphatase activity (Montessuit *et al.*, 1991). It has been shown that Na^+-dependent P_i uptake across intestinal brush border membrane is markedly increased within hours of dietary phosphate restriction (Cheng *et al.*, 1983). The maximum velocity (V_{max}) of Na^+/P_i-transport is 2.7 fold greater in fibroblasts from patients with vitamin D resistant rickets than the normal controls (Escoubet *et al.*, 1992). A decrease in the number of Na^+-P_i cotransporters in the renal proximal tubule can occur within hours of high P_i-diet feeding in rats (Levi *et al.*, 1994). The Na^+/P_i-cotransport in the rachitic MV can be attributed to a similar increased expression of the cotransporter. The non-Na^+-dependent P_i uptake by MV isolated from the normal growth plate needs further clarification.

Once the apatite seed is formed, the autocatalytic growth of the crystal would cause a continuous influx of Ca^{2+} and P_i into MV. There is an urgent need for proper understanding of the exact point of apatite nucleation in the process of MV formation by chondrocytes. Without this knowledge, the interpretation of data on the MV calcification will remain inconclusive. Further, without a full knowledge of the mechanism of ion transports across the MV membrane, the kinetics of ion uptake by MV would be difficult to interpret. Little is known about ion transport by the MV membrane. The mechanism of MV calcification remains a wide open field.

Alkaline phosphatase theory

Of the enzymes implicated in calcification, alkaline

phosphatases (**AP**) are representative. Demonstrable AP activity on the outer surface of MV has led to an extensive search for its role in calcification. Inhibition of enzyme activity prevented calcification of epiphyseal plates and of isolated MV *in vitro*. Calcification of isolated MV can be enhanced by the addition of artificial substrates for AP. Incorporation of AP into the membranes of reconstituted MV restored its calcifiability. The AP activity has been demonstrated in both physiological and pathological calcifications and *in vitro* experiments. The theory that this enzyme plays an important role in calcification has therefore gained wide acceptance (see, Anderson, 1988, 1989). Of the numerous theories of calcification which have come and gone, the AP theory is the only one that has survived from the time of its conception. Interestingly enough, AP activity has been implicated in calcification of glutaraldehyde fixed porcine aortic valve xenografts (Levy *et al.*, 1991).

Pyrophosphate (**PP_i**), a metabolic product of nucleotides and a known inhibitor of calcification, is perhaps the best recognized substrate for AP (Anderson and Reynolds, 1973). Hydrolysis of PP_i by AP may promote calcification by elimination of its inhibitory activity and by a local increase in P_i. An active P_i transport by AP across the cell or MV membranes has been considered but it has been proved otherwise (Montessuit, 1991).

Mammalian AP is a dimeric zinc-containing glycoprotein, which requires Mg^{2+} for hydrolysis of a wide range of phosphomonoesters (Simko, 1991). Although the optimum pH range for its activity is 9.3-10.3, it can also act at physiological pH (Wachstein and Meisel, 1957). Four isoenzymes encoded by distinct genetic loci are known in humans; placental, intestinal, tissue nonspecific, and "Regan" or placenta-like AP. The placenta-like AP are established markers for many cancers (Fishman, 1974). It is interesting that levamisol, an AP inhibitor, is regularly used for the treatment of colon carcinoma. The tissue non-specific isoenzyme is expressed in most types of tissues but seen predominantly in the liver, bone, and kidney. The presence of AP activity in bone cells has long been known and metabolic bone disorders increase serum concentrations of AP. Bone AP is heat labile, which distinguishes it from the hepatic AP (Simko, 1991). Elevation of bone AP in serum also occurs in periods of bone growth, fracture and osteomalacia (Epstein, 1988).

In mammals, AP are generally present extracellularly either free in the circulation or anchored to plasma membranes via a COOH-terminal glycosyl phosphatidylinositol moiety (Ferguson and Williams, 1988). The enzyme is also present in microsomes, nucleus and Golgi apparatus (Zijlstra *et al.*, 1970). Within the cell, AP is believed to regulate P_i metabolism (Alpers *et al.*, 1989). AP is a stable enzyme and its activity survives formalin or glutaraldehyde fixation. For this reason, AP is extensively used as an immunohistochemical marker.

The AP-theory of calcification has certain limitations; AP is widely distributed in non-calcifying tissues, as well and its physiological role or substrate has yet to be identified. It is difficult to establish a relationship between apatite nucleation within the lumen and the presence of AP on the outer surfaces of MV. Further, calcification of MV *in vitro* does not necessarily require AP activity (Register *et al.*, 1984). Certain preparations of MV do not calcify unless supraphysiological concentrations of the substrates are added (Register *et al.*, 1986). The enzyme activity was shown to have an inverse relationship with the rate of calcium accumulation by isolated MV (Genge *et al.*, 1988). A removal of AP from MV by treatment with phospholipase C had little effect on P_i or Ca^{2+} uptake by MV; hydrolysis of AP substrate was not linked to the P_i uptake by MV; and the treatment of MV with proteases decreased Ca^{2+} uptake but had minimal effect on the AP activity of MV (Wuthier, 1988).

In a recent study, placental AP conjugated to collagen calcified *in vivo* whereas collagen without AP did not (Beertsen and van den Bos, 1992). AP binding to collagen has been shown to occur (Wu *et al.*, 1991). AP activity has been demonstrated histochemically in the cartilage matrix and the uncalcified collagen of osteoid (Bonucci *et al.*, 1992). These findings raise interesting points; AP may not need MV for calcification, non-skeletal AP can cause calcification, and structural macromolecules may be able to induce calcification with the aid of coexistent AP. There is a pressing need for the identification of the physiological substrate(s) and the molecular mechanism on the role of AP in calcification.

ATPase theory

Along with AP, MV contain a constellation of cell derived enzymes. A number of other phosphatases has been identified in isolated MV. ATP pyrophosphohydrolase, which hydrolyzes ATP and releases PP_i has been identified in MV (Hsu, 1983). There is a view that neutral proteolytic enzyme(s) that are released by chondrocytes digest(s) glycoproteins in the extracellular matrix which normally inhibits calcification. The removal of the inhibitory activity may promote calcification (Kawabe *et al.*, 1986). Carbonic anhydrase in MV may enhance calcification via the increase in pH (Sauer *et al.*, 1994). However, carbonyl anhydrase II deficient strain of mice have been shown to develop extensive vascular calcinosis (Spicer *et al.*, 1989). Of the enzymes of MV, the potential role of ATPase, which is localized on both the inner and outer surfaces of MV membrane deserves a comment.

Hydrolysis of ATP by ATPase has been theorized

to increase P_i in MV and to bring about calcification (Kanabe *et al.*, 1983). Recently, the serum level of ATP (0.6 mM) was shown to induce calcification in isolated MV. This was attributed to the entry of ATP into MV, where it may serve as a substrate for ATPase or may modify the MV membrane structure and initiate calcification (Hsu, 1992).

Cytoplasmic ATP in most cells is greater than 5 mM, and a large proportion can be released without significant effects upon cell viability (Gordon, 1986). Exocytosis from platelets is thought to be the primary source of circulating ATP. The release of ATP has been shown to take place in ischemic heart (Clemens and Forrester, 1981), isolated hypoxic cardio-myocytes (Forrester and Williams, 1977), hypoxic renal cortex and ascites tumor cells (Pentilla and Trump, 1974). Extracellular ATP ($[ATP]_o$) is eliminated by ectonucleotidases that have widespread existence in mammalian tissues (Gordon, 1986). The lungs eliminate most of the circulating ATP. Non-specific phosphatases do not participate in the elimination of $[ATP]_o$ by endothelial cells; the metabolism of ATP, ADP or AMP is unaltered in the presence of a large excess of beta-glycerophosphate or paranitrophenyl phosphate (Pearson *et al.*, 1980).

Extracellular ATP has a variety of physiological and pathological effects including cell death on susceptible cells (Avery *et al.*, 1992). $[ATP]_o$ increases cell membrane permeability of susceptible cells. The resultant influx of $[Ca^{2+}]_o$ appears to mediate cell injury and death (Zheng *et al.*, 1991). Of nucleoside-5'-tri-phosphates, only ATP and UTP causes an increase in $[Ca^{2+}]_i$ that is followed by cytoplasmic condensation, blebbing, DNA fragmentation, and eventual cell death (Conigrave and Jiang, 1995; Gordon, 1986). ATP induced increases in $[Ca^{2+}]_i$ are dependent upon extracellular P_i. Extracellular ATP appears to stimulate Na^+/P_i-cotransport, which in turn activates the Na^+/Ca^{2+}-exchange (De Young and Scarpa, 1991).

Extracellular ATP and UTP binds with purinoreceptors on the surfaces of many different cells (Conigrave and Jiang, 1995; Gordon, 1986) followed by breakdown of phosphoinositides and resultant increases in IP_3 and $[Ca^{2+}]_i$. P_2-purinergic receptor agonists also stimulate the breakdown of PC in cells, catalyzed by phospholipases C and D. Hydrolysis of PC is attributable in part to an increase in $[Ca^{2+}]_i$ which is induced by $[ATP]_o$ and other Ca^{2+}-mobilizing agonists in cells (Exton, 1990).

Since hydrolysis of ATP is the most readily available source of P_i which is needed for calcification, the theoretical role of ATPase in calcification appears attractive. In light of its capacity to induce apoptosis and to alter membrane permeability of cells equipped with P_2-purinoceptors, the potential role of ATPase in calcification deserves further evaluation. Chondrocytes have been shown to possess P_2-purinoreceptors (Leong *et al.*, 1994). It will be interesting to know if MV retain the receptors.

Phospholipids

Since the discovery of a zone of lipid deposition along the calcification front, the role of lipids as a potential nucleator of apatite has been considered (Irving and Wuthier, 1968). Because of their affinity for Ca^{2+}, phospholipids drew particular attention and have led to the subsequent discovery of MV in the epiphyseal cartilage and pathological calcifications.

Phospholipids occupy less than one half of the plasma membrane surface area. The rest of the area is occupied by proteins (Yeagle, 1989). Because of their hydrophobicity, fatty acid chains are repelled by water and PL in aqueous suspensions spontaneously form a bilayer or micelles exposing only hydrophilic head groups to water. The PL bilayer is virtually impermeable to water and water-soluble substances, and their transport across cell membranes is accomplished through intrinsic proteins (Deuticke and Haest, 1987). Membranes which bound the territory of the cell and regulate transports, play a vital role for the maintenance of the structure and function of the cells. The assembly of PL bilayers is likely to be the earliest event in the evolutionary steps of forming life on earth.

In eukaryotic cells, PL are synthesized in the ER, passed onto the Golgi apparatus, bound to carrier proteins, and transported to the plasma membrane via vesiculation and fusion (Sleight, 1987). The composition of PL in the plasma membrane varies with cell cycle, cell aging and a variety of environmental stimuli including dietary fats (Clandinin, 1991; Schroeder, 1984). Dietary lipid also modifies fatty acid composition of MV (Xu *et al.*, 1994a). Fatty acid chains of PL are heterogeneous; they vary considerably even in the same class of PL in the membrane. Phospholipids and embedded proteins diffuse laterally and individual PL molecules spin constantly (Jacobson *et al.*, 1987). Phospholipids are asymmetrically distributed in the membranes. The maintenance of PL asymmetry is dependent upon ATP, Ca^{2+} and Mg^{2+}. ATP-dependent flippases are primarily responsible for the asymmetry. The asymmetry of PL in the intracellular organelles are reversed; aminophospholipids face the cytoplasmic surface (Devaux, 1991). It has been proposed that cytoskeletal proteins (spectrin and band 4.1) may also play a role in the maintenance of PL asymmetry (Williamson *et al.*, 1982). There is a constant turnover of PL in response to exo- and endocytosis and agonist-induced PL breakdown (Kent *et al.*, 1991).

The molecules of each group of PL are clustered or

form domains within each PL layer of the membrane (Simons and van Meer, 1988). Proteins in the membrane and Ca^{2+} on the surface have been shown to be involved in the domain formation. Phospholipids in turn have a significant effect on the function of embedded proteins (Deuticke and Haest, 1987). The lipid-protein interaction frequently boosts the function of the intrinsic proteins, i.e., ion pumps and channels (Yeagle, 1989). Cytoskeletal proteins are attached to the inner surfaces of the plasma membrane via phosphatidylinositol and provide additional stability to the membranes.

The distribution of PL in the plasma membrane is disturbed by cell injury. In addition to reduction of PC by PLA_2, overloaded $[Ca^{2+}]_i$ has been shown to scramble the PL asymmetry in the red cell membrane. Red cell ghosts prepared in the presence of Mg^{2+} as the only divalent cation retain the normal phospholipid asymmetry. However, the presence of 5-10 μM Ca^{2+} during the ghost preparation results in ghosts in which lipid asymmetry had been abolished (Williamson *et al.*, 1992). Experimental increases in $[Ca^{2+}]_i$ with agents, such as, ionophores in red cells have been shown to abolish PL asymmetry by internalization of PC and sphingomyelin (**SM**) and externalization of phosphatidylserine (**PS**) and phosphatidyl-ethanolamine (**PE**; Williamson *et al.*, 1992). The scrambling of the asymmetry is independent from, but is reversible by, the action of PL-flippase (Verhoven *et al.*, 1992). When red cell ghosts are resealed in the absence of Mg^{2+}-ATP or when the aminophospholipid translocase is inhibited by vanadate or Ca^{2+}, a complete randomization of PC, PE, PS and SM analogues is demonstrable by NMR. If the ghosts are resealed with Mg^{2+}-ATP inside, the asymmetry is reestablished (Schrier *et al.*, 1992).

Observations of apatite formation mainly in the lumina of MV have been attributed to the presence of PS domains on the inner surfaces of MV as in the normal plasma membrane (Majeska *et al.*, 1979). The PL scrambling caused by increased $[Ca^{2+}]_i$ raises a question concerning the speculated role of PS in the intraluminal calcification of MV, because PS may no longer be on the inner layer of the membrane. *In vitro*, PS has been shown to inhibit apatite nucleation under certain circumstances (Boskey and Dick, 1991).

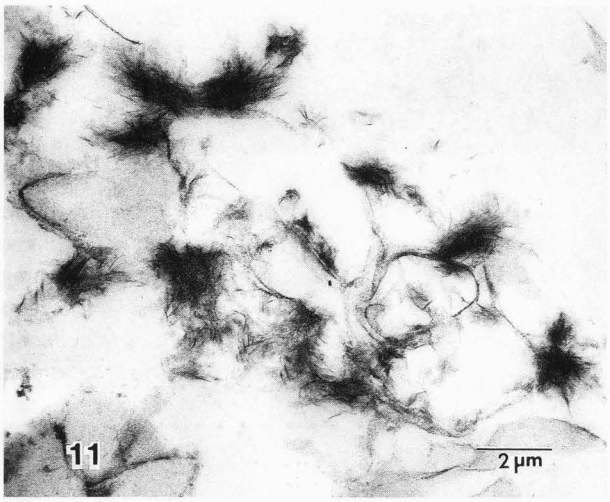
Interesting studies concerning the role of PL in calcification have been the extraction of proteolipids, and the calcium-phospholipid-phosphate complex (**Ca-PL-P_i**) from calcified tissues. These complexes are able to nucleate apatite *in vitro* and *in vivo* (Boyan *et al.*, 1989; Boskey *et al.*, 1988). Proteolipids are complexes of hydrophobic proteins and phospholipids derived from cell membranes which are extractable by organic solvents. Calcifiable proteolipids are associated with PS and the protein components are heterogenous. Interestingly, annexins of MV were claimed to exhibit properties similar to proteolipids (Genge *et al.*, 1991). Proteolipids and Ca-PL-P_i are coextracted from calcified tissues. Proteolipids incorporated into an artificial membrane act as a proton ionophore. A subset of proteolipids are incorporated into Ca-PL-P_i (Swain and Boyan, 1988). Calcifiable proteolipids are said to form prior to the Ca-PL-P_i formation in the process of an *in vitro* calcification. Thus these two groups of PL complexes appear to be closely related (Boyan *et al.*, 1989).

The Ca-PL-P_i complex has been isolated only from calcified tissues, both physiological and pathological, and are not extractable from non-calcified tissues (Boskey *et al.*, 1988). The complex has been extracted from isolated MV, that calcify *in vitro*, as well (Wuthier and Gore, 1977). Peculiarly, insertion of Ca-PL-P_i into liposomes inhibits their calcifiability (Boskey and Dick, 1991). The extraction of Ca-PL-P_i requires the presence of P_i in the solution. This has been interpreted that the complex formation rather than Ca^{2+}-binding to PL is prerequisite for lipid induced calcification (Boskey and Posner, 1982). Contrarily, one can raise the possibility that this represents the formation of an artificial complex during the extraction and the PL component of the complex is specific for calcified tissues. The marker for these complexes appears to be not yet available and it is not clear where and how these complexes exist *in vivo*. Cell membranes are the most likely origin of the complexes.

There is another line of evidence that PL can cause calcification: liposomes made of PS or PL similar to MV membrane have been shown to nucleate apatite in metastable solutions (Eanes and Hailer, 1987; Skrtic and Eanes, 1992) and in isolated serum (Kim, 1993). PL-bilayers thus have emerged as a candidate for apatite nucleator.

The mechanism of apatite nucleation by PL is far from clear. One possible mechanism would be that PL or their complexes serve as a substrate for heterogeneous nucleation of apatite. However, any theory should take into account that PL in membranes are highly mobile. Apatite deposits on liposome membranes tend to be vertically oriented in the "hair on the end" fashion on the surfaces of the membrane and do not alter the trilamellar structure of the membrane (Kim, 1993; **Fig. 11**). Similar deposition of apatite was noted in fat necrosis *in vivo* (**Fig. 12**). Heterogeneous nucleation offers the best explanation for the phenomenon.

It has been noted for some time that the outer surface of the plasma membrane electrostatically attracts Ca^{2+} and forms a condensed layer of Ca^{2+} (Tatulian, 1987; Seelig, 1990). The Ca^{2+} concentration in the condensed layer is estimated to be two orders of magnitude greater than that of the surrounding solution. The



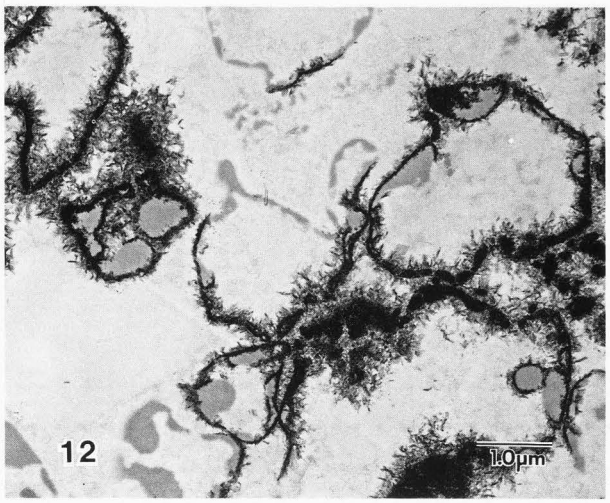


Figure 11 (at left). Liposomes incubated in MEM-2.5 for a week. Needle shaped apatite deposits in the hair-on-the end fashion on the membrane surface (see, Kim, 1993).

Figure 12. Apatite deposits in human mesenteric fat necrosis similar to that of liposomes.

---

concentration of Ca^{2+} in the high density layer appears to increase exponentially in relation to the Ca^{2+} concentration in the surrounding environment. The Ca^{2+} binding also brings about a vertical orientation of the polar head groups of PL, which are normally parallel to the membrane surface. The conformational change of the head groups enhances the dipolar electric fields, which may in turn regulate the activity of membrane bound proteins and their functions (Seelig, 1990). Any increase in P_i in this layer would be sufficient to bring about apatite nucleation.

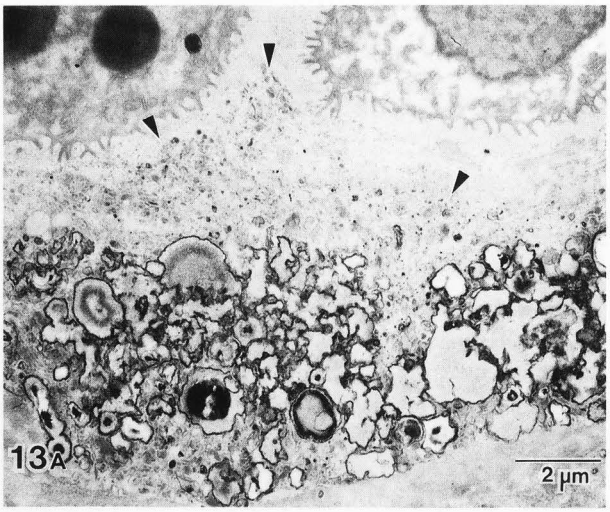
The likely reversal of the PL asymmetry due to an increased $[Ca^{2+}]_i$ and the formation of a similar layer of Ca^{2+} condensation along the inner surfaces of MV and blebs may provide an ideal milieu in which to nucleate apatite. There is evidence that Ca^{2+} binding indeed occurs on the inner surface of the plasma membrane (Long and Mouat, 1971).

Apoptosis theory

The primary function of epiphysis is growth of long bones. Lacking an ability to grow, long bones depend mainly upon their epiphyseal cartilages for growth. Growth is accomplished by cell divisions in the proliferating zone of the epiphysis, which is stimulated by pituitary growth hormone through IGF-I (Boehme *et al.*, 1992; Ohlsson *et al.*, 1992). Cell proliferation in a limited space calls for elimination of an equivalent number of cells or a cell turnover by apoptosis. Cell proliferation exceeding elimination will result in growth or a neoplasm (Williams, 1991). Dead cells in the epiphysis are incorporated into the zone of preliminary calcification which is further remodeled by bone cells into the completed bone tissue. This continuous addition of calcification to the bone enables it to grow longitudinally until puberty, when the secretion of pituitary growth hormone abates. Upon completion of growth, the epiphyseal plate vanishes altogether. Bonucci (1970) observed frequent degenerated cells in the resting zone of guinea pig epiphysis and advanced that MV may originate from cell degeneration. Kim and Huang (1971) and Kim (1976) demonstrated that calcifying MV in vascular tissue originate from senescent cell degeneration. A review of various mechanisms of calcification led Kardos and Hubbard (1982) to theorize that MV are apoptotic bodies.

There is morphological and chemical evidence that MV originate from the plasma membrane. Electron microscopy on serial sections of the growth plates have demonstrated the formation of MV by budding of the cell processes of chondrocytes (see, Anderson, 1989). The chemical profile of phospholipids in isolated MV is similar but not identical to that of the plasma membrane; MV contain greater amounts of cholesterol, PS and PE in contrast to intracellular membranous organelles, e.g., mitochondria (Wuthier, 1988).

Many studies have indicated that hypertrophic chondrocytes in the epiphysis undergo cell death. Morphological studies have demonstrated the changes of cell death towards the zone of provisional calcification (Brighton *et al.*, 1973; Holtrop, 1972). As cells reach the hypertrophic zone, they cease to synthesize DNA and RNA (Mankin *et al.*, 1968; Farnum and Wilsman, 1993). A measurement of nucleotides in the epiphyseal



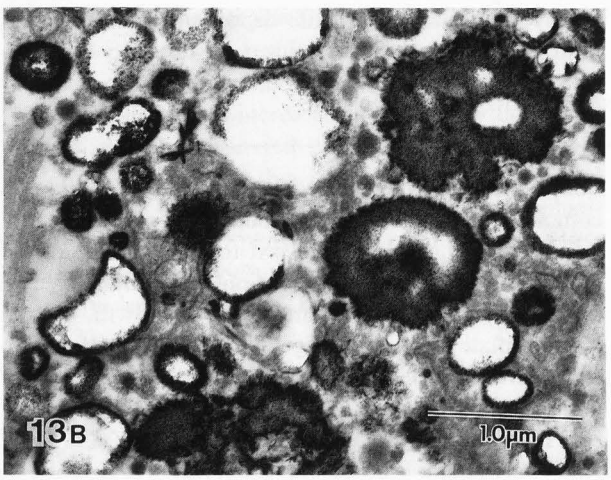


Figure 13. (**a**) Disintegration of chondrocytes *in toto* in aging human bronchial cartilage. CDP derived from cellular organelles apparently calcified. Discrete particles (MV) are also seen in the intercellular septum (arrowhead). The cell appears to have shed MV prior to its eventual death. (**b**) A closer view of an area showing that many CDP became thick-walled and had embedded needles. Membrane bilayers are no longer visible.

---

plate has demonstrated a decline in the activities of ATP and oxidoreductase associated with an increase in di- and monophosphates in the hypertrophic and calcifying zones (Shapiro *et al.*, 1983). Electron probe microanalysis (**EPM**) of the epiphyseal plate demonstrated intracellular accumulations of Ca and P towards the hypertrophic zone (Shapiro and Boyde, 1984). A similar gradient increase in $[Ca^{2+}]_i$ towards the hypertrophic zone has been demonstrated in isolated chondrocytes (Iannotti and Brighton, 1989). The increased $[Ca^{2+}]_i$ appears to correlate with MV formation (Buckwalter and Schaffer, 1987). Furthermore, Ca^{2+}-ionophore treatment causes MV formation by cultured hypertrophic chondrocytes (Iannotti *et al.*, 1994).

One study demonstrated an increased rate of phospholipid synthesis by hypertrophic chondrocytes and this result was taken as evidence against the cell death (Wuthier *et al.*, 1977). However, it has been shown that cells injured by anoxia and toxic agents display an accelerated phospholipid turnover (Farber and Young, 1981; Glende and Pushpendran, 1986). Isolated chondrocytes from degenerative arthritis have been shown to synthesize a greater amount of proteoglycans, as well (Lafeber *et al.*, 1993).

Chondrocytes secrete and embed themselves in a glycosaminoglycan rich matrix, which is avascular. Compared with capillary-rich tissues, cartilage is low in oxygen tensions (Maroundas, 1972). In fact, mesenchymal cells cultured under lower oxygen tensions favor a chondrocytic differentiation (Pawelek, 1969). Chondrocytes are well adapted to hypoxia by means of glycolysis (Meyer and Kunis, 1969). A large glycogen reserve in the cytoplasm is characteristic of chondrocytes. Towards the zone of mineralization, there is a further decrease in pO_2 with concomitant changes in the redox status signifying a hypoxic effect on the cells (Brighton and Heppenstall, 1971; Shapiro *et al.*, 1982).

Hypertrophic chondrocytes are terminally differentiated; they secrete type X collagen and are no longer able to divide (Boheme *et al.*, 1995; Kato *et al.*, 1988; Pacifici *et al.*, 1990). Hypertrophic chondrocytes express high levels of transglutaminase, TGF-β and c-myc, that have been shown to be involved in terminal differentiation and apoptosis of hypertrophic chondrocytes (Aeschilimann *et al.*, 1993; Boehme *et al.*, 1995; Kato *et al.*, 1988; Loveridge *et al.*, 1993). Hypertrophic chondrocytes are thus predestined to undergo apoptosis. In addition to a higher $[Ca^{2+}]_i$ detected by EPM in hypertrophic chondrocytes, isolated hypertrophic chondrocytes and MV have been shown to contain much higher concentrations of $[Ca^{2+}]_i$ and $[P_i]_i$ and decreased ATP than resting cells (Wuthier, 1988). A recent fluorescence image analysis and NMR of cultured hypertrophic chondrocytes demonstrated high concentrations of $[Ca^{2+}]_i$ and $[P_i]_i$ and a decrease in ATP prior to their formation of MV (Wuthier, 1993). The phenomenon is remarkably similar to that of blebbing in cell injury and apoptosis. The formation of MV in the epiphyseal cartilage may be construed as a form of blebbing by chondrocytes that are in the process of apoptosis.

It is common observation that chondrocytes which persist and age in non-epiphyseal cartilages regularly undergo cell death and calcification (Bonucci and Dearden, 1976). In addition to accumulations of discrete CDP, disintegration of the dead cell in its entirety occurs

frequently in aging cartilages (**Fig. 13**). Not only scattered blebs but freed cell organelles of the disintegrated cells participate in calcification. The major difference between the epiphyseal chondrocytes and other chondrocytes is the ability of the former to respond to growth hormone. Apoptosis of the senescent chondrocytes and their calcification in the resultant CDP are akin to epiphyseal calcification in slow motion.

It can be said that through the suicidal mission of hypertrophic chondrocytes, the epiphyseal plate continuously adds to the zone of calcification of the long bone to promote its growth. It is noteworthy that hypertrophic chondrocytes isolated from murine fetal bone have been shown to dedifferentiate and transdifferentiate into osteoblasts when pieces of cerebrum are added to the culture (Thesingh *et al.*, 1991). Growth factors play the key role for the regulation of transdifferentiation and extracellular matrix appears to stabilize the differentiated state of the cells (Eguchi and Kodama, 1993). However, the occurrence and the extent of such transdifferentiation *in vivo* remain to be determined.

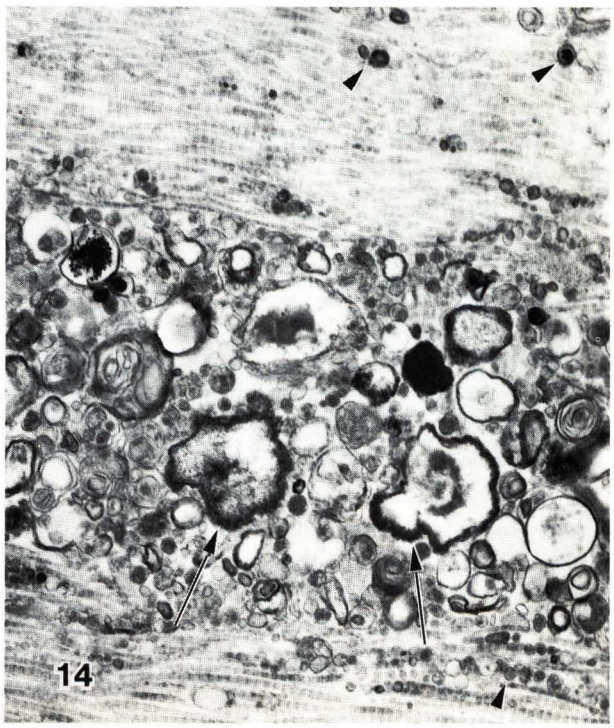
Pathological Calcification

The literature on pathological calcification is extensive and their review is beyond the scope of this article. Three areas of calcinosis: vascular, neoplastic, and renal calcinosis, in which the author has experience, will be reviewed in some detail.

Of dystrophic calcifications, vascular calcinosis has been most extensively studied because of their prevalence and clinical significance (Kim, 1983a). Vascular calcinosis will be discussed under three major headings; calcification in the heart valves, aorta and cardiovascular prostheses.

Calcinosis in aortic valves

Calcinosis in human aortic valves begins in the young, progressively increases with age and may give rise to calcific aortic stenosis (Sell and Scully, 1965). Therefore, the valvular calcinosis gained particularly early attention. Mönkeberg (1904) ascribed sclerosis of the aortic valves in old age to continuous mechanical stress and degeneration. Subsequent studies have concurred with the theory and shown that age related degeneration of the valve most likely underlies the mechanism of age associated calcification in the aortic valve (Angrist, 1964; McMillan and Lev, 1964). Sell and Scully (1965) observed a progressive decrease in the number of fibroblasts and a gradual accumulation of calcific deposits along with lipids in the fibrosa of aging aortic valves. Kim and Huang (1971) demonstrated that the lipid deposition in aging valves corresponds to the accumulation of CDP derived from disintegration of



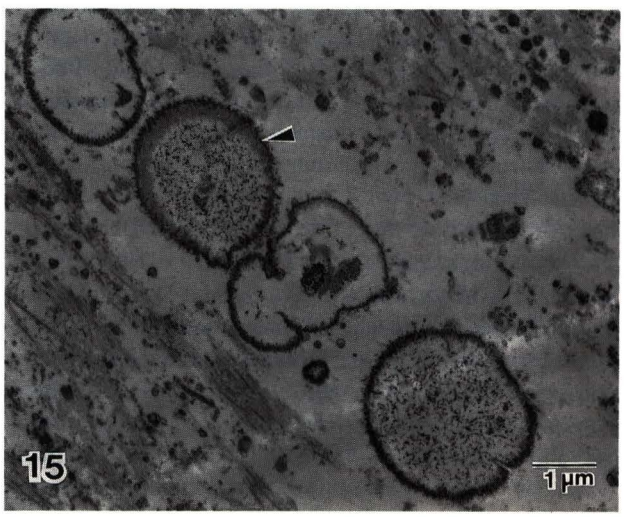


Figure 14 (top). A degenerated fibroblasts in a 12-year-old canine aortic valve. Calcific deposits are seen in the thickened wall of disintegrated cell organelles (arrow). Discrete CDP (arrowhead) are presumably formed by blebbing.

Figure 15 (bottom). Thick-walled vesicles in human aortic valve. Needle shaped apatite crystals are radially embedded in the thick-wall. The lumen contains granular particles. A peculiar layer of electron lucent condensation is seen in a vesicle (arrowhead). In view of their large size, these vesicles are assumed to have originated from the plasma membrane. From Kim *et al.*, 1986, with publisher's permission.

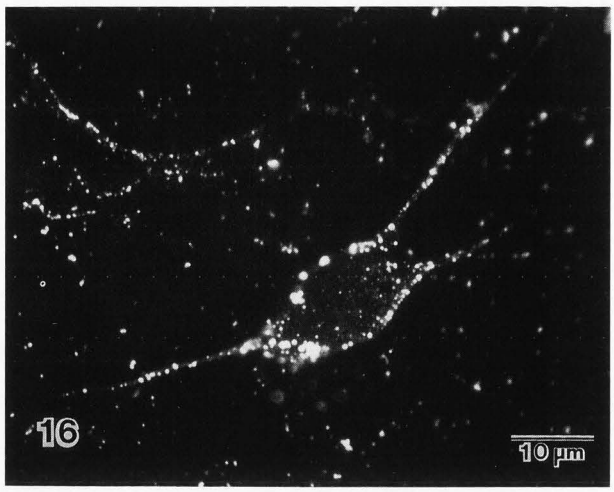


Figure 16. Anoxically injured canine valvular fibroblasts. Fluorescent micrograph of cells stained with 0.03% calcein. Bright spots are seen in the cell especially along the plasma membrane, that are consistent with early stages of blebbing. Extracellular bright spots indicate calcified blebs. From Kim, 1994, with publisher's permission.

---

senescent fibroblasts. These CDP were the primary site of calcification (Kim, 1976; Kim *et al.*, 1976; 1986).

Of the tissue components, fibroblasts in human aortic valve display most conspicuous age changes. In senescent fibroblasts, the number of mitochondria has an inverse relationship with that of residual bodies. Many cells in the valves were filled with residual bodies and mitochondria were scanty to absent (Kim, 1976; Kim *et al.*, 1976). Mitochondrial decay due to oxidative damage and their subsequent autophagocytosis, that eventuate in residual bodies, are thought to be a mechanism of cell aging (Brunk *et al.*, 1992; Shigenaga *et al.*, 1994). Autophagocytosis of mitochondria is common in aging valvular fibroblasts (Kim, 1976). A variety of structural and functional deterioration including those of mitochondria by senescent cells has been demonstrated (Cristofalo *et al.*, 1994; Dice 1993). Although blebbing is likely to have occurred, disintegration of the cells in their entirety appeared to be the major source of CDP in the valves (**Fig. 14**). The fate of the plasma membrane of the disintegrated cells is obscure. However, large sized, thick-walled vesicles are frequently encountered amid CDP suggesting their origination from the plasma membrane (**Fig. 15**).

Canine aortic valves reveal changes nearly identical to that of human (Kim *et al.*, 1986). Fibroblasts in the aortic valves were larger in their size, fewer in number, and contained a greater amount of residual bodies compared to the pulmonic valves. An accumulation of numerous membranous CDP with frequent calcific deposits was noted regularly in the aortic and mitral valves, whereas the pulmonic and tricuspid valves were devoid of such cell debris. This disparity is attributable to the difference in the rate of population doublings and of exhaustion of the cellular renewal potential between the right and the left sided valves. Were there no limit in cell replication, such loss of cells should not occur. To test that the loss of cell renewal is involved in valvular aging, colony size of fibroblasts cultured from aortic and pulmonic valves obtained from 5 dogs were compared by the method of Smith *et al.* (1978). In every dog, the colony size of fibroblasts from the pulmonic valve was larger than that of the aortic valve (**Table 2**; Kim *et al.*, 1985b). The findings further substantiate that the gradual loss of cells in the valves results from an exhaustion of cellular renewal potential as observed *in vitro* (Hayflick, 1977).

Direct evidence of cell death of fibroblasts as the cause of calcification was demonstrated using cultured cells. When cultured canine valvular fibroblasts were injured by anoxia and freeze-thaw, the cells calcified in MEM-2.5, whereas uninjured control cells did not (Kim, 1994; **Fig. 16**). Extracellular Ca^{2+} was required for calcification. Calcification of anoxically treated cells occurred in mitochondria and extracellular blebs. Inhibition of Ca^{2+}-pump and ATP synthesis, and ionophoric treatment of fibroblasts similarly resulted in calcification. The findings were taken as evidence that the loss of ATP and Ca^{2+}-pump activity can cause calcification.

Terminal differentiation, aging and apoptosis of fibroblasts is widespread (Bayreuther *et al.*, 1992). The reason for the accumulation of calcified CDP peculiar to valvular tissue is attributable to avascularity and the lack of scavenging as discussed above. There has been evidence that gradual loss of Ca^{2+} regulation occurs in aging cells (Eckert *et al.*, 1994; Peterson and Goldman, 1986). A gradual decline in ion regulation as a part of generalized deterioration in cell aging thus may further exacerbate aging and apoptosis of fibroblasts. In senescent fibroblasts filled with residual bodies, effective ATP synthesis is unlikely. Although calcification of blebs formed by senescent fibroblasts are likely to be due to increased $[Ca^{2+}]_i$ and $[P_i]_i$, calcification occurring in the whole cell disintegration may involve a different mechanism. The fate of increased $[Ca^{2+}]_i$ in senescent cells (Peterson and Goldman, 1986; Shapiro and Lam, 1982) is not known but its potential contribution to the subsequent calcification of degenerated cells is likely. Alterations of the membrane phospholipids and their potential roles in calcification are discussed above.

Aortic calcinosis

Despite frequent calcification in atherosclerosis, and

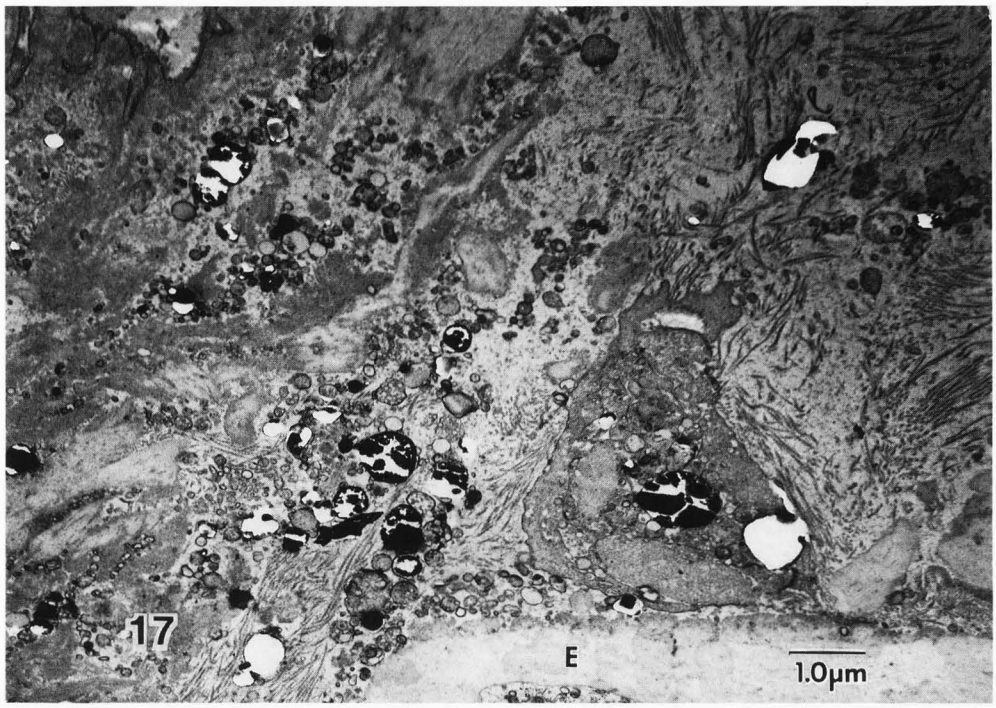


Figure 17. Accumulation of CDP with calcification in "normal" aged human aorta. Elastic fibers (E) are free of calcification.

Table 2. Colony size of aortic and pulmonic valve fibroblasts (values ± standard deviation).

	No. of colonies per flask	% of flask area	Mean area of colonies (mm^2)	Mean diameter of colonies (mm)
Pulmonic valve	64.5 ± 23.6	8.9 ± 3.81	10.29 ± 0.97	3.35 ± 0.15
Aortic valve	10.2 ± 7.7	1.1 ± 1.2	6.46 ± 1.65	2.77 ± 0.39

presumably due to the complexity of the changes therein, detailed studies on atheromatous calcinosis are relatively rare. In experimental and human atherosclerosis, calcification was claimed to be associated with membranous vesicles similar to MV (Tanimura *et al.*, 1986a, 1986b). Similarly, atheromatous plaques from carotid arteries that were surgically removed revealed that membranous CDP were the predominant loci of calcification (**Fig. 2**). In addition to atherosclerosis, aortic media calcifies commonly in association with age.

Aortic medial calcinosis precedes intimal atherosclerosis and has been thought to have a role in the development of atherosclerosis (Blumenthal *et al.*, 1950; Kramsch *et al.*, 1981). The medial calcinosis begins in the second decade and increases with age. In light of the abundance of elastin and the earlier study results, it has been entertained that elastin serves as the nidus for vascular calcinosis (Schiffman and Marytin, 1962; Urry *et al.*, 1974). However, subsequent studies disclosed that membranous vesicles (CDP) are the primary loci of calcification in aging human aortic media and atheromatous plaques (Gardner and Blankenhorn, 1968; Hoff, 1973; Kim, 1976; Paegle, 1969) and spontaneously aged rats (Morgan, 1980; **Fig. 17**). Rats fed with high doses of vitamin D developed calcification in association with lipid deposition in the aorta (Irving and Wuthier, 1968). Cliff (1970) described calcified membranous particles originating from "necrotic" cells in aging rat aorta. Age-dependent calcification also occurs in a variety of tissues, e.g., pineal gland, brain, cartilage, kidneys and senile cataract, just to list a few examples (Bonucci and Dearden, 1976; Haggit and Pitcock, 1971; Hightower and Reddy, 1982; Zimmerman and Bilaniuk, 1982).

Calcification of elastic fibers in pseudoxanthoma elasticum has been one of the main reasons for implicating the role of elastin in calcification (Martinez-Hernandez and Huffer, 1974). In accordance with previous observations, an extensive calcification of elastic fibers was seen in skin biopsies of pseudoxanthoma elasticum. However, closer examination revealed large numbers of membranous vesicles in apposition to elastic fibers. Calcific deposits were seen in both the vesicles and elastic fibers (**Fig. 18**). The presence of calcified CDP in the proximity of elastic fibers attests the likely role of

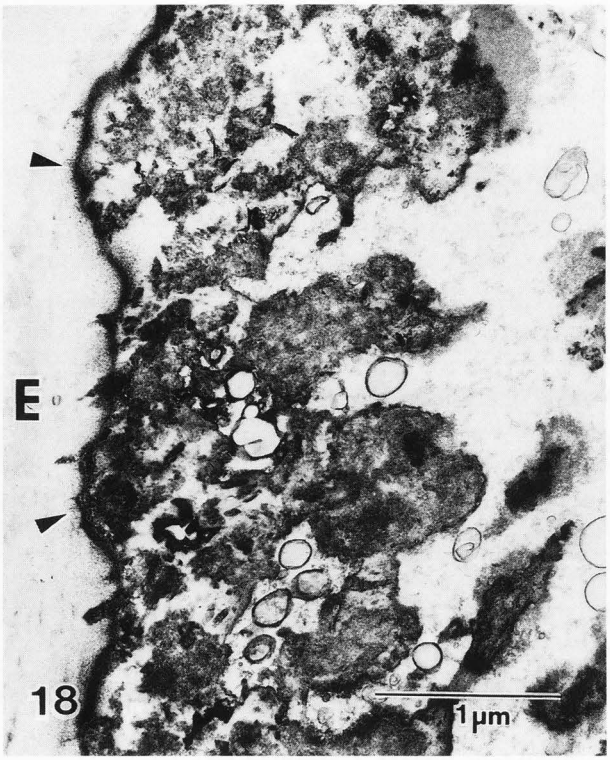


Figure 18. A skin biopsy of pseudoxanthoma elasticum. A massive accumulation of CDP in apposition to an elastic fiber is present. Calcific deposits are seen in both CDP and elastic fiber (arrowheads).

CDP for calcification in pseudoxanthoma elasticum, as well. An accelerated cell death or defect in scavenging may be involved in pseudoxanthoma elasticum. In an allograft study of the vascular tissue in the anterior chamber of rat eye, whole aortic segments calcified, whereas isolated and washed bovine elastic fibers did not (Urist and Adams, 1967). When isolated collagen and elastin were incubated in MEM-2.5, they failed to calcify, whereas, liposomes and red cell ghosts did (Kim, 1993).

Vascular calcinosis also occurs commonly following tissue injury. In experimental anastomosis of piglet aorta, calcification occurred at the necrotic site of anastomosis and clamp injury (Berry *et al.*, 1970). In experimental hypervitaminosis D, aortic calcification may not necessarily be related to hypercalcemia. Calcification occurred in foci of aortic degeneration (Hass *et al.*, 1958). When segments of rat aorta were grafted subcutaneously and incubated in millipore chambers in the peritoneum of inbred rats, freeze thawed aorta calcified more intensely than fresh aorta (Kim, 1978). Similarly, organ cultured rat aorta developed calcification in occasional CDP, whereas freeze-thawed aorta calcified intensely (Kim, 1984).

Vascular calcification has features in common with epiphyseal calcification. In both, membranous vesicles (CDP, MV) derived from apoptotic cells are the loci of calcification, and both tissues are avascular. Avascularity and the lack of scavenging appear to be the common denominator of calcifying tissues. It can be said that fibroblasts and smooth muscle cells in aging aortic valves and aortae undergo apoptosis one by one and give rise to CDP, that serve as the nidus for calcification. Of the programmed cell deaths, cell aging is representative.

Cardiovascular prosthesis

Calcific aortic stenosis is commonly treated by surgical replacement of the valves with glutaraldehyde-fixed porcine valve. Unfortunately, the prostheses frequently develop calcification and subsequent failure. The mechanism of the prosthetic calcification, therefore, has been of interest (Schoen *et al.*, 1988).

Interestingly enough, bioprosthetic calcification has been shown to occur in the cells. A certain capacity to induce calcification by the cells evidently survives the glutaraldehyde fixation. Fibroblasts in the prosthesis were shown to retain alkaline phosphatase activity. Inhibition of AP activity was said to inhibit calcification in the prostheses (Levy *et al.*, 1991). In view of devitalization of the cells by the fixation, increases in Ca^{2+} and P_i as the mechanism of the prosthetic calcinosis are questionable.

In order to simulate cellular calcification in porcine valve bioprosthesis, canine valvular fibroblasts in culture were fixed in 0.5% glutaraldehyde for 5 days followed by their incubation in MEM-2.5. The fixed cells calcified in a week, whereas live cells did not. Glutaraldehyde fixation may be construed as a form of cell injury that results in calcification (Kim, 1994). It is interesting that porcine valves subjected to ischemia for 24 hours prior to fixation led to severe calcification. In the valves treated by ischemia at 4°C, calcification was significantly reduced in comparison to the valves with ischemic treatment at 25°C. Retardation of metabolic activities in cold ischemia was presumed to account for the lesser degree of calcification (Neethling *et al.*, 1992). Considering the time spent for the removal of the valves and diffusion of glutaraldehyde to the cells, some ischemic injury to the cells in the valves is inevitable (Maranto and Schoen, 1988). Theoretically, elimination of Ca^{2+} from the tissue prior to glutaraldehyde fixation may reduce their subsequent calcification. For human aortic valve allografts, cell viability has been shown to correlate with improved clinical performance of the grafts (McNally and Brockbank, 1991).

Setting aside the incompletely understood mechanism of prosthetic calcification, interest has lately been shifted to the prevention of prosthetic calcinosis. A

significant progress in retardation by pharmaceutical means of the prosthetic calcinosis is being made (Schoen *et al.*, 1992).

In addition to bioprosetheses, synthetic prostheses, i.e., artificial hearts, frequently calcify and fail. In artificial hearts, a layer of neointima is formed on the surface of the pump and embed cells. Cells adherent to the pulsatile area of the pump membrane regularly undergo cell death and calcification (Harasaki *et al.*, 1985). The mechanism is remarkably similar to calcification in heart valves and aortic media.

Nephrocalcinosis

Because of the likely causal relationship with urolithiasis, nephrocalcinosis gained an early attention. Nephrocalcinosis is very common in humans. Microscopic foci of calcinosis are seen in every adult kidney (Anderson and McDonald, 1946; Burry *et al.*, 1976). There has been evidence that nephrocalcinosis results from various tissue injuries. In patients who had recovered from acute tubular necrosis, calcification in the necrotic cortex is visible through radiography (Heptinstall, 1974). Diffuse nephrocalcinosis is frequently observed in rejected renal transplants (Harrison and Vaughan, 1978). Furthermore, calcinosis can be the cause of the transplant failure (Hartman *et al.*, 1991). Anderson (1968) observed a strong correlation between calcification at the cell debris in the duct of Bellini and urinary stones in man. Ramzy and Elwi (1968), in a histochemical study, considered hyaline cast formation to be precalcific. When dogs were bled to induce shock followed by re-transfusion of autologous blood in 24 hours, and the kidneys were examined in a week, extensive tubular necrosis and calcinosis were noted mainly in the proximal tubules. Calcific deposits were seen in CDP and flocculent densities in mitochondria that were free in the tubular lumina (**Fig. 5**).

Nephrocalcinosis is common in end-stage kidney diseases of various etiologies with normal serum Ca^{2+} x P_i. Calcific deposits are mainly observed in the tubules (Ibels *et al.*, 1981). A high incidence of nephrocalcinosis has been noted in very low birth weight infants. Tubular injury and cell death are believed to increase the probability of crystal formation in these infants (Adams and Rowe, 1992). Calcinosis of renal tubules is common in neonates with fibrocystic disease; flocculent densities in mitochondria of proximal tubule cells were frequent in these kidneys (Katz *et al.*, 1988). Recently, an increase in the incidence of nephrocalcinosis in systemic infections especially in AIDS patients has been noted (Bargman *et al.*, 1991; Falkoff *et al.*, 1987; Seney *et al.*, 1990). Nephrocalcinosis in hyperprostaglandin E syndrome apparently results from hypercalciuria secondary to bone resorption (Shoemaker *et al.*, 1993).

In the face of its strategic location, the concept that Randall's plaque gives rise to a stone nidus was considered (Randall, 1937). However, poor association between the incidences of the plaque and urolithiasis has aroused an alternative view (Anderson and McDonald, 1946; Heptinstall, 1974). Resnick and Boyce (1979) recovered calcospherites from stone former's urine and considered them as an embryonic stage of urinary calculi. Jordan *et al.* (1978) suggested that the majority of calcium crystallization occurs in the nephron.

Presumably due to artificial ingredients in the diet, nephrocalcinosis is common in experimental animals, especially in female rats. In addition to hypercalcemia, nephrocalcinosis in rats can be induced by electrolyte imbalance, e.g., Mg or F deficiencies and changes in dietary composition (Ritskes-Hoitinga and Beynen, 1992). In experimental hypercalcemia, including hyperparathyroidism, calcification began in mitochondria and cytoplasmic vacuoles of degenerated tubules (Caulfield and Schrag, 1964). Calcium gluconate injection to rats resulted in calcification in necrotic proximal tubules. Calcification was observed in mitochondria of necrotic cells (Ganote *et al.*, 1975). In nephrocalcinosis due to chloride depletion (Sarkar *et al.*, 1973), in rats fed with semi-purified diet (Woodard, 1971), and in rats fed with caries-reducing diet (Collan *et al.*, 1972), calcification was mainly intraluminal frequently in association with changes of cell injury. In magnesium deficiency, proximal tubular degeneration and calcification was followed by similar calcification in the Henle's loop (Ko *et al.*, 1962). In $HgCl_2$ poisoning of rats, calcification occurred in sloughed off necrotic cell debris in the tubular lumen (Siegel and Bulger, 1975).

In experimental nephrocalcinosis, nodular calcific deposits were commonly observed in the basement membrane (Ganote *et al.*, 1975). In drug induced medullary necrosis, calcification of membranous vesicles were seen in the basement membrane (Shimamura *et al.*, 1974). Similar membrane bound calcific deposits are common in human placenta (Varma and Kim, 1985). However, in nephrocalcinosis *in vitro*, the basement membrane did not appear to participate in calcification (Kim, 1983c). The basement membrane probably sequesters CDP that give rise to the deposits.

To further study the role of cell injury and identify the initial loci of calcification, rat renal cortical tissue was anoxically incubated in a metastable solution. Calcification began in 24 hours in membranous CDP and flocculent densities in mitochondria. After a few weeks of incubation, calcium level in the solution plateaued at 1/3 of the serum level (Kim, 1983c).

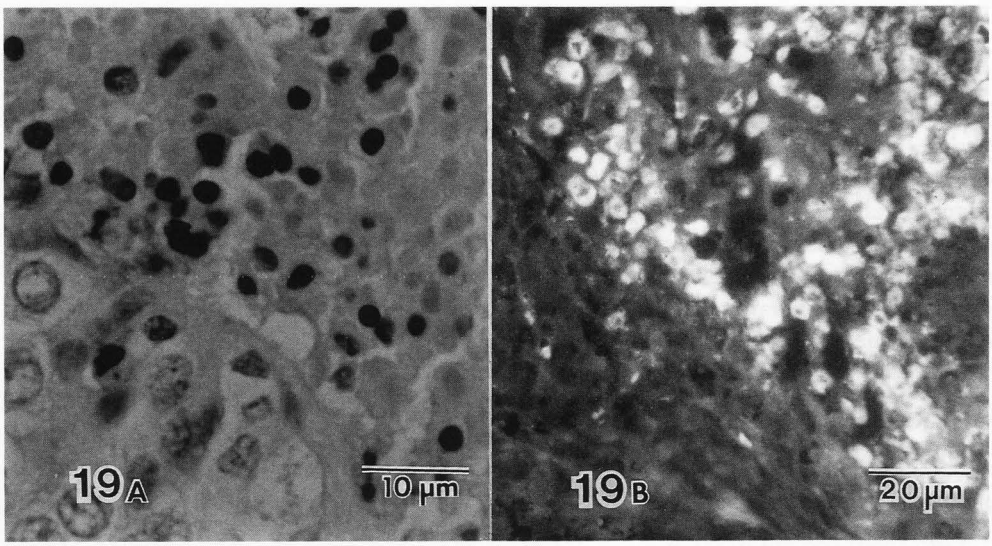


Figure 19. (**a**) A large cell carcinoma of the lung with "tumor necrosis." Desquamated cells are shrunken and their nuclei are pyknotic (chromatin condensation). Hematoxylin and eosin stain. (**b**) Fluorescent micrograph of the same area stained with 0.03% calcein for 1 minute showing fluorescence in dead cells.

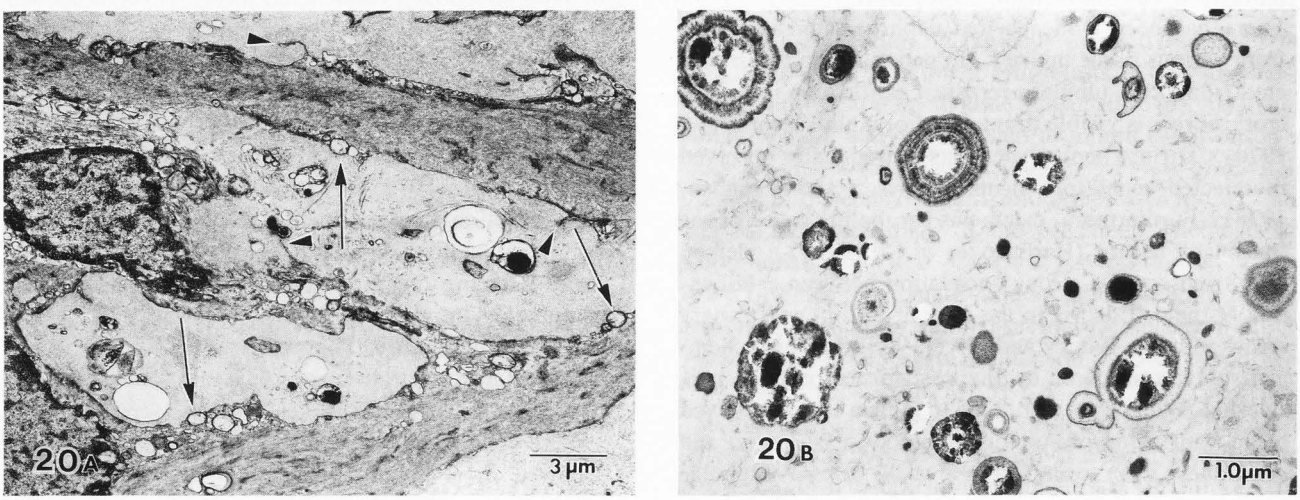


Figure 20. (**a**) Discrete CDP in a gastric leiomyosarcoma. Calcific deposits are seen in CDP. Adjacent cells show villous projections of the plasma membrane (arrowheads). It also appears that membranous vesicles are being exocytosed (arrow). (**b**). An area of calcific deposits in the same tumor. Morphology of CDP and their calcific deposits are complex.

There has been a view that urinary stones may originate from intraluminal calcinosis (Drach and Boyce, 1972). Cell debris of necrotic tubular epithelial cells including mitochondria have been observed to serve as nidi of nephrocalcinosis (Anderson, 1968; Kim, 1983c; Trump *et al.*, 1980). Like other tissues, renal tubule cells turnover constantly (Cameron, 1971). Dead cells are excreted as urinary casts. The urinary tract thus has a constant supply of potential nidus for calcification. In decalcified urinary stones, membranous CDP including thick-walled vesicles and bacteria were recovered (Kim, 1983d). Calcium phosphate crystalluria is seen in every adult (Werness *et al.*, 1981). Calcium phosphates are the most common component of urinary stones. When closely examined, every stone contains some apatite (Kim, 1982). Calcium phosphates form 80% of the cores of calcium stones (Leusmann, 1982), especially whewellite stones (Kim *et al.*, 1985a). Programmed cell death of renal tubules is likely to have an important role in urinary stone formation. Abnormal sequestration of calcified CDP in the urinary tract may eventuate in many stones. It is interesting that CDP also serve as a substrate for heterogeneous nucleation of calcium oxalate in urine (Kahn, 1995).

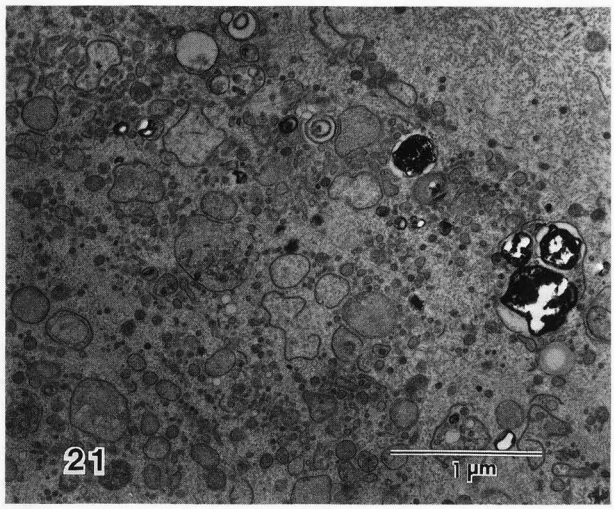


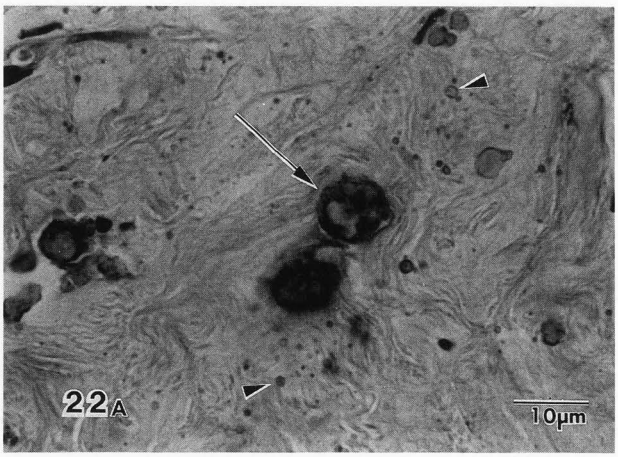
Figure 21. A total cell disintegration in a medullary carcinoma of the thyroid. Calcific deposits are seen in CDP. Trilamellar structure of the membrane is intact. These CDP are presumed to be of earlier stage than that seen in Figures 12 and 13.

Neoplastic Calcinosis

Presumably due to the rapid turnover of cells, apoptosis is commonly observed in neoplasms (Kerr *et al.*, 1994). Sequestration of dead cells as a result of the loss of their normal excretory mechanism in neoplasms may account for the high incidence of apoptosis in neoplasms. There is an increasing view that neoplasms are a manifestation of the disturbance in the rate of apoptosis (Wright *et al.*, 1994).

Calcinosis is also prevalent in neoplasms and frequently serves as a diagnostic landmark, i.e., in mammography. Examples of neoplastic calcinosis in the literature are too numerous to cite. The so-called psammoma bodies are frequently seen in papillary adenocarcinomas and meningiomas. Calcification of psammoma bodies begins in MV (Ferenczy *et al.*, 1977; Lipper *et al.*, 1979). In certain tumors, such as gonadoblastoma, calcific deposits are the intrinsic part of the tumor (Scully, 1970). It is not an overstatement that practically every known tumor may calcify in microscopic foci.

It is common observation that neoplastic calcinosis occurs in association with cell death (**Fig. 19**). In calcifying epithelial odontogenic tumor of Pindborg, breast carcinomas and pituitary adenomas, calcification was observed to occur in degenerate cells and their debris (Anderson *et al.*, 1969; Kim, 1983a; Landolt and Rothenbuhler, 1977; Stegner and Pape, 1972). In experimental malakoplakia, calcification appeared to begin in association with degenerated macrophages (Csapo *et al.*, 1975).



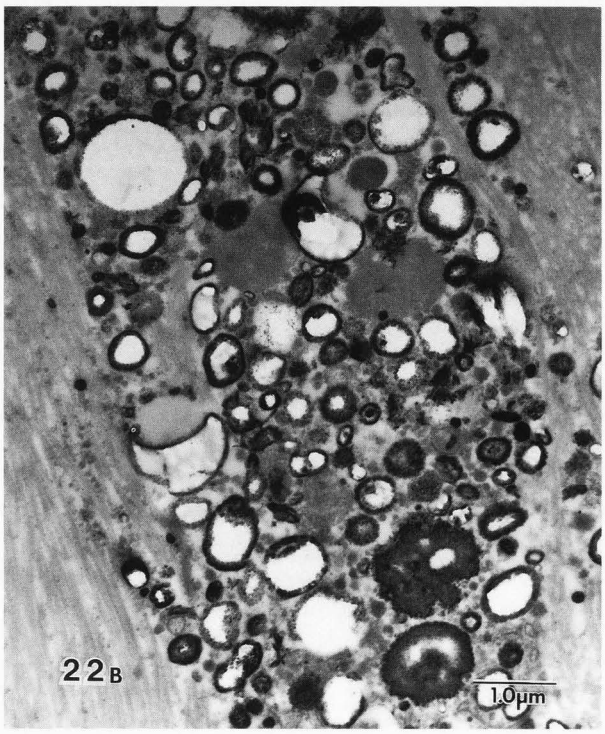
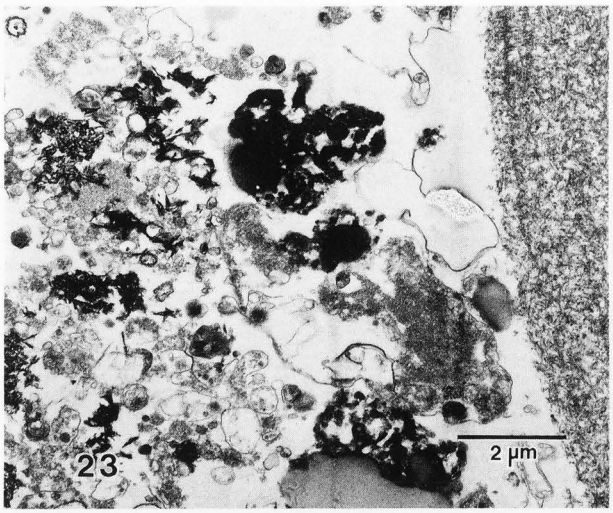


Figure 22. (**a**) An area of sclerosis in a fibroadenoma, the same tumor shown in Figure 7. In addition to numerous CDP (arrowheads) in dense collagenous tissue, calcification of total cells is seen (arrow). (**b**). A closer view of an area similar to Figure 21a. Trilamellar membrane is no longer visible. Many calcified CDP became thick-walled.

With the advent of radiation and cytotoxic agents for the treatment of cancer, increased incidence of calcinosis following tumor necrosis has appeared in the literature. When a disseminated ovarian carcinoma was treated with irradiation, the tumor disappeared but was replaced by diffuse calcification of the peritoneum (Menuck, 1976).



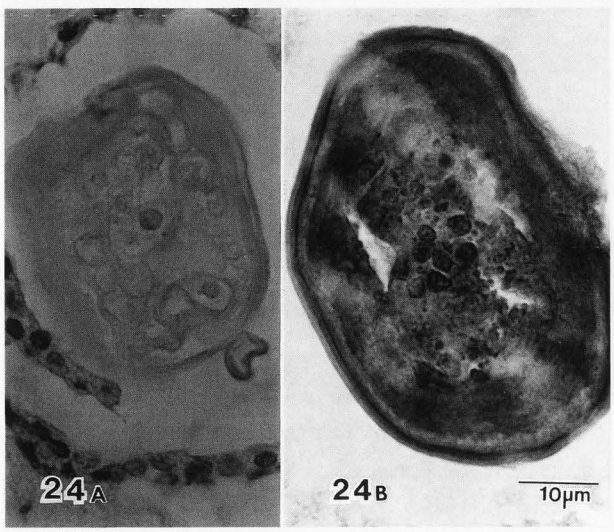


Figure 23. Calcified CDP in an organ cultured human prostate gland lumen. A portion of corpus amylaceum (amyloid body) is seen in the right, that is free of calcification. From Kim, 1982, with publisher's permission.

Figure 24. (**a**) A prostatic amyloid body retaining dead cells. Hematoxylin and eosin stain. (**b**) A calcified amyloid body in the same prostate. Mummified cells are visible.

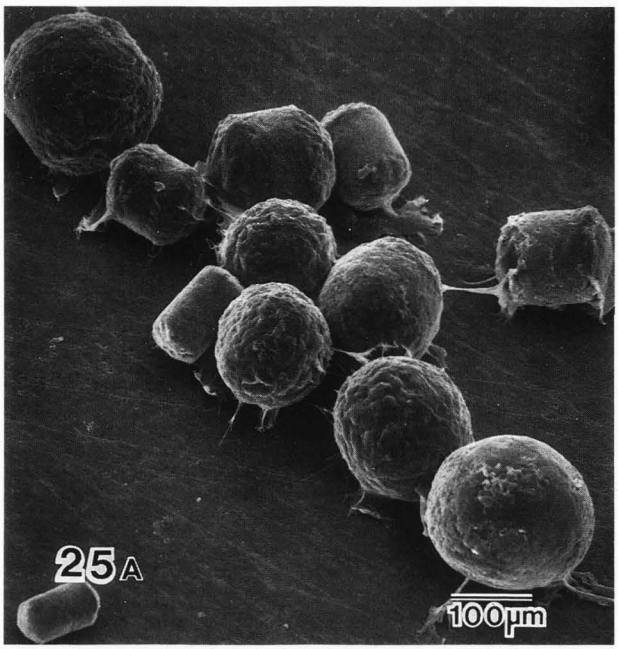
Irradiation or chemotherapy of various cancers frequently causes symptomatic calcification (Bertrand *et al.*, 1977; Jones *et al.*, 1979). In a childhood leukemia treated with cytotoxic agents, calcification developed at the site of previous tumor infiltration in the brain (Moir and Bale, 1976). Tumors resected following local irradiation frequently reveal extensive cell death. These irradiated tumors frequently display calcification in association with CDP (Kim, 1983a).

Table 3. Break-down of 54 human tissues studied.

Specimens	Number
Adenocarcinomas (lung, stomach, ovary, colon and Kidney	12
Squamous Cell Carcinoma, Larynx	2
Basal Cell carcinoma, Skin	1
Epidermal Cysts (including 2 Malherbe's)	5
Leiomyosarcoma, Gastrointestinal	3*
Meningioma	3
Spindle Cell Lipoma	1*
Odontogenic Cyst	1
Carcinoid, small intestine	3
Medullary Carcinoma, Thyroid	1*
Fibroadenoma, Breast	1
Transitional Cell Carcinoma, Bladder	1
Seminoma, Testis	1
Osteochondroma, Toe	1
Calcifying Tendinitis, Shoulder	1
Tracheal Cartilage	2*
Fat Necrosis, Mesentery	2
Pseudoxanthoma Elasticum, Skin	2
Tuberculosis, Pulmonary	1
Pelvic Lymph Nodes	4*
Atheromatous Plaques, Carotid	4*
Intervertebral Disc	1
Ligamentum Flavum	1

*Cases in which thick-walled vesicles are seen.

To study the extent of cell death involved in neoplastic calcinosis, a series of surgically removed human neoplasms were studied. The results were compared with non-neoplastic calcinosis (**Table 3**). The mode of calcification was remarkably similar regardless of the type of neoplasms or tissues involved. In every calcified tissue, there were scattered membranous CDP in the extracellular space. Adjacent cells frequently displayed villous projections of the plasma membrane suggesting the formation of CDP by blebbing. The smallest calcific deposit was invariably seen in membranous CDP (**Fig. 20**). In addition to discrete CDP, there frequently were clusters of CDP retaining the same approximate size and shape as the cells in the vicinity, indicating disintegration of the cells *in toto*. Freed organelles of the disintegrated cells also participate in calcification (**Fig. 21**). Calcification in recognizable mitochondria was rarely seen in neoplastic calcinosis.



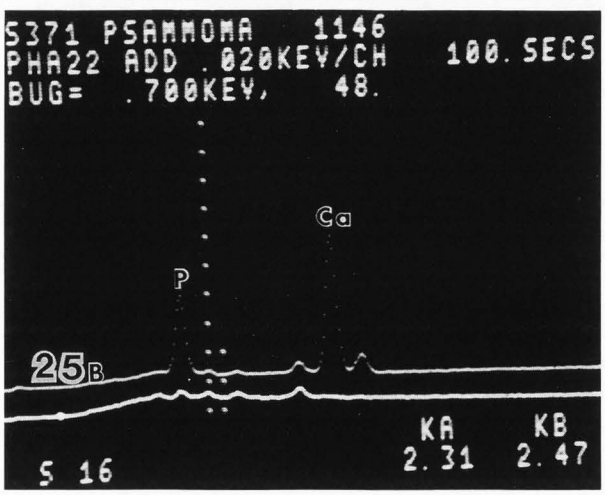


Figure 25. (**a**) Scanning electron micrograph of psammoma bodies isolated from a meningioma. PB are either spherical or cylindrical. The tissue was digested in concentrated NaOH solution and PB were separated by centrifugation. (**b**) EPM of psammoma bodies (upper spectrum) and adjacent microfibrillar (amyloid) stroma (lower spectrum). The presence of S in both indicates that the stroma is incorporated in the body.

Mammary calcinosis occurs commonly in the duct lumen in association with CDP. Calcification of the breast stroma is believed to be rare (Anastassiades *et al.*, 1984). However, in densely fibrotic areas, especially in neoplasms, where scavenging would be ineffective, calcinosis in blebs and the disintegrated cells is common (**Fig. 22**). Similarly, calcification of CDP is common in

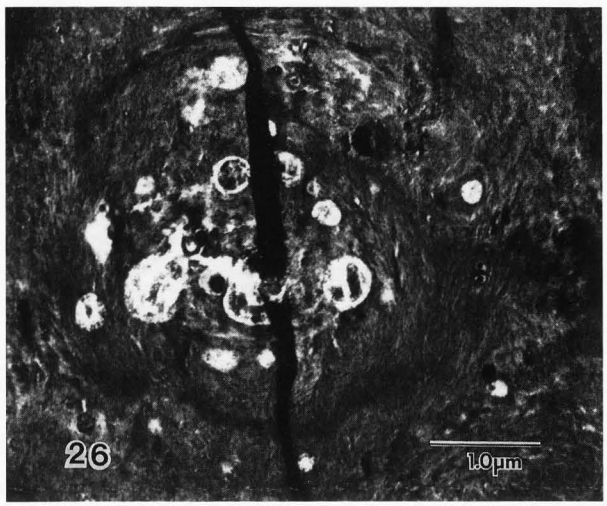


Figure 26. The central core of a psammoma body in a meningioma. Cell debris occupy the core.

---

prostatic gland lumina (**Fig. 23**). Apparently, calcified CDP in the lumina are normally excreted. However, dead cells and CDP are frequently incorporated in corpora amylacea and are thus retained. In calcified corpora amylacea, calcified cell ghosts are often seen (**Fig. 24**). It appears that incorporation and retention of apoptotic cells in corpora amylacea and resultant calcification leads to the formation of prostatic calculi.

Isolated psammoma bodies (**PB**) frequently assume cylindrical as well as spherical shapes (**Fig. 25a**). PB in meningiomas frequently contained sulphur by EPM. However, surrounding microfibril rich matrix also contained sulphur (**Fig. 25b**). Concentric layering of microfibrils with properties of amyloid (corpora amylacea) has been shown to incorporate in PB (Foschini *et al.*, 1993; Kepes, 1961). Dead cells and cell debris frequently occupy the center of PB (**Fig. 26**). Corpora amylacea are common in various neoplasms (see, David and Kim, 1982). It can be said that cell death underlies the mechanism of calcification in psammomatous neoplasms and the prostatic glands, and corpora amylacea serve as the template for PB and prostatic stone formations.

In massively calcified specimens, every structure in the tissue was obscured by calcific deposits and their relationship with organic structures was not possible to determine. Calcification of collagen fibers was seen only in heavily calcified tissues.

Role of Membranes in Calcification

The mechanism of CDP calcification in various calcinosis is complex. Three modes of calcification were commonly noted; intraluminal, juxtamembranous and in-

tramural. Intraluminal calcification is most prevalent and can be attributed to increases in Ca^{2+} and P_i that were acquired through blebbing of apoptotic and injured cells. However, increased ions can hardly account for the other modes of calcification. Juxtamembranous calcific deposits refers to the deposition of apatite along the membrane surfaces. Such deposits were especially notable in fat necrosis and experimental calcification of liposomes (**Figs. 7 and 8**). Juxtamembranous deposits imply that membranes may serve as a substrate for heterogeneous nucleation of apatite. The above mentioned layer of Ca^{2+} condensation on the surface of the membranes may contribute to the juxtramembranous deposition of apatite. Most intraluminal deposits are juxtramembranous. Intramural calcification refers to radially arranged needles embedded in the thickened wall of large sized vesicles measuring up to several micrometers in diameter (**Fig. 15**). Needle-shaped apatite deposition in the thick-wall is frequently seen in vascular and neoplastic calcinosis and decalcified urinary stones (Kim, 1976, 1983b, 1983d; **Table 3**). Within the whole cell disintegration in senescent hyaline cartilage, vascular tissue and neoplasms, what appeared to be altered cellular membranous organelles frequently develop the thick-wall with calcification, as well (**Figs. 13, 14 and 22b**). Identical thick-walled vesicles with calcification were seen in heart valve xenograft (Maxwell *et al.*, 1989). When red cell ghosts were placed in Millipore chambers and incubated in rat peritoneum, thick-walled vesicles with calcification developed in 4 weeks, whereas liposomes did not. The thick-wall formation is apparently a slow process (Kim, 1993). Certain complex formations by the membrane with osmiophilic substance appear to form the thick-wall. Alteration of the membranes by phospholipases and non-membranous components of the cells are likely to be involved in the thick-wall formation. The role of CDP in calcification is not limited to compartmentalized increases in Ca^{2+} and P_i.

Summary

A comparison of published data on calcification in the growth plate with various dystrophic calcinosis furnishes evidence that cell death is the common underlying mechanism for both physiological and pathological calcifications. Cellular Ca^{2+} overload has been noted in both apoptosis and necrosis for decades and has been shown to be responsible for cell morbidity. Recent studies demonstrate that multi-fold increases in $[P_i]_i$ also occurs as a result of decreased ATP synthesis in injured and apoptotic cells. Concomitant increases in $[Ca^{2+}]_i$ and $[P_i]_i$ in cell death and their conveyance to blebs and MV evidently play the key role in calcification. It is also theorized that MV formation is a form of blebbing by terminally differentiated hypertrophic chondrocytes. The exact point at which apatite nucleation takes place in the process of cell death and blebbing remains to be determined. In view of multi-fold increases in $[Ca^{2+}]_i$ and $[P_i]_i$, apatite nucleation is likely to occur in the cytosol prior to blebbing. Little is known about cytosolic and organelle changes that accompany cell death and may affect nucleation and growth of apatite. However, the occurrence of calcification in CDP derived from apoptotic and necrotic cells indicates that increases in Ca^{2+} and P_i far outweigh any inhibitory effect that may be elicited during cell death. In addition to blebbing, CDP originated from cell organelles due to the cell disintegration *in toto* frequently calcify.

In view of the congestive engorgement of residual bodies, ATP in the senescent cells is likely to be depleted. Abrupt surges of $[Ca^{2+}]_i$ and $[P_i]_i$ as seen in acute cell injury is unlikely in these cells. The fate of the demonstrated increase in calcium and the likely high $[P_i]_i$ derived from ATP in senescent cells remains to be determined. The mechanism of calcification in senescent cells presumably differs from that of acute cell death.

The mode of CDP calcification and morphology of calcific deposits *in vivo* are complex. In addition to increases in ion concentrations, the membranes of CDP also appear to serve as a substrate for heterogeneous nucleation of apatite. Complex interaction of the membrane with organic substances and the formation of various calcific deposits in the tissues may be but a few phenomena that demonstrate the complexity of calcification *in vivo*. The potential role of Ca^{2+} condensation along the surface of membranes in calcification remains to be determined. Mounting evidence in the literature indicates that cell death, both necrosis and apoptosis, is primarily responsible for both pathological and physiological calcifications.

References

Adams ND, Rowe JC (1992) Nephrocalcinosis. Clin Perinatol **19**: 179-195.

Aeschilimann D, Wetterwald A, Fleisch H, Paulsson M (1993) Expression of tissue glutaminase in skeletal tissues correlates with events of terminal differentiation of chondrocytes. J Cell Biol **120**: 1461-1470.

Alpers DH, Eliakim R, DeSchruyver-Kecskemeti K (1989) Physiologic release of tissue alkaline phosphatases. Secretion of hepatic and intestinal alkaline phosphatases: Similarities and differences. Clin Chim Acta **186**: 211-224.

Anastassiades OTh, Bouropoulou V, Kontogeorgos G, Rachmanides M (1984) Microcalcifications in benign breast diseases. A histological and histochemical study. Path Res Pract **178**: 237-242.

Anderson HC (1967) Electron microscopic studies of induced cartilage development and calcification. J Cell Biol **35**: 81-101.

Anderson CK (1968) Renal histological changes in stone formers and non-stone formers. In: Renal Stone Research Symposium. Hodgkinson A, Nordin BEC (eds.). J and A Churchill, Ltd., London. pp. 133-136.

Anderson HC (1988) Mechanisms of pathologic calcification. Rheumat Dis Clin N Amer **14**: 303-319.

Anderson HC (1989) Mechanism of mineral formation in bone. Lab Invest **60**: 320-330.

Anderson L, McDonald JR (1946) The origin, frequency, and significance of microscopic calculi in the kidney. Surg Gynecol Obst **82**: 275-282.

Anderson HC, Reynolds JJ (1973) Pyrophosphate stimulation of calcium uptake into cultured embryonic bones: Fine structure of matrix vesicles and their role in calcification. Dev Biol **34**: 211-227.

Anderson HC, Kim BH, Minkowitz S (1969) Calcifying epithelial odontogenic tumor of Pindborg. An electron microscopic study. Cancer **24**: 585-596.

Angrist A (1964) Aging heart valves and a unified pathological hypothesis for sclerosis. J Gerontol **19**: 135-143.

Arends MJ, Wyllie AH (1991) Apoptosis: Mechanisms and roles in pathology. Internat Rev Exp Pathol **32**: 223-254.

Arends MJ, Morris RG, Wyllie AH (1990) The role of endonuclease. Am J Pathol **136**: 593-608.

Arispe N, Rojas, Pollard HB (1993) Alzheimer disease amyloid beta protein forms calcium channels in bilayer membranes: Blockade by tromethamine and aluminum. Proc Natl Acad Sci USA **90**: 167-571.

Avery RK, Bleier, KJ, Pasternack MS (1992) Differences between ATP-mediated cytotoxicity and cell-mediated cytotoxicity. J Immunol **149**: 1265-1270.

Barac-Nieto M, Spitzer A (1994) NMR-visible intracellular P_i and phosphoesters during regulation of Na^+-P_i cotransport in opossum kidney cells. Am J Physiol **267**: C915-C919.

Bargman JM, Wagner C, Cameron R (1991) Renal cortical nephrocalcinosis: A manifestation of extrapulmonary pneumocystis carinii infection in the acquired immuno-deficiency syndrome. Am J Kidney Dis **17**: 712-715.

Bayreuther K, Francz PI, Gogol J, Kontermann K (1992) Terminal differentiation, aging, apoptosis, and spontaneous transformation in fibroblast stem cell systems *in vivo* and *in vitro*. Ann NY Acad Sci **663**: 167-179.

Beertsen W, van den Bos T (1992) Alkaline phosphatase induces the mineralization of sheets of collagen implanted subcutaneously in the rat. J Clin Invest **89**: 1974-1980.

Bennet J, Weeds A (1986) Calcium and cytoskeleton. Br Med Bul **42**: 385-390.

Berendes R, Voges D, Demange P, Huber R, Burger A (1993) Structure-function analysis of the ion channel selectivity filter in human annexin V. Science **262**: 427-430

Berridge MJ (1993) Inositol triphosphate and cell signalling. Nature **341**: 197-205.

Berry CL, Stark J, Anderson C (1970) Histopathological changes after aortic anastomosis: Their effect on the assessment of structure materials. J Pathol **102**: 213-217.

Bertrand M, Chen JTT, Libslutz HI (1977) Lymphnode calcification in Hodgkin's disease after chemotherapy. Am J Roentgenol **129**: 1108-1110.

Block ER, Patel JM, Edwards D (1989) Mechanism of hypoxic injury to pulmonary artery endothelial cell plasma membranes. Am J Physiol **257**: C223-C231.

Bloss FD (1971) Crystallography and Crystal Chemistry. An Introduction. Hold, Reinhart and Winston, Inc., New York. pp. 284-342.

Blumenthal NC (1989) Mechanism of inhibition of calcification. Clin Orthop **247**: 279-289.

Blumenthal HT, Lansing AJ, Gray SH (1950) The interrelation of elastic tissue and calcium in the genesis of arteriosclerosis. Am J Pathol **26**: 989-1009.

Bode AP, Orton SM, Frye MJ, Udis BJ (1991) Vesiculation of platelets during *in vitro* aging. Blood **77**: 887-895.

Boden SD, Kaplan FS (1990) Calcium homeostasis. Orthop Clin N Amer **21**: 32-42.

Boe R, Gjertsen BT, Vintermyr OK, Houge G, Lanotte M, Dosekeland SO (1991) The protein phosphatase inhibitor okadaic acid induces morphological changes of apoptosis in mammalian cells. Exp Cell Res **195**: 237-246.

Boehme K, Conscience-Egli M, Tschan T, Winterhalter KH, Bruckner P (1992) Induction of proliferation or hypertrophy of chondrocytes in serum-free culture: The role of insulin-like growth factor-I, insulin, or thyroxine. J Cell Biol **116**: 1035-1042.

Boehme K, Winterhalter KH, Bruckner P (1995) Terminal differentiation of chondrocytes in culture is a spontaneous process and is arrested by transforming growth factor-beta 2 and basic fibroblast growth factor in synergy. Exp Cell Res **21**: 191-198.

Boistelle R (1986) The concept of crystal growth from solution. Adv Nephrol **15**: 173-217.

Bonga SEW, Pang PKT (1991) Control of calcium regulating hormones in the vertebrates: Parathyroid hormone, calcitonin, prolactin, and stanniocalcin. Internat Rev Cytol **128**: 139-213.

Bonucci E (1967) Fine Structure of early cartilage calcification. J Ultrastruc Res **20**: 33-50.

Bonucci E (1970) Fine structure and histochemistry of "calcifying globules" in epiphyseal cartilage. Z Zellforsch **103**: 192-217.

Bonucci E, Dearden LC (1976) Matrix vesicles in aging cartilage. Fed Proc **35**: 163-168.

Bonucci E, Sadun R (1972) An electron microscopic study of experimental calcification of skeletal muscle. Clin Orthop **88**: 197-217.

Bonucci E, Silvestrini G, DiGrezia R (1987) The ultrastructure of the organic phase associated with the inorganic substance in calcified tissues. Clin Orthop Rel Res **233**: 243-261.

Bonucci E, Silverstrini G, Bianco P (1992) Extracellular alkaline phosphatase activity in mineralizing matrices of cartilage and bone; ultrastructural localization using a cerium-based method. Histochemistry **97**: 313-327.

Borrelli MJ, Wong RSL, Dewey WC (1986) A direct correlation between hyperthermia-induced membrane blebbing and survival in synchronous G1 CHO cells. J Cell Physiol **126**: 181-190.

Boskey AL (1992) Mineral-matrix interactions in bone and cartilage. Clin Orthop Rel Res **281**: 244-274.

Boskey AL, Dick BL (1991) The effect of phosphatidylserine on *in vitro* hydroxyapatite growth and proliferation. Calcif Tissue Int **4**: 193-196.

Boskey AL, Posner AS (1982) Optimal conditions for Ca-acidic Phospholipid-PO_4 formation. Calcif Tissue Int **34**: S1-S7.

Boskey AL, Bullough PG, Victoria V, di Carlo E (1988) Calcium-acidic phospholipid-phosphate complexes in human hydroxyapatite-containing pathologic deposits. Am J Pathol **133**: 22-29.

Boyan BD, Schwartz Z, Swain LD, Khare A (1989) Role of lipids in calcification of cartilage. Anat Rec **229**: 211-219.

Bredt DS, Snyder SH (1994) Nitric oxide: A physiological messenger molecule. Annu Rev Biochem **63**: 175-195.

Brighton CT, Heppenstall RB (1971) Oxygen tension in zones of the epiphyseal plate, the metaphysis and diaphysis. An *in vitro* and *in vivo* study in rats and rabbits. J Bone Joint Surg **53A**: 719-728.

Brighton CT, Sugioka Y, Hunt RM (1973) Cytoplasmic structure of epiphyseal plate chondrocytes: Quantitative evaluation using electron micrographs of rat costochondral junctions with special reference to the fate of the hypertrophic cells. J Bone Joint Surg **55A**: 771-784.

Brimble KS, Ananthanarayanan VS (1992) Induction of Ca^{2+} transport in liposomes by insulin. Biochem Biophys Acta **1105**: 319-327.

Brown EM, Pollak M, Seidman CE, Seidman JG, Chou Y-HW, Ricardi D, Hebert SC (1995) Calcium-ion-sensing cell-surface receptors. New Engl J Med **333**: 234-240.

Brown WE, Mathew M, Tung MS (1981) Chemistry of octacalcium phosphate. Prog Crystal Growth Charact **4**: 59-87.

Brunk UT, Jones CB, Sohal RS (1992) A novel hypothesis of lipofuscinogenesis and cellular aging based on interactions between oxidative stress and autophagocytosis. Mutation Res **275**: 395-403.

Buckwalter JA, Schaffer MD (1987) Differences in matrix vesicle concentration among growth plate zones. J Orthop Res **5**: 157-163.

Buja LM, Hagler HK, Parsons D, Chien K, Reynolds RC, Willerson JT (1985) Alterations of ultrastructure and elemental composition in cultured neonatal rat cardiac myocytes after metabolic inhibition with iodoacetic acid. Lab Invest **53**: 397-412.

Burry AF, Axelsen RA, Trolove P, Saal JR (1976) Calcification in the renal medulla: A classification based on prospective study of 2261 necropsies. Human Pathol **7**: 435-449.

Cameron IL (1971) Cell proliferation and renewal in mammalian body. In: Cellular and Molecular Renewal in the Mammalian Body. Cameron IL, Thrasher JD (ed.). Academic Press, New York. pp. 45-85.

Carafoli E (1987) Intracellular calcium homeostasis. Annu Rev Biochem **56**: 395-433.

Carafoli E (1994) Biogenesis: Plasma membrane ATPase: 15 years of work on the purified enzyme. FASEB J **8**: 993-1002.

Carbantchik ZI, Greger R (1992) Chemical probes for anion transporters of mammalian cell membranes. Am J Physiol **262**: C803-C827.

Carley WW, Rucker E (1982) ATP-dependent phosphate transport in sarcoplasmic reticulum and reconstituted proteoliposomes. Biocheim Biophys Acta **680**: 187-193.

Caulfield JB, Schrag BA (1964) Electron microscopic study of renal calcification. Am J Pathol **44**: 365-381.

Cheng L, Liang CT, Sacktor B (1983) Phosphate uptake by renal membrane vesicles of rabbits adapted to high and low phosphorus diet. Am J Physiol **245**: F175-F180.

Clandinin TR (1991) Dietary fat: Exogenous determination of membrane structure and cell function. FASEB J **5**: 2761-2769.

Clapham DE (1995) Calcium signaling. Cell **80**: 259-268.

Clarke PGH (1990) Developmental cell death: Morphological diversity and multiple mechanisms. Anat Embryol **181**: 195-213.

Clemens MG, Forrester T (1981) Appearance of adenosine triphosphate in the coronary sinus effluent

from isolated working rat heart in response to hypoxia. J Phsiol (London) **296**: 229-243.

Cliff WJ (1970) The aortic tunica media in aging rats. Exp Mol Path **13**: 172-189.

Cohen GM, Sun X-M, Snowden RT, Dinsdale D, Skilleter DN (1992) Key morphological features of apoptosis may occur in the absence of internucleosomal DNA fragmentation. Biochem J **286**: 331-334.

Collan Y, Luoma H, Ylinen A, Teir H (1972) Histological and ultrastructural features of nephrocalcinosis caused by a caries reducing diet. Calcif Tissue Res **8**: 247-257.

Conigrave AD, Jiang L (1995) Review: Ca^{2+} mobilizing receptors for ATP and UTP. Cell Calcium **17**: 111-119.

Cristofalo VJ, Gerhard GS, Pgnolo RJ (1994) Molecular biology of aging. Surg Clin N Amer **74**: 1-21.

Crompton M, Ellinger H, Costi A (1988) Inhibition by cyclosoporin A of a Ca^{2+} dependent pore in heart mitochondria. Biochem J **255**: 357-360.

Csapo Z, Kuthy E, Lantos J, Ormos J (1975) Experimentally induced malakoplakia. Am J Pathol **79**: 453-464.

Dainiak N (1991) Surface membrane-associated regulation of cell assembly, differentiation, and growth. Blood **78**: 264-276.

Dargel R (1992) Lipid peroxidation - a common pathogenetic mechanism? Exp Toxicol Pathol **44**: 169-181.

David R, Kim KM (1982) β-fibrillary bodies in low-grade adenocarcinoma of parotid gland: A histochemical and ultrastructural study. Human Pathol **13**: 1028-1038.

Davidson AM, Halestrap AP (1989) Inhibition of mitochondrial-matrix inorganic pyrophosphatase by physiological $[Ca^{2+}]$ and its role in the hormonal regulation of mitochondrial matrix volume. Biochem J **258**: 817-821.

DeMeis L, Hasselbach W, Machada RD (1974) Characterization of calcium oxalate and calcium phosphate deposits in sarcoplasmic reticulum vesicles. J Cell Biol **62**: 505-509.

Denton RM, McCormack JG (1990) Ca^{2+} as a second messenger within mitochondria of the heart and other tissues. Annu Rev Physiol **52**: 451-466.

Deuticke B, Haest WM (1987) Lipid modulation of transport proteins in vertebrate cell membranes. Annu Rev Physiol **49**: 221-235.

Devaux PF (1991) Static and dynamic lipid asymmetry in cell membranes. Biochemistry **30**: 1163-1173.

De Young MB, Scarpa A (1991) Extracellular ATP activates coordinated Na^+, P_i, and Ca^{2+} transport in cardiac myocytes. Am J Physiol **260**: C1182-C1190.

Dice JF (1993) Cellular and molecular mechanisms of aging. Physiol Rev **73**: 149-159.

Dillaman RM, Roer RD, Gay DM (1991) Fluid movement in bone: Theoretical and empirical. J Biomechanics **24** (Suppl 1): 163-177.

Drach GW, Boyce WH (1972) Nephrocalcinosis as a source for renal stone nuclei. Observation on humans and squirrel monkeys and hyperparathyroidism in the squirrel monkey. J Urol **107**: 897-904.

Eanes ED, Hailer AW (1987) Calcium phosphate precipitation in aqueous suspensions of phosphatidylserine-containing anionic liposomes. Calcif Tissue Int **40**: 43-48.

Eanes ED, Termine JD (1983) Calcium in mineralized tissues. In: Calcium in Biology. Spiro TG (ed.). Wiley-Interscience Publ., New York. pp. 201-233.

Eckert A, Hartmann H, Foerstl H, Mueller W (1994) Alterations of intracellular calcium regulation during aging and Alzheimer's disease in non-neuronal cells. Life Sci **55**: 2019-2029.

Eguchi G, Kodama R (1993) Transdifferentiation. Cur Opinion Cell Biol **5**: 1023-1028.

Enright H, Hebbel RP, Nath KA (1994) Internucleosomal cleavage of DNA as the sole criterion for apoptosis may be artifactual. J Lab Clin Med **124**: 63-68.

Epstein S (1988) Serum and urinary markers of bone remodeling: Assessment of bone turnover. Endocr Rev **9**: 437-449.

Escoubet B, Silve C, Balsan S, Amiel C (1992) Phosphate transport by fibroblasts from patients with hypophosphataemic vitamin-D-resistant rickets. J Endocrinol **133**: 301-309.

Evans VG (1993) Multiple pathways to apoptosis. Cell Biol Int **17**: 461-476.

Exton JH (1990) Effects of extracellular ATP on phosphatidylcholine phosohplipase signaling systems. Ann NY Acad Sci **603**: 246-255.

Fagan PJ, Ward MD (1992) Building molecular crystals. Sci Amer **July**, 48-54.

Fagan JM, Wajnberg EF, Culbert L, Waxman L (1992) ATP depletion stimulates calcium-dependent protein breakdown in chick skeletal muscle. Endocrinol Metab **25**: E637-E643.

Falkoff GE, Rigsby CM, Rosenfield AT (1987) Partial, combined cortical and medullary nephrocalcinosis: US and CT patterns in AIDS-associated MAI infection. Radiology **162**: 343-344.

Farber JL (1990) The role of calcium ions in toxic cell injury. Environ Health Persp **84**: 107-111.

Farber E (1994) Programmed cell death: Necrosis versus apoptosis. Mod Pathol **7**: 605-609.

Farber JL, Young EE (1981) Accelerated phospholipid degradation in anoxic hepatocytes. Arch Biochem Biophys **221**: 312-320.

Farge E, Devaux PF (1992) Shape changes of giant liposomes induced by an asymmetric transmembrane

distribution of phospholipids. Biophys J **61**: 347-357.

Farnum CE, Wilsman NJ (1993). Determination of proliferative characteristics of growth plate chondrocytes by labeling with bromodeoxyuridine. Calcif Tissue Int **52**: 110-119.

Ferenczy A, Talens M, Zoghby M (1977) Ultrastructural studies on the morphogenesis of psammoma bodies in ovarian serous neoplasia. Cancer **39**: 2451-2459.

Ferguson MAJ, Williams AF (1988) Cell surface anchoring proteins via glycosyl-phosphatidyl inositol structures. Annu Rev Biochem **57**: 285-320.

Ferrel JE Jr, Lee K-J, Huestis WH (1985) Membrane bilayer balance and erythrocyte shape: A quantitative assessment. Biochemistry **24**: 2849-2857.

Fishman WH (1974) Perspectives on alkaline phosphatase isoenzymes. Am J Med **56**: 595-600.

Fiskum G, Lehninger AL (1982) Mitochondrial regulation of intracellular calcium. In: Calcium and Cell Function, vol. II. Cheung WY (ed.). Academic Press, New York. pp. 39-80.

Fleisch H (1980) Experimental basis for the clinical use of diphsophonates in Paget's disease of bone. Arth Rheum **23**: 1162-1171.

Florine-Casteel K, Lemasters JJ, Herman B (1991) Lipid order in hepatocyte plasma membrane blebs during ATP depletion measured by digitized video fluorescence polarization microscopy. FASEB J **5**: 2078-2084.

Forrester T, Williams CA (1977) Release of adenosine triphosphate from isolated heart cells in response to hypoxia. J Physiol (London) **268**: 371-390.

Foschini MP, Adda TD, Bordi C, Eusebi V (1993) Amyloid stroma in meningiomas. Virchows Arch A Path Anat **422**: 53-59.

Futai M, Noumi T, Maeda M (1989) ATP synthase (H^+-ATPase): Results by combined biochemical and molecular biological approaches. Annu Rev Biochem **58**: 111-136.

Ganote CE, Phillipson DS, Chen E, Carone FA (1975) Acute calcium nephrotoxicity. An electron microscopical and semi-quantitative light microscopical study. Arch Pathol **99**: 650-657.

Gardner MB, Blankenhorn DH (1968) Aortic medial calcification. An ultrastructural study. Arch Pathol **85**: 397-403.

Garlick PB, Brown TB, Sullivan RH, Ugerbil K J (1983) Observation of a second phosphate pool in the perfused heart by ^{31}P-NMR: Is this the mitochondrial phosphate? Mol Cell Cardiol **15**: 855-858.

Gasbarrini A, Borle AB, Farghali H, Bender C, Francavilla A, Thiel DV (1992) Effect of anoxia on intracellular ATP, Na^+_i, Ca^{2+}_i, Mg^{2+}_i, and cytotoxicity in rat hepatocytes. J Biol Chem **267**: 6654-6663.

Genge BR, Sauer GR, Wu LNY, McLean FM, Wuthier RE (1988) Correlation between loss of alkaline phosphatase activity and accumulation of calcium during matrix vesicle-mediated mineralization. J Biol Chem **263**: 18513-18519.

Genge BR, Wu LN, Adkisson HD, Wuthier RE (1991) Matrix vesicle annexins exhibit proteolipid-like properties. Selective partitioning into lipophilic solvents under acidic conditions. J Biol Chem **266**: 10678-10685.

Genge BR, Cao X, Wu LN, Buzzi WR, Showman RW, Arsenault AL, Ishikawa Y, Wuthier RE (1992) Establishment of the primary structure of the major lipid-dependent Ca^{2+} binding proteins of chicken growth plate cartilage matrix vesicles: Identity with anchorin CII (Annexin V) and Annexin II. J Bone Miner Res **7**: 807-819.

George M, Chenerey RJ, Krishna G (1982) The effect of ionophore A23187 and 2,4-dinitrophenol on the structure and function of cultured liver cells. Toxicol Appl Pharmacol **66**: 349-660.

Glende EA Jr, Pushpendran KC (1986) Activation of phospholipase A_2 by carbon tetrachloride in isolated rat heaptocytes. Biochem Pharmacol **35**: 3301-3307.

Glimcher MJ (1989) Mechanism of calcification: Role of collagen fibrils and collagen-phosphoprotein complexes *in vitro* and *in vivo*. Anat Rec **224**: 139-153.

Gordon JL (1986) Extracellular ATP: Effects, source and fate. Biochem J **233**: 309-319.

Gunter TE, Pfeifer DR (1990) Mechanisms by which mitochondria transport calcium. Am J Physiol **258**: C755-C786.

Gunter TE, Gunter KK, Sheu S-S, Gavin CE (1994) Mitochondrial calcium transport: Physiological and pathological relevance. Am J Physiol **267**: C313-339.

Haggit RG, Pitcock JA (1971) Renal medullary calcifications: A light and electron microscopic study. J Urol **106**: 342-347.

Halestrap AP (1989) The regulation of volume of mammalian mitochondria *in vivo* and *in vitro* and its role in the control of mitochondrial metabolism. Biochem Biophys Acta **973**: 355-382.

Hammar SP, Krous H (1977) Myocardial mitochondrial calcification in Reye's syndrome. Human Pathol **8**: 95-98.

Harasaki H, McMahon J, Richards T, Goldcamp J, Kiraly R, Nose Y (1985) Calcification in cardiovascular implants: Degraded cell related phenomena. Trans Amer Soc Artif Intern Organs **31**: 489-494.

Harrison RB, Vaughean ED (1978) Diffuse cortical calcification in rejected renal transplants. Radiology **126**: 635-636.

Hartman A, Charania B, Sund S, Holdaas H, Fauchald P, Flatmark A (1991) Interstitial nephritis with extensive calcium deposits as a cause of renal allograft failure. Nephrol Dialysis Transpl **6**: 984-988.

Hass GM, Trueheart RE, Taylor CB, Stumpe M (1958) An experimental histologic study of hypervitaminosis D. Am J Pathol **34**: 395-431.

Hayflick LH (1977) The cellular basis for biological aging. In: Handbook of The Biology of Aging. Finch CE, Hayflick L (eds.). Van Nostrand Reiniholt Co., New York. pp. 159-188.

Heptinstall RH (1974) Calcium and the kidney: Stone formation. In: Pathology of Kidney, 2nd ed. Heptinstall RH (ed.). Little, Brown and Co., Boston. p. 242; pp. 1091-1122.

Hightower KR, Reddy VN (1982) Calcium content and distribution in human cataract. Exp Eye Res **34**: 413-421.

Hoff HF (1973) Human intracranial atherosclerosis. An ultrastructural study of atheromatous plaques. Virchow Arch A Path Anat **361**: 97-108.

Holtrop ME (1972) The ultrastructure of epiphyseal plate. II. The hypertrophic chondrocyte. Calcif Tissue Res **9**: 140-151.

Hricak H (1989) Phosphorus-31 MRS of the kidney. Invest. Radiology **24**: 993-996.

Hsu HHT (1983) Purification and partial characterization of ATP pyrophohydrolase from fetal bovine epiphyseal cartilage. J Biol Chem **258**: 3486-3488.

Hsu HHT (1992) Further studies on ATP-mediated Ca deposition by isolated matrix vesicles. Bone Miner **17**: 279-283.

Humes HD, Nguyen VD, Hunt DA (1986) High energy phosphates, phospholipids, and calcium in ischemic renal tubular injury. Adv Exp Med Biol **208**: 3-7.

Humphrey SM, Garlick PB (1991) NMR-visible ATP and P_i in normoxic and reperfused rat hearts: A quantitative study. Am J Physiol **260**: H6-H12.

Iannotti JP (1990) Growth plate physiology and pathology. Orthop Clin N Amer **21**: 1-17.

Iannotti JP, Brighton CT (1989) The cytosolic ionized calcium concentration in isolated chondrocytes from each zone of the growth plate. J Orthop Res **7**: 511-518.

Iannotti JP, Naidu S, Noguchi Y, Hunt RM, Brighton CT (1994) Growth plate matrix vesicle biogenesis. Clin Orthop Rel Res **306**: 222-229.

Ibels LS, Alfrey AC, Huffer WE, Craswell PW, Weil R (1981) Calcification in end-stage kidneys. Am J Med **71**: 33-37.

Irving JT, Wuthier RE (1968) Histochemistry and biochemistry of calcification with a special role of lipids. Clin Orthop **56**: 237-260.

Jacobson K, Ishihara A, Inman R (1987) Lateral diffusion of proteins in membranes. Annu Rev Physiol **49**: 163-175.

Johnson HB (1986) Foreign substances in tissues. In: Histochemistry in Pathological Diagnosis. Spicer SS (ed.). Marcell Dekker, Inc., New York. pp. 103-114.

Jones WA, Miller EV, Sullivan LD, Chapman WH (1979) Severe prostatic calcification after radiation therapy for cancer. J Urol **21**: 828-830.

Jordan WR, Finlayson B, Hackkett RL (1978) Kinetics of early time calcium oxalate nephrolithiasis. Invest Urol **15**: 465-468.

Jurkowitz-Alexander MS, Altschuld RA, Hohl CM, Johnson JD, McDonald JS, Simmons TD, Horrocks LA (1992) Cell swelling, blebbing, and death are dependent on ATP depletion and independent of calcium during chemical hypoxia in a glial cell line (ROC-1). J Neurochem **59**: 344-352.

Kahn SR (1995) Heterogeneous nucleation of calcium oxalate crystals in human urine. Scanning Microsc **9**: 597-616.

Kanabe S, Hsu HHT, Cecil RNA, Anderson HC (1983) Electron microscopic localization of adenosine triphosphate (ATP)-hydrolyzing activity in isolated matrix vesicles and reconstituted vesicles from calf cartilage. J Histochem Cyotochem **31**: 462-470.

Kardos TB, Hubbard MJ (1982) Are matrix vesicles apoptotic bodies? Prog Clin Biol Res **101**: 45-60.

Kato Y, Iwamoto M, Koike T, Suzuki F, Takano Y (1988) Terminal differentiation and calcification in rabbit chondrocyte cultures grown in centrifuge tubes: Regulation by transforming growth factor beta and serum factors. Proc Natl Acad Sci USA **85**: 9552-9556.

Katz SM, Krueger LJ, Falkner B (1988) Microscopic nephrocalcinosis in cystic fibrosis. New Engl J Med **319**: 263-266.

Kawabe N, Ehrlich MG, Mankin HJ (1986) *In vivo* degradation systems of the epiphyseal cartilage. Clin Orthop **211**: 244-251.

Kay MM (1994) Regulatory autoantibody and cellular aging and removal. In: Immunobiology of Proteins and Peptides VII. Atassi MZ (ed.). Plenum Press, New York. pp. 161-192.

Kemp GJ, Blumsohn A, Morris BW (1992) Circadian changes in plasma phosphate concentration, urinary phosphate excretion, and cellular phosphate shifts. Clin Chem **38**: 400-402.

Kent C, Carman GM, Spence MW, Dowhan W (1991) Regulation of eukaryotic phospholipid metabolism. FASEB J **5**: 2258-2266.

Kepes J (1961) Electron microscopic studies of meningiomas. Am J Pathol **39**: 499-510.

Kerr JF, Wyllie AH, Currie AR (1972) Apoptosis: A basic biological phenomenon with wide ranging implications in tissue kinetics. Br J Cancer **26**: 239-257.

Kerr JF, Winterford CM, Harmon BV (1994) Apoptosis. Its significance in cancer and cancer therapy. Cancer **73**: 2013-2016.

Kiang JG, Doenig ML, Smallridge RC (1992) Heat shock increases cytosolic free Ca^{2+} concentration via

Na^+-Ca^{2+} exchange in human epidermoid A431 cells. Am J Physiol **263**: C30-C38.

Kim KM (1976) Calcification of matrix vesicles in human aortic valve and aortic media. Fed Proc **35**: 156-162.

Kim KM (1978) Matrix vesicle calcification of rat aorta in millipore chambers. Met Bone Dis Rel Res **1**: 213-217.

Kim KM (1982) The stones. Scanning Electron Microsc **1982**; IV: 1635-1660.

Kim KM (1983a) Pathological calcification. In: Pathobiology of Cell Membranes. Trump BF, Arstilla AA (eds.). Academic Press, New York. pp. 117-155.

Kim KM (1983b) Role of membranes in calcification. Surv Synth Pathol Res **2**: 215-228.

Kim KM (1983c) Nephrocalcinosis *in vitro*. Scanning Electron Microsc **1983**; III: 1285-1292.

Kim KM (1983d) Lipid matrix of dystrophic calcification and urinary stone. Scanning Electron Microsc **1983**; III: 1275-1284.

Kim KM (1984) Cell injury and calcification of rat aorta *in vitro*. Scanning Electron Microsc **1984**; IV: 1809-1818.

Kim KM (1993) Calcification of liposomes and red cell ghosts *in vitro* and *in vivo*. Cells Mater **3**: 293-304.

Kim KM (1994) Cell death and calcification of canine fibroblasts *in vitro*. Cells Mater **4**: 247-261.

Kim KM, Huang S-H (1971) Ultrastructural study of calcification of human aortic valve. Lab Invest **25**: 357-366.

Kim KM, Valigorsky JM, Mergner WJ, Jones RT, Pendergrass RE, Trump BF (1976) Aging changes in the human aortic valve in relation to dystrophic calcification. Human Pathol **7**: 47-60.

Kim KM, Alpaugh HB, Johnson FB (1985a) X-ray microanalysis of urinary stones, a comparison with other methods. Scanning Electron Microsc **1985**; III: 1239-1246.

Kim KM, Resau JH, Cottrell JR, Bingham SP, Monticone RE, Trump BF (1985b) Aging changes of canine heart valves. Lab Invest **52**: 35 (abstract).

Kim KM, Chang SH, Trump BF, Spurgeon H (1986) Calcification in aging canine aortic valve. Scanning Electron Microsc **1986**; III: 1151-1156.

Kirsch T, Wuthier RE (1994) Stimulation of calcification of growth plate cartilage matrix vesicles by binding to type II and X collagens. J Biol Chem **269**: 11462-11469.

Ko KW, Fellers FX, Craig JM (1962) Observations on magnesium deficiency in the rat. Lab Invest **11**: 294-305.

Kramsch DM, Aspen AJ, Rozler LJ (1981) Atherosclerosis: Prevention by agents not affected by abnormal levels of blood lipids. Science **213**: 1511-1512.

Kretzinger RH (1990) Why cells must export calcium? In: Intracellular Calcium Regulation. Bronner F (ed.). Wiley-Liss, New York. pp. 439-450.

Krieger NS (1991) Evidence for sodium-calcium exchange in rodent osteoblasts. Ann NY Acad Sci **639**: 660-662.

Kunkler KJ, Everett LM, Breedlove DK, Kempson SA (1991) Insulin stimulates sodium-dependent phosphate transport by osteoblast-like cells. Am J Physiol **260**: E751-E755.

Lafeber FP, van der Kraan PM, van Roy JL, Vitters EL, Huber-Bruning O, van den Berg WB, Bijlsma JW (1993) Local changes in proteoglycan synthesis during culture are different for normal and osteoarthritic cartilage. Am J Pathol **140**: 1421-1429.

Landolt AM, Rothenbuler V (1977) Pituitary adenoma calcification. Arch Pathol **101**: 22-27.

LeGeros RZ (1981) Apatite in biological systems. Prog. Cryst Growth Charact **4**: 1-45.

Lemaster JJ, DiGuiseppi J, Nieminen A-L, Herman B (1987) Blebbing, free Ca^{2+} and mitochondrial membrane potential preceding cell death in hepatocytes. Nature **325**: 78-81.

Leong WS, Russel RGG, Caswell AM (1994) Stimulation of cartilage resorption by extracellular ATP acting at P_2-purinoceptors. Biochem Biophys Acta **1201**: 298-304.

Leusmann DB (1982) Routine analysis of urinary calculi by SEM. Scanning Electron Microsc **1982**; I: 387-396.

Levi M, Loetscher M, Sorribas V, Custer M, Arar M, Kaissling B, Murer H, Biber G (1994) Cellular mechanisms of acute and chronic adaptation of rat renal P_i transporter to alterations in dietary P_i. Am J Physiol **267**: F900-F908.

Levy RJ, Schoen FJ, Flowers WB, Staelin ST (1991) Initiation of mineralization in bioprosthetic heart valves: Studies of alkaline phosphatase activity and its inhibition by $AlCl_3$ or $FeCl_3$ preincubations. J Biomed Mat Res **25**: 905-935.

Liepins A, Younghusband HB (1985) Low temperature-induced cell surface membrane vesicle shedding is associated with DNA fragmentation. Exp Cell Res **161**: 525-532.

Lingrel JB, Kuntzweiler T (1994) Na^+,K^+-ATPase. J Biol Chem **269**: 19659-19662.

Lipper S, Dalzell JC, Watkins PJ (1979) Ultrastructure of psammoma bodies of meningioma in tissue culture. Arch Pathol Lab Med **103**: 670-675.

Long C, Mouat B (1971) The binding of calcium ions by erythrocytes and "ghost"-cell membranes. Biochem J **123**: 829-836.

Loveridge N, Farquharson C, Hesketh JE, Jakowlew SB, Whitehead CC, Thorp BH (1993) The

control of chondrocyte differentiation during endochondral bone growth *in vivo*: Changes in TGF-β and the proto-oncogene c-myc. J Cell Sci **105**: 949-956.

MacLennan DH, Toyofuku T, Lytton J (1992) Structure-function relationship in sarcoplasmic or endoplasmic reticulum type Ca^{2+} pumps. Ann NY Acad Sci **671**: 1-10.

Mahmood U, Alfieri AA, Ballon D, Traganos F, Koutcher JA (1995) *In vitro* and *in vivo* ^{31}P nuclear magnetic resonance measurements of metabolic changes post radiation. Cancer Res **55**: 1248-1254.

Majeska RJ, Holwerda DL, Wuthier RE (1979) Localization of phosphatidylserine in isolated chick epiphyseal cartilage matrix vesicles with trinitrobenzene sulfonate. Calcif Tissue Int **27**: 41-46.

Malis CD, Bonventre JV (1989) Mechanism of calcium potentiation and oxygen free radical injury in renal mitochondria. J Biol Chem **16**: 14201-14208.

Mankin HJ, Revak C, Lippiello L (1968) Ribonucleic acid synthesis in the epiphyseal plate of the rat: An autoradiographic study. Bull Hosp Joint Dis **29**: 111-118.

Maranto AR, Schoen FJ (1988) Effect of delay between tissue harvest and glutaraldehyde pretreatment on mineralization of bovine pericardium used in bioprosthetic heart valves. J Biomed Mater Res **22**: 819-825.

Markowitz M, Rotkin L, Rosen JF (1981) Circadian rhythms of blood minerals in humans. Science **213**: 672-674.

Maroundas A (1972) Physical chemistry and structure of cartilage. J Physiol (London) **223**: 21P-22P.

Martinez-Hernandez A, Huffer WE (1974) Pseudoxanthoma elasticum: Dermal polyanions and the mineralization of elastic fibers. Lab Invest **31**: 181-186.

Maxwell L, Gavin JB, Barrat-Boyes BG (1989) Differences between heart valve allograft and xenografts in the incidence and initiation of dystrophic calcification. Pathology **21**: 5-10.

McConkey DJ, Hartzell P, Nicotera P, Orrenius S (1989) Calcium activated DNA fragmentation kills immature thymocytes. FASEB J **3**: 1843-1849.

McCormack JG (1985) Characterization of the effects of Ca^{2+} on the intramitochondrial Ca^{2+} sensitive enzymes from rat liver and within intact rat liver mitochondria. Biochem J **231**: 581-595.

McCormack JG, Denton RM (1993) Mitochondrial Ca^{2+} transport and the role of intramitochondrial Ca^{2+} in the regulation of energy metabolism. Develop Neurosci **15**: 165-173.

McCormack JG, Daniel RL, Osbaldeston NJ, Rutter GA, Denton RM (1992) Mitochondrial Ca^{2+} transport and the role of matrix Ca^{2+} in mammalian tissues. Biochem Soc Trans **20**: 153-159.

McGraw CF, Somlyo AV, Blaustein MP (1980) Localization of calcium in presynaptic nerve terminals. An ultrastructural and electron microprobe analysis. J Cell Biol **85**: 228-241.

McMillan JB, Lev M (1964) The aging Heart, II. The valves. J Geront **19**: 1-14.

McNally RT, Brockbank GM (1991) The correlation between improved cellular viability and clinical performance in 5,000 cryopreserved human heart valves. Trans Amer Soc Artif Intern Organs **37**: M355-M356.

Meldolesi J, Villa A (1993) Endoplasmic reticulum and the control of Ca^{2+} homeostasis. Sub-Cellular Biochem **21**: 189-207.

Menuck L (1976) Intraabdominal calcification in treated disseminated carcinoma of ovary. Obstet Gynecol **49** (Suppl): 56-58.

Meyer WL, Kunis AS (1969) Decreased glycolytic enzyme activity in epiphyseal cartilage of cortisone treated rats. Arch Biochem Biophys **129**: 431-437.

Mikkelsen RB, Reinlib L, Donowitz M, Zahniser D (1991) Hyperthermia effects on cytosolic $[Ca^{2+}]$: Analysis at single cell level by digitized imaging microscopy and cell survival. Cancer Res **51**: 359-364.

Milner RE, Famulski KS, Michalak M (1992) Calcium binding proteins in the sarcoplasmic/endoplasmic reticulum of muscle and non-muscle cells. Mol Cell Biochem **112**: 1-13.

Min KW, Wheeler WS (1985) Mitochondrial calcification of myocardial cells in idiopathic cardiomyopathy. Am J Clin Path **85**: 383 (abstract).

Moir DH, Bale PM (1976) Necropsy findings in childhood leukemias, emphasizing neutropenic enterocolitis and cerebral calcification. Pathology **8**: 247-258.

Mönkeberg JG (1904) Der normal histologische Bau und die Sklerose der Aortenklappen (The histological structure of normal and sclerotic aortic valves). Virchows Arch Path Anat **176**: 472-513.

Montessuit C, Caverzasio J, Bonjour J-P (1991) Characterization of P_i transport system in cartilage matrix vesicles. Potential role in the calcification process J Biol Chem **266**: 17791-17797.

Montessuit C, Bonjour J-P, Caverzasio J (1994) P_i transport regulation by chicken growth place chondrocytes. Am J Physiol **267**: E24-E31.

Morgan AJ (1979) Non-freezing techniques preparing biological specimens for electron microprobe X-ray microanalysis. Scanning Electron Microsc **1979**; II: 635-648.

Morgan AJ (1980) Mineralized deposits in the thoracic aorta of aged rats: Ultrastructural and electron probe X-ray microanalysis. Exp Gerontol **15**: 563-573.

Nakagawa J, Kitten GT, Nigg EA (1989) A somatic cell-derived system for studying both early and late mitotic events *in vitro*. J Cell Sci **94**: 449-462.

Nancollas GH (1976) The kinetics of crystal growth

and renal stone-formation. In: Urolithiasis Research. Fleisch H, Robertson WG, Smith LH, Wahlensieck W (eds.). Plenum Press, New York. pp. 5-23.

Nancollas GH, Zawacki SJ (1989) Calcium phosphate mineralization. Conn Tiss Res **21**: 239-246.

Neethling WML, Van Den Heever JJ, Meyer JM, Barnard HC (1992) Processing factors as determinants of tissue valve calcification. J Cardiovasc Surg **33**: 285-291.

Neuman WF, Neuman MW (1958) The Chemical Dynamics of Bone. University of Chicago Press, Chicago. pp. 169-187.

Nicotera P, Orrenius S (1992) Ca^{2+} and cell death. Ann NY Acad Sci **648**: 17-27.

Nicotera P, Hartzell P, Davis G, Orrenius S (1986) The formation of plasma membrane blebs in hepatocytes exposed to agents that increase cytosolic Ca^{2+} is mediated by activation of a non-lysosomal proteolytic system. FEBS (Fed Europ Biol Societies) Lett **209**: 139-144.

Nicotera P, Orrenius S, Nilsson T, Berggren P-O (1990) An inositol 1,4,5-triphosphate-sensitive Ca^{2+} pool in liver nuclei. Proc Natl Acad Sci USA **87**: 6858-6862.

Nicotera P, Bellomo G, Orrenius S (1992) Calcium-mediated mechanisms in chemically induced cell death. Annu Rev Pharmacol Toxicol **32**: 449-470.

Ogiso BO, Hughes FJ, Melcher AH, McCulloch CAG (1991) Fibroblasts inhibit mineralized bone nodule formation by rat bone marrow stromal cells *in vitro*. J Cell Physiol **146**: 442-450.

Ohlsson C, Nilsson A, Isakson OGP, Lindahl A (1992) Effect of growth hormone and insulin-like growth factor-I on DNA synthesis and matrix production in rat epiphyseal chondrocytes in monolayer culture. J Endocrinol **133**: 291-300.

Ohya K, Meyer JL, Felix R, Fleisch H (1988) Production of calcification inhibitors by rat calvaria and bone cells in culture. Clin Orthop **232**: 119-126.

Orrenius S, McKonkey DJ, Nicotera P (1991) Role of calcium in toxic and programmed cell death. Adv Exp Med Biol **283**: 419-425.

Pacifici M, Golden EB, Oshima O, Shapiro IM, Leboy PS, Adams SL (1990) Hypertrophic chondrocytes. The terminal stage of differentiation in the chondrogenic cell lineage? Ann NY Acad Sci **599**: 45-57.

Paegle RD (1969) Ultrastructure of calcium deposits in arteriosclerotic human aortas. J Ultrastruc Res **26**: 412-423.

Pawelek JM (1969) Effects of thyroxine and low oxygen tension on chondrogenic expression in cell culture. Dev Biol **19**: 52-72.

Pearson JD, Carleton JS, Gordon JL (1980) Metabolism of adenine nucleotides by enzymes of vascular endothelial and smooth muscle cells in culture. Biochem J **190**: 421-429.

Pentilla A, Trump BF (1974) Extracellular acidosis protects Erlich ascites tumor cells and rat renal cortex against anoxic injury. Science **185**: 277-278.

Peterson C, Goldman JE (1986) Alterations in calcium content and biochemical processes in cultured skin fibroblasts from aged and Alzheimer donors. Proc Natl Acad Sci USA **83**: 7999-8001.

Pfeiffer DR, Schmid PC, Beatrice MC, Schmid HHO (1979) Intramitochondrial phospholipase activity and the effects of Ca^{2+} plus N-ethyl-maleimide on mitochondrial functions. J Biol Chem **254**: 11485-11494.

Phelps PC, Smith MW, Trump BF (1989) Cytosolic ionized calcium and bleb formation after acute cell injury of cultured rabbit renal tubule cells. Lab Invest **60**: 630-642.

Poole AR, Rosenberg LC (1986) Chondrocalcin and the calcification of cartilage. A review. Clin Orthop Rel Res **208**: 114-118.

Pozzan T, Rizzuto R, Volpe P, Meldolesi J (1994) Molecular and cellular physiology of intracellular calcium stores. Physiol Rev **74**: 595-636.

Quamme GA, Shapiro RJ (1987) Membrane controls of epithelial phosphate transport. Can J Physiol Pharmacol **65**: 275-286.

Quamme G, Pfeilschifter J, Murer H (1989) Parathyroid hormone inhibition of Na^+/phosphate cotransport in OK cells: Requirement of protein kinase C-dependent pathway. Biochem Biophys Acta **1013**: 159-165.

Raisz LG, Pilbeam CC, Fall PM (1993) Prostaglandins: Mechanism of action and regulation of production in bone. Osteoporosis Int **1** (Suppl): 136-140.

Ramp WK (1975) Cellular control of calcium movement in bone. Interrelationship of the bone membrane, parathyroid hormone and alkaline phosphatase. Clin Orthop **106**: 311-322.

Ramzy I, Elwi AM (1968) Renal calcification and the preceding tissue alterations. A pathological and histochemical study. Int Surg **50**: 393-402.

Randall A (1937) Origin and growth of renal calculi. Ann Surg **105**: 1009-1027.

Register TC, Warner GP, Wuthier RE (1984) Effect of L- and D-tetramisole on $^{32}P_i$ and ^{45}Ca uptake and mineralization by matrix vesicle-enriched fractions from chicken epiphyseal cartilage. J Biol Chem **259**: 922-928.

Register TC, McLean FM, Low MG, Wuthier RE (1986) Roles of alkaline phosphatase and labile internal mineral in matrix vesicle-mediated calcification. Effect of selective release of membrane-bound alkaline phosphatase and treatment with isosmotic pH 6 buffer. J Biol Chem **261**: 9353-9360.

Resnick MI, Boyce WM (1979) Spherical calcium bodies in stone forming urine. Invest Urol **15**: 449-451.

Ritskes-Hoitinga J, Beynen AC (1992) Nephrocalci-

nosis in rat: A literature review. Prog Food Nutr Sci **16**: 85-124.

Rojas E, Arispe N, Haigler HT, Burns AL, Pollard HB (1992) Identification of annexins as calcium channels in biological membranes. Bone Miner **17**: 214-218.

Roomans GM (1991) Cryopreparation of tissue for clinical application of X-ray microanalysis. Scanning Microsc Suppl **5**: S95-S106.

Sarkar K, Tolani G, Levine DZ (1973) Nephrocalcinosis in chloride depleted rats. An ultrastructural study. Calcif Tissue Res **12**: 1-7.

Sauer GR, Wuthier RE (1988) Fourier transform infrared characterization of mineral phases formed during induction of mineralization by collagenase-released matrix vesicles *in vitro*. J Biol Chem **263**: 13718-13724.

Sauer GR, Genge BR, Wu LN, Donachy JE (1994) A facilitative role for carbonic anhydrase activity in matrix vesicle mineralization. Bone Miner **26**: 69-79.

Savill J, Fadok V, Henson P, Haslett C (1993) Phagocyte recognition of cells undergoing apoptosis. Immunol Today **14**: 131-136.

Scarpace PJ, Neuman WF (1976a) The blood: bone disequilibrium. I. The active accumulation of K^+ into the bone extracellular fluid. Calcif Tissue Res **20**: 137-149.

Scarpace PJ, Neuman WF (1976b) The blood: bone disequilibrium. II. Evidence against the active accumulation of calcium or phosphate into the bone extracellular fluid. Calcif Tissue Res **20**: 151-158.

Schiffman E, Martin GR (1962) *In vitro* calcification of rat aorta in serum. Nature **194**: 189-190.

Schoen FJ, Harasaki H, Kim KM, Anderson HC, Levy RJ(1988) Biomaterial-associated calcification: Pathology, mechanisms, and strategies for prevention. J Biomed Mater Res **22** (Suppl A1): 11-36.

Schoen FJ, Levy RJ, Hilbert SL, Bianco RW (1992) Antimineralization treatment for bioprosthetic heart valves. Assessment of efficacy and safety. J. Thorc. Cardiovasc. Surg. **104**: 1285-1288.

Schrier SL, Zachowski A, Herve P, Kader JC, Devaux PF (1992) Transmembrane redistribution of phospholipids of the human red cell membrane during hypotonic hemolysis. Biochim Biophys Acta **1105**: 170-176.

Schroeder F (1984) Role of membrane lipid asymmetry in aging. Neurobiol. Aging **5**: 323-333.

Schwartz LM, Smith SW, Jones MEE, Osborne BA (1992) Do all programmed deaths occur via apoptosis? Proc Natl Acad Sci USA **90**: 980-984.

Schwartz Z, Boyan B (1988) The effects of vitamin D metabolites on phospholipase A_2 activity of growth zone and resting zone cartilage cells *in vitro*. Endocrinology **122**: 2191-2198.

Scully RE (1970) Gonadoblastoma. A review of 74 cases. Cancer **6**: 1340-1350.

Seelig J (1990) Interaction of phospholipids with Ca^{2+} ions. On the role of the phospholipid head groups. Cell Biol Internat Rep **14**: 353-360.

Sell S, Scully RE (1965) Aging changes in the aortic and mitral valves. Histologic and histochemical studies, with observations on the pathogenesis of calcific aortic stenosis and calcification of the mitral annulus. Am J Pathol **46**: 345-365.

Seltz T, Caverzasio J, Bonjour J-P (1991) Fluoride selectively stimulates Na-dependent phosphate transport in osteoblast-like cells. Am J Physiol **260**: E833-E838.

Seney FD Jr, Burns DK, Silva FG (1990) Acquired immuno-deficiency syndrome and the kidney. Am J Kidney Dis **16**: 1-13.

Shapiro IM, Boyde A (1984) Microdissection-elemental analysis of the mineralizing growth cartilage of the normal and rachitic chick. Metab Bone Dis Rel Res **5**: 317-326.

Shapiro BL, Lam F-H (1982) Calcium and age in fibroblasts from control subjects and patients with cystic fibrosis. Science **216**: 417-419.

Shapiro IM, Golub EE, Kakuta S, Hazelgrove J, Harvey J, Chance B, Frasca P (1982) Inhibition of endochondral calcification is related to changes in redox state of hypertrophic chondrocytes. Science **217**: 950-952.

Shapiro IM, Golub EE, May M, Rabinowitz JL (1983) Studies of nucleotides of growth-plate cartilage: Evidence linking changes in cellular metabolism with cartilage calcification. Biosci Rep **3**: 345-351.

Shigenaga MK, Hagen TM, Ames BN (1994) Oxidative damage and mitochondrial decay in aging. Proc Natl Acad Sci USA **91**: 10771-10778.

Shimamura T, Aogaichi M, Liu CY (1974) Drug induced renal medullary necrosis. II. Mode of calcification in the kidney. Exp Mol Pathol **20**: 109-114.

Shoemaker L, Welch TR, Bergstrom W, Abrams SA, Yergey AL, Vieira N (1993) Calcium kinetics in the hyperprostaglandin E syndrome. Pediat Res **33**: 92-96.

Siegel FL, Bulger RE (1975) Transmission and scanning electron microscopy of mercury chloride-induced acute tubular necrosis in rat kidney. Virchow Arch B Cell Path **18**: 243-262.

Simko V (1991) Alkaline phosphatases in biology and medicine. Dig Dis **9**: 189-209.

Simons K, van Meer G (1988) Lipid sorting in epithelial cells. Biochemistry **27**: 6197-6202.

Skrtic D, Eanes ED (1992) Membrane-mediated precipitation of calcium phosphate in model liposomes with matrix vesicle-like lipid composition. Bone Miner **16**: 109-119.

Sleight RG (1987) Intracellular lipid transport in eukaryotes. Annu Rev Physiol **49**: 193-208.

Smith JR, Pereira-Smith OM, Schneider EL (1978) Colony size distribution as a measure of *in vivo* and *in*

vitro aging. Proc Natl Acad Sci USA **75**: 1353-1356.

Smith MW, Phelps PC, Trump BF (1991) Cytosolic Ca^{2+} deregulation and blebbing after $HgCl_2$ injury to cultured rabbit proximal tubule cells as determined by digital imaging microscopy. Proc Natl Acad Sci USA **88**: 4926-4930.

Somlyo AP, Bond M, Somlyo AV (1985) Calcium content of mitochondria and endoplasmic reticulum in liver frozen rapidly *in vivo*. Nature **314**: 622-625.

Spicer SS, Lewis SE, Tahsian RE, Schulte BA (1989) Mice carrying a CAR-2 null allele lack carbonic anhydrase II immunohistochemically and show vascular calcification. Am J Pathol **134**: 947-954.

Stefanova HI, Jane SD, East JM, Lee AG (1991) Effects of Mg^{2+} and ATP on the phosphate transporter of sarcoplasmic reticulum. Biochim Biophys Acta **1064**: 329-334.

Stegner HE, Pape C (1972) Beitrag zur Feinstruktur der sog. Mikrokalzifikation in Mammatumoren (A contribution to the fine structure of microcalcification in mammalian tumors). Zentralbl Allg Pathol Path Anat **115**: 106-112.

Stewart BW (1994) Mechanisms of apoptosis: Integration of genetic, biochemical, and cellular indicators. J Natl Cancer Inst **86**: 1286-1296.

Swairjo MA, Seaton BA (1994) Annexin structure and membrane interactions: A molecular perspective. Annu Rev Biphys Biomol Struct **23**: 193-213.

Swain LD, Boyan BD (1988) Ion-translocating properties of calcifiable proteolipids. J Dent Res **67**: 526-530.

Tanaka M, Ito H, Adachi S, Akimoto H, Nishikawa T, Kasajima T, Marumo F, Hiroe M (1994) Hypoxia induces apoptosis with enhanced expression of Fas antigen messenger RNA in cultured neonatal rat cardiomyocytes. Circ Res **75**: 426-433.

Tani M (1990) Mechanisms of Ca^{2+} overload in reperfused ischemic myocardium. Annu Rev Physiol **52**: 543-559.

Tanimura A, McGregor DH, Anderson HC (1986a) Calcification in atherosclerosis. I. Human studies. J Exp Med **2**: 261-273.

Tanimura A, McGregor DH, Anderson HC (1986b) Calcification in atherosclerosis. II. Animal studies. J Exp Pathol **2**: 275-297.

Tatulian SA (1987) Binding of alkaline-earth metal cations and some anions to phosphatidylcholine liposomes. Eur J Biochem **170**: 413-420.

Termine JD (1972) Mineral chemistry and skeletal biology. Clin Orthop **85**: 207-241.

Thesingh CW, Groot CG, Wassenaar AM (1991) Transdifferentiation of hypertrophic chondrocytes into osteoblasts in murine fetal metatarsal bones, induced by co-cultured cerebrum. Bone Miner **12**: 25-40.

Thomas DB, Whitehead JW, Dorse C, Threatt BA, Gilbert FI, Present AJ, Carlile T (1993) Mammographic calcifications and risk of subsequent breast cancer. J Nat Cancer Inst **85**: 230-235.

Trump BF, Berezesky IR (1992) The role of cytosolic Ca^{2+} in cell injury, necrosis and apoptosis. Curr Opinion Cell Biol **4**: 227-232.

Trump BF, Valigorsky JM, Dees JH, Mergner WJ, Kim KM, Jones RT, Pendergrass RE, Garbus J, Cowley RA (1973) Cellular changes in human disease. A new method of pathological analysis. Human Pathol **4**: 89-109.

Trump BF, Berezesky IK, Laiho KU, Osorino AR, Mergner WJ, Smith MW (1980) The role of calcium in cell injury. A review. Scanning Electron Microsc. **1980**; II: 437-462.

Tsien RW (1990) Calcium channels, stores, and oscillations. Annu Rev Cell Biol **6**: 715-760.

Tyson CA, Zande HV, Green DE (1976) Phospholipids as ionophores. J Biol Chem **251**: 1326-1332.

Ucker DS, Obermiller PS, Eckhart W, Apgar JP, Berger NA, Meyer J (1992) Genome digestion is a dispensable consequence of physiological cell death mediated by cytotoxic T lymphocytes. Mol Cell Biol **12**: 3060-3069.

Urist MR, Adams JM (1967) Localization mechanism of calcification in transplants of aorta. Ann Surg **166**: 1-18.

Urry DW, Starcher BC, Ohnishi T, Long MM, Cox BA (1974) On the elastic fiber of the arterial wall as a site of molecular pathogenesis. Path Biol **22**: 701-706.

Vallance P, Collier J (1994) Biology and clinical relevance of nitric oxide. Br Med J **309**: 453-457.

van der Meulen J, Verhoeven MC, Koerten HK (1993) Mouse peritoneal cavity: A model compartment for degradation studies. Eur J Morphol **31**: 9-12.

Varma VA, Kim KM (1985) Placental calcification: Ultrastructural and X-ray microanalytic studies. Scanning Electron Microsc **1985**; IV: 1567-1572.

Verhoven B, Schlegel RA, Williamson P (1992) Rapid loss and restoration of lipid asymmetry by different pathways in resealed erythrocyte ghosts. Biochim Biophys Acta **1104**: 15-23.

Virchow R (1855) Cellular Pathology. Virchows Archiv. 8 (1. Translated from the original German). In: Disease, Life and Man, with an Introduction by R Virchow. Translated with an introduction by T Rather. Stanford Univ. Press. p. 100.

Wachstein M, Meisel E (1957) Histochemistry of hepatic phosphatases at a physiological pH, with special reference to the demonstration of bile canaliculi. Am J Clin Pathol **27**: 13-23.

Webb MR (1992) A continuous spectrophotometric assay for inorganic phosphate and for measuring phos-

phate release kinetics in biological systems. Proc Natl Acad Sci USA **89**: 4884-4887.

Wehrle JP, Pedersen PL (1989) Phosphate transport processes in eukaryotic cells. J Membr Biol **111**: 199-213.

Wendt-Gallitelli MF (1986) Presystolic calcium-loading of the sarcoplasmic reticulum influences time to peak force of contraction. X-ray microanalysis on rapidly frozen guinea-pig ventricular muscle preparation. Basic Res Cardiol **81**: 25-32.

Werness PG, Bergert JH, Smith LH (1981) Crystalluria. In: Urolithiasis, Clinical and Basic Research. Smith LH, Robertson WG, Finlayson B (eds.). Plenum Press, New York. pp. 17-27.

Westheimer FH (1987) Why nature chose phosphates. Science **235**: 1173-1178.

Williams GT (1991) Programmed cell death: Apoptosis and oncogenesis. Cell **65**: 1097-1098.

Williamson P, Bateman J, Kozarsky K, Mattocks K, Hermanovicz N, Choe HR, Schlegel RA (1982) Involvement of spectrin in the maintenance of phase state asymmetry in the erythrocyte membrane. Cell **30**: 725-735.

Williamson P, Kulick A, Zachowski A, Schlegel RA, Devaux PF (1992) Ca^{2+} induces transbilayer redistribution of all major phospholipids in human erythrocytes. Biochemistry **31**: 6355-6360.

Wohlrab H (1986) Molecular aspects of inorganic phosphate transport in mitochondria. Biochim Biophys Acta **853**: 115-134.

Woodard JC (1971) A morphological and biochemical study of nutritional nephrocalcinosis in female rats fed semipurified diets. Am J Pathol **65**: 253-268.

Wright SC, Zhong J, Larrick JW (1994) Inhibition of apoptosis as a mechanism of tumor promotion. FASEB J **8**: 654-660.

Wu LNY, Genge BR, LLoyd GL, Wuthier RE (1991) Collagen binding proteins in collagenase released matrix vesicles from cartilage. Interaction between matrix vesicle proteins and different types of collagen. J Biol Chem **266**: 1195-1203.

Wutheir RE (1988) Mechanism of Matrix Vesicle-mediated mineralization of cartilage. ISI Atlas Sci, Biochemistry/**1988**. Inst. Sci. Info., Philadelphia. pp. 231-241.

Wuthier RE (1993) Involvement of cellular metabolism of calcium and phosphate in calcification of avian growth plate cartilage. J Nutr **123**: 301-309.

Wuthier RE, Gore ST (1977) Partition of inorganic ions and phospholipids in isolated cell, membrane, and matrix vesicle fractions: Evidence for Ca-P_i-acidic phospholipid complexes. Calcif Tissue Res **24**: 163-171.

Wuthier RE, Majeska RJ, Collins GM (1977) Biosynthesis of matrix vesicles in epiphyseal cartilage. I. In vivo incorporation of ^{32}P orthophosphate into phospholipids of chondrocyte, membrane and matrix vesicle formation. Calcif Tissue Res **23**: 135-139.

Xu H, Watkins BA, Adkisson HD (1994a) Dietary lipids modify the fatty acid composition of cartilage, isolated chondrocytes and matrix vesicles. Lipids **29**: 619-625.

Xu X, Star RA, Tortorici G, Muallem S (1994b) Depletion of intracellular Ca^{2+} stores activates nitric-oxide synthase to generate cGMP and regulate Ca^{2+} influx. J Biol Chem **269**: 12645-12653.

Yeagle PL (1989) Lipid regulation of cell membrane structure and function. FASEB J **3**: 1833-1842.

Zheng LM, Zychlinsky A, Liu C-C, Ojcius DM, Young JD (1991) Extracellular ATP as a trigger for apoptosis or programmed cell death. J Cell Biol **112**: 279-288.

Zijlstra JB, Torringa JL, Stinson RA (1970) Intracellular distribution of alkaline phosphatase. Gynecol Invest **1**: 161-168.

Zimmerman RA, Bilaniuk LT (1982) Age related incidence of pineal calcification detected by computed tomography. Radiology **48**: 652-659.

Zollinger HU (1948) Cytosolic studies with the phase microscope. I. The formation of "blisters" on cells in suspension (potocytosis), with observations on the nature of the cellular membrane. Am J Pathol **24**: 545-567.

Discussion with Reviewers

G.M. Roomans: In Figure 25b, the presence of sulfur could be due to any protein (cell debris?). This is no proof that this is stroma.

Author: I agree. However, it has been known that fibrillar amyloid matrix is incorporated into psammoma bodies. The presence of S in both the matrix and PB concurs with the incorporation of the matrix. Sulfur containing proteins exist in small quantities in tissues. Sulfur is usually not detectable by EPM both in tissues and calcific deposits.

E. Bonucci: Besides MVs originating from apoptotic chondrocytes, most of the cartilage MV are formed by swelling of the tip of chondrocyte processes and successive detachment of the swollen portion. Is this process due to apoptosis?

Author: As seen in blebbing, condensation of Ca^{2+} in hypertrophic chondrocytes occurs in cell processes prior to their formation of MV (Wuthier, 1993). It is not yet known how cells are able to concentrate Ca^{2+} in certain locations of the cytosol. There have been limited studies on the movement of $[Ca^{2+}]_i$ in the cytosol (Clapham, 1995). What is unique to cartilage is that the cell processes give rise to a relatively homogeneous population of

MV. This may be attributable in part to the viscous matrix of the cartilage.

E. Bonucci: It has been reported that MV are of two different types which possibly have different role. Have all apoptotic blebs the same composition, and are all of them able to induce calcification?

Author: Calcifying CDP are morphologically heterogeneous. They are likely to vary in their structural compositions and enzyme, Ca^{2+} and P_i contents. The complexities of calcific deposits in CDP indicate that the microenvironment in which the deposits are formed is variable. Of the theories of calcification, ion deregulation in cell death, leading to high concentrations of Ca^{2+} and P_i in blebs (and MV), affords the most logical explanation for their calcification. In my opinion, the failure to link calcification to cellular events is the main reason for the slow progress made in the field of calcification.

E. Bonucci: Do you think that all MV are formed through cell apoptosis? Have you considered that there are tissues, as for instance, embryonic woven bone, where MV are numerous although oxygenation and scavenging are very effective, and apoptosis (at least to my knowledge) are rare or lacking?

Although in epiphyseal cartilage apoptosis is frequent and MV are the loci of initial calcification, there are other tissues which physiologically calcify in spite of the rare presence or complete absence, of apoptosis and MVs, as for instance, compact bone (where MV are rare) or tooth enamel (where MV are lacking). Do you not think this invalidates your conclusion that "... cell death is the common underlying mechanism for both physiological and pathological calcifications"?

Reviewer VI: It is stated that mounting evidence indicates that cell death (apoptosis) is primarily responsible for both pathological and physiological calcification, but not true in the case of physiological calcification of bones and teeth. With the exception of the growth plate where programmed cell death occurs, there is no evidence that apoptosis and cell disintegration leads to calcification of the periosteum or endosteum in bone development and repair, or of the initial stages of calcification of dentin. In all of these examples, the cells shed calcifiable vesicles into the matrix in a polarized fashion but then survive the blebbing experience and persist as living cells in the bone and predentin.

Author: It is not possible to generalize a phenomenon with the results obtained from limited studies. Since the discovery of MV three decades ago, there has not been a satisfactory explanation for the mechanism of MV formation. Overloaded $[Ca^{2+}]_i$ in cell death best explains the formation and calcification of blebs and MV.

The formation of compact bone and dental enamel is an issue related to apatite crystal growth and proliferation and bone modeling; it has no direct relevance to apatite nucleation. It is a mystery how bone cells mold calcific deposits into cancellous and compact bones. Mechanical stress has long been noted to affect bone remodeling. This may occur through intercellular signal exchanges as a result of compression of bone fluid (Turner and Forwood, 1995). It is tempting to speculate that teeth have evolved through millions of years of chewing. There is evidence that calcification in cultured dental pulp cells is related to cell death and its degradation products (Hayashi *et al.*, 1993). To my knowledge, apoptosis in embryonic bone has not been studied. Theoretically, some cells are likely to survive the blebbing experience, perhaps depending on the degree of injury and cellular resilience. However, the occurrence and the extent of such survival *in vivo* remain to be determined. In general, cellular turnover is faster in developing tissues than their mature counterparts. Without the knowledge of the cells' total life span versus their residual life span after blebbing, it will be difficult to reach a conclusion. The "polarized deposition" of the vesicles may result from effective scavenging on the marrow side surface of the endosteal layer.

D.J. McConkey: What are the physiological consequences of calcification?

Author: Calcification is an important biological phenomenon. According to the apoptosis theory, there would be no bones and teeth if there were no apoptosis. Skeletal mineral also serves as the main Ca^{2+} and P_i storage. In plants, calcium is stored mainly in the leaves as calcium oxalate. Calcification occurring in a strategic location, i.e., calcific aortic stenosis, can be fatal. The degree of vascular calcinosis correlates with the prognosis in atherosclerosis, especially of coronary arteries. Further, heavily calcified vascular tissue, e.g., aortic aneurysm, makes its surgical repair difficult. Stones containing calcium phosphate are a form of abnormal calcification.

D.J. McConkey: Is calcification always initiated within cells? What concentration of calcium ion would one expect to require for the process to be initiated?

Author: Cell death presumably plays the major role in calcification. With the current status of art, it is not possible to determine the exact point of apatite nucleation in relation to blebbing and CDP formation. It is likely that a large number of apatite nuclei are formed within the cell and packaged into blebs. There has been many studies on the potential role of the extracellular matrix in calcification. None, thus far, offers a better explanation for calcification than cell death. It is im-

portant to keep in mind that in heavily calcified tissues that are frequently used for the study of calcification, it is not possible to determine where calcification began.

In simple synthetic solutions, calcification can occur at the total Ca X P_i products much lower than that of serum. *In vitro*, calcification occurring at the product lower than 2.0 mM^2 is usually considered "physiological." There has not yet been a detailed correlation between the level of $[Ca^{2+}]_i$ (and $[P_i]_i$) in cell death and calcification. In light of several fold increases in $[P_i]_i$, that may exceed 10 mM in cell injury and apoptosis, micromolar concentrations of $[Ca^{2+}]_i$ would be sufficient for apatite nucleation.

D.J. McConkey: It is currently thought that the recognition, uptake, and disposal of apoptotic cells by neighboring cells and macrophages is an extremely efficient and rapid phenomenon. How would the author reconcile this concept with the idea that apoptotic cells might promote calcification (a presumably more protracted response)?
Author: Apparently, not every tissue benefits from the efficient scavenging by macrophages, as evidenced by the presence of CDP in aging connective tissues and neoplasms. Since tissue macrophages are delivered and removed through capillaries and lymphatics, the lack of their network in certain tissues may account for the accumulation of CDP and the resultant calcification.

D.J. McConkey: It would seem that calcification might be better promoted by necrosis than apoptosis. What contribution does the author think secondary necrosis of apoptotic cell plays in calcification?
Author: Changes in $[Ca^{2+}]_i$ in apoptosis of anti-Fas mAb treated FMO cells takes place in multiple steps. Increases in $[Ca^{2+}]_i$ as high as 2 μM in the later half of apoptotic process coincides with the development of "secondary necrosis" (Oshimi and Miyazaki, 1995). It is likely that $[Ca^{2+}]_i$ will further increase even after the secondary necrosis. Needless to say, higher $[Ca^{2+}]_i$ in later stages of apoptosis, provided there is no loss of $[P_i]_i$, will be favorable for calcification.

D.J. McConkey: Early work by Bowen and his colleagues (see his book: Programmed Cell Death in Tumors and Tissues, Chapham and Hall, 1990, and in several papers referenced therein) suggested that upregulation of alkaline phosphatase activity is linked to programmed cell death *in vivo*. This observation appears to support the author's thesis.
Author: Thank you for the comment.

Reviewer III: Why is phosphate difficult to measure?
Author: As opposed to Ca^{2+}, the ion selective electrode for P_i and fluorescent probes for intracellular $[P_i]_i$ have not been available. The measurement of P_i in the past depended mainly on a color reaction with, i.e., malachite green. Furthermore, three different species of P_i exists in equilibrium, $H_2PO_4^-$, HPO_4^{2-}, and PO_4^{3-}. There is not yet a satisfactory means that detects these ion species. With the advent of NMR, there has been an increasing number of studies on $[P_i]_i$ homeostasis.

Reviewer V: The author discusses the basic morphologic features of apoptosis and necrosis and the potential overlap between the two states. Is there morphological and biochemical evidence for early changes of apoptosis in the conditions cited and illustrated by the author as representing calcification in apoptotic states?
Author: The process of cell death is perhaps as complex as life phenomena. Although cell death can be easily recognized in cell cultures, it is difficult to define properly. The pursue of the likely initial step of apoptosis caused by a genetic mechanism is promising. However, current attempts to define apoptosis by a few simple morphological and chemical characteristics are, in the author's opinion, an oversimplification for the immensity of the phenomenon of cell death. As pointed out, naturally occurring cell death *in vivo* without notable cause is considered apoptotic in this discussion.

Reviewer VI: An insufficient distinction is drawn between the features of physiological calcification, and the various forms of pathological calcification which are the main concern of this paper. Only features of pathological calcification are presented in detail. Therefore, it is inappropriate and uncritical to equate the features of calcification in growth plate as mediated by matrix vesicles with the process of pathological calcification as depicted in the aortic valve. Since no adequate description of normal biological calcification is put forth, an unfortunate confusion is created leading the reader to believe that the two processes are identical, but they are not. Specifically, the types of mineral ultrastructure described here in pathologically calcifying aortic valve as "porous particles", "fine fibrils", and "solid deposits" have not been recognized in normal growth plate calcification.
Author: Existing concepts of MV calcification, the mainstay of physiological calcification, are reviewed. The similarity between calcification of MV and blebs and the involvement of cell death in both physiological and pathological calcification are stressed. Theoretically, even in the same tissue, the microenvironment in which calcification occurs is likely to be variable. Not every MV or CDP in tissues displays calcific deposits. The possibility that differences in the rate of calcifying process instead of the fundamental mechanism of calcification may dictate the subtle difference between physio-

logical and pathological calcification should be considered. For instance, the changes observed in the slowly progressing cell death and calcification in aging tracheal cartilage may be difficult to observe in the epiphysis where it occurs in fast motion and calcific deposits are rapidly incorporated in to bone.

Porous particles have been observed in skeletal mineral (Molnar, 1959). The occurrence of carbonate apatite has been described in bone, in which the Ca/P ratio is consistently lower than 1.67. Solid whitlockite particles were observed in cartilage. OCP formation by isolated MV is mentioned in the text.

It would be wise to take into account the possible role of cell death in studies of calcification involving MV.

Reviewer VI: Several of the studies of MV calcification *in vitro* by Wuthier's group (1988) may be invalid because they used MV's taken from chicken growth plate, many of which are already precalcified *in vivo*. Thus, the calcification rate observed is partly due to initial mineralization and partly due to the growth of pre-existing mineral, and it may be incorrect to conclude that removal of alkaline phosphatase (ALP) had "little effect on P_i or Ca^{2+} uptake by MV". In fact in recent reports, Hsu *et al.* (1993) and Hsu and Anderson (1995a, 1995b) have found the opposite when studying non-precalcified rachitic rat MVs. In short, Dr. Hsu's studies indicate that physiological levels of ATP (not-supraphysiological) are optimal to support the calcification of non-precalcified MV's, and that inhibitors of ALP (such as PIPLC and Levamisole) inhibit MV calcification at least partially and in a dose dependent fashion.

Author: Thank you for the comment.

Reviewer VI: Long bones do not lack "an ability to grow" except at the growth plate. In fact, the ability to grow periosteally and endosteally persists into late adult life. This occurs during normal metabolic turnover of bone and at sites of fracture healing.

Author: The primary function of bone cells is remodeling and modeling (Freemont, 1993). Mechanical stress, especially of weight bearing, has been known to affect the remodeling (Turner and Forwood, 1995). Bone cells maintain a close relationship with marrow cells and they both may share the common stem cells. The polarized deposition of MV in osteoid can be attributed to the highly vascular marrow. It is true that bone cells retain some capacity to respond to growth hormone. Acromegaly develops in response to an excessive growth hormone secretion by pituitary adenoma. However, the phenomenon may be viewed as a derangement in remodeling. The trivial growth of mature bone is not a match for the dramatic consequences of epiphyseal malfunctions, e.g., dwarfism and gigantism. Fracture healing, in my opinion, is a form of wound healing rather than growth. Bone growth is generally associated with an enlargement of the bone that increases the height during the growth period rather than the cellular activity therein. In regard to dental pulp cells, calcification in rat pulp cells in culture has been shown to occur in association with cell death (Hayashi *et al.*, 1993).

Reviewer VI: The definition of "dystrophic calcification" is insufficient and should incorporate the fact that dystrophic calcification occurs in normocalcemia in "injured tissue."

Author: Although injury frequently results in dystrophic calcification, the reverse may not be true. For instance, calcinosis demonstrated in aging connective tissue, i.e., tracheal cartilage, or in fibroadenoma of the breast cannot be attributed to injury.

Additional References

Freemont AJ (1993) Basic bone cell biology. Int J Exp Path **74**: 411-416.

Hayashi Y, Imai M, Goto Y, Murakami N (1993) Pathological mineralization in a serially passaged cell line from rat pulp. J Oral Pathol Med **22**: 175-179.

Hsu HHT, Anderson HC (1995a) Effect of Zinc and cation chelators on ATP hydrolysis and Ca-deposition by rachitic rat matrix vesicles. Bone, in press.

Hsu HHT, Anderson HC (1995b) A role for ATPase-dependent Ca and phosphate deposition by isolated rachitic matrix vesicles. Int J Biochem Cell Biol, in press.

Hsu HHT, Morris DC, Davis L, Moylan P, Anderson CJ (1993) Calcium deposition by rat matrix vesicles: The membrane association of alkaline phosphatase is essential for matrix vesicle-mediated calcium deposition. Int J Biochem **25**: 1737-1742.

Molnar Z (1959) Development of the parietal bone of young mice. I. Crystals of bone mineral in frozen dried preparation. J Ultrastruct Res **3**: 39-45.

Oshimi O, Miyazaki S (1995) Fas antigen-mediated DNA fragmentation and apoptotic morphologic changes are regulated by elevated cytosolic Ca^{2+} level. J Immunol **154**: 599-609.

Turner CH, Forwood MR (1995) What role does the osteocyte network play in bone adaptation? Bone **16**: 283-285.