Interstitial Fluid Calcium, Magnesium and Phosphorus Concentrations in Bone, Muscle and Subcutaneous Tissue Sampled with Ultrafiltration Probes

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Specialized ultrafiltration probes make it possible to measure calcium, magnesium and phosphorus in bone, muscle and subcutaneous tissue in freely moving sheep.

In vivo sampling using membrane probes has provided a tool for investigating the chemistry of the interstitial space. It has provided data which have expanded the understanding of both physiological and pathological conditions. Under a contract with NASA, we developed ultrafiltration probes which could be implanted in bone, muscle and subcutaneous tissue to sample the interstitial fluid. In this project, sheep were used as the animal model. This permitted the investigation of calcium, magnesium and phosphorus concentrations in locations not previously accessible to direct in vivo measurement. The goal of this project was to develop tools to study mineral metabolism. These tools will facilitate the development of a better understanding of bone physiology and pathology.

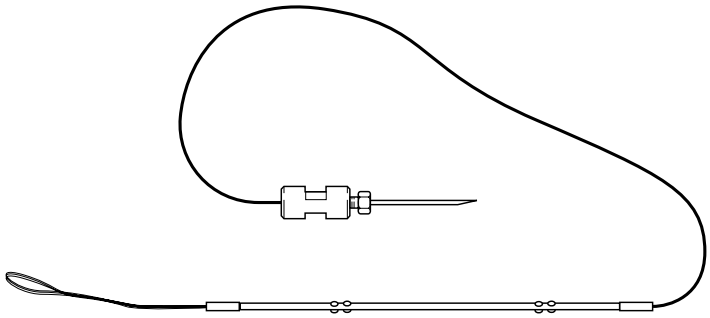
Calcium, magnesium and phosphorus are the minerals present in the largest quantities in bone and are important in giving strength and shape to the bone. However, they have important physiological functions in other parts of the body as well. Calcium is an essential element in intercellular regulation and metabolism. Extracellular calcium is an essential component of cofactors required for bone formation, blood clotting, adhesions molecules, and as a first messenger in signaling functions (1). Many of its actions within cells are dependent on a constant extracellular pool of available calcium. The parathyroid hormone-Vitamin D-calcitonin system and the parathyroid gland "set point" tightly regulate extracellular calcium concentrations. Magnesium is involved in neuromuscular transmission and is a cofactor in various enzyme reactions. It is important in ribosomal protein synthesis and ATP energy transfer. Phosphorus is ubiquitous throughout the body. Phosphates are a major component of the blood buffering system. Phosphorus is found in DNA, RNA, proteins and phospholipids and is important in energy metabolism. It therefore affects all aspects of physiology (2).

One of the benefits of membrane probe sampling is that different tissues can be sampled simultaneously. This provides a tool to study the differences in distribution of these minerals. In this study, calcium, magnesium and phosphorus concentrations were compared in the different tissues and in blood.

Calcium exists in blood in three forms: protein bound, complexed and ionized. Magnesium is also found in the blood in the protein bound or ionized forms. Complexed and ionized calcium can pass though the probe membrane but protein bound calcium cannot. The recovered calcium is called the ultrafilterable calcium. Ultrafiltrate membrane probes can also be used in vitro to separate protein bound calcium and magnesium from other forms of these minerals in blood.

F1

The Bone Ultrafiltration Probe has three 12 cm looped ultrafiltration fibers. A reinforcing sheath prevents kinking of the tubing at the bone exit site. Two tissue ingrowth cuffs and suture retainers help to stabilize the position of the probe.



Materials and Methods

Probe Development

Ultrafiltration probes are small implantable devices consisting of semipermeable hollow fiber membranes attached to a conducting tube. The fibers were implanted in the tissue to be sampled. The tubing exited through the skin and was attached to a needle hub. The needle was inserted into a VACUTAINER™, which created a negative pressure within the probe. The semipermeable fibers do not allow substances with a molecular weight higher than 40,000 daltons through their pores. Small molecules and ions pass though the membrane while large molecules such as proteins are excluded. The probes developed for this project were modifications of the BAS large animal probe (PN MF-7028). All probes in this study were constructed with three loops of semi-permeable fiber with a 40,000 molecular weight cut-off. Each fiber was 12 cm long. The bone probe (***F1***) had a reinforcing sheath to prevent kinking of the tubing as it made a 90° bend when exiting the bone. It also had an additional porous cuff at the end of the sheath to promote tissue ingrowth and increase positional stability. Additional suture retainers were added to anchor the probe in place and prevent dislodging with the normal movement of the sheep. For the muscle and subcutaneous probes, only one cuff was used, but additional suture retainers were added for anchoring.

In Vitro Recoveries. Before the probes were used for in vivo studies, in vitro recoveries were performed for calcium, magnesium and phosphorus to verify that they would cross the membrane (3). In vitro recoveries were: calcium 100%±3%, magnesium 99%±7% and phosphorus 102%±4%.

Sheep. Five female mixed-breed sheep (age range 1 to 4 years old) were obtained from the Purdue Sheep Farm. Protocols for this study were approved by the Purdue Animal Care and Use Committee. Each sheep was surgically implanted with bone, muscle and subcutaneous ultrafiltration probes. Free choice access to mixed grass/alfalfa hay and water was available throughout the experimental period.

Probe Implantation. A detailed description of the probe implantation procedure has been published previously (4). The sheep were anesthetized and maintained under isoflurane general anesthesia during the implantation procedure. Strict aseptic technique was used for all surgical procedures. For bone probe implantation, a hole was drilled into the medullary cavity via the greater trochanter. A second hole was drilled into the medullary cavity at the distal portion of the shaft of the femur. A looped wire was then inserted into the proximal hole and, using a guide in the distal hole, directed out of the femur. A length of suture was attached to the wire and the wire was withdrawn. The suture was then affixed to the probe tubing and the probe was carefully drawn into place. When the procedure was finished, the UF probe fibers were in the marrow cavity of the femur with the collection tubing exiting from the great trochanter, and then subsequently from the skin.

For implantation into the muscle, the probe was placed into a curved introducer. Skin incisions were made at the entrance and exit points to facilitate placement. The introducer was inserted through the skin and about 7 cm into the quadriceps muscle. The curved introducer penetrated the body of the muscle and curved back to the second incision. The introducer was pulled out, leaving the probe in place. The subcutaneous probe was inserted by a similar procedure. However, a straight introducer was used since the placement was immediately under the skin. After placement of the probes, the incisions were sutured, and a suture was placed around the probes to hold them in place until the tissue ingrowth into the cuff could anchor them in place. Needle hubs were then placed on the ultrafiltration probes and the needles were inserted into Vacutainers. Fitted jackets were put on the sheep, and the Vacutainers were placed into pockets in the jackets.

Sample Collection. Samples were collected continuously from the UF probes. The Vacutainers were changed at least once a day. During weekdays, they were changed twice a day. Samples were aliquoted immediately and frozen at -80° C until assayed. Heparinized blood samples were collected twice a week in the morning when the ultrafiltrate tubes were changed. Samples were centrifuged and plasma aliquoted and stored at -80° C until assayed.

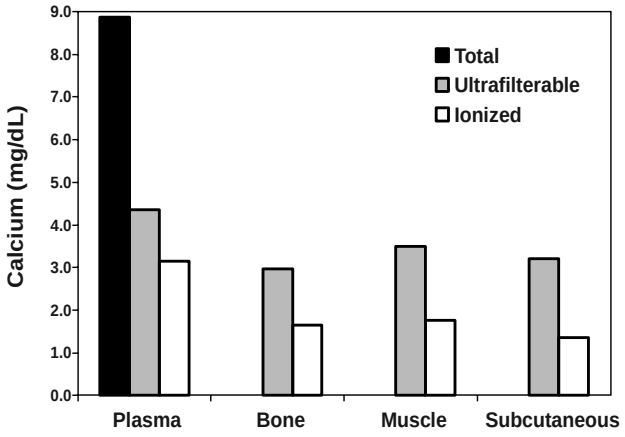
Sample Analysis. Plasma and ultrafiltrate samples were analyzed for calcium by spectrophotometric analysis using the o-cresolphthalein complexone method from Sigma Diagnostic Kit No. 587. In plasma this analysis yields the total calcium, which includes protein bound calcium, complexed calcium and ionized calcium. In the ultrafiltrate samples, this assay measures the sum of ionized and complexed calcium. In order to determine the complexed and ionized calcium in plasma, the plasma samples were ultrafiltered using U 3-2 probes. Ion-

T1

Calcium concentrations in plasma, and in bone, muscle and subcutaneous interstitial space.

	Bone	Muscle	Subcutaneous	Plasma
Total				8.87 ± 0.14 n=35
Ultrafilterable	2.97 ± 0.20 n=63	3.50 ± 0.09 n=139	3.21 ± 0.10 n=113	4.35 ± 0.15 n=33
Ionized	1.66 ± 0.20 n=70	1.77 ± 0.14 n=139	1.37 ± 0.15 n=85	3.16 ± 0.15 n=33

F2



In all tissues and in plasma, the ultrafilterable calcium was significantly higher than the ionized calcium ($p < .0001$). In plasma, the total calcium was significantly higher than the ionized and ultrafilterable ($p < 0.0001$). Muscle ultrafilterable calcium was significantly higher than both bone ($p = 0.02$) and subcutaneous tissue ($p = 0.03$). Plasma ultrafilterable calcium was significantly higher than interstitial ultrafilterable calcium in all tissues ($p < 0.0001$). There were no significant differences between ionized interstitial calcium concentrations in any of the tissues.

ized calcium was determined by ion-selective electrode. Magnesium was analyzed spectrophotometrically by the magnon method using Sigma Diagnostic Kit No. 596, and phosphorus was analyzed spectrophotometrically by the formation of a phosphomolybdate complex using Sigma Diagnostic Kit No. 360.

Data Analysis. Data was analyzed using the General Linear Models program of the SAS Statistical Program. The model used for analysis of the calcium data was: Concentration=Sheep|Tissue|Form, where form was ionized, ultrafilterable or total. For magnesium and phosphrus the model was: Concentration= Sheep|Tissue. Means and standard errors and p differences were determined. Differences with $p < 0.05$ was considered significant.

Results

In this study we determined the total, ultrafilterable and ionized calcium in blood, and ultrafilterable and ionized calcium in bone, muscle and subcutaneous tissue, using sheep as a model. In the blood, the spectrophotometric value represented total calcium or the sum of all three types of calcium: protein bound, complexed and ionized. The ion-selective electrode concentration represented the ionized calcium. In the probe samples, the spectrophotometric value measured the ultrafilterable calcium consisting of the complexed and ionized forms, and the ion-selective electrode value represented the ionic calcium.

The average lifetime of the bone probes was 35 days. The average lifetime for the muscle probe was 40 days and for the subcutaneous probe 37 days.

Calcium Concentrations: **T1** gives the means and standard errors of the plasma and tissue total, ultrafilterable and ionized calcium concentrations.

The ultrafilterable calcium concentrations for the muscle, subcutaneous and bone sites were 3.50 mg/dl, 3.21 mg/dl, and 2.97 mg/dl, respectively (**F2**). In plasma, the total, ultrafilterable and ionized calcium concentrations were 8.87 mg/dL, 4.35 mg/dL, and 3.16 mg/dL, respectively. There were some differences in ultrafilterable calcium among the different tissues. Muscle had the highest level of ultrafilterable interstitial calcium, and bone the lowest. Muscle was significantly higher than both bone ($p = 0.02$) and subcutaneous tissue ($p = 0.03$). Subcutaneous ultrafilterable calcium was not significantly higher than bone. Plasma ultrafilterable calcium was significantly higher than interstitial ultrafilterable calcium in all tissues ($p < 0.0001$). Ionized interstitial calcium concentrations in muscle, bone and subcutaneous averages were 1.77 mg/dL, 1.66 mg/dL and 1.37 mg/dL, respectively. There were no significant differences between ionized interstitial calcium concentrations in any of the tissues. The plasma ionized calcium was significantly different from the ionized calcium in the other tissues ($p = 0.0001$).

In plasma, calcium is protein bound, but there is very little protein in the interstitial fluid and therefore, probe samples reflect the total concentration of bone minerals within the interstitial space. With the use of UF probes, protein bound was separated in vitro from the non-protein bound in plasma samples. The ultrafilterable plasma calcium consists of the complexed and ionized calcium. By subtracting the ionized calcium from the ultrafilterable calcium it is possible to calculate the amount of complexed calcium. For calcium, it was therefore possible to determine all three forms in plasma (**F3**).

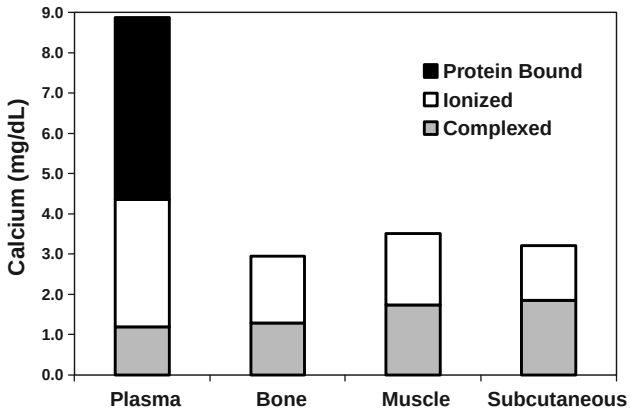
Magnesium Concentrations

T2 gives the means and standard errors of the plasma and tissue total and ultrafilterable magnesium concentrations.

Plasma total and ultrafilterable magnesium were 2.25 mg/dL and 1.20 mg/dL, respectively. The ultrafilterable magnesium concentrations for the bone, muscle and subcutaneous sites were 1.73 mg/dl, 1.58 mg/dl, and 1.62 mg/dl, respectively. Bone interstitial magnesium

F3

Calcium in plasma exists in three forms: protein bound, complexed and ionized. The total calcium represents the sum of these three forms. Ultrafilterable calcium represents the sum of ionized and complexed calcium.



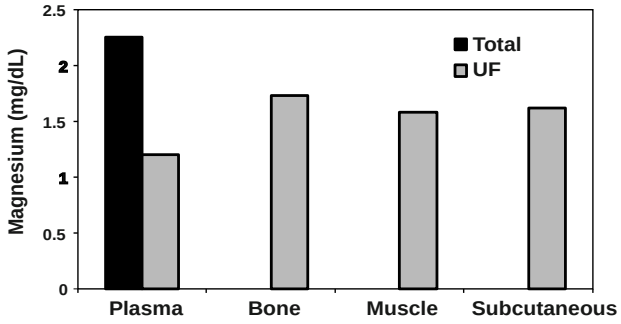
T2

Magnesium concentrations in plasma, and bone, muscle and subcutaneous interstitial space.

	Bone	Muscle	Subcutaneous	Plasma
Total				2.25 ± 0.01 n=42
Ultrafilterable	1.73 ± 0.04 n=124	1.58 ± 0.04 n=177	1.62 ± 0.03 n=208	1.20 ± 0.07 n=41

F4

Figure 4. In all tissues the interstitial magnesium was significantly higher that the plasma ultrafilterable magnesium ($p < .01$). This pattern is the reverse of what is found for calcium.



was significantly greater than muscle and subcutaneous interstitial magnesium ($p < 0.01$). There was no difference between muscle and subcutaneous interstitial magnesium. In all tissues the interstitial magnesium was significantly higher than the plasma ultrafilterable magnesium ($p < .01$) (***F4***).

Phosphorus Concentrations

The mean concentrations of phosphorus in bone, muscle and subcutaneous tissue were 4.30 mg/dL, 4.35 mg/dL, and 4.45 mg/dL, respectively. There were no significant differences between any of the tissues. Interstitial phosphorus in each of the tissues was significantly lower than the plasma phosphorus (6.11 mg/dL, $p < 0.0001$).

Discussion

Homeostasis in multi-celled organisms is achieved by maintaining the concentration of ions, cell constituents, and water within cells at a constant level. This, in turn, is maintained by homeostatic mechanisms that ensure the cells are bathed in extracellular fluid that has constant concentrations of many constituents (5). The extracellular calcium and magnesium concentrations are not identical in all tissues, however. There are clear examples of subcompartments within the larger extracellular pools, which differ in concentration from the blood. Extracellular calcium levels are different from blood in many tissues including kidney, bone, lung, gastrointestinal tract and skin. The extreme example would be the fluid in the resorptive lacunae of the bone that may reach calcium concentrations of 76 mM (6). Direct sampling of the extracellular fluid is necessary to determine calcium and magnesium concentrations at different sites, as they are not reflected accurately by sampling the vascular space. Knowledge of the extracellular calcium and magnesium concentrations in a given tissue is particularly important in studies where perturbations may not be reflected in the blood concentrations, such as in disease states or in conditions of microgravity. The changes in extracellular concentrations within tissues and compartments are unknown in these instances.

The ultrafiltrate probe is the ideal tool to investigate interstitial fluid chemistry in both large and small animals (3,4,7-13). It has the advantage that it can be used for long periods without loss of recovery (7,8). Previously, most ultrafiltration sampling had been done from subcutaneous tissue, although muscle in horses (11) and spine in dogs (10) have also been sampled. In this study, we demonstrated that the technique is also useful in studying bone and muscle in sheep. Sheep were used as a model of bone research because of their low cost, docile dispositions, and large bones. The sheep in this study were all older. This is an advantage when comparing them to humans, as older animals have Haversian remodeling. Ovariectomized ewes show decreased bone mass along with increased biochemical markers of bone turnover. Despite these advantages, sheep are not a widely used animal in mineral research, and questions about how they resemble human conditions remain.

This study demonstrated that there were differences in the relationship of plasma and interstitial bone mineral concentrations for the different bone minerals. For calcium, the concentration of ultrafilterable calcium is less in the interstitial fluid of bone, muscle and subcutaneous tissue than in the plasma (***F2***). For magnesium, the

situation is reversed (***F3***) and the interstitial magnesium in all tissues studied was greater than the plasma ultrafilterable magnesium.

Variations in concentration from one tissue to another also differed for the various bone minerals. For phosphorus, there were no significant differences among the interstitial concentrations of the different tissues studied. For magnesium, bone interstitial magnesium was significantly greater than subcutaneous and muscle interstitial magnesium. For calcium, the relationship was reversed and bone interstitial calcium was lower than the other tissues, although the difference was significant only for muscle. Although there were significant differences among ultrafilterable calcium concentrations in the different tissues, the ionized calcium concentrations were not different. The fact that there is a difference in UF calcium in different tissues, but not in ionized, is consistent with homeostatic control of ionized calcium, which is to be expected since ionized calcium is the physiologically active form. The complexing agents may be acting as a buffering system to help maintain constant ionized calcium concentrations.

Capillary ultrafiltration probes were shown to be a useful tool for monitoring chemical dynamics in the intercellular fluid. These probes have potential applications in the development of anti-osteoporosis drugs and countermeasures to microgravity-induced bone loss. They will also be useful tools in nutritional studies and in the study of bone physiology and pathology.

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