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The Size exclusion characteristics of collagen and elastin fibers and role of fetuin in their calcification

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UNIVERSITY OF CALIFORNIA, SAN DIEGO

The Size Exclusion Characteristics of Collagen and Elastin Fibers and the Role of Fetuin in their Calcification.

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Biology by Damon Armen Toroian

Committee in charge:

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Randy Y. Hampton
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2007
The dissertation of Damon Armen Toroian is approved, and it is acceptable in quality and form for publication on microfilm:

Chair

University of California, San Diego

2007
# TABLE OF CONTENTS

Signature Page................................................................................................. iii

Table Of Contents............................................................................................ iv

List Of Symbols................................................................................................ v

List Of Acronyms.............................................................................................. vi

List Of Abbreviations......................................................................................... vii

List Of Figures................................................................................................... ix

List Of Tables.................................................................................................... xi

Acknowledgements............................................................................................ xii

Curriculum Vitae............................................................................................... xiv

Abstract............................................................................................................ xv

Chapter I. Introduction....................................................................................... 1

Chapter II. The Size Exclusion Characteristics of Type I Collagen: Implications for the Role of Non-Collagenous Bone Constituents In Mineralization.................................................................................. 26

Chapter III. The Essential Role of Fetuin in the Serum-Induced Calcification of Collagen........................................................................................................ 65

Chapter IV. The Essential Role of Fetuin in the Serum-Induced Calcification of Elastin Matrices........................................................................................... 94

Chapter V. Discussion......................................................................................... 124
LIST OF SYMBOLS

α  Alpha
%
±  Plus or minus
+  Plus
>  Greater-than
≥  Greater-than or equal to
<  Less-than
≤  Less-than or equal to
=  Equals
~  Approximately
&  And
°  Degrees
×  Multiplication sign
[x]  Concentration of x
©  Copyright
®  Registered
Å  Angstroms
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tbody>
<tr>
<td>BGP</td>
<td>Bone Gla Protein</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetate</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>MGP</td>
<td>Matrix Gla Protein</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular Weight</td>
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<tr>
<td>NBCS</td>
<td>Newborn Calf Serum</td>
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<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
</tr>
<tr>
<td>NRS</td>
<td>Normal Rat Serum</td>
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<td>PAGE</td>
<td>Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>RIA</td>
<td>Radioimmunoassay</td>
</tr>
<tr>
<td>RPM</td>
<td>Revolutions per Minute</td>
</tr>
<tr>
<td>RT</td>
<td>Room Temperature</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission Electron Microscopy</td>
</tr>
<tr>
<td>SD</td>
<td>Standard Deviation</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulfate</td>
</tr>
<tr>
<td>UCSD</td>
<td>University of California San Diego</td>
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# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>µg</td>
<td>Microgram</td>
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<tr>
<td>µl</td>
<td>Microliter</td>
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<td>µM</td>
<td>MicroMolar</td>
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<tr>
<td>µmol</td>
<td>Micromoles</td>
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<tr>
<td>A&lt;sub&gt;xxx&lt;/sub&gt;</td>
<td>Absorbance at xxx nm</td>
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<tr>
<td>C</td>
<td>Celsius</td>
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<tr>
<td>Ca</td>
<td>Calcium</td>
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<td>cm</td>
<td>Centimeter</td>
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<tr>
<td>cpm</td>
<td>Counts per minute</td>
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<td>And others</td>
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<td>Fig.</td>
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<td>g</td>
<td>Gravity or Grams</td>
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<td>h</td>
<td>Hour</td>
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<tr>
<td>kDa</td>
<td>Kilodaltons</td>
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<td>Liter</td>
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<td>M</td>
<td>Molar</td>
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<td>mg</td>
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<td>Minutes</td>
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<td>ml</td>
<td>Milliliter</td>
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<td>Symbol</td>
<td>Definition</td>
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<tr>
<td>mM</td>
<td>Millimolar</td>
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<tr>
<td>N</td>
<td>Normal</td>
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<td>nm</td>
<td>Nanometer</td>
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<tr>
<td>pH</td>
<td>Acidity</td>
</tr>
<tr>
<td>Pi</td>
<td>Inorganic Phosphate</td>
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<tr>
<td>t</td>
<td>Time</td>
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LIST OF FIGURES

Chapter I

Figure 1.1: The hierarchical structure of collagen, from peptide chains to supermolecular arrangement. Modified from Orgel, JP et. al “Microfibrillar structure of type I collagen in situ”, PNAS 103: 9001-9005 .................................................................5

Chapter II

Figure 2.1: Separation of fetuin and glucose by passage over a column packed with purified type I collagen from bovine achilles tendon .......................38

Figure 2.2: Effect of demineralization in 0.6N HCl on the gross appearance of steer bone: photograph and radiograph ..........................40

Figure 2.3: Separation of fetuin and glucose by passage over a column packed with demineralized bovine bone collagen ..................................44

Figure 2.4: Separation of fetuin and glucose by passage over a column packed with non-demineralized bovine bone ........................................46

Figure 2.5: Effect of hydration on the packing of collagen molecules in the lateral plane of a collagen fibril .......................................................54

Chapter III

Figure 3.1: Radioimmunoassay of bovine fetuin, and detection of bovine fetuin antigen in adult bovine serum ..................................................75

Figure 3.2: Removal of fetuin from bovine serum by antibody affinity chromatography .................................................................77

Figure 3.3: Evidence that fetuin is required for the serum-induced re-calcification of demineralized bone: analysis for Ca and P ......................78

Figure 3.4: Evidence that fetuin is required for the serum-induced calcification of demineralized bone: Alizarin red and von Kossa staining ..........81
Figure 3.5: Evidence that fetuin is required for the serum-induced calcification of rat tail tendon……………………………………………………………82

Figure 3.6: Evidence that fetuin is required for the serum-induced calcification of purified bovine type I collagen………………………………………………83

Figure 3.7: Evidence that fetuin depletion unmasks a potent serum initiator of mineral formation…………………………………………………………………………………85

Chapter IV

Figure 4.1: Evidence that fetuin is required for the serum-induced calcification of devitalized arteries: analysis for Ca and P……………………………………106

Figure 4.2: Evidence that fetuin is required for the serum-induced calcification of devitalized arteries: Alizarin red and von Kossa staining………………107

Figure 4.3: Evidence that fetuin is required for the serum-induced calcification of purified ligament elastin…………………………………………………………108

Figure 4.4: Separation of fetuin and glucose by passage over a column packed with purified ligament elastin…………………………………………………110

Figure 4.5: Correlation between the volume of liquid from which fetuin is excluded, and the mineral formed in serum in the matrices of elastin and bone………………………………………………………………………………115
LIST OF TABLES

Chapter II

Table 2.1: The water content of bovine Achilles tendon fibers..........................36
Table 2.2: The size exclusion properties of purified bovine Achilles tendon
Collagen........................................................................................................38
Table 2.3: Effect of demineralization on the gross dimensions and water content of
bovine bone..................................................................................................40
Table 2.4: The water content of demineralized bovine bone............................41
Table 2.5: Characterization of columns packed with demineralized and non-
demineralized bovine bone.........................................................................42
Table 2.6: The Size Exclusion properties of demineralized bovine bone collagen...45
Table 2.7: The impact of mineral on the size exclusion properties of bone
collagen.........................................................................................................47
Table 2.8: The size exclusion properties of deineralized bovine bone collagen:
23 ml column experiments.................................................................49

Chapter III

Table 3.1: The concentration of fetuin in the experimental calcification solutions
used in these studies..............................................................................76

Chapter IV

Table 4.1: The size exclusion properties of purified elastic fibers from bovine
ligament....................................................................................................111
Table 4.2: The size exclusion properties of purified elastic fibers from bovine
aorta........................................................................................................112
Table 4.3: Comparison of the fetuin excluded volume in elastin and
demineralized bone...........................................................................114
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PUBLICATIONS

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PRESENTATIONS

ABSTRACT OF THE DISSERTATION

The Size Exclusion Characteristics of Collagen and Elastin Fibers and the Role of Fetuin in their Calcification.

by

Damon Armen Toroian

Doctor of Philosophy in Biology

University of California, San Diego, 2007

Professor Paul A. Price, Chair

The diffusion of molecules into a mineralizing matrix may affect the process of serum-initiated mineralization. We theorized that biological matrices that mineralize in serum must have molecular exclusion characteristics due to the large number of inhibitors of mineralization present in serum. For our initial tests, we developed a gel filtration-like procedure using columns packed with collagen from tendon or bone. The elution volumes of test molecules from these columns show the volume within each column that is accessible to the test molecules, and reveal the size exclusion characteristics of the collagen within the column. Molecules smaller than a 6 kDa protein are shown to diffuse into all of the space occupied by water within the collagen fibrils, while molecules larger than a 40 kDa protein are excluded from this space.
We hypothesized that fetuin, a 48 kDa serum calcification inhibitor, promotes fibril calcification by selectively inhibiting apatite growth outside the fibril. This hypothesis was tested by examining the impact of removing fetuin on serum’s ability to mineralize collagen. The presence of fetuin in serum is shown to determine the location of serum-driven mineralization- in fetuin’s presence, mineral forms only within the collagen fibril; in fetuin’s absence, mineral forms only outside the fibril.

Calcification of the elastin fibers of the artery media is also a serum driven process. We hypothesize that elastin’s mineralization is dependent on fetuin preventing mineral growth outside, but not inside the elastin fiber. This hypothesis was tested by examining the impact of removing fetuin on serum’s ability to mineralize elastin. By subsequently running molecules over a column packed with elastin fibers, we show that the fiber has size exclusion characteristics comparable to those of collagen, and is dependent on fetuin for mineralization in serum.

These observations show that serum calcification activity consists of at least two large proteins. One unidentified protein generates crystal nuclei outside of the collagen fibril or elastin fiber, which then diffuse in. The other protein, fetuin, binds crystal nuclei outside the collagen or elastin matrices, inhibiting their growth, and paradoxically promoting matrix mineralization. This mineralization mechanism provides insight into the ways biological matrices calcify in vivo.
Chapter I

Introduction
The mineralization of the type I collagen of bone, and the pathological mineralization of the elastic lamellae of arteries are physiologically important, but poorly understood processes. The mineralization of bone is a normal physiological process that provides mechanical strength to the skeleton. The positive and negative regulation of this mineralization are necessities, as both over- and under-mineralization of bone can compromise its mechanical strength[1, 2]. The mineralization of the elastic lamellae of arteries, on the other hand, is entirely pathological and occurs most commonly during chronic renal failure [3]. This arterial mineralization implies that at least one inhibitory mechanism preventing lamellar mineralization has been compromised.

There are several proteins and small molecules that have been implicated in the regulation of matrix mineralization. This group of molecules includes, but is certainly not limited to: bone sialoprotein [4], alkaline phosphatase [5] (considered positive regulators of mineralization), pyrophosphate [6, 7], and matrix Gla Protein [8] (considered negative regulators of mineralization). However, examining potential regulatory elements without taking into account the physical location in which they act only allows part of the story to be told. We hypothesize that the mechanisms underlying the mineralization of collagen or elastin matrices must be closely tied to the physical structures of these matrices. Specifically, the ability of any regulators of mineralization to access the physical structure in type I collagen and elastin matrices in which mineralization occurs must strongly influence their effect on the process of mineralization. The locations of several molecules relative to the aqueous
mineralizing compartments in collagen and elastin were determined in the experiments presented in this thesis. Based on the results of these experiments, hypotheses were formed and tested to determine that these regulators’ location of action is as important to their effect on matrix mineralization as their activities as positive or negative regulators of mineralization.

**Statement of Goal:** In this thesis I will test the hypothesis that the matrices of collagen and elastin have size exclusion characteristics which allow the access of some molecules to the space in which mineral forms, but disallow this access to others. I further hypothesize that the entry into or exclusion from this space has important implications for the function of these molecules in the serum driven mineralization of both of these physiologically important matrices. By using fetuin depleted serum in an *in vitro* serum-driven mineralization assay this second hypothesis will be tested. In the following sections of this introduction I will present both the background for, and the hypothesis to be tested in each chapter of this thesis.

**Chapter II: The Size Exclusion Characteristics Of Type I Collagen: Implications For The Role Of Non-Collagenous Bone Constituents In Mineralization.**

Collagen is the most abundant protein in the bodies of both vertebrates and invertebrates. There are several varieties of collagen that can be divided into subcategories based on their appearance by electron microscopy,
immunohistochemical differences, location and specific function. Collagens can, for example, form long fibrous structures (Type I, II, III, V, XI) [9], dense meshworks (Types IV, VII and X), or appear as transmembrane proteins (Type XII and XVIII) [10, 11]. Several other collagen types (such as FACIT collagens) have been discovered which have many diverse functions. Fibrillar collagens are, however, particularly interesting, as they are a main structural element of most of the body’s connective tissues including, but not limited to, the tissues of tendon and bone.

Both tendon and bone are primarily made of type I collagen [12]. In this section I will briefly review common thoughts about the composition and ultrastructure of the type I collagen fibril, the structural unit of type I collagen matrices. Finally I will discuss the formation of mineral in a type I collagen matrix, and the location of that mineral in relation to the collagen fibril.

**Type I collagen fibril molecular structure:** The mature type I collagen molecule is approximately 1.1nm in diameter and 300nm in length. This molecule is formed by the spontaneous association of two alpha-1 and one alpha-2 polypeptide chains to create a triple helical structure [9]. These triple helical molecules are considered the significant subunits of the Type I collagen fibril, which itself is the structural unit of all Type I collagenous matrices. The spontaneous association of hundreds of collagen triple helices through both hydrophobic and ionic interactions is responsible for the assembly of the fibril.

The physical structure of the type I collagen fibril can be viewed in two dimensions, the axial (or longitudinal) and lateral (or equatorial). The fibril is
composed of collagen molecules, each 1.1 x 300 nm in size and formed by the association of two alpha 1 and one alpha 2 polypeptide chains to create a rope-like triple helical structure. The fibril assembles by the non-covalent association of collagen molecules, each offset by 67 nm with respect to its lateral neighbors [9, 13, 14]. There is an axial repeat within the collagen fibril of 5x67nm (335 nm), which is longer than the ~300 nm collagen molecule. This results in a ‘gap’ between each collagen molecule and its nearest axial neighbors. (Figure 1.1) This gap is responsible for the fact that the fibril has alternating differences in electron density and diameter with a 67 nm repeat corresponding to the gap and overlap regions of the fibril [14-16].

Figure 1.1. The hierarchical structure of collagen, from peptide chains to supermolecular arrangement. Modified from Orgel, JP et. al “Microfibrillar structure of type I collagen in situ”, PNAS 103: 9001-9005 [17]
The lateral structure of the collagen fibril consists of collagen molecules arranged in a quasihexagonal lattice [17-24]. The final type I collagen fibril is a cylindrical structure with a diameter varying from 20-400nm depending on the tissue [25]. The fibril is stabilized by covalent cross-links between collagen molecules. Any one collagen molecule has four cross-links with neighboring molecules. Two of these cross-links occur at each end of the collagen molecule [26-28]. These covalent cross-links, in addition to non-covalent interactions help maintain fibrillar strength and structure, even under significant stress. [29-31].

A “microfibril” is thought to be the basic building block of the collagen fibril [17, 18, 20, 23, 24, 32], but the relationship of the microfibril structure to the molecular packing of collagen molecules in the fibril is sometimes unclear (see [17] for references). A recent fiber x-ray crystallographic determination of the collagen type I supermolecular structure has clarified the role of the microfibril in collagen structure by examining for the first time the detailed packing arrangement of collagen molecules from their N- to C-termini[17]. This study shows that each collagen molecule associates with its packing neighbors to form a super-twisted, right-handed, pentameric microfibril that interdigitates with neighboring microfibrils.

**The Impact of Hydration on the Collagen Fibril**: In the dry fibril, Bragg spacing (or center to center distance) between adjacent collagen molecules is approximately 1.1nm. However, Fullerton has shown that at physiological levels of hydration, the type I collagen fibril is approximately 30% collagen and 70% water. At this level of
hydration the Bragg spacing increases to ~1.8 nm, with water occupying the space between the collagen molecules [33]. Therefore, at physiological levels of hydration each collagen molecule in the lateral plane is separated from its neighbors by a water layer 6-7 Å thick. This change in Bragg spacing caused by hydration increases the diameter of the fibril without altering its length or changing the axial order, which has the same 67nm periodicity in both dry and hydrated fibrils [33, 34]. By showing that this fibrillar swelling directly corresponds to the swelling of a tendon hydrated to the same extent, Fullerton also argues that all of the water in a collagen matrix is within the collagen fibrils.

The thickness of the water layer in the fibril argues against non-covalent associations along the full length of adjacent collagen molecules. Due to this, one may speculate that while fibrils may have a microfibrillar topology, it is possible that each collagen triple helix has some flexibility and freedom to move relative to its neighbors, creating aqueous spaces of fluctuating size within the fibril. Torchia’s studies uphold this view, showing that collagen molecules within the fibril are free to re-orient within a hydrated fibril, but not in a dry or mineralized fibril. This motion within the hydrated fibril is unimpeded by the N- and C-terminal covalent cross-links [35]. This movement and the thick water layer introduce significant disorder into the microfibrillar models that is unable to be portrayed in any static image of collagen, though this disorder has been speculated to exist by proponents of these models [36]. Dextrans of 3 kDa MW can diffuse into the water within the collagen fibril, an event
impossible in a rigid structure, supporting the idea of a non-static, non-rigid collagen fibril [37].

All these data taken together suggest that the collagen fibril should not be considered a solid or impenetrable structure, despite the numerous interactions between its subunits. There is a significant amount of water within the fibril, which produces a transverse space between collagen molecules. This space is accessible to molecules at least as large as a 3kDa dextran, suggesting that small molecules have access to the interior of the fibril.

**Properties of bone mineral:** Bone is unique among type I collagenous matrices in that it is thoroughly and specifically infiltrated with mineral *in vivo* while other collagenous matrices, such as tendon, are only mineralized in special cases [38]. Bone mineral is a calcium-phosphate compound, commonly thought to be apatitic [39][40]

The precise location of this apatitic mineral relative to collagen fibrils has been studied for decades, and the field has come to accept that most to all bone mineral is located inside the collagen fibril.

Early work showed that mineralization of bone replaces the water within collagen with an equivalent volume of mineral [41, 42] a finding that was verified recently by nuclear magnetic resonance (NMR) [43]. Robinson states that fibrils are not grossly distorted by mineral deposition, and asserts that the extrafibrillar space is not large enough to contain the formed mineral crystals [12]. Some or all mineral must therefore reside in the fibrils. In an early paper, Katz claimed that 70-80% of bone
mineral is located within fibrils [44] a conclusion come to by analysis of X-ray diffraction data. Glimcher used transmission electron microscopy (TEM) to argue that almost all mineral is within fibrils [45]. Sasaki and Sudoh used X-Ray pole analysis to determine that approximately 80% of mineral must be intrafibrillar [46]. A model was also devised by Jager and Fratzl [47] demonstrating that bone mineral could be wholly intrafibrillar and that a collagen matrix mineralized in this manner could duplicate the mechanical and elastic properties of bone without distorting the fibrils. Finally, if as Fullerton’s evidence suggests, all water in a collagen matrix is within the collagen fibrils, then when this water is replaced by mineral, the fibril must logically be the location of that formed mineral.

**Mechanisms of collagen mineralization:** The *in vivo* mineralization of the type I collagen fibril of bone has been extensively studied, but the underlying mechanisms controlling that mineralization are not yet understood. One common theory proposes that mineralization is controlled by specialized structures called matrix vesicles, which bud from osteoblasts (specialized bone cells that create the collagen matrix) or chondrocytes. Mineral initially forms within these vesicles, eventually penetrating the vesicle membrane and depositing within the collagenous matrix, where it can then grow [48].

Another proposed mechanism of mineralization suggests that mineral formation is a process that is driven by specific proteins such as bone sialoprotein [4] or alkaline
phosphatase [49]. This mechanism must also include the recently discovered serum calcification activity discovered by Price et. al [50].

Price et. al showed that the incubation of demineralized newborn rat tibia or rat tail tendon in rat serum, or in DMEM containing as little as 1.5% rat serum caused these matrices to mineralize. By histological examination, the mineralization of bone matrices was specific to those locations which were calcified prior to demineralization. Mineralization only ceased when the amount of mineral introduced into the matrix during incubation in serum was comparable to that present in the matrix prior to demineralization. The mineral density and distribution after remineralization are also comparable to those properties before demineralization. While tail tendon does not mineralize in rats in vivo, possibly due to a lack of vascularization [38], it does mineralize in this in vitro system when it is exposed to serum. This suggests that the serum calcification factor must be able to act on all type I collagen matrices with which it comes in contact. This serum calcification factor has been shown to be present in all vertebrates, and consists of an as yet unidentified, 50-150 kDa protein or proteins.

The matrix of bone is highly vascularized and thus, presumably, constantly in contact with this serum factor. It is therefore impossible to discount this serum calcification factor as a major in vivo mechanism of bone mineralization. [50-53].

**Hypothesis:** We believed that there had to be a way to reconcile the fact that collagen matrices mineralize in serum with the fact that serum contains very high
concentrations of calcification inhibitors necessary to prevent mineral formation from occurring throughout the bloodstream. The most abundant of these inhibitors is fetuin [54, 55], and fetuin is responsible for approximately 70% of serum’s ability to inhibit mineralization when calcium and phosphate concentrations are increased above physiological norms [54]. One possible explanation for serum fetuin’s inability to prevent collagen mineralization is that it cannot access the interior of type I collagen fibrils where serum-initiated mineralization occurs. We hypothesized that the physical structure of the collagen fibril determines the size of the molecules that can diffuse into the fibril interior, thereby dictating which molecules might directly interact with growing mineral crystals, and which must interact with mineral nuclei outside the fibril.

In order to investigate the size exclusion characteristics of the collagen fibril we developed the first experimental technique that can be used to investigate these characteristics. This novel, gel filtration-like procedure uses columns packed with type I collagen from different bovine tissues. The elution volumes of the test molecules show the volume within the packed column that is accessible to the test molecules, and therefore reveal the size exclusion characteristics of the collagen in the column.
Chapter III: The Essential Role of Fetuin in the Serum Induced Calcification of Collagen

Characteristics and Proposed Function of Fetuin: Because fetuin (Fetuin-A, termed α-Heremens-Schmidt glycoprotein in humans) is the cornerstone of this portion of this thesis, it is useful to review what is known about the structure and inhibitory activity of the protein. Fetuin, an approximately 48 kDa glycoprotein member of the cystatin superfamily, contains two N-terminal cystatin domains that themselves contain several calcium binding sites which may be involved in binding apatitic mineral. [56].

Fetuin is produced in the liver by parenchymal cells, but fetuin does not stay at the site of production [57]. Instead it escapes to and circulates through the bloodstream [58], and accumulates at high concentrations in bone [59-61]. In adult mammals, fetuin concentration ranges from 0.5 to 1.5 mg/ml in serum, while the concentration in fetal and newborn serum is much higher. [62]

Fetuin is found throughout the mineralized collagenous matrix of bone [59-61, 63, 64] with an approximately 1mg/g concentration of fetuin in mammalian bone [64], though there is no evidence for the production of fetuin in bone. This suggests that fetuin accumulates in bone by binding bone mineral during its circulation though blood [59, 65], a theory supported by the fact that fetuin strongly binds apatite in vitro [61]. There is no obvious localization of fetuin to any particular substructure of bone [66], indicating that whatever its impact, it operates uniformly throughout the tissue.
For these reasons, it was thought that fetuin might play some role as a positive or negative regulator of mineral formation.

Work performed in this lab has shown that fetuin forms a complex with a calcium phosphate mineral in both in vivo and in vitro experimental systems [55], and in vitro retards the formation of a mineral precipitate [55]. This work is consistent with Schinke’s evidence that fetuin is a potent inhibitor of mineral formation in serum containing high concentrations of calcium and phosphate[68], and Heiss’s studies of fetuin, which have demonstrated that fetuin engulfs forming calcium-phosphate mineral to temporarily form a soluble colloid, thus retarding mineral precipitation. [56]

Fetuin, therefore, has in vitro and in vivo been shown to be associated with mineral and has in vitro proven to be a strong inhibitor of mineral precipitation. [55, 68]. As mentioned earlier, fetuin is present at a very high concentration in the blood of animals. Fetuin’s circulation through the bloodstream is important in preventing the pathological formation of mineral in the circulatory system, or vascularized tissues. In fetuin knockout mice, the muscles, vasculature, and kidneys all develop small foci of pathological mineralization, possibly caused by small mineral crystals circulating through the bloodstream and infiltrating these tissues [54]. Serum obtained from fetuin knockout animals also shows an approximately 70% loss of the ability to prevent the formation of mineral in vitro when compared to serum from wild type animals. [54]
**Hypothesis:** The non-collagenous proteins of bone are thought to be important for several processes. These include bone fibril assembly [69], maintenance of fibril structure [13], and regulation of mineralization [8, 70]. The protein of the most relevance to my research was the mineralization inhibitor fetuin. We show in Chapter II that fetuin cannot penetrate the collagen fibril, while molecules the size of BGP can. However, fetuin is prevalent in both mammalian serum [58, 62] and bone [60, 61], and is an *in vitro* inhibitor of mineralization. Because of this, we hypothesized that fetuin must play an important role in ensuring that mineralization occurs within the collagen fibril by preventing mineral formation outside the fibril.

Recent experiments have demonstrated that the type I collagen fibrils of tendon and demineralized bone calcify when incubated in serum, and that this is due to a proteinaceous and as yet unidentified serum calcification factor [50] consisting of one or more proteins that have a molecular weight of 50-150 kDa. Because these molecules are too large to penetrate the fibril, they must generate small crystal nuclei outside the collagen fibril some of which then diffuse into the fibril and grow. In serum, therefore, there must be some inhibitory element or elements that disallow the growth of mineral outside the collagen fibril, paradoxically favoring mineral growth inside the fibril.

In this set of experiments, the mechanism of serum driven mineralization is dissected by removing fetuin from serum by antibody affinity chromatography. Because we believe that fetuin promotes mineralization within the collagen fibril by inhibiting mineral growth outside the fibril, we hypothesize that by removing fetuin
from serum, mineral will preferentially form in the far greater volume of liquid outside the collagen fibril rather than inside the fibril. Fetuin will, therefore, determine the location of mineral formed by serum driven mineralization.

**Chapter IV: The Essential Role of Fetuin in the Serum-Induced Calcification of Elastin Matrices**

**Mineralization of the elastic lamellae of arteries:** Two major types of arterial calcification have been observed in human patients [71, 72]. One affects the collagen rich intimal layer of arteries and is associated with atherosclerotic plaques. The other occurs within the elastin-rich elastic lamellae of the medial layer of arteries. This second type of vascular calcification is common in patients with chronic kidney disease or diabetes mellitus. Each type of arterial calcification has different physiological consequences, with clear-cut evidence for adverse hemodynamic changes due to medial calcification of the artery wall, but not to atherosclerotic plaque calcification.

There exists evidence for an association between bone metabolism and artery calcification (see [73] for references. While the nature of the link between bone metabolism and artery calcification has not yet been established, we have recently proposed that a causative agent (or agents) for artery calcification arises in bone metabolism, travels in blood, and then induces calcification in the elastic lamellae of the artery media [73]. This theory predicts that serum contains a causative agent for
medial artery calcification, and that the elastic lamellae of the artery media should therefore calcify when devitalized arteries are incubated in serum.

The elastic lamellae of devitalized arteries do indeed calcify when incubated in rat, bovine, or human serum [74]. This calcification is due to a potent serum calcification factor, one that causes devitalized arteries to calcify when incubated in DMEM containing at least 1.5% serum. The serum calcification activity that initiates medial elastin calcification has the same 50-150kDa size and protease sensitivity as the serum calcification activity previously shown to initiate calcification of type I collagen [50]. Our working hypothesis is that the same serum calcification activity initiates calcification of collagen and elastin.

**Elastic Fiber: Components and Structure:** Just as with collagen, understanding the structure of the major component of the artery media, the elastic fiber, may be important in understanding any size-exclusion and mineralization properties of that component. Elastic fibers, though thoroughly studied, still have a poorly understood ultrastructure. The components of the fiber structure, however, have been determined. Elastic fibers are composed of two separate materials; approximately 90% of the elastic fiber consists of the amorphous, highly dynamic protein elastin [75]. Most of the remaining 10% of the fiber’s mass is a fibrous scaffolding protein, fibrillin [75]. Elastin is initially synthesized as a monomeric 72 kDa protein, tropoelastin. Mechanical testing suggests that the structure of tropoelastin is nearly random [76]. Cross-links are formed between neighboring tropoelastin molecules giving the elastic
fiber structural integrity, despite elastin's amorphous character [75]. Elastin is highly dynamic even after this cross linking, with a high mobility of any specific elastin molecule relative to its neighbors [77], and a propensity of elastin molecules to convert between several different stable structures [78]. This dynamism gives elastin its extraordinary elasticity, resilience and durability, and recalls the dynamic motion of the triple-helical molecules of the collagen fibril. As in the collagen fibril, this dynamism gives the elastic fiber a very high degree of disorder. Also as with collagen, these physical properties of the matrix are dependent on hydration [77, 79]. This disorder within the elastic fiber has made understanding the fiber ultrastructure very difficult.

The commonly accepted mechanism for elastic fiber assembly is the deposition of soluble tropoelastin on fibrillin. The 350 kDa, linear glycoprotein fibrillin is thought to form a scaffolding with which tropoelastin molecules spontaneously associate. After this association, tropoelastin molecules become insoluble, and a filamentous elastin structure is formed due to cross-linking and interaction with the fibrillar scaffolding [75]. Though it is known that fibrillin monomers are linked together in an axial array with an observable 56 nm periodicity [80] the lateral organization of the microfibrillar scaffold is very poorly understood. At this point no consensus has been reached in the field about the nature of this organization, the exact microfibril diameter, or even the number of fibrillin molecules per microfibril.
**Hypotheses:** In recognizing that the causes of mineralization in bone and the pathological mineralization of arterial elastic fibers may very well be the same, one must ask the question, “What is the similarity between the type I collagen of bone and tendon, and the elastin of artery which allows them both to be mineralized in serum?”

**Hypothesis 1:** We hypothesize that in order to be mineralized in serum both collagen and elastin must have as a major component a water containing structure that has the capacity to exclude fetuin, while allowing the penetration of smaller molecules. If this is true, then the removal of fetuin from serum should have the same impact on the serum-induced mineralization of the elastin fiber as it does on the serum-induced mineralization of collagen.

**Hypothesis 2:** Because the arterial lamellae mineralize in serum, we hypothesized that the elastic fiber must have size exclusion characteristics. Several test molecules were run over a column packed with purified elastic fibers. As in columns packed with purified collagen, the elution volumes of the test molecules show the volume within the packed column that is accessible to the test molecules, and therefore reveal the size exclusion characteristics of the collagen in the column.

**References**


Chapter II

The Size Exclusion Characteristics of Type I Collagen: Implications for the Role of Non-Collagenous Bone Constituents in Mineralization
Abstract

The mineral in bone is located primarily within the collagen fibril, and during mineralization the fibril is formed first and then water within the fibril is replaced with mineral. The collagen fibril therefore provides the aqueous compartment in which mineral grows. Although knowledge of the size of molecules that can diffuse into the fibril to affect crystal growth is critical to understanding the mechanism of bone mineralization, there have been as yet no studies on the size-exclusion properties of the collagen fibril.

To determine the size-exclusion characteristics of collagen, we developed a gel filtration-like procedure that uses columns containing collagen from tendon and bone. The elution volumes of test molecules show the volume within the packed column that is accessible to the test molecules, and therefore reveal the size exclusion characteristics of the collagen within the column. These experiments show that molecules smaller than a 6 kDa protein diffuse into all of the water within the collagen fibril, while molecules larger than a 40 kDa protein are excluded from this water.

These studies provide an insight into the mechanism of bone mineralization. Molecules and apatite crystals smaller than a 6 kDa protein can diffuse into all water within the fibril and so can directly impact mineralization. Although molecules larger than a 40 kDa protein are excluded from the fibril, they can initiate mineralization by forming small apatite crystal nuclei that diffuse into the fibril, or can favor fibril mineralization by inhibiting apatite growth everywhere but within the fibril.
**Introduction**

Most present evidence shows that the mineral in bone is located primarily within the type I collagen fibril (1-6), that the fibril is formed first and then mineralized [1, 2], and that mineralization replaces water within the fibril with mineral [1, 3, 4]. The collagen fibril therefore plays an important role in mineralization, providing the aqueous compartment in which mineral grows. Our working hypothesis is that the physical structure of the collagen fibril may also play a critical additional role in mineralization: the role of a gatekeeper that determines the size of the molecules that can penetrate the fibril to affect apatite crystal growth. The present experiments were carried out to test this hypothesis.

The physical structure of the type I collagen fibril can be viewed in two dimensions, the axial (or longitudinal) and lateral (or equatorial). The fibril is composed of collagen molecules, each 1.1 x 300 nm in size and formed by the association of two alpha 1 and one alpha 2 polypeptide chains to create a rope-like triple helical structure. The fibril assembles by the non-covalent association of collagen molecules, each offset by 67 nm with respect to its lateral neighbors [5-7]. An axial repeat is 5 x 67 = 335 nm in length, which is longer than the 300 nm collagen molecule. This difference results in a 35 nm ‘gap’ between each collagen molecule and its nearest axial neighbors, and is responsible for the fact that the fibril has alternating differences in electron density [7] and diameter [8, 9] with a 67 nm repeat that corresponds to the gap and overlap regions of the fibril. The lateral structure of the collagen fibril consists of collagen molecules arranged in a quasihexagonal lattice.
The final fibril can be from 20 to 400 nm in diameter [17, 18] and is stabilized by four covalent cross links per collagen molecule, two at either end of the molecule [19, 20].

A “microfibril” is thought to be the basic building block of the collagen fibril [6, 11, 14-16, 21], but the relationship of the microfibril structure to the molecular packing of collagen molecules in the fibril is sometimes unclear (see [14] for references). A recent fiber x-ray crystallographic determination of the collagen type I supermolecular structure has clarified the role of the microfibril in collagen structure by examining for the first time the detailed packing arrangement of collagen molecules from their N- to C-termini [14]. This study shows that each collagen molecule associates with its packing neighbors to form a super-twisted, right-handed, pentameric microfibril that interdigitates with neighboring microfibrils.

At physiological levels of hydration, the type I collagen fiber is about 30% collagen and 70% water by volume (see [22] and references therein). Micro CT measurements have shown convincingly that the progressive hydration of a collagen fiber increases the diameter of the fiber but not its length. This observation shows that hydration affects the lateral structure of the fiber, but not the axial structure [22]. X ray structural analyses support this conclusion. Hydration has no measurable impact on the axial structure of the fibril, which has the same 67 nm periodicity in dry and fully hydrated collagen fibrils [23]. In contrast, hydration progressively increases the Bragg spacing between adjacent collagen molecules in the lateral plane, from 1.1 nm in the dry fibril to 1.8 nm when the fibril is fully hydrated [24]. In the lateral plane, each
collagen molecule is therefore separated from its neighbors by a water layer 6 to 7 Å thick [22].

We have recently shown that the chemically identical type I collagen fibrils of tendon and demineralized bone calcify when incubated in rat or bovine serum for 6 days at 37°C [25, 26]. Among the more puzzling aspects of the serum induced calcification of collagen fibrils is that calcification occurs in spite of the presence of potent serum calcification inhibitors, the best characterized and most abundant of which is fetuin [27-29]. A possible explanation for this observation is that fetuin (and other large calcification inhibitors) may not be able to penetrate into the interior of the type I collagen fibril where serum-initiated calcification occurs [26]. Our general working hypothesis is that the physical structure of the collagen fibril determines the size of the molecules that can diffuse into the water that lies within the fibril and thereby affect apatite crystal growth.

In the course of evaluating our working hypothesis, we became aware that there is no experimental evidence that shows what types of molecules can and cannot penetrate the type I collagen fibril. We accordingly developed the first experimental technique that can be used to investigate the size exclusion characteristics of the collagen fibril. This novel, gel filtration-like procedure uses columns packed with type I collagen from different bovine tissues. The elution volumes of the test molecules show the volume within the packed column that is accessible to the test molecules, and therefore reveal the size exclusion characteristics of the collagen in the column.
The results of these experiments provide the first experimental evidence that the collagen fibril has size exclusion characteristics. Small molecules such as bone Gla protein (BGP; a 5.7 kDa vitamin K-dependent protein also called osteocalcin), calcium, phosphate, citrate, pyrophosphate, and etidronate have free access to the aqueous compartment within the collagen fibril where mineral is deposited, while macromolecules such as fetuin (48 kDa), albumin (66 kDa), and dextran (≥ 5,000 kDa) are excluded from this aqueous compartment.

The size exclusion characteristics of collagen defined in this study reveal some of the ways that molecules of different size might function in bone mineralization (see Discussion). The companion paper in this Journal shows how the size exclusion characteristics of collagen explain the observed effects of fetuin depletion on serum-induced collagen mineralization.

**Experimental Procedures**

**Materials:** Purified type I collagen from bovine Achilles tendon, bovine serum albumin, bovine fetuin, ovalbumin, rabbit immunoglobulin, soy bean trypsin inhibitor, cytochrome c, low molecular weight dextran, anthrone, and heptaose were purchased from Sigma. Methemoglobin and riboflavin were obtained from Calbiochem; and high molecular weight dextran, and 1-14C-glucose were obtained from ICN. BGP was purified from bovine bone as described [30].

**Determination of water content of bovine achilles tendon:** Bovine achilles tendon fibers were dissected from a steer, thoroughly cleaned of all adhering non-
collagenous tissue, and separated into two approximately equal masses. Both masses
of tendon fibers were treated to remove non-collagenous constituents as described [31]
and then dried in a lyophilizer at $\leq 50$ milli Torr and weighed. The purified collagen
fibers were rehydrated overnight at room temperature in 20mM Tris pH 7.4 containing
2M NaCl, briefly blotted with a paper towel to remove excess liquid, and immediately
weighed. This procedure was repeated twice, with a 20 minute equilibration in 20mM
Tris pH 7.4 containing 2M NaCl between measurements. Liquid weight in the fibers is
determined by subtracting the dry weight from the wet weight; liquid volume in the
fibers is the liquid weight divided by 1.07 g/cc, the buffer density.

**Gel filtration procedures: Tendon collagen:** Purified type I collagen from
bovine achilles tendon (Sigma) was fractionated by size to obtain particles between
0.833mm and 2.36 mm. 14 g of this collagen was hydrated, degassed under vacuum,
and packed into a 2 x50cm column to a final volume of 91ml. The column was then
washed extensively with a 20 mM Tris pH 7.4 equilibration buffer that contained 2M
NaCl in order to minimize non-specific ionic interactions between test molecules and
the collagen matrix; the final effluent absorbance at 280 nm was less than 0.01.
Samples were dissolved in 2 ml of equilibration buffer containing about 160,000 cpm
of $^{14}$C-glucose as an internal reference; the load was 20 mg of albumin or fetuin, 10
mg of bone Gla protein, or 30 mM phosphate. A constant flow rate of 6.7 ml/h was
maintained using a Fisher Variable Speed Peristaltic Pump, and the fraction size was
approximately 1 ml. The true volume of each effluent fraction was determined from
the weight of the fraction contents and the density of the column buffer (1.07 g/ml).
The elution position of test substances was determined as follows: proteins, absorbance at 280 nm; 1-\(^{14}\)C-glucose, liquid scintillation counting; phosphate, as described [32].

**Effect of demineralization on the shape, mineral volume, and water volume in bovine bone segments:** To obtain the data shown in Table 2.3, a cylindrical bone segment was cut from the midshaft of a two-year-old steer’s femur and cleaned of marrow and non-mineralized connective tissue. The mean length, thickness, and wet weight of the resulting bone ring were measured, and the ring was freeze dried and weighed. The ring was then demineralized in 840 ml of 0.6 N HCl at room temperature; the 0.6 N HCl was replaced with fresh solution daily. The wet weight, physical properties of the ring, and the calcium and phosphate released into the demineralization solution were determined periodically in order to monitor the progress of demineralization. The demineralized bone ring was photographed and X rayed (Figure 2.2). The bone ring was extensively washed with water, its mean length and thickness were determined and its wet and dry weights were measured.

To determine the volume of water within the collagen of demineralized bone (Table 2.4), two cylindrical steer bone segments were demineralized as described above. Three equilibration solutions were tested: water, 20mM Tris pH 7.4 with 0.15M NaCl (density, 1.016 g/ml), and 20mM Tris pH 7.4 with 2 M NaCl (density, 1.07 g/ml). For each solution, the bone wet weight was measured three times with a two hour equilibration in the solution between measurements and the length and thickness of each segment was determined. Bone was then washed in 50mM HCl and
lyophilized to determine dry weight. The volume of each liquid in bone was determined using the difference between the wet and dry weights, and the liquid densities.

**Preparation of columns packed with demineralized and non-demineralized bovine bone:** To obtain the data shown in Table 2.5, bovine bone sand with a median diameter of 0.5 mm was prepared from the midshaft of tibias from 2-year-old steers as described [33] and divided into two portions of 242 g each. One portion was demineralized with a 10-fold excess of 10% (v/v) formic acid for 72h at 4°C, washed with water and dried; the final dry weight was 51g. High temperature ashing of this acid-extracted bone sand demonstrated that these procedures removed all traces of calcium and phosphate from the collagenous bone matrix (data not shown). Empty 2 X 100 cm columns were weighed, packed with the 51 g of demineralized bovine bone sand or the 242 g of non-demineralized bovine bone sand, and equilibrated with water. Excess water was removed to the surface of the packed matrix, the height of the packed sand was measured (for volume calculation), and the columns were re-weighed. The wet weight of the column contents is the difference between the weights of the packed and empty columns; the amount of water in the packed column is the difference between the wet and dry weights of the column contents; the amount of mineral in the bone sand is the difference between the dry weights before and after demineralization; and the volume of the packed column was determined by measuring the volume of water needed to fill an empty column to the same height as the packed column (see Table 2.5).
**Gel filtration procedures- bone collagen:** The 227 ml columns of non-decalcified and decalcified bone sand prepared for the measurements shown in Table 2.5 were equilibrated with a 20 mM Tris pH 7.4 buffer that contained 2M NaCl in order to minimize non-specific ionic interactions between test molecules and the collagen matrix; the final effluent absorbance at 280 nm was less than 0.01. A constant flow rate of 18 ml/h was maintained and the fraction size was approximately 3 ml. Samples were dissolved in 5 ml of column buffer containing about 400,000 cpm of 1-14C-glucose as an internal reference; the load was 50 mg of the test protein or carbohydrate, 10 mg dimethyl sulfoxide, or 30 mg calcium chloride. The volume of each effluent fraction was determined from the weight of the fraction contents and the density of the column buffer (1.07 g/ml).

In experiments using a column containing 23 ml of decalcified bovine bone sand, the sample volume was reduced to 0.5 ml, the flow rate to 7.2 ml/h and the fraction volume to 0.5 ml. The amounts of sample loaded were: 5 mg protein; 40,000 cpm of 1-14C-glucose; 0.5 mg riboflavin; 10 mg sodium citrate; 4 mg disodium etidronate; and 30 mM phosphate or pyrophosphate. Certain samples were also run over the column at a flow rate of 0.72 ml/hr (Table 2.8).

The elution position of test substances was determined as follows: proteins, absorbance at 280 nm; 1-14C-glucose, liquid scintillation counting; high and low molecular weight dextrans, heptaose, and triose, as described [34, 35]; dimethyl sulfoxide and citrate, absorbance at 220nm; calcium, cresolphthalein complexone (JAS Diagnostic, Miami, FL); phosphate, as described [32]; pyrophosphate, enzymatic
assay with NADH (Sigma); riboflavin, absorbance at 450 nm; and etidronate, by Ceric IV sulfate assay [36].

Results

The size exclusion characteristics of tendon collagen: The initial experiment was carried out to determine whether there is a measurable volume of liquid in hydrated tendon collagen. Purified type I collagen fibers were prepared from bovine Achilles tendon as described [31], and their dry and hydrated weights were measured. When equilibrated in 20 mM Tris pH 7.4 containing 2 M NaCl, purified bovine achilles tendon collagen fibers took up 2.12 ml liquid per gram dry collagen (Table 2.1). Essentially identical hydration values were found for fibers equilibrated in 20 mM Tris pH 7.4 containing 0.15 M NaCl (data not shown). These observations show that hydrated tendon collagen fibers are about 2/3 liquid by weight.

Table 2.1: The water content of bovine achilles tendon fibers

Bovine achilles tendon fibers were dissected from a steer, and thoroughly cleaned of all adhering tissue. Fibers were extracted to remove non collagenous constituents, and then dried, weighed, and re-hydrated in 20mM Tris pH 7.4 containing, 2M NaCl. The fibers’ wet weights were measured three times with a 20 minute equilibration in 20mM Tris pH 7.4 containing 2M NaCl between measurements. Liquid volume in fibers is the liquid weight divided by 1.07 g/cc, the buffer density. (See Experimental Procedures for details.)

<table>
<thead>
<tr>
<th>Bovine achilles tendon</th>
<th>Sample 1</th>
<th>Sample 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wet weight of tendon fibers</td>
<td>1.330 ± 0.003 g</td>
<td>1.251 ± 0.008 g</td>
</tr>
<tr>
<td>Dry weight of tendon fibers</td>
<td>0.408 g</td>
<td>0.381 g</td>
</tr>
<tr>
<td>Weight of liquid in tendon fibers</td>
<td>0.922 g</td>
<td>0.870 g</td>
</tr>
<tr>
<td>Volume of liquid in tendon fibers</td>
<td>0.862 ml</td>
<td>0.813 ml</td>
</tr>
<tr>
<td>Volume liquid : Dry weight tendon fibers</td>
<td>2.11 ml/g</td>
<td>2.13 ml/g</td>
</tr>
</tbody>
</table>
A novel, gel filtration-like method was developed to determine which molecules can access the liquid in tendon collagen. Purified type I collagen from bovine achilles tendon [31] was purchased from Sigma, hydrated in column buffer, and packed in a 2 by 50cm glass column. The size exclusion characteristics of this tendon collagen were then evaluated by filtering a mixture of glucose and fetuin (a 48 kDa glycoprotein) over this column. As can be seen in Figure 2.1, ¹⁴C-labeled glucose eluted at a volume of about 80 ml, which is comparable to the 79.5 ml volume of liquid in the column bed. This observation shows that glucose has free access to essentially all liquid within the packed column. Fetuin eluted at a volume of about 51 ml, which is 29 ml less than the elution volume of glucose. This shows that fetuin is excluded from a 29 ml volume of liquid in the packed column that glucose is able to freely access. Because this 29 ml volume is comparable to the 29.7 ml liquid estimated to lie within collagen (14g collagen X 2.12 ml/g tendon fibers, Table 2.1), the simplest explanation for the lower elution volume of fetuin is that the protein cannot access the liquid within tendon collagen while glucose can.

Additional filtration experiments were carried out to further characterize the molecular exclusion characteristics of tendon collagen. As seen in Table 2.2, phosphate and bone Gla protein (BGP; osteocalcin) co-elute with glucose, while albumin co-elutes with fetuin. These observations indicate that there may be a molecular weight cut off for access to the liquid inside tendon collagen, a cut off that lies between the 5.7 kDa BGP and the 48 kDa fetuin.
Figure 2.1. Separation of fetuin and glucose by passage over a column packed with purified type I collagen from bovine achilles tendon. Purified type I collagen from bovine achilles tendon [31] (Sigma) was fractionated by size to obtain particles between 0.83 mm and 2.36 mm. 14 g of this collagen was hydrated in 20 mM Tris pH 7.4 containing 2M NaCl, packed into a 2 x50cm column to a final volume of 91 ml, and washed extensively with 20 mM Tris pH 7.4 containing 2M NaCl. A 2 ml volume of equilibration buffer containing 20 mg bovine fetuin and 160,000 cpm of \( ^1\)C-glucose was applied to the column, and buffer was pumped through the column at a constant flow rate of 6.7 ml/h. The fraction size was approximately 1 ml. The liquid volume in the packed column bed was obtained by subtracting the weight of dry collagen in the column from the wet weight of the packed column bed; the volume inside tendon collagen was estimated by multiplying the liquid content of hydrated tendon collagen, 2.12 ml/g (Table 2.1), times the weight of collagen in the column, 14g. (See “Experimental Procedures.”)

Table 2.2: The size exclusion properties of purified bovine achilles tendon collagen. The packed column whose preparation is described in the Figure 2.1 legend was equilibrated with 20mM Tris pH 7.4 and 2M NaCl. A 2 ml volume of equilibration buffer containing the test molecule, and 160,000 cpm of \( ^1\)C glucose was then applied to the column. Flow rate, 6.7 ml/hour; fraction size, 1 ml. The elution volume of glucose for these 4 runs was 80±0.95 ml (Mean ±SD). The results show the elution volume of each test molecule. (See Experimental Procedures for details)

<table>
<thead>
<tr>
<th>Test molecule</th>
<th>MW (Da)</th>
<th>Elution volume, ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albumin</td>
<td>66,000</td>
<td>52</td>
</tr>
<tr>
<td>Fetuin</td>
<td>48,000</td>
<td>51</td>
</tr>
<tr>
<td>Bone Gla Protein</td>
<td>5,700</td>
<td>80</td>
</tr>
<tr>
<td>Glucose</td>
<td>180</td>
<td>80</td>
</tr>
<tr>
<td>Phosphate</td>
<td>95</td>
<td>80</td>
</tr>
</tbody>
</table>
Evidence that the demineralization of bone replaces mineral with a comparable volume of water. Bone and tendon are composed of essentially identical type I collagen fibrils [6], and it therefore seemed likely that bone collagen would have size exclusion properties that are similar to those observed with tendon collagen. The goal of our next experiments was to test this hypothesis. Bone is 70% mineral by weight, however, and it was apparent that the presence of mineral in collagen will have a profound effect on its size exclusion characteristics. Any study of the size exclusion characteristics of bone collagen would therefore require comparison of bone before and after removal of mineral.

Several experiments were first carried out to determine the impact of demineralization on the water content and shape of bone. In the initial experiment, a cylindrical bone segment was cut from the midshaft of a two year old steer’s femur and demineralized in 0.6 N HCl at room temperature for 10 days. The gross shape of the resulting demineralized bone ring was comparable to the same bone ring prior to demineralization (Table 2.3), its radiographic density was dramatically and uniformly reduced (Figure 2.2), and the bone ring was flexible (personal observations). The data in Table 2.3 also show that the demineralization of the bone ring is accompanied by a 9.7 ml increase in the volume of water in the bone, and that this increased water volume is comparable to the 9.4 ml volume originally occupied by mineral in the bone prior to demineralization. Demineralization therefore replaces mineral with a comparable volume of water.
Table 2.3: Effect of demineralization on the gross dimensions and water content of bovine bone.
A cylindrical bone segment was cut from the midshaft region of a femur from a two-year-old steer, and was then cleaned of marrow and connective tissue. The length, thickness and wet and dry weights were obtained before demineralization for 10 days at room temperature in 0.6N HCl. After demineralization, the bone was washed with 20mM Tris, 0.15M NaCl pH 7.4, and equilibrated in this buffer overnight. The length, thickness, wet and dry weights were again determined. The weight of mineral in bone is the difference in dry weights due to demineralization. (See Experimental Procedures for details.)

<table>
<thead>
<tr>
<th>Mean Thickness</th>
<th>Bovine bone segment before demineralization (A)</th>
<th>The same segment after demineralization (B)</th>
<th>Change due to demineralization (B-A)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean Length</td>
<td>2.03 cm</td>
<td>2.05 cm</td>
<td>+0.02 cm</td>
</tr>
<tr>
<td></td>
<td>1.77 cm</td>
<td>1.74 cm</td>
<td>-0.03 cm</td>
</tr>
<tr>
<td>Wet Weight of bone</td>
<td>40.50 g</td>
<td>21.16 g</td>
<td>-19.34 g</td>
</tr>
<tr>
<td>Dry weight of bone</td>
<td>37.30 g</td>
<td>8.10 g</td>
<td>-29.2 g</td>
</tr>
<tr>
<td>Weight of liquid in bone (Wet minus dry weight)</td>
<td>3.20 g</td>
<td>13.06 g</td>
<td>9.86 g (+9.70 ml)³</td>
</tr>
</tbody>
</table>

a. Assuming a density of 1.016 g/cc  
b. Assuming a density of 3.1 g/cc

Figure 2.2. Effect of demineralization in 0.6 N HCl on the gross appearance of steer bone: photograph and radiograph. Cylindrical bone segments were cut from the midshaft region of a steer’s femur and cleaned of marrow and connective tissue. One segment was set aside as a control, and the other was demineralized in 0.6 N HCl for 10 days at room temperature. This figure shows the photograph and radiograph of these bone rings; other data for this demineralized bone ring is presented in Table 2.3.
An additional experiment was carried out to examine the impact of the composition of the hydration liquid on the shape and water content of demineralized bone rings. As seen in **Table 2.4**, demineralized bone retains its shape and water content when equilibrated in water, in 20mM Tris pH 7.4 containing 0.15 NaCl, and in 20mM Tris pH 7.4 containing 2 M NaCl. The average liquid content of demineralized bone is 1.58 ± 0.02 ml/g dry ring; essentially all of this water lies within collagen.

**Table 2.4: The water content of demineralized bovine bone**

To determine the volume of water within the collagen of demineralized bone, two cylindrical bone segments were demineralized for 10 days at room temperature in 0.6N HCl, then washed extensively in water. Three equilibration solutions were tested: water, 20mM Tris, pH 7.4 with 0.15M NaCl (density 1.016 g/ml), and 20mM Tris pH 7.4 with 2 M NaCl (density, 1.07 g/ml). For each solution, the bone wet weight was measured three times with a two hour equilibration in the solution between measurements, and the length and thickness of each segment was determined. Bone was then washed in 50mM HCl and lyophilized to determine dry weight. The volume of each liquid in bone was determined using the difference between the wet and dry weights, and the liquid densities.

<table>
<thead>
<tr>
<th>Segment 1</th>
<th>Equilibration Solution</th>
<th>Water</th>
<th>20mM Tris, 2M NaCl</th>
<th>20mM Tris, 0.15M NaCl</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean Thickness</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.10 cm</td>
<td>2.02 cm</td>
<td>2.05 cm</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.69 cm</td>
<td>1.72 cm</td>
<td>1.74 cm</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mean Length</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.10 cm</td>
<td>2.02 cm</td>
<td>2.05 cm</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.69 cm</td>
<td>1.72 cm</td>
<td>1.74 cm</td>
<td></td>
</tr>
<tr>
<td>Wet Weight of bone</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>21.03 ± 0.03 g</td>
<td>21.81 ± 0.03 g</td>
<td>21.16 ± 0.03 g</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dry Weight of bone</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8.10 g</td>
<td>8.10 g</td>
<td>8.10 g</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight of liquid in bone (wet minus dry weight)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12.93 g</td>
<td>13.71 g</td>
<td>13.06 g</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Volume of liquid in bone</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12.93 ml</td>
<td>12.81 ml</td>
<td>12.85 ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liquid volume : Dry Weight</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.60 ml/g</td>
<td>1.58 ml/g</td>
<td>1.59 ml/g</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Segment 2</th>
<th>Equilibration Solution</th>
<th>Water</th>
<th>20mM Tris, 2M NaCl</th>
<th>20mM Tris, 0.15M NaCl</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean Thickness</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.23 cm</td>
<td>2.24 cm</td>
<td>2.25 cm</td>
<td></td>
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<tr>
<td></td>
<td>1.54 cm</td>
<td>1.58 cm</td>
<td>1.59 cm</td>
<td></td>
</tr>
<tr>
<td>Wet Weight of bone</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20.53 ± 0.03 g</td>
<td>20.97 ± 0.01 g</td>
<td>20.58 ± 0.02g</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dry Weight of bone</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.90 g</td>
<td>7.90 g</td>
<td>7.90 g</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight of liquid in bone (wet minus dry weight)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12.60 g</td>
<td>13.07 g</td>
<td>12.68 g</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Volume of liquid in bone</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12.60 ml</td>
<td>12.21 ml</td>
<td>12.48 ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liquid volume : Dry Weight</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.59 ml/g</td>
<td>1.55 ml/g</td>
<td>1.58 ml/g</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The size exclusion characteristics of bovine bone before and after demineralization: The size exclusion characteristics of bovine bone before and after demineralization were evaluated using the gel filtration-like procedure developed with bovine tendon collagen. Bone from the midshaft region of steer tibias was ground to the consistency of coarse sand (median diameter 0.5 mm) as described [33] and divided into two portions of 242 g each. One portion was then demineralized with 10% formic acid for 3 days at 4°C [33], washed with water, dried, and weighed. The demineralized and non-demineralized bone portions were hydrated in water and separately packed into 2 X 100 cm columns. The final packed volumes of the two columns were the same, which indicates that demineralization does not alter the shape or volume of the bone sand particles. As can be seen in Table 2.5, demineralization of bovine bone sand replaced mineral (62 ml) with a comparable volume of water (67 ml).

Table 2.5: Characterization of columns packed with demineralized and non-demineralized bovine bone. Bone from the midshaft region of steer tibias was ground to the consistency of coarse sand and divided into two portions of 242 grams each; one portion was then demineralized with 10% formic acid for 3 days at 4°C, dried and weighed. Both materials were hydrated in water and separately packed into 2x100cm glass columns. The volume of each packed column was then determined. The wet weight of the column contents is the difference between the weights of the packed and empty columns. Weight of mineral in packed column is the difference in the dry weight of column contents due to demineralization. (See Experimental Procedures for details)

<table>
<thead>
<tr>
<th></th>
<th>Non-demineralized bone sand (A)</th>
<th>Demineralized bone sand (B)</th>
<th>Change due to demineralization (B-A)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total volume of packed column</td>
<td>227 ml</td>
<td>227 ml</td>
<td>---</td>
</tr>
<tr>
<td>Wet weight of column contents</td>
<td>367 g</td>
<td>243 g</td>
<td>-124 g</td>
</tr>
<tr>
<td>Dry weight of column contents</td>
<td>242 g</td>
<td>51 g</td>
<td>-191 g</td>
</tr>
<tr>
<td>Weight of water in packed column (Wet minus dry weight)</td>
<td>125 g</td>
<td>192 g</td>
<td>+67 g (+67 ml)</td>
</tr>
<tr>
<td>Weight of mineral in packed column</td>
<td>191 g</td>
<td>0 g</td>
<td>-191 g (-62 ml)²</td>
</tr>
</tbody>
</table>

a. Assuming a density of 3.1 g/cc
Figure 2.3 shows the result obtained when a mixture of $^{14}$C-labeled glucose and fetuin are filtered over the column of demineralized bovine bone sand. As can be seen, $^{14}$C-labeled glucose eluted from the demineralized bone sand column at a volume of 191 ml, which is comparable to the 192 ml volume of liquid in the column bed. This observation shows that glucose has free access to essentially all liquid within the packed column. In contrast, fetuin eluted at a volume of 111 ml, which is approximately 80 ml less than the elution volume of glucose. This shows that fetuin is excluded from an 80 ml volume of liquid in the packed column that glucose is able to freely access. Because the volume of liquid inside bone collagen is estimated to be about 81 ml (51 g collagen X 1.58 ml/g collagen; Tables 4 and 5), the simplest explanation for the lower elution volume of fetuin is that the protein cannot access the aqueous solution within bone collagen while glucose can. The type I collagen matrices of tendon and demineralized bone are therefore comparably accessible to glucose and inaccessible to fetuin.

Additional experiments were carried out to further characterize the molecular exclusion characteristics of the demineralized bone sand column. As can be seen in Table 2.6, glucose, dimethyl sulfoxide, and calcium elute at approximately the bed volume, and therefore have access to essentially all liquid within the packed column. In contrast, fetuin, ovalbumin, albumin, and high molecular weight dextran elute at the approximate volume of liquid estimated to lie outside of collagen (the excluded volume), and therefore are probably equivalently unable to access the volume of liquid
within collagen. Trypsin inhibitor (21.5 kDa), low molecular weight dextran (10.2 kDa), and heptaose (1.15 kDa) elute from the demineralized bone sand column between glucose and fetuin, and consequently appear to have partial access to the volume of liquid in collagen.

Figure 2.3. Separation of fetuin and glucose by passage over a column packed with demineralized bovine bone collagen. The demineralized bovine bone sand column described in Table 2.3 was equilibrated with 20 mM Tris pH 7.4 containing 2M NaCl until the absorbance at 280 nm was < 0.01. A 5ml volume of equilibration buffer containing 50 mg bovine fetuin and 400,000 cpm of 1\(^{14}\)C-glucose was applied to the column. Flow rate, 18 ml/h; fraction size, 3 ml. The liquid volume in the packed column bed is from Table 2.5; the volume inside collagen was estimated by multiplying the liquid content of hydrated bone, 1.58 ml/g (Table 2.4), by the weight of collagen in the column, 51g (Table 2.5). (See "Experimental Procedures").
Table 2.6: The size exclusion properties of demineralized bovine bone collagen
The demineralized packed bone sand column whose preparation is described in the Table 2.3 legend was equilibrated at room temperature with 20mM Tris pH 7.4 containing 2M NaCl. A 5 ml volume of equilibration buffer containing the test molecule and 400,000 cpm of 1-14C-glucose was then applied to the column. Flow rate, 18 ml/hour; fraction size, 3 ml. The elution volume for glucose for these nine runs was 191 ± 2.5 ml (Mean ± SD). The results show the elution volume of the indicated test molecule. (See Experimental Procedures for details).

<table>
<thead>
<tr>
<th>Molecules eluting at excluded volume</th>
<th>MW (Da)</th>
<th>Elution volume, ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>High MW Dextran</td>
<td>5-40x10^6</td>
<td>110</td>
</tr>
<tr>
<td>Albumin</td>
<td>67,000</td>
<td>113</td>
</tr>
<tr>
<td>Fetuin</td>
<td>48,000</td>
<td>110</td>
</tr>
<tr>
<td>Ovalbumin</td>
<td>43,000</td>
<td>119</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Molecules eluting in fractionation range</th>
<th>MW (Da)</th>
<th>Elution volume, ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trypsin inhibitor</td>
<td>21,500</td>
<td>154</td>
</tr>
<tr>
<td>Low MW Dextran</td>
<td>10,200</td>
<td>130</td>
</tr>
<tr>
<td>Heptaose</td>
<td>1,152</td>
<td>160</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Molecules eluting at bed volume</th>
<th>MW (Da)</th>
<th>Elution volume, ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>180</td>
<td>191</td>
</tr>
<tr>
<td>Dimethylsulfoxide</td>
<td>78</td>
<td>191</td>
</tr>
<tr>
<td>Calcium</td>
<td>40</td>
<td>191</td>
</tr>
</tbody>
</table>

We next examined the size-exclusion characteristics of a column made with non-demineralized bone sand. Comparison of Figures 3 and 4 shows that the presence of mineral in the same amount of collagen dramatically reduces the elution volume of glucose but does not comparably affect the elution volume of fetuin. The reduced separation volume between glucose and fetuin on the two columns, 71 ml, is therefore a direct measure of the impact of mineral on the volume in collagen that glucose can access. Table 2.7 shows that the reduced separation between glucose and test
molecules due to the presence of mineral is comparable for fetuin, albumin, and high molecular weight dextran. The average reduced separation due to the presence of mineral, 70 ml, is comparable to the reduced volume of water in the column bed (67 ml, Table 2.7), and the reduced volume of water is comparable to the increased volume occupied by mineral (62 ml, Table 2.7). Mineral therefore occupies a space in bone collagen that is occupied by water in demineralized bone collagen, and this water compartment is accessible to glucose but not fetuin, albumin, or high molecular weight dextran.

Figure 2.4. Separation of fetuin and glucose by passage over a column packed with non-demineralized bovine bone. The non-demineralized bovine bone sand column characterized in Table 2.5 was equilibrated at room temperature with 20 mM Tris pH 7.4 containing 2M NaCl. A 5 ml volume of equilibration buffer containing 50 mg bovine fetuin and 400,000 cpm of $\text{^{14}C}$-glucose was then applied to the column. Flow rate, 18 ml/h; fraction size, 3 ml. The liquid volume in the packed column bed is from Table 2.5. (See "Experimental Procedures").
Table 2.7: The impact of mineral on the size exclusion properties of bone collagen

The packed bone sand columns whose preparation is described in the Table 2.5 legend were equilibrated at room temperature with 20mM Tris pH 7.4 containing 2M NaCl. A 5 ml volume of equilibration buffer containing 50 mg of the test protein or carbohydrate and 400,000 cpm of $1^{14}$C-glucose was then applied to each column. Flow rate, 18 ml/hour; fraction size, 3 ml. The results show the elution volume separating glucose from the indicated test molecule for each column. (See Experimental Procedures for details).

<table>
<thead>
<tr>
<th>Test molecule</th>
<th>MW (Da)</th>
<th>Volume separating test molecule from glucose, ml</th>
<th>Difference due to demineralization (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Demineralized Bone Sand</td>
<td>Non-demineralized Bone Sand</td>
</tr>
<tr>
<td>High MW dextran</td>
<td>5-40x10$^6$</td>
<td>81</td>
<td>10</td>
</tr>
<tr>
<td>Albumin</td>
<td>66,000</td>
<td>78</td>
<td>11</td>
</tr>
<tr>
<td>Fetuin</td>
<td>48,000</td>
<td>81</td>
<td>10</td>
</tr>
</tbody>
</table>

| Volume of liquid in column bed, ml (Table 2.5) | 192 | 125 | 67 |
| Volume of mineral, ml (Table 2.5) | 0 | 62 | -62 |

The size exclusion characteristics of demineralized bovine bone sand: 23 ml column experiments. Additional experiments were carried out to determine whether a smaller bone sand column could be used to obtain information on the size exclusion characteristics of bone collagen without the need for the large sample amounts and long filtration times required for the 227 ml column. The volume of demineralized bone sand in the column was reduced by about 1/10 (to 23 ml from 227 ml), the sample volume was reduced by 1/10 (to 0.5 ml from 5 ml), and the flow rate was reduced to 7.2 ml/h in order to give an equivalent flow per unit of cross sectional column area. This 23 ml demineralized bone sand column gave a 7.6 ml separation
volume between glucose and fetuin, which is about 1/10 of the 81 ml separation volume previously found using the 227 ml bone sand column (Table 2.7). The filtration time required for a single determination with this 23 ml column was 3 h compared to about a day with the 227 ml column. The size exclusion characteristics of bone collagen were further evaluated by passing a number of additional substances over this 23 ml demineralized bone sand column (see Table 2.8). The most significant new information obtained in these experiments is the discovery that the 5.7 kDa bone Gla protein (BGP; osteocalcin) is able to penetrate bone collagen to the same extent as glucose, calcium, phosphate, pyrophosphate, and citrate.

Because of the reduced filtration times needed with the 23 ml bone sand column, it was feasible to use this column to explore the effect of reducing the buffer flow rate on the size exclusion characteristics of bone collagen. These experiments showed that reducing the flow rate from 7.2 ml/h to 0.72 ml/h did not significantly affect the elution volumes of fetuin, cytochrome C, BGP, riboflavin, or glucose (not shown). The elution volumes obtained using the standard flow rates (Tables 6 and 8) therefore reflect differences in the absolute ability of molecules to penetrate the bone collagen, not differences in the time needed to diffuse into collagen. A final experiment was carried out to evaluate the effect of salt concentration on elution volume. This experiment showed that reducing the NaCl content of the equilibration buffer from 2M to 0.15M did not significantly affect the elution volume of fetuin or glucose (not shown).
Table 2.8: The size exclusion properties of demineralized bovine bone collagen: 23 ml column experiments. Demineralized bovine bone sand (4.3g dry weight) was hydrated and packed into a 1.25 cm diameter column to a volume of 23 ml and equilibrated at room temperature with 20mM Tris pH 7.4 containing 2M NaCl until the absorbance at 280nm was <0.01. A 0.5 ml volume of equilibration buffer containing the test molecule and 40,000 cpm of 1-14C glucose was then applied to the column. Flow rate, 7.2 ml/h; fraction size, 0.5 ml. The results show the elution volume separating glucose from the indicated test molecule. The elution volume of glucose for these 14 runs was 18.9 ± 0.4 ml (Mean ± SD). (See Experimental Procedures for details.)

<table>
<thead>
<tr>
<th>Test molecule</th>
<th>MW (Da)</th>
<th>Volume separating test molecule from glucose, ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit IgG</td>
<td>152,000</td>
<td>7.4</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>64,000</td>
<td>8.0</td>
</tr>
<tr>
<td>Fetuin</td>
<td>48,000</td>
<td>7.6</td>
</tr>
<tr>
<td>Cytochrome C</td>
<td>12,300</td>
<td>4.3</td>
</tr>
<tr>
<td>BGP</td>
<td>5,700</td>
<td>0</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>376</td>
<td>0.5</td>
</tr>
<tr>
<td>Etidronate</td>
<td>192</td>
<td>0</td>
</tr>
<tr>
<td>Citrate</td>
<td>189</td>
<td>0.9</td>
</tr>
<tr>
<td>Pyrophosphate</td>
<td>174</td>
<td>0.6</td>
</tr>
<tr>
<td>Phosphate</td>
<td>95</td>
<td>0</td>
</tr>
</tbody>
</table>

Discussion

Our study is the first to demonstrate that the chemically identical type I collagen matrices of tendon and demineralized bone have the ability to exclude large molecules but not small, and it is important to examine the results of our study from an empirical as well as a theoretical perspective. For clarity, the sections below begin with the simpler case of the size exclusion characteristics of tendon collagen, proceed to a discussion of the impact of demineralization on the shape and water content of the
bone collagen, and then to a discussion of the more complex case of the size exclusion properties of bone collagen and the impact of mineralization on these properties. The Discussion ends with a brief analysis of the implications of the size exclusion characteristics of the collagen fibril for the possible functions of non-collagenous bone constituents in bone mineralization.

**The size exclusion characteristics of tendon collagen:** The method we developed to investigate the size exclusion characteristics of tendon collagen is an adaptation of the biochemical procedure used to separate macromolecules by size, a procedure termed gel filtration chromatography. It is useful to briefly review this biochemical procedure before discussing the empirical interpretation of our results. In gel filtration chromatography, a cylindrical column is packed with an insoluble matrix that consists of minute, spherical beads with a porous skin that encloses an interior aqueous compartment. The packed column therefore has two aqueous volumes, one outside the beads and the other inside. In a typical gel filtration experiment, a solution containing molecules of different size is applied to the column, and the elution volume of each molecule is measured. The results of these experiments show that some molecules are sufficiently small that they can rapidly penetrate the skin of the beads and so achieve the same concentration in the water inside the bead as they do outside. These small molecules elute at the liquid volume in the column bed (volumes outside plus inside the beads). Other molecules are sufficiently large that they cannot penetrate the skin of the beads; these large molecules elute at the smaller volume of liquid outside the beads [37].
In the initial study, we packed a column with purified type I collagen from bovine tendon and then determined the elution volume of different test molecules from this collagen column. The results of this experiment show that molecules that range in size from the 95 dalton phosphate to the 5,700 dalton bone Gla protein elute at an ~80 ml volume that is identical to the liquid volume in the column bed. As they pass through the column, each of these molecules is therefore able to access all of the water in the column bed. In contrast, molecules the size of fetuin (48,000 daltons) and albumin (66,000 daltons) both elute at 51 ml, which is 29 ml less than the elution volume of the small molecule group. The simplest explanation for these observations is that the type I collagen in the column contains 29 ml of water that is accessible to BGP, glucose, and phosphate, and inaccessible to fetuin and albumin.

Where in the ~80 ml volume of water in the collagen column is the 29 ml water that is freely accessible to small molecules but not large? Two observations indicate that this 29 ml volume lies within the collagen fibril: 1. A comparable, 29.7 ml volume of water was calculated to lie in the 14g of collagen fibers in the column bed (see Table 2.1). 2. Collagen fibers consist of densely packed collagen fibrils [5, 6], and it has been demonstrated that most or all of the water in collagen fibers lies within the individual collagen fibrils ([22] and references therein).

Why do small molecules such as phosphate, glucose, and the 5,700 dalton BGP elute at the 80 ml volume of total liquid in the column, in spite of the fact that 29 ml of this water lies within the collagen fibrils? Each of these molecules must be able to attain the same concentration in the water that lies inside the collagen fibrils of the
packed column (~29 ml, Figure 2.1) as it does in the water that lies outside of the fibrils (~ 50 ml, Figure 2.1); each molecule therefore elutes at the same volume it would from a 80 ml column of water with no collagen. This result is surprising, as it indicates that the collagen molecules in the fibril have no influence on the ability of small molecules in the buffer to attain the same concentration in the entire aqueous volume that lies within the collagen fibril. This result is even more surprising when one considers that these small molecules must attain this equivalent concentration in the < 10 millisecond interval in which a given concentration of solute is in contact with the fibril (see Footnote 2).

As a first step to understanding the molecular basis for the ability of small molecules to reach concentration equilibrium with all of the water within the collagen fibril, we have constructed a model of the lateral structure of a typical collagen fibril in the fully hydrated and dry states (Figure 2.5). In this model, collagen molecules are represented by 1.1nm hard disks that are arranged in a quasihexagonal lattice [14] at packing densities corresponding to those seen for fully hydrated and dry collagen fibrils [22, 24]. It is readily apparent from this model that molecules the size of glucose can freely diffuse into all of the water in the lateral plane of the hydrated fibril. In contrast, the water in the hydrated fibril appears to be inaccessible to BGP. How then are both glucose and BGP able to attain equilibrium concentration in all of the water within the fibril? The likely explanation is that the quasihexagonal packing of collagen molecules observed in x-ray crystallographic studies (and reproduced in Figure 2.4) is the average position of these molecules in the lateral plane of the fibril.
structure, and that the actual position of a collagen molecule varies rapidly in time. As reviewed in the Introduction, hydration of the collagen fibril separates adjacent collagen molecules in the lateral plane by a water layer 7 Å thick (see Figure 2.5). The thickness of this water layer argues against non-covalent lateral associations along the full length of adjacent collagen molecules in the fibril, and suggests that collagen molecules have the flexibility to move relative to their neighbors to create aqueous cavities of rapidly fluctuating size within the fibril. As can be seen in Figure 2.5, minimal movements of collagen molecules are sufficient to accommodate BGP within the quasihexagonal lattice of the fibril.

Several studies support the hypothesis that collagen molecules have substantial freedom to move within the fibril. $^{13}$C nuclear magnetic resonance studies have shown that the polypeptide backbone of the collagen molecule is free to reorient within a fully hydrated collagen fibril in less than 0.1 milliseconds [38]. These motions are not observed in dry fibrils or in mineralized collagen fibrils, and are not affected by covalent cross links at the N and C termini of the collagen molecule [38]. Atomic force microscopy studies further show that collagen molecules are free to move relative to their neighbors when the fibril is bent or folded [8]. Finally, recent studies show that a 3 kDa fluorescently labeled dextran can diffuse along the length of the collagen fibril [39]. Diffusion of such a relatively large molecule within the fibril is consistent with the present observation that BGP can freely access all of the water within the collagen fibril, and further supports the hypothesis that individual collagen molecules have substantial freedom to move in the lateral plane of the fibril.
Figure 2.5: Effect of hydration on the packing of collagen molecules in the lateral plane of a collagen fibril. The collagen molecules in a cross section (overlap region) of a single collagen fibril are represented by 521 hard disks whose 1.1 nm diameter provides the scale factor of the model. The collagen molecules are arranged in a quasihexagonal lattice, the arrangement of collagen molecules seen in the lateral plane of the collagen fibril [14]. The hydrated fibril has a diameter of 44 nm and is 70% water by volume (Bragg spacing, 1.8 nm; packing fraction, ~0.7). The dry fibril has a diameter of ~30 nm (Bragg spacing, 1.1 nm; packing fraction, ~0.3). The maximum hard disk cross section of albumin, BGP, and glucose are drawn to scale in order illustrate the size difference between molecules that can fully penetrate (BGP and glucose) or not penetrate (albumin) the hydrated fibril. The fibril depicted here has the diameter [40] and water content (Table 2.4) of a typical bone collagen fibril. Since tendon fibrils are 75% water by volume (Table 2.1), a hydrated tendon fibril with the same number of collagen molecules would have a diameter of 48 nm.

Why are fetuin and albumin completely excluded from the volume of water that lies within the collagen fibril? As is apparent in the model shown in Figure 2.5, molecules the size of albumin (~60 Å diameter) and fetuin (probably >60 Å diameter, owing to the fact that it is 25% carbohydrate) are far too large to be
accommodated within the collagen fibril without crowding collagen molecules in the lateral plane (see Figure 2.5) and substantially reducing their freedom of motion (entropy).

**Impact of demineralization on the size, shape, and water content of bone:** Our next objective was to determine the size exclusion characteristics of the collagen matrix of bone, and to accomplish this goal it was clear that it would be first necessary to remove mineral from bone collagen, since the presence of mineral is an obvious barrier to the penetration of molecules into collagen. Experiments were accordingly carried out to determine the effect of demineralization on the water content and shape of bone. These experiments showed that bone shape and volume are not affected when an intact steer bone segment is demineralized in 0.6 N HCl at 20°C (Figure 2.2), or when a sample of ground steer bone sand is demineralized in 10% formic acid at 4°C (Table 2.5). These experiments also showed that demineralization of bone consistently replaced mineral with a comparable volume of water (Tables 2.3 and 2.5). These observations are logically connected, since the absence of a change in bone volume associated with the removal of mineral requires that the volume occupied by mineral be replaced with an equivalent volume of water. To our knowledge, the present study is the first to show that demineralization of bone replaces mineral with a comparable volume of water.

Several investigators have studied the effects of the reverse process, normal bone mineralization, on bone structure. In his seminal studies on bone, Robinson presented evidence that the collagenous matrix is first formed in its final shape and volume, and
then mineralized, and that the deposition of mineral is associated with the loss of a comparable volume of water from the collagenous bone matrix [1, 3]. Subsequent studies of bones with differing degrees of mineralization further showed that, for a fixed amount of bone collagen matrix, there is an inverse correlation between mineral content and water content [4].

The mineralization and demineralization of bone therefore appear to be reciprocal processes; one replaces water in collagen with mineral and the other mineral with water. The volume of water in collagen prior to mineralization is comparable to the volume of mineral in after demineralization, and the volume and shape of the bone prior to mineralization are comparable to the volume and shape of the collagen matrix after demineralization. Demineralized bone is therefore likely to be a good model for investigating the size exclusion characteristics of bone collagen prior to mineralization.

**The size exclusion characteristics of demineralized bone collagen:** The same biochemical procedures used to determine the size exclusion characteristics of tendon collagen were also used for demineralized bone collagen. The results of these experiments show that tendon and demineralized bone collagen have essentially identical size exclusion characteristics. Small molecules that range in size up to the 5,700 dalton bone Gla protein elute at the same volume as glucose. With the 227 ml column, this glucose elution volume is 191 ml, which is identical to the liquid volume in the column bed (Figure 2.2). In contrast, molecules the size of fetuin (48,000 daltons), albumin (66,000 daltons), and high molecular weight dextran (5-40 x 10^6"
57 daltons) elute at about 111ml, which is 80 ml less than the elution volume of glucose, BGP, and other small molecules. The simplest explanation for these observations is that the demineralized bone collagen in the column contains 80 ml of water that is accessible to molecules the size of the 5.7 kDa BGP or smaller, and inaccessible to molecules the size of the 48 kDa fetuin or larger.

The 80 ml volume of water in the demineralized bone collagen column that can be freely accessed by small molecules but not by large probably lies within the collagen fibril. The collagen location of this water is supported by the fact that an 80 ml volume of water is calculated to lie within the collagen of the demineralized bone column (see Results and Table 2.4). The fibril location of this collagen water is in turn supported by X ray diffraction studies that show that hydration produces a comparable increase in the Bragg spacing of collagen molecules in the lateral plane of tendon and demineralized bone collagen fibrils [41].

The comparable Bragg spacing in the fully hydrated fibrils in tendon and demineralized bone shows that both have a comparable layer of water separating adjacent collagen molecules in the lateral plane of the fibril. Because the internal structure of the collagen fibrils in both tissues are therefore essentially identical [42], the fibrils in both tissues would be expected to impose a comparable barrier to the penetration of large molecules but not small and give rise to indistinguishable size exclusion properties (Figure 2.5).

**The size exclusion characteristics of non-demineralized bone collagen:** In order to evaluate the impact of mineral on the size exclusion properties of bone collagen, we
prepared a column of non-demineralized bone that contained the same amount of collagen as the demineralized bone column (see Table 2.5). We then compared the elution volume of different test molecules on the columns packed with non-demineralized and demineralized bone collagen. The results of these experiments showed that the presence of mineral in the same amount of collagen dramatically reduces the elution volume of glucose but does not comparably affect the elution volume of fetuin, albumin, and high molecular weight dextran. The average reduced separation due to the presence of mineral, 70 ml, is comparable to the reduced volume of water in the column bed (67 ml, Table 2.7), and the reduced volume of water is due to the volume occupied by mineral (62 ml, Table 2.7). Mineral therefore occupies a space in bone collagen that is occupied by water in demineralized bone collagen, and this water compartment is accessible to glucose but not fetuin, albumin, or high molecular weight dextran.

The size exclusion characteristics of the collagen fibril: insights into the function of non-collagenous bone constituents in bone mineralization: The type I collagen fibril plays several critical roles in bone mineralization. The mineral in bone is located primarily within the fibril (1-6), and during mineralization the fibril is formed first and then water within the fibril is replaced with mineral [1, 2]). The collagen fibril therefore provides the aqueous compartment in which mineral grows. The present study shows that the physical structure of the collagen fibril plays an important additional role in mineralization: the role of a gatekeeper that allows molecules smaller than a 6 kDa protein to freely access the water within the fibril.
while preventing molecules larger than a 40 kDa protein from entering the fibril. Molecules smaller than a 6 kDa protein can therefore interact directly with apatite crystals growing within the fibril while molecules larger than a 40 kDa protein cannot. Proteins that are too large to penetrate the collagen fibril can still have important roles in bone mineralization. Some large bone proteins, such as osteopontin [43, 44] and fetuin (25-27,42), potently inhibit apatite formation or growth in vitro. We propose that such large protein inhibitors of calcification may paradoxically promote mineralization of the collagen fibril by selectively inhibiting apatite growth everywhere but within the fibril. Other proteins that are too large to penetrate the fibril may nucleate mineral formation, proteins such as bone sialoprotein [45, 46] and the recently discovered serum nucleator of collagen calcification [26] as well as large structures such as matrix vesicles [47]. We propose that such proteins generate apatite crystal nuclei outside of the collagen fibril, and that some of these small crystals can then diffuse into the interior of the fibril and grow. Since BGP diffuses into all of the water within the collagen fibril, it seems likely that apatite crystals up to the size of BGP (about 12 hydroxyapatite unit cells; see Footnote 3) can also diffuse throughout the fibril. Experiments are in progress to test both of these hypotheses for the function of large proteins in mineralization of the collagen fibril.

Chapter II Acknowledgement:

Chapter II, the manuscript (published online), Toroian, DA; Lim, JE; and Price, PA “The size exclusion characteristics of type I collagen: Implications for the role of non-collagenous bone constituents in mineralization” J. Biol. Chem., Jun 2007;
doi:10.1074/jbc.M700591200 is used with the permission of the my co-authors, Dr. Paul A. Price, my dissertation advisor, and Ms. Joo Eun Lim. The dissertation author was the primary investigator and author of this paper.

References


Footnotes

1. The fraction of demineralized bone water calculated to lie within osteocyte lacunae is less than 1% of the total [48].

2. The diameter of a typical fibril is 50 nm. At a flow rate of 6.7 ml/h, it takes 8 milliseconds for a layer of water to travel 50 nm in the 2 cm diameter column.

3. Because the volume of BGP (~6500 Å³) is over 12 times greater than the volume of a hydroxyapatite unit cell (529.2 Å³) [49], a hydroxyapatite crystal the size of BGP contains about 12 hydroxyapatite unit cells.
Chapter III

The Essential Role of Fetuin in the Serum-Induced Calcification of Collagen
Abstract

We carried out the present experiments in order to understand the biochemical mechanism that causes type I collagen fibrils to calcify rapidly when incubated in serum. The serum activity that induces collagen calcification consists of proteins that are too large to penetrate the collagen fibril where mineral growth occurs (see companion paper). We therefore investigated how proteins that act only outside the fibril can cause calcification to occur specifically within the fibril.

Our hypothesis was that fetuin, a potent calcification inhibitor that is too large to penetrate the fibril, promotes fibril calcification by selectively inhibiting apatite growth outside of the fibril. We tested this hypothesis by examining the impact of removing fetuin on the ability of serum to mineralize collagen. The results of this test show that the presence of fetuin in serum determines the location of serum-driven mineralization: in the presence of fetuin, mineral forms only within the collagen fibril; in the absence of fetuin, mineral forms only in the solution outside the fibril.

These observations show that serum calcification activity consists of at least two large proteins. One as yet unidentified protein generates crystal nuclei in the solution outside the fibril, some of which diffuse into the fibril. The other protein, fetuin, inhibits the growth of crystal nuclei that remain in the solution outside the fibril, thereby freeing calcium and phosphate ions for crystal growth within the fibril. This mineralization mechanism provides an insight into the way collagen calcifies in vivo.
Introduction

The type I collagen fibril plays several critical roles in bone mineralization. The mineral in bone is located primarily within the fibril [1-6], and during mineralization the fibril is formed first and then water within the fibril is replaced with mineral [7, 8]). The collagen fibril therefore provides the aqueous compartment in which mineral grows. We have recently shown that the physical structure of the collagen fibril plays an important additional role in mineralization: the role of a gatekeeper that allows molecules smaller than a 6 kDa protein to freely access the water within the fibril while preventing molecules larger than a 40 kDa protein from entering the fibril (Chapter 2). Molecules smaller than a 6 kDa protein can therefore enter the fibril and interact directly with mineral to influence crystal growth. Molecules larger than a 40 kDa protein cannot enter the fibril and so have no ability to act directly on the apatite crystals growing within the fibril.

Molecules too large to enter the collagen fibril can still have important effects on mineralization within the fibril. We have suggested that large inhibitors of apatite growth can paradoxically favor mineralization within the fibril by selectively preventing apatite growth outside of the fibril (Chapter 2). We have also proposed that large nucleators of apatite formation may generate small crystal nuclei outside of the collagen fibril and that some of these nuclei subsequently diffuse into the fibril and grow (Chapter 2). Because the size exclusion characteristics of the fibril allow rapid penetration of molecules the size of a 6 kDa protein, apatite crystals up to 12 unit cells
in size should in principle be able to freely access all of the water within the fibril (Chapter 2). The present study tests these hypotheses for the possible function of large molecules in mineralization.

The calcification assay we have employed to test the function of large proteins in collagen mineralization is based on our discovery that the type I collagen fibrils of tendon and demineralized bone calcify when incubated in serum (or plasma) for 6 days at 37° C and pH 7.4 [9, 10]. The calcification activity responsible for collagen mineralization in serum consists of one or more proteins that are 50 to 150 kDa in size [10]. Because these molecules are too large to penetrate the collagen fibril, they must be able to act outside the fibril to cause calcification within the fibril. The serum-driven calcification of a collagen fibril is therefore an excellent model system to explore the mechanisms by which molecules too large to penetrate the collagen fibril can nonetheless cause the fibril to calcify.

Although serum-driven collagen calcification is an in vitro, cell-free assay, there are several reasons to believe that it could be relevant to understanding mechanisms by which collagen fibrils are mineralized in normal bone formation. 1. The assay conditions are physiologically relevant: collagen added to serum calcifies when incubated at the temperature and pH of mammalian blood, without the need to add anything to serum to promote mineralization, such as β glycerophosphate or phosphate (see [9] and references therein). 2. Serum is relevant to bone mineralization: osteoblasts form bone in a vascular compartment [11], and proteins in serum have direct access to the site of collagen fibril formation and mineralization while proteins
secreted by the osteoblast appear rapidly in serum. 3. **Serum-driven calcification is evolutionarily conserved:** the serum calcification activity appeared in animals at the time vertebrates acquired the ability to form calcium phosphate mineral structures, with no evidence for a similar activity in the serum of invertebrates [12]. 4. **Serum-driven calcification is specific:** calcification is restricted to those structures that were calcified in bone prior to demineralization, with no evidence of calcification in cartilage at the bone ends or in cell debris [9, 10]. 5. **Serum-driven calcification can achieve the total re-calcification of demineralized bone:** serum-driven calcification progresses until the re-calcified bone is comparable to the original bone prior to demineralization in mineral content and composition, radiographic density, and powder X-ray diffraction spectrum [10].

The initial goal of the present experiments was to examine the possible function of the 48 kDa protein fetuin in the serum-driven calcification of collagen matrices. Our working hypothesis was that fetuin promotes calcification within the collagen fibril by selectively inhibiting apatite growth outside of the fibril. This hypothesis is supported by the observation that fetuin is the most abundant serum inhibitor of apatite crystal growth [13, 14], and by the observation that fetuin is too large to penetrate the interior of the collagen fibril (Chapter 2) where serum-induced collagen calcification occurs [10]. The present study tests this hypothesis by examining the impact of removing fetuin from serum on the ability of serum to mineralize the collagen fibril. The results of this test show that the presence of fetuin in serum determines the location of serum-driven mineralization: in the presence of fetuin, mineral forms only within the
collagen fibril; in the absence of fetuin, mineral forms only in the solution outside the fibril.

Because fetuin is the subject of this study, it is useful to review briefly its structure, occurrence, and calcification-inhibitory activity. Fetuin is a 48 kDa glycoprotein that consists of 2 N-terminal cystatin domains and a smaller C-terminal domain. The five oligosaccharide moieties of the protein account for ~25% of fetuin’s mass and, because of their disordered structures, give fetuin an apparent size in SDS gel electrophoresis and Sephacryl gel filtration of about 59 kDa. Fetuin is synthesized in the liver and is found at high concentrations in mammalian serum [15, 16]and bone [17-22]. The serum fetuin concentration in adult mammals ranges from 0.5 to 1.5 mg/ml, while the serum fetuin concentration in the fetus and neonate is typically far higher [16]. Fetuin is also one of the most abundant non-collagenous proteins found in bone [17-22], with a concentration of about 1 mg fetuin per g bone in rat [21], bovine [17], and human [19, 23] bone. In spite of the abundance of fetuin in bone, however, it has not been possible to demonstrate the synthesis of fetuin in calcified tissues, and it is therefore presently thought that the fetuin found in bone arises from hepatic synthesis via serum [20, 22]. This view is supported by the observation that fetuin binds strongly to apatite, the mineral phase of bone, and is selectively concentrated from serum onto apatite in vitro [18].

In vitro studies have demonstrated that fetuin is an important inhibitor of apatite growth and precipitation in serum containing increased levels of calcium and phosphate [14], and that targeted deletion of the fetuin gene reduces the ability of
serum to arrest apatite formation by over 70% [13]. More recent studies have shown that a fetuin-mineral complex is formed in the course of the fetuin-mediated inhibition of apatite growth and precipitation in serum containing increased calcium and phosphate [24]. Purified bovine fetuin has also been shown to be a potent inhibitor of the growth and precipitation of a calcium phosphate mineral phase from supersaturated solutions of calcium phosphate [14], and recent studies have shown that a fetuin mineral complex is formed in the course of this inhibition [25].

**Experimental Procedures**

**Materials:** Forty-day-old and newborn albino rats (Sprague-Dawley derived) were purchased from Harlan Labs. Adult bovine serum was purchased from Invitrogen. Each 500ml volume of Dulbecco's modified eagle medium (DMEM; Gibco) was supplemented with 5ml of penicillin-streptomycin (Gibco) and 1ml of 10% sodium azide to prevent bacterial growth. Unless otherwise stated, the concentration of phosphate in DMEM was increased from the basal 0.9mM to a final 2mM by the addition of 0.5 M sodium phosphate buffer pH 7.4. When prepared as described [26], DMEM containing 2mM phosphate is stable for at least 3 weeks at 37°C, with no evidence for loss of calcium or phosphate from the medium or formation of a mineral phase. Bovine fetuin, purified type I collagen from bovine achilles tendon, and Alizarin red S were purchased from Sigma.

Rats were killed by exsanguination while under isoflurane anesthetic; the UCSD Animal Subjects Committee approved all animal experiments. Tail tendons were
dissected from 40-day-old rats and tibias were dissected from newborn rats. Both
tissues were extracted with a 1000-fold excess (v/w) of 0.5 M EDTA pH 7.5 for 72h at
room temperature to kill cells and remove any mineral that might be present; the
tissues were then washed exhaustively with ultra pure water to remove all traces of
EDTA and stored at –20°C until use.

**Calcification procedures:** Experiments to examine the calcification of collagen
matrices were carried out using 24-well cell culture clusters (Costar 3524, Corning) in
a humidified incubator at 37°C and 5% CO₂. Each well contained a 1 ml volume of
DMEM alone or of DMEM containing 10% by volume bovine serum or fetuin-
depleted bovine serum. The amount of matrix added to each 1 ml volume was: a
single hydrated, demineralized newborn rat tibia; a portion of tail tendon (3 mg dry
weight; hydrated before use); or a portion of type I collagen (3 mg dry weight;
hydrated before use). Each tissue was then incubated for 6 days.

**Biochemical analyses:** The procedures used for Alizarin red staining have been
described.[27] For histological analyses, tibias were fixed in 100% ethanol for at
least 1 day at room temperature; San Diego Pathology Inc. (San Diego, CA) sectioned
and von Kossa stained the tibias. For quantitative assessment of the extent of
calcification, Alizarin red stained matrices and precipitates formed outside the matrix
were extracted for 24h at room temperature with 1 ml of 0.15 M HCl, as described
[27]. Calcium levels in culture media and in the acid extracts of tissues and
precipitates were determined colorimetrically using cresolphthalein complexone (JAS
Diagnostics, Miami FL) and phosphate levels were determined colorimetrically as described [28].

**Immunological procedures:** Rabbits were immunized against purified bovine fetuin. The procedures employed for the bovine fetuin radioimmunoassay used this antiserum at a final 1:2000 dilution. The radioimmunoassay diluent, sample volumes, and procedures are identical to those used in the rat fetuin radioimmunoassay [24]. For affinity purification of anti fetuin antibody, 16 mg of purified bovine fetuin were covalently attached to 5 ml of cyanogen bromide activated Sepharose 4B (Amersham Biosciences) and packed into a column. 10 ml of anti fetuin antiserum was than passed over this fetuin affinity column, and the bound antibody was eluted with 100 mM glycine pH 2.5. An anti-fetuin antibody column was subsequently prepared by covalently attaching 7 mg of purified anti fetuin antibody to 5ml of CNBr-activated Sepharose 4B. The anti-fetuin antibody column was then equilibrated with the DMEM calcification buffer, and bovine serum was dialyzed against the same buffer. Adult bovine serum was freed of fetuin by passing 0.85 ml aliquots of dialyzed serum over the column at room temperature. The absorbance at 280 nm of each 0.8 ml fraction was then determined, and the fetuin content of the fractions was measured by radioimmunoassay. The 4 fractions with the highest absorbance were pooled, and then diluted with DMEM until the absorbance at 280 nm equaled that of 10% bovine serum. Protein bound to the column was removed by washing the column with 100 mM glycine pH 2.5. The desorbed protein was dialyzed against 5 mM ammonium
bicarbonate and dried; a portion of the desorbed protein was electrophoresed using a 4 to 12% polyacrylamide gel, as described [24].

The 10% control serum used in these studies was prepared by the same procedures, with the sole exception being that the control column was prepared by covalently attaching 7 mg of purified rabbit IgG (Sigma) to 5 ml of CNBr-activated Sepharose 4B rather than 7 mg of rabbit anti-bovine fetuin antibody. 0.85 ml aliquots of dialyzed adult bovine serum were passed over the control column at room temperature, and the 4 fractions with the highest absorbance were pooled and diluted with DMEM until the absorbance at 280 nm equaled that of 10% bovine serum.

**Results**

**Removal of fetuin from bovine serum by antibody affinity chromatography:**

We developed procedures to remove fetuin from bovine serum by antibody affinity chromatography in order to evaluate the possible role of the protein in serum-induced calcification. Rabbits were immunized with purified bovine fetuin, and the resulting antisera were used to construct a radioimmunoassay for bovine fetuin that could be used to monitor the effectiveness of fetuin depletion procedures (Figure 3.1). Polyclonal anti fetuin antibodies were purified from the rabbit antiserum using Sepharose 4B with covalently attached bovine fetuin, and the resulting purified anti fetuin antibodies were then attached covalently to Sepharose 4B and packed into a column.
Figure 3.1: Radioimmunoassay of bovine fetuin, and detection of bovine fetuin antigen in adult bovine serum. Relative fraction of $^{125}$I labeled bovine fetuin bound to antibody ($B/B_0$) at increasing amounts of purified bovine fetuin, and at increasing volumes of adult bovine serum.

Because the goal of fetuin removal from serum was to test its role in serum-induced calcification, we used a suitable buffer for study of serum-induced calcification [9, 10], DMEM culture medium, to equilibrate the anti fetuin antibody column. Adult bovine serum was then dialyzed against DMEM and passed over this column to remove fetuin. The results of a typical experiment are shown in Figure 3.2. As can be seen, there is a massive peak of unbound serum protein absorbance that elutes at the column volume; this unbound protein peak accounts for about 98% of the A280 applied to the column and is devoid of fetuin. The four fractions with the highest absorbance were pooled; calcification solutions containing fetuin-depleted bovine serum were then prepared by diluting these pooled fractions with DMEM to yield a final serum concentration of 10% by absorbance. The 10% control bovine serum used in these studies was prepared by a similar procedure, with the sole difference being that the control column was prepared by covalently attaching purified normal rabbit
IgG to Sepharose rather than rabbit anti-bovine fetuin antibody. Figure 3.1 shows that the fetuin content of the resulting fetuin-depleted 10% bovine serum is over 1000-fold lower than the fetuin content of the 10% control bovine serum.

**Table 3.1** The concentration of fetuin in the experimental calcification solutions used in these studies. The concentrations of bovine fetuin were determined by radioimmunoassay in each of the experimental solutions employed in this study: 10% adult bovine serum in DMEM culture medium; 10% fetuin-depleted bovine serum in DMEM; and 10% fetuin-depleted bovine serum in DMEM containing 130 µg/ml of purified bovine fetuin. Each sample was assayed in triplicate.

<table>
<thead>
<tr>
<th>Additions to DMEM</th>
<th>Fetuin (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10% Control serum</td>
<td>126.0 ± 3.2</td>
</tr>
<tr>
<td>10% Fetuin depleted serum</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>10% Fetuin depleted serum, supplemented with purified fetuin</td>
<td>139.8 ± 9.9</td>
</tr>
</tbody>
</table>

After elution of those proteins that did not bind to the column, the anti fetuin antibody column was washed with DMEM until the absorbance at 280nm was less than 0.01, and bound fetuin was then eluted from the column by washing with acid (Figure 3.2). The resulting small peak of A280nm absorbance (not evident in the scale used for Figure 3.2) accounted for about 1% of the initial serum absorbance. The amount of fetuin immunoreactivity in this peak corresponded to the fetuin content of the serum applied to the column, and the SDS gel of the bound protein fraction revealed a single major component in the 59 kDa position expected for fetuin [24].
Figure 3.2. Removal of Fetuin from bovine serum by antibody affinity chromatography. In order to prepare fetuin-depleted bovine serum for tests of the role of fetuin in serum-induced mineralization, adult bovine serum was dialyzed against a buffer suitable for calcification (DMEM) and then passed over a column that containing 7 mg of affinity purified rabbit anti bovine fetuin antibody attached covalently to 5ml of Sepharose 4B (see Methods). Elution buffer, DMEM; fraction volume, ~0.8 ml; fetuin concentration was determined by radioimmunoassay (Figure 3.1). Inset: Fractions 19-24 were pooled and 10 µg protein from this pool was electrophoresed on a 4-12% SDS polyacrylamide gel and stained with coomassie brilliant blue. Note that the major band is in the 59 kDa position expected for bovine fetuin [24].

**Evidence that fetuin is required for the serum-induced re-calcification of demineralized bone.** In the initial study, the impact of fetuin depletion on serum-induced calcification was evaluated by incubating demineralized newborn rat tibias for 6 days at 37°C in DMEM alone, in DMEM containing 10% control bovine serum, or in DMEM containing 10% fetuin-depleted bovine serum. In agreement with earlier studies [9, 10], demineralized tibias calcified after incubation in DMEM containing 10% serum but did not calcify after incubation in DMEM alone (Figures 3 and 4). The pattern of Alizarin red staining in the tibias incubated in DMEM containing 10%
control serum matches that seen in the original tibia prior to demineralization (not shown; see [9, 10] for examples).

In contrast to tibias incubated in 10% control serum, tibias incubated in 10% fetuin-depleted serum did not have significant incorporation of calcium and phosphate (Figure 3.3) and did not stain for calcification by Alizarin red (Figure 3.4); histological sections of these tibias also revealed no von Kossa staining for calcification (Figure 3.4). Removal of fetuin from serum therefore eliminates the serum-induced re-calcification of demineralized bone.

To confirm the essential role of fetuin in serum-induced calcification, we added sufficient purified bovine fetuin to the fetuin-depleted bovine serum in order to attain a final fetuin concentration comparable to that found in the original serum prior to fetuin depletion and in the 10% bovine serum control (see Table 3.1). The calcification of tibias incubated in this fetuin-repleted serum was indistinguishable from the calcification of tibias incubated in the 10% bovine serum control: the pattern of Alizarin red staining was identical (Figure 3.4), the amount of calcium and phosphate incorporated was comparable (Figure 3.3), and the von Kossa staining was restricted to the collagen matrix (Figure 3.4). Comparable results were obtained when fetuin purified during the course of the preparation of fetuin-depleted serum (see Figure 3.2 inset) was substituted for commercial fetuin (data not shown). The addition of purified fetuin therefore fully restores the ability of fetuin-depleted serum to induce the re-calcification of a demineralized tibia.
In the course of these experiments, we noticed the presence of a fine precipitate coating the entire bottom of each culture well that contained a tibia incubated in DMEM plus 10% fetuin-depleted serum (not shown); no precipitate could be detected in wells that contained a tibia incubated in DMEM alone, in wells that contained a tibia incubated in DMEM plus 10% control bovine serum, or in wells that contained a tibia incubated in DMEM plus 10% fetuin-depleted serum supplemented with purified bovine fetuin. To assess the nature of this precipitate, the precipitate was collected, stained with Alizarin red, and analyzed for calcium and phosphate. This analysis showed that the precipitate isolated from the wells containing 10% fetuin-depleted serum stained intensely with Alizarin red and that the amounts of calcium and phosphate recovered from the precipitate were comparable to the amounts incorporated into tibias that had been incubated in DMEM containing 10% serum or 10% fetuin-repleted serum (Figure 3.3). This result suggests that the role of fetuin in the serum-induced re-calcification of demineralized bone is to direct mineral formation into the collagen matrix of bone.
Figure 3.3. Evidence that fetuin is required for the serum-induced re-calcification of demineralized bone: analysis for Ca and P. In order to evaluate the possible role of fetuin in the serum-induced re-calcification of bone, demineralized newborn rat tibias were separately incubated for 6 days at 37°C in 1 ml DMEM containing 2 mM Pi and: no serum; 10% control bovine serum; 10% fetuin-depleted bovine serum; 10% fetuin-depleted bovine serum plus 130 µg/ml of purified bovine fetuin. Tibias were removed, stained with Alizarin red, photographed, and then analyzed for calcium and phosphate. The media and any precipitate were removed from the well, centrifuged to pellet any precipitate, and the pellet fraction was analyzed for calcium and phosphate (see Methods). This experiment was performed in triplicate. The data show the average calcium and phosphate in the tibia and the pellet fraction from each well; the error bars show the standard deviations. * = Ca or Pi in the extract is less than 0.01 µmol.

A final experiment was carried out to evaluate the effect of reducing the phosphate concentration of the DMEM medium from 2 mM to 0.9 mM [9]. This experiment showed that tibias do not calcify when incubated in DMEM (0.9 mM Pi) containing 10% control bovine serum, 10% fetuin-depleted bovine serum, or 10% fetuin-depleted serum plus added fetuin (not shown). There was also no evidence for a mineral precipitate in any condition. These results demonstrate that the serum-induced
formation of a mineral phase in DMEM will not occur unless the phosphate content of the DMEM medium is at the 2 mM concentration found in bovine serum.

Figure 3.4. Evidence that fetuin is required for the serum-induced calcification of demineralized bone: Alizarin red and von Kossa staining. Demineralized newborn rat tibias were separately incubated for 6 days at 37°C in 1 ml DMEM containing 2mM Pi and: 10% control bovine serum; 10% fetuin depleted bovine serum; 10% fetuin depleted bovine serum containing 130 µg/ml of purified bovine fetuin. After incubation, the tibias were either stained for calcification with Alizarin red or fixed in ethanol, cut in 5 micron thick sections, stained for calcification with von Kossa (stains calcification black), and counter stained with nuclear-fast red.

Evidence that fetuin is required for the serum-induced calcification of tendons and purified collagen. Additional experiments were carried out to further explore the role of fetuin in the serum-induced calcification of collagenous matrices. One test examined the role of fetuin in the serum-induced calcification of rat tail tendon, a type I collagen matrix that is chemically identical to the type I collagen matrix of bone but
does not normally calcify in rats. Tendons incubated in 10% control bovine serum calcified; tendons incubated in 10% fetuin-depleted serum did not calcify, and tendons incubated in 10% fetuin-depleted serum containing purified fetuin calcified (Figure 3.5). There was again a fine precipitate coating the bottom of all wells containing fetuin-depleted serum, and the amount of calcium and phosphate in this precipitate was comparable to that found in tendons incubated in 10% serum that contained fetuin (Figure 3.5).

Figure 3.5. Evidence that fetuin is required for the serum-induced calcification of rat tail tendon. To test the role of fetuin in the serum-induced calcification of tendon, a type I collagen matrix that does not normally calcify, rat tail tendons (dry weight, 3mg) were separately incubated for 6 days at 37°C in 1 ml DMEM containing 2mM Pi and: no serum; 10% control bovine serum; 10% fetuin-depleted bovine serum; 10% fetuin-depleted bovine serum plus 130 µg/ml of purified bovine fetuin. Tendons were removed, stained with Alizarin red, photographed, and then analyzed for calcium and phosphate. The media and any precipitate were removed from the well, centrifuged to pellet any precipitate, and the pellet fraction was analyzed for calcium and phosphate (see Methods). This experiment was performed in triplicate. The data show the average calcium and phosphate in the tendons and the pellet fraction from each well; the error bars show the standard deviations. * = Ca or Pi in the extract is less than 0.01 µmol.
Another test examined the role of fetuin in the serum-induced calcification of purified type I collagen fibers from bovine Achilles tendon. Purified collagen fibers incubated in 10% control bovine serum calcified; fibers incubated in 10% fetuin-depleted serum did not calcify, and fibers incubated in 10% fetuin-depleted serum containing purified fetuin calcified (Figure 3.6). There was a fine precipitate coating the entire bottom of all wells containing fetuin-depleted serum, and the amount of calcium and phosphate in this precipitate was comparable to that found in collagen fibers incubated in 10% serum containing fetuin (Figure 3.6).

Figure 3.6. Evidence that fetuin is required for the serum-induced calcification of purified bovine type I collagen. To assess the role of fetuin in the serum-induced calcification of collagen fibers, 3 mg amounts of purified bovine type I collagen were separately incubated for 6 days at 37°C in 1 ml DMEM containing 2mM Pi and: no serum; 10% control bovine serum; 10% fetuin-depleted bovine serum; 10% fetuin-depleted bovine serum plus 130 µg/ml of purified bovine fetuin. Collagen fibers were removed, stained with Alizarin red, photographed, and then analyzed for calcium and phosphate. The media and any precipitate were removed from the well, centrifuged to pellet any precipitate, and the pellet fraction was analyzed for calcium and phosphate (see Methods). This experiment was performed in triplicate. The data show the average calcium and phosphate in the purified collagen and the pellet fraction from each well; the error bars show the standard deviations. * = Ca or Pi in the extract is less than 0.01 µmol.
Taken together, these results show that fetuin plays a similar essential role in the serum-induced calcification of the type I collagen fibers in a tissue that was once calcified (demineralized bone), a tissue that does not normally calcify (tendon), and in purified collagen. In each case the essential role of fetuin in the serum-induced calcification is to direct mineral formation into the collagen matrix, and it appears to do this by preventing mineral precipitation outside of this matrix.

Evidence that the removal of fetuin from serum unmasks a potent serum nucleator of mineral formation: In each of the above experiments, the removal of fetuin from serum prevented the calcification of the collagen matrix, but led to the formation of a fine precipitate of a calcium phosphate mineral on the bottom of the well. In order to see if the formation of this precipitate is dependent on the presence of a matrix, this experiment was repeated using the same calcification solutions but no matrix. A fine precipitate coated the entire bottom of all wells that contained DMEM plus 10% fetuin-depleted serum, while no precipitate could be detected in the wells that contained DMEM alone, DMEM plus 10% control bovine serum, or DMEM with 10% fetuin-depleted serum plus added purified fetuin. This precipitate stained intensely with Alizarin red and chemical analysis showed that it contained calcium and phosphate (Figure 3.7) in amounts comparable to those previously seen in wells that contained fetuin-depleted serum and a collagen matrix. This result demonstrates that the formation of a precipitate in DMEM containing 10% fetuin-depleted serum is not dependent on the presence of a collagen matrix. The removal of fetuin from serum
therefore appears to unmask a potent serum initiator of calcium phosphate mineral formation.

**Figure 3.7. Evidence that fetuin depletion unmasks a potent serum initiator of mineral formation.** To determine whether a calcium phosphate mineral phase forms spontaneously in fetuin-depleted serum even in the absence of a collagen matrix, the following solutions were prepared that contained 1 ml DMEM with 2mM Pi and: no serum; 10% control bovine serum; 10% fetuin depleted bovine serum; 10% fetuin depleted bovine serum containing 130 µg/ml of purified bovine fetuin. The solutions were incubated for 6 days at 37°C in the absence of a collagen matrix. The media and any precipitate were removed from the well, centrifuged to pellet any precipitate, and the pellet fraction was analyzed for calcium and phosphate (see Experimental Procedures for details). This experiment was performed in triplicate. The data show the average calcium and phosphate in the pellet fraction from each well; the error bars show the standard deviations. * = Ca or Pi in the extract is less than 0.01 µmol.

**Discussion**

This study and the companion study that precedes it in this journal were both carried out with the goal of understanding the biochemical basis for the ability of serum to induce the calcification of a type I collagen fibril. The first study demonstrates that the physical structure of the collagen fibril is such that molecules
smaller than a 6 kDa protein can freely access all of the water within the fibril while molecules larger than a 40 kDa protein cannot enter the fibril. This study therefore shows that molecules smaller than a 6 kDa protein can enter the fibril and interact directly with mineral to influence crystal growth, while molecules larger than a 40 kDa protein cannot enter the fibril and so have no ability to act directly on the apatite crystals growing within the fibril.

The serum calcification activity that induces calcification of the collagen fibril consists of one or more proteins that are 50 to 150 kDa in molecular weight. Since these molecules are too large to penetrate the collagen fibril, there must be mechanisms by which proteins that act only outside the fibril can cause calcification to occur specifically within the fibril. One possibility is that large inhibitors of apatite growth favor mineralization within the fibril by selectively preventing apatite growth outside of the fibril. In addition, large nucleators of apatite formation may generate small crystal nuclei outside of the collagen fibril that subsequently diffuse into the fibril and grow. The present study tests these hypotheses for the possible function of large molecules in mineralization.

Our working hypothesis was that the serum protein fetuin promotes calcification within the collagen fibril by selectively inhibiting apatite growth outside of the fibril, and we tested this hypothesis by examining the impact of removing fetuin from serum on the ability of serum to mineralize the collagen fibril. The results of this study reveal that removing fetuin from serum completely prevents the serum-driven calcification of a type I collagen matrix. Removing fetuin from serum does not prevent the serum-
driven formation of mineral, however, because a comparable amount of apatite mineral consistently forms on the bottom of all wells that contain fetuin-depleted serum. The results of these experiments therefore support our working hypothesis, namely that large protein inhibitors of apatite growth such as fetuin can favor mineralization of the collagen fibril by selectively preventing apatite growth outside of the fibril. The net effect of this fetuin activity is extraordinary: all of the calcium and phosphate ions that, in the absence of fetuin, are incorporated into a mineral that forms throughout the ~1 ml volume that lies outside the fibril are, in the presence of fetuin, incorporated into a mineral that forms within the ~5 ul volume of water that lies within the 3 mg collagen in the well.

Previous in vitro studies using pure fetuin in solutions containing high levels of calcium and phosphate provide an insight into how fetuin may act to direct apatite growth within the collagen fiber. In these experiments, solutions were prepared that substantially exceed the calcium phosphate ion product required for homogeneous formation of an apatite-like mineral phase, and in the absence of fetuin a mineral phase forms in minutes [25]. When fetuin is added to these solutions, no mineral phase precipitates, no mineral phase can be sedimented by high speed centrifugation, and the solution remains clear for about 24 hours. At this time the solution becomes opalescent and a fetuin-mineral complex can, for the first time, be sedimented from the solution by centrifugation [25]. Measurement of ionic calcium and phosphate levels during the first 24 hours further show that small amounts of a mineral phase still form in the presence of fetuin, and that the role of fetuin is to form a complex with
these nascent mineral nuclei that retards their growth and prevents their precipitation (or sedimentation in a centrifuge) [25]. Purified fetuin therefore does not prevent mineral nuclei from forming in this homogeneous nucleation system. It traps the nascent mineral nuclei and dramatically retards their growth.

We believe that the role of fetuin in serum-driven calcification of a type I collagen matrix is similar to its action on a homogeneous apatite nucleation system: fetuin traps mineral nuclei and retards their growth. The major difference is that mineral nuclei are generated by the serum nucleator activity, not by a high calcium phosphate ion product. The serum nucleator elutes from a gel filtration column in the position expected for proteins 50 to 150 kDa in size, and is therefore clearly too large to physically penetrate the collagen fibril. The product of nucleator action outside the fibril are presumably small crystal nuclei, however, and even apatite crystals up to 12 unit cells in size should in principle be able to freely access all of the water within the fibril (see Introduction). Since fetuin can only trap those nuclei that it can access, the crystal nuclei that penetrate the fibril are free to grow far more rapidly than those nuclei trapped by fetuin outside of the fibril, and the collagen fibril therefore selectively calcifies. When fetuin is removed from serum, the same number of mineral nuclei still form, and some of these no doubt still penetrate the fibril. All crystal nuclei are now free to grow, however. Because the vast majority of the nuclei are in the solution outside of the fiber, the only mineral formed in amounts that can be detected is the mineral precipitate found on the bottom of the well, not mineral within the fibril.
Summary and conclusion: a hypothesis for the mechanism of normal bone mineralization: The present study was carried out to understand the mechanism by which a serum calcification factor activity consisting of proteins 50 to 150 kDa in size is able to drive the calcification of a collagen fibril. The results of this study show that serum calcification factor activity consists of at least two large proteins, neither of which can penetrate the collagen fibril. One as yet unidentified protein generates crystal nuclei outside of the fibril, some of which then diffuse into the fibril. The other protein, fetuin, inhibits the growth of crystal nuclei that remain in the solution outside of the fibril, thereby freeing calcium and phosphate ions for crystal growth within the fibril.

It seems likely that mineralization of the collagen fibril occurs by a similar mechanism in vivo. Nucleators too large to penetrate the fibril, such as the serum calcification factor activity, generate small crystals near the mineralization front, some of which penetrate the fibril. Crystal growth inhibitors, such as fetuin, bind to crystals that remain in the solution outside of the fibril, thereby ensuring that only crystals within the fibril can grow. As with many other critical processes in biochemical physiology, there are probably multiple layers of redundancy in the process of normal bone mineralization. This redundancy ensures the complete replacement of water within the fibril with mineral in order to achieve a composite material with exceptional mechanical properties [29]. In addition to the serum nucleator activity, nucleators may include large proteins such as bone sialoprotein [30, 31] as well as large
structures such as matrix vesicles [32]. Inhibitors of apatite crystal growth are likely to include a number of other non-collagenous bone proteins, in addition to fetuin.

The fetuin-depleted serum assay employed in the present study may prove to be a powerful tool in the search for the biochemical mechanism by which different molecules inhibit the pathway of mineral nucleation, growth, and fibril calcification. Some molecules may prove to inhibit crystal growth but not crystal nucleation; when added to fetuin-deficient serum, these molecules will restore the serum-driven calcification of the collagen fibril and prevent the growth and precipitation of mineral outside of the fibril. Other molecules may prove to inhibit crystal nucleation; when added to fetuin-depleted serum, these molecules will inhibit both the precipitation of mineral outside of the fibril and the calcification of the fibril. Studies are in progress to use this fetuin-depleted serum assay in a search for those molecules that can substitute for the activity of fetuin in promoting crystal growth within the collagen fibril.

**Chapter III Acknowledgement:**

Chapter III, the manuscript (unpublished) “*The Essential Role of Fetuin in the Serum-Induced Calcification of Collagen*” is used with the permission of my co-author and dissertation advisor, Dr. Paul A. Price. The dissertation author was the primary investigator and author of this paper.

**References**


Chapter IV

The Essential Role of Fetuin in the Serum-Induced Calcification of Elastin Matrices
Abstract

We carried out the present experiments to understand the biochemical mechanism that causes the mineralization of elastin matrices in serum. Serum contains a high concentration of the mineralization inhibitor fetuin. These experiments were performed to understand the rapid and highly reproducible mineralization of elastin in the presence of serum fetuin.

Our hypothesis was that fetuin promotes elastin calcification by selectively inhibiting apatite growth outside of the elastic fiber. We tested this hypothesis by examining the impact of removing fetuin on the ability of serum to mineralize elastin matrices. The results of these tests show that the presence of fetuin in serum determines the location of serum-driven mineralization: in the presence of fetuin, mineral forms only within the elastin fiber; in the absence of fetuin, mineral forms only in the solution outside the fiber. These observations suggested that there is an aqueous compartment within the elastin fiber that fetuin cannot access, but calcium, phosphate or small apatite crystals can.

Using a modification of gel filtration chromatography we have observed and characterized the size exclusion characteristics of the aqueous compartment suggested by our mechanism of mineralization. Molecules 5.7 kDa or smaller can enter this compartment while molecules 48 kDa or larger, such as fetuin, cannot. The amount of mineral formed in a matrix is also directly related to the volume of this fetuin exclusion compartment. This role of fetuin in mineralization and structural property...
of the mineralizing matrix of elastin may provide an insight into the way biological matrices calcify in vivo.

Introduction

Two major types of arterial calcification have been observed in human patients [1, 2]. One affects the intimal layer of arteries and occurs within atherosclerotic plaques. The other involves the artery media, and initially occurs within the elastic lamellae. This second type of vascular calcification is common in patients with chronic kidney disease and in patients with diabetes mellitus. Each type of arterial calcification has different physiological consequences, with clear-cut evidence for adverse hemodynamic changes due to medial wall calcification [3].

One of our long term goals is to understand the mechanisms that initiate calcification of the elastic lamellae of the artery media and the mechanisms that inhibit this calcification. In the course of our investigations we have become intrigued with the evidence for an association between bone metabolism and artery calcification (see [4] for references), an association which led us to propose that medial artery calcification is linked to bone resorption. One prediction of this hypothesis is that inhibitors of bone resorption should inhibit artery calcification [4]. In previous studies we tested this prediction using three different types of bone resorption inhibitors, each with an entirely different mode of action on the osteoclast, the amino bisphosphonates alendronate and ibandronate [4-7], the cytokine osteoprotegerin [8], and the V-H+-ATPase inhibitor SB 242784 [9]. These bone resorption inhibitors proved to potently
inhibit medial artery calcification in each of the three rat models tested. Ibandronate, osteoprotegerin, and SB 242784 are each highly specific inhibitors of the osteoclast at the concentrations used in these studies, and have no known actions on vascular cells. Their ability to potently inhibit artery calcification therefore strongly supports the hypothesis that medial artery calcification is linked to bone resorption.

While the nature of the link between bone metabolism and artery calcification has not yet been established, we have recently proposed the theory that a causative agent (or agents) for artery calcification arises in bone metabolism, travels in blood, and then induces calcification in the elastic lamellae of the artery media [4]. This theory predicts that serum contains a causative agent for medial artery calcification, and that the elastic lamellae of the artery media should therefore calcify when devitalized arteries are incubated in serum.

We have recently demonstrated that the elastic lamellae of devitalized arteries do indeed calcify when incubated in rat, bovine, or human serum [10]. This calcification is due to a potent serum calcification factor, one that causes devitalized arteries to calcify when incubated in DMEM containing as little as 1.5% serum. The serum calcification activity that initiates medial elastin calcification is 50-150kDa in size and protease sensitive [10]. When devitalized arteries are incubated in serum, von Kossa staining shows that mineral formation is restricted to the elastic lamellae of the arteries with no evidence for mineral formation between the lamellae or on the interior or exterior artery surfaces.
It was curious to us that a devitalized artery could mineralize in serum, given that serum contains a very high concentration of the mineralization inhibitor fetuin. Fetuin is a 48 kDa glycoprotein that consists of 2 N-terminal cystatin domains and a smaller C-terminal domain. Fetuin is synthesized in the liver and is found at high concentrations in mammalian serum [11, 12]. The serum fetuin concentration in adult mammals ranges from 0.5 to 1.5 mg/ml, while the serum fetuin concentration in the fetus and neonate is typically far higher [12]. Fetuin binds strongly to apatite, the mineral phase of bone, and is selectively concentrated from serum onto apatite in vitro [13].

In vitro studies have demonstrated that fetuin is an important inhibitor of apatite growth and precipitation in serum containing increased levels of calcium and phosphate [14, 15]. A fetuin-mineral complex is formed during the course of the fetuin-mediated inhibition of apatite growth in serum [16]. Formation of this complex is thought to be involved in retarding mineral precipitation [17]. Purified bovine fetuin has been shown to be a potent inhibitor of the growth and precipitation of a calcium phosphate mineral phase from supersaturated solutions of calcium phosphate [14], and recent studies have shown that, as in serum, a fetuin mineral complex is formed in the course of this inhibition.[16]

Fetuin knock-out mice have been generated, and show a dramatic phenotype. Mineral foci form throughout the soft tissues of these animals, most notably within the kidneys, heart, and lungs [15, 18, 19]. Although mineral also forms within the vasculature, none of this formed mineral can be localized to the elastic lamellae of
arteries, even in fetuin knockout animals with chronic kidney disease.[19] Because artery media mineralize in serum, and during chronic kidney disease but do not mineralize in fetuin knockouts, this suggests a possible role of fetuin in promoting the pathological mineralization of the elastic lamellae of arteries, while inhibiting mineralization elsewhere.

The goal of the experiments presented in this paper is to determine the possible role of fetuin in the serum-initiated calcification of arterial elastic lamellae. Our working hypothesis was that fetuin promotes calcification within the elastic fibers that compose the elastic lamellae of arteries by selectively inhibiting mineralization in the solution outside the elastic fibers. By incubating devitalized arteries or purified elastin in serum depleted of fetuin by immunoaffinity chromatography, or in serum containing fetuin we demonstrate that removing fetuin from serum eliminates the ability of serum to induce the calcification of an elastin matrix.

The hypothesis that fetuin is necessary to specifically cause the mineralization of arterial elastin is dependent on there being an aqueous compartment in the elastin fiber that allows the entry of small molecules but excludes the mineralization inhibitor fetuin. Additional experiments were run to define the molecular exclusion characteristics of the elastin fiber. To do so, we used a modification of gel-filtration chromatography to determine the molecular exclusion characteristics of aorta and ligament elastin. Our results indicate that there is an aqueous compartment in the elastin matrix from which fetuin is excluded, but into which small molecules such as phosphate, pyrophosphate or bone Gla protein can freely diffuse.
Experimental Procedures

**Materials:** Albino rats (Sprague-Dawley derived) were purchased from Harland Labs. Adult bovine serum was purchased from Invitrogen. Each 500ml volume of Dulbecco's modified eagle medium (DMEM; Gibco) was supplemented with 5ml of penicillin-streptomycin (Gibco) and 1ml of 10% sodium azide to prevent bacterial growth. Unless otherwise stated, the concentration of phosphate in DMEM was increased from the basal 0.9mM to a final 2mM by the addition of 0.5 M sodium phosphate buffer pH 7.4. When prepared as described [10], DMEM containing 2mM phosphate is stable for at least 3 weeks at 37°C, with no evidence for loss of calcium or phosphate from the medium or formation of a mineral phase. Bovine fetuin, cytochrome C, and Alizarin red S were purchased from Sigma. BGP was purified from bovine bone as described [20].

Rats were killed by exsanguination while under isoflurane anesthetic; the UCSD Animal Subjects Committee approved all animal experiments. Carotid artery segments were dissected from 40-day-old rats and extracted with a 1000-fold excess (v/w) of 0.5 M EDTA pH 7.5 for 72h at room temperature to kill cells and remove any mineral that might be present. The arteries were then washed exhaustively with ultra pure water to remove all traces of EDTA. The midshaft region of tibias from 22-day-old rats were demineralized in 0.5M EDTA, washed exhaustively with water, dried and weighed [21].

Collagen-free elastin fibers from bovine neck ligament were purchased from Elastin Products Co. Elastin was purified from the media of bovine aorta [22, 23],
ground to the consistency of coarse sand, and freed of collagen [24]. The possible presence of collagen in the purified ligament and aortic elastin used in this study was assessed by digestion with elastase. In brief, 10 mg of elastin was incubated for 24 hours at 37°C in 1 ml of 0.2 M Tris pH 8.7 containing 1 mg elastase (Elastin Products Co.). Both purified elastins were completely dissolved by elastase, while purified bovine tendon collagen was not affected by elastase treatment.

**Calcification procedures:** Experiments to examine the serum-induced calcification of elastin matrices (Figures 1-3) were carried out using 24-well cell culture clusters (Costar 3524, Corning) in a humidified incubator at 37°C and 5% CO₂. Each well contained a 1 ml volume of DMEM alone or of DMEM containing 10% control bovine serum or fetuin-depleted adult bovine serum. The amount of matrix added to each 1 ml volume was: a 1 cm segment of hydrated, devitalized rat carotid artery; or a portion of purified ligament elastin (3 mg dry weight; hydrated before use). Each tissue was then incubated for 6 days.

The experiment to determine whether the fetuin-excluded volume of a matrix influences the amount of mineral deposited within it (Figure 4.5) was carried out in nine 50 ml conical tubes. Each tube contained a 50 ml volume of newborn calf serum containing 0.02% sodium azide. The following dry weights of tissues were then hydrated and added to a tube: a 4mg segment of demineralized tibia midshaft (n=3); 4 mg of purified ligament elastin (n=3); or 19 mg of ligament elastin (n=3). The tubes were placed uncovered for 2h in an incubator at 37°C and 7.5% CO₂ and then sealed and mixed end over end at 37°C for 18d.
**Biochemical procedures:** The procedures used for Alizarin red staining have been described [8]. For histological analyses, arteries were fixed in 100% ethanol for at least 1 day at room temperature; San Diego Pathology Inc. (San Diego, CA) sectioned and von Kossa stained the arteries. For quantitative assessment of the extent of calcification, Alizarin red stained arteries and precipitates formed outside the matrix were extracted for 24h at room temperature with 1 ml of 0.15 M HCl, as described [7]. Calcium levels in culture media and in the acid extracts of tissues and precipitates were determined colorimetrically using cresolphthalein complexone (JAS Diagnostics, Miami FL) and phosphate levels were determined colorimetrically as described [25].

**Immunological procedures:** Fetuin-depleted serum was prepared as previously described (Chapter 3). In brief, a column packed with rabbit anti- bovine fetuin antibodies conjugated to Sepharose 4B was equilibrated with DMEM, and bovine serum was dialyzed against the same solution. The dialyzed bovine serum was then freed of fetuin by passing a 0.85 ml serum aliquot over the column at room temperature. The absorbance at 280 nm of each 1 ml fraction was determined; the 4 fractions with the highest absorbance were then pooled and diluted with DMEM until the absorbance equaled that of 10% bovine serum. The fetuin content of the resulting 10% fetuin-depleted serum was measured by radioimmunoassay (Chapter 3), and was consistently over 1000-fold lower than that of 10% bovine serum. Protein bound to the column was removed by washing the column with 100 mM glycine pH 2.5.

The 10% control serum used in these studies was also prepared as described in chapter 3. A control column was prepared by covalently attaching 7 mg of purified
rabbit IgG (Sigma) to 5ml of CNBr-activated Sepharose 4B. 0.85 ml aliquots of dialyzed adult bovine serum were passed over the control column at room temperature, and the 4 fractions with the highest absorbance were pooled and diluted with DMEM until the absorbance at 280 nm equaled that of 10% bovine serum.

**Gel filtration procedures:** The procedures used to determine the size exclusion characteristics of elastin are a modification of the gel filtration like methods developed to establish the size exclusion properties of collagen (Chapter 2). Purified collagen-free elastin from bovine neck ligament or from the media region of bovine aorta was fractionated by size to obtain particles between 0.833 mm to 2.36 mm. For the ligament elastin column, 53.8 g of the elastin was hydrated, degassed under vacuum, and packed into a 2 x50 cm column to a final volume of 124 ml. For the aortic elastin column, 32.5 g of the elastin was hydrated, degassed under vacuum, and packed into a 2 x50 cm column to a final volume of 125 ml. Both columns were then washed extensively with a 20 mM Tris pH 7.4 equilibration buffer that contained 2M NaCl in order to minimize non-specific ionic interactions between test molecules and the elastin matrix; the final effluent absorbance at 280 nm was less than 0.01. Samples were dissolved in 2 ml of equilibration buffer containing about 160,000 cpm of 1-14C-glucose as an internal reference: the load was 20 mg of fetuin, cytochrome C, or high molecular weight dextran; 10 mg of bone Gla protein; 3 mg matrix Gla protein; 30 mM phosphate pH 7.4; or 30 mM tetra sodium pyrophosphate. A constant flow rate of 6.7 ml/h was maintained using a Fisher Variable Speed Peristaltic Pump, and the fraction size was approximately 1 ml. The true volume of each effluent fraction was
determined from the weight of the fraction contents and the density of the column buffer (1.07 g/ml). The elution position of test substances was determined as follows: proteins, absorbance at 280 nm; dextran, as described [26]; 1-14C-glucose, liquid scintillation counting; pyrophosphate, enzymatic assay with NADH (Sigma); phosphate, as described [25].

**Results**

**Evidence that fetuin is required for the serum-induced calcification of arteries:** The importance of fetuin to the serum-induced calcification of devitalized arteries was determined by removing fetuin from serum. Fetuin was removed from bovine serum by chromatography over a Sepharose 4B matrix containing covalently attached rabbit anti-fetuin antibodies, as described previously (Chapter 3). The fetuin content of the resulting fetuin-depleted serum was over 1000-fold lower than the fetuin content of the original bovine serum, as measured by fetuin radioimmunoassay (Chapter 3).

The impact of fetuin depletion on serum-induced calcification was evaluated by incubating devitalized rat carotid artery segments for 6 days at 37°C in DMEM alone, in DMEM containing 10% control bovine serum, or in DMEM containing 10% fetuin-depleted bovine serum. In agreement with earlier studies [10], devitalized arteries calcified after incubation in DMEM containing 10% control serum by the criteria of calcium and phosphate uptake (Figure 4.1) and Alizarin red staining (Figure 4.2). Von Kossa staining of artery sections again showed that calcification was confined to
the elastic lamellae of the artery media (Figure 4.2). Also in agreement with earlier studies, there was no evidence for calcification in arteries incubated in DMEM alone, a result that further documents the serum-dependence of artery calcification.

In contrast to arteries incubated in 10% control serum, arteries incubated in 10% fetuin-depleted serum did not have significant incorporation of calcium and phosphate (Figure 4.1) and did not stain for calcification by Alizarin red (Figure 4.2); histological sections of these arteries also revealed no evidence for von Kossa staining for calcification in the elastic lamellae of the artery media (Figure 4.2). Removal of fetuin from serum therefore eliminates the serum-induced calcification of devitalized arteries.

To confirm the essential role of fetuin in serum-induced calcification, we added sufficient purified bovine fetuin to the 10% fetuin-depleted bovine serum in order to attain a final fetuin concentration comparable to that found in 10% bovine serum (Chapter 3). The calcification of devitalized arteries incubated in this fetuin-repleted serum was indistinguishable from the calcification of arteries incubated in the original serum: the amount of calcium and phosphate incorporated was comparable (Figure 4.1), the intensity of Alizarin red staining was similar (Figure 4.2), and the von Kossa staining for calcification was again confined to the elastic lamellae of the artery media (Figure 4.2). The addition of purified fetuin therefore fully restores the ability of fetuin-depleted serum to induce the calcification in the elastic lamellae of a devitalized artery.
Figure 4.1. Evidence that fetuin is required for the serum-induced calcification of devitalized arteries: analysis for Ca and P. One cm segments of devitalized rat carotid artery were separately incubated for 6 days at 37°C in 1 ml DMEM containing 2mM Pi and: no serum; 10% control bovine serum; 10% fetuin-depleted bovine serum; or 10% fetuin depleted bovine serum plus 130 µg/ml of purified bovine fetuin. Arteries were removed, stained with Alizarin red, photographed, and then analyzed for calcium and phosphate. The media and any precipitate were removed from the well, centrifuged to pellet any precipitate, and the pellet fraction was analyzed for calcium and phosphate (see Experimental Procedures). This experiment was performed in triplicate. The data show the average calcium and phosphate in the artery and the pellet fraction from each well; the error bars show the standard deviations. * = Ca or Pi in the extract is less than 0.01 µmol.
Figure 4.2. Evidence that fetuin is required for the serum-induced calcification of devitalized arteries: Alizarin red and von Kossa staining. One cm segments of devitalized rat carotid arteries were separately incubated for 6 days at 37°C in 1 ml DMEM containing 2mM Pi and: 10% control bovine serum; 10% fetuin depleted bovine serum; 10% fetuin depleted bovine serum containing 130 µg/ml of purified bovine fetuin. After incubation, arteries were either stained for calcification with Alizarin red and photographed, or fixed in ethanol, cut in 5 micron thick sections, stained for calcification with von Kossa (stains calcification black), and counter stained with nuclear-fast red. Note that von Kossa staining is located in the elastic lamellae of the artery media.

**Evidence that fetuin is required for the serum-induced calcification of purified ligament elastin:** The serum-induced calcification of devitalized arteries is invariably confined to the elastic lamellae of the artery media (Figure 4.2) [10], which suggests that elastin matrices may be uniquely sensitive to serum-induced calcification. To confirm the elastin-matrix specificity of serum-induced artery calcification, an additional experiment was carried out using purified, collagen-free elastin fibers from bovine neck ligament. As seen in Figure 4.3, ligament elastin fibers incubated in 10%
control serum calcified; elastin fibers incubated in 10% fetuin-depleted serum did not calcify; and elastin fibers incubated in 10% fetuin-depleted serum containing purified fetuin calcified (Figure 4.3). There was again a fine precipitate coating the bottom of all wells containing fetuin-depleted serum, and the amount of calcium and phosphate in this precipitate was comparable to that found in elastin fibers incubated in 10% fetuin-replete serum (Figure 4.3).

Figure 4.3. Evidence that fetuin is required for the serum-induced calcification of purified ligament elastin: Ca and P. Four mg amounts of purified bovine ligament elastin (Elastin products Co) were separately incubated for 6 days at 37°C in 1 ml DMEM containing 2mM Pi and: no serum; 10% control bovine serum; 10% fetuin-depleted bovine serum; 10% fetuin-depleted bovine serum plus 130 µg/ml of purified bovine fetuin. Elastin fibers were removed, stained with Alizarin red, photographed, and then analyzed for calcium and phosphate. The media and any precipitate were removed from the well, centrifuged to pellet any precipitate, and the pellet fraction was analyzed for calcium and phosphate (see Methods). This experiment was performed in triplicate. The data show the average calcium and phosphate in the elastin fibers and in the pellet fractions; the error bars show the standard deviations. * = Ca or Pi in the extract is less than 0.01 µmol.

Taken together, these results show that fetuin plays a similar essential role in the serum-induced calcification of the elastic lamellae of devitalized arteries and of
purified neck ligament elastin. In both cases, the essential role of fetuin in the serum-induced calcification is to direct mineral formation into the elastin fiber, and it appears to do this by preventing mineral precipitation outside of elastin.

**The size exclusion characteristics of purified elastin from bovine ligament:**

The next experiments were carried out in order to understand the molecular mechanism by which fetuin directs serum-induced calcification into the elastin fiber. Our working hypothesis was that fetuin favors calcification within the elastin fiber by selectively preventing apatite crystal growth outside the fiber. This mechanism requires that there be a volume of water within the matrix that is accessible to calcium, phosphate, and small crystals, but is not accessible to fetuin. The next experiments were carried out to determine whether elastin fibers have the ability to exclude molecules based on size.

A gel filtration-like method (Chapter 2) was employed to determine the size of molecules that can access the water in elastin. Purified, collagen-free elastin from bovine neck ligament was hydrated in column buffer and packed in a 2 by 50 cm glass column. The size exclusion characteristics of ligament elastin were then evaluated by filtering a mixture of glucose and fetuin (a 48 kDa glycoprotein) over this column. As can be seen in Figure 4.4, 14C-labeled glucose eluted at a volume of 80 ml, which is comparable to the 83.5 ml volume of liquid in the column bed. This observation shows that glucose has free access to essentially all liquid within the packed column. Fetuin eluted at a volume of about 62 ml, which is 18 ml less than the elution volume
of glucose. These observations show that fetuin is excluded from an 18 ml volume of liquid in the packed column that glucose is able to freely access.

Figure 4.4. Separation of fetuin and glucose by passage over a column packed with purified ligament elastin. Purified collagen-free elastin from bovine neck ligament was fractionated by size to obtain particles between 0.833 mm and 2.36 mm. 53.8 g of this elastin was hydrated in 20 mM Tris pH 7.4 containing 2M NaCl, packed into a 2 x50cm column to a final volume of 124 ml, and washed extensively with 20 mM Tris pH 7.4 containing 2M NaCl. A 2 ml volume of equilibration buffer containing 20 mg bovine fetuin and 160,000 cpm of 1-14C-glucose was applied to the column, and buffer was pumped through the column at a constant flow rate of 6.7 ml/h. The fraction size was approximately 1 ml. The liquid volume in the packed column bed was obtained by subtracting the weight of dry elastin in the column from the wet weight of the packed column bed. (See “Experimental Procedures.”)

Additional filtration experiments were carried out using this column in order to further characterize the molecular exclusion characteristics of ligament elastin. As seen in Table 4.1, phosphate, pyrophosphate, and the 5.7 kDa bone Gla protein (BGP) co-elute with glucose at the approximate volume of water in the packed column, which shows that molecules up to the size of BGP have access to essentially all of the
water in the column. Matrix Gla protein and cytochrome C elute from the ligament elastin column between glucose and fetuin, and consequently appear to have only partial access to the volume of liquid in elastin that is accessible to glucose and BGP.

Table 4.1: The size exclusion properties of purified elastic fibers from bovine ligament. The packed column whose preparation is described in the Figure 4.4 legend was equilibrated with 20mM Tris pH 7.4 containing 2M NaCl. A 2 ml volume of equilibration buffer containing the test molecule and 160,000 cpm of \(^{14}\)C glucose was then applied to the column. Flow rate, 6.7 ml/hour; fraction size, 1 ml. The elution volume of glucose for these 7 runs was 80±0.94 ml (Mean ±SD). The results show the elution volume of each test molecule. (See Experimental Procedures for details)

<table>
<thead>
<tr>
<th>Test molecule</th>
<th>MW (Da)</th>
<th>Elution volume, ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>HMW Dextran</td>
<td>5-40x10^6</td>
<td>51</td>
</tr>
<tr>
<td>Fetuin</td>
<td>48,000</td>
<td>62</td>
</tr>
<tr>
<td>Cytochrome C</td>
<td>12,300</td>
<td>69</td>
</tr>
<tr>
<td>MGP</td>
<td>10,000</td>
<td>71</td>
</tr>
<tr>
<td>BGP</td>
<td>5,700</td>
<td>80</td>
</tr>
<tr>
<td>Glucose</td>
<td>180</td>
<td>80</td>
</tr>
<tr>
<td>Pyrophosphate</td>
<td>174</td>
<td>80</td>
</tr>
<tr>
<td>Phosphate</td>
<td>95</td>
<td>80</td>
</tr>
</tbody>
</table>

Volume of Liquid in Column 83.5 ml
The size exclusion characteristics of purified elastin from bovine aorta: The same gel filtration-like method was used to determine the size of molecules that can access the water in elastin purified from the media region of bovine aorta. As seen in Table 4.2, bone Gla protein elutes with glucose at the volume of water in the packed column, which shows that both molecules have access to essentially all of the water in the column. In contrast, fetuin eluted at a volume that is 11 ml less than the elution volume of glucose, which shows that fetuin is excluded from an 11 ml volume of liquid in the packed column that glucose and BGP are able to freely access.

Table 4.2: The size exclusion properties of purified elastic fibers from bovine aorta
Bovine aortic elastin was freed of collagen and ground to the consistency of coarse sand. 32.5 g of this elastin was hydrated in 20mM Tris, pH 7.4 containing 2M NaCl, packed into a 2x50 cm column to a final volume of 125 ml, and washed extensively with 20mM Tris pH 7.4 containing 2M NaCl. A 2 ml volume of equilibration buffer containing the test molecule and 160,000 cpm of 1-14C glucose was then applied to the column. Flow rate, 6.7 ml/hour; fraction size, 1 ml. The results show the elution volume of each test molecule. (See Experimental Procedures for details)

<table>
<thead>
<tr>
<th>Test molecule</th>
<th>MW (Da)</th>
<th>Elution volume, ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fetuin</td>
<td>48,000</td>
<td>89</td>
</tr>
<tr>
<td>BGP</td>
<td>5,700</td>
<td>100</td>
</tr>
<tr>
<td>Glucose</td>
<td>180</td>
<td>100</td>
</tr>
</tbody>
</table>

Volume of Liquid in Column 100.1 ml
Comparison of the fetuin-excluded volume in elastin and collagen. Elastin and collagen appear to have similar size exclusion characteristics: Glucose and BGP elute at the total volume of water in the elastin and collagen columns, and so have comparable ability to access all of the water in the two matrices. In contrast, fetuin always elutes well before glucose, and is therefore excluded from a volume of water in the packed column that glucose and BGP are able to freely access. Our working hypothesis is that the critical role of fetuin in serum-induced mineralization is to favor calcification within the fetuin-excluded volume of the elastin fiber by selectively preventing apatite crystal growth outside the fiber. The fetuin-excluded volume of water within a matrix is therefore the volume in which crystals grow.

We have compared the fetuin-excluded volume per gram for different matrices in Table 4.3 in order to better understand the properties of organic matrices that are required for serum-induced mineralization. As can be seen, elastin purified from bovine ligament and aorta have identical fetuin-excluded liquid volumes, while the fetuin-excluded volume of demineralized bone collagen (Chapter 2) is about 5 times greater.
Table 4.3: Comparison of the fetuin-excluded volume in elastin and demineralized bone

The liquid volume that excludes fetuin but not glucose and the dry weights for the matrices were obtained as follows: demineralized bone collagen, (Chapter 2); ligament elastin, Table 4.1; and aortic elastin, Table 4.2. This table shows the volume that separates fetuin from glucose (or BGP) per gram of matrix in column. (See Experimental Procedures for details).

<table>
<thead>
<tr>
<th></th>
<th>Demineralized Bone Collagen</th>
<th>Ligament Elastin</th>
<th>Aortic Elastin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume that excludes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fetuin but not</td>
<td>81 ml</td>
<td>18 ml</td>
<td>11 ml</td>
</tr>
<tr>
<td>Glucose or BGP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dry Weight of Matrix</td>
<td>51 g</td>
<td>53.8 g</td>
<td>32.5 g</td>
</tr>
<tr>
<td>in Column</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fetuin-Excluded</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Volume per gram matrix</td>
<td>1.59 ml/g</td>
<td>0.33 ml/g</td>
<td>0.34 ml/g</td>
</tr>
</tbody>
</table>

Evidence that the fetuin-excluded volume of a matrix influences the amount of serum-induced mineral deposited within it. The next experiment was carried out to determine if the fetuin-excluded volume of water within a matrix determines the amount of mineral that is formed within it during incubation in serum. In order to remove possible limitations on the extent of mineral formation within each matrix, a 50 ml volume of neat serum was used instead of a 1 ml volume of 10% control serum and the incubation time was increased to 18 days from the standard 6 day incubation. As can be seen in Figure 4.5, the amount of mineral formed within collagen and elastin matrices is in direct proportion to the total fetuin-excluded volume within each matrix, and not to the total dry weight of the matrix. This result further illustrates the critical importance of the fetuin-excluded volume to the mechanism of serum-induced: crystals can only grow in that volume of water within the matrix that excludes fetuin.
Figure 4.5: Evidence that the fetuin-excluded volume of a matrix influences the amount of serum-induced mineral deposited within it. The indicated dry weights of tissues were separately incubated in 50 ml volumes of newborn calf serum for 18 days at 37°C. Tissues were removed from serum, stained with Alizarin red, and analyzed for calcium and phosphate. This experiment was performed in triplicate. The data show the average mass of calcium plus phosphate in each matrix, and the calculated fetuin-excluded volume in the matrix (see Table 4.3). The error bars show the standard deviations. (See “Experimental Procedures.”)

**Discussion**

In earlier studies, it was hypothesized that a factor responsible for calcification of the elastic lamellae of arteries arises during bone resorption and acts systemically by travelling through the blood. This was demonstrated in two ways. First through testing the *in vivo* effect of resorption inhibitors on arterial mineralization in uremic animals.
it was shown that inhibiting bone resorption inhibited arterial mineralization. Second, an *in vitro*, serum-based mineralization system was used to demonstrate that serum contains a factor that causes the mineralization of the elastic lamellae of devitalized arteries; the same mineralization phenotype as observed in uremic animals.

The calcification activity is shown to be sufficiently potent that it can promote calcification of the elastic lamellae in DMEM containing as little as 1.5% serum, and consists of at least one protein between 50 and 150 kDa molecular weight [10]. These studies provided the first biochemical characterization of the molecular basis for the calcification of devitalized arteries in serum. In the experiments presented in this paper we have attempted to refine our understanding of the mechanism by which arteries become calcified in serum.

**The role of fetuin in the serum initiated mineralization of the elastin fiber:**

Serum contains, as briefly reviewed in the introduction, a high concentration of fetuin, a potent inhibitor of mineralization. Because serum can promote arterial mineralization, serum fetuin must not be able to inhibit arterial mineralization. We hypothesized that fetuin paradoxically promotes calcification within the elastic lamellae by selectively inhibiting apatite growth outside, but not inside the elastin fibers that compose these structures. We tested this hypothesis by examining the impact of removing fetuin from serum on the ability of serum to mineralize the elastic lamellae of arteries and purified elastin fibers. The results of this study reveal that removing fetuin from serum completely prevents the serum-driven calcification of
elastin containing matrices. Removing fetuin from serum does not prevent the serum-driven formation of mineral, however, because apatite mineral consistently forms on the bottom of all wells that contain fetuin-depleted serum. The net effect of this fetuin activity is extraordinary: all of the calcium and phosphate ions that, in the absence of fetuin, are incorporated into a mineral that forms throughout the ~1 ml volume that lies outside the artery or elastin are, in the presence of fetuin, incorporated into a mineral that forms within the much smaller volume of water that lies within the elastic fiber.

These results are consistent with those seen in vivo in the fetuin knock-out mouse [15]. In a fetuin knock-out, multiple small foci of mineral form in the soft tissues of the animal, most notably in the kidney, lungs, and heart. This mineral apparently does not form within the structural molecules (such as collagen or elastin fibers) that these tissues are composed of, but instead is located outside these structures. In these knockout mice there is also no evidence of mineral formation within the elastic lamellae of arteries [19], again suggesting that fetuin is required for this specific structure to be mineralized.

How, then might fetuin be acting to promote mineral formation within arterial elastin? Previous work has shown that fetuin traps any nascent mineral nuclei and dramatically retards their growth [16, 17]. The easiest explanation for the mineralization of elastin fibers in serum is that there is a space within the fiber from which the mineralization inhibitor fetuin is excluded but to which small molecules such as calcium, phosphate and mineral crystals have access. The mineralization
inhibitor fetuin would, in that circumstance paradoxically allow the growth of mineral within the elastic fiber by acting to prevent mineral formation outside it. We therefore hypothesize that when fetuin is absent from serum, mineral can form throughout the serum volume, explaining the presence of a precipitate in fetuin depleted serum, and the multiple foci of mineral found in the soft tissues of the fetuin knockout mouse. However when fetuin is present in serum, mineral can only form in that volume from which fetuin is excluded- that volume inside the elastic fiber.

**The size exclusion characteristics of the elastin fiber:** The present experiments were performed in order to determine if, as hypothesized, there is a liquid containing compartment in the elastin fiber from which fetuin is excluded, and what the molecular exclusion characteristics of any such compartment are. The method we developed to investigate the molecular exclusion characteristics of the elastic fiber is an adaptation of gel filtration chromatography, a procedure typically used to separate macromolecules by size. Columns were packed with either purified elastin fibers from bovine ligament, or elastin fibers from bovine aorta. Mixtures of a test molecule and $^{14}$C glucose were then run over these columns. In all cases we found that glucose elutes from the column at a liquid volume identical to the volume of water in the column bed. Fetuin, in both cases elutes at a volume of liquid less than that found in the column bed. The simplest explanation for this is that there is an aqueous volume within the elastin fiber that glucose can enter but fetuin cannot. The volumes of separation between fetuin and glucose per gram of matrix in each column are
comparable for both elastin sources at 0.33 ml and 0.34 ml/g, suggesting that both ligament elastin and aortic elastin can similarly separate fetuin from glucose. Therefore both elastin matrices have a similar fetuin-exclusion property.

The molecular exclusion characteristics of elastin were further analyzed. It was found that both the 12.3 kDa cytochrome C and the 10 kDa matrix Gla protein elute between fetuin and glucose indicating partial diffusion into the fetuin excluding space. This may help explain why MGP, a known inhibitor of calcification of the artery media is able to act within elastin matrices in vitro [10, 27] and in vivo [28]- MGP has at least some access to growing mineral crystals. Small molecules such as phosphate (a component of apatite mineral), pyrophosphate (a proposed inhibitor of mineralization), and the 5.7 kDa bone Gla protein (BGP, the most abundant non-collagenous protein in bone) co-elute with glucose. All of these molecules must, in contrast to fetuin, freely diffuse into the aqueous space inside the elastin fiber.

How might a matrix that excludes proteins as large as fetuin become mineralized by a serum factor 50-150 kDa in size? The serum mineralization factor is far too large to enter the fiber and must therefore act outside the mineralizing volume (as suggested by the formation of a precipitate outside the elastin matrix in fetuin-depleted serum.) However, the crystals that it produces may diffuse into the matrix. Because BGP (~6500 A³) diffuses into all of the water within the elastic fiber, it seems likely that apatite crystals the size of BGP (approximately 12 unit cells of hydroxyapatite[29] can diffuse in as well. Once inside the elastin fibers, these crystals are inaccessible to fetuin and are therefore able to grow.
Implications for the role of proteins in regulating the pathological mineralization of arteries:

The presented data show that the mineralization inhibitor fetuin is necessary for mineralization of an elastin matrix. Fetuin inhibits mineral formation everywhere but within the elastic fiber, and therefore it paradoxically and preferentially allows mineral to grow in that space. This is due to fetuin’s exclusion from a space that small mineral crystals can enter.

This allows us to form a more general hypothesis regarding the mechanism of matrix mineralization in serum. Any matrix that can be mineralized by the activity of the serum calcification factor must therefore contain a space that excludes fetuin, but allows the entry of small mineral crystals. As shown in Table 4.3 and Figure 4.5, the amount of mineral formed in a matrix directly relates to the volume of this space; the larger the volume that excludes fetuin, the greater the amount of mineral that can form in the matrix. Elastin, which has 1/5 the fetuin exclusion volume of bone (Table 4.3) can be mineralized to 1/5 the capacity of bone (Figure 4.5). Therefore, different matrices can have different capacities for mineralization based solely on the volume of this fetuin-exclusion space.

If fetuin is allowed entry into all of the liquid in a matrix, or mineral crystals are not allowed into any, the matrix will not mineralize. By examining the size exclusion characteristics of several matrices one might predict which will be targets of the serum calcification activity. Comparing this expectation with the actual ability of these matrices to mineralize in serum will allow us to more fully understand the normal and pathological mechanisms of biological matrix mineralization.
Chapter IV Acknowledgement:

Chapter IV, the manuscript (unpublished) “The Essential Role of Fetuin in the Serum-Induced Calcification of Elastin Matrices” is used with the permission of my co-author and dissertation advisor, Dr. Paul A. Price. The dissertation author was the primary investigator and author of this paper.

References


Chapter V

Discussion
The *in vivo* mineralization of both collagen and elastin matrices has been extensively studied, but the mechanisms underlying that mineralization have yet to be understood. In this thesis we hypothesized that there is a connection between the physical structure of a matrix and the molecules that can enter that matrix. Furthermore, we hypothesized that the location of a molecule relative to the mineralizable volume in a matrix informs how that molecule might impact the process of mineralization. These hypotheses were tested in the experiments outlined in chapters II-IV. I have concluded that there is a size exclusion characteristic of the type I collagen fibrils of bone and tendon, and of the elastic fiber of the artery media. These structures allow the penetration of apatite crystals, but disallow the penetration of nucleators or inhibitors of mineralization larger than 40 kDa. One of these large mineralization inhibitors, fetuin, was subsequently shown to ensure mineral grows only within the collagen fibril or elastic fiber. The results of these experiments also suggest that the serum calcification factor must act outside the mineralizable volume within a matrix, while the apatitic product of that nucleation can diffuse into and grow within the matrix.

**Chapter II: The Size Exclusion Characteristics Of Type I Collagen: Implications For The Role Of Non-Collagenous Bone Constituents In Mineralization.**

*The size exclusion characteristics of collagen.* In Chapter II, I have attempted to show that molecules are either excluded from, or can access the water within the type I collagen fibril. While it has been theorized that certain non-collagenous proteins have
the ability to affect the process of mineral formation in bone or other collagenous tissues, there has been little work examining how the physical structure of the collagen matrix might, itself, affect mineralization. Collagenous matrices have been shown to mineralize *in vitro* in serum or dilutions of serum in DMEM [1-3]. Because serum also contains a very high concentration of the mineralization inhibitor fetuin, we hypothesized that fetuin could not access the space in collagen matrices that mineralized, while other, smaller, non-collagneous molecules could.

The method that was developed to determine which molecules could access the water space within type I collagen is an adaptation of gel filtration chromatography. Columns were packed with either purified collagen from bovine Achilles tendon or with demineralized bone, and the elution volume of different test molecules from these columns were determined. The results of these experiments show that molecules that range in size from the 95 dalton phosphate to the 5,700 dalton bone Gla protein elute at a volume comparable to the liquid volume in the column bed, and therefore have access to all of that liquid. In contrast, molecules the size of fetuin (48,000 daltons) or larger elute at a volume equal to the volume of liquid in the collagen fibrils subtracted from the total volume of liquid in the bed. The simplest explanation for these observations is that the type I collagen fibrils in the column contain water that is accessible to small molecules that were tested, such as BGP, glucose, and phosphate, and inaccessible to fetuin and other large molecules.

It has been demonstrated that most or all of the water in type I collagen lies within the individual collagen fibrils ([4] and references therein). Small molecules such as
phosphate, glucose, and the 5,700 dalton BGP must be able to attain the same concentration in the water that lies inside the collagen fibrils of the packed column as in the water that lies outside of the fibrils. The comparable Bragg spacing in the fully hydrated fibrils in tendon and demineralized bone shows that both have a comparable layer of water separating adjacent collagen molecules in the lateral plane of the fibril. Because the internal structure of the collagen fibrils in both tissues are essentially identical [5], the fibrils in both tissues would be expected to impose a comparable barrier to the penetration of large molecules but not small and give rise to indistinguishable size exclusion properties (Figure 2.5), and this is what the data show.

Because the collagen fibril is both the mineralized structure, and hypothetically the space that has size exclusion characteristics, one would expect to see no size exclusion characteristics in mineralized collagen. To evaluate the impact of mineral on the size exclusion properties of bone collagen, we prepared a column of non-demineralized bone that contained the same mass of collagen as the demineralized bone column (see Table 2.5). We then examined the elution volume of different test molecules from this column. The results demonstrate that the presence of mineral in collagen dramatically reduces the elution volume of glucose (Figure 2.4) but does not comparably affect the elution volume of fetuin, albumin, and high molecular weight dextran. The average reduced separation due to the presence of mineral is comparable to the reduced volume of water in the column bed and the reduced volume of water is comparable to the volume now occupied by mineral.
Insights into the function of non-collagenous bone constituents in bone mineralization

The type I collagen fibril plays several critical roles in bone mineralization. The mineral in bone is located primarily within the fibril [6-11], and during mineralization the fibril is formed first and then water within the fibril is replaced with mineral [12, 13]. The collagen fibril therefore provides the aqueous compartment in which mineral grows. The present study shows that the physical structure of the collagen fibril plays an important additional role in mineralization: disallowing large molecules to diffuse into the aqueous space within the fibril, while allowing small molecules to enter that space. Molecules small enough to enter can therefore interact directly with apatite crystals growing within the fibril.

Proteins that are too large to penetrate the collagen fibril can still have important roles in bone mineralization. Some large bone proteins, such as fetuin [14-17], potently inhibit apatite formation or growth in vitro. The data presented in this section of my thesis support the view that such large protein inhibitors of calcification promote mineralization of the collagen fibril by inhibiting apatite growth everywhere but within the fibril. Other proteins that are too large to penetrate the fibril such as bone sialoprotein, and the recently discovered serum nucleator of collagen calcification may nucleate mineral formation [1]. Such proteins may generate apatite crystal nuclei outside of the collagen fibril, and some of these small crystals then diffuse into the interior of the fibril and grow. Since BGP diffuses into all of the water within the collagen fibril, it seems likely that apatite crystals up to the size of BGP...
(about 12 hydroxyapatite unit cells; see Chapter II) can also diffuse throughout the fibril.

**Chapter III: The Essential Role of Fetuin in the Serum Induced Calcification of Collagen**

In Chapter III, a study was described which had the goal of understanding the biochemical basis of serum’s ability to induce the calcification of a type I collagen fibril. The serum factor that induces calcification of the collagen fibril consists of one or more proteins that are 50 to 150 kDa in molecular weight, and therefore, as suggested by the data presented in Chapter II, cannot enter the collagen fibril. This factor may therefore act to generate small crystal nuclei outside of the collagen fibril that themselves subsequently diffuse into the fibril and grow. Because nuclei are generated outside the fibrils of collagen, there must be mechanisms ensuring that the serum calcification factor causes mineralization to occur only within bone collagen fibrils in vivo, preventing the pathological mineralization of the vasculature and soft tissues. One possibility is that inhibitors of apatite growth excluded from the fibril favor mineralization within the fibril by selectively preventing apatite growth outside of the fibril. One such fibril-excluded inhibitor of apatite growth is fetuin.

This study revealed that removing fetuin from serum completely prevents the serum-induced calcification of a type I collagen matrix, but does not prevent the serum-driven formation of mineral (Figures 3.3-3.7). An apatite mineral consistently
forms throughout fetuin-depleted serum and not in serum containing fetuin. The results of these experiments therefore support our working hypothesis, namely that large protein inhibitors of apatite growth such as fetuin can favor mineralization of the collagen fibril by selectively preventing apatite growth outside of the fibril.

Previous *in vitro* studies using pure fetuin in solutions containing high levels of calcium and phosphate provide an insight into how fetuin may act to direct apatite growth within the collagen fiber. In these experiments, solutions were prepared that substantially exceeded the calcium phosphate ion product required for formation of an apatite-like mineral phase. In the absence of fetuin a mineral phase forms in minutes [16]. In fetuin’s presence, no mineral precipitate can form for 24 hours, even though a slight decline in ionic calcium indicates that mineral nucleation still occurs. Fetuin engulfs these nuclei and by doing so slows mineral growth and precipitation. [16] [18]

We believe that the role of fetuin in serum-driven calcification of a type I collagen matrix is similar to its action in the just described homogeneous apatite nucleation system: fetuin traps mineral nuclei formed by the serum activity and slows their growth. The products of nucleator action are presumably small crystal nuclei, but even apatite crystals up to 12 unit cells in size should in principle be able to freely access all of the water within the fibril (see Chapter II). Since fetuin can only trap those nuclei that it can access, the crystal nuclei that penetrate the fibril are free to grow far more rapidly than those nuclei trapped by fetuin outside of the fibril, and the collagen fibril selectively calcifies. When fetuin is removed from serum, the same number of mineral nuclei still form, but the large number formed outside the fibril are
free to grow without fetuin to retard their growth. The only mineral formed in amounts that can be detected is, accordingly, a mineral precipitate found in the fetuin depleted serum, not mineral within the fibril (Figure 3.3, 3.5, 3.6).

As with many other critical physiological processes, there are probably multiple layers of redundancy of those factors that affect the process of normal bone mineralization. Inhibitors of apatite crystal growth are likely to include a number of other non-collagenous bone proteins in addition to fetuin. These might include molecules such as the 32.6 kDa protein osteopontin ([19] and included references), or the 10 kDa matrix Gla protein. MGP and osteopontin knockout animals have both been generated, and show an increase in ectopic mineralization [20, 21]. These molecules are significant components of mineralized bone, and their presence could be indicative of a role in specifically promoting fibril mineralization while inhibiting mineral formation outside the fibril.

In addition to the serum calcification factor activity, nucleators may include large proteins such as bone sialoprotein [22, 23], and alkaline phosphatase [24] as well as large structures such as matrix vesicles [25]. Theoretically these proteins and structures can all support each other in the process of matrix mineralization.

Chapter IV: The Essential Role of Fetuin in the Serum-Induced Calcification of Elastin Matrices

In Chapter IV, we wanted to determine whether those characteristics and factors necessary for the mineralization of a type I collagen matrix were required
characteristics of any matrices that mineralize in serum. In addition to type I collagen, the elastin of the artery media also mineralizes \textit{in vivo}. Arterial mineralization is one of the consequences of chronic renal failure, as illustrated in [26]. In uremic rats the mineralization of the abdominal and thoracic aorta as well as the associated branch arteries has been demonstrated, a condition enhanced by a low protein diet [27].

Our lab has recently demonstrated that the elastic lamellae of devitalized arteries calcify when incubated in rat, bovine, or human serum [28]. This calcification is due to a potent serum calcification factor, one that causes devitalized arteries to mineralize when incubated in DMEM containing as little as 1.5% serum, but not in DMEM alone. The serum calcification activity that initiates medial elastin calcification has the same 50-150kDa size and protease sensitivity as the serum calcification activity previously shown to initiate calcification of type I collagen [1]. Our working hypothesis is that the same serum calcification activity initiates calcification of collagen and elastin, and that this calcification activity is present in general circulation.

If the mineralization of elastic lamellae in serum were to occur through the same mechanism as the mineralization of collagen fibers there must be a compartment, as there is in collagen, that excludes fetuin and other large inhibitors of mineralization but allows entry to calcium, phosphate, and small apatite crystals. One might predict that in this circumstance, the removal of fetuin from serum would have the same effect on mineralization of an elastin matrix as it does on a collagen matrix. In agreement with this prediction, we found that in fetuin depleted serum there was no mineralization of the elastic lamellae of arteries or of pure elastin fibers. Instead, as
occurred when collagen matrices were incubated in fetuin depleted serum, mineral precipitated outside the matrix. In serum containing fetuin, however, the elastic fibers mineralized and no mineral formed outside the matrix (Figure 1,2, Chapter IV). In fetuin knockout mice it is seen that mineral forms within the artery. However, the mineral that forms is not a specific and thorough mineralization of the artery media, but instead is a non-specific distribution of mineral foci throughout the soft tissues of the animal. This suggests that in the absence of fetuin, mineral nuclei may be formed in blood, and these mineral nuclei then grow, precipitating in the vasculature of several different tissues.

Because pure elastin and the elastic lamellae of carotid artery mineralize in serum, we hypothesized that there must be a space in the elastin fibers that has size exclusion characteristics- a space that serum fetuin can’t penetrate that calcium, phosphate and apatite crystals can. We modified the gel filtration procedure described in Chapter II, substituting purified, collagen-free elastin fibers for the purified type I collagen matrix. Different test molecules were filtered over this column. Molecules as large as BGP were able to access the water in that compartment of the elastin fiber that has size exclusion characteristics. Molecules the size of fetuin were unable to penetrate all of the water in the fiber (Table 4.1, Table 4.2). It is apparent from these experiments that only a fraction of the water in the elastic fiber is in a compartment that has size exclusion characteristics (data not shown), as opposed to the water in the collagen fibril, all of which is contained in a structure that has size exclusion characteristics (Chapter II). This observation is supported by the differing amounts of mineral
formed in each matrix, amounts that directly relate to the volume of water in the matrix that fetuin is excluded from. Elastin contains a fetuin-exclusion space approximately 5-fold smaller than that in bone collagen, and approximately 5-fold less mineral is formed in elastin as opposed to bone during incubation in serum. (Table 4.3, Figure 4.5).

While our data do show that there is a compartment in the elastin fiber that has size exclusion characteristics they, unfortunately don’t reveal the exact nature of that compartment. The elastic fiber contains both fibrillin microfibrils and elastin, and therefore it is possible that the mineralizing compartment is within either elastin matrix, or the fibrillin scaffolding. Based on work performed in other labs, we speculate that the space lies solely, or to a large extent in the elastin itself. When insoluble elastin containing no microfibrils is subcutaneously implanted in a rat, it mineralizes as quickly and thoroughly as microfibril containing elastin [29]. Therefore it is not a necessarily a fibrillar structure that must be the location of mineralization (i.e. the collagen fibril or fibrillin microfibril). Instead, this suggests that any biological matrix might contain an aqueous space from which fetuin is excluded, and any matrix that contains such a space may be able to mineralize in serum.

**General Conclusion** This thesis describes and tests the hypotheses that the matrices of collagen and elastin have size exclusion characteristics that limit access to the aqueous space in which mineral forms. The ability of molecules to access this
space is shown to have important implications for the regulation of serum-induced mineralization.

The results of these experiments suggest that there are only two necessary criteria for mineralization of any matrix in serum. First, the matrix must be able to exclude fetuin and other large inhibitors of mineralization from an internal water-containing space. Second, calcium, phosphate and stable mineral nuclei produced outside the matrix must be able to diffuse into that space. Once there, mineral is able to grow and fill this aqueous space.

**References**


