

Osteoporosis—New Insights

by JOHN KINNEY

Synchrotron radiation is giving us new insights into causes and treatments for osteoporosis.

OSTEOPOROSIS IS A DISEASE characterized by fragile bones that results in fractures. Half of women over seventy will have a fracture as a result of osteoporosis. For many of them, this will lead to a decline in their quality of life and independence. These fractures, which often occur with little or no injury,

are usually a result of reduced bone mass. Accordingly, research on osteoporosis has focused on those factors that affect bone mass, such as estrogen deprivation, age, and physical activity. Treatments to control osteoporosis have been largely based on trying to maintain or to increase skeletal mass; however, significant overlap exists between the bone mass of normal individuals and those with osteoporosis. This overlap has resulted in investigation into factors other than low bone mass, such as structural properties and bone quality, to explain the higher frequency of fractures among individuals with osteoporosis.

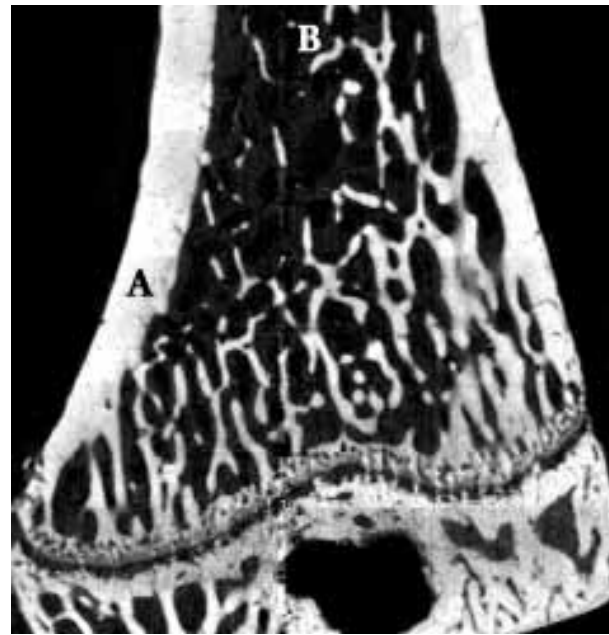
Bone is the hard, marrow-filled calcified tissue that forms the skeleton. Near the joints a porous spongy-like bone fills much of the marrow space as seen in the illustration on the right. This spongy bone aids in transferring the stresses of motion to the hard outer shell called the cortex. The spongy bone (frequently referred to as trabecular bone) is composed of an interconnected structure of curved plates and struts. One of the age-related changes that accompanies bone loss is a decrease in the amount of spongy bone. In normal people the volume occupied by this spongy bone decreases with age. This decrease in bone volume is apparently due to a loss in the number of struts and a severing of the interconnections, as opposed to a general uniform thinning of the elements. Many questions regarding the development and treatment of osteoporosis could be answered if the architecture of the spongy bone—and how it changes with disease and age—were better understood.

BONE STRUCTURE CHARACTERISTICS

About a hundred years ago, an English reverend named Abbott published a small book titled **Flatland**. It was a story of a two-dimensional world occupied by polygons. The protagonist was a square who, one day, was visited by a sphere. The sphere carried the square above Flatland, and allowed him to view his three-dimensional counterpart, the cube. Upon returning to Flatland, the square set about to enlighten his two-dimensional world about the existence of a three-dimensional universe. Unfortunately, the square was never able to provide convincing proof of the third dimension to his fellow inhabitants of Flatland and was eventually labeled a heretic and spent his remaining days in prison.

Though this book was intended as a social satire, it points out the difficulties describing three-dimensional structures using only two-dimensional views. The science of reconstructing three-dimensional information from two-dimensional views is called stereology. With stereological methods it is possible to estimate volume fractions and surface areas of particles or voids from just a few flat microscopic sections cut from an object.

Stereological methods are currently used to estimate three-dimensional properties of bone from two-dimensional or planar sections. These methods allow calculation of the volume fraction and surface areas of the spongy bone. It is also possible to quantify other structural features of the bone required to explain bone formation, resorption, and



An X-ray tomographic microscope image of a thin section of leg bone in a rat. The thick bone (A) surrounding the marrow cavity is the cortical bone, or cortex. The thin, web-like bone (B) within the marrow cavity is the spongy bone. The earliest stages of osteoporosis involve the resorption of spongy bone and a breaking of its interconnections.

structure. The application of stereological principles has provided important clues as to how a bone's structure affects its properties and is altered by disease and aging. Unfortunately, as we understand better the complex structures and properties of biological systems, we begin to exceed the ability of stereological methods to describe the critical aspects of the structure. In particular, conventional stereological methods begin to collapse when questions arise regarding how the spongy bone networks are connected. For example, one of the easiest questions to pose about any structure is to ask how many objects are contained within it. Indeed, it is impossible to answer the basic question "how many?" from a limited number of two-dimensional views or sections. A complete three-dimensional visualization is necessary.

Questions such as "how many" relate to the topological structure of an object. For any three-dimensional structure, the topology can be described with three variables: *i*) the number of separate particles ("how many"); *ii*) the interconnectedness of the particles (number of handles or pathways connecting different parts of the object); and *iii*) the number of enclosed surfaces (for example, bubbles, voids, or entrapped particles). These variables are extremely valuable for describing the process of a disease like osteoporosis and the mechanical behavior of calcified tissues.

In the past, quantifying these topological variables required time-consuming, artifact-prone serial sectioning of entire specimens. In serial sectioning, a specimen is imbedded

in a rigid holder, and a shallow cut is made that just exposes the interior. The surface is polished, stained, and photomicrographed. Then another cut is made parallel to the original one. This fresh surface is then polished, stained, and photomicrographed. This procedure is repeated until the entire specimen has been examined. The micrographs from each section are then digitized, and a volumetric image is created. In order to quantify the interconnectedness of the spongy bone in a small animal, it would be necessary to obtain 50 to 100 sections from each one. This explains why this method is rarely used.

Another disadvantage with sectioning is that it can only be performed on dead animals. This requires a large number of animals to study at each time point in an experiment. For example, in an experiment that tests the effectiveness of a bone-growth drug at a single dose, it is necessary to have control animals, estrogen-deficient animals (induced by removing the animal's ovaries), and treated animals. If six animals are required at each time point for statistical accuracy, and if we examine bone loss in the ovariectomized animals at three time points, and then examine treated, controlled, and ovariectomized animals at three additional time points after treatment, a minimum of 78 animals would be required for this simple study. If, on the other hand, it were possible to examine living animals, then time-point sacrifice would no longer be necessary. In this case, only 18 animals would be required, and the results would have greater statistical significance because repeated

measures would be performed on the same animal. The savings in the number of animals becomes even greater as the sophistication of the experiment increases. Hence, there is a strong motivation for developing imaging methods that provide the same information as serial sectioning—but on living animals.

An alternative method for reconstructing three-dimensional images is X-ray computed tomography or CT (see "Positron Emission Tomography" in the Summer 1993 *Beam Line*, Vol. 23, No. 2). Because little or no sample preparation is required for CT, tomographic methods provide a cost-effective alternative to serial sectioning, and can, in principle, be used on living animals. CT is most frequently used as a medical diagnostic (higher resolution CT scanners have been developed, but they have not been approved for use on humans). Unfortunately, the present resolution is not high enough to image spongy bone with the accuracy required for quantitative measurements. To be useful as a substitute for conventional methods, the resolution of the CT method must be improved a hundred-fold over the newest instruments. This requires a new type of X-ray source and improved detectors.

COMPUTED TOMOGRAPHY WITH SYNCHROTRON RADIATION

The attenuation of X rays through a sample is a sensitive measure of atomic composition and density. By measuring the X-ray attenuation coefficient as a function of position within a sample with CT, a three-dimensional image of the sample can

be obtained. Subtle compositional and structural changes from one position to the next appear as differences in the X-ray attenuation. As long as the spatial resolution is small with respect to the features of interest, the three-dimensional X-ray images obtained from these measurements will provide valuable structural information.

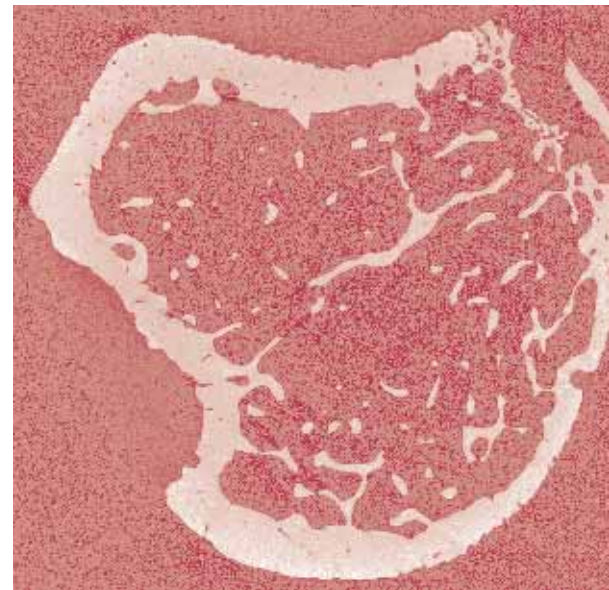
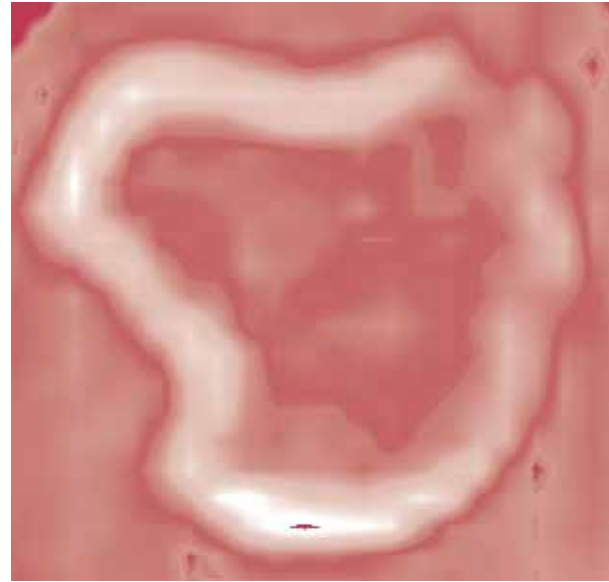
High spatial resolution depends upon *i*) a sufficient X-ray intensity, so that enough X rays reach the detector to provide good measurement statistics; *ii*) a detector with sufficient spatial resolution to discriminate between closely separated X-ray paths; and *iii*) monochromatic (single energy) radiation. Synchrotron radiation sources, because of their high brightness and natural collimation, have allowed the development of CT systems that have spatial resolutions approaching $1\ \mu\text{m}$. For this study we modified the X-ray tomographic microscope (XTM) developed at Lawrence Livermore National Laboratory in Livermore, California, for use on beam line 10-2 at the Stanford Synchrotron Radiation Laboratory (SSRL) at Stanford Linear Accelerator Center in order to image the three-dimensional structure and mineral density of bone in living animals. We wanted to demonstrate that the XTM with synchrotron radiation can detect microscopic changes in the spongy bone structure and connectivity in estrogen-deficient rats, an important animal model for osteoporosis. The improved resolution available with synchrotron radiation over the highest resolution CT scanners is shown in the two figures on the right.

EXPERIMENTAL STUDY OF POST MENOPAUSAL BONE LOSS

In a recent study, the leg bones (tibias) of living female rats were imaged with the XTM at SSRL. Imaging times with the nearly monochromatic X-ray wiggler beam at 25 keV were less than 30 minutes per animal. Shuttering of the direct beam reduced actual exposure times to less than two-and-a-half minutes.

The rats were anesthetized, and while unconscious they were secured to a rotating platform with their right hind limbs elevated into the X-ray beam. On the day following the initial scans, rats were chosen at random and their ovaries were removed. Five weeks after removal of the ovaries, all animals were imaged a final time with the same imaging parameters.

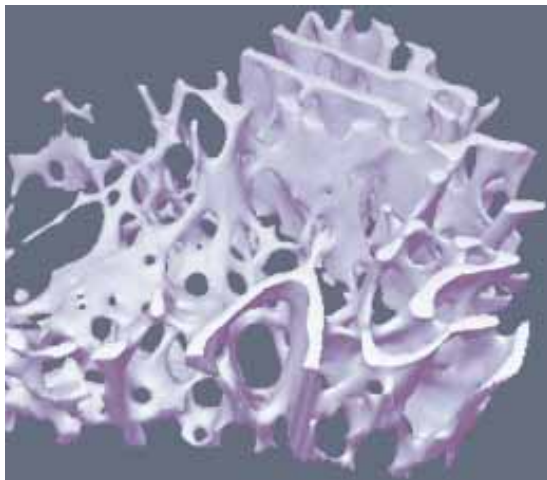
Once the data were acquired, the three-dimensional images of the tibias were reconstructed on a computer workstation. The volumetric data were analyzed by the following method. Cluster analysis was performed on the spongy bone structures in the three-dimensional images; it identified all of the spongy bone that was continuously interconnected, and also identified any isolated structures that were disconnected from the surrounding cortical bone and spongy structure. Cluster analysis provided a direct measure of the topological variables that quantify the number of isolated bone fragments and the number of imbedded pores. For the interconnected cluster, the connectivity was calculated from the three-dimensional image.



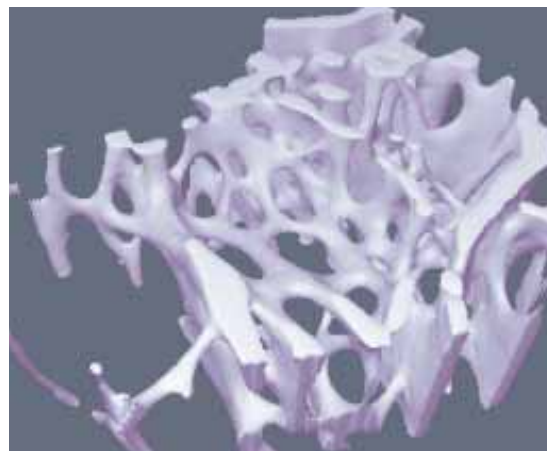
Conventional X-ray tomography instruments do not have the resolving power required to detect structural features within the spongy bone and cortex. Above is the highest resolution image of a rat leg bone with specialized commercial instrumentation. Below is the same bone imaged with the X-ray tomographic microscope and synchrotron radiation. Variations in the color of the bone correspond to small differences in the calcium concentration.



Top: An X-ray tomographic microscope image of a small region of spongy bone in the leg bone of an osteoporotic rat. The normal plate and strut-like structure of the bone has been greatly eroded, and several isolated bone fragments can be seen suspended in the marrow. Though contributing to the total bone mass, these isolated fragments do not contribute to the bones' strength and resistance to fracture.



Middle: The spongy bone in a rat leg at the earliest stages of osteoporosis showing the beginning of perforations to the plate structures.



Bottom: The spongy bone of an osteoporotic rat given parathyroid hormone after osteoporosis has been established. New bone is formed, and the spongy bone is significantly thicker than the original bone; however, the original plate and strut-like structure of the bone is not reestablished.

Three-dimensional images of the tibias just prior to removal of ovaries and five weeks after surgery showed that a 60 percent loss in bone volume occurred in the five weeks following estrogen loss (see "Biological Applications of Synchrotron Radiation" in the Fall/Winter 1994 *Beam Line*, Vol. 24, No. 3, page 21). In addition, the three-dimensional images demonstrated a significant change from an interconnected plate- and strut-like structure to one that is mostly disconnected struts. Also, dangling (or dead-end) elements are seen only in the estrogen-deficient animals. These dangling elements, although still contributing to the total bone mass, probably do not contribute to the strength of the bone.

A small region of spongy bone at higher magnification in a rat with ovaries removed is shown in the top figure on the left. Of particular interest is the small bone fragment that is isolated from the surrounding bone and supported only by marrow. We have only observed bone fragments such as this in animals with osteoporosis, where they account for about 1.5 percent of the total spongy bone volume. These isolated bone fragments, as well as the more significant fraction of dangling bone, may be responsible for the overlap in bone mass between individuals with osteoporotic fractures and individuals without fractures.

CAN WE REGROW LOST BONE?

Intermittent parathyroid hormone therapy has been shown to increase bone mass and improve biomechanical strength in osteoporotic rats.

Also, intermittent parathyroid therapy both alone and in combination with estrogen therapy has been reported to preserve spongy bone connectivity. Because the reports of connectivity have been based on two-dimensional data, we have been using synchrotron radiation with X-ray tomographic microscopy to determine if intermittent parathyroid therapy actually does increase bone mass and connectivity. What we have found is that spongy bone volume and connectivity significantly decreased after 8 and 12 weeks of estrogen loss. Parathyroid treatment appears to increase bone mass by thickening existing bone, not by forming new connections (see middle and bottom figures on the left). From our results, we hypothesize that to re-establish connectivity in the spongy bone, treatment would have to be given before significant bone loss has developed, or treatment with a bone-forming agent such as parathyroid would be less effective. Further studies need to evaluate critical time points for the administration of bone-forming compounds and for assessing the effects of these treatments on improving bone strength.

New insights gained from these studies using synchrotron radiation are allowing us to develop more rapid screening of new clinical treatments for osteoporosis, a major public health problem responsible for over one million fractures a year in the United States alone.

