THE DISTRIBUTION OF BONE SIALOPROTEIN IN BONE MINERALIZATION

THESIS

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Master of Science

By

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ABSTRACT

Bone consists of an organic matrix and hydroxyapatite (HA) crystals. The mechanism by which bone mineralizes the organic matrix is not clearly understood. Collagen provides the framework of bone and may even play a role in the nucleation of the HA crystals through the hole zones within collagen fibers. The hole zones are created by the alternating arrangement of the individual collagen fibrils. Phosphoproteins that are only found in bone are thought to play an important role in the nucleation and organization of the crystals. Bone sialoprotein (BSP) is a bone matrix protein that possesses an Arg-Gly-Asp (RGD) cell attachment region and three polyglutamic acid regions. These two regions of the protein are thought to have an active role in bone mineralization. The RGD region has been shown to bind integrins on the surface of osteoblasts. The negative charge of the glutamic acid regions can bind to HA crystals and also may attract calcium ions that are incorporated into the HA crystals. BSP is heavily phosphorylated through associations between BSP's serine and threonine content and inorganic phosphate ions. These phosphate ions may be available for use in the mineralization process. It is thought that BSP is aiding in the nucleation of the crystals. In the literature, there exist little histological characterizations of this protein in mature bone. The organization of BSP within already ossified bone may help to ascertain its function. Fish, chicken and pork bone were chosen for their differences in their organization of mineralized bone and growth patterns. Immunohistological sections were made of each bone type to try to identify the organization of the BSP in each. BSP was found to be a component of the organic matrix. Moreover, BSP was more concentrated in areas where there was active mineralization.

INTRODUCTION

Mineralized tissues are necessarily a composite material consisting of inorganic crystals within an organic matrix (1). The necessity of having an organic matrix is well understood in terms of providing a nucleating site and concentrations of both cations and anions at supersaturation levels to initiate and maintain mineralization. Thus it has been well documented in invertebrates that the organic matrix plays a key role in the mineralization process (2). Calcium and magnesium are supplied either by specified tissues such as the mantle or hypodermis or from the environment via seawater. The matrix acts via its negatively charged regions to attract and thus concentrate the cations. Anions maybe supplied via the action of an enzyme mediated process (carbonic anhydrase located on the matrix) or via cell or pseudopod secretions. In this way, a site directed mineralization process can be assured with localized crystal formation.

Bone originates in the Ordovician Period among the ostracoderm agnathan fishes (3). Early records indicate that calcium carbonate skeletons were forming at least 4-500 million years earlier. There are two types of bone: dermal and endochondral. This designation is based upon how bone develops. Dermal bone forms directly within a membrane via cellular deposition of cations and anions. Endochondral bone is preformed in cartilage, and then is replaced by bone. The manner in which bone grows determines whether high levels of enzyme activity are constantly occurring and whether nucleating proteins are continuously laid down. In adult mammals, only re-working of existing bone or thickening of the bone occurs throughout life. In reptiles and birds, bone may continue to grow at the ends where cartilage is replaced by bone. Thus, in these articular areas, one would expect to find active cartilage-bone transitions. Such areas may be a prime target

for immunohistological analysis of proteins that may be involved in bone formation. Fish bone is a special case because dermal fish bone is acellular (3). This condition lends itself to immunohistological preparation because only exported proteins can be found in the acellular bone matrix.

In bone, the mineral crystals are hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$] (HA) and the organic matrix consists of primarily type I collagen. Mineralized bone is characterized by the presence of HA crystals in the "hole zones" of the collagen fibrils and the orientation of the crystals parallel to the fibril axis (4). It has been theorized that the nucleation of the HA crystals is aided by an anionic phosphoprotein that is bound to type I collagen fibrils (4). For many years much work has been focused on sialic acid-rich proteins which appear to only be found in relatively large quantities in bone (5). One of these proteins is bone sialoprotein.

Bone sialoprotein (BSP) is a 70,000-80,000 kDa protein found in mineralized bone (6). This protein was first described by Herring in the 1960's (5). BSP is a phosphorylated protein of about 300 amino acid residues, and in developing bone, is ~50% protein with the remainder of the weight being carbohydrates (12% sialic acid) (6). The sequence for BSP consists of a series of sulfated tyrosines flanking a Arg-Gly-Asp (RGD) cell attachment site (6). There are also three polyglutamic acid regions that are thought to aid in the HA-binding capabilities of the protein by way of its negative charge at physiological pH. Bone sialoprotein has been shown to be produced by osteoblasts (8), developing osteocytes, osteoclasts, hypertrophic chondrocytes (9), odontoblasts, and cementoblasts (10).

The first attempt at a true function of BSP was to examine its function as an integrin binding protein. BSP is thought to bind to integrins due to the RGD sequence. The RGD zone has affinity for the vitronectin cell receptor (11). BSP has also been shown to have affinity for collagen in vitro (12). The possibility of BSP being a cell adhesion molecule between cells and collagen exists. Various mammalian cells bind to BSP. This adhesion event is generally regarded to occur by way of the RGD fragment to cell surface integrins (13). Mintz and coworkers have isolated fragments that do not contain the RGD tripeptide and still facilitated cell adhesion. This suggests that other amino acid fragments or post-translational modifications may also play an integral role in BSP (13).

The affinity of BSP for HA crystals has long been considered to be an indication that BSP is directly involved with HA crystal formation or growth and, therefore, mineralization. It has been demonstrated in vitro that there is an upregulation (140 fold) of BSP in osteoblasts just before a wave of mineralization occurs (14). BSP can nucleate HA crystal formation without cells in a cell free agarose gel system (15). This ability to initiate nucleation adds further evidence that BSP is more than just a cell adhesion molecule and may play an important role in the mineralization of bone. BSP also has been shown to regulate the osteblastic phenotype of bone cells (16). A possible function of BSP is that BSP is exported to the cell surface in a complex that may involve integrins; and upon release it orchestrates the initiation of crystal nucleation and therefore, mineralization.

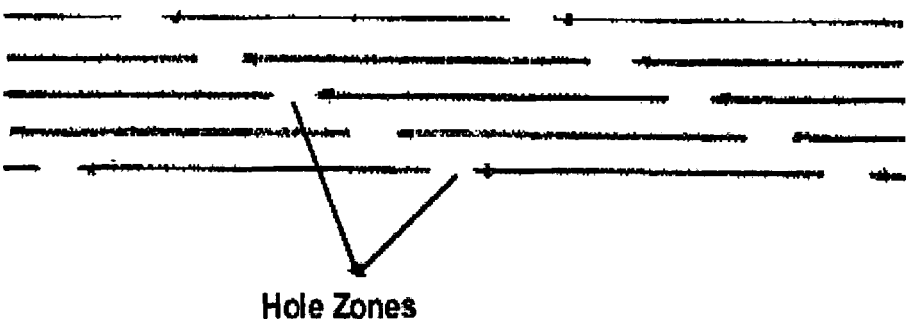


Figure 1: Collagen fibril arrangement and holes zones (Miller 1984)

To understand the possible point of action of BSP one must have an understanding of both bone mineralization and the organic matrix. Type 1 collagen is arranged in an alternating pattern that leaves small regularly spaced gaps (holes). These "hole zones" have been shown to have the ability to nucleate HA crystal formation. In one experiment, native type collagen fibrils were obtained from the skin of rats, rabbits, and guinea pigs, these fibrils were purified and reconstituted into native type fibrils, unstructured fibrils and fibrous long spacing fibrils which were then placed (intraperitoneally and subcutaneously) back into the animals (17). Deposition of apatite crystals occurred within the hole zone regions of only the native type collagen fibrils.

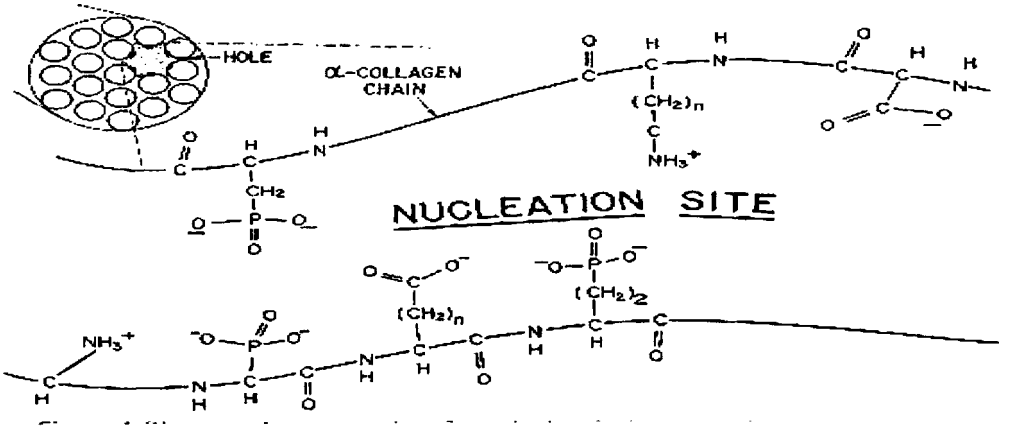


Figure 2: Possible sight of nucleation within collagen fibril (Glimcher 1984)

There is strong evidence to support the hypothesis that the native type aggregation of the collagen macromolecules is necessary for the nucleation of HA crystals within the hole zones for the fibrils (4). It is not known if the collagen molecules are sufficient biologically to facilitate mineralization in a biological system. The native collagen, without any aid from other components may not be able to mineralize rapidly enough in functioning tissue. (4) So even if extracellular conditions are adequate and stable, other factors that are in close relation to the collagen, may play an important role to constitute the necessary biologically sufficient conditions for HA crystal nucleation. Phosphoproteins are a possible addition to the equation to aid in HA nucleation.

Phosphoproteins were first isolated and purified in the 1970's from chicken and bovine bone (18). The partially purified proteins were shown to both contain O-phosphoserine and O-phosphothreonine. These two phosphate-associated amino acids accounted for 100% of the organic phosphate content of the proteins. The two phosphoproteins identified were characterized by their high levels of aspartic and glutamic acid composition which accounted for roughly 40% of the total amino acid composition (19).

BSP has been found to be consistent in its composition with the glutamic and aspartic acid composition being 165.8 and 204.3 (mol of residue/ 1000 mol of residue) respectively (20). Threonine and serine levels were also found to be in abundance at 83.5 and 95.3 (mol of residue/ 1000 mol of residue). Carboxylic acid side chain groups and organic phosphate groups Ser(P) and Thr(P) account for ~50% of the total amino acid residue content of the protein. This provides an abundance of calcium ion binding sites.

BSP is the strongest apatite binding protein known (15). The ability for a protein or molecule to bind ions can prove to be more detrimental than beneficial if the ions are bound in a way that makes them unavailable for use. The ion must be bound in an electrochemically or stereochemically way to facilitate the use of the ion. For example EDTA strongly binds ions and will prevent crystal growth; it will dissolve calcium based crystals such as HA crystals.

To determine whether calcium ions bound to the Ser(P) groups of the phoshphoproteins were capable of further reaction with inorganic orthophosphate ions, the phosphoprotein was titrated with calcium chloride in the presence of inorganic orthophoshate ions and examined by ^{31}P n.m.r (21). In the absence of $CaCl_2$ the addition of inorganic orthophosphate ions causes neither a shift nor a broadening of the phosphomonoester peak. When increasing amounts of $CaCl_2$ were added to a solution containing both the phosphoprotein and inorganic orthophosphate ions, the phosphomonoester peak and the inorganic orthophosphate peak were both markedly broadened. This indicates that ternary complexes are formed between protein phosphomonoester groups, the ions and the inorganic orthophosphate ions. It also demonstrates that the phosphoproteins possess the physiochemical properties necessary for them to participate in the nucleation of a Ca-P solid phase from solution (21).

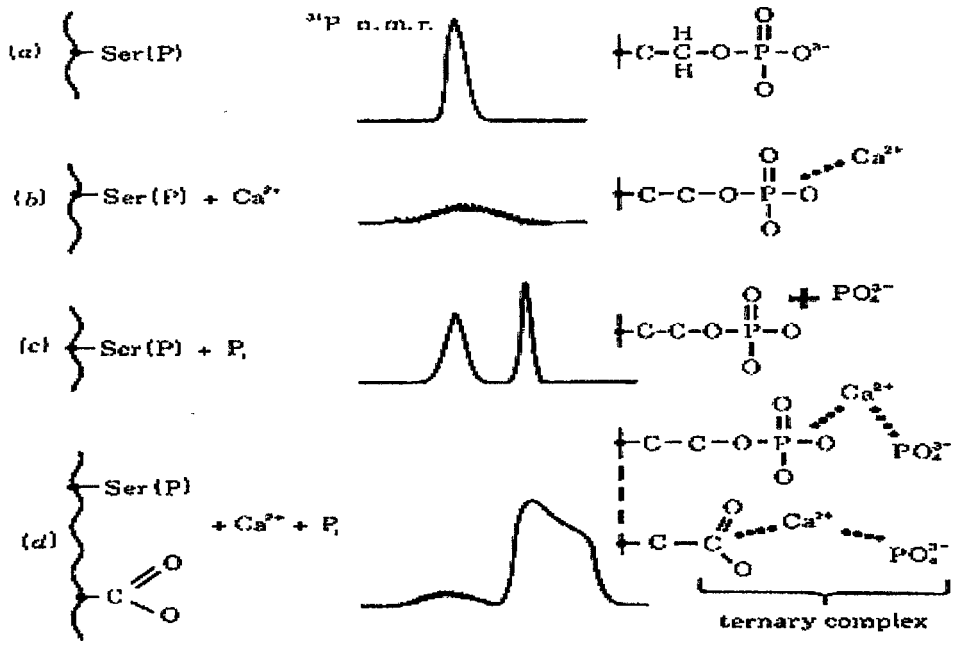


Figure 3: Graph of N.M.R. results showing availability of ions (Lee and coworkers, 1983)

From the physiological , biochemical, structural and physiochemical points of view, an organic component that could play a significant and beneficial role in the mineral phase formation should ideally meet four criteria: it should be synthesized by the cells of the tissue and probably by those cells that synthesize the major structural components within which the mineral is deposited; it should be located ultra structurally at the sites where mineralization occurs; it should interact with the appropriate mineral ions constituting the inorganic crystals; and bind the ions in such a way that the bound ion remains active and can further interact with other inorganic ions to eventually form the inorganic crystals (4).

BSP has been shown in previous research to fulfill these properties and is one of the leading candidates for the initiation of nucleation and mineralization. It is interesting to note that much recent research has been conducted into the apparent rise of BSP in

blood serum levels of breast cancer patients that have metastasized to the bone or are likely to have bone metastases. These new findings also support the current hypothesis that BSP is playing an active role in bone deposition and should be one of the leading researched phosphoproteins if mineralization is to be understood.

In my research of the literature I have found little histological preparations of mature bone, with the identification and characterization of the organization of BSP within mature bone. All of the sections I have come across have been in fetal bone and the concentration of the BSP has been around the osteoblasts. This is not a fair representation of the organization of BSP, in that the bone is not yet mineralized and would not have BSP present except in direct association with cells if BSP is playing an active role in the deposition of bone.

In this research I will use histological sections of pork, avian, and fish bone to examine the role that BSP may play in the mineralization of each. By studying the different ways the bone is organized and the different organization of the BSP, I hope to gain insight into its possible function.

MATERIALS

The materials used in this study are as follows for the individual protocols. For the ELISA's that were performed, bovine serum albumin (BSA) Sigma B-4287 was used as a blocking agent. Chemicon AB1854 rabbit anti-BSP primary antibodies and Sigma A-3687 anti-rabbit alkaline phosphatase conjugate secondary antibodies were used in the assay. The substrate used was Sigma N-2765 p-nitrophenyl phosphate tablets. The plate was read with an EL311 Bio-Tek microplate reader.

The immunohistological sections were sectioned on a Microm HM 505 N cryotome. BSA was used as above as a blocking agent and the previously mentioned anti-BSP were used as primaries. The Sigma QUIK-3 Rabbit/ Mouse Rapid Staining Kit was used for development of the primary antibody location.

The procedure followed for the Western Blot was taken from *Protein Methods* by Bollog and Edestein. The same primary and secondary antibodies were used as in the ELISA and the sections. Development of the blot was by way of Sigma B-1911 BCIP/NBT liquid substrate system.

METHODS

ELISA (Enzyme Linked Immunosorbency Assay)

Beef, pork and rainbow trout were obtained from a grocery store. The soft tissue was cleaned off a beef rib, the rib of a pig and the jaw bone of a rainbow trout. The bones were then cut into four pieces, each approximately 1 cm^2. Grooves were cut into the pieces to expose more surface area. The pieces were then placed in a 12-well titer plate that had been blocked with 5% BSA for 2 hours. Each piece was washed vigorously three times with PBS-T (phosphate buffered saline with 0.05% tween 20) to clean the specimens. A 1:2500 dilution (PBS-T) of rabbit anti-BSP (Chemicon AB 1854) was then added to each well containing bone fragments except for one well for each that served as a negative control. The samples were then rocked on a rocker at room temperature for 2-3 h. The pieces were then washed 3X with PBS-T and transferred to another blocked titer plate that contained alkaline phosphatase-linked goat anti-rabbit antibodies (1:1000). This was rocked and incubated at room temperature for 2-3 h. After incubation, the specimens were washed vigorously 3X as above and 2 ml p-nitrophenyl phosphate substrate (Sigma N-2765) was added to each well. After 10, 15, 20 and 25 minutes 100 µl of the substrate was taken out of each well and put into a 96 well flat bottom microtiter plate and read in a plate reader at 405 nm. The absorbance readings were then recorded.

The above procedure was changed to account for endogenous alkaline phosphotases by the addition of levamisole to the final washes and the substrate solution.

Paraffin Embedded Immunohistological slide preparation:

-Embedding

Pork, avian and fish bone were decalcified in 10% formic acid for 2 days and then fixed in Bouins solution for 24 hours. The tissue samples were then washed with water and run through a dehydration series consisting of 50%, 70%, 95% and absolute ethanol for 30-45 minutes each. The samples were then placed in two consecutive xylene soaks for 30 min. each. Liquid paraffin prepared to 58°C and the tissues were placed into two consecutive paraffin dips for 45 min. each. The tissues were then placed in a mold and the paraffin was allowed to solidify. The blocks were then trimmed and cut at 5-10 μm in thickness. The sections were placed on a slide that was prepared with egg albumin (for section adhesion) and placed on a warming tray at 50°C overnight.

-Immunolabeling

The slides were deparaffinized in xylene for 4-5 min. and rehydrated in an ethanol series that consisted of absolute, 95% and 75% each for 2-3 min. A final wash in water for 2-3 min complered the hydration series. The slides were then stained using the Sigma QUIK-3 Rabbit/Mouse Rapid Staining Kit (protocol as follows). Endogenous peroxidases were quenched using 2 drops of 3% hydrogen peroxide for 5 minutes. The slides were washed with PBS-T 3X. The slides were then incubated with 1° rabbit antiBSP antibody 1:2500 in PBS 1% BSA at 4C° overnight. The slides were then washed as above and incubated with biotinylated secondary antibody from the kit for 5 min. Slides were washed as above and 2 drops of peroxidase reagent were added and

incubated for 5 min. Slides were washed and substrate was prepared from kit and 2 drops were added to each section. After 3 min. the sections were washed and mounted with glycerol mounting medium.

Cryotome Immunohistological Preparation

Pork, chicken, and rainbow trout were obtained from local grocery store and their bone was removed and cleaned as above. Approximately 1 cm pieces were decalcified in formic acid (pH 3.5-4.0) for 2-3 days. The bone fragments were then soaked in deionized water for 5 min and frozen in the cryotome at -30°C for 1 h. The specimens were sectioned at 5-10 μm and placed on cover slips that had been coated with a .5% gelatin solution. After the sections were allowed to sit overnight, they were then stained with the QUIK-3 Rabbit/Mouse Rapid Staining Kit (Sigma). Endogenous peroxidases were quenched with 2 drops hydrogen peroxide for 5 minutes. The coverslips were washed in PBS-T 3x for 5 min. each wash and incubated overnight at 4°C in the primary antibone sialoprotein made in a rabbit antibody diluted 1:2500 in PBS-T with 1% bovine serum albumin. The sections were then washed 3X as before and the secondary biotinylated antibody (QUIK-3 Kit) was added and incubated for 2 hours at room temperature. The sections were washed as before and 2 drops of peroxidase reagent (QUIK-3 Kit) were added and allowed to incubate at room temperature for 5 min. The sections were washed and the substrate (prepared from the kit) was added. The sections were then incubated for 3 min, rinsed with deionized water and mounted with a glycerol mounting media.

Western Blot

Rainbow trout, chicken femur, and pork rib were cleaned to remove all visible flesh and ground in a blender in formic acid (pH 3.5-4) until it became a slurry. Tissues were then allowed to incubate for two days and centrifuged overnight at 3000 X g. The lipids at the top were poured off and the remaining supernatant was retained. The supernatant was then dialyzed against tris buffer overnight. The samples were then mixed with 25 µl of NuPAGE sample buffer in the following volumes: chicken 20 µl, 5 µl; fish 30 µl, 10 µl; pork 35µl, 15µl. Water was then added to make up volumes to 90 µl. The samples were then incubated at 70°C for 10 minutes, spun to collect all fluid into the bottom of the containers, and 30 µl of each sample were loaded into wells of a NuPAGE gel and NuPAGE Running buffer was added to the chambers. The gel was run at 200 V for approx. 30 min. The gel was removed from plastic cartridge and placed in a "sandwich against nitrocellulose paper. The sandwich was then placed in an electrophoretic chamber that included frozen transfer buffer along with liquid transfer buffer and allowed to run at 30 V for 8 h. The nitrocellulose paper was removed and blocked with 2% gelatin in TBS for 3 h. The paper was then washed in TBS 2X for 10 min. each time. The primary rabbit anti-BSP antibodies were diluted 1:5000 into 0.5% gelatin in TBS and added to the nitrocellulose paper. The primary antibodies were incubated for 3 h and then washed as above. The alkaline phosphatase conjugated anti-rabbit secondary antibodies were diluted 1: 30,000 in 0.5% gelatin and TBS and added to the paper. These were incubated for 3 h and the paper was washed 3X for 10 min.

BCIP/NBT substrate was prepared, added, and developed for 45 min. The nitrocellulose paper was then washed with TBS and observed.

RESULTS

The altered ELISA using the bone fragments were performed numerous times using mammal, avian and fish bone. The first ELISA runs were problematic as reactions with the antibodies and substrate occurred in both positive and negative wells. However, the fish samples were the only sections that did not produce equivalent or near equivalent results from the absorbance reading of the negative vs. the positive samples (Fig. 12). This was originally thought to be because of inadequate washes of the more odd-shaped fragments of pork and beef bone. I tested the hypothesis that reactions in the negative controls were the result of endogenous alkaline phosphatases being present in large quantities in mammalian and avain bone samples where bone cells are numerous. In fish bone, cells are absent or have become part of the matrix by cell death (3) thus the amount of endogenous phosphatases would be less than found in corresponding avian or mammalian bone. To inactivate phosphatases, levamisole was added to block endogenous alkaline phosphatases and the negative fragments reduced their absorbance. By trial and error, levels of levamisole were determined to be 5 mg/ml that would maintain an unreactive negative control while allowing reactions with positive samples. In this way endogenous phosphatases were neutralized.

Paraffin sections were made and the results were inconclusive against the negative controls and were not added to this paper. It appears that the decalcification process to the extent necessary for paraffin embedding, as well as, the dehydration and embedding process denatured and leached BSP. This could have resulted in the lack of antibody binding on the positive controls. This led me to use the cryotome to cut sections,

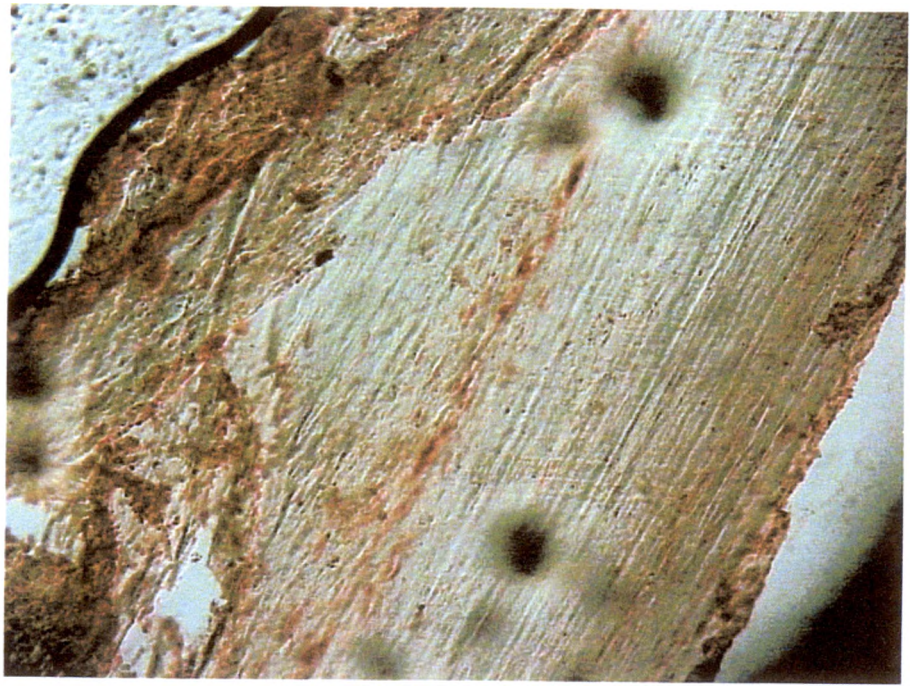
since this apparatus allows cutting of bone samples with less decalcification and no embedding process.

The cryotome sections on different samples of bone were examined under a compound microscope and resulted in findings presented below for both positive staining and. negative controls. In the fish and chicken sections, staining was more concentrated at the edges of the bone sections. In a fish sample, heavy dense staining was evident in one slide along what looks to be a line of mineralization (Fig. 6). The pork sections had some dense staining at the edges but also had more stain throughout the bone matrix (Fig. 7). The chicken bone samples where the bone cartilage interface was located, showed positive staining in the surface mineralized portions, but the underlying cartilage showed little if any staining especially in the deeper cartilage (Figs. 9, 10, 11).

The Western Blot showed a band within both the chicken and pork lanes. The fish lanes were negative, most likely due to a low concentration of protein. Spectrophotometer analyses of the samples support this, in that the fish sample was the lowest in its protein content. A picture of the blot is not included because of the faintness of the bands. Moreover, some background staining made it impossible to see the bands from a scanned figure.

Figure 4: (top) Fish bone stained with anti-bone sialoprotein. (bottom) Negative control with the absence of primary antibodies. 50X

Positive Fish



Negative Fish

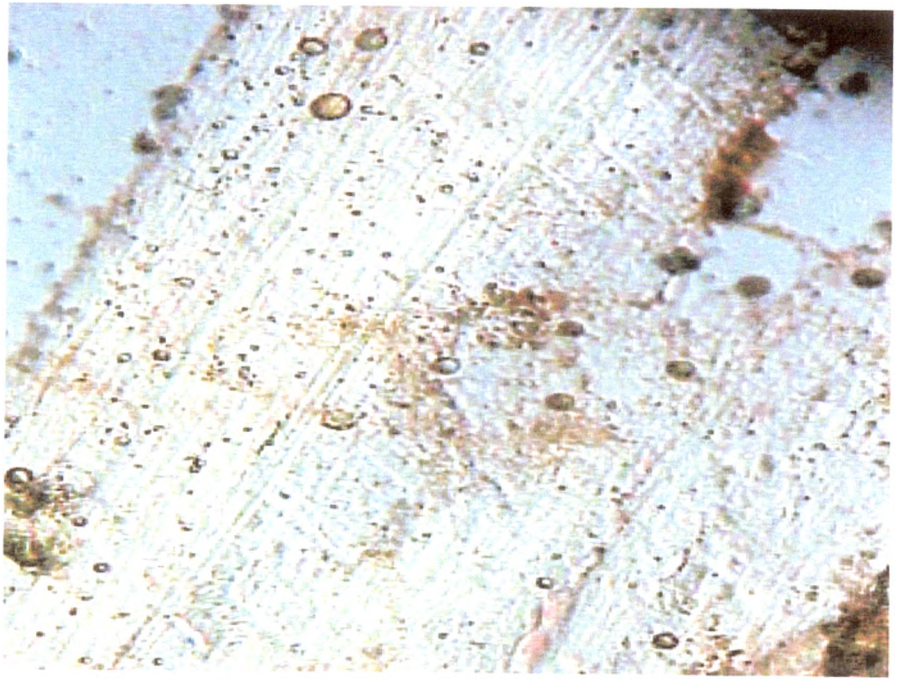


Figure 5: (top) Fish bone stained with BSP antibodies. (bottom) Fish bone without primary antibodies. 100X

Positive Fish

Negative Fish

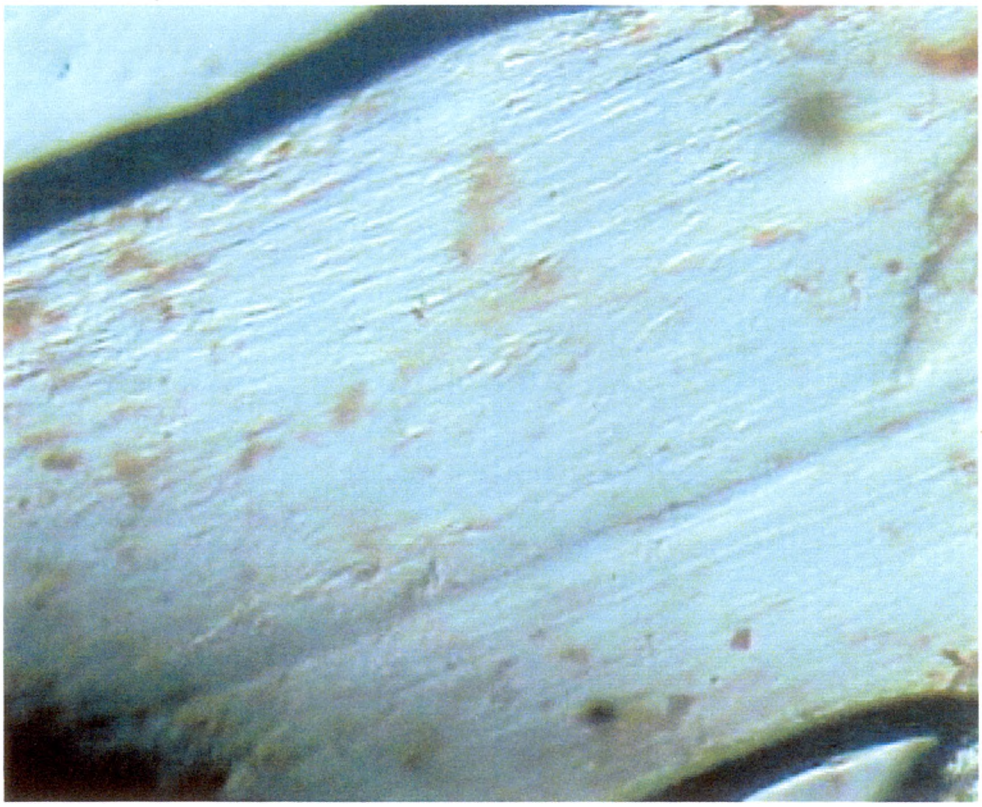
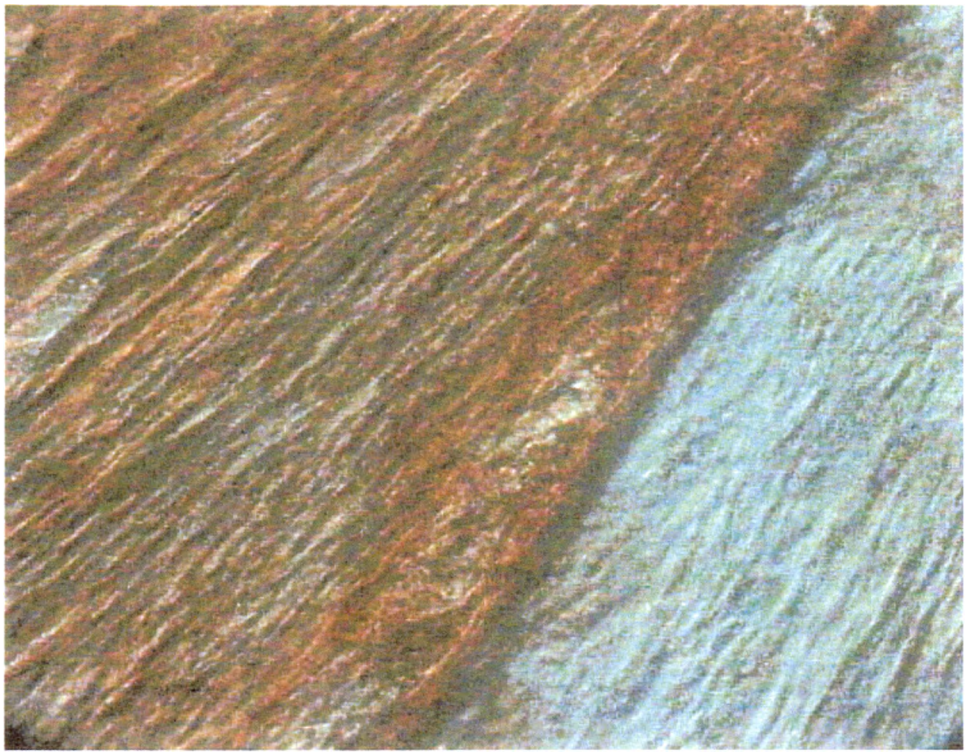


Figure 6: (top) Fish stained with BSP antibodies. (bottom) Fish bone without primary antibody. 400X

Positive Fish



Negative Fish

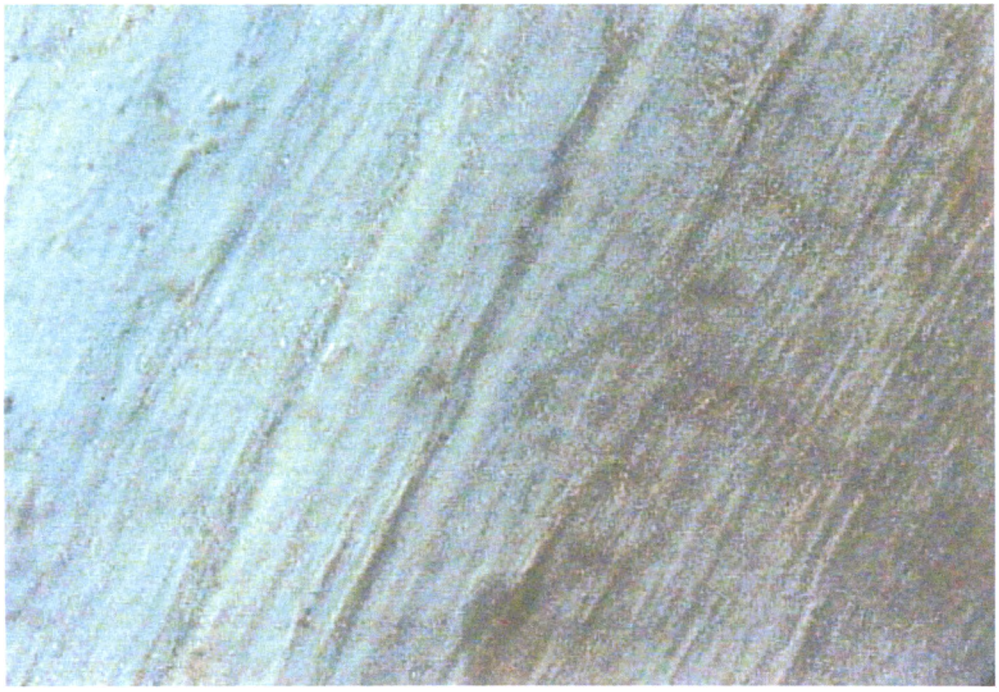
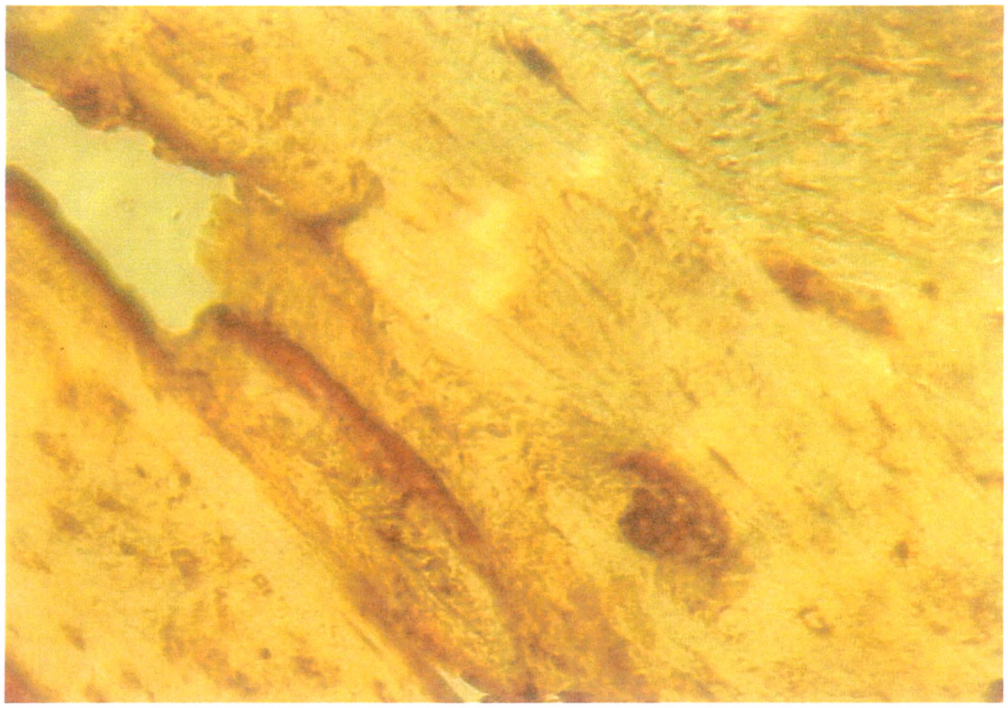


Figure 7: (top) Pork bone stained with anti-BSP antibodies. (bottom) Pork bone without primary antibodies. 100X

Pork Positive



Pork Negative

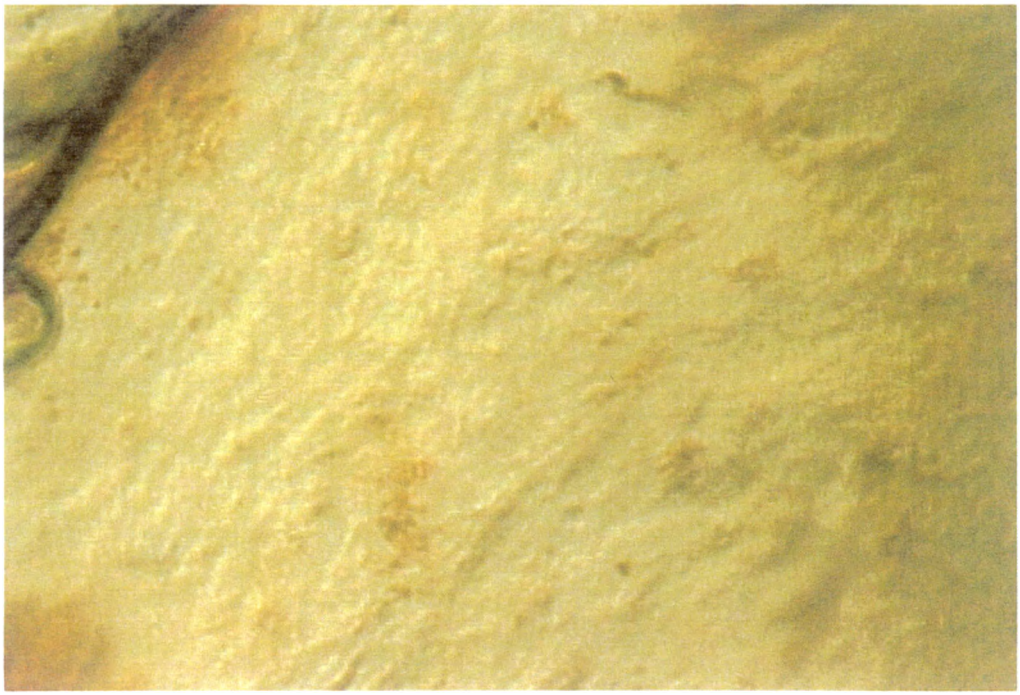
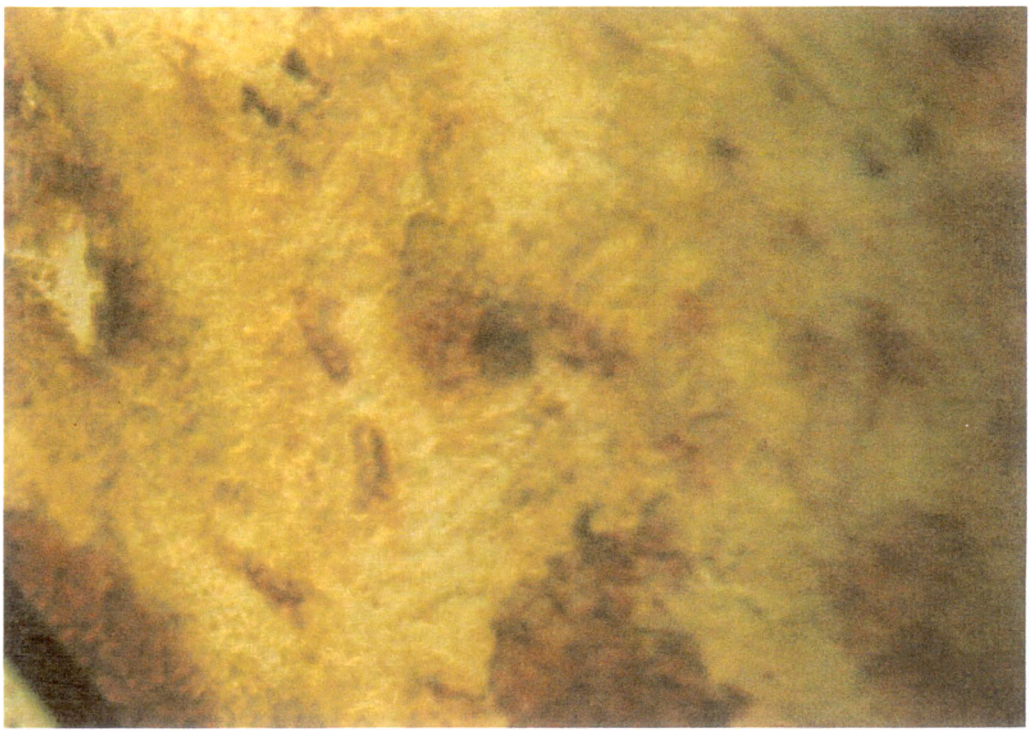


Figure 8: (top) Pork bone with anti-BSP antibodies. (bottom) Pork bone without primary antibodies. 200X

Pork Positive



Pork Negative

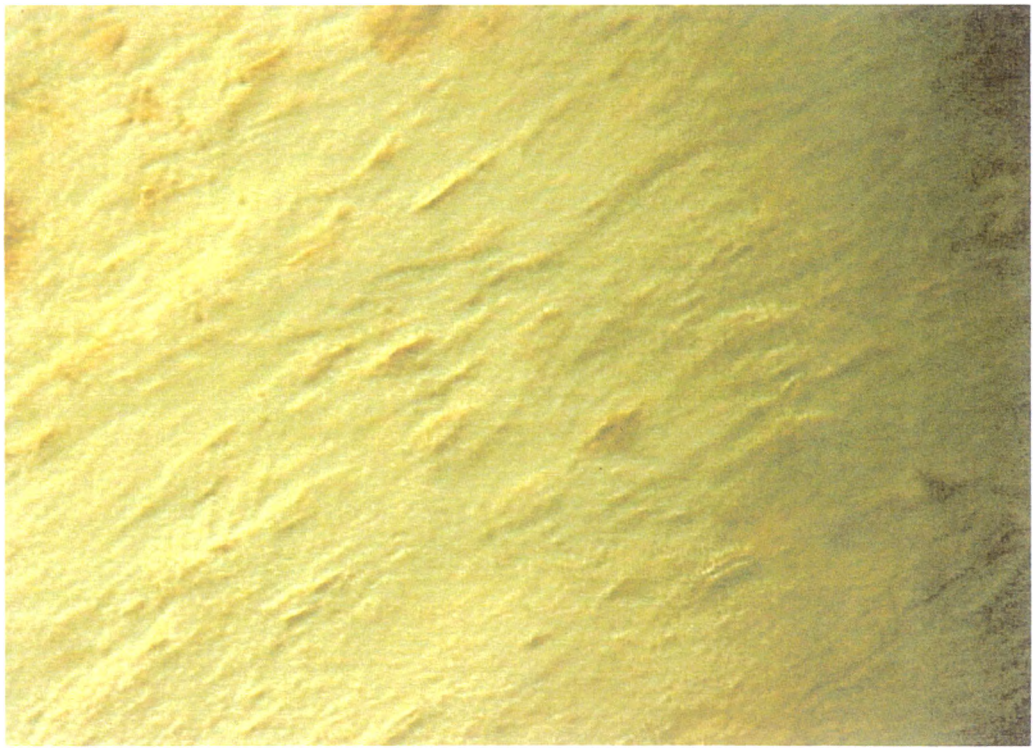
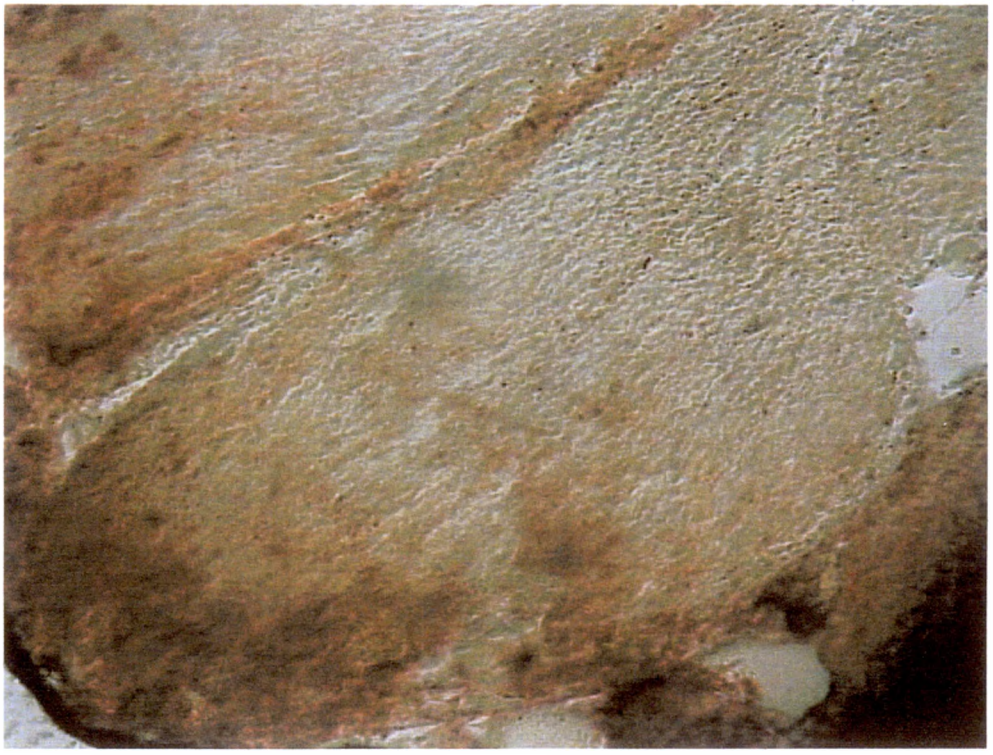


Figure 9: (top) Chicken bone with anti-BSP antibodies. 50X (bottom) Chicken bone without primary antibodies. 100X

Chicken Positive



Chicken Negative

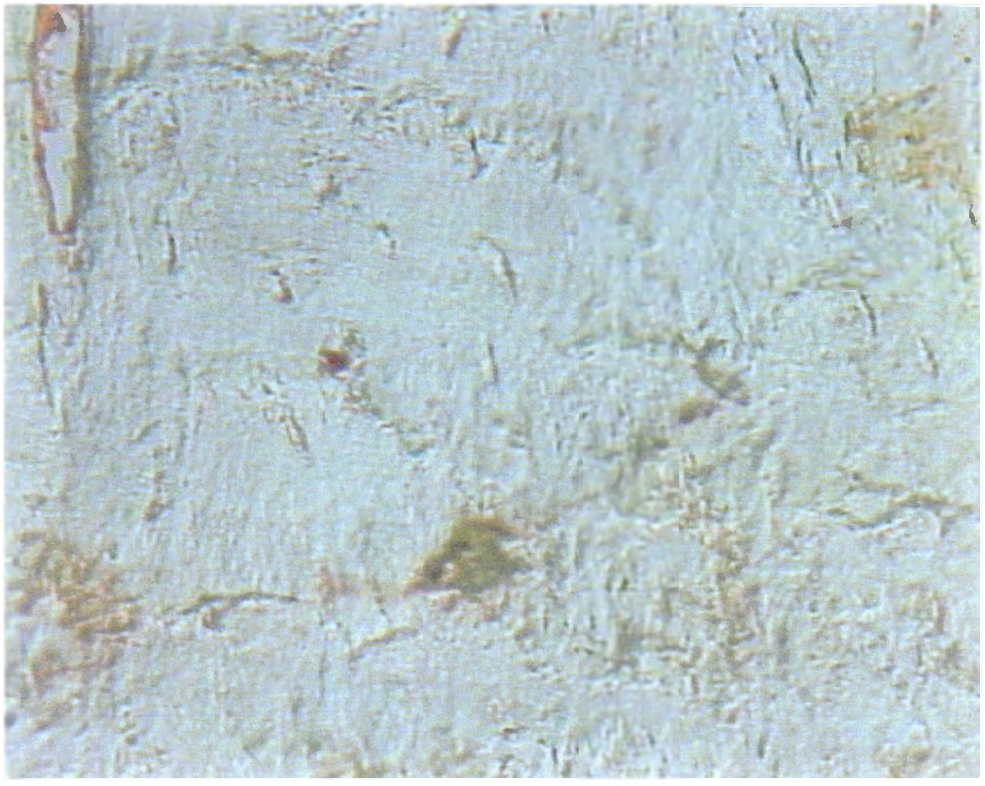
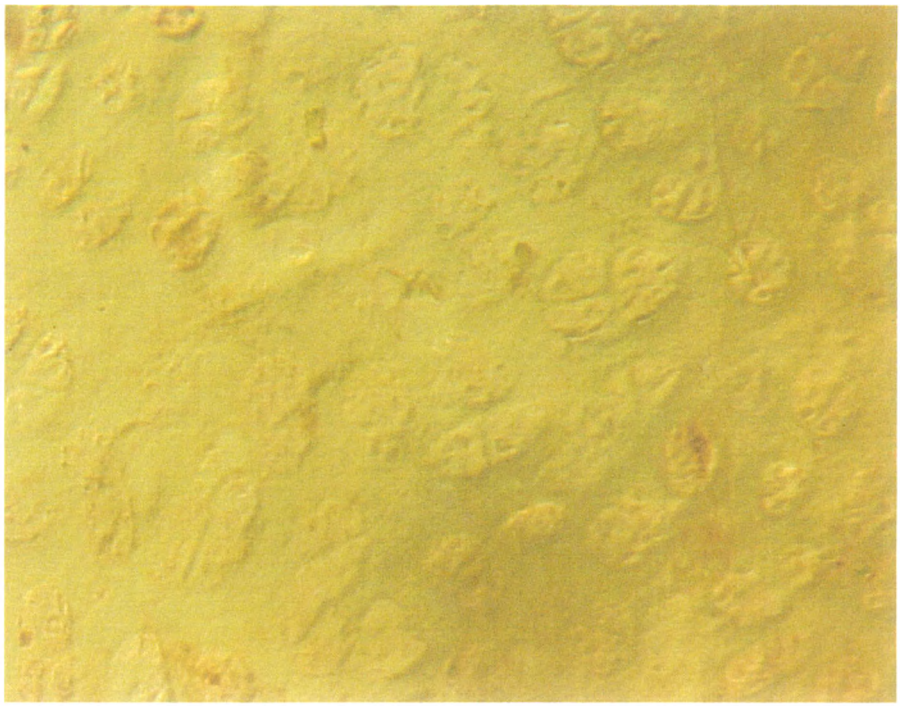


Figure 10: Chicken cartilage stained with anti-BSP antibodies. 100X

Chicken Deep Cartilage Positive



Chicken Surface and Shallow Cartilage (Positive)

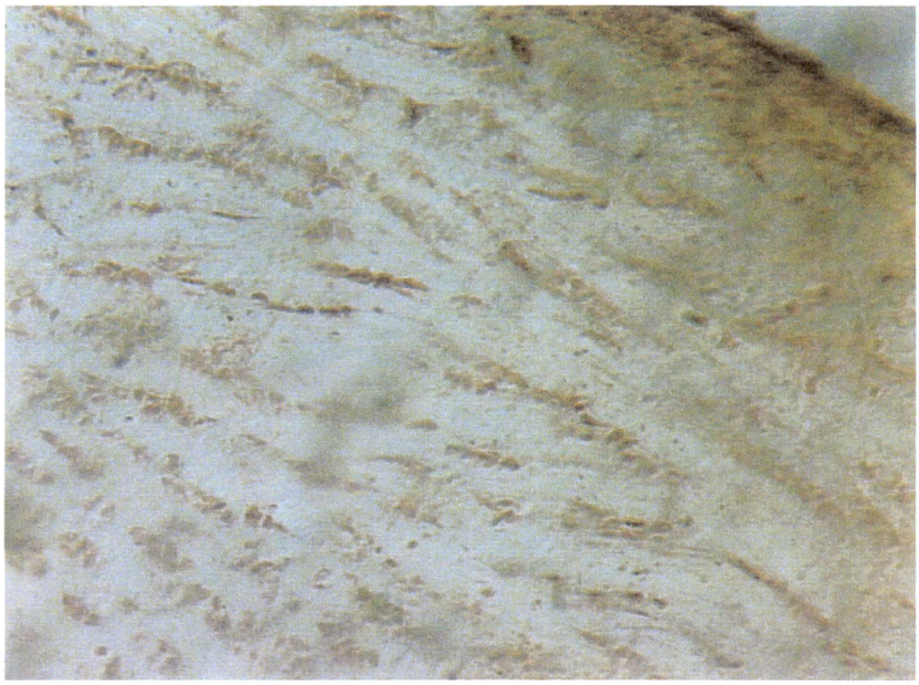


Figure 11: Chicken bone stained with anti-BSP antibody. 100X

Chicken Positive

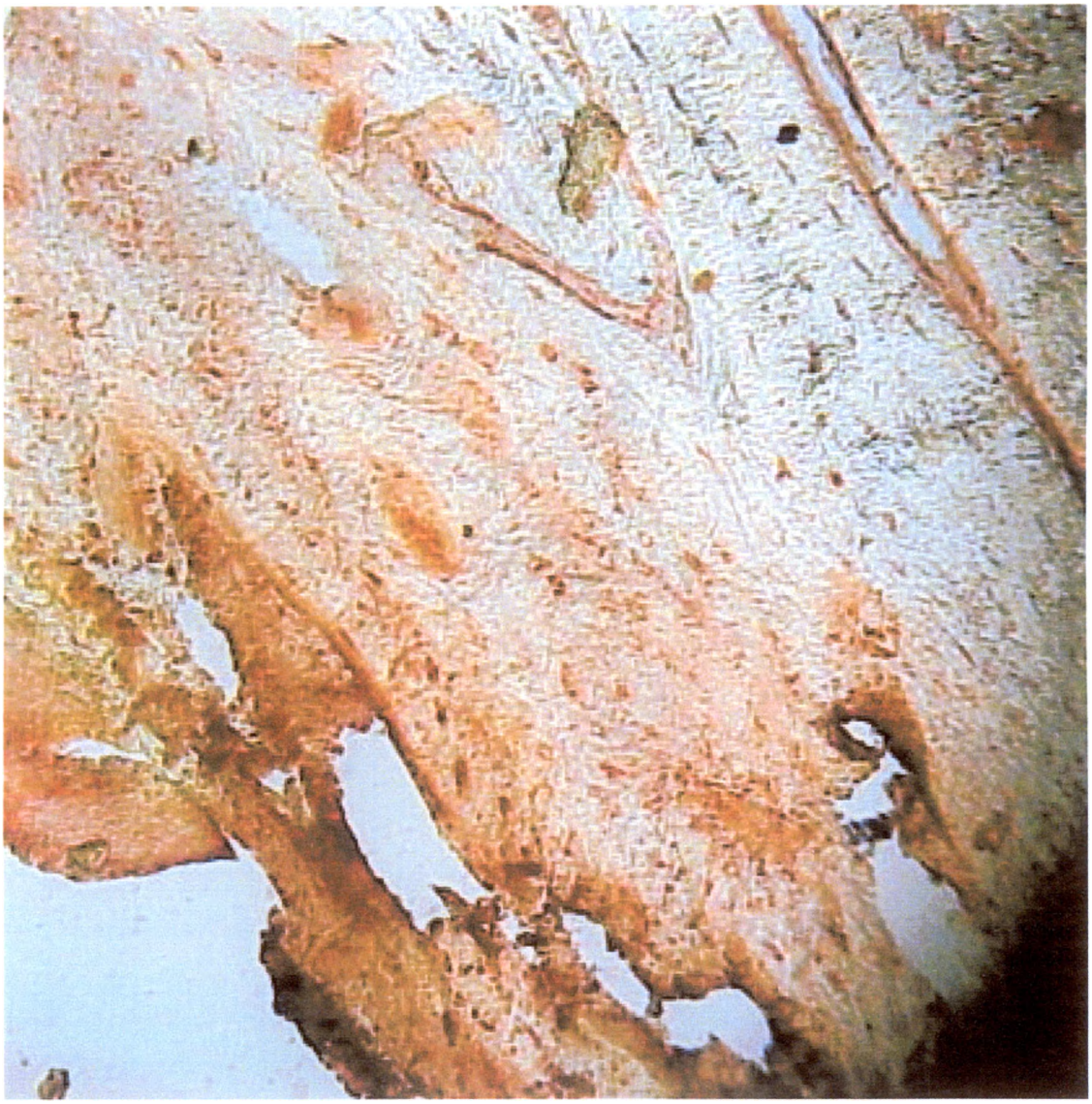
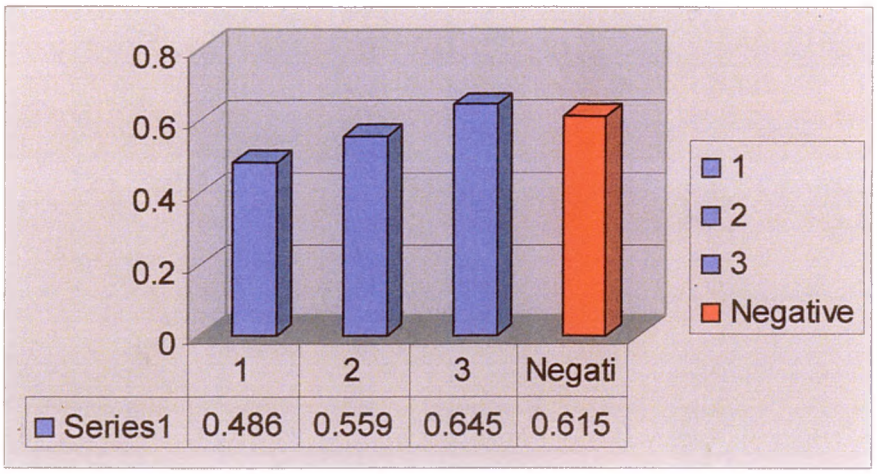
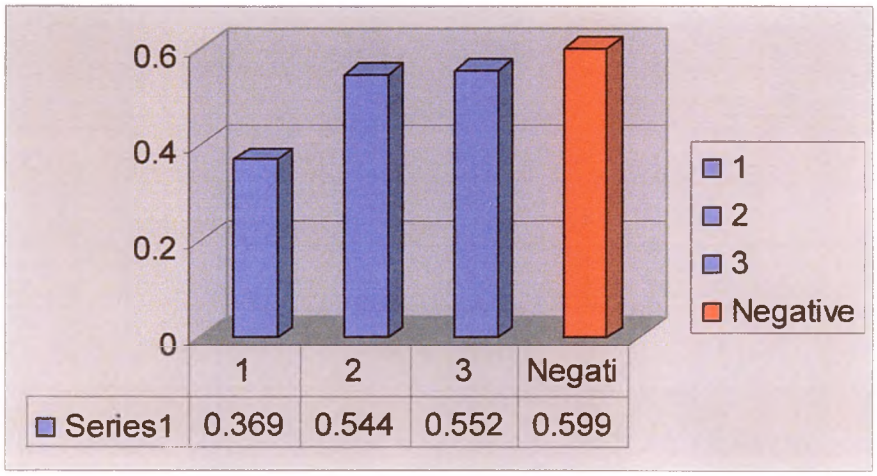


Figure 12: Results of modified ELISA's run on beef (1), pork (2) and fish (3) bone pieces.

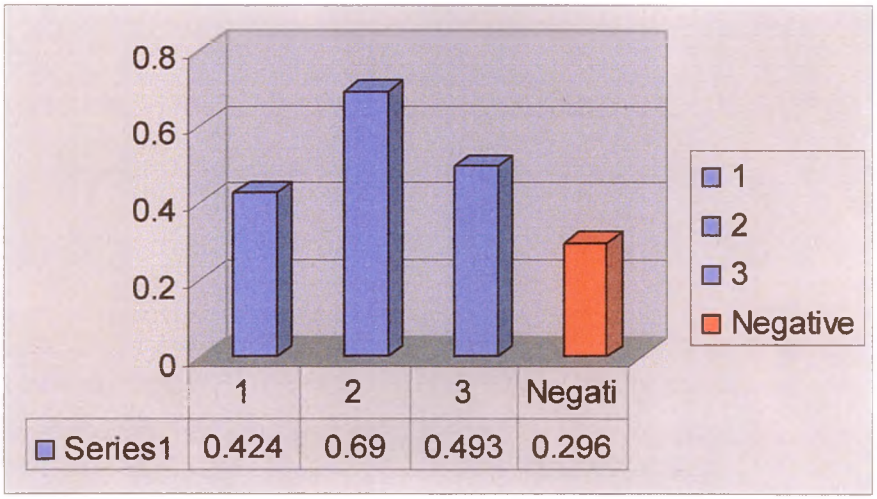
Beef Samples (1)



Pork Samples (2)



Fish Samples (3)



DISCUSSION

Mineralization of tissues appears to occur in specifically defined patterns regardless of the phylum studied. While there may be variations as to how the mineralization is carried a matrix is always involved in the nucleation process. This matrix is usually either chitin or protein or a mixture of the two. The question that is discussed here is how does bone fit the pattern of mineralization seen among the invertebrate and protist phyla. In the invertebrate phyla, a fiber matrix is exported from an epithelial tissue as seen in the Mollusca and Arthropoda. Sponges may make spicules within cells using a fiber matrix as a nucleating site. The spicules are then exported from the cell where further mineralization occurs. Thus, in all cases, crystallization does not proceed unless there is a nucleating fiber matrix present. There are two components of crystal formation: a cation (normally calcium) and an anion (usually carbonate among invertebrates). Calcium can be supplied by the cells involved in mineralization either through environmental uptake or food. The limiting factor in mineralization appears to be the supply of the anion. Among most invertebrate phyla this seems to be either supplied directly by the epithelial tissue (22) or indirectly supplied via the action of carbonic anhydrase acting on carbon dioxide. The earliest skeletons found in the fossil record are either silica or calcium carbonate and arise in the Precambrian. Bone does not evolve until the Ordovician Period, well over two hundred million years later. The reason for this delay remains obscure, but may be due to either energetic requirements of phylogenetic constraints. Along with arthropods, bone has an extremely high organic matrix content reaching percents of over 40% by volume. The role of this matrix has been investigated (4). Proteins found only in and around forming bone raise questions

about their function. These proteins (i.e. BSP) may be used as binding proteins, essentially hold cells to the matrix while calcium and phosphates were dumped, or were more directly involved in the mineralization process by either acting as nucleating sites or enzymes that would supply the phosphate anions.

BSP has been shown to possibly have both cell attachment and phosphate donation capabilities. BSP may nucleate crystal formation through the sequestering of Calcium ions via its polyglutamic regions and the carrying of available phosphate groups through the O-phospherine serine and threonine content. The nucleation of crystals along the collagen matrix with a specific orientation would promote crystal growth in the same organized and directional manner. The function of the cell adhesion (RGD) region may stimulate cells to exhibit the osteoblastic phenotype (16) and to pump phosphate and Ca ions for crystal growth once nucleation has been established.

The question that is yet to be answered is why is BSP only seen in actively mineralizing section of bone and not to any large degree in established bone matrix? BSP may be reabsorbed by the cell after nucleation of the crystals, or, given its highly charged regions, it may be soluble in demineralized bone. The solubility problem of BSP is supported by the small amounts of BSP that were seen within the fish bone mineralized matrix. If fish bone is acellular and BSP is reabsorbed by the cells, one would not expect to find BSP within established mineralized fish bone. More evidence indicating the loss of some of the BSP was the positive detection of BSP in the supernatant of the decalcified pig and chicken slurry by the Western Blot. This could account for some of the results or lack of results seen in some of the paraffin sections. The paraffin sections were more extensively decalcified to allow sectioning with a standard microtome. In

addition, the paraffin slides had to be rehydrated before they could be treated with the antibody protocol. This process could have leached the protein out of decalcified areas that were accessible to the antibodies. The cryotome sections did not need to be decalcified to the same degree and were not exposed to the same hydration protocols that may have placed BSP in solution.

BSP was shown to be in high concentrations in the mineralized matrix of the ends of the chicken bone and on the edges of the fish bone specimens. The pork bone had dense staining loci of mineralization throughout the sections. This makes sense from the growth standpoint of the animals. Fish bone grows in a lamellar fashion and would have active mineral deposition on the surface of established bone. Avian bone grows more from the ends and this is where the highest concentration of staining was observed. Mammalian haversian bone is constantly being remodeled and bone is actively reorganized to account for growth and new stresses that are being imposed on the bone. This may account for the organization or lack there of that was observed in the pig bone.

There is little doubt that BSP is playing a role in the deposition of HA crystals. The fact that this protein is conserved through many different classes of $CaPO_4$ skeletoned organisms is further evidence of its involvement in the mineralization equation. The lack of stain in the unossified cartilage of the chicken bone suggests that this is not a protein that is found throughout the matrix of tissues unless there is mineralization.

The histological slides give some insight on the role of BSP in that they show markedly higher concentrations in bone that is being mineralized. The association of BSP with the collagen matrix must be further investigated to make the assumption that

BSP is the phosphoprotein that is aiding in the nucleation of the HA crystals in the mineralization process of bone.

CONCLUSION

The immunohistological sections and the Western bBlot showed evidence of antigen-antibody recognition of BSP in the pork and chicken bone. The fish also had positive results in the histological sections and the ELISA. The fact that this protein seems to be conserved over different classes of organisms with $Ca_3(PO_4)_2$ skeletons adds further evidence to the vital role that BSP is playing in the mineralization of bone. The role of BSP is still not fully identified, but the evidence indicates BSP is playing an active role in calcification.

The histological sections give us some insight in the role BSP is playing because of the different organizations and styles of growth. The antibody staining of the sections was where one would expect to find active mineralization. BSP could possibly be involved in nucleating crystal formation and aiding in the organized deposition of the crystals.

Answering the question of how tissues are mineralized has far reaching consequences. As mentioned before, BSP has been found at relatively high levels in the serum of breast cancer patients that have advanced to bone metastisis (23). Other diseases such as osteoporosis can be impacted by a greater understanding of the building of bone at a molecular level. The problems with space travel and the degradation of bone in 0 g and the repair of fractures to name a few, all could be better understood and possibly answered if bone deposition is understood.

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VITAE

I was born in Honolulu, Hawaii in 1974. My father was military and moved to Abilene, Texas at the age of two. I graduated from Cooper High School in 1993 and attended SWTSU in the fall of 1993. I received my B.S. in biology from SWTSU in 1998 and have attended the graduate school since 1999.

I have been a lab instructor in Histology, Anatomy and Physiology, Virology, Immunology, and General Biology. I have done research under Dr. Tarsitano and Dr. Horne. Under Dr. Tarsitano I have conducted numerous histological studies on the evolution and development of flight feathers along with work on bone mineralization on a NASA JOVE grant. I have also played an active role in Dr. Horne's lab studying the activity of Carbonic Anhydrase in the mineralization of invertebrate exoskeletons.

I have supported my education through work on the Guadalupe River in the summers. I am currently a manager at Gruene River Company in New Braunfels Tx.

I enjoy any outdoor activities especially if they involve water. Fishing and rafting has become my outlet for the stresses school can put on me. Hunting is a big part of my life in the winter when the water is not an option.