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Morphologic Features and Nuclide Composition of Infarction-associated Cardiac Myocyte Mineralization in Humans

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Low dietary Mg results in Ca loading of cardiac myocytes, which increases the likelihood of myocyte calcification in the event of acute myocardial infarction (AMI), and possibly increases myocyte vulnerability to necrosis. Bloom and Peric-Golia[1] previously reported an autopsy study of cases from the Washington, D.C. area (a region with low levels of Mg in the drinking water), demonstrating AMI-associated mineralization in myocytes with histologically normal nuclei and cross striations, as well as in obviously necrotic myocytes. The authors have re-examined mineralized myocytes from the same autopsy material, using electron probe microanalysis, light microscopy, and transmission electron microscopy. Microprobe analysis identified Ca and P as the nuclides composing the inorganic phase of the mineral deposits. Ultrastructurally, all Ca deposits, regardless of size or intracellular location, were composed of aggregates of needlelike hydroxyapatite crystals. The mildest form of intracellular Ca deposition was observed as small Ca deposits limited to some mitochondria of myocytes, which demonstrated intact nuclei and regular sarcomere pattern. More advanced stages of intracellular calcification, in the form of Ca deposits associated with mitochondria, Z-band regions and nuclei, were observed in other myocytes that also retained intact nuclei and sarcomeres. Massive Ca deposits were associated with myocytes which showed morphologic features of advanced necrosis, including loss of nuclei, disruption of sarcomere structure and masses of cellular debris. These observations support the theory originally proposed by Bloom and Peric-Golia[1] suggesting that Ca loading of myocytes, possibly related to Mg deficiency in humans, increased vulnerability of the myocytes to subsequent AMI-associated necrosis and dystrophic calcification. In addition, the light microscopic impression of calcification of otherwise normal myocytes is contradicted by the electron microscopic identification of hydroxyapatite crystals free in the sarcoplasm, a condition unlikely to be compatible with viability. Lastly, the fact that all Ca deposits were in the form of hydroxyapatite supports the view that they were formed in a Mg-poor environment, which favors conversion of the more common amorphous form of Ca phosphate into the needlelike crystals of hydroxyapatite. (Am J Pathol 1991, 139:565–572)

In experimental animals, administration of a low magnesium diet has been shown to result in decreased serum Mg levels, increased myocardial Ca levels, and, if the dietary Mg level is very low, focal myocardial necrosis associated with myocyte calcification.[2–4] Less severe Mg deficiency in dogs is associated with increased myocardial Ca and Na, and increased vulnerability to ischemic necrosis.[5] Likewise, low dietary Mg in humans may be associated with an increased acute myocardial infarction (AMI) death rate[6–11] and an increased tendency for AMI-associated myocyte mineralization.[1] It thus appears that myocyte injury in severe Mg deficiency is due to severe Ca-loading of myocytes. Likewise, the increased myocardial vulnerability to injury found in subclinical Mg deficiency may be secondary to increased intracellular levels of Ca, which facilitate Ca overload associated with injury and leads to the compromise of energy metabolism.[12–17]

Bloom and Peric-Golia[1] observed AMI-associated myocyte mineralization in a series of autopsy cases in Washington, D.C., an area with low levels of Mg in the drinking water. A corresponding group of cases from the

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Salt Lake City, Utah area, where the level of Mg in the drinking water is high and the AMI death rate is low, did not show any morphologically recognizable AMI-associated myocyte mineralization. This study further characterizes the mineralized myocytes in the aforementioned cases from the Washington, D.C. area. Detailed information is presented about the nuclide composition, ultrastructural appearance, and intracellular localization of the mineral deposits. Detailed information is also presented about variations in morphologic characteristics of the mineralized myocytes. The present findings, together with previous studies, support the hypothesis that myocyte Ca loading varies inversely with dietary Mg intake, and that the level of Ca loading of functioning cardiac myocytes is a determinant of cardiac myocyte vulnerability to AMI-associated necrosis and calcification.

Methods

Myocardial tissues from 15 selected autopsy cases done in 1986, at George Washington University Medical Center, Washington, D.C., were used in this study. Bloom and Peric-Golia[11] previously identified the presence of mineralized myocytes associated with acute myocardial necrosis in these specimens. The current study used this autopsy material to re-examine the calcified myocytes by means of light microscopy, transmission electron microscopy, and electron microprobe analysis.

Light Microscopy

Light microscopic sections were obtained from samples of myocardium, 2 to 4 mm thick, fixed in phosphate-buffered 4% formaldehyde at pH 7, and embedded in paraffin. From each case, one slide was stained with hematoxylin-eosin for routine examination. Additional slides were stained by the von Kossa[18] and the Alizarin red S[19] methods, using hematoxylin-eosin as a counterstain.

Transmission Electron Microscopy

Myocardial tissue selected for examination by electron microscopy was removed from paraffin, placed in acetone for 8 hours, then absolute ethanol, followed by rehydration by passage through progressively reduced concentrations of ethanol. The tissue was then divided into 1-mm cubes, fixed in phosphate-buffered 1% osmium tetroxide, dehydrated in graded concentrations of ethanol, and embedded in Epon 812. Semithin sections were stained with Paragon stain to identify specific areas of interest. Ultrathin sections were stained with uranyl acetate and lead citrate before examination in a Zeiss 109 transmission electron microscope. As a result of obtaining myocardial tissues for electron microscopy from previously paraffin-embedded blocks, ultrastructural preservation was imperfect, but satisfactory for evaluation of the mineral deposits and many morphologic features of interest.

STEM Electron Probe Microanalysis

Electron probe microanalysis was done to specifically identify inorganic nuclides comprising the intracellular mineral deposits observed in myocytes. Unstained, ultrathin sections of myocardium, mounted on copper grids, were examined in a Hitachi H-7000 STEM coupled to a Kevex Microanalyst 8000 EDS. A Kevex 30mm quantum detector at 75 KV was used to obtain x-ray spectra of the predominant atomic elements present in the deposits and to perform fast x-ray mapping of the ions.

Results

Nuclide Composition of the Inorganic Phase of Intracellular Mineral Deposits

Figures 1a through 1d show results of electron probe microanalysis and X-ray mapping studies that identified Ca and P as major inorganic components of intracellular electron-opaque mineral deposits observed in myocytes. In all of the cases studied, X-ray spectra of mineral deposits revealed prominent Ca and P peaks, regardless of deposit size or intracellular location (Figure 1b). No other significant ion was identified in the deposits. Independent X-ray mapping of the Ca and P ions showed the consistent presence and similar locations of both ions in the deposits (Figures 1c, 1d). These data imply that the mineral deposits primarily consist of some form of Ca phosphate.

Morphology of Calcified Myocytes

Examination of sections of myocardium by light and electron microscopy showed the presence of calcified myocytes with varying morphologic appearances. In 4 of the 15 autopsy cases studied, Ca deposits were observed in myocytes that had visible nuclei and intact sarcomere structure. These calcified myocytes were scattered throughout the left ventricular myocardium, including ar-

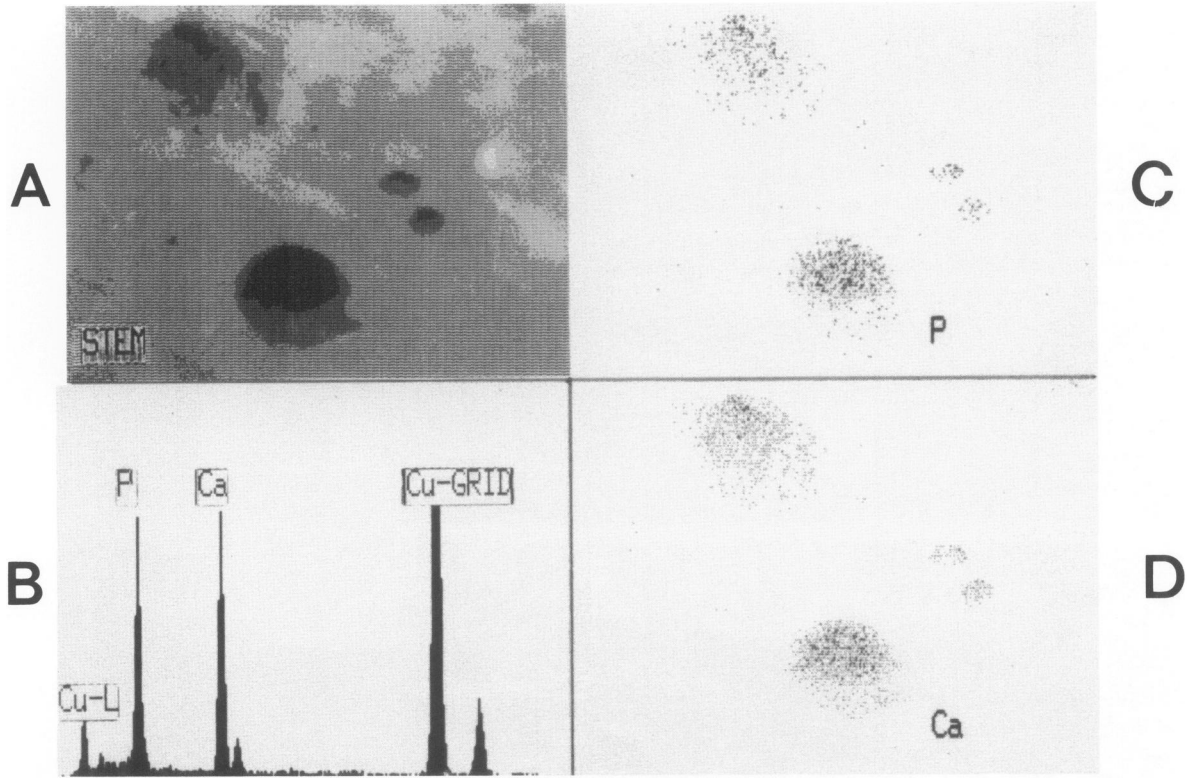

Figure 1. *Electron microprobe analysis and fast X-ray mapping of Ca deposits scattered throughout the sarcoplasm of a cardiac myocyte, ×10,000.* **A:** *Digitized electron microscopic image of Ca deposits analyzed.* **B:** *X-ray spectra of the crystalline deposits showed prominent Ca and P peaks.* **C, D:** *Independent, fast X-ray mapping of the Ca and P revealed the consistent presence and similar locations of both ions in the deposits.*

eas that were remote from those showing AMI. That is, calcified myocytes that did not appear necrotic by light microscopy were found in areas showing no evidence of necrosis.

Myocardial sections from all 15 of the autopsy cases showed the presence of calcified myocytes demonstrating obvious morphologic features of necrosis. The necrotic, calcified myocytes were located in areas of AMI, frequently directly beneath the endocardium. Calcified myocytes with visible nuclei and intact sarcomeres, as well as calcified myocytes that were obviously necrotic, were usually located adjacent to normal-appearing myocytes. Often, calcified myocytes showed especially prominent Ca deposits along an intercalated disk, on the other side of which was a normal-appearing noncalcified myocyte.

Myocytes that showed Ca deposition and retained visible nuclei and intact sarcomere structure showed the following morphologic appearances, depending on the amount and intracellular location of the Ca deposition. 1) Some myocytes appeared normal by light microscopy except for the presence of small, discrete Ca deposits randomly scattered throughout the sarcoplasm (Figure 2a). Electron microscopic examination of these myocytes showed the small Ca deposits to be limited exclusively to some mitochondria (Figure 2b). The mitochondrial Ca deposits were composed of aggregates of filament or needlelike crystals (Figure 2b, inset). Individual Ca crystals measured approximately 5 nm in diameter and 20 to 30 nm in length. Some mitochondria contained small aggregates of Ca crystals associated only with the membranes of cristae, whereas other mitochondria contained larger irregular aggregates that occupied most of the mitochondrial matrix. 2) A second morphologic group of calcified myocytes, demonstrating visible nuclei and intact sarcomeres, was observed by light microscopy to contain fine Ca deposits scattered throughout the entire sarcoplasm and also within nuclei (Figure 3a). Electron microscopy showed the Ca deposits, which were composed of the same needlelike crystals, to be present in some mitochondria, in Z-band regions and to a lesser extent between myofilaments. Crystalline Ca deposits within nuclei of these myocytes were closely associated with chromatin material (Figure 3b). 3) A third morphologic group of myocytes with visible nuclei and intact sarcomere structure showed a light microscopic appearance characterized by the presence of relatively heavy Ca deposits distributed predominantly along Z bands and intercalated disks and present within nuclei. In addition, small Ca deposits were also observed throughout

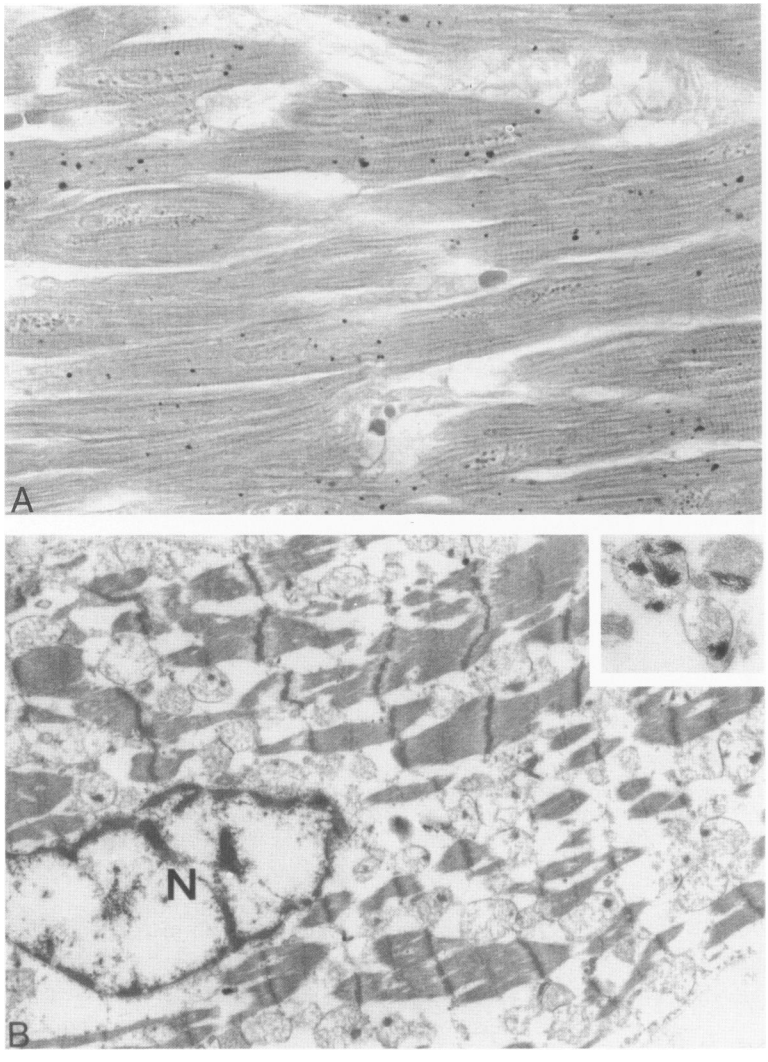

Figure 2. A: *Light micrograph of myocytes with visible nuclei and sarcomeres with few Ca deposits randomly scattered throughout the sarcoplasm, von Kossa and H&E, ×320.* **B:** *Electron micrograph of myocyte, showing Ca deposits limited to mitochondria, ×11,000. Inset-mitochondrial Ca deposits composed of needle-like crystals, uranyl acetate and lead citrate, ×17,000.*

the sarcoplasm (Figure 4a). Electron microscopy confirmed the presence of large accumulations of needlelike crystalline Ca deposits in regions of Z bands and associated with nuclear chromatin (Figure 4b, inset). Smaller crystalline Ca deposits were present in some mitochondria and scattered among the myofilaments (Figure 4b).

Calcified myocytes that showed severe necrotic changes appeared on light microscopy as masses of necrotic debris, masked by large flocculent Ca deposits. These necrotic myocytes showed no evidence of nuclei or recognizable sarcomere structure (Figure 5a). Electron microscopic examination showed the necrotic myocytes to consist of disorganized masses of myofibrils and cellular debris containing large Ca deposits (Figure 5b). Ultrastructurally, the massive Ca deposits appeared to have dense central areas composed of compact aggregates of needlelike crystals, bordered by more loosely arranged crystals at the periphery (Figure 5b, inset).

Discussion

The results of this study suggest or impinge on three questions: 1) Why are the Ca deposits noted here all in the form of hydroxyapatite, when dystrophic myocardial calcification is usually amorphous? 2) Are the cells that contain Ca deposits, but which have intact nuclei and ordered sarcomeres, viable? 3) Why is AMI-associated myocyte calcification seen in patients from the Washington, D.C. area but not from the area of Salt Lake City, Utah?

Biological Ca deposits exist in many forms. In vertebrates, Ca is usually deposited as the phosphate salt, either as amorphous Ca phosphate or as hydroxyapatite, which is $Ca_{10}(PO_4)_6(OH)_2$. There are, of course, other salts of Ca that may be deposited in vertebrates, as demonstrated by the $CaCO_3$ of eggshells and otoliths whose composition varies with species.[20] The electron micro-

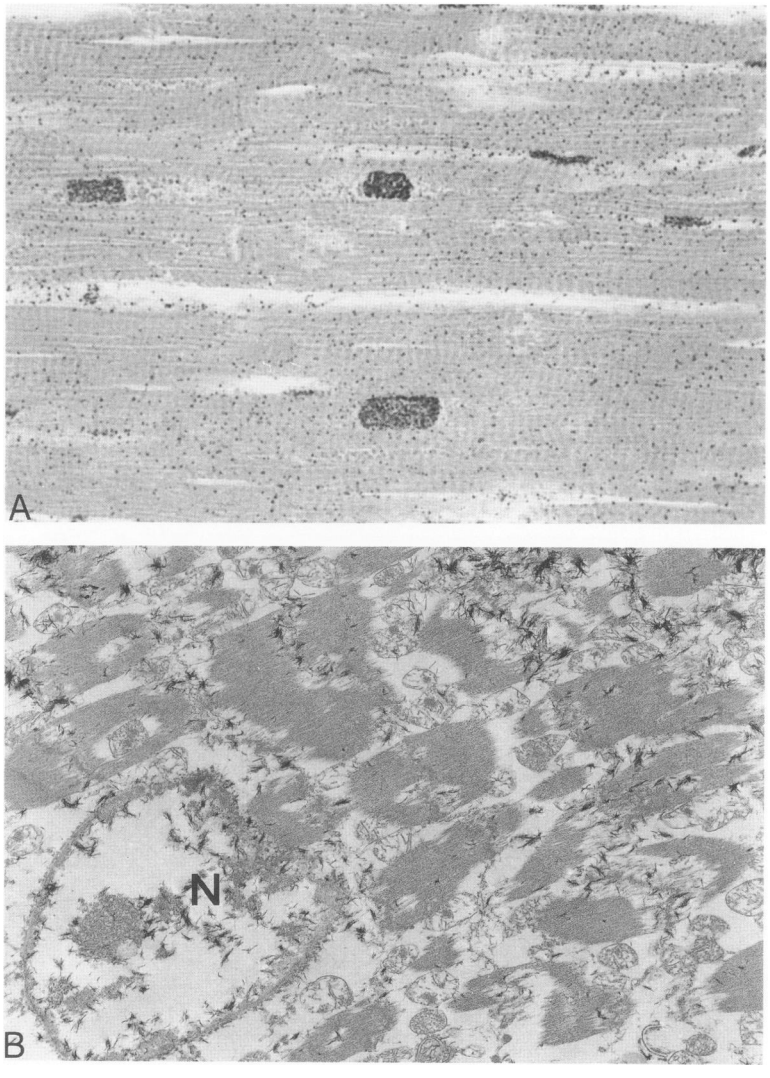

Figure 3. **A:** *Light micrograph of myocytes with visible nuclei and sarcomeres with Ca deposits scattered throughout the entire sarcoplasm and within nuclei, von Kossa and H&E, ×320.* **B:** *Electron micrograph of myocyte, showing crystalline Ca deposits in some mitochondria, in Z-band regions, between myofilaments, and in nuclei. Uranyl acetate and lead citrate, ×11,000.*

probe studies carried out in the present work show that we are dealing with deposits containing Ca and P, whereas the electron microscope studies show that we are specifically concerned with hydroxyapatite.

In its pure state, hydroxyapatite is a needlelike monoclinic structure with a crystal size of about 20 × 5 × 5 × nm,[20] comparable to that observed in our study. Other anions may partially replace P, but when this happens, different crystalline forms result. Precipitation of Ca phosphate from a supersaturated solution at 37°C leads to the formation of an amorphous precipitate of CaPO4 with no crystalline structure. This amorphous form gradually converts, spontaneously, to crystalline hydroxyapatite.

Intracellular deposits of Ca phosphate also exist in these two forms, with the amorphous form being the most common.[21–26] In pathologic calcification, both forms of precipitated Ca are apparently associated with an organic nucleator consisting of phospholipids which may be derived from damaged cell membranes.[27–30] Amorphous Ca deposits, which are believed to represent the first stage of Ca precipitation, remain stabilized in that form in the presence of physiologic levels of Mg and ATP.[31–33] Even levels of Mg found in necrotic cells are commonly adequate to sustain the amorphous form of Ca phosphate. Crystalline Ca deposits form as a result of transformation of the amorphous deposits when stabilizing factors such as Mg are at a low level. In the current study, the consistent presence of intracellular crystalline Ca deposits might be explained by reduced levels of Mg in the environment of the forming mineral deposits. This conclusion is not based on actual knowledge of cytosolic Mg levels. However, Ca deposits are usually amorphous, but those found in this study were crystalline, an observation consistent with a low cytosolic Mg level. This crystallinity thus buttresses the hypothesis given later that attempts to explain why patients in the Washington, D.C.

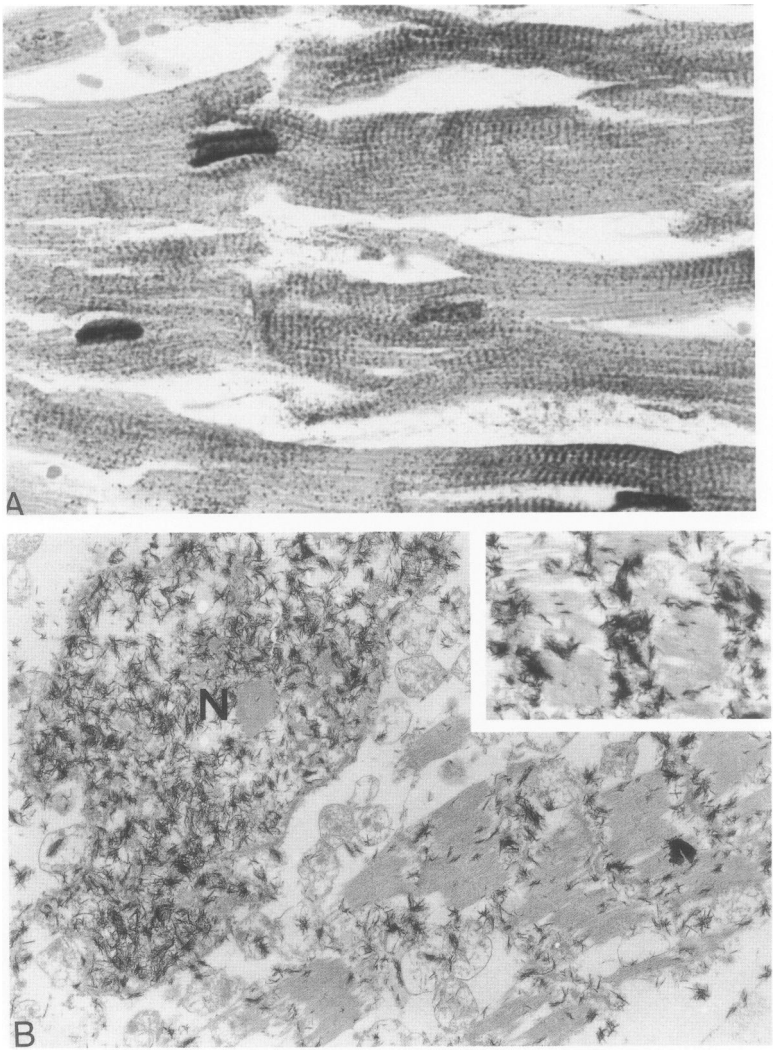

Figure 4. A: *Light micrograph of myocytes with visible nuclei and sarcomeres with heavy Ca deposits in Z band regions and nuclei, and finer Ca deposits scattered throughout the sarcoplasm, von Kossa and H&E, ×320.* **B:** *Electron micrograph of myocyte, showing heavy crystalline Ca in Z band regions and associated with nuclear chromatin, and fine crystalline Ca deposits in some mitochondria and between myofilaments, ×11,000. Inset-heavy, crystalline Ca deposits in Z band regions, uranyl acetate and lead citrate, ×11,000.*

area are more likely to form AMI-associated myocyte calcification than are patients in the Salt Lake City, Utah area.

The viability of cells that contain deposits of hydroxyapatite is something that can not be answered with certainty by morphologic methods. However, it is unlikely that myocytes with Ca deposits free in their cytoplasm, or associated with the Z bands and nuclei, could be viable. Since movement of large amounts of Ca into cells that are injured by ischemia probably requires reflow (i.e., a continuing source of Ca), and since reflow is associated with rapid morphologic deterioration,[34] the present observations are difficult to explain. If we accept the conclusion that such myocytes are not viable, then we must conclude that persistence of an apparently intact nucleus and a regular sarcomere pattern do not establish viability. Since disappearance of nuclei and disruption of the sarcomere pattern are widely used criteria for myocyte necrosis, the present observations seem to indicate an unusual circumstance. The presence of hydroxyapatite crystals within the nuclei and associated with Z bands may prevent dissolution of these structures.

The last of the three questions, that of why AMI-associated myocyte calcification should be found more commonly in patients in the Washington, D.C. area than in patients in the Salt Lake City, Utah area is also not answerable with the data that were obtained in this investigation. However, the current studies, by confirming that the mineral deposits noted by Bloom and Peric-Golia[1] are hydroxyapatite, lend some support to the hypothesis presented earlier that Ca deposition may have occurred because of increased Ca loading during life. The occurrence of myocardial Ca loading in Mg-deficient animals has been described[2,35] and the lower Mg content of the drinking water in the area of Washington, D.C. than in Salt Lake City, has also been noted.[1] Furthermore, the occurrence of the Ca phosphate deposits in the form of hydroxyapatite specifically supports the notion of reduced

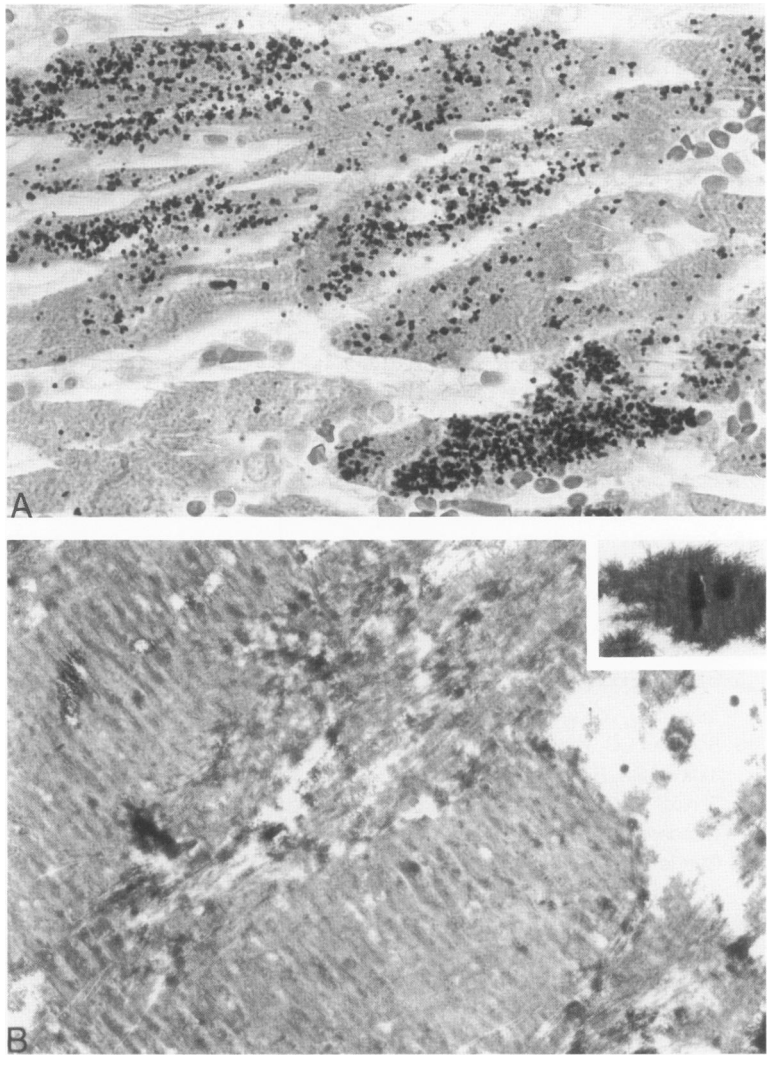

Figure 5. A: *Light micrograph of necrotic myocytes with large flocculent Ca deposits, von Kossa and H&E, ×320.* **B:** *Micrograph of necrotic myocyte with massive crystalline Ca deposits, ×8,000. Inset-massive Ca deposits with dense central areas composed of compact aggregates of crystals and more loosely arranged crystals at the periphery, uranyl acetate and lead citrate, ×11,000.*

Mg in the cytosol during the time of myocyte injury. Presumably, the cytosolic Mg varies with the serum Mg in injured (leaky) cells, and it is known that serum Mg is a reflection of dietary intake,[8,33,35] and that drinking water is an important source of dietary Mg.[6,9,11,36] If the aforementioned is true, the mean serum Mg and myocardial Ca should be lower and higher, respectively, in the Washington, D.C. area than in the Salt Lake City area. These differences would probably be slight, but in any event, the question remains untested.

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