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A Novel Assay that Analyzes the Calcification Activity in Serum and How this Activity Relates to Bone Metabolism

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A Novel Assay that Analyzes the Calcification Activity in Serum and How this Activity Relates to Bone Metabolism

A Thesis submitted in partial satisfaction of the requirements for the Degree Master of Science

in

Biology

by

Erin Kathleen Hourigan

Committee in charge:

Professor Paul A. Price, Chair
Professor Nigel Crawford
Professor Susan Golden

2014
The Thesis of Erin Kathleen Hourigan is approved and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California, San Diego

2014
DEDICATION

This thesis is dedicated to my parents, two older sisters (Ann and Michal), and friends for their unwavering support over the years.
A mature person is one who does not think only in absolutes, who is able to be objective even when deeply stirred emotionally, who has learned that there is both good and bad in all people and in all things, and who walks humbly and deals charitably with the circumstances of life, knowing that in this world no one is all knowing and therefore all of us need both love and charity.

*Eleanor Roosevelt*
# TABLE OF CONTENTS

Signature Page ........................................................................................................... iii  
Dedication ................................................................................................................. iv  
Epigraph .................................................................................................................... v  
Table of Contents ...................................................................................................... vi  
List of Figures ........................................................................................................... vii  
List of Tables .......................................................................................................... vii  
Acknowledgements ................................................................................................. ix  
Abstract .................................................................................................................. x  

Introduction ............................................................................................................. 1  
Materials and Methods .......................................................................................... 6  
Results ...................................................................................................................... 11  
Discussion ................................................................................................................. 38  
References ................................................................................................................. 49
LIST OF FIGURES

Figure 1. The mineralization mechanism in collagen ................................................. 3

Figure 2. Apatite crystals grow in the presence of calcium and phosphate .................. 12

Figure 3. The addition of 2% rat serum into calcification buffer forms a cloudy suspension after a 24-hour incubation at 37°C ................................................................. 16

Figure 4. Alizarin staining confirms that the cloudiness seen in a 2% rat serum solution is due to apatite crystals .............................................................................................................. 18

Figure 5. Calcium levels monitor the formation of mineral in a 50-fold dilution of rat serum in calcification buffer .......................................................................................... 20

Figure 6. Three age-equivalent normal rats have comparable serum calcification activity ........................................................................................................................................ 22

Figure 7. Alkaline phosphatase accelerates the formation of mineral in 2% normal rat serum ............................................................................................................................... 24

Figure 8. Uremic rats has more serum calcification activity than age-equivalent normal rats .................................................................................................................................... 27

Figure 9. Alkaline phosphatase similarly accelerates mineral formation in 2% normal and 2% uremic rat serum ....................................................................................................... 30

Figure 10. Ibandronate treatment does not affect the calcification activity in uremic rat serum ................................................................................................................................. 33

Figure 11. A visual comparison of the rat serum dilutions after 24 hours in 37°C ........ 35

Figure 12. Alizarin staining of suspended mineral grown by serum from the three rat treatment groups ...................................................................................................................... 36

Figure 13. Stained mineral grown by serum from the three rat treatment groups sediments out of solution .................................................................................................................. 37

Figure 14. An example of an experiment using the 96-well plate system ............... 48
LIST OF TABLES

Table 1. A comparison of two potential calcification buffers ........................................... 14
Table 2. Serum biochemical measurements ........................................................................ 45
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I would first like to thank my advisor, Dr. Price, for his guidance throughout my years of research in his lab.

I would also like to thank all the fellow researchers that I have worked with in the Price Lab. A special thanks to Bryan Lam, who developed the buffer for my assay, and Wendy Chen who have both become like family.
ABSTRACT OF THE THESIS

A Novel Assay that Analyzes the Calcification Activity in Serum and How this Activity Relates to Bone Metabolism

by

Erin Kathleen Hourigan

Master of Science in Biology

University of California, San Diego, 2014

Professor Paul A. Price, Chair

Our long-term goal is to understand the biochemical mechanism responsible for the calcification of collagen fibrils in normal bone formation. Previous studies in our lab have shown that purified type I collagen and demineralized bone matrix both calcify rapidly when incubated in serum in the absence of cells [1-4]. Therefore, the calcification of collagen is due to the presence of a serum calcification activity. Molecules smaller
than a 6kDa protein can diffuse into the fibril matrix and can directly impact mineralization. While molecules larger than a 40kDa 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 matrix [5]. Therefore, without a size-excluding matrix, inhibitors of apatite growth prevent us from studying the calcification activity in serum.

We developed a simple method where we can examine the calcification activity in serum without the need of a size excluding matrix. Diluting serum 50-fold into a system with temperature, pH and calcium and phosphate levels comparable to blood enables us to isolate the serum calcification activity by reducing inhibitors of apatite growth down to concentrations where they can no longer prevent mineral formation. With this dilution method, we gain more insight into the calcification activity in serum and how this activity relates to bone metabolism.
INTRODUCTION

Mineral in bone is mostly located within the fibrils of Type I collagen [6-11]. During mineralization, the fibril is formed first and then water within the fibril is replaced with mineral [12, 13]. Consequently, the collagen fibril provides the aqueous compartment in which mineral grows. Previous studies in our lab have shown that the physical structure of the collagen fibril plays an additional role in mineralization: the role of a gatekeeper that allows molecules smaller than a 6kDa protein to freely access the water within the fibril while molecules larger than a 40kDa protein are secluded from the fibril [5]. Molecules smaller than a 6kDa protein can diffuse into the fibril matrix and can directly impact mineralization. Although molecules larger than a 40kDa 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 matrix (Figure 1) [5].

Our long-term goal is to understand the biochemical mechanism responsible for the calcification of collagen fibrils in normal bone formation. Previous studies in our lab have shown that purified type I collagen and demineralized bone matrix both calcify rapidly when incubated in serum in the absence of cells [1-4]. The calcification of collagen is due to the presence of a serum calcification activity, one sufficiently potent that collagen calcifies when incubated in media containing as little as 1.5% serum but not in serum-free media alone [1-4].
The serum mechanism that drives the mineralization of type I collagen within bone and tendon was found to have four key requirements (Figure 1): 1) tissue non-specific alkaline phosphatase (TNAP) is required to activate a serum nucleator of apatite formation [14]. The serum nucleator consists of one or more proteins that are 50-150kDa in size [14]. This nucleator has been found in all vertebrates tested including humans, rats, cows, cartilaginous fish, bony fish, and lamprey [2]. 2) The activated serum nucleator is necessary to generate small apatite crystals (<6kDa in size) [14]. 3) The calcifying matrix (the collagen fibril) must have size exclusion characteristics that allow molecules less than 6kDa (such as calcium, phosphate, and small apatite crystals) to diffuse into the water within the collagen fibrils while excluding large molecules greater than 40kDa [5]. 4) A large protein, such as fetuin (59kDa), that inhibits the growth of crystals outside of the matrix [15]. Most small apatite crystals form complexes with serum fetuin that inhibit their growth, forming a fetuin-mineral complex. A few of the crystals are free and because of their small size, can diffuse into the collagen fibrils. Since fetuin is too large to pass through, the crystals inside the matrix are free of fetuin, and grow rapidly in the presence of serum calcium and phosphate [16].
It has been a goal in our lab to develop a method that allows us to examine the calcification activity (nucleator) that forms apatite crystals in serum, but without the presence of a size excluding matrix, fetuin prevents mineral from forming in serum [16]. Therefore, to study the activity that forms apatite crystals in serum, we looked to develop
a method that would reduce the inhibitory effects of fetuin. A previous study in our lab removed fetuin from serum using rabbit anti-bovine fetuin antibodies. Removing fetuin eliminated the ability of serum to calcify Type I collagen, but caused mineral to precipitate in serum whether or not a collagen matrix was present [16]. This study robustly demonstrated that removing fetuin from serum leads to mineral growth in the solution outside the matrix. However, while this method added some insight into the activity that forms apatite crystals in serum, it was an arduous, energy intensive and time-consuming process to remove fetuin with antibodies.

My project focused on developing a simpler method to reduce the inhibitory effects of fetuin in serum so that I could more easily study the calcification activity in serum. Rather than directly removing fetuin from serum, as with the antibody method, we hypothesized that we could similarly reduce the inhibitory effects of fetuin and enable apatite growth by simply diluting serum into a system with temperature, pH and calcium and phosphate levels comparable to mammalian blood. If fetuin is diluted down to a concentration where it could no longer inhibit mineral growth, we could allow the apatite crystals that are formed by serum to grow without the need of a size excluding matrix.

For my project, we chose to analyze serum samples that were the product of a previous study in our lab. This previous study explored the association between bone loss and artery calcification in uremic rats [17]. Adult male rats that were made uremic through a synthetic 0.75% adenine diet developed extensive artery calcification in the aorta and associated arteries while the age-equivalent control rats failed to show any signs of abnormal artery calcification [17]. This study also showed that the medial artery calcification in the uremic-induced rats was prevented by a dose of ibandronate that was
previously shown to inhibit bone resorption in other rat models [18-21]. Ibandronate is a highly specific inhibitor of the osteoclast at the concentration used in this study and has no known effects on vascular cells. We wanted to develop an assay that would allow us to analyze the calcification activity in both uremic and normal rat serum. By doing so, we hoped to find an association between uremia and the amount of calcification activity in serum. Also, understanding how inhibiting bone resorption affects the growth of apatite crystals from serum could help us find an association between the calcification activity in serum and bone metabolism.

The initial goal of this present study was to first develop a simple assay that would allow us to gain more insight into the calcification activity in serum. We next wanted to use this assay to analyze the calcification activity in uremic and normal rat serum. We hoped to see whether we could use the serum calcification activity to distinguish between normal and uremic rats. By doing so, we could demonstrate that the serum calcification activity could be used as an indicator for disease. Lastly, we wanted to see how inhibiting bone resorption affects the serum calcification activity in uremic rats so we could better understand how this calcification activity in serum relates to overall bone metabolism.
MATERIALS AND METHODS

Materials

Dulbecco’s Modified Eagle Medium (DMEM) was purchased from Gibco (Grand Island, NY). Penicillin-Streptomycin was purchased from Invitrogen (Carlsbad, CA). Sodium azide was purchased from EM Science (Gibbstown, NJ). A 500 ml volume of DMEM was supplemented with 5 ml of penicillin/streptomycin, 1 ml of 10 % sodium azide, and 1.1 ml of 0.5 M Na$_2$HPO$_4$. HEPES were purchased from Fischer (Fair Lawn, NJ). Intestinal alkaline phosphatase was purchased from Calzyme (San Luis Obispo, CA). Calcium levels were determined colorimetrically using cresolphthalein complexone (JAS Diagnostics, Miami FL). Alizarin was purchased from Sigma (St. Louis, MO).

Methods

Preparation of calcification buffer

The calcification buffer used to investigate the calcification activity in serum was prepared at room temperature utilizing a procedure that was designed to achieve the near instantaneous mixing of calcium and phosphate. 75ml of 0.2M HEPES, pH 7.4 with 4mM CaCl$_2$ was placed into one 300ml beaker and 75ml of 0.2M HEPES pH 7.4 with 4mM Na$_2$HPO$_4$ was placed into a second 300ml beaker. A stir bar was placed in the CaCl$_2$-HEPES solution and stirring was initiated. Next, the Na$_2$HPO$_4$–HEPES solution was quickly poured into the CaCl$_2$-HEPES solution. While stirring continued, 300ul of 10% (w/v) sodium azide was added to the final solution to prevent bacterial growth. This would achieve a final calcification buffer containing 0.2M HEPES, pH 7.4,
2mM calcium and phosphate, and 0.02% azide. The final calcification buffer was stored in 50ml conicals at 2°C until use.

**Preparation of alkaline phosphatase**

5mg of 2000U/mg intestinal alkaline phosphatase was added to 5ml of Milli-Q water in a 15ml conical. The conical was rotated end-over-end for 10 minutes until the powder was fully dissolved. The final 2000U/ml alkaline phosphatase solution was stored in 0.5ml aliquots at -70°C until use.

**Source of rat sera**

Rat serum samples were the product of a previous study in our lab [17]. Thirteen-week-old male rats (Sprague–Dawley derived) were purchased from Harlan Sprague–Dawley (San Diego, CA, USA). Rats were fed the diets containing either 25% protein or 0.75% adenine and 2.5% protein for 4 weeks ad libitum. A subset of the rats fed the 2.5% protein diet received once daily subcutaneous injections of ibandronate at a dose of 0.25 mg/kg body weight/day. Ibandronate injections began 12 days after the start of the adenine diet. Animals were killed by exsanguination while under ether anesthetic and blood was allowed to clot for 30 minutes at room temperature. Serum was collected by centrifugation at 1400g for 10 min and was stored in 0.5ml aliquots at -70°C until use.
Calcium analysis

At specified time points, 50ul aliquots of sample were removed and spun down in an Eppendorf Centrifuge 5415C for 5 minutes at 7,000 rpm. 25ul of the supernatant solution was added to 25ul of 150mM HCl and then quantitatively assayed for calcium by a colorimetric assay purchased from JAS Diagnostics Inc. Calcium levels were determined at a wavelength of 575nm using a Infinite® 200 PRO 96-well plate reader from TECAN Group Ltd.

Alizarin staining to indicate the presence of mineral

Alizarin staining was utilized to detect apatite mineral precipitated in our experiments. Conicals were spun in a Fischer Scientific Centra 228 Centrifuge at 3,400 rpm (x1,300 g) for five minutes and the supernatants were discarded. The mineral pellets were then immediately resuspended in 3ml of 0.01% Alizarin Red in 0.05% KOH. Conicals were tightly sealed and placed at room temperature overnight. Solutions were again spun down in a Fischer Scientific Centra 228 Centrifuge at 3,400 rpm (x1,300 g) for five minutes and the supernatants were discarded. The stained mineral pellets were resuspended in 3ml of 0.05% KOH. This step was repeated until all excess stain was removed. Conicals were tightly sealed and stored at room temperature.

Mineral formation by 2% normal rat serum

2ml of calcification buffer (0.2M HEPES buffer, pH 7.4 containing 2mM calcium and phosphate; See Preparation of Calcification Buffer) was aliquotted into 15ml polyethylene terephthalate conicals. 40ul of sera from three 17-week-old normal rats
were separately diluted into calcification buffer and the concials were immediately flicked to mix. In three additional conicals, dilutions of the same three rat sera were repeated and 20U/ml of alkaline phosphatase was added to each of those three dilutions. The conicals were flicked again to ensure mixing. 20U/ml of alkaline phosphatase was also added to 2ml of serum-free calcification buffer to verify that the calcification buffer did not form mineral spontaneously. All conicals were tightly capped and incubated at 37°C for 24 hours. Each dilution was performed in triplicate.

Aliquots of each solution were taken at hours 3, 4, 5, 6, 7, 11, 12, 13, 14, 15 and 24 and analyzed for calcium (See Calcium Analysis). At 24 hours, the remainder of the solutions were spun down with a Fischer Scientific Centra 228 Centrifuge at 3,400 rpm (x1,300 g) for five minutes and the supernatants were discarded. Mineral was resuspended in 2ml of ethanol and stored tightly capped at room temperature.

The calcium data was analyzed as follows: For each data point, the calcium levels in the three replicates of the serum-free calcification buffer were averaged (Figures 5, 6, 7). The calcium levels from the three dilutions containing serum from normal rat 1 were averaged (Figure 5). Next, calcium levels were averaged according to the normal rat serum that was diluted (Figure 6). Calcium levels in the three replicate solutions of each of the three separate normal rat serum dilutions (total of 9 solutions) were all collectively averaged together. The same was done with the calcium levels from all the serum dilutions with alkaline phosphatase (Figure 7). Each data point in Figure 6 is the average calcium level. Each data point in Figure 5 and 7 is the average calcium level ± standard deviation.
Comparison of calcification activity in normal and uremic rat serum

The same general protocol used in the previous experiment was followed except the following modifications were made. Serum from three 17-week-old uremic rats and serum from one of the normal rats used in the previous experiment were separately diluted. In four additional conicals, the dilution of the same four rat sera was repeated and alkaline phosphatase was added to each of those four solutions. Aliquots of each solution were taken at hours 2, 3, 4, 5, 7, 8, 9, 11, 12, 13 and 24 and analyzed for calcium (See Calcium Analysis). Calcium levels were averaged based on experimental group and data points were shown as the mean calcium level ± standard deviation (Figures 8 and 9).

The effect of ibandronate treatment on the calcification activity in uremic rat serum

The same general protocol used in the two previous experiments was followed except the following modifications were made. In seven conicals, the normal rat serum and three uremic rat serum used in the previous experiment and the serum from three 17-week-old ibandronate-treated uremic rats were separately diluted into calcification buffer. Immediately after the dilution step, alkaline phosphatase was added to each of the seven solutions. Aliquots of each solution were taken at hours 2, 3, 4, 6, and 7 and analyzed for calcium (See Calcium Analysis). Calcium levels were averaged based on experimental group and data points were shown as the mean calcium level (Figure 10).
RESULTS

Development of a buffer to study the calcification activity in serum

The first goal of my project was to develop a system that allows us to study the calcification activity that forms apatite crystals in serum. However, the mineralization mechanism in serum is very complex and involves many ill-defined entities. Additionally, without a size excluding structure, such as collagen, fetuin forms small complexes with apatite crystals, preventing mineral formation in serum [18]. Therefore, we wanted to develop a simple system where we could suppress the inhibitory effects of fetuin on the formation of apatite crystals. By diluting serum, we hoped to reduce the inhibitory effects of fetuin and isolate the calcification activity that forms apatite crystals.

To accomplish this, we first needed to develop an appropriate calcification buffer to function as a diluent in our system. We sought to design a simple buffer that met the following criteria: 1) The buffer had to be stable and not form mineral spontaneously. 2) The calcium and phosphate levels of the buffer had to be comparable to those of normal rat serum. (The concentrations of calcium and phosphate in normal rat serum are 2.5mM [14]). 3) Lastly, at 37°C and pH 7.4, the physiological temperature and pH of blood, the addition of a mineral-forming catalyst had to lead to apatite crystal growth.

In initial pilot studies, we used Dulbecco’s Modified Eagle Medium (DMEM) with phosphate boosted to 2mM to study the calcification activity in serum (See Materials and Methods). We found that 2% normal rat serum in this phosphate-boosted DMEM buffer formed mineral after 8-9 days incubated at 37°C. Adding 20U/ml of alkaline phosphatase accelerated mineral formation in this system to only 2-3
days (Table 1). Without the addition of serum, the phosphate-boosted DMEM buffer remained stable and free of mineral for weeks.

The onset of mineral formation in phosphate-boosted DMEM was indicated when the initially clear solution turned a powdery white. Throughout the course of our experiments, we discovered that this cloudiness was a reliable and robust indicator of mineral formation. 1-2 days after the onset of cloudiness, the mineral in solution began to settle and form a pellet. Since apatite crystals grow from ionic calcium and phosphate (See Figure 2) and can be removed by centrifugation, we could quantitatively monitor mineral formation with the decline of calcium and phosphate in the supernatant. For our studies, we found that measuring calcium levels in the supernatant solutions was a sufficient and reliable gauge for monitoring this reaction.

**Figure 2. Apatite crystals grow in the presence of calcium and phosphate.** The high molecular weight nucleator forms small apatite crystals from ionic calcium and phosphate. In our system, these apatite crystals become very apparent when the initially clear calcification buffer turned into a cloudy, powdery white solution. 1-2 days after the onset of mineral formation, the originally suspended crystals settle to the bottom of the solution and form a pellet. Since apatite crystals grow at the expense of calcium and phosphate and can be removed by centrifugation, we can quantitatively monitor mineral formation with the decline of ionic calcium and phosphate in the supernatant [14].
Even though we were pleased that this phosphate-boosted DMEM system allowed us to qualitatively and quantitatively analyze the activity that forms apatite crystals in serum, we still wanted to simplify our system even further. While DMEM is a common cell culture media, it contains components that are not involved in mineral growth and the carbonate buffering requires a 5% CO₂ atmosphere, which made maintaining the pH of our system difficult. We, therefore, sought to design a simpler buffer that only contained materials necessary for mineral formation.

Bryan Lam, a fellow researcher in our lab, developed a 0.2M HEPES buffer, pH 7.4 containing 2mM calcium and phosphate (See Materials and Methods). When incubated at 37°C, a solution of 2% normal rat serum in this calcification buffer formed mineral in 11 hours and the reaction was accelerated to 5-6 hours with the addition of 20U/ml of alkaline phosphatase (Table 1). Without the addition of serum, the HEPES calcification buffer remained free of mineral for weeks. In addition to the increased speed of mineral formation in this calcification buffer, maintaining the pH of our solutions was no longer problematic. Since HEPES replaced the carbonate buffering agent, the pH of our solutions always remained at 7.4. We were confident that with this HEPES calcification buffer, we developed a simple system that would allow us to easily and efficiently study the formation of apatite crystals in a variety of sera.
Table 1. A comparison of two potential calcification buffers.

In initial pilot studies, we used two different calcification buffers to examine the calcification activity that forms apatite crystals in serum. First, we used a DMEM buffer with added phosphate. Next, we studied apatite crystals with a 0.2M HEPES buffer, pH 7.4 containing 2mM calcium and phosphate. Both buffers 1) were stable and did not form mineral spontaneously, 2) had phosphate and calcium levels comparable to normal rat serum, and 3) at the physiological temperature and pH of blood, formed mineral with the addition of a mineral-inducing catalyst. However, the increased speed of mineral formation and the simplicity of the system with 0.2M HEPES made it the preferred calcification buffer.

<table>
<thead>
<tr>
<th>Buffer</th>
<th>[Ca] (mM)</th>
<th>[Pi] (mM)</th>
<th>Buffering agent</th>
<th>Time till onset of mineral formation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>No addition of serum</td>
</tr>
<tr>
<td>DMEM</td>
<td>1.8</td>
<td>2.0</td>
<td>Carbonate pH 7.4</td>
<td>--</td>
</tr>
<tr>
<td>0.2M HEPES</td>
<td>2.0</td>
<td>2.0</td>
<td>HEPES pH 7.4</td>
<td>--</td>
</tr>
</tbody>
</table>

Mineral formation in a 50-fold dilution of normal rat serum in calcification buffer

We first wanted to test if serum from a single normal rat could reliably and reproducibly form mineral in our calcification system. The degree of reproducibility of this reaction determines the validity of our assay and results. To test this, serum from a single 17-week-old rat was diluted to 2% into 2ml of calcification buffer (0.2 M HEPES buffer at pH 7.4 containing 2mM calcium and 2mM phosphate; See Materials and Methods) and incubated at 37°C for 24 hours. Serum-free calcification buffer was also incubated at 37°C for 24 hours to verify that our calcification buffer did not form mineral spontaneously. Both solutions were performed in triplicate. Aliquots of each solution
were taken at specified time points and analyzed for calcium (See Materials and Methods).

At the start of the experiment, the solutions with and without serum were clear. By hour 11, while the serum-free calcification buffer remained clear, the 2% normal rat serum solutions became slightly cloudy. As the experiment continued, the 2% normal rat serum solutions became progressively cloudier and were nearly opaque at 24 hours (Figure 3). At this time, the mineral was in the form of many small crystals that remained completely suspended in solution. The serum-free calcification buffer, however, remained clear and free of any detectable mineral throughout the entire 24-hour experiment. A conical of 2% normal rat serum and a conical of serum-free calcification buffer were centrifuged to sediment any mineral from solution. After centrifugation, the previously cloudy 2% normal rat serum solution formed a white pellet at the bottom of the conical, leaving the remaining supernatant solution clear (Figure 3). The serum-free calcification buffer, however, did not form a pellet after centrifugation.
Figure 3. The addition of 2% rat serum into calcification buffer forms a cloudy suspension after a 24-hour incubation at 37°C. At the start of incubation, the serum-free calcification buffer and 2% normal rat serum solutions were clear. Solutions were incubated at 37°C for 24 hours. After 11 hours, the 2% normal rat serum solutions began to show signs of cloudiness. The serum-free calcification buffer remained clear for the entire 24 hours. At hour 24, a conical of 2% normal rat serum and a conical of serum-free calcification buffer were centrifuged. The 2% normal rat serum solution formed a white pellet at the bottom of the conical, leaving the supernatant solution clear. The serum-free solution, however, remained clear and free of any detectable mineral.

Alizarin staining allowed us to verify the presence of apatite mineral in our system (See Materials and Methods). Alizarin Red is a dye that binds specifically to apatite mineral. Therefore, with alizarin, any apatite mineral formed in our experiments would become a rich dark red. After alizarin staining, the previously powdery white
mineral formed by 2% normal rat serum became a dark red. The conical that contained the serum-free calcification buffer, however, remained clear after staining (Figure 4).

The conicals were centrifuged for a second time to confirm that the dramatically colored solution was a result of suspended stained mineral (Figure 4). After centrifugation, a large red pellet sedimented at the bottom of the conical that contained mineral from 2% normal rat serum, leaving the supernatant solution clear. This pellet is similar in size and location to the white pellet formed in the same conical before staining (Figure 3). This staining verifies that apatite crystals are responsible for the extensive cloudiness seen within the 2% normal rat serum solution. The conical that contained the serum-free calcification buffer showed no signs of staining after centrifugation, demonstrating that our calcification buffer failed to form mineral without the addition of serum.
Figure 4. Alizarin staining confirms that the cloudiness seen in a 2% rat serum solution is due to apatite crystals. After the centrifugation step shown in Figure 3, the supernatants were removed and the pellet fraction was stained with Alizarin (See Materials and Methods). The stained mineral was dispersed resulting in a dramatically red solution. After a subsequent centrifugation step, a large red pellet formed at the bottom of the previously colored solution, leaving the remaining solution clear. Since alizarin stains apatite crystals red, these results demonstrate that apatite crystals cause the cloudiness seen in a 2% normal rat serum solution.

To support our visual observations quantitatively, the precipitation of apatite crystals was monitored throughout the experiment by the decline of calcium in the supernatant solutions (See Materials and Methods). We also wanted to use calcium analysis to examine the reproducibility of the rate of mineralization from a single rat serum source. The degree of reproducibility of calcium decline in our system determines the reliability of our results and the consistency of this reaction.
The supernatants of the three replicate 2% normal rat serum solutions began to decline in calcium at hour 11, which was also the time that the solutions began to turn slightly cloudy (Figure 5). Calcium levels continued to slowly but steadily decline until the end of the experiment at 24 hours. This calcium decline closely correlates with the onset and progression of cloudiness seen in the solutions. The supernatant of the serum-free calcification buffer never declined in calcium throughout the 24 hour experiment and calcium levels remained at 2mM.

This data demonstrates that calcium decline closely correlates with the onset and progression of mineralization in our calcification system, making it a reliable quantitative gauge of apatite crystal growth. The calcium analysis also demonstrates that in this calcification system, the onset and progression of mineralization from serum of a single rat is highly consistent and reproducible. The robust reproducibility of these findings suggests that the calcification activity in serum is not a random phenomenon, but a characteristic of the serum itself.
Figure 5. Calcium levels monitor the formation of mineral in a 50-fold dilution of rat serum in calcification buffer. Serum from a 17-week-old rat was diluted to 2% in a 2ml volume of calcification buffer (0.2 M HEPES buffer at pH 7.4 containing 2mM calcium and 2mM phosphate; See Materials and Methods). All dilutions were incubated at 37°C for 24 hours. The precipitation of apatite mineral was monitored by the decline of calcium in the supernatant solutions. Solutions were performed in triplicate. Aliquots of each solution were removed at the indicated times and analyzed for calcium. (See Materials and Methods). Each time point is the average calcium level in the three replicate solutions ± standard deviation. The dotted line is the theoretical calcium level of normal rat serum.

After demonstrating the robust consistency of mineral formation with a single sample of rat serum, we wanted to analyze the serum calcification activity in multiple normal rats of the same age. Our goal was to examine the variation of serum calcification activity among the age-equivalent normal rats. Serum from three 17-week-old rats were separately diluted 50-fold into calcification buffer and incubated for 24 hours at 37°C.
These dilutions were performed in triplicate. Aliquots of each dilution were removed during specified time points and analyzed for calcium (See Materials and Methods).

Dilutions of all three rat sera first showed slight signs of cloudiness at hour 11 and calcium levels in the supernatants began to decline between hours 11 and 12 (Figure 6). The supernatants of the rat serum dilutions also showed comparable calcium decline throughout the entire 24-hour experiment. The serum-free calcification buffer never formed mineral and calcium levels in the supernatants again remained at 2mM. The comparable onset of mineral formation and decline in calcium levels show that the serum calcification activity is similar in all three normal rats. These results demonstrate that this amount of calcification activity in serum is not a unique characteristic of a single rat, but of a collection of these age-equivalent normal rats, suggesting that the amount of calcification activity in serum could be a characteristic of a given treatment group.
Figure 6. Three age-equivalent normal rats have comparable serum calcification activity. Serum from three 17-week-old normal rats were separately diluted to 2% into 2ml of calcification buffer. All dilutions were incubated at 37°C for 24 hours. The precipitation of mineral was monitored by the decline of calcium in the supernatant solutions. Solutions were performed in triplicate. Aliquots of each solution were removed at specified time points and analyzed for calcium (See Materials and Methods). Each data point is the average calcium level according to the rat serum that was diluted.

Alkaline phosphatase accelerates mineral formation in 2% normal rat serum

In our general model of the mineralization of collagen, TNAP plays a vital role in activating the nucleator that forms apatite crystals in serum (Figure 1). As a result, we wanted to examine how alkaline phosphatase affects the calcification activity that forms apatite crystals in normal rat serum. Therefore, we repeated the previous experiment, but with the addition of alkaline phosphatase. Serum from the same three 17-week-old
normal rats in the previous experiment were separately diluted into calcification buffer and 20U/ml of alkaline phosphatase was added to each of those solutions. All solutions were incubated at 37°C for 24 hours. Aliquotes of each solution were taken at specified time points and analyzed for calcium (See Materials and Methods).

In the previous experiment, the 2% normal rat serum solutions began to show signs of cloudiness at hour 11. With the addition of alkaline phosphatase, however, dilutions of the same normal rat sera became slightly cloudy by hour 5. Calcium began to decline in the supernatants around the same time as the onset of cloudiness (Figure 7). Therefore, the addition of alkaline phosphatase comparably accelerated the onset of mineral formation with all three normal rat sera by about 2 fold. The 2% normal rat serum solutions with alkaline phosphatase were nearly opaque by hour 7. By 24 hours, apatite crystals were still completely suspended in solution and there was no observable change in cloudiness from hour 7 to hour 24. At the end of the 24-hour experiment, there was no significant difference between the cloudiness in the normal rat serum dilutions with or without alkaline phosphatase. The serum-free calcification buffer showed no signs of mineral formation or calcium decline throughout the entire 24-hour experiment.

In addition to a sooner onset of crystal formation, the solutions with alkaline phosphatase also declined in calcium at a faster rate (Figure 7). It is difficult to determine at this point whether this difference in rate of calcium decline is a reflection of the calcification activity of the serum sample, or whether a sooner mineral onset in our calcification system results in a faster decline in calcium.
Figure 7. Alkaline phosphatase accelerates mineral formation in 2% normal rat serum. To determine the impact of alkaline phosphatase on the formation of mineral, the experiment described in Figure 6 was repeated and 20U/ml of alkaline phosphatase was added immediately after the serum dilution step. The data from Figure 6 was incorporated into this figure. (See Material and Methods). For each time point from Figure 6, the calcium levels in the 3 replicate solutions of each of the 3 separate normal rat serum dilutions (total of 9 solutions) were collectively averaged together. The same was done with calcium levels from the serum dilutions with alkaline phosphatase. Each time point is the average calcium level ± standard deviation.

Uremic rat serum has more calcification activity than normal rat serum

The rat serum samples for this project were the product of a previous study in our lab. In this previous study, our lab investigated medial artery calcification in adult uremic rats to examine the correlation between bone loss and artery calcification [17]. The uremic-induced rats developed extensive calcification in the aorta and associated arteries.
while the age-equivalent normal rats showed no signs of abnormal artery calcification. Since we previously demonstrated that three age-equivalent normal rats have comparable serum calcification activity, we next wanted to analyze the serum from uremic rats. By analyzing uremic rat serum, we hoped that the diseased rats would have a different serum calcification activity, which would allow us to make an association between the animal’s disease and the calcification activity in serum. To test this, serum from three 17-week-old uremic rats and serum from one of the age-equivalent normal rats used in the previous experiment were separately diluted into calcification buffer. Solutions were performed in triplicate and incubated at 37°C for 24 hours. Aliquots of each sample were removed during specified time points and analyzed for calcium (See Materials and Methods).

Again, the 2% normal rat serum solutions began to turn slightly cloudy at hour 11 and calcium began to decline in the supernatants around the same time. The uremic rat serum dilutions, however, turned faintly cloudy at hour 6 and calcium in the supernatants began to decline between hours 6 and 7 (Figure 8). This data indicates that, in our calcification system, 2% uremic rat serum forms apatite crystals 1.5 to 2 times faster than 2% normal rat serum. In addition to a sooner onset of crystal formation, the uremic rat serum dilutions also declined in calcium more rapidly than the normal rat serum dilutions (Figure 8). Also, while the previous experiment indicated that serum from the three normal rats have comparable calcification activity, there was greater variation in calcium decline among the solutions with uremic rat serum. This variation could be a result of the differing severities of disease in the uremic rats.

At 24 hours, the cloudiness of the uremic serum solutions was comparable to the cloudiness seen in the normal rat serum solutions. Based on visual observations and
calcium analysis, the serum-free calcification buffer never formed apatite crystals throughout the entire 24-hour experiment. While it is difficult to quantify the magnitude, the visual observations and calcium analysis show that uremic rat serum forms apatite crystals ~1.5 times faster than normal rat serum. Since all three 2% uremic rat serum dilutions formed apatite mineral faster than the 2% normal rat serum dilutions, our data indicates that uremic rat serum has more calcification activity than normal rat serum. By demonstrating that uremic rat serum has more calcification activity than normal rat serum, these results establish that there is an association between the disease of the uremic rats and their serum calcification activity.
Figure 8. Uremic rats have more serum calcification activity than age-equivalent normal rats. Serum from three 17-week-old uremic rats and from one of the normal rats in the previous experiment were separately diluted into calcification buffer. All dilutions were performed in triplicate and incubated at 37°C for 24 hours (See Materials and Methods). Calcium levels in 3 replicate solutions of each of the 3 separate uremic rat serum dilutions (total of 9 solutions) were collectively averaged together. For the normal rat serum dilutions, each time point is the average calcium level in the three replicate solutions. Each time point is the average calcium level ± standard deviation.

We just demonstrated that uremic rat serum has more calcification activity than normal rat serum. Since one of our previous experiments showed that alkaline phosphatase accelerated the growth of apatite crystals formed by normal rat serum (Figure 7), we wanted to see if alkaline phosphatase has a similar effect on the growth of crystals formed by uremic rat serum. To do so, we repeated the previous experiment, but with the addition of alkaline phosphatase. Serum from the same normal rat and three
uremic rats used in the previous experiment were separately diluted into calcification buffer. 20U/ml of alkaline phosphatase was added to each solution and all solutions were incubated at 37°C for 24 hours. Dilutions were performed in triplicate. Aliquots were taken at specified time points and analyzed for calcium (See Materials and Methods).

The uremic rat serum solutions with alkaline phosphatase began to turn slightly cloudy between hours 2 and 3. Calcium began to decline in the supernatants of these solutions around the same time as the onset of cloudiness. As seen in our previous experiments, the 2% normal rat serum solutions with alkaline phosphatase became slightly cloudy at hour 5 and the supernatants started to decline in calcium at the same time (Figure 9). This data indicates that alkaline phosphatase accelerated the growth of apatite crystals formed from uremic rat serum by about 2 fold. Therefore, alkaline phosphatase has a similar acceleratory effect on the growth of apatite crystals formed by both uremic and normal rat serum. Also, while there was a slight variation in calcium decline among the uremic rat serum solutions in the previous experiment (Figure 8), the addition of alkaline phosphatase caused the uremic rat serum dilutions to decline in calcium more uniformly.

At 24 hours, the cloudiness of the 2% uremic rat serum solutions with alkaline phosphatase was similar to the cloudiness seen in the 2% normal rat serum solutions with alkaline phosphatase. Based on our visual observations and calcium analysis, the serum-free calcification buffer never formed apatite crystals throughout the entire 24-hour experiment. These results robustly demonstrate that alkaline phosphatase has a similar acceleratory effect on the growth of apatite crystals formed by both normal and uremic rat serum.
The calcium analysis also allows us to make a few additional observations regarding the formation of apatite crystals in our system. Solutions with a sooner onset of crystal formation also have a faster rate in calcium decline and a larger over decline in calcium (Figure 9). Therefore, in our system, greater calcification activity in serum is associated with 1) a sooner onset of mineral formation, 2) a faster rate of calcium decline and 3) a greater overall decline in calcium. These correlations provide us with some insight into the overall behavior of the calcification activity in serum.
Figure 9. Alkaline phosphatase similarly accelerates mineral formation in both 2% normal and 2% uremic rat serum. To compare the effect of alkaline phosphatase on mineral formation in the normal rat and uremic rat serum dilutions, the experiment in Figure 5 was repeated and 20U/ml of alkaline phosphatase was added immediately after the serum dilution step. (See Materials and Methods). The calcium levels in 3 replicate solutions of each of the 3 separate uremic rat serum dilutions (total of 9 solutions) were collectively averaged together. The calcium levels of the three replicate normal rat serum dilutions were also averaged. Each time point is the average calcium level ± standard deviation.
Ibandronate treatment does not affect the calcification activity in uremic rat serum

The previous study in our lab that produced the serum samples for the present project investigated the effect of inhibiting bone resorption on medial artery calcification in uremic rats. The results of the previous study demonstrated that the medial artery calcification in uremic rats is prevented by a dose of ibandronate that inhibits bone resorption [17]. Since we previously showed that uremic rat serum has more calcification activity than normal rat serum, we wanted to analyze serum from the uremic rats that received ibandronate treatment to see how inhibiting bone resorption affects the calcification activity in uremic rat serum.

Serum from the same four rats used in the previous experiment and serum from three 17-week-old ibandronate-treated uremic rats were separately diluted into calcification buffer. 20U/ml of alkaline phosphatase was added to each solution and conicals were incubated at 37°C for 24 hours. Each solution was performed in triplicate. Aliquots of each sample were removed during specified time points and analyzed for calcium (See Materials and Methods).

The uremic rat serum solutions with alkaline phosphatase turned slightly cloudy at hour 2 and the normal rat serum solutions with alkaline phosphatase began to turn slightly cloudy by hour 5. Based on visual observations and calcium analysis, the serum-free calcification buffer never formed apatite crystals throughout the entire experiment. These results repeated those seen in our previous experiments.

The solutions with serum from the ibandronate-treated uremic rats and alkaline phosphatase began to turn slightly cloudy by hour 2 or 3. The supernatants of these solutions also began to decline in calcium around the same time. In fact, the progression
of calcium decline was nearly identical to that seen in the uremic rat serum solutions with alkaline phosphatase. Therefore, according to these results, uremic rats with and without ibandronate treatment have comparable serum calcification activity. Our results show that inhibiting bone resorption does not affect the calcification activity in uremic rat serum.
Figure 10. Ibandronate treatment does not affect the calcification activity in uremic rat serum. We wanted to determine whether treating a uremic rat with ibandronate affects the calcification activity in serum. Serum from one 17-week-old normal rat, three 17-week-old uremic rats and three 17-week-old ibandronate–treated uremic rats were separately diluted into calcification buffer. Immediately after the dilution step, 20U/ml of alkaline phosphatase was added to each solution. (See Materials and Methods). The calcium levels in 3 replicate solutions of each of the 3 separate uremic rat serum dilutions (total of 9 solutions) were collectively averaged together. The same was done with the calcium levels from the ibandronate-treated uremic rats. The calcium levels from three replicate normal rat serum dilutions were also averaged.
According to the calcium analysis and visual observations, ibandronate treatment does not affect the timing or rate at which apatite crystals formed by uremic rat serum. Since we could not quantitatively distinguish between the two treatment groups using the onset and progression of calcium decline in the supernatant solutions, we wanted to see if there was a qualitative difference between the apatite crystals formed by the serum of the three rat treatment groups.

First, we compared the 2% serum solutions of all three treatment groups after 24 hours of incubation at 37°C (Figure 11). After 24 hours at 37°C, while the serum-free calcification buffer remained clear and free of any detectable apatite crystals, the 2% serum solutions of the three treatment groups were nearly opaque and comparable in cloudiness. The solutions were then centrifuged to sediment any mineral from solution. After centrifugation, all of the 2% serum solutions formed small white pellets that were comparable in size and color, leaving the remaining supernatant solutions clear (Figure 11). The serum-free calcification buffer remained clear and did not form a pellet after centrifugation.
Figure 11. A visual comparison of the rat serum dilutions after 24 hours in 37°C. We compared the 2% serum solutions of all three treatment groups after 24 hours of incubation at 37°C. While the serum-free calcification buffer remained clear and free of any detectable apatite crystals, the 2% serum solutions from all three rat treatment groups were nearly opaque and comparable in cloudiness. After centrifugation, the 2% serum solutions all formed small white pellets that were comparable in size and color, leaving the remaining supernatant solutions clear. The calcification buffer did not form a pellet after centrifugation.

Next, we wanted to stain the apatite crystals with Alizarin Red to see if the staining could reveal any differences in the crystals formed by the serum of the three rat treatment groups (See Materials and Methods). After staining with Alizarin, the crystals formed from the uremic and ibandronate-treated uremic rat sera were a comparable vibrant red (Figure 12). The crystals formed by the normal rat serum, however, were observably more faint. The calcification buffer remained clear and free of any stain. The conicals were centrifuged to confirm that the dramatically colored solutions were a result of suspended stained mineral (Figure 13). After centrifugation, a large red pellet
sedimented at the bottom of the previously red solutions, leaving the supernatants clear. The red pellets were similar in size and color among the three treatment groups. The difference in intensity of crystal staining, however, suggests that we could gain more insight into the calcification activity of serum by studying the morphology of these apatite crystals.

Figure 12. Alizarin staining of suspended mineral grown by serum from the three rat treatment groups. Alizarin staining was used to compare the apatite crystals formed by the serum from the three rat treatment groups (See Materials and Methods). While the serum-free calcification buffer did not show any stain, the solutions containing rat serum from the three treatment groups all showed an intense red stain. The stained mineral from the uremic and ibandronate-treated uremic rat serum solutions, however, was much more vibrant than that of the mineral formed by normal rat serum. The difference in staining suggests that we could gain some insight into the calcification activity of apatite crystals in serum by studying the morphology of these crystals.
Figure 13. Stained mineral grown by serum from the rat treatment groups sediments out of solution. After staining, the crystals formed by the 2% serum solutions of the three rat treatment groups were centrifuged. The centrifugation caused a large red pellet to sediment at the bottom of the previously red solutions and left the supernatants clear. The red pellets were similar in size and location among the three rat treatment groups. The conical that contained the serum-free calcification buffer remained clear and did not form any pellet or show any staining.
DISCUSSION

A system that forms apatite crystals with 2% serum

The goal of our study was to develop an assay that would allow us to easily and efficiently study the calcification activity in serum. The calcification of collagen is due to the presence of a serum calcification activity. Yet, since the growth of apatite crystals is inhibited in neat serum, we had to develop a method that would enable us to unmask and analyze this calcification activity. A previous study in our lab showed that a massive mineral precipitate forms during the incubation of fetuin-depleted serum but not during the incubation of serum containing fetuin [16]. This same study also found that removing fetuin from serum eliminates the ability of serum to induce the calcification of a type I collagen matrix and that adding purified fetuin to fetuin-depleted serum restores this activity [16]. Therefore, we knew that reducing the concentration of fetuin in serum enables apatite growth and reveals the calcification activity in serum.

The findings of the previous study provided great insight into the calcification activity in serum. However, this previous study removed fetuin from serum using antibodies, which was an arduous, energy intensive and time-consuming process. My project focused on developing a simpler method to study the calcification activity in serum. Rather than directly removing fetuin from serum, as with the antibody method, we hypothesized that we could similarly reduce the inhibitory effects of fetuin and enable apatite growth by simply diluting serum into a system with temperature, pH and calcium and phosphate levels comparable to mammalian blood.

The present study proved that, at physiological temperature (37°C), diluting serum 50-fold into a buffer with pH and calcium and phosphate levels comparable to mammary
blood consistently and reproducibly formed a vast number of apatite crystals at a remarkably fast rate. Even though the rate of mineral growth differed in phosphate-boosted DMEM and 0.2M HEPES, the mineral formation phenomenon was still extremely similar in both calcification buffers (Table 1). In both types of calcification buffers, a 2% rat serum solution formed a cloudy precipitate and became progressively cloudier as the experiment continued (Figure 3). The mineral in solution appeared powdery white and was in the form of a vast number of small apatite crystals. 1-2 days after the onset of mineral formation, the apatite crystals settled out of solution and formed a pellet. Since apatite crystals sediment out of solution, we were able to quantitatively monitor the growth of apatite mineral by measuring calcium decline in the supernatant. We found that calcium decline in the supernatant closely correlated with the onset and progression of cloudiness in solution, making it a reliable quantitative gauge of apatite growth in our system. Alizarin staining confirmed that the cloudiness in the 2% serum solutions was due to the presence of many small apatite crystals suspended in solution (Figure 4). Therefore, with our system, we have laid the foundations for an assay that allows us to analyze the calcification activity in serum.

The formation of apatite crystals in our system is the result of an activity in serum

The simplicity of our calcification system allowed us to associate the growth of apatite mineral in our experiments to a calcification activity in serum. Both the phosphate-boosted DMEM and 0.2M HEPES calcification buffers had a pH (7.4), temperature (37°C), and calcium and phosphate ion product (3.6 to 4 mM²) comparable to mammalian blood. Without the addition of serum, both the phosphate-boosted and
HEPES calcification buffers did not form apatite crystals and remained stable for weeks. Yet, a 50-fold dilution of serum in either calcification buffer quickly formed a vast number of apatite crystals. Since the addition of serum was required for apatite crystals to grow in our system, this calcification activity we were monitoring must have been the result of an activity in serum.

Another advantage of our method was that we never manipulated the serum samples in any way. After the rats were killed by exsanguination while under ether anesthetic, blood was allowed to clot for 30 minutes at room temperature. Serum was collected after centrifugation and stored at -70°C until dilutions were performed for our experiments [17]. In pilot studies, we have also observed the formation of apatite crystals with 2% human serum and the human serum samples were acquired using standard medical practices. Therefore, the formation of apatite crystals in our system is not a result of how the serum samples were handled. This simple, physiologically relevant system coupled with the unaltered serum samples enabled us to directly correlate the growth of apatite crystals in 2% serum to an activity within serum itself.

*Why do apatite crystals form in 2% serum, but not in undiluted serum?*

Previous studies in our lab have shown that apatite crystals do not grow in undiluted serum [14, 16]. Yet, our study clearly demonstrated that apatite crystals grow robustly and consistently in 2% serum. These findings cause us to question why apatite forms with a 2% serum solution, but not in neat serum. Our data and previous studies in our lab have shown that the calcification activity in serum is highly active [14, 16], but somehow this activity is being suppressed in neat serum. As we initially hypothesized,
our dilution method could be reducing fetuin down to a concentration where it can no longer inhibit apatite growth.

Even though previous studies have shown that mineral precipitates rapidly in fetuin-depleted serum [14, 16], it is still unclear how apatite crystals grow in our system. There are two reasonable theories: 1) Apatite crystals are completely absent from blood and diluting serum enables the nucleation and growth of these crystals at a dramatically fast rate or 2) small crystal nuclei are already present in blood and diluting serum enables their growth. However, regardless of whether crystal nuclei are present in blood or not, the speed and robust consistency of mineral formation in our system suggests that the absence of inhibitors of apatite growth in serum would quickly lead to dramatically adverse effects in blood. Our present findings and the previous studies in our lab demonstrate that reducing the inhibitors of apatite growth in serum dramatically exposes the highly active calcification activity in serum.

Evidence that calcification activity in serum is linked to bone metabolism

Serum is relevant to bone mineralization: osteoblasts form bone in a vascular compartment 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 [22]. Therefore, there is a constant exchange of materials between bone and serum.

TNAP has a long and extensive history of association with normal bone calcification [23]. Despite its nonspecific expression in tissues genetic deficiency in tissue - nonspecific alkaline phosphatase in mice and humans (called hypophosphatasia) is primarily associated with defects in bone mineralization [23, 24, 25]. This observation,
together with the abundant expression of alkaline phosphatase at sites of bone mineralization [26, 27] in association with the outer surface of osteoblasts [28] and matrix vesicles [29], supports the role of the enzyme in normal bone mineralization.

When we tested the effect of alkaline phosphatase on the formation of apatite crystals in our system, we found that the enzyme accelerated the formation of apatite crystals grown in both 2% normal and 2% uremic rat serum by 2 fold. Since alkaline phosphatase has such a potent influence on apatite growth in a 2% serum solution, our findings strongly support the hypothesis that the formation of apatite crystals in serum is related to bone metabolism. Yet, how this calcification activity in serum relates to bone metabolism is still unclear.

With our system, we have shown that a solution of 2% normal rat serum with the addition alkaline phosphatase forms apatite crystals in about 5 hours. In pilot studies, 2% adult human serum with alkaline phosphatase takes 4 days for apatite to form and the same reaction takes 5 hours to occur in a solution of 2% human cord blood with alkaline phosphatase. These results cause us to question why a human fetus and a 17-week-old normal rat, animals of two different species, have similar serum calcification activity.

Bone turnover could explain why human cord blood and serum from a 17-week-old rat have comparable calcification activity. The same biological mechanisms are responsible for increasing bone mass in both growing rats and human children [30]. Young human children replace their skeleton over a two-year period [31]. By the time the human skeleton reaches peak bone mass in the early 20’s, the adult human skeleton is replaced every 10 years [31]. On the other hand, a rat doubles the size of its skeleton between weeks 7 and 11 and reaches peak bone mass by 10 months [32, 33]. Therefore,
the bone turnover rate of a rat’s skeleton is much faster than that of a human skeleton. These observations together with our present data suggest that animals with a higher rate of bone turnover and growth have greater serum calcification activity.

Our present findings also show that uremic rats have greater serum calcification activity than age-equivalent normal rats. We already presented evidence that there is a relationship between serum calcification activity and the rate of bone turnover. Since bone loss is a common symptom associated with uremia, our results suggest that animals with bone loss could have more serum calcification activity than age-equivalent control animals. In our present study, ibandronate treatment did not normalize or diminish the elevated calcification activity in uremic rat serum. Since ibandronate is a potent inhibitor of osteoclast activity, our findings indicate that the calcification activity in serum is not influenced by bone resorption. Rather, the serum calcification activity could be a reflection of bone formation. Since inhibition of osteoclast activity did not affect the serum calcification activity in uremic rats, the elevated serum calcification activity in the uremic rats could be a result of a reduction in bone mineralization.

We previously established that there is a constant exchange of materials between bone and serum. There could also be a distribution of calcification activity between bone and serum as well. Diminished osteoblast activity results in a decrease in bone mineralization, and could consequently cause calcification activity to mostly remain in serum. Our present findings show that uremic rats have more serum calcification activity than age-equivalent normal rats, suggesting that animals with severe bone loss, or a reduction in bone mineralization, will have elevated serum calcification activity.
Therefore, the serum calcification activity appears to be dependent on the animal’s skeletal turnover and the distribution of calcification activity between bone and serum.

*Why does uremic rat serum have more calcification activity than normal rat serum?*

The biochemical analysis of the rat serum samples shows that serum phosphate levels of the uremic rats with and without ibandronate treatment are more than double those of the normal rats (Table 2). By diluting serum 50-fold, we rule out the possibility that serum chemistry could influence the calcification activity observed in our system. Even though in neat serum the difference in phosphate levels is significant, at a 50-fold dilution, this difference in phosphate levels becomes trivial. Therefore, high serum phosphate levels could not simply accelerate mineral formation in our system. However, elevated phosphate levels in the uremic rat serum could be indicative of a greater presence of crystal nuclei or of a crystal-forming catalyst in blood.

Elevated phosphate levels in blood typically indicate a decline in renal function and are commonly associated with a variety of kidney diseases, such as uremia [34-36]. High serum phosphate leads to elevated parathyroid hormone (PTH), which typically results in more bone resorption. Since the data from the present study proved that bone resorption does not affect the serum calcification activity, PTH is unlikely to be responsible for the elevated calcification activity in uremic rat serum. However, PTH receptors are also found on the surface of osteoblast. Therefore, while it is unlikely, PTH could still affect mineral formation in bone, and affect the serum calcification activity.

Greater concentrations of calcium and phosphate in blood also favor crystal nucleation [37-39]. Consequently, the higher phosphate levels in uremic rat serum could
have driven the formation of more crystal nuclei or could indicate the presence of more of a mineral-forming catalyst in blood, resulting in more calcification activity in serum. The presence of more crystal nuclei or more of a mineral-forming catalyst in uremic rat serum could explain why, after staining, the mineral formed by 2% uremic rat serum was darker than the mineral formed by 2% normal rat serum. Alizarin is a highly specific stain that binds to the surface of apatite mineral. Consequently, solutions with more stained crystals would appear darker than the solutions with fewer stained crystals. Therefore, 2% uremic rat serum may have formed more apatite crystals than 2% normal rat serum.

**Table 2: Serum biochemical measurements.**
BUN, blood urea nitrogen; PTH, parathyroid hormone. Blood was obtained from rats in each of the four indicated treatment groups 4 weeks after rats had been placed on to an adenine-containing diet in order to induce uremia. Blood was allowed to clot for 30 min at room temperature; serum was obtained by centrifugation, and stored at -70°C until analyzed. The values presented are the mean ± s.d. For the measurement of PTH, n=6 for the control group, n=6 for the 2.5% protein group, and n=5 for the 2.5% protein + ibandronate group. Corrected calcium is calcium normalized for differences in serum albumin [17].

<table>
<thead>
<tr>
<th>Serum Measurement</th>
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<th>Uremic</th>
<th>Uremic + Ibandronate</th>
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<td>Calcium (mM)</td>
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<td>Albumin (g/dl)</td>
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<tr>
<td>Phosphate (mM)</td>
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<td>PTH (pg/ml)</td>
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<td>2554.8 ± 1054.0</td>
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Future Experiments

Our lab has performed some pilot experiments that have analyzed the calcification activity of 2% human serum. Even though the rate of mineral formation is much slower with 2% human serum, the mineral formation phenomenon is extremely similar in both 2% human and 2% rat serum. We would eventually like to use our system to analyze the serum from humans with uremia to see if there is an elevated calcification activity in the serum of these diseased individuals. If uremic patients also have elevated serum calcification activity, then this assay could provide insight into how the calcification activity in serum relates to uremia and the overall bone metabolism in uremic patients. After analyzing the serum of uremic patients, this assay could be used to study the serum from patients of a variety of diseases, such as osteoporosis. By doing so, this assay could provide useful insight into how disease affects the calcification activity in serum and the overall bone metabolism of the patient.

Some of our pilot studies have analyzed 2% rat serum in a 96-well plate system (Figure 14). In one of our pilot experiments, serum from three normal and three uremic rats were separately diluted 50-fold into the same calcification buffer that was used in our original system and alkaline phosphatase was added to each serum solution. Alkaline phosphatase was also added to serum-free calcification buffer to serve as a negative control. A plastic seal was placed over the plate to prevent gas exchange and the plate was incubated in an automated plate reader at 37°C for 15 hours. This 96-well plate system has the same calcification buffer, pH and temperature as our original calcification system. However, with this system, 2% serum solutions are analyzed at a 200ul volume rather than a 2ml volume.
Since in our original calcification system we found that cloudiness was a robust and reliable gauge of mineral formation, we wanted to monitor the rate of mineralization by measuring the absorbance of the solutions. Rather than monitoring mineralization through calcium decline in the supernatant solutions, we could directly monitor the growth of mineral by measuring the absorbance of apatite crystals formed in each well. We are very enthusiastic with the speed and efficiency of this system since we can analyze multiple serum samples simultaneously and test various perturbations of our system in the same plate. Also, by using an automated plate reader to collect our data, we drastically reduce the influence of human error and are able to collect more precise data. Transferring our assay to this 96-well plate system would enable us to collect a tremendous amount of data in a relatively short period of time and would allow us to gain a better understanding of how this serum calcification activity relates to bone metabolism. In addition, this 96-well plate system could make our assay practical for clinical purposes and a potentially useful tool in medicine.
Figure 14. An example of an experiment using the 96-well plate system. We performed a pilot experiment in the 96-well plate system where we analyzed the rate of mineral formation in 2% normal and 2% uremic rat serum with alkaline phosphatase. Serum from 3 normal and 3 uremic rats were analyzed at a 50-fold dilution with the same calcification buffer as in our original system. Alkaline phosphatase was added to each of the 2% serum solutions and to serum-free calcification buffer. Each condition was preformed in quadruplicate. A plastic seal was placed over the plate to prevent gas exchange and the plate was incubated at 37°C for 15 hours. Absorbance values of each solution were taken every 30 minutes. Absorbance values were averaged based on treatment group.
REFERENCES


