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THE SIZE EXCLUSION CHARACTERISTICS OF TYPE I COLLAGEN: IMPLICATIONS FOR THE ROLE OF NON-COLLAGENOUS BONE CONSTITUENTS IN MINERALIZATION.

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Running title: Size exclusion properties of type I collagen fibrils

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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.

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 (6,10-16). The final fibril can be from 20 to 400nm 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 1 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-^{14}C-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 ≤ 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 1-^{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 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 (**Supplemental Figure A**). 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 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 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 5**).

Gel filtration procedures: bone collagen. The 227 ml columns of non-demineralized and demineralized bone sand prepared for the measurements shown in **Table 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-^{14}C-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 demineralized 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-^{14}C-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 8)**.

The elution position of test substances was determined as follows: proteins, absorbance at 280 nm; 1-^{14}C-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 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.

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 1**, ^{14}C-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 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,** 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.

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 3),** its radiographic density was dramatically and uniformly reduced (**Supplemental Figure A**), and the bone ring was flexible (personal observations). The data in **Table 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.

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 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[1].

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 5**, demineralization of

bovine bone sand replaced mineral (62 ml) with a comparable volume of water (67 ml).

Figure 2 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 (51g 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 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.

We next examined the size-exclusion characteristics of a column made with non-demineralized bone sand. Comparison of **Figures 2 and 3** 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 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 7**), and the reduced volume of water is comparable to the increased volume occupied by mineral (62 ml, **Table 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 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 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 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).

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 to 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 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 1) as it does in the water that lies outside of the fibrils (~ 50 ml, Figure 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 4**). 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 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 4**). 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 4, 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 futher supports the hypothesis that individual collagen molecules have substantial freedom to move in the lateral plane of the fibril.

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 4**, 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 4**) 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 (**Supplemental Figure A**), or when a sample of ground steer bone sand is demineralized in 10% formic acid at 4°C (**Table 5**). These experiments also showed that demineralization of bone consistently replaced mineral with a comparable volume of water (**Tables 3 and 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**). In contrast, molecules the size of fetuin (48,000 daltons), albumin (66,000 daltons), and high molecular weight dextran (5-40 x 10^6 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 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 (40).

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 (41), 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 4**).

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 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 7**), and the reduced volume of water is due to the volume occupied by mineral (62 ml, **Table 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 (42,43) 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. The companion paper in the Journal tests this hypothesis by examining the impact of fetuin-depletion on the serum-induced calcification of the collagen fibril. The results of this test show that the presence of fetuin in serum determines the location of serum-induced mineralization: in the presence of fetuin, mineral forms within the collagen fibril; in the absence of fetuin, a comparable amount of mineral forms outside the fibril.

Other proteins that are too large to penetrate the fibril may nucleate mineral formation, proteins such as bone sialoprotein (44,45) and the recently discovered serum nucleator of collagen calcification (26) as well as large structures such as matrix vesicles (46). 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. The companion paper

in the Journal demonstrates that the serum nucleator of collagen calcification does indeed generate crystal nuclei outside of the fibril, and provides evidence that some of these crystal nuclei subsequently diffuse into the collagen fibril and grow.

REFERENCES

1. Robinson, R. A., and Elliott, S. R. (1957) *J. Bone and Joint Surg.* **39A**, 167-188
2. Boivin, G., and Meunier, P. (2002) *Calcif Tissue Int* **70**, 503-511
3. Robinson, R. A. (1958) Chemical analysis and electron microscopy of bone. In. *Bone as a tissue; proceedings of a conference, October 30-31, 1958.*, McGraw-Hill, New York
4. Blitz, R. M., and Pellegrino, E. D. (1969) *J. Bone and Joint Surg.* **51-A**, 456-466
5. Ottani, V., Martini, D., Franchi, M., Ruggeri, A., and Raspanti, M. (2002) *Micron* **33**, 587-596
6. Wess, T. J. (2005) *Adv. Protein Chem.* **70**, 341-374
7. Hodge, A., and Petruska, J. (1963) *Recent studies with the electron microscope on ordered aggregates of the tropocollagen molecule*, Academic Press, New York
8. Gutsmann, T., Fantner, G., Manuela, V., Ekani-Nkodo, A., Thompson, J., Kindt, J., Morse, D., Fygenson, D., and Hansma, P. (2003) *Biophys J* **84**, 2593-2598
9. Revenko, I., Sommer, F., Minh, D., R, G., and Franc, J. (1994) *Biol Cell* **80**, 67-69
10. Fraser, R. D. B., MacRae, T. P., Miller, A., and Suzuki, E. (1983) *J. Mol. Biol.* **167**, 497-521
11. Holmes, D. F., Gilpin, C. J., Baldock, C., Ziese, U., Koster, A. J., and Kadler, K. E. (2001) *Proc. Natl. Acad. Sci. USA* **98**, 7307-7312
12. Hulmes, D. J. S., and Miller, A. (1979) *Nature* **282**, 878-880
13. Lees, S., Pineri, M., and Escoubes, M. (1984) *Int. J. Biol. Macromolecules* **6**, 133-136
14. Orgel, J. P. R. O., Irving, T. C., Miller, A., and Wess, T. J. (2006) *Proced. Natl. Acad. Sci. U.S.A.* **103**(24), 9001-9005
15. Orgel, J. P. R. O., Miller, A., Irving, T. C., Fischetti, R. F., Hammersley, A. P., and Wess, T. J. (2001) *Structure* **9**, 1061-1069
16. Piez, K. A., and Trus, B. L. (1981) *Bioscience Reports* **1**, 801-810
17. Moeller, H., Bosch, U., and Decker, B. (1995) *J. Anat* **187**, 161-167
18. Parry, D. C. A. (1984) *Growth and Development of Collagen Fibers in Connective Tissues*
19. Reiser, K., Mccormick, R., and Rucker, R. (1992) *FASEB* **6**, 2439-2449
20. Knott, L., and Bailey, A. (1998) *Bone* **22**, 181-187
21. Raspanti, M., Ottani, V., and Ruggeri, A. (1989) *Int. J. Biol. Macromol.* **11**, 367-371
22. Fullerton, G., and Amurao, M. (2006) *Cell Biology INternational* **30**, 56-65
23. Price, R., Lees, S., and Kirschner, D. (1997) *Int J Biol Macromol* **20**, 23-33
24. Fratzl, P., Fratzl-Zelman, N., and Klaushofer, K. (1993) *Biophys J* **64**, 260-266
25. Hamlin, N. J., and Price, P. A. (2004) *Calcif.Tiss. Internat.* **75**, 231-242
26. Price, P. A., June, H. H., Hamlin, N. J., and Williamson, M. K. (2004) *J. Biol. Chem.* **279**(18), 19169-19180
27. Jahnen-Dechent, W., Schinke, T., Trindl, A., Muller-Esterl, W., Sablitzky, F., Kaiser, S., and Blessing, M. (1997) *J. Biol. Chem.* **272**, 31496-31503
28. Price, P. A., and Lim, J. E. (2003) *J. Biol. Chem.* **278**(24), 22144-22152
29. Schinke, T., Amendt, C., Trindl, A., Poschke, O., Muller-Esterl, W., and Jahnen-Dechent, W. (1996) *J. Biol. Chem.* **271**, 20789-20796
30. Price, P. A., Otsuka, A. S., Poser, J. W., Kristaponis, J., and Raman, N. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 1447-1451
31. Einbinder, J., and Schubert, M. (1950) *Journal of Biological Chemistry* **188**, 335-341

32. Chen, P. S., Toribara, T. Y., and Warner, H. (1956) *Anal. Chem.* **28**(11), 1756-1758
33. Hale, J. E., Williamson, M. K., and Price, P. A. (1991) *J. Biol. Chem.* **266**, 21145-21149
34. Hemmelder, M. H., De Jong, P. E., and De Zeeuw, D. (1998) *J. Lab Clin. Med.* **132**, 390-403
35. Scott, T. A., and Melvin, E. H. (1953) *Anal. Chem.* **25**, 1656-1661
36. Taha, E. A., and Youssef, N. R. (2003) *Cliem. Pharm. Bull.* **51**(12), 1444-1447
37. Voet, D., and Voet, D. G. (2004) *Biochemistry*, 3rd Ed., John Wiley & Sons Inc., New York
38. Torchia, D. (1982) *Methods in Enzymology* **82**, 174-186
39. Ekani-Nkodo, A., and Fygenson, D. (2003) *Phys Rev E Stat Nonlin Soft Matter Phys* **67**, 021909
40. Bonar, L. C., Lees, S., and Mook, H. A. (1985) *J. Mol. Biol.* **181**, 265-270
41. Ottani, V., Raspanti, M., and Ruggeri, A. (2001) *Micron* **32**, 251-260
42. Boskey, A. L., Maresca, M., Ullrich, W., Doty, S. B., Butler, W. T., and Prince, C. W. (1993) *Bone Miner.* **22**, 147-159
43. Hunter, G. K., Kyle, C. L., and Goldberg, H. A. (1994) *Biochem. J.* **300**, 723-728
44. Midura, R. J., Wang, A., Lovitch, D., Law, D., Powell, K., and Gorski, J. P. (2004) *J. Biol. Chem.* **279**(24), 25464-25473
45. Tye, C. E., Rattray, K. R., Warner, K. J., Gordon, J. A. R., Sodek, J., Hunter, G. K., and Goldberg, H. A. (2003) *J. Biol. Chem.* **278**(10), 7949-7955
46. Anderson, H. C. (1995) *Clinical Orthopaedics and Related Research* **314**, 266-280
47. Skedros, J. (2005) *Cells Tissues Organs* **181**, 23-37
48. Bhatnagar, V. M. (1969) *Contr. Mineral. and Petrol.* **22**, 375-378
49. Tzaphlidou, M. (2005) *Micron* **36**, 593-601

FOOTNOTES

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1. The fraction of demineralized bone water calculated to lie within osteocyte lacunae is less than 1% of the total (47).
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 A^3) is over 12 times greater than the volume of a hydroxyapatite unit cell (529.2 A^3 (48))**,** a hydroxyapatite crystal the size of BGP contains about 12 hydroxyapatite unit cells.

FIGURE LEGENDS

Figure 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 91ml, 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-^{14}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 1**), times the weight of collagen in the column, 14g. (See "Experimental Procedures.")

Figure 2. 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 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 5**; the volume inside collagen was estimated by multiplying the liquid content of hydrated bone, 1.58 ml/g (**Table 4**), by the weight of collagen in the column, 51g (**Table 5**). (See "Experimental Procedures").

Figure 3. 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 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 1-^{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 5**. (See "Experimental Procedures").

Figure 4. 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, 1.8nm; packing fraction, ~ 0.7). The dry fibril has a diameter of ~ 30 nm (Bragg spacing, 1.1nm; 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 lower

right diagram shows that albumin would interfere with collagen packing far more than BGP; these effects on packing may explain why albumin can't penetrate the fibril while BGP can. The fibril depicted here has the diameter (49) and water content (Table 4) of a typical bone collagen fibril. Since tendon fibrils are 75% water by volume (Table 1), a hydrated tendon fibril with the same number of collagen molecules would have a diameter of 48 nm.

Table 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.)

	Bovine achilles tendon	
	Sample 1	Sample 2
Wet weight of tendon fibers	1.330 ± 0.003 g	1.251 ± 0.008 g
Dry weight of tendon fibers	0.408 g	0.381 g
Weight of liquid in tendon fibers	0.922 g	0.870 g
Volume of liquid in tendon fibers	0.862 ml	0.813 ml
Volume liquid : Dry weight tendon fibers	2.11 ml/g	2.13 ml/g

Table 2: The size exclusion properties of purified bovine achilles tendon collagen

The packed column whose preparation is described in the Figure 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-^{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 4 runs was 80±0.95 ml (Mean ±SD). The results show the elution volume of each test molecule. (See Experimental Procedures for details)

Test molecule	MW (Da)	Elution volume, ml
Albumin	66,000	52
Fetuin	48,000	51
Bone Gla Protein	5,700	80
Glucose	180	80
Phosphate	95	80

Table 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.)

	Bovine bone segment before demineralization (A)	The same segment after demineralization (B)	Change due to demineralization (B-A)
Mean Thickness	2.03 cm	2.05 cm	+0.02 cm
Mean Length	1.77 cm	1.74 cm	-0.03 cm
Wet Weight of bone	40.50 g	21.16 g	-19.34 g
Dry weight of bone	37.30 g	8.10 g	-29.2 g
Weight of liquid in bone (Wet minus dry weight)	3.20 g	13.06 g	9.86 g (+9.70 ml)[a]
Weight of mineral in bone	29.20 g	0.0 g	-29.20 g (-9.42 ml)[b]

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

Table 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.

Segment 1

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

Segment 2

Equilibration Solution	**Water**	**20mM Tris, 2M NaCl**	**20mM Tris, 0.15M NaCl**
Mean Thickness	2.23 cm	2.24 cm	2.25 cm
Mean Length	1.54 cm	1.58 cm	1.59 cm
Wet Weight of bone	20.53 ± 0.03 g	20.97 ± 0.01 g	20.58 ± 0.02g
Dry Weight of bone	7.90 g	7.90 g	7.90 g
Weight of liquid in bone (wet minus dry weight)	12.60 g	13.07 g	12.68 g
Volume of liquid in bone	12.60 ml	12.21 ml	12.48 ml
Liquid volume : Dry Weight	1.59 ml/g	1.55 ml/g	1.58 ml/g

Table 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)

	Non-demineralized bone sand (A)	Demineralized bone sand (B)	Change due to demineralization (B-A)
Total volume of packed column	227 ml	227 ml	---
Wet weight of column contents	367 g	243 g	-124 g
Dry weight of column contents	242 g	51 g	-191 g
Weight of water in packed column (Wet minus dry weight)	125 g	192 g	+67 g (+67 ml)
Weight of mineral in packed column	191 g	0 g	-191 g (-62 ml)[a]

a. Assuming a density of 3.1 g/cc

Table 6: The size exclusion properties of demineralized bovine bone collagen

The demineralized packed bone sand column whose preparation is described in the Table 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-^{14}C-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).

Molecules eluting at excluded volume	**MW (Da)**	**Elution volume, ml**
High MW Dextran	5-40x10^6	110
Albumin	67,000	113
Fetuin	48,000	110
Ovalbumin	43,000	119
Molecules eluting in fractionation range	**MW (Da)**	**Elution volume, ml**
Trypsin inhibitor	21,500	154
Low MW Dextran	10,200	130
Heptaose	1,152	160
Molecules eluting at bed volume	**MW (Da)**	**Elution volume, ml**
Glucose	180	191
Dimethylsulfoxide	78	191
Calcium	40	191

Table 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 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).

Test molecule	MW (Da)	Volume separating test molecule from glucose, ml		Difference due to demineralization (ml)
		Demineralized Bone Sand	Non-demineralized Bone Sand	
High MW dextran	5-40x10^6	81	10	71
Albumin	66,000	78	11	67
Fetuin	48,000	81	10	71
Volume of liquid in column bed, ml (Table 5)		192	125	67
Volume of mineral, ml (Table 5)		0	62	-62

Table 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-^{14}C 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.)

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

FIGURE 1

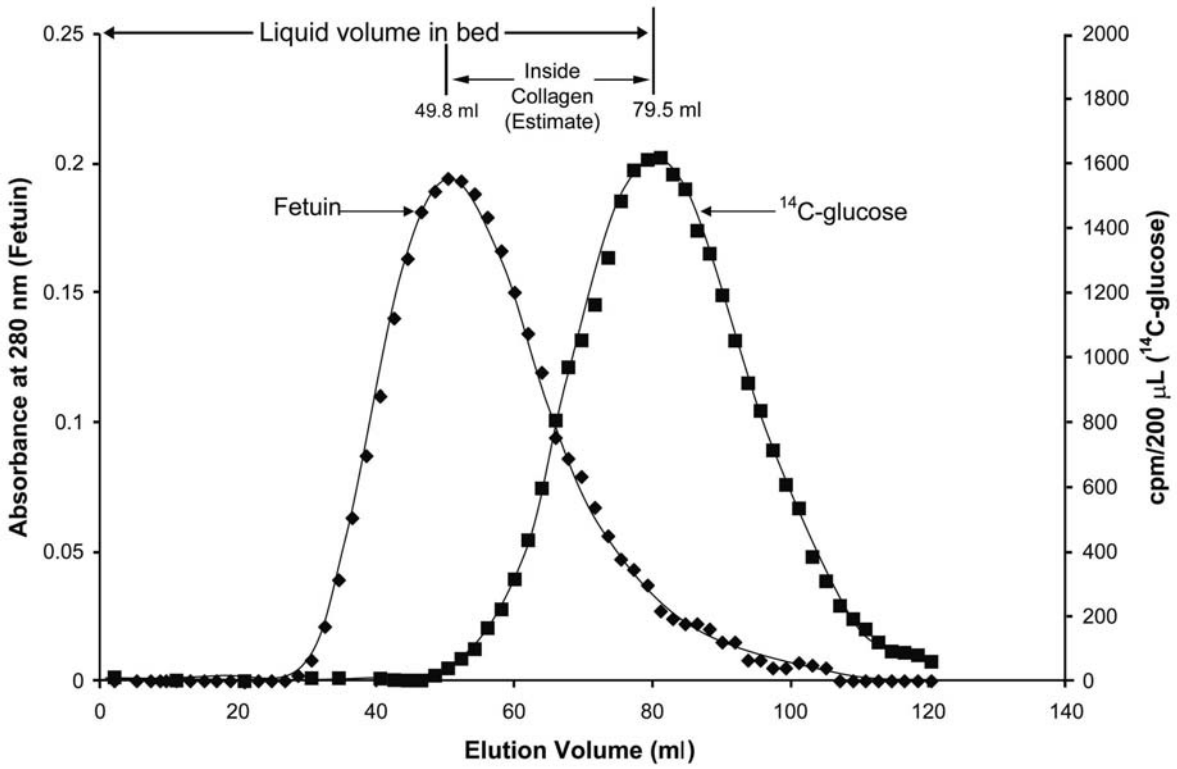

FIGURE 2

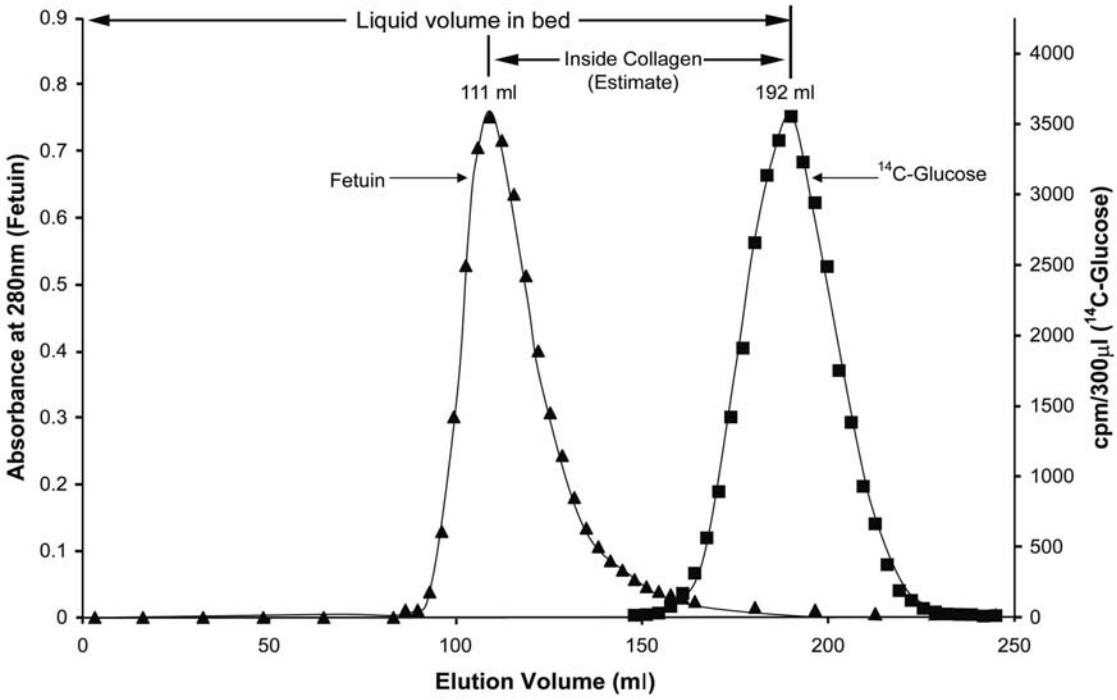

FIGURE 3

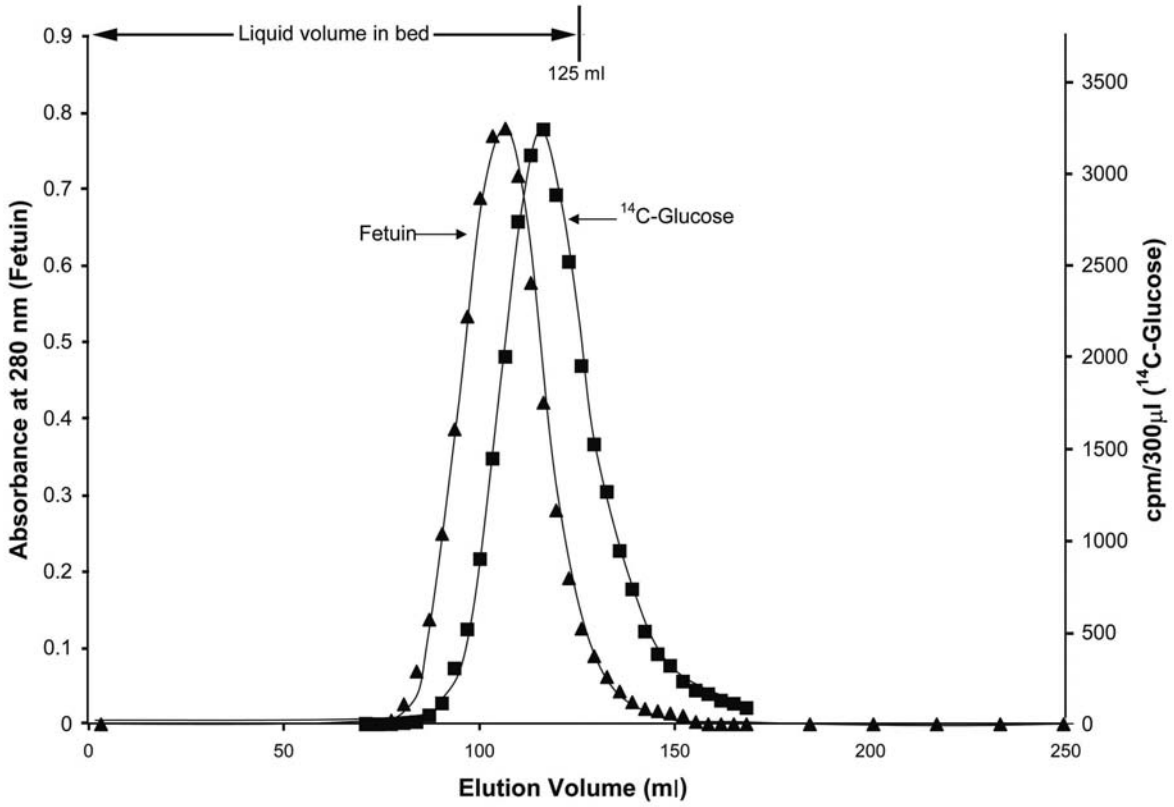

FIGURE 4