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Elements of Bone Biophysics

1.1 Introduction

Bone is a dynamic tissue that, when properly organized and distributed in a whole bone, acts as both a mechanically competent skeletal structure and a physiological unit. Bone is a very rigid anisotropic material and a specialized connective tissue, which forms the basis of the skeleton, and, as such, its functions are manifold and at times too complex to understand by any simplistic model. One of its apparent roles is to provide support to the body, which is carried out by cortical bone throughout the skeleton and by peripheral cancellous bone. While it is well established that geometrical characteristics contribute to bone strength, their dependence upon specific behavioral, hormonal and metabolic factors is unknown. In combination with the associated musculature, the bones of the skeleton also provide a means of physical support, locomotion and related movement. Evolution has led to a complex, multiphase, heterogeneous and anisotropic microstructure. Another important role is that as a reservoir for a multitude of inorganic ions (calcium, phosphorous, etc.) that are subsequently recruited by various physiological systems. Subsidiary functions are participation in plasma calcium homeostasis and support of hematopoiesis, which is carried out mainly by central cancellous bone (Parfitt, 2001). It is well known that in the event of calcium deprivation it is the mass of the skeleton that is sacrificed at the expense of other functions.

Bone is never static, it is a living structure, responding and adapting itself to the applied load and has the capacity to remodel. The cells of the skeleton act continuously to maintain the remodeling (Figure 1.1). It is thus in a constant state of dynamic equilibrium both in terms of its composition and structure and responds to external mechanical forces (or their absence) by adopting changes in its normal architecture. The mechanical properties of bone depend on the load direction. The loading regimen should be dynamic (Figure 1.2). Bone possess various physical, solid-state and electro-mechanical properties. These properties are characteristics of bone and are modified under the action of external stimulus and changes of Ca and P metabolism. However, the mechanism at work and their biological significance are not completely understood. Also, the functional significance of bone renewal is still a matter of speculation.

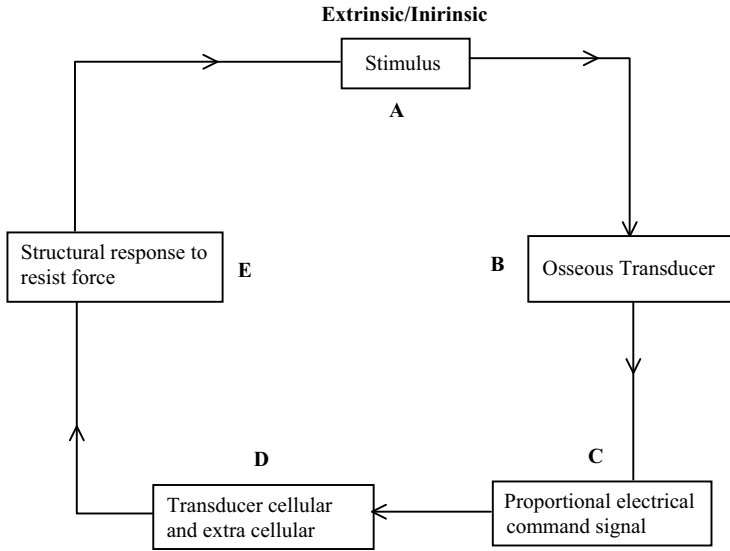


Figure 1.1 Basic response pattern of bone cells to extrinsic/intrinsic stimulus

The mechanical competence of bone is determined by its density, the architecture and its intrinsic material properties. The conventional view holds that the intrinsic chemical, structural and mechanical properties of bone remain invariant and, therefore, that the old and young, or osteoporotic and normal bone, are not substantially different. It has been suggested that once the bone architecture is determined (possibly by genetic processes) its mechanical behavior can

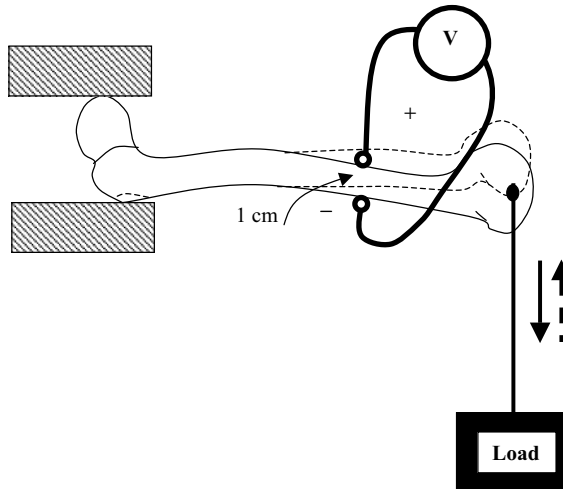


Figure 1.2 Basic model experimental design for detecting electrical polarization of living/non-living bone subjected to dynamic loading. The magnitude and resulting wave shape depend upon the duration and type of loading

be extrapolated – a view that is still open to question. The general understanding is one of morphological adaptation. Namely, that peak mechanical strains in the tissue trigger a biological response. *In vitro* results support the notion that bone formation *in vivo* is stimulated by dynamic rather than static loads (Rubin and Lanyon, 1987) and that low magnitude, high frequency mechanical stimuli may act as stimulator for high amplitude, low frequency stimuli, provided that the cells are stimulated in a preconditioned state. Bioelectrically, it is now accepted that bone remodeling is dependent upon load-induced voltages and, hence, also that understanding the ability of bone to generate piezoelectricity is one of the keys to understanding bone physiology: an issue discussed in Chapter 2.

The physical hardness, which is a unique characteristic of bone, poses particular problems requiring special laboratory procedures to achieve a high standard of tissue section preparation. For instance, before attempting conventional histological methods, it is generally necessary to “soften” bone (and other calcified tissue) by removing the mineralized component. This procedure requires special treatment, and the time it adds to the overall tissue processing cycle will inevitably cause some delays in the assessment of diagnostic specimens. There are specific techniques that enable the preparation of sections from calcified tissue, without the need to remove the mineralized phase. Apart from bone, many other kinds of mineralized specimens may be encountered since virtually any normally soft tissue can become calcified, resulting in a disease process.

Indeed, human bone has a complex hierarchical microstructure (Yeni *et al.*, 1997) that can be considered at several dimensional scales (Rho, Kuhn Spearing and Zioupos, 1998). At the shortest length of scale it is composed of type-I mineralized collagen fibers (up to 15 μm long, 50–70 nm in diameter) bound and impregnated with carbonated apatite nanocrystals (tens of nm long and wide, 2–3 nm thick). Several skeletal tissues participate in this mechanical objective of transmission and protection: bone cartilage, tendons, ligaments and muscles. Bone mainly determines the global structural stiffness and strength, whereas other tissues transmit loads between bones. The mechanical properties of bone are a result of a compromise between the need for a certain stiffness (to reduce strain and achieve a more efficient kinematics) and the need for enough ductility to absorb impacts (to reduce the risk of fracture and minimize skeletal weight).

1.2 Structural Aspect of Bone

It is now commonly accepted that bone tissue changes its morphology in response to mechanical forces. An important role of the musculoskeletal system is to transmit forces from one part of the body to another under controlled conditions of stress and strain. This also offers protection to vital organs (e.g., lungs, brain, etc.) in addition to its other more specified functions. Several skeletal tissues participate in this mechanical objective of transmission and protection: bone, cartilage, tendons, ligaments and muscles. Bone exists in two main forms: cortical and trabecular bone. Trabecular bone has a greater surface area than cortical bone – mineral is drawn from the former during short-term calcium and phosphorous deficiencies to maintain equilibrium within the body. Trabecular bone is located on the end of long bones, in vertebral and envelopes the marrow (Figure 1.3a and b) (Jee, 1988; Wasserman, 1984; Hays and Swemson, 1984). Bone contains an outer casing of cortical bone, surrounding the medullary cavity. The latter contains a network of bone fibers, the trabecular bone. The spaces within the trabecular structure are filled with red hematopoietic or yellow (fatty) marrow. The relative

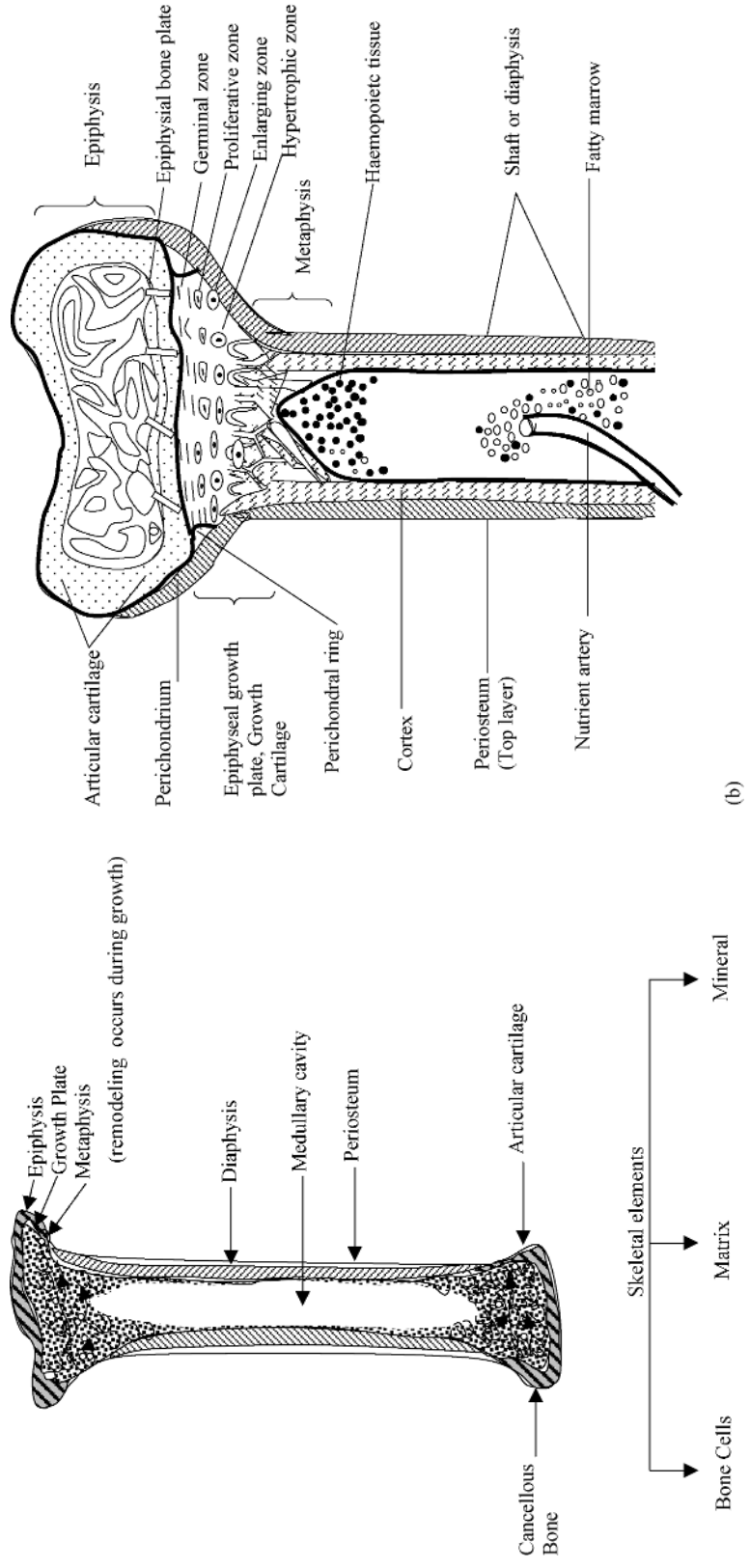


Figure 1.3 (a) Diagrammatic representation of the parts of a long bone. (b) Features in developing long bone. Adopted from Little, 1973 (Reproduced with permission from K. Little, *Bone Behavior*, Academic Press, New York. ©1973, Elsevier B.V.)

composition of these four components is dependent on the bone location and also on the age of the skeleton.

Long bones are made up of the diaphysis, or shaft and the epiphysis, or end of the bone (Figure 1.3b). The diaphysis and epiphysis are connected by the metaphysis. In young animals, the epiphysis and metaphysis are separated by a thick cartilaginous tissue known as the growth plate. The long plate elongates the growth plate, as new bone is formed at the base of the plate where blood vessels infiltrate the cartilage matrix. In mature animals, the growth plate is replaced by trabecular bone and the bone ceases to grow in length. Both cortical and trabecular bone are made up of lamellae, which consist of circular collagen fibers and mineralized components.

In contrast to trabecular bone, which is more like a woven net, cortical bone has a solid mass, has a very low porosity and its anisotropy is mainly controlled by lamellar and osteonal orientation (Figure 1.4a and b). Bone anisotropy is defined by means of a second-order tensor (fabric tensor) that defines the principal values and directions of the bone mass distribution (Odgaard, Jensen and Gundersen, 1990; Whitehouse, 1974; Whitehouse and Dyson, 1974; Odgaard, 2001). In fact, structural anisotropy has a direct influence on stiffness properties as well as on strength. The average strength of a compact human bone in longitudinal compression tests is 105 MPa, and in transversal compression test it is 131 MPa (Reilly and Burnstein, 1975). There is evidence that resting cells in the cambium layer of the periosteum are coupled through junctional complexes to osteocytes within the underlying, osseous substance (Weinger and Holtrop, 1974). These junctions are very sensitive to minute changes in their electrical or calcium ion environment (Cooper and Keller, 1984; Flagg-Newton and Lowenstein, 1980; Lowenstein *et al.*, 1978; Ravel *et al.*, 1980; Sheridan *et al.*, 1978). Furthermore, they can act as a conduit both for macromolecular transfer and, probably, electrical signaling. When a local change in the strain pattern reaches a threshold level, which is strain rate, cycle and duration dependent, these cells are activated. A transition, actively from a sub-divided to an active state (or vice versa), will stimulate an appropriate adaptive remodeling response (Woo *et al.*, 1981), while localized adaptation can be seen within a single individual subjected to differential exercise (Jones *et al.*, 1997). The mechanism of this adaptive phenomenon remains unknown, though it has been suggested that microdamage within the extracellular matrix, which might accumulate following long periods of exercise, could stimulate a reparative process (Burr *et al.*, 1993). A cascade of events can be visualized from a single-threshold stimulus to produce a new bone (modeling form of osteogenesis) (Tornberg and Bassett, 1977).

Human long bone is composed of woven bone before an individual reaches the age of 4. During this time, as later, the bone diameter at the mid-diaphyses increases principally by new bone deposition at periosteal surfaces and old bone resorption at endosteal surfaces. Thus, microstructural information for growing woven bone may be studied discretely by examining the mid-diaphyses at different locations across the thickness of bone from periosteum to endosteum and representing different stages of both mineral and tissue age and maturation. Collagen provides the fundamental organic matrix framework for the deposition of apatite in all vertebrate mineralized tissue with the exception of dental enamel and eggshell (Lowenstam and Weiner, 1989). The overall organization of collagen fibrils in woven bone differs considerably from those in lamellar bone. In the latter, each lamella consist of highly oriented, densely packed collagen fibrils. The collagen fibrils adjacent lamellae differ by 90°. Woven bone, in contrast, has no lamellar structure. The collagen fibrils do not lie parallel to one

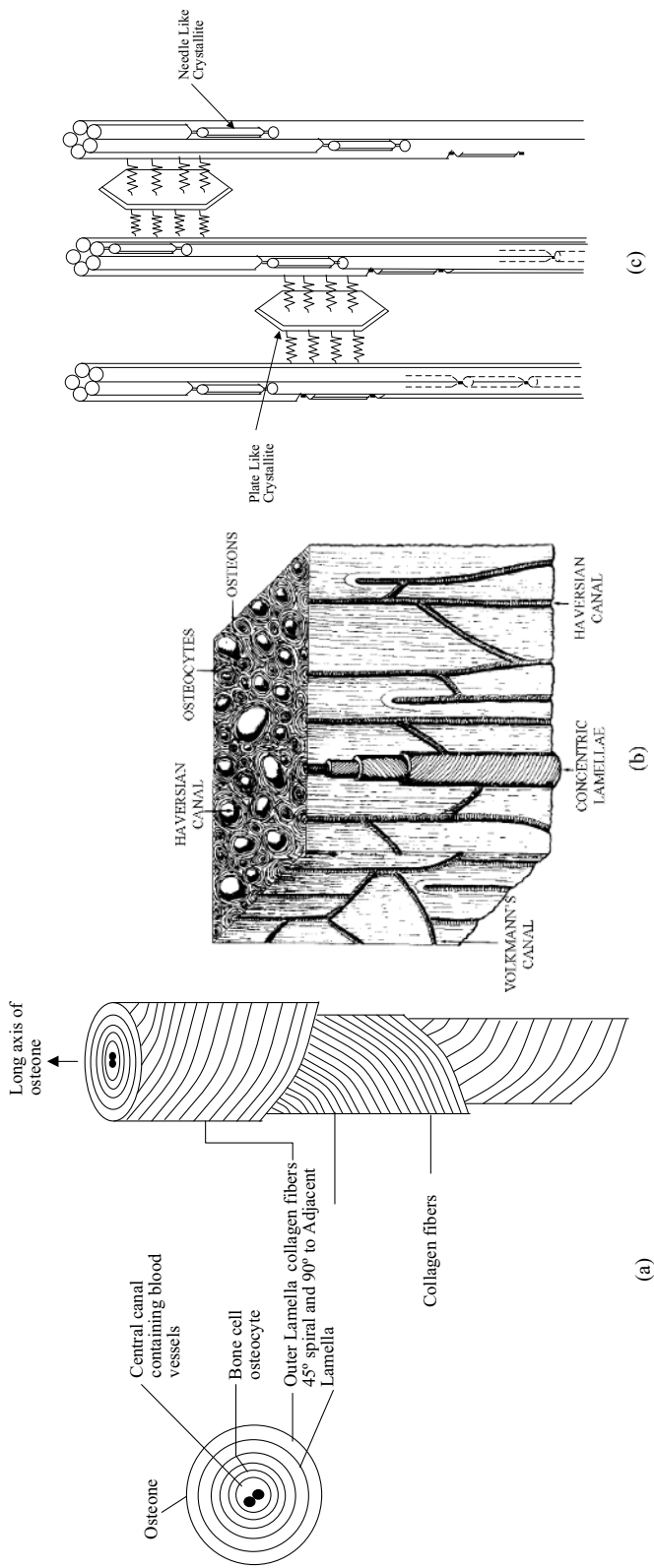


Figure 1.4 (a) Microscopic structure of compact bone; (b) microstructure of adult human compact bone (vertical axis is parallel to the long axis of the bone) (Saha, 1977); (c) mineralizing bone collagen crystallites embedded in the crosslinks (Reproduced with permission from S. Lees and C. Davidson. "A theory relating sonic velocity to mineral content in bone." In: Linzer, M, ed. *Ultrasonic Tissue Characterization* Vol. II, Special Publication 525. Government Printing Office, Washington, DC © 1979)

another, though they usually show some degree of preferential orientation parallel to the long axis of the bone. In newly deposited woven bone the collagen fibrils are interwoven and dispersed (Su *et al.*, 1997). The study of longitudinal and cross-sectional woven bone sections by transmission electron microscopy has confirmed the existence of apatite crystals in the extrafibrillar spaces of collagen (Figure 1.4c). Within the layer of collagen, lacunae, canaliculi, the cavities and channels comprise the communication system for bone. One concentric unit of lamellae and its associated lacunae and canaliculi is known as an osteon or Haversian system (Jee, 1988; Lawrence and Fowler, 1997).

The properties of the cells in bone are primarily controlled by agents that affect cell membranes. These membranes are of two main types: (i) The rough membranes, which are known as the endoplasmic reticulum; these are so-called because of an array of ribosome particles on the surfaces of the membranes. (ii) The smooth membranes, which comprise the cell surface, nuclear envelope and their connecting membranes. The endoplasmic reticulum is responsible for the production of intercellular matrices. The surface membranes are for cell mobility and the smooth membranes for cell division. The stages of division are pre-division (inter phase), followed by prophase, metaphase, anaphase and telophase. The remainder of the division process is dependent upon the pre-division stage, which is the only one that requires a source of energy.

The structural integrity of bone, like any other form of matter, living or dead, is maintained by atomic forces, which are electrical in nature. The various physical properties of bone show that there may be a close relationship between biophysical properties and physiological processes involved in bone growth and remodeling (Cochran, 1966; Frost, 1980; Glimcher, 1976; Bassett, 1971). The present concept of bone as consisting of collagen fibers, hydroxyapatite crystal, a small amount of proteoglycans, non-collagenous proteins and water (Weiner and Wagner, 1998; Robinson and Elliot, 1957; Martin, 1984; Lucchinetti, 2001) is expected to explain mineralization, along with suggestions of new composite materials. The intervention of external agents to artificially control the growth pattern of bone by applying mechanical stress or electrostimulation or treatment of Ca and P minerals in isolation or in combination provides another reason for basic studies on its structure and properties.

1.2.1 Elementary Constituents of Bone

Bone is a highly specialized form of connective tissue. The main elements of bone tissue may be considered to consist of, besides osteocytes, a crystalline mineral phase (hydroxyapatite), an amorphous mineral phase, a crystalline organic phase (collagen), an amorphous organic phase and liquids. The biphasic behavior of bone composite materials arises from inorganic crystals of calcium phosphate disposed within the collagen fibril of an organic matrix (Glimcher and Krane, 1968). Inorganic components (mainly hydroxyapatite) are mainly responsible for the compression strength and stiffness, while organic components provide the corresponding tension properties. This composition varies with species, age, sex, the specific bone and its pathogenic state. An important aspect that also characterizes this peculiar mechanical behavior of bone is its hierarchical organization. Weiner and Wagner (1998) have described this, stating from the nanometric level and ending at the macroscopic level, relating the latter to the mechanical properties.

1.2.2 The Fibers

The organic matrix has two chief components: the collagenous fibers and ground substances. The ratio of organic to inorganic substance in bone is 35–65% (Glimcher, 1959). The organic component is often called osteoid. The organic part of bone is about 95% collagen by volume. Collagen is a generic term for a class of protein that make up much of the body tissue and are found in bone and cartilage. Osteoid consists of proteins and carbohydrates that are secreted outside cells. These proteins and carbohydrates include type I collagen fibers, proteoglycans and glycoproteins. These components are found throughout the connective tissues of the body and provide tissues with strength and also flexibility. The mineral occupies 35% of the volume. Since collagen is such an important body constituent its chemistry has been studied extensively. Because of the need to determine the presence of collagen in tissues and to identify its extent, the sonic and elastic properties of some types of collagen have remained an attractive subject of investigation.

Collagen provides connective tissue with strength and makes up a major portion of bone. A portion of cartilage supramolecular aggregate is associated with the extracellular matrix and is stabilized by the triple helix characteristic of collagen (Wasserman, 1984; Lawrence and Fowler, 1997; Marks and Popoff, 1988; Van der Rest and Garrone, 1991). Collagen molecules have a repeating pattern of glycine-proline-hydroxyproline-glycine-x-y amino acids, where x and y denote any other amino acid. Hydroxyproline and hydroxylysine are two amino acids found predominantly in connective tissue and are prevalent in collagen. For static reasons glycine is the only amino acid that can be in the center of the super helix of a collagen molecule.

All collagens are highly structured materials in a multilevel hierarchal order. The molecular weight of the basic collagen molecule, defined as tropocollagen, is approximately 300 000 daltons, which is a very large molecule even in organic chemistry. The molecule is much like a piece of spaghetti, being about 300 nm long and having a wet diameter of 1.5 μm . Bone collagen differs from other body tissue collagens by an extensive network of intermolecular crosslinks that render it insoluble in even the most potent of solvents. In contrast, tendon collagen is reported to be mostly lacking in intermolecular bonds but has many hydrogen bonded intramolecular links, and so that it can be dissolved and readily reconstituted. Figure 1.4b shows a schematic diagram. While other tissues like arteries and joints calcify, the special structural characteristics of bone collagen cause it to calcify in a unique manner (Lees and Davidson, 1977).

The collagen molecule has a triple-helix structure. The triple helix itself shows in a right-handed spiral around a common central axis (Ramachandran, 1963) that gives rise to an X-ray diffraction pattern indicating the structural similarity of collagen in bone, tendon and skin (Figure 1.5a–c). The collagen macromolecules are organized to form fibrils, which in turn are organized into fibers. Fibers are woven into lamellae, which in human compact bone tend to form cylindrical Haversian systems. The other components in the organic matrix include some other proteins, along with the acid mucopolysaccharides, and these constitute a part of ground substance. Some of the mucopolysaccharides are present in the form of mucoproteins (non-collagenous). The exact anatomical location of these components at a macromolecular level is not certain and their state of aggregation and polymerization is not well known (Glimcher, 1959). Collagen fibers, which are composed of 400–1200 Å diameter fibrils with a cross bonded structure, account for nearly one-third of the dry weight of the bone matrix

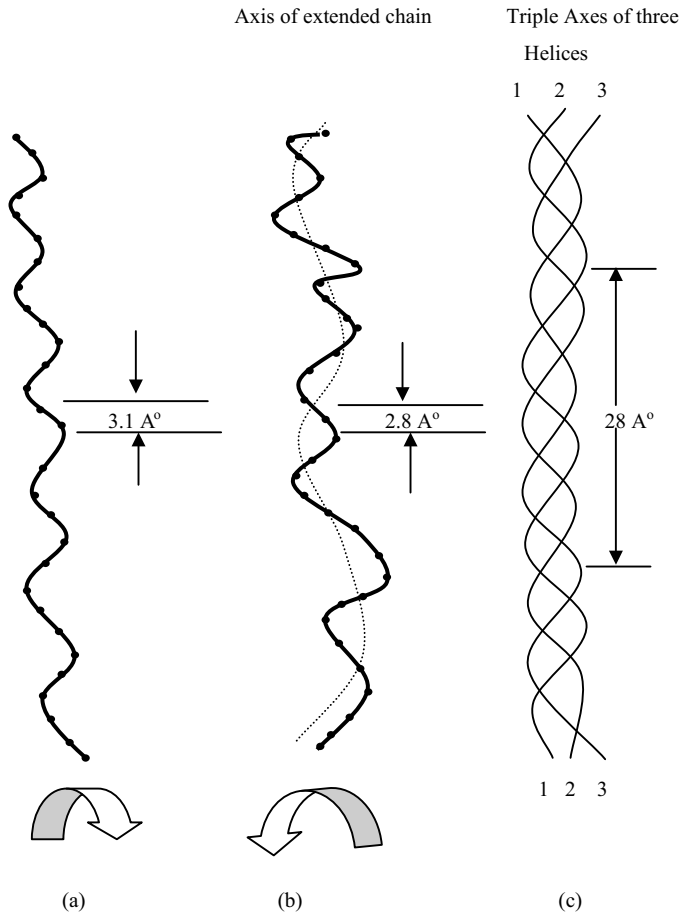


Figure 1.5 (a) The α -helix is left handed. Each spot is a residue and the repeat length is 3.1 Å. (b) The α -helix is twisted to the right and the length between residues is reduced to 2.8 Å; the repeat of the supercoil is 10 times the residue length (28 Å). (c) Three α -helices rethreaded to form a single tropocollagen (TC) unit. (Adapted from Lees and Davidson, 1977/79)

(Figure 1.6). Collagen molecules form the tropocollagen macromolecules, which consist of three polypeptide chains, each being twisted into a left-handed helix, and the three chains being wound round a common axis in the form of a right-handed superhelix. It forms a long, thin rod about 2800 Å long and 14 Å in diameter. Tropocollagen molecules are organized into fibrils extracellularly. The lamellae are built by collagen fibers and the inorganic salts are deposited into this organic polymerized protein crystals. An understanding of the nature of bone at the molecular level is important not only for a basic understanding but also for the treatment of degenerative bone diseases.

About 2% of the volume mature compact bone consists of cells, the remainder being extracellular matrix. Of this matrix 70% is occupied by mineral, about 2% is organic and

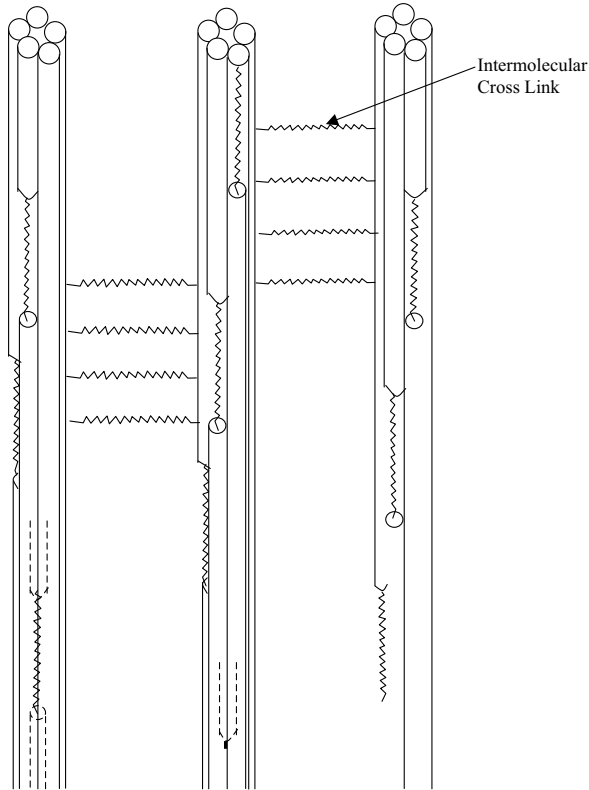


Figure 1.6 Schematic representation of the ultrastructure of collagen showing intermolecular crosslinks (Lees and Davidson, 1979)

the remainder is water. Of the organic material 90% is collagen, about 1% is proteoglycan and the rest is a series of matrix proteins. The collagen of bone is almost all type I. The proteoglycans of bone are all of the small non-aggregating type (decorin and biglycan) and contain only chondroitin sulfate. The matrix proteins are characterized by their anionic nature, being rich in phosphate (phosphoprotein, e.g., osteonectin) sialic acid (sialoproteins, e.g., osteonectin) or γ -carboxyglutamic acid (Gla proteins, e.g., osteocalcin). Growth factors such as TGF- β and BMP (bone morphogenic protein) are also stored within the bone matrix.

As collagen is a polymer, bone may be considered to be a two-phase, mineral-filled polymer. Collagen is laid down first and then it becomes mineralized when the HAP crystallites are deposited (Figure 1.4c). When bone is demineralized the collagen matrix can be recovered as a rubbery solid, having the form and shape of the original bone. However, when collagen is removed the mineral structure left behind is a weak solid that easily crumbles into a powder. It may be concluded that the collagen forms a continuous medium but the mineral does not. The mineral fills voids in the well-laid collagen structures.

Collagen has long been studied and much is now understood about its chemistry and ultrastructure. Type I collagen is the most abundant form of the molecule; it is found in bone, tendon and skin and many other tissues. This molecule has an [a-1(I)2a-2(I)] conformation. Type II collagen is largely found in cartilage and between vertebral and has an [a-1(II)]₃ structure. Type I and II collagens are examples of fibril-forming collagens. Other collagen types, such as basement membrane microfibrillar, anchoring fibril, hexagonal network-forming fibril associated with interrupted triple helices transmembrane and multiplexin collagens, have different types of structures. These collagens have various subunits that help to differentiate each type of collagen (Lawrence and Fowler, 1997; Van der Rest and Garrone, 1991; Van der Mark, 1999). Type I collagen is the most prevalent type (90%) in bone. Additionally, collagen makes up about one-third of the dry weight of bone organic matrix (Pritchard, 1972; Wasserman, 1984; Lawrence and Fowler, 1997). Collagen molecules have many complex characteristics: such as three-dimensional order, the chemical character of the intermolecular crosslinks and the location (Ramachandran, 1967; Gallop and Paz, 1975; Veiss, 1974).

1.2.3 Collagen Synthesis

Collagen is synthesized intracellularly as procollagen, followed by hydroxylation of proline and glycosylation of hydroxylysine to yield procollagen. The ends are cleaved and procollagen is expelled from the cell (Wasserman, 1984). Extracellularly, the procollagen molecules undergo hydrolyses to form tropocollagen, which is the basis for matrix collagen fibrils. The polypeptide chains of tropocollagen are wound in a left-handed fashion, formed when every third amino acid residue is turned into the center (Van der Rest and Garrone, 1991).

Fibril forming types of collagen molecules like those in bone are arranged parallel to each other, with spaces strategically placed between fibers once the molecules have been cleaved at the N- and C-telopeptides to form collagen fibrils extracellularly. The ends of the fibers can then become crosslinked. Crosslinkage between collagen molecules at hydroxylysine and lysine sites provides collagen with its stabilization and forms the striation in the collagen fibrils. Collagen, in conjunction with its crosslinking, yields a scaffold for minerals to bind in the matrix (Wasserman, 1984; Van der Rest and Garrone, 1991; Lawrence and Fowler, 1997; Marks and Popoff, 1988).

Pyridinoline (PYD) is a 3-hydroxypyridinium derivative consisting of three amino acids and carboxyllysine residues; lysyl oxidase converts these amino acid residues into an aldehyde, (Last *et al.*, 1990). An analog crosslink, deoxypyridinoline (DPD), is formed in a similar manner as PYD, but the helix rather than hydroxylysine (Ogawa *et al.*, 1982). Eyre *et al.* (1988) have found that hydroxypyridinium residues persist in human bone through adulthood, thereby establishing them as final crosslinks.

Collagen exhibits many levels of order, terminating in a fibrous structure. The successive levels of organization have been explained by Lees and Davidson (1977). The smallest element is the α -helix, which spontaneously combines in triplets to form a superhelix molecule, the tropocollagen (TC) unit. In turn, TC forms microfibril ropes, which in their turn form three-dimensional fibrils. Some characteristics of these elements are listed below:

- The basic element, the α -helix chain, has a molecular weight of about 100 000 daltons. Several types of chain have been identified, depending on the amino acid composition.

The α_1 chain has 1052 amino residues, of which 1011 are in triplet sets where the first term is glycine. The N-terminus has 16 nonhelical residues beginning with an amino (NH_2) group while the C end has 25 nonhelical residues terminating in a COOH group. Gallop and Paz (1975) as well as Hulmes *et al.* (1973) have prepared the residue map.

- The hydrogen-bonded state between the α -helices, of which TC is the basic molecule, is stable.
- A TC unit is 4.4D units long and packs into a structure exhibiting a repeated 0.6D unit gap between TC ends. According to Hodge (1967) no two quarter stagger gaps can be adjacent, which puts a restriction on the three-dimensional arrangement (Segrest and Cunningham, 1973).
- The nonhelical chains at each end of the TC unit and some of the adjacent glycine coded triplets are probably the links between molecule ends but may also serve to link helices within ends – they may also serve to link helices within a single molecule, indicating that the, inter- and intramolecular links are similar. However, many of the intramolecular links are hydrogen bonds, while many of the intermolecular links between parallel molecules are chains covalently bonded as side chains to residues in the backbone of the molecule.
- Several three-dimensional structures have been proposed for the microfibrils, fibrils and fibers. Figure 1.5c shows a TC unit (Smith, 1968) as modified by Miller and Parry (1973). The model shows a 67 nm axial stagger, 72° azimuthal displacement between axes of nearest neighbors and a 43 nm hole between collinear TC units. It incorporates a repeat pattern after 5×67 nm and a helix with a fivefold screw axis (five subunits per screw turn characterized by the holes). The pentagonal unit is 4 nm in diameter while the diameters of the wet TC unit and the lumen are 1.5 and 1.1 nm, respectively.
- Miller and Parry (1973) have indicated that the five-strand rope must be twisted to produce a fourfold symmetrical supercoil, where the holes are 90° apart. However, there is still a pentagonal packing of the strands. The collinear TC units are inclined about 2.5° to the rope axis to accommodate the twist.
- The pattern of successive helical structures suggests that the microfibrils are coiled about each other while maintaining the four unit cell.

One way to characterize a molecule is to look for its resonance frequency. Enemeto and Krimm (1962) calculated a value for the macromolecular resonance of a very similar molecule, polyglycine II, which they found to be 41 GHz – a value far in excess of any reported for collagen or even for bone (26 GHz). Polyglycine II has a triple helix, hydrogen-bonded molecule and closely resembles collagen in its structure (Ramachandran, 1967).

1.2.4 Bone Matrix (Inorganic Component)

Apatite crystals are one of the major constituents in bone and other mineralized vertebrate tissues (Lowenstam and Weiner, 1989). Their presence, which accounts for about 65 wt% of bone, provides most of the stiffness and strength of bone (Glimcher, 1992). With the normal increase of apatite deposition during tissue aging and maturation, bone mechanical properties increase greatly, an observation confirmed by measurement of the elastic modulus and microhardness of growing human long bone (Su *et al.*, 1997). Correlative to the increase in the diameter of the middiaphyses of long bone, occurring principally by periosteal bone

deposition and the later resorption (Enlow, 1991), endosteal bone becomes more mineralized than the former. The elastic modulus and microhardness of endosteal bone can be up to three times higher than that of periosteal bone (Su *et al.*, 1997). Lees and Davidson (1977, 1979) suggested that mineralization causes additional crosslinkages to be set up, some between TC units but more between the mineral, whether crystalline or amorphous, and the TC units. The collagen is stiffened by decreasing the crosslink length and the mineral fills. The mineral TC links may be reversible so that in the event of demineralization the collagen becomes less stiff.

The first role of the mineral component in calcified tissue in this hypothesis is to stiffen the collagen by decreasing the length of the crosslinks and increasing the crosslinking density proportionally to the amount of mineral present. At 34% volume, all the crosslinking is shortened to the minimum length and the maximum crosslinking density is attained. The additional strength that is obtained by additional mineral content is in accordance with the Reuss model (Chapter 2).

The general structure of the inorganic component of the bone matrix is mainly hydroxyapatite; a small amount of non-crystalline form of calcium phosphate also exists in the mineral phase. The plate-like hydroxyapatite crystals, roughly 400 Å long, with a comparable width (200–350 Å), and 25–50 Å thick (Robinson and Watson, 1955, 1952; Johansen and Stone, 1997), are embedded in the collagen fibers with orientations approximately parallel to the fibers. This molecule can generally be found around the collagen fibers in a crystalline structure. The organic matrix of the bone and the apatite crystals make a coherent unit that is vital to bone tissue function. The tropocollagen molecules create heterogeneous centers for crystallization of the bone minerals. These centers are usually placed at the points where the more polar heads of collagen fibrils are located.

The structure of hydroxyapatite can be derived from the spatial organization of a small number of the constituent ions (Posner, Perloff and Diorio, 1958). The unit cell for this is a right rhombic prism, when stacked in the manner of a simple hexagonal lattice. The length along an edge of the basal plane of the cells (a) is 9.432 Å and the cell height (C) is 6.881 Å. The spatial symmetry is symbolized as $P6_3/m$. There is considerable evidence indicating that biological factors are important in establishing the size, shape and orientation of bone crystals. The C -axis of the apatite crystallites is parallel to the collagen fibers (Engstrom and Zetterstrom, 1951).

The structural basis for the observed chemical stoichiometry of bone mineral involves the two major constituents, Ca and phosphate. The hydroxyapatite model would predict that the ideal composition of bone apatite should be $\text{Ca}_5(\text{PO}_4)_3\text{OH}$, with Ca and phosphate in Ca/P molar ratio of 1.67, 1.74 and 1.57 (Woodard, 1962; Eastoe, 1961). In studies on the dynamics of mineralization, the calcium/phosphorous ratio is adopted as best reflecting the formation of hydroxyapatite crystals. The calcium to phosphorous ratio in an ideal hydroxyapatite crystal is 1.67 : 1 and is slightly lower *in vivo* conditions (McLean and Budy, 1964). Diet has a significant effect on the mineralization of bone tissue. The carbon/calcium plus phosphorous ratio reflects the degree to which the bone matrix is saturated with hydroxyapatite crystals (Krawczyk *et al.*, 2007). A value of this ratio exceeding 10 is typical and is indicative of poorly or non-mineralized status. When fibrous bone tissue predominates the value is usually below 5 and for most advanced remodeling process the ratio is close to 9.

The physical character of bone, including the morphology (Fratzl *et al.*, 1993; Heywood *et al.*, 1990), dimensions and distribution of its composite apatite crystals, affects

its mechanical properties. These parameters from different mineralized tissues have been investigated over many years. Isolated fracture apatite crystals studied with transmission electron microscopy have been shown to be platelet-like and, on average, ~ 50 – 100 nm or more long and 25 – 50 nm wide. Relatively newly deposited crystals are ~ 4 – 6 nm thick. The crystals are associated with collagen in bone and most other calcifying vertebrate tissues, but the precise spatial relationship between these two components is difficult to ascertain. A major problem in this regard is that electron microscopy, used to image the constituents, provides two-dimensional information. Three-dimensional aspects can be incorporated by applying low-voltage electron microscopic tomography and graphic insertions. These methods have demonstrated that apatite platelets are oriented approximately parallel to the other end to the collagen fibrils. The crystals are separated by a minimum of 4.2 nm.

While information concerning the morphology and dimensions of apatite crystals and their location and distribution, in relation to collagen, can be obtained, such data have been mostly obtained from avian tendon and lamellar bone. Comparatively less data are available regarding these points in woven bone and particularly in human woven bone. Woven bone differs from lamellar bone in the organization of its collagen fibrils, its cell population and its mechanical properties. Woven bone is defined as having randomly distributed collagen fibrils and is deposited only during initial formation in fracture repair. The tissue physical, chemical and biological nature of woven bone, including the morphology and detailed organization of its composite crystals, needs to be considered to elucidate possible mineralization mechanisms. This has applications in biomechanics and biomedicine. It has been reported that bone modulus and microhardness increase from periosteal bone to endosteal woven bone in human fetal tissue.

The experimental results (TEM and X-rays) appear to be in good agreement in that the smallest dimension of the bone apatite crystal is about 50 \AA . Considerable variation in the length appears to exist; the values reported from TEM are consistently higher than those obtained from X-ray bone broadening studies. A possible explanation for this observation is that the bone crystals appear subdivided in the direction of elongation. There is periodicity of 50 – 60 \AA (Fernandez-Moran and Engstrom, 1957) along the crystal length as a rod exhibited subunit of about 50 \AA (Molnar, 1960). These observations (Ascenzi and Bonucci, 1966; Molnar, 1960) suggest that bone crystals are composed of chains of microcrystals fused in an end to end relationship. Such a fusion process would account for the variability in reported lengths and may also account for the divergent view on shape. The X-ray diffraction study (Posner *et al.*, 1963) reported that the largest dimension is probably less than 100 \AA , which is consistent with this view of bone apatite as a mosaic of microcrystals rather than a continuously uniform single crystal.

Changes in the size of bone crystals with age have been noted on X-ray powdered rat bones (Menezel, Posner and Harper, 1965). The mean size increases with age of the animal up to maturity. The initial growth of the apatite crystals is quite rapid, occurring in a matter of minutes or less (Eanes and Posner, 1965), with the average crystal size at the end of the precipitation being generally smaller than that of the crystals in mature bone. The synthetic crystals, however, gradually increase in size with subsequent aging, until they exceed the average size of bone crystals (Eanes, 1965). The crystal growth with age in synthetic systems continues indefinitely, though at progressively reduced rates. Water is necessary for the ripening to proceed in these synthetic systems. Reducing the water/mineral ratio retards

secondary growth and, at a sufficiently low ratio, ripening can no longer be detected. Robinson (1966) has emphasized the fact that with maturation the mineral content per unit volume of whole bone increases at the expense of displaced tissue water. The water/mineral ratio, therefore, decreases with an increase in the degree of mineralization. This drop in water content with maturation could stop the ripening of bone crystals and cause the constancy of crystal size in mature bone.

Macroscopically, bone consists of tissue and anisotropic amorphous non-crystalline mineral phase (Hancox and Boothroyd, 1965; Molnar, 1959; Fitton-Jackson and Randall, 1956; Robinson and Watson, 1955). Porosity varies between 5 and 95%, with most bone tissues having either very low or very high porosity. Accordingly, there are two types of bone tissue. One is trabecular or cancellous bone with 50–95% porosity, which is usually found in cuboidal bones, flat bones and at the end of long bones. The pores are interconnected and filled with a tissue composed of blood vessels, nerves and various type of cells. While the main function is to produce the basic blood cells, the bone matrix has the form of plates and struts called trabecular with a thickness of about 200 μm and a variable arrangement (Martin, Burr and Sharkey, 1998). The second type is cortical bone, with 5–10% porosity and a different type of pores (Cowin, 1999). The first evidence to indicate that this amorphous phase is a major component of bone mineral came from X-ray diffraction (Termine and Posner, 1967; Harper and Posner, 1966) by analyzing the intensity pattern of the crystalline portions. It has also been supported by several other authors (Robinson, Doty and Copper, 1973; White *et al.*, 1977). It is estimated that 40% (Harper and Posner, 1966) of the mineral in the femur of adult human, cow and rat is non-crystalline. The amorphous content of bone, however, varies with age. This age dependency in the fraction of amorphous mineral has also been demonstrated by IR techniques (Termine and Posner, 1966). The amorphous phase of bone, like apatite, is supposed to be composed of Ca, phosphate and carbonate. Certain X-ray diffraction studies indicate that the crystallites are needle like, others that they are platelets. There is evidence indicating that both forms are present, depending on the site of formation. There are three levels of porosity, all containing bone fluid. These include the vascular porosity associated with Volkmann canals and the Haversian lumens ($\sim 10 \mu\text{m}$) and the lacunar canalicular porosity associated with the fluid space surrounding the osteocytes ($\sim 0.1 \mu\text{m}$) and the collagen–hydroxyapatite porosity associated with the spaces between the crystallites of the mineral hydroxyapatite ($\sim 20\text{--}60 \text{ nm}$). Movement of the bone fluid in the collagen–hydroxyapatite porosity is negligible because most of the bone water in that porosity is bound by interaction with ionic crystals (Neuman and Neuman, 1958).

One cannot understand the structure of an amorphous particle from a knowledge of the spatial organization of a small group of its constituent atoms. This is because the atoms are not arranged in a regular, periodic array that would enable one to define the whole space occupied by the particle by simple translational repetitions of a basic structure of atoms and hence define the lattice type. This does not mean that local ordering of ions does not exist in amorphous calcium phosphate. However, there are sufficient random variations in this order, on going from one local co-ordination ensemble to the next, that long-range periodic order is absent. Nonetheless, the spatial organization of the ions comprising the amorphous material in bone mineral is unknown.

Bone mineral not only has a high surface area per unit weight, but its surface is chemically reactive with its environment. Low-temperature gas adsorption methods and small angle X-ray scattering have shown that the surface area of human and bovine deproteinated bone mineral

ranges from 100 to 200 m² g⁻¹. The high magnitude of surface area of bone mineral provides a large interface for mineral reactions.

By measuring the heats of adsorption of small molecules on bone surface, it is shown that bone tends to bond strongly with certain molecules. In fact, poorly crystallized synthetic hydroxyapatite has long been used in chromatographic adsorption columns because of the high bonding capacity its surfaces have for specific proteins and polynucleotides. Studies on surface bonding (Posner, Betts and Blumenthal, 1979) suggest that a chemical linkage probably exists between the mineral in bone and certain free polar groups of collagen. Bone mineralization is a cell-mediated process in which complex events take place in sequentially (Wuthier, 1982). It has been reported (Lawrence *et al.*, 1994) that the mineralization process of equine bone is almost complete (76%) by twelve months of age and that the bone mineral content of the equine third metacarpal does not differ by sex.

The fluid shear stress amplitudes and frequencies in bone can be determined theoretically from known physiological loading parameters. By applying Biot's theory of poroelasticity to bone, the predicted range of *in vivo* fluid shear stress ranges from 0.8 to 3 Pa due to loading, with induced strains ranging between 1000 and 3000 $\mu\epsilon$ (Cowin, 1999; Weinbaum *et al.*, 1994). Several *in vitro* studies (Bacabac *et al.*, 2004; Bakker *et al.*, 2001; Chen *et al.*, 2000; Frangos and Johnson, 1995; Jacobs *et al.*, 1998; Johnson, McAllister and Frangos, 1996; Klein-Nulend *et al.*, 1995a, 1995b, 1995c) have confirmed that this range of fluid shear stress magnitudes is able to stimulate bone cells.

It has been suggested that the rate (determined by the frequency and amplitude) rather than the magnitude alone of the applied loading stimulus correlates to bone formation (Bacabac *et al.*, 2004; Mosley and Lanyon, 1998; Turner, 1998). This implies that bone formation is enhanced by dynamic rather than purely static loading. Thus, both the amplitude and the frequency of loading seem to be important parameters for bone formation. Indeed, it has been shown that low magnitude (<10 $\mu\epsilon$), high frequency (10–1000 Hz) loading can stimulate bone growth and inhibit disuse osteoporosis (Rubin *et al.*, 2001). High amplitude, low frequency stimuli are rare in the activities of daily life, whereas high frequency, low amplitude stimuli are more common (Fritton *et al.*, 2000). Weinbaum *et al.* (1994) suggested that low amplitude postural strains due to muscular contraction could be more effective than high-amplitude low-frequency strains due to locomotion, in maintaining bone mass. This suggests that bone cells are likely to be excited by low-amplitude postural strains due to muscular contractions, and also by high amplitude, low frequency strains due to locomotion. Such behavior might explain why astronauts in a microgravity environment lose bone mass despite exercise. The rate of loading seems to be a decisive factor in bone formation and maintenance. However, the nature of bone cells response to the rate of loading remains to be fully understood.

High impact physical activity, including jumps in unusual directions, has a great osteogenic potential in humans (Nordstrom, Pettersson and Lorentzon, 1998) and in ovariectomized rats (Tanaka, Alam and Turner, 2002). High impact drop jumps significantly increase bone formation rates compared to that of baseline walking (Judex and Zernicke, 2000). Furthermore, an initial high stress rate, as in step wise increases fluid shear stress, has been shown to stimulate neonatal rat calvarial bone cells. Therefore, the osteogenic response to high impact activity might be related to the response of bone cells to a sudden increase (i.e., stress-kick) in fluid shear stress. The osteogenic benefits of high impact activity imply that the bone cell response to fluid shear stress is nonlinear.

1.3 Classification of Bone Tissues

Two types of bone are observed in the normal, mature human skeleton: compact bone and cancellous bone. Although macroscopically and microscopically different, the two forms are identical in their chemical composition.

1.3.1 Compact Bone

Compact (cortical) bone is a solid mass that is found along the shafts of the long bones (femur, tibia, radius, ulna) and is the principal component of the flat bones (skull and ribs). It has an extremely dense physical structure arranged around Haversian systems and Volkmann's canals, which are responsible for providing cellular nutrition. Because of its strength, cortical bone plays a significant role in the support of body weight and in the protection of internal organs. Approximately 80% of skeletal mass is cortical bone. In general, the compact bone is the ivory-like surface layers of the mature bone.

The compact or cortical bone can be divided into three main categories: primary osteon bones, secondary osteon bones and surface bones. Primary osteon bones are formed in the vascular tunnels of the cancellous bone as cylinders and they are structured due to the merging of the fine cancellous cartilage and membrane bones of young subjects. Secondary osteon bones are established during the remodeling process in the tunnels that are opened by osteoclastic activities. These gradually increase throughout adult life as a result of remodeling. The internal remodeling is very active in childhood but slows during the older ages, being lowest at about the age of 30 (Pauwels, 1948). Surface bones are the primary formation of solid bone on the medullary and periosteal surfaces of any existing bone shaft.

The cross section of the shaft of a long bone can be divided into the following areas. One tenth of the outermost of the thickness of the shaft consists of approximately circumferential lamellae, the inside, next to the bone marrow tend, to be cancellous. The thickness between the outer and the inner portion consists of the secondary osteons, Haversian canals, interstitial lamellae, blood vessels, and so on. The secondary osteons are also called the Haversian system – mostly they look like hollow cylinders in cross section. The inner tube of the osteon is called the Haversian canal and the concentric lamellae structure. Usually, osteons are made of 5–30 concentric lamellae. In the regeneration or remodeling processes, the existing bone is first removed by osteoclasts in a circular fashion and then lined with partly mineralized concentric lamellae. The mineral content of these lamellae increases with increasing time and complete mineralization may take a very long time. Therefore, the cross section of a compact bone contains osteons of various ages. During the formation of new osteon, some of the old osteons with irregular shapes lie in the interstitial lamellae sections. Some of the older osteons may have been partly excavated and are replaced during the new osteon formation.

1.3.2 Fine Cancellous Bone

Compared to the cortical bone, cancellous (trabecular/spongy) bone is mesh like, considerably finer and more delicate in appearance. Its physical arrangement of broad plates connected by thin struts provides for maximum support but with a minimum of raw material. Cancellous

bone is found principally in the vertebral of the spinal column and at the epiphyses of the long bones.

Fine cancellous bones are found in the second ossification centers of the fetal skeleton and induce new bones of pathological nature. Generally, they consist of bone bundles with a honeycomb structure spanning the intertrabecular spaces containing both blood vessels and cells. The small spicules of solid matrix in spongy bone are called trabeculae; therefore, the spongy bone is often called trabecular bone.

In general, there are two kinds of the cancellous bones. The difference in their structure depends on the place where the bone is formed. If it is formed in the cartilage, it is called fine cancellous cartilage bone, while if it is situated near the membrane it is called fine cancellous membrane bone. The internal cancellous structure of many bones, especially the long bones of the leg, contributes to their strength with a little amount of material.

1.3.3 Coarse Cancellous Bone

Coarse cancellous bone has a similar structure to that of compact bone, with some exceptions. It contains less bone cells and less osteons. The cells are nourished from the vessels in the intertrabecular marrow spaces, because the Haversian canal system is not capable of supplying blood to the cells.

Figure 1.3a shows the arrangement of cortical and trabecular bone in the proximal femur, where the outer dense cortical bone of the femoral shaft encases the finer trabecular structure of the cancellous bone. The trabeculae have adopted a preferential alignment along the direction of osteogenic response to mechanical loading. This is because electronegativity is an indication of the region of growth. It has been documented in compact bone in diaphyseal cross sections (Pauwels, 1976; Radin *et al.*, 1982; Currey, 1964; Lanyon and Rubin, 1985; Martin and Burr, 1989; Hou *et al.*, 1990; Gross *et al.*, 1997; Lieberman and Crompton, 1998; Martin, Burr and Sharkey, 1998).

1.4 Lamellation

Within each lamellae of the osteons the collagen fibers have one dominant direction; this direction varies from one lamellae to the next, giving the effect of a family of co-axial helices of different helix angles. In general it is assumed that there are three types of arrangement of fibers in the osteons. The first has the fibers in all lamellae roughly parallel to the axis of the osteon (90° helix angle). The second has the fibers in alternate lamellae longitudinal and circumferential (helix angle alternates between 90 and 0°). The third has the inclined fibers in each lamellae but in opposite directions in successive lamellae (the helix angle alternates between plus and minus approximately 45°).

Bones are heterogeneous in their organization and may be divided into two distinct structural regions. At the outer surface is the periosteum, which is the source of the cells responsible for growth in bone width. Adjacent to this is cortical bone and on the inner surface of the bone, adjacent to the marrow cavity, is trabecular bone. The inner surfaces of the bone are covered by cells that form the endosteum. Both trabecular and cortical bone are made of calcified lamellae in which osteocytes are embedded. The osteocytes are linked by a network of unclassified channels termed canaliculi (Figure 1.7a and b).

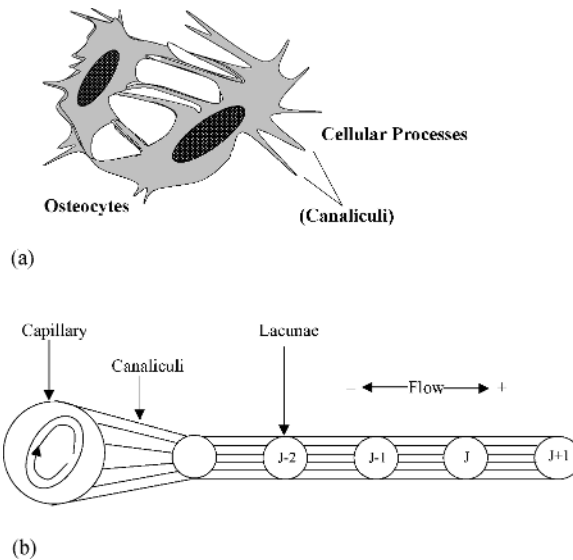


Figure 1.7 (a) Schematic of osteocytes; (b) model of the Haversian canal-lacunae-canalicular system

In cortical bone, the canaliculi are linked to the Haversian canals in which the bone vasculature resides (Figure 1.8a). Surrounding the blood vessels are concentric rings of lamellar bone, which form a unit termed an osteon or Haversian system. The Haversian canals run parallel to the axis of the bone and arise through bone remodeling. The vessels penetrate the bone from the periosteum to the marrow cavity through transverse Volkmann's canals. The bone surfaces are covered with cells, which include dormant bone lining cells, osteoblasts and osteoclasts. Like bone lining cells, osteocytes are inactive osteoblasts that are not buried in new bone. They remain on the surface during bone formation steps and can be reactivated in response to chemical and or mechanical stimulus (Miller and Jee, 1992). They are located in lacunae (Cowin, 1999) and communicate with the rest of cells via canaliculi (Figures 1.7b and 1.8b). Many authors (Cowin, Moss-Salentijn and Moss, 1991; Lanyon, 1993; Burger, 2000; Skerry *et al.*, 1989) suggest that osteocytes are mechanoreceptor cells that control bone remodeling, but this needs further confirmation. Furthermore, it is assumed that osteocytes, the only cells embedded in the bone matrix, are affected by processes that damage the bone matrix, interrupting their communication through canaliculi and thereby affecting their metabolic exchange. Fatigue microdamage may therefore create a situation resembling disuse at the level of the osteocyte cell body and lead to bone remodeling starting with osteoclast recruitment.

A cartilage model of the bone known as the anlage cartilage is formed, which is approximately the same shape as the final bone. The anlage cartilage is made up of cartilage cells with a surrounding immature intercellular matrix. In the long bones, during the first stages of conversion into bones, cartilage cells in the central part of the shaft increase their rate of proliferation, enlarge and become hypertrophic. Blood vessels penetrate into this altered cartilage and promote osteogenic activity. The osteogenic cells have the capacity to change

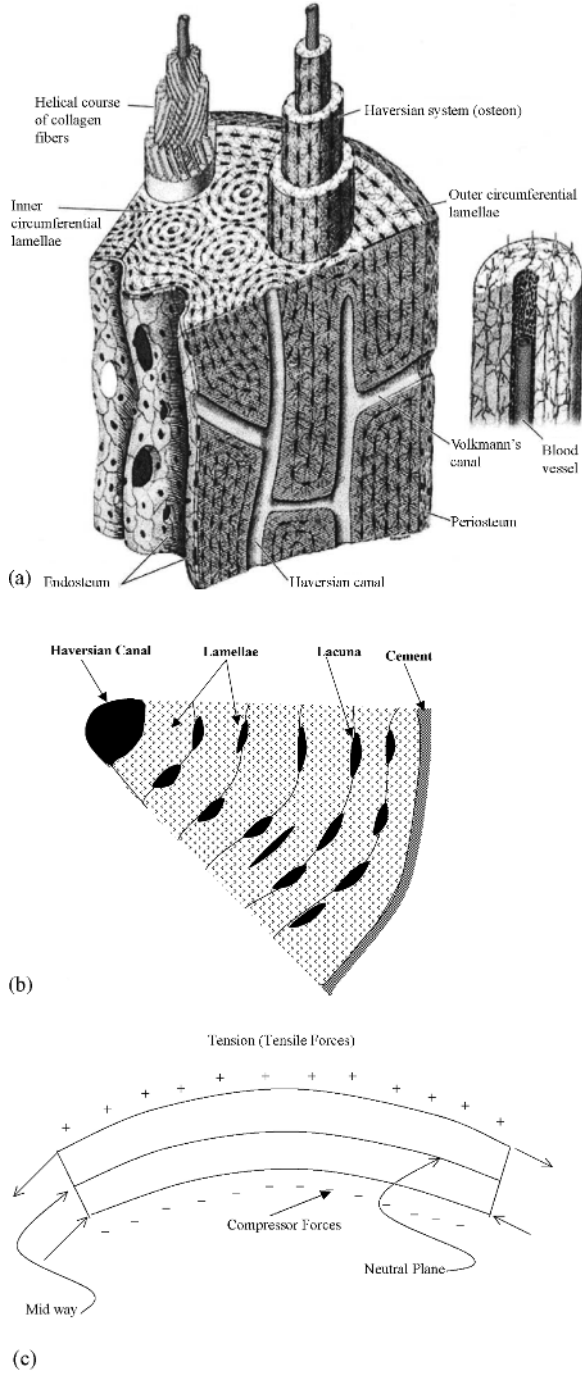


Figure 1.8 (a) and (b) Schematic representation of bone anatomy at the microscopic level; (c) bone under bending, showing a negative charge on the concave surface and a positive charge on the convex surface – the polarities will be reversed if the direction of bending is reversed

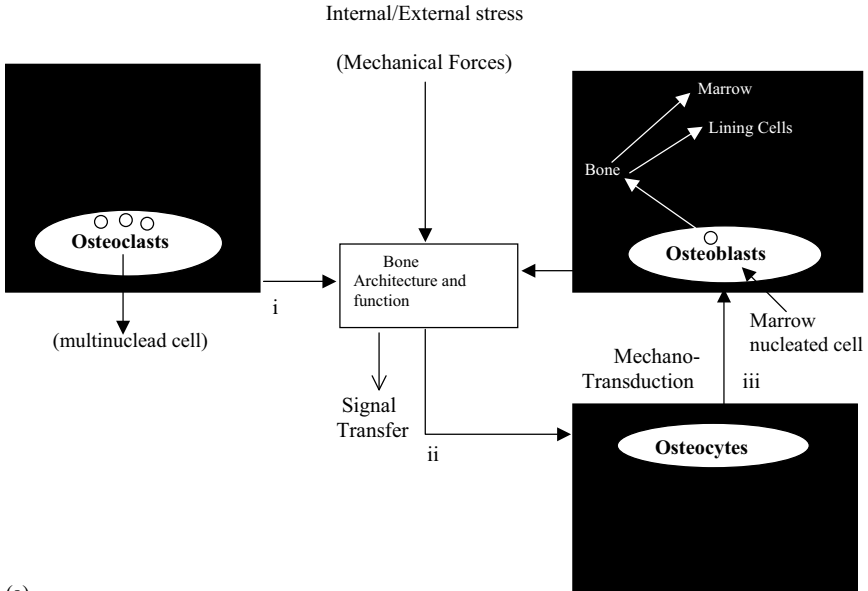
their function, and the name given to a cell depends on its activity at the time. Cells in the process of laying down an osteoid matrix, which rapidly calcifies, are the osteoblasts. When the cells coalesce to form multinucleated cells, which remove bone, they are known as osteoclasts (Figure 1.9a and b). Their precursor cells have been given various names, one is macrophage.

The process of replacing cartilage by bone after vascular penetration is known as enchondral ossification. This proceeds from the central regions of the shaft towards the ends, the epiphyses. When the advancing ossification front reaches the epiphyseal region, then ossification commences separately within the epiphyses, to form the secondary bone nucleus. During childhood a region of cartilage remains at either end of the long bones, between the epiphysis and the metaphysis. It is in a highly organized state, contains vigorously proliferating cells and is known as the epiphyseal growth cartilage, or sometimes the epiphyseal growth plate. Regulating this process is the application of physical forces, which may either act directly or indirectly.

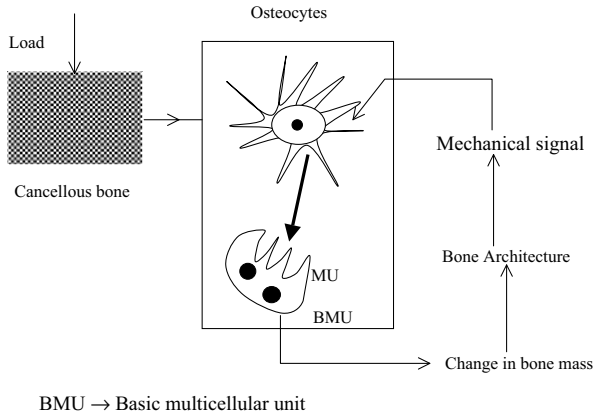
The zone of growth cartilage nearest the epiphysis is the germinal or resting zone. It is similar to the analage cartilage. Then comes the proliferative zone with columns of active rapidly dividing cells. These enlarge and form a calcifiable matrix that is laid down over the main structural part of the intercellular matrix. In the stages of most rapid growth they enlarge further to form the hypertrophic zone. As enchondral ossification takes place each hypertrophic space is invaded by an advancing sinusoid vessel. When the rate of growth allows, cartilage may be removed without enlargement of the cells to the hypertrophic state. The mode of removal takes place by multinucleated cells known as chondroclasts. These coalesce from similar cells to those that formed the osteoclasts. The different names denote the different function. In other cases enchondral ossification takes place with the formation of bone trabeculae. They may be very regularly arranged during the period of rapid growth which is accompanied by chondroclast activity. These trabeculae occupy the metaphyseal region of the bone.

During growth, while the epiphyseal growth cartilage provides for the growth in length, there is also an increase in diameter of the shaft of the long bones. New bone is laid down on the outer part of the cortex, the periosteal surface, while at the same time bone is removed from the inner surface of the cortex, the endosteal surface. In the case of periosteal bone formation cartilage is not formed as an intermediate stage in the process. Changes in the shape and distribution of the bone tissue occur both before and after growth has ceased, by the process of remodeling. One of the main factors regulating this process is the application of physical forces, which may act either directly on the walls of sinusoidal vessels in the bone or indirectly by altering blood flow and pressure. In the former case the remodeling consists of the removal of trabeculae followed by the formation of new ones in rather different positions. The latter frequently leads to the formation of osteons in the cortical bone in the shaft. More generally, this type of bone is known as compact bone. During osteon formation there is a proliferation of vessels, with accompanying osteoclasts. This channel – a Haversian canal – is then partly filled with new bone laid down concentrically around the vessels.

When cortical or trabecular bone is laid down the osteoblasts arise from the vessels walls, and are arranged in rows. Cortical bone that has been so formed in a regular array is known as lamellar bone. It is present in children, but when they take sufficient exercise it is all converted into osteonal bone by the time the adolescent changes are complete. Small animals, such as rabbits, tend to retain a lamellar bone structure throughout their life.



(a)



(b)

Figure 1.9 (a) Probable signal transduction pathways: (i) osteoclasts resorb bone on trabecular surface locations; (ii) osteocytes sense a mechanical signal due to external load transfer through the architecture; the signal is transferred to the trabecular surface, where (iii) osteoblasts are initiated from bone. (b) Cellular response to bone stimulus

Bone, both the marrow, among the trabeculae and Haversian canals in compact bone are sinusoid vessels, which are of varying diameter. In normal marrow only about 1 in 10 of these is open at any given time. These blood vessels have a sheath of basement membrane or reticulum and flattened endothelial cells are seen at intervals on their walls. Wherever these sinusoid vessels are formed in the body, whether in bone or in the spleen and liver, their hemopoietic activity is possible. Their cells only act as bone precursors in the presence of a substance known as the osteogenic factor. Other blood vessels, whether arteries or veins, have more substantial walls. Even small capillaries tend to have a continuous layer of cells over their basement membrane. During proliferation, sinusoid vessels and capillaries display similar characteristics. Nerves are found accompanying many arteries.

The end of the long bones are covered by articular cartilage, while between the two articular surfaces, in a joint, is the synovial fluid, the remaining surfaces of the fluid-containing cavity being occupied by the synovial membrane. Cells on the surface of the synovial membrane secrete the synovial fluid. The shaft of the bone is covered by a sheath of connective tissue called the periosteum and outside this again there are usually muscles. Points of attachment of muscle to bone are known as muscle insertions. The tissue surrounding the growth cartilage is known as the perichondrial ring and fibrous tissue surrounding those parts of the articular cartilage that are not in contact with the synovial fluid is called the perichondrium.

Arrangements around other bones are of a related type. Between the vertebral bodies in the spine are the intervertebral discs. Each side of a cartilage end plate, in the growing child, acts as a growth cartilage, while in the adult it has many of the characteristics of articular or hyaline cartilage. The central part of the disc is a type of fibrocartilage known as the nucleus pulposus and surrounding it is an oriented fibrous tissue known as the annulus fibrosus of the bone in the vertebral bodies. A part of this is formed by enchondral ossification, while the remainder is formed by periosteal ossification. The whole area of a normal vertebral body is occupied by trabecular bone surrounded by hemopoietic tissue.

1.4.1 The Cement

The cement can be considered as the amorphous continuum phase in which the discrete fibers and inorganic crystals are embedded. In essence, what remains after removing the fibers and the inorganic phase constitutes the cement. This cement is composed mostly of mucopolysaccharides, glycoproteins, lipids, carbonate and citrate. The other constituents include sodium, magnesium and fluoride. The ground substance and the cement lines are made of cement and they show viscous and plastic behavior.

1.5 Role of Bone Water

Next to osteoid and mineral, water occupies the largest volume fraction in bone. It is probably because of this that the nature and role of water in calcified tissues has been a subject of serious investigations (Neuman and Neuman, 1958; Robinson and Elliot, 1957; Timmins and Wall, 1977).

Water not only resides within the vascular canals, lacunae and canaliculi but also exists within the collagen matrix and the mineral apatite (i.e., extracellular matrix). The estimated

maximum water content in the vascular-lacunar-canalicular space is 12% of the total volume of bone, a value higher than the 8% calculated by others (Zhang, Weinbaum and Cowin, 1998). Robinson (1960) reported that water could exist in two fractions, one driven off at 50 °C, associated with marrow-vascular-osteoid, and the other driven off at 100 °C, associated with the calcified matrix. Water distribution in bone not only exists as mobile water in pores but also has other forms, which interact with bone tissue at different energy levels.

Water interacts with the collagen and mineral phases of bone in several ways. Firstly, the polarity of water facilitates its bonding with the hydrophilic groups of the collagen protein (glycine, hydroxyproline, carboxyl and hydroxylysine) and the charged groups, PO_4^- or Ca^{2+} , of bone mineral. Secondly, studies on the hydration of collagenous tissue (human dura mater and rat-tail tendons) with dynamic mechanical spectroscopy indicate that water does bond with collagen at two levels (Nomura *et al.*, 1977; Pineri, Escoubes and Roche, 1978). Thus, collagen has structural water and loosely bound water. The former results from hydrogen bonding within the triple helix of collagen molecules (due to the hydroxyl group of hydroxyproline) and requires more energy to remove than the latter, which arises from hydrogen bonding with the polar side chains of collagen fibrils. Thirdly, there are two types of water interaction in the mineral phase, lattice water and surface bound water. Both X-ray diffraction and infrared (IR) spectroscopy of heat-treated synthetic, precipitated apatites found that water bound to surface crystals is lost at a lower temperature (<200 °C) than water inserted into the lattice structure (between 200 and 400 °C) (Le Geros, Bonel and Legros, 1978). Nonetheless, the spectra intensities from nuclear magnetic resonance of both surface water and lattice water decrease when bone dries up to 120 °C, with more lattice water remaining at higher temperatures (Casciani, 1971). Based on the energy characteristics of water with collagen and mineral, it is suggested that water removal from bone is related to energy level as follows: (i) mobile water molecules require less energy to evaporate than water on bone surfaces, (ii) the removal of the loosely bound water (via hydrogen bonding) requires less energy than the water molecules trapped inside collagen molecules, which in turn requires similar or less energy than water molecules bound to the surface charges of mineral apatite (more ionic in nature) and (iii) water that is imbedded in the lattice of hydroxyapatite (about 35 mg of water per g mineral more covalent in nature) requires the highest energy to dislodge (Neuman and Neuman, 1958).

This water cannot be displaced by simple drying at 100 °C. The work of Timmins and Wall (1977) indicates that removal of water by thermal dehydration is rather gradual. Neuman and Neuman (1958) have further pointed out that there is a 10 nm thick hydration layer (bound water) retained at centrifuges of 10 000g around each hydroxyapatite crystal.

In the unmineralized state, as the bone matrix is laid down by osteoblasts, the collagen fibers produced contain a large volume fraction of water (up to 60%). During calcification, while apatite crystals are deposited in the organic matrix, the osteoid water is gradually displaced and reduced down to a 20% volume fraction. The spin–lattice and spin–spin relaxation times of proton and deuteron (water adsorbed on collagen fibers) decrease with the decrease in water content. By comparing these changes with the change in the quadrupole splitting of heavy water, it is concluded that shorter relaxation times correspond to a higher degree of ordering for water in collagen. This fraction drops to 10% in senile bone (Robinson, 1952), corresponding to reduced impact strength in old age. It is thus plausible that the volume occupied by mineral cannot vary in a manner independent of that of collagen bound water since the total volume has

to be preserved. Hence, any change in the mineral fraction will result in a corresponding change in the water fraction.

It has been suggested (Fung and Tautmann, 1971; Chapman *et al.*, 1971) that a small fraction of water adsorbed on collagen is highly ordered and the rest moves isotropically. It may be speculated that the ordered water molecules would have a longer correlation time and relax must faster than the isotropic water molecules and a change in their ratio would cause a change in the observed relaxation times. The spin–lattice relaxation time (T_1) decreases with decreasing amount of water in collagen. The spin–spin relaxation time (T_2 , for $^2\text{H}_2\text{O}$ on collagen) also decreases with decreasing water content. The relaxation times are strongly frequency dependent.

The loosely associated water is what is referred to as “bulk water” (free water) that fills the pores of the calcified matrix making up the Haversian and lacuno-canalicular system. It is this fraction of water that has been shown to confer the unique viscoelastic properties of bone, which are largely lost after drying. In its natural fully hydrated state, stress-induced deformation upon application of a load is damped by the resistive forces experienced by the fluid in the lacuno-canalicular system (Garner *et al.*, 2000). Notably, the binding state of pore water depends on its proximity to the surface and thus is expected to be greatest in the canaliculi measuring less than $1\ \mu\text{m}$ in diameter. This water has an additional critical role in that during mineralization and demineralization ions need to be transported to and from the osteoid sites. Water thus provides a medium for flow and diffusion driven ion transport (Neuman and Neuman, 1958). The study of pore water dynamics can provide detailed insight into transport phenomena occurring within the matrix. It is also possible that pressure-induced fluid flow through the lacuno-canalicular system is one possible mechanism of mechanotransduction regulating bone formation (Figure 1.10).

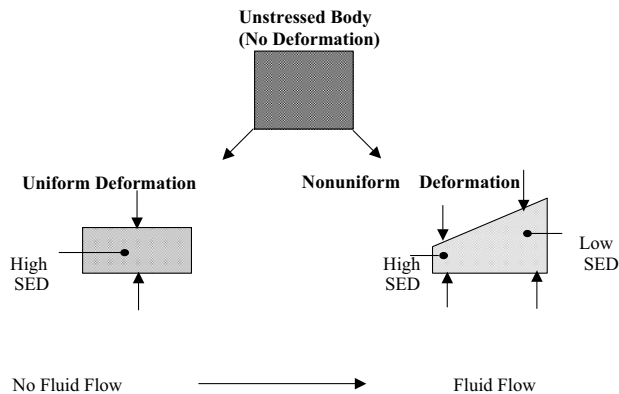


Figure 1.10 Effects of different mechanical parameters and their gradients, for strain energy density (SED). A high volumetric strain gradient favors liquid flow (Ruimerman *et al.*, 2005)

1.6 Bone Metabolism

Bone metabolism is regulated by a wide variety of hormones, cytokines and other factors. Metabolism is also regulated by the activity of five different type of bone cells: osteoprogenitor, bone lining cells, osteoblasts, osteocytes and osteoclasts. Osteoprogenitor cells consist of preosteoblasts, which undergo mitosis and differentiation to become osteoblasts.

1.6.1 Ca and P Metabolism

Calcium and phosphorus are essential elements required for various physiological and biochemical functions. Calcium is needed for processes such as bone formation, blood clotting, nerve conduction, muscle contraction, intercellular communication secretion and membrane permeability. Phosphorus is required for bone formation and the phosphoryl moiety is part of biologically significant molecules, such as ATP, DNA and RNA. The source of Ca and P for human beings and animals is food.

The level of Ca in the plasma is maintained by the diet and varies from 9.1 to 10.7 mg per 100 mL (Smith *et al.*, 1960; Peacock and Nordin, 1973). There are considerable diurnal variations in any individual. The value goes up in the day when calcium is ingested in the diet and it falls during the night when there is no dietary intake. The variation may be as much as 10% of 1 mg per 100 mL. The normal calcium level is maintained by control of absorption in the gut and excretion in the urine. Bone calcium acts only as an emergency reservoir in the state of calcium deficiency and possibly during the early morning fast calcium is mobilized from the skeleton to maintain the plasma calcium concentration.

Calcium is present in plasma in three forms: ionized, protein bound and combined with citrate and other organic acids. The ionized calcium is diffusible, the citrate calcium is not ionized but is also diffusible, while the calcium proteinate is neither ionized nor diffusible. The distribution of calcium (Lingarde, 1972; Walser, 1961) and its fall is affected by age. The P_H (i.e. $[H^+]$ ion concentration) level is a controlling parameter which affects Ca^{2+} . Increased protein calcium binding counteracts Ca^{2+} homeostatic control mechanism as the Ca^{2+} decreases. The amount of diffusible non-ionized calcium is small and is generally believed to be of little clinical interest except in patients (Lingarde, 1973).

Interpretation of variations in the total plasma calcium depends on how much calcium is bound by the plasma proteins. The plasma protein involved is largely albumin; 1 g of albumin binds approximately 0.7 mg calcium and is proportional, depending on the P_H and other factors. Some 80–90% of protein bound calcium is an albumin chelate (Lingarde, 1973). The fact that albumin may also move into bone suggests that consideration may also need to be given to the bound calcium (Owen, Triffitt and Melick, 1973).

In the contrast with calcium, most of the phosphate in plasma is in a diffusible form as HPO_4 or H_2PO_4 and about 12% is protein bound (Bijvoet, Froeling and Sluys Veer, 1972). The normal plasma level varies from 2.4 to 4.04 mg in different clinical states. The relative proportions of these two ions types depends on the plasma P_H . There are many biological uses of phosphate, extending from its role as an inorganic to its multiple involvements as organic phosphate in both enzymatic and structural proteins as well as in nucleic acids. The maintenance of calcium and phosphorus homeostasis involves delicate interrelationships of absorption by intestine, accretion and reabsorption by bone tissue and, finally, urinary excretion by the kidneys.

The calcium and phosphate are made available in the plasma – again a delicate balancing operation occurs, between accretion and mobilization in the bone and excretion by the kidney. The dietary intake or availability of calcium and phosphorus can diminish or be increased. It is possible to tip the balance in favor of increased bone mobilization or increased bone accretions to meet the stringent prerequisite of a constant serum calcium level. Thus the serum calcium may become elevated through stimulation of bone calcium mobilization or through PTH stimulation of the tubular reabsorption of Ca in the kidney. The PTH stimulates phosphate excretion, so that as serum calcium concentration increases there is usually an associated fall in the plasma phosphate level. In contrast, if serum calcium levels become too elevated, the action of calcitonin may come into play.

The dynamics of phosphate metabolism are not particularly different from those of calcium. Some 70% of the phosphate in the diet is absorbed. Absorption of phosphate is interrelated in a complex fashion with the presence of calcium and can be stimulated by a low calcium diet as well as vitamin D and its metabolites. The intestinal absorption of phosphate is inhibited by high dietary calcium levels. Phosphate in the body is also partitioned among three major pools: kidney ultrafiltrate, the readily exchangeable fraction of bone and the intercellular compartment in various soft tissues.

The central consideration of Ca and phosphorus homeostatic is the fact that the endoskeleton of higher organisms serves two important functions (Cummings *et al.*, 1995): A metabolic role concerned with the maintenance of dynamic steady state not only of Ca and P but also of many other ionic constituents for the body's extracellular fluids (Kanis *et al.*, 1994). This provides a mechanical or supportive function to locomotion and protection of the organism. A characteristic feature of bone that enables it to fulfill both these functions is that the skeleton normally undergoes a process of continual remodeling throughout life. This remodeling process is governed by changes in the mechanical stresses on the skeleton as well as by effects of metabolic regulators.

1.7 Osteoporosis

Osteoporosis is defined mainly according to pathological and anatomical principles and is considered a clinical entity caused by aging (Pommer, 1885; Albright, Smith and Richardson, 1941). The progressive diminution in bone volume starts at the beginning of adult life (Atkinson, 1964), is accelerated after middle age and appears to involve, sooner or later, both trabecular and compact bone. This is identified relating to metabolic bone disease. Therefore, this type of aging osteoporosis constitutes a major problem and has an immense social dimension.

Bone is a body of uniform strength and its construction seem to be optimally designed. The adaptational processes are similar to a feedback mechanism, which is controlled by mechanical stress. They concern the remodeling of the tissue as well as the exchange of mineral salts. After initial formation of bone, the tissue remains very dynamic. During long bone formation certain areas need to be shaped as the bone continues to grow. Therefore, in modeling, formation and resorption are separate and may occur at different sites. If the two processes are not coupled, a drift of the bone can occur, resulting in an eccentric shaft. During growth, modeling occurs continuously and there is an ultimate net gain of bone via this process (Jee, 1988; Lawrence and Fowler, 1997; Wasserman, 1984). Modeling and remodeling increases bone strength

and stiffness by adding bone to areas where deformation (stress gradient) will be the greatest, the periosteum (Kimmel *et al.*, 1993).

The balance between bone architecture and mechanical loading is a dynamic one, where bone is constantly resorbed and new bone is formed. In osteoporosis, however, bone is inadequately maintained, leading to inferior quality of bone architecture and osteopenia (Kleerekoper *et al.*, 1985; Parfitt, 1984). This is characterized by decreased bone density, which correlates to a decrease in estrogen levels. In the postmenopausal stage, the production of follicle steps are the primary source of estrogen. In the absence of estrogen, bone resorption increases due to an increased number of active osteoclasts. In males the corresponding role is that of testosterone. In osteoporosis there is a loss of both hydroxyapatite and osteoid components and bone becomes much thinner and more fragile. It may be that many serious fractures occur in the vertebrae of osteoporotic patients, resulting at times in a bent-over posture.

Most workers now agree that aging osteoporosis results from an increased rate of bone resorption. This can be inferred from the normal rates of bone formation or accretion that most workers have observed in osteoporosis (Dymling, 1964; Heaney and Whedon, 1958). Jowsey (1960) demonstrated an increase in the resorbing surfaces of bone in the elderly. Moreover, Bordier (1964) has shown increased osteoclasts in the iliac crest biopsies of patients with osteoporosis. The cause of this increased bone resorption is uncertain, but there are two main hypothesis. The first is that there is a primary change in the bone matrix, which leads to an inevitable bone breakdown analogous to that occurs in other tissues with age. It has been pointed out that with pathologic osteoporosis the skeleton is, as it were, aging prematurely. This hypothesis describes certain biochemical (Casuccio, 1962), histologic and biophysical (Little and Kelley, 1962) changes in osteoporotic bone that might be interpreted in this sense, as well as certain associated skin changes (McConkey *et al.*, 1963).

Many types of genes are involved in bone metabolism. Such genes include those coding for structural proteins, such as type I collagen and the vitamin D receptor and genes involved in sex steroid metabolism. These categories of genes and others are referred to as osteoporosis candidate genes (Gennari and Brandi, 2001). Polymorphism has been discovered in these genes and investigations into their associations with a male osteoporotic phenotype is sought (Melton, 2002).

A loss of bone mass is accompanied by a negative balance of bone remodeling. However, different parts of the skeleton are not affected to the same degree. The clinical syndrome of spinal osteoporosis is characterized by the occurrence of nontraumatic vertebrae fractures. The observation that bone mass in the general population decreases with age has led to the suggestion that osteoporosis represents an extreme form of the normal aging process (Newton-John and Morgan, 1968). Therefore, we speak of a deficiency in bone mass in comparison with a healthy control group of corresponding age and sex, while the quality of bone substance is normal. According to this definition, the term osteoporosis clearly marks a pathological condition, which has to be sharply distinguished from physiological bone loss with increasing age. The age-dependent atrophy is sometimes called physiological osteoporosis, as opposed to a pathological osteoporosis (McLean and Wrist, 1968). The breaking strength of bone is linearly related to its mineral content and porosity (Saha, 1977), hence the measurement of bone mineral content at the actual site of fracture appears to be the most accurate method of determining fracture risk (Arnold, 1973; Chalmers and Weaver,

1966). Several methods of quantifying BMC (bone mineral content) of the appendicular skeleton have been developed (Mazess, 1979). These measurements, however, have not provided good discrimination between subjects with spinal osteoporosis and age and sex matched controls (Johnson *et al.*, 1968). Because appendicular bone is predominantly trabecular, several authors (Nordin, 1971; Mazess, 1979; Wahner, Riggs and Beabout, 1977) have hypothesized that subjects with spinal osteoporosis have lost disproportionately large amounts of trabecular bone.

1.8 Bone Cells

Cellular dynamics play a major role in natural and stress induced bone remodeling and consequently are also important in normal and in bone diseases. There are five different type of cells controlling bone metabolism, namely, osteoprogenitor, bone lining cells, osteoblasts, osteocytes and osteoclasts. Hormones growth factors, ion concentrations and nutrition regulate all five bone cells. Mesenchymal stem cells can also differentiate into bone marrow cells, which can then differentiate into blood forming cells (Marks and Popoff, 1988; Lawrence and Fowler, 1997). The role of cell dynamics in bone remodeling and repair of bone fracture is of major concern (Binderman, Somjen and Shimshoni, 1986; Frost, 1964a). Bone adapts to changes in its mechanical environment based on the principle of cellular accommodation. For bone to adapt to a new mechanical loading state, bone cells must have some memory of their previous state in order to determine that the new state is different and to respond accordingly (Schriefer *et al.*, 2005). Bone cells process loading information locally because of poor innervations of bone tissue and, unlike many mechano receptor cells, they do not depend on the central nervous system to integrate and distribute information about mechanical signals. Frost (1983) has proposed that adaptive changes in bone shape occur when minimum effective strain (MES) thresholds are surpassed. It is assumed that when a strain threshold is surpassed the sensor cells will gradually accommodate to the new state, either by cytoskeletal reorganization or by changing the extracellular microenvironment. Using this definition of cellular accommodation, a set point can be determined simply by summation of the past history of daily strain stimuli (Turner, 1999). Bone formation (or resorption) would then be dependent upon the difference between the new strain stimulus and the ever changing set point. The temporal skeletal adaptive response will depend, to some extent, upon how the strain history is integrated into cellular memory. It is further assumed that the memory of the cells previous loading decays exponentially. Lanyon (1992) assumes a value of the MES that sets the threshold for a response. However, for the cellular response to be bone specific the MES must vary from bone to bone. Furthermore, the MES is location dependent within bones. Most long bones are loaded in bending (Bertham and Biewener, 1988), creating a strain gradient across bone section that has a value of zero along the neutral axis (Figure 1.8c). To avoid excessive bone resorption at the neutral axis, the MES varies across the bone section.

Each mechanosensitive cell must have some strain threshold above which a mechanical signal causes a cellular response. It is well known that many cell types, including osteoblasts, recognize their cytoskeletons in response to a mechanical stimulus. Cytoskeletal reorganization in turn changes a cell's mechanosensitivity, allowing the cells to accommodate to the strain environment. In addition, cellular mechanosensitivity may be

altered through reworking the local environment around the cell (Rubin, Judex and Hodjiargyrou, 2002). Osteocytes are considered to be part of the mechanosensory apparatus in bone and hence any change in their extracellular microenvironment could affect their mechanosensitivity. The extracellular environment of osteocytes does not remain constant as these cells actively form and remove layers of matrix on the surface of their lacunae (McKee and Nanci, 1996).

In principle, there are four types of functionally distinct cells that are known to play major roles in bone physiology: mesenchymal cells, osteoclast, osteoblast and osteocyte. Figure 1.9a and b shows their role in controlling bone dynamics. The mesenchymal cells have an outstanding capacity for proliferation and are capable of further differentiation into osteoclasts and osteoblasts (Hall, 1965).

Bone cells respond to mechanical stimuli with various biological signals, and a number of these signals have been utilized as measures of their responsiveness to oscillatory fluid flow (OFF) (You *et al.* 2000, 2001; Kurokouchi *et al.*, 2001). Batra *et al.* (2005) have reported that insertion of 10 and 155 rest periods into an OFF profile results in greater $[Ca^{2+}]_i$ response magnitudes than those found in cells exposed to continuous OFF. The intracellular calcium magnitude has been shown to control cell processes in other cell types (Thomas *et al.*, 1996; Dolmetsch *et al.*, 1997; Berridge *et al.*, 1998; Dolmetsch, 2003).

1.8.1 Osteoblasts

At the bone surface, osteoblasts are recruited from the environment or by reactivation of lining cells (Chow *et al.*, 1998; Dobnig and Turner, 1995) to form bone in response to the total stimulus they receive. Bone lining cells cover the surface of bone. They also serve as ion barriers between the canaliculi and interstitial fluids. Distraction osteogenesis is predominantly achieved by intramembraneous bone formation that is characterized by direct differentiation of mesenchymal cells into osteoblasts without the occurrence of cartilage tissues (Aronson and Harp, 1990; Bizarov *et al.*, 1984). The osteoblast can then secrete matrix, which will become calcified. This increases the amount of bone in the area. This differs principally from secondary bone healing where perivascular mesenchymal cells are induced to differentiate into chondroblasts (Brighton, 1984), and with further development of the callus undergo endochondral ossification (Schenk, 1992). Mesenchymal stem cells concentrate in the area of new bone formation and differentiate into osteoblasts. This then increases the amount of bone in the area.

Osteoblasts, the bone-forming cells, become entrapped in the lacunae and canaliculi and secrete intracellular bone matrix, known as osteoid. Osteoblasts originate from osteogenic lineage and secrete minerals, the amorphous ground substance osteocalcin, osteonectin, water and type I collagen into the matrix; osteoblasts have rough endoplasmic reticulum. Osteoblasts surround themselves with osteoid, mature into osteocytes and reside in the lacunae till death (Jee, 1988; Lawrence and Fowler, 1997). Osteoblasts make all the proteins and carbohydrates in osteoid and secrete them to form a gel in the extracellular environment around the cell.

Osteoblasts are derived from stromal stem cells (fibroblast colon forming units, F-CFU) that differentiate into osteoprogenitor cells. The same stem cells can also give rise to fibroblasts, endothelial cells or adipocytes. In more mature bone, osteoprogenitor cells are present at the periosteum and stem cells are present in bone marrow. The number of stromal stem cells in the

bone marrow decreases with age, while the number of adipocytes increases. The osteoprogenitor cells may differentiate into chondrocytes or osteoblasts depending to a large degree on the environmental conditions. In regions with a good blood supply (via the presence of capillaries) the development of osteoblasts is promoted, whereas in regions of inadequate blood supply differentiation into chondrocytes takes place.

1.8.2 Osteoblast Differentiation

Studies of the pathophysiology and genetics of skeletal cell differentiation have provided insight into the mechanism by which osteoblasts arise from mesenchymal precursors and osteoclasts arise from hematopoietic precursors (Ducy *et al.*, 1997; Karsenty and Wagner, 2002; Karsenty, 2003). The differentiation of osteoblasts is controlled by the transcription factor Runx2 and also the transcription factor osterix, which functions downstream of Runx2 (Karsenty and Wagner, 2002; Nakashima *et al.*, 2002). In the absence of either Runx2 or osterix, no osteoblasts are formed. Mice and humans that carry only a single copy of Runx2 have abnormalities that are specific to certain bones rather than the entire skeleton. Overexpression of Runx2 leads to a decrease in bone mass (Geoffroy *et al.*, 2002). A role for polymorphism of these transcription factors in osteoporosis has not yet been identified.

LRP5 (low density lipoprotein receptor-related protein 5) deficiencies lead to the development of osteoporosis in both mice and human (Gong *et al.*, 2001; Kato *et al.*, 2002) and also to chronic inflammatory disorders (Raisz, 2003). There are also suggestions that some of the anabolic effects of parathyroid hormone (PTH) are exerted through suppression of Dickkopf1 expression (Guo, Bringhurst and Kronenberg, 2004). Mutations in human LRP5 that interfere with the Dickkopf1 inhibition are suggested to cause a high bone mass phenotype (Boydin *et al.*, 2002).

The function of mature osteoblasts, including the ability to synthesize extracellular matrix proteins, also requires LRP5 as well as the signaling protein ATF4 (Yang *et al.*, 2004) and bone morphogenetic proteins (BMPs). Members of a family of secreted growth factors provide important tissue specific signals to preosteoblasts that are essential for full osteogenic differentiation (Chen, Zhao and Mundy, 2004; Mundy, 2002). Thus, it has been possible to use a reporter system driven by the BMP-2 promoter to screen a set of chemical compounds and natural products for potential bone anabolic agents (Mundy, 2002). There is evidence for interactions among the possible pathways (Mbalaviele *et al.*, 2005; Yang *et al.*, 2004).

A changing pattern of cell adhesion molecules (CAMs) expression may also characterize the aging process at the tissue level. One can speculate that age-related changes in the type and/or amount of CAM expressed may impair cell-cell communication, either directly or through abnormal expression of connexins. Cell-cell communication is necessary to maintain the work of osteoblasts synchronous within basic multicellular units. An improper signal exchange may cause the remodeling units to become asynchronous. This may disrupt or slow down bone turnover. Ultimately, age-related abnormalities in the expression of CAMs and/or connexins may impair the ability of osteoblasts precursors to differentiate, with a consequent decrease in osteoblast number. This is a commonly accepted cause and phenomena in osteoporosis. This phenomena of aging on the skeletal tissue may lead to decreased bone formation. Additionally, because cell-cell contact and communication may be required for osteoclast development

(Suda, Takahashi and Martin, 1992), abnormal expression of CAMs may also lead to decreased osteoclast activity and translate into reduced activation frequency of the remodeling cycle at the tissue level.

The identification of the critical role for the Wnt signaling pathway in regulating osteoblast function is of particular interest. It plays an important role in determining bone mass and strength (Little *et al.*, 2002; Boyden *et al.*, 2002; Van Wesenbreeck *et al.*, 2003; Gong *et al.*, 2001). LDL receptor related protein 5 (LRP5) interacts with the frizzled receptor to transduce signaling by Wnt ligands. A mutation of LRP5 that leads to constitutive inactivation can result in an increase in bone density (Little *et al.*, 2002; Boyden *et al.*, 2002). Another key predictor is the rate of bone remodeling, increased rates of osteoclastic bone resorption, measured by the level of collagen breakdown products, and increased bone formation, measured by bone-specific alkaline phosphatase and osteocalcin. Procollagen peptide levels are also associated with an increase in risk of bone loss and fragility fractures.

1.8.3 Osteoclast

Osteoclasts are identified as multinucleated cells that can move about in tissue, are physiologically specialized, do not undergo cell division and are considered the daughter cells of a mesenchymal cell (Frost, 1963). They are monocyte-macrophage derivatives that are responsible for degrading bone, and are derived from preosteoclasts in the bone marrow. Active osteoclasts cells are usually found in resorption pits or Howship's lacunae. Their specialized role is central to the process that continuously removes and replaces segments of the skeleton in the higher vertebrates. Osteoclasts allow skeletal mineral to be used to manage extracellular calcium activity. This allows solid skeletal structure to be replaced by hollow architecture that has a superior strength-to-weight ratio. Osteoclasts tend to dissolve bone mineral by acid secretion and secrete specialized proteinases that in turn degrade the organic matrix, mainly type I collagen. Osteoclastic differentiation is normally balanced with bone formation, which is a function of stromal cell-derived osteoblasts. Interactions between osteoclast precursors and bone-forming cells are believed to control osteoclast differentiation under most circumstances, preserving bone architecture over many cycles of bone replacement. The junction of osteoclast and bone forms a ruffled border, which forms an impermeable microenvironment and enhances resorption.

Osteoclasts are assumed to be recruited by osteocyte apoptosis due to microdamage or possibly cracks (Bronckers *et al.*, 1996; Noble *et al.*, 1997; Parfitt *et al.*, 1983; Verborgt, Gibson and Schaffler, 2000). Ruimerman *et al.* (2005) assumed that, due to daily loading conditions, microcracks and damage occur at spatially random locations, occurring anywhere at any time. This describes the coupling between formation and resorption as an effect of mechanical stress transfer. Regulatory biomechanical factors related to this are still to be elucidated.

Osteoclasts are apparently giant cells with the ability to resorb mineralized tissue (Katagiri and Takahashi, 2002; Suda *et al.*, 1999). They evolve out of the fusion of mononuclear cells of extrasosseal origin (Suda *et al.*, 1999). The osteoclast initiates bone resorption by demineralizing the bone (Remedios, 1999). Osteoclasts appear only rarely in intact adults bone tissue (Miyamoto and Suda, 2003). However, areas with high bone turnover are characterized by an increasing number of osteoclasts. This regularly occurs in growth plates of juvenile bones but is especially pronounced in bone fractures.

The bone resorbing osteoclasts participate in bone healing. The osteoclasts are drawn to a resorption area by signals sent by osteoblasts (Bonewald, 2002). Osteoclasts are always found at or near the bone surface (Amling and Delling, 1996). In contrast to osteoblasts, which are densely populated and normally appear in layers on the bone surface, osteoclasts resorb misaligned or redundant bone tissue during bone healing (Cruess and Dumont, 1975). A single osteoclast can resorb the amount of mineralized tissue synthesized by 100 osteoblasts (Remedios, 1999).

1.8.4 Osteoclast Differentiation

Osteoclast differentiation requires the binding of macrophage colony-stimulating factor to its receptor as well as the binding of the soluble differentiation factor receptor activator of NFB ligand (RANKL) to its receptor (RANK) on osteoclast precursor cells (Teitelbaum and Ross, 2003). This process is also regulated by a secreted decoy receptor of RANKL, osteoprotegerin (OPG), which functions as a paracrine inhibitor of osteoclast formation (Udagawa *et al.*, 2000). Osteoprotegerin, originally identified as a novel secreted member of the TNFR super family, was later found to inhibit spontaneous or induced bone resorption and cause osteopetrosis (the converse of osteoporosis). Osteoprotegerin acts as a decoy receptor that binds to RANKL and prevents it from interacting with its receptor RANKL and osteoprotegerin, which are both produced by osteoblasts at different stages of maturity (Gori *et al.*, 2000), thereby accounting for some of the signals in osteoblast–osteoclast communication. In conjunction with macrophage colony-stimulating factor, the RANK-RANKL-osteoprotegerin system regulates osteoclast differentiation. Thus, mice and humans deficient in osteoprotegerin have a high rate of bone loss (Whyte *et al.*, 2002). As in other high turnover states, anti-resorptive agents can still reduce both bone formation and resorption and compensate for osteoprotegerin deficiency. The mechanism of how the signal(s) couples resorption and formation remains elusive, although several anabolic ligands, such as BMPs and TGF, are stored in bone matrix as one is formed and are released at the sites of bone resorption and can thus act on osteoblasts and precursors in the vicinity. Signals from osteoblasts to osteoclasts can be provided by RANKL and osteoprotegerin. In this case preosteoblasts express a high level of RANKL relative to osteoprotegerin, which stimulates osteoclast differentiation and function. More mature osteoblasts, by contrast, express high levels of osteoprotegerin relative to RANKL, which inhibits osteoclast differentiation and function (Boyd *et al.*, 2002). The production of RANKL and OPG is regulated by agents that signal through CAMP, the vitamin D receptor (VDR) and gp130 (Suda *et al.*, 1999; Boyle *et al.*, 2003). RANKL produced by cells of the osteoblast lineage binds to its receptor.

The precise nature of the mechanism by which osteocytes translate mechanical signals into bone formation stimuli is still unknown. Ruimerman *et al.* (2005) have used the strain energy density (SED) rate as the relevant osteocyte signal (Figure 1.10). That alternative choices of the osteocyte stimulus signal may affect the results considerably is obvious as the precise distribution of the mechanical signals throughout the structure can differ significantly. The osteoclast can transform into an osteoblast and vice versa (Frost, 1963) (Figure 1.11). The osteocyte has been referred to as the inhabiting cell of formed bones. Coupled with the bone formation process is osteoclastic activity on the innermost surface of the cortical bone. This results in an increased diameter of the diaphyses, without altering the width of the cortical bone (Wasserman, 1984).

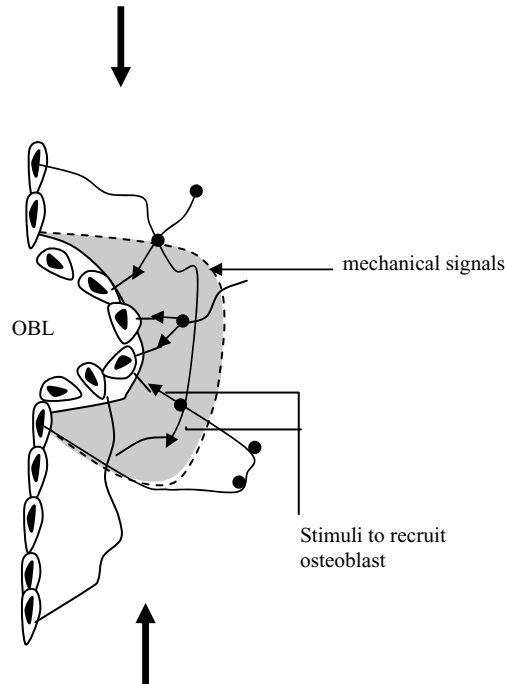


Figure 1.11 A schematic diagram of Osteocytes recruiting osteoblasts cells. (Adapted from Ruimerman *et al.*, 2005)

It can thus be said that stresses applied from time to time play a definite role in the development of bone. Basically, these induce an electrical activity that is sensed as a command signal by cells or their environment that then respond accordingly. However, the nature of the command signal remains to be understood. Elevated external loads increase trabecular thickness (Jee *et al.*, 1990; Li, Luidier and Schaffler, 2003) and reduced loads cause trabecular thinning and loss of connectivity (Mosekilde, 1990). Evidently, bone is a part of system controlled by feedback from the applied stresses. The intrinsic and extrinsic control mechanism in bones, based on the generation of piezoelectric changes emanating from changes in pressures on or movements of crystals, remains, therefore, an attractive point of investigation. As an extrapolation of this, experimental and theoretical studies have shown that bone cells residing in intact matrix may be activated to initiate bone adaptation processes by interstitial fluid flow through the lacuno-canalicular network (Weinbaum *et al.*, 1994; Cowin *et al.*, 1995; Knothe Tate *et al.*, 2000).

During life, bones adapt their mass and structure to the average prevailing mechanical loads, to resist mechanical failure with a minimum of material expense. It is presently believed that this process of adaptation is governed by osteocytes, which respond to the loading induced flow of interstitial fluid through the lacuno-canalicular network (Burger and Klein-Nulend, 1999). When bones are loaded, the resulting deformation will drive the thin layer of interstitial fluid surrounding the network of osteocytes within the calcified bone matrix to flow from regions under high pressure to regions under low pressure (Piekarski and Munro, 1977; Weinbaum *et al.*, 1994). The loading-induced movement of labeled molecules demonstrates fluid flow in

the mineralized matrix of bone both *in vivo* (Knothe Tate, Niederer and Knothe, 1998) and in *ex vivo* (Knothe Tate and Knothe, 2000; Klein-Nulend *et al.*, 1995a, 1995b). Osteocytes, and to a lesser extent also osteoblasts, respond to fluid flow stimulation *in vitro* (biochemical messengers) that is transduced through the canalicular network to the trabecular surface (Bacabac *et al.*, 2004; Bakker *et al.*, 2001; Chen *et al.*, 2000; Frangos and Johnson, 1995; Jacobs *et al.*, 1998; Johnson, McAllister and Frangos, 1996; Klein-Nulend *et al.*, 1995a, 1995b, 1995c; Burger and Klein-Nulend, 1999; Cowin, Moss-Salentijn and Moss, 1991; Knothe Tate *et al.*, 2002; Martin, 2000; Mullender and Huijskes, 1995; Nicoletta and Lankford, 2002; Skerry *et al.*, 1989; Ruimerman *et al.*, 2005).

Osteoblasts are sensitive to fluid shear stress (Bakker *et al.*, 2001; Jacobs *et al.*, 1998) rather than to a streaming potential mediated by the transport of ions with the flow (Bakker *et al.*, 2001; Hung *et al.*, 1996). Bone cells are also more responsive to shear stress by fluid flow stimulation than to direct mechanical strain by substrate stretching (Mullender *et al.*, 2003; Owan *et al.*, 1997; Smalt *et al.*, 1997). Bone cells respond to fluid flow with increased nitric oxide (NO) and prostaglandin E₂ production, which are essential for the induction of new bone formation in relation to mechanical loading *in vivo* (Forwood, 1996; Turner *et al.*, 1996). In particular, MC3T3-E1 osteoblastic cells produce NO in response to fluid shear stress in a rate-dependent manner (Bakker *et al.*, 2001). However, how bone cell sensitivity is modulated by the various flow parameters (amplitude, frequency, duration, etc.) is yet to be fully understood. It is suggestive that, as an alternative pathway, mechanical loading induces fluid flow due to the cyclic nature of the applied loads *in vivo*. This indicates that flow through the canalicular network is the actual trigger for metabolism (Burger and Klein-Nulend, 1999). Osteocytes are stimulated by a relatively small fluid shear stress acting on the membranes of their osteocytic processes (Weinbaum *et al.*, 1994). These authors have shown that in the physiological frequency range (1–20 Hz), associated with either locomotion (1–2 Hz) or the maintenance of posture (15–20 Hz), the fluid shear stress is nearly proportional to the product of frequency and strain. The impact of losing osteocytes in bone may be enormous (Parfitt, 1993). In human bone, osteocyte cell death can occur in association with age and both osteoporosis and osteoarthritis, leading to increased bone fragility (Dunstan *et al.*, 1990; Dunstan, Somers and Evans, 1993; Frost, 1960).

Several *in vivo* studies have shown that bone can recover its responsiveness and can respond to mechanical stimuli with the same magnitude as earlier exposures to loading with the insertion of an appropriate length of rest periods (Forwood *et al.*, 1996; Robling *et al.*, 2000). Modulating loading parameters such as duration and magnitude may be a key factor in allowing bone to recover its mechanosensitivity. Understanding the mechanism required to restore mechanosensitivity in bone will be important for optimizing the osteogenic potential of mechanically induced bone formation. Robling *et al.* (2000) found, in a four-point bending model of rat tibia, that the insertion of a recovery period between loading cycles led to significantly higher relative bone formation rates than with continuously loaded limbs. Srinivasan *et al.* (2002) found that although continuous low magnitude loading is a non-osteogenic stimulus, the insertion of a 10s rest period between each loading cycle results in a potent anabolic response. These studies show that the introduction of short-term recovery periods, introduced into mechanical loading regimens *in vivo*, can have an osteogenetic effect at the tissue level. This corresponds to the fact that static loading (or approximating to that) has an effect on bone behavior. However, the cell–cell response to this loading pattern

remains to be fully understood (Batra *et al.*, 2005). The quantum difference in the effect between the dynamic and near static loading on osteogenesis may be important to examine: if it resets the modeling pattern.

Ramnaraine, Pan and Clonisy (2006) found that treatment of NCD (cytosine deaminase fusion gene) transduced osteoclasts can promote killing of cancer cells. This introduces the possibility of developing osteoclast based treatment of primary bone cancers and breast cancers.

1.8.5 The Osteocytes

Osteocytes are mature bone cells that have many long cytoplasmic branches projecting from the main body (Figure 1.7a). Osteocytes are cells of osteoblast lineage that reside within the lacunae in the bone matrix. They communicate with each other and with the bone surface via cellular processes extending through canaliculi. While the genetic influence may dictate the general structural plan of bone but the environmental influence and growth modify it significantly. The osteocytes have a round cell body and many long fine extensions for cytoplasmic processes. Many types of osteocytes exist in the different parts of the bone matrix. They may vary in geometry and cytoplasmic detail as well as in the regularity and density with which they are placed in the matrix. Their main functional role is to facilitate the exchange of materials between tissue fluids and the bone matrix. In the normal adult skeleton, a balance exists between bone resorption and bone formation. The osteoclastic and osteoblastic activity cause a continuous turnover in the bone tissue but not an appreciable change in bone mass or a significant change in bone tissue structure. In a pathological case (e.g., osteoporosis), these activities become unbalanced and one of the processes dominates (resorption) and in turn causes a change in the bone structure.

1.8.6 Mathematical Formulation

The interrelationship of bone cell types and a summary of the theoretical formulation can be illustrated with a regulation scheme. Mathematical equations are introduced to quantify the relationship (Huiskes *et al.*, 2000; Ruimerman *et al.*, 2001). The change in bone mass at a particular trabecular surface location x at time t is determined by:

$$\frac{dm_{\text{tot}}(x, t)}{dt} = \frac{dm_{\text{bl}}(x, t)}{dt} - \frac{dm_{\text{cl}}(x, t)}{dt} \quad (1.1)$$

with osteoblast bone formation, $dm_{\text{bl}}(x, t)/dt$, and osteoclast bone resorption, $dm_{\text{cl}}(x, t)/dt$.

Osteoblast activity at the trabecular surface is controlled by osteocyte bone formation stimuli. For a total stimulus P ($\text{mol mm}^{-2} \text{day}^{-1}$) that exceeds a certain threshold value, k_{tr} ($\text{mol mm}^{-2} \text{day}^{-1}$), the osteoblast tissue formation rate becomes:

$$\frac{dm_{\text{bl}}(x, t)}{dt} = \tau [P(x, t) - K_{\text{tr}}] \quad (1.2)$$

where τ ($\text{mm}^5 \text{mol}^{-1}$) is a proportionality factor that regulates the formation rate relative to the formation stimulus. All osteocytes N within the influence region contribute to the bone

formation stimulus P on trabecular surface to cation X , depending on their mechanosensitivity μ_i , their distance d to surface location x and the signal R (SED rate) the osteocytes sense in their location x_i ; hence:

$$\begin{aligned} P(x, t) &= \sum_{i=1}^N f(x, x_i) \mu_i R(x, t), \quad \text{with} \\ f(x, x_i) &= e^{-d(x, x_i)/D} \end{aligned} \quad (1.3)$$

where $f(x, x_i)$ is an exponential function that describes the signal intensity relative to distance d and a decay parameter D .

The overall osteoclast bone resorption is described by:

$$\frac{dm_{cl}(x, t)}{dt} - r_{cl} \quad (1.4)$$

where r_{cl} ($\text{mm}^3 \text{ day}^{-1}$) represents a stochastic function that describes that portions of tissue are removed randomly from the surface.

Bone essentially receives all its nutrition via its extensive vascular system and when blood flow ceases the osteocytes entrapped in their calcified matrix rapidly die. Nutrients move to the osteocytes through the canaliculi by diffusion from the Haversian canals. Fluid flow through the canaliculi is not very efficient but osteocytes may be within 0.1–0.2 mm of blood vessel for adequate nutrition.

Examined in this way bone behaves like a dynamic structure in which the structural elements and the overall architectural plans are being continuously modified by proper cells, proper stimulations and proper nutrition. Bone undergoes substantial changes in structure, shape and composition according to the mechanical and physiological environment. Bone adaptability allows for efficient repair, which in turn helps to prevent fractures. Bone adaptation to mechanical loading depends on the duration and magnitude of the applied loads (Rubin and Lanyon, 1984; Forwood and Turner, 1994; Forwood *et al.*, 1996). However, application of continuous dynamic mechanical loads *in vivo* in animal models suggests that the osteogenic response of bone saturates with continued long-term mechanical loading (Turner *et al.*, 1994; Umemura *et al.*, 1997; Turner, 1998). However, the mechanisms behind bone's osteogenic saturation in bone to loading are not well understood. Bone as living tissue consists of cells (e.g., osteoblasts, osteoclasts and osteocytes) and their products, the extracellular substance (Hancox, 1972), and the cells that produce it belong to the connective series. The activity observed during the formation of bone is carried out by osteoblasts and is known as "osteogenic activity." The osteogenic cells have the capacity to change their function – the name given to a cell depends on its activity at that time (Little, 1973). Osteoblasts are the differentiated mesenchymal cells that produce bone, and are created at the periosteum layer or stromal tissue of bone marrow. Bone lining cells are inactive osteoblasts, remain on the surface when bone formation stops and can be reactivated in response to chemical and/or mechanical (electrical) stimuli (Miller and Jee, 1992). Like bone lining cells, osteocytes are former osteoblasts and are buried in the bone matrix and are located in lacunae (Figure 1.11) and communicate with other cells via canaliculi (Figure 1.7a and b). Look at in this way, matrix disruption may be expected to directly injure osteocytes, disrupting their attachments to bone matrix. This intervention is expected to interfere with their communi-

cation through canaliculi or alter their metabolic exchange. Fatigue micro damage may therefore create a situation resembling disuse at the level of the osteocyte cell body and lead to bone remodeling. These are responsible for the maintenance of bone, as a living tissue.

The presence of nerve terminals in bone is well established (Chenu, 2002), and the interactions between neurons and bone cells have come under intense study (Spencer, Hitchcock and Genever, 2004; Chenu, 2002; Madsen *et al.*, 1996; Kingery *et al.*, 2003; Suyama *et al.*, 2003). Bone cells express a wide range of neurotransmitter receptors and transporters. This includes glutamate, γ -aminobutyric acid, purines, pyrimidines, (*S*)-hydroxytryptamine, catecholamines and neuropeptides (Chenu, 2002).

Intramembranous growth occurs on bone surfaces or “in membrane” such as under the periosteum of long and flat bones. There are two forms of bone growth – endochondral and intramembranous ossification. The compound (bone or cartilage) that provides the starting material for bone formation characterizes each of these forms of osteogenesis. Endochondral ossification indicates growth occurring in the cartilage, which is made up of chondrocytes and occurs in long bones at the growth plate. The growth plate is composed of zones. The first zone, known as the resting zone, is near the epiphysis of the bone and is made up of hyaline cartilage formed by chondrocytes. Although the exact function of this zone is unclear, chondrocytes are the likely recruit into the maturation process during growth. The hypertrophic zone begins when chondrocytes differentiate. Finally, in the zone of calcification, terminally differentiated chondrocytes, near the metaphysis, begin to lay down mineral into the matrix (Jee, 1988). The chondrocytes in this region undergo apoptosis. Endothelial and phagocytic cells initiate vascularization in the empty lacunae (Jacobson, Weil and Raff, 1997). In the chondro-osseous function, where calcification and chondrocyte cell death have begun, the marrow begins to form the primary spongiosa. In this region, osteoblast and surrounding matrix of the woven bone continue to release osteoid. Additionally, the marrow carries osteoclasts and resorbs the bone. This synergistic effect of formation and resorption helps to shape the long bone.

1.9 Bone Remodeling

The growth and development of bone to a stage of maturity is controlled by the combination of intrinsic and extrinsic forces. The mass and architecture in bones are governed, to some extent, by adaptive mechanisms sensitive to their mechanical environment.

The idea that mechanical forces shaped the architecture of the skeleton emerged in the work of Roux (1905) and Meyer (1967). However, Wolff (1892) stated that when bone is bent under a mechanical load it modifies its structure so as to resist external pressure by bony apposition in the concavity and by resorption in the convexity. Supported by much experimental data, the idea that mechanical stresses affect the form of bone is now generally accepted (Thompson, 1917; Frost, 1964b, 1964c). A reduction in bone formation (Wronski and Morey, 1983; Shaw *et al.*, 1988; Morey and Baylink, 1978), mineral content (Vico *et al.*, 1987; Rambout and Goode, 1985; Russell and Simmons, 1985; Turner *et al.*, 1985; Cann and Adachi, 1983), and bone matrix protein production (Patterson-Buckendahl *et al.*, 1985) result from the skeletal unweighting associated with spaceflight. Conversely, increased skeletal loading through exercise has been shown to increase bone mass (Bassey and Ramsdale, 1994; Smith and Gilligan, 1990; Eisman *et al.*, 1990) and retard bone loss caused by postmenopausal osteoporosis (Krolner *et al.*, 1983;

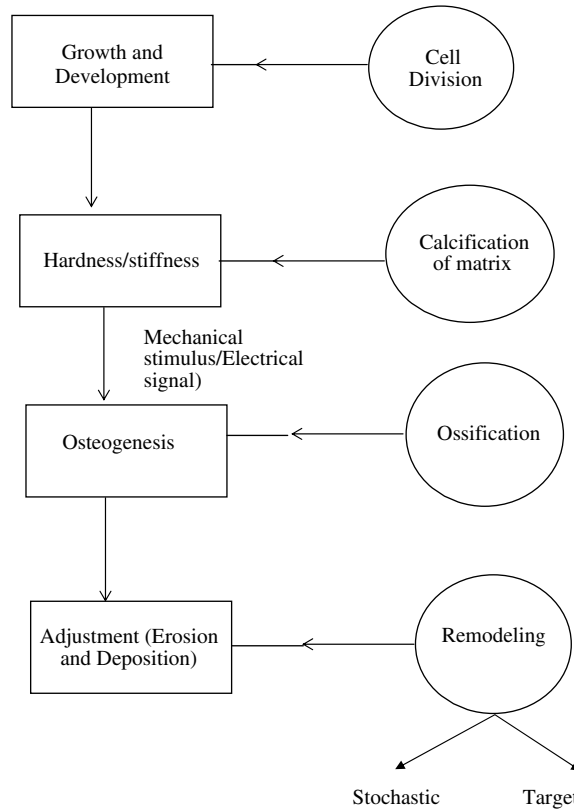


Figure 1.12 A schematic presentation of broad steps in Broad steps in bone growth, development and in remodeling

Simkin, Ayalon and Leichter, 1987; Prince *et al.*, 1991; Chesnut, 1993). Figure 1.12 shows a sequence of bone mechanical stimuli leading to consequent bone remodeling.

The general hypothesis is that animals that experience higher levels of load-bearing activity are predicted to have larger articular surface areas (ASAs) relative to the body mass. This may be summarized in the modified Wolff’s law as:

The form of a bone being given, the bone elements place or displace themselves in the direction of functional forces and increase or decrease their mass to reflect the amount of functional forces (Figure 1.1).

The results indicate that the effects of mechanical loading from moderate exercise are not only complex but also differ substantially in diaphyses and the subchondral articular surfaces of epiphyses (Lieberman, Devlin and Pearson, 2001). Bone is not homogenous, both morphologically and mechanically, but interestingly inhomogeneities do not disturb the functional adaptation (Bennok, 1972). On the contrary, they contribute towards it. Most experiments and theory on bone adaptation are concerned with the size and shape of the bone. This is termed modeling and is probably produced by uncoordinated activity of bone cells. In contrast,

“remodeling” occurs in cancellous bone, in which osteoclasts and osteoblasts work together in coordinated sequence to replace bone (Figure 1.9b). This leaves the total amount of bone unaltered, in the form of secondary osteons (Haversian systems). There has been much less concern about the adaptations that bone may have, in particular there has been little consideration of whether hard bone material properties may be related to the loads falling on the bone (Currey, 2003). The mechanical properties of whole bones depend both on the material properties of the bone and the architecture of the whole bone. There may be adaptive links between these two features. This feature is better seen during growth: the material properties of bone can change in synergy with the architecture as the function of the bone either changes or is about to change. In this case the material properties are determined over a long, evolutionary, time, while the architecture of the bones may also respond to the strains in the bone over a correspondingly short period.

Bone remodeling can conserve and reduce bone strength and mass but does not increase the same. Remodeling process achieves three goals (Burn, 2002). First it provides a way for the body to alter the balance of essential minerals by increasing or decreasing the concentration of these in serum. Second it provides a mechanism for the skeleton to adapt to its mechanical environment, reducing the risk for fracture and increasing the organisms chances for passing its genes to the next generation. Third, it provides a mechanism to repair damage created in bone by repetitive cycles of mechanical loading. The mechanical properties of bone are a result of a compromise between the need for certain stiffness and the need for enough ductility to absorb impacts. Bone undergoes substantial changes in structure, shape and composition according to the mechanical and physiological environment.

It is now widely accepted that bone remodeling is the mechanism of bone replacement in the vertebrate skeleton. One of the primary reasons for replacement is the functional capacity of bone, which in some way is compromised if it is allowed to become too old (Parfitt, 2001). The remodeling process arranges a given amount of calcified material to support the naturally occurring loads with the largest possible safety factor. The remodeling apparatus can accommodate circadian fluctuations in calcium balance (Parfitt, 1993) and can also cater to a temporary need for additional calcium lasting for a few months (Parfitt, 1981) and accomplishes slow thickening of trabeculae during growth (Parfitt, 2000). This also entails slow elimination of surplus bone in response to the age related decline in physical activity (Parfitt, 1990). Frost’s mechanostat theory is unique in its distinction between modeling and remodeling processes, the threshold for activating lamellar or woven bone formation and its application to the etiology of osteopenia and osteoporosis. Frost (1987) suggested that certain hormones and biochemical agents might overcome this mechanical setting of bone to alter the boundaries of the pathological window. This allows normal mechanical usage to increase bone mass and bone strength in a significant manner.

Modeling can increase bone “mass” and hence its strength. It increases these when bone strains equal or exceed a threshold range. Formation and resorption drifts use osteoblasts and osteoclasts, respectively, to determine the cross sectional shape and size of bones and trabeculae and their longitudinal shapes and to increase their strength and mass. The modeling is best in evidence during growth and slows down on attainment of adulthood. If strain is greater than a particular threshold the modeling takes place otherwise it does not:

$$\begin{aligned} \text{Strain} < \text{Threshold} &< \text{off} \\ \text{Strain} > \text{Threshold} &> \text{on} \end{aligned}$$

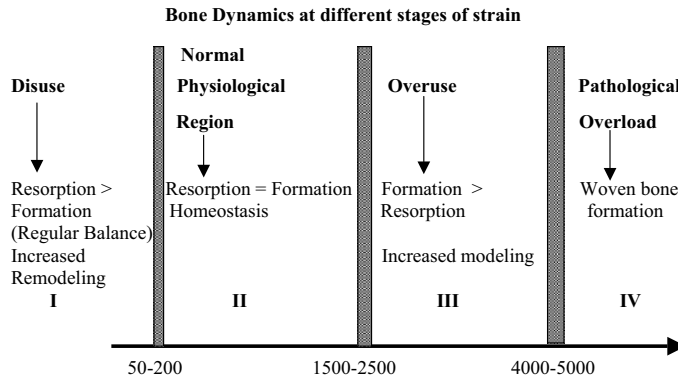


Figure 1.13 Mechanostat theory according to Burr and Martin (1992). In the disuse window (I), strains are low and bone is lost due to increased remodeling. In the normal physiological window, bone is at a state of normal turnover and bone equilibrium is maintained (II). In overuse (III), bone formation exceeds resorption. The site depends upon strain. This causes laying down of woven bone (IV)

Bone that is strong enough to keep strains from reaching or exceeding the threshold would satisfy the above criterion (Frost, 1997). Peak bone strains from voluntary activities should be larger in growing subjects than in adults. These range between 2000 and 4000 microstrain in most growing subjects, but between 800 and 1300 microstrain in most adults (Frost, 1997) (Figure 1.13).

The mechanisms by which the mechanostat works are not fully understood; however, they require some form of cellular mechanotransduction. Mechanotransduction, or the conversion of a biophysical force into a cellular response, is an essential mechanism for a wide variety of physiological functions that allow living organisms to respond to the mechanical environment. Mechanotransduction in bone necessarily includes four distinct phases: (i) mechanocoupling, the transduction of mechanical force applied to the bone into a local mechanical signal perceived by a sensor cell; (ii) biochemical coupling, the transduction of a local mechanical signal into a biochemical signal and, ultimately, gene expression; (iii) transmission of signal from the sensor cell to the effector cell, that is, the cell that will actually form or remove bone; and (iv) the effector cell response, the final tissue level response. on the basis of mechano-transduction mechanics, forms the model for bone adaptation.

Bone remodeling is essentially a surface based event – the metabolic activity of cancellous bone is tenfold greater than at the cortical sites. At any given instant, approximately 10–15% of the bone surface is undergoing remodeling, the remaining surface being relatively quiescent. In the stage of dynamic equilibrium the process of calcium resorption (5 mmol) and new matrix formation are equal, as in the case of healthy adults (normal bone). Mineralization of bone occurs after matrix production and hence the skeletal demands for calcium are governed by the rate of matrix synthesis. This is consistent with clinical findings that severe calcium deficiency is associated with severe osteomalacia, but not with the formation of new bone. There is evidence that low calcium diets decrease formation in growing rats, but in adults most evidence suggests that calcium deficiency increases bone formation by increasing the turnover of bone. This is suggestive that, under normal

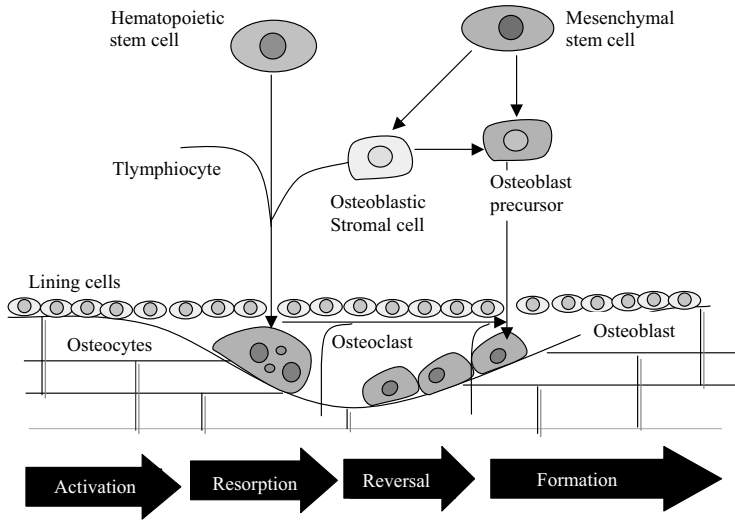


Figure 1.14 BMU with possible estrogen action. Bone remodeling on the surface of trabecular bones is illustrated (Raisz, 2005). (Reproduced with permission from Lawrence G. Raisz, Pathogenesis of osteoporosis: concepts, conflicts, and prospects, *Journal of Clinical Investigation*, **115**(12), 3318–3325 ©2005, The American Society for Clinical Investigation)

conditions, the skeletal requirement of calcium is governed by the rate of matrix synthesis rather than by the availability of calcium.

From Wolff's law it can be easily extrapolated that living cells are constantly subjected to mechanical stimulations arising from the external environment, mechanical properties of bone and internal physiological conditions (Figure 1.14). Depending on the magnitude, direction and distribution of these mechanical stimuli, cells can respond in various ways. The mechanism by which the mechanical signal is translated into biological and chemical responses in a cell is a matter of continuing investigation (Wang, Liu and Shiau, 1993; Ingber, 2003). Apart from mechanical loads induced within or outside the body many chemicals also affect the mechanical properties of living cells. In turn, the mechanical properties of individual cells can control the structural integrity of the whole tissue. This may arise from the mechanical interactions between cells and the surrounding extracellular matrix (Wakatsuki *et al.*, 2000). On the other hand, mechanical loads exerted at the tissue level are possibly transmitted to individual cells and can influence their physiological functions (Guilak, Ratcliffe and Mow, 1995; Guilak and Mow, 2000).

The remodeling process is not performed by each cell but by group of cells functioning as organized unit, which are named "basic multicellular units" (BMU) (Frost, 1963). They operate on bone periosteum, endosteum, trabecular surfaces and cortical bone, replacing old bone with new bone in discrete packets. The cycle of remodeling stages may be described as:

Activation → Resorption → Reversal → Formation → Quiescence

Upon activation of bone surface, osteoclasts are recruited from the bone marrow and migrate to the site of activation, where they remove mineral and matrix in the resorption period

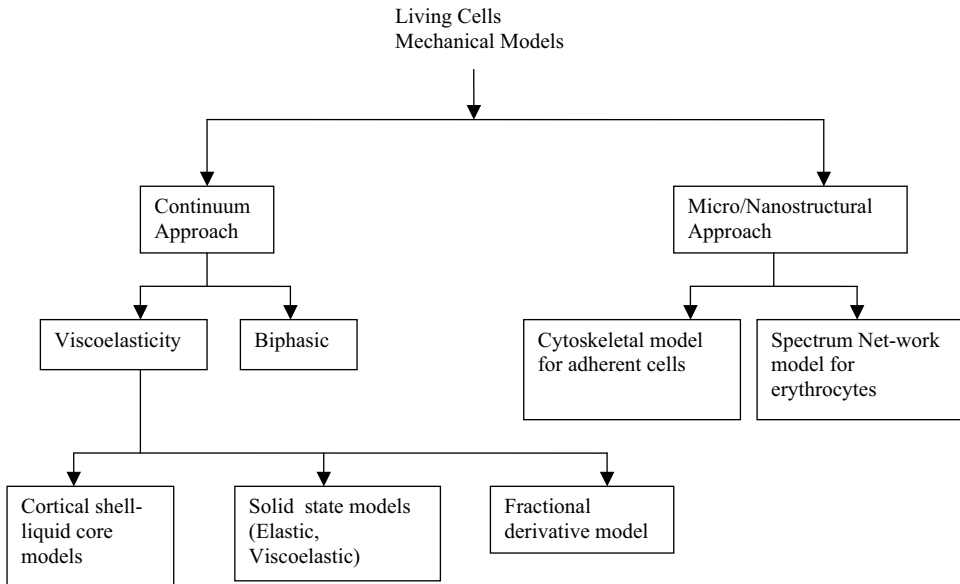


Figure 1.15 Living cells subjected to mechanical stimulations and their broad response. (Adopted from Lin *et al.*, 2006)

(Figure 1.15). The reversal phase is characterized by the repression of osteoclastic activity and deposition of cement into the resorption cavity. Finally, new bone formation begins with matrix formation and sequential mineralization. Once this cycle is completed the surface of the bone will return to the quiescence level (Parfitt, 1984). The site of resorption and formation, where the remodeling occurs, is referred to as the bone remodeling unit. Furthermore, this turnover cycle is usually completed in about four months. Trabecular bone remodeling occurs on the surface, while cortical bone remodeling occurs by osteoblastic apposition occurring during Haversian canal formation (Jee, 1988). Initiation of the remodeling cycle begins with activation of osteoprogenitor cells in a localized area in bone. The stimulus produces mitotic division in the precursor cells, producing osteoclasts that gradually resorb bone. After resorption subsides, the reversal phase begins. New osteoblasts appear and lay down similar amounts of bone as previously resorbed. On the completion of the cycle, cellular activity returns to its resting state, leaving the remaining osteoblasts as inactive lining cells or surface osteocytes.

The process from activation to quiescence involves the disappearance of bone lining cells and their replacement by osteoclasts that generate resorption lacunae on the endosteal surface of bone over a 2–4-week interval. The resorption phase is then terminated, probably by osteoclast apoptosis and, after a brief reversal phase, osteoblasts are recruited that fill in the resorption cavity with new bone (Riggs, Khosla and Melton, 2002). The net result, the replacement of a packet of old bone with new bone, may be due to electrostimulation, mechanical stimulus, hormonal changes (estrogen deprivation or in response to endogenous parathyroid hormone uses), cytokine stimulation, growth hormone surges, glucocorticoid excess or changes in serum calcium level, and may be a combination of some of these.

Bone formed by modeling during the growth phase tends to be structurally inferior to the bone that is formed during remodeling. Additionally, adult bone tends to change over time, becoming damaged by microfractures as a result of loading or reduction in quality as it ages. For these reasons remodeling is an important mechanism adopted by the body to replace and repair bone. Remodeling is a different process than modeling, whereby formation and resorption events are coupled and isolated to the same location. Remodeling occurs to replace the Haversian systems upon their demise and to respond to altered stress and strains placed on the bone (Jee, 1988; Lawrence and Fowler, 1997; Wasserman, 1984; Lanyon, 1994).

There are three types of strain: tensile, compressive and shear. The cells that make bone up undergo these strains with normal use. Therefore, loaded bones must adapt by continually altering their shape and architecture (Lanyon, 1994). This is also in the spirit of the Wolff's law. Remodeling may strengthen bone by replacing damaged areas caused by strain, without altering bone mass (Kimmel *et al.*, 1993). Remodeling can also occur during times of disuse and acts to decrease the total amount of bone present, thereby decreasing the strength of bone.

The bone remodeling cycle begins with the activation of resting osteoblasts on the surface of bone and stromal cells in the marrow. This is followed by a cascade of signals to osteoclasts designed to stimulate the recruitment and differentiation of these multinucleated cells from hemopoietic stem cells. After osteoclast-induced bone resorption, matrix components such as transforming growth factor-beta (TGF- β) and insulin-like growth factor-1 (IGF-I), as well as collagen and osteocalcin, are released into the micro-environment. The growth factors released by resorption contribute to the recruitment of new osteoblasts to the bone surface, which begin the process of collagen synthesis and biomineralization. In healthy adults, as many as two million remodeling sites may be active at any given time, and it is estimated that nearly one quarter of all cancellous bone is remodeled each year. In general, resorption takes only 10–13 days, while formation is a comparatively longer process, taking upwards of 3 months. In an ideal equilibrium situation the amount of bone resorbed by the end of the cycle equals the amount reformed. It is, however, also apparent that some individuals have impaired peak bone and acquisition. This scenario may be more common than previously appreciated and almost certainly represents inherited or acquired alterations in the rate of either bone formation or bone resorption during a critical period when several hormones acting synchronously leads to a marked increase in bone mass.

Some investigators (Mori and Burr, 1993; Parfitt, 1996a, 1996b) have suggested that there may be two kinds of bone remodeling: one that is stochastic (site independent though not totally random) and a second that is target specific. However, related aspects and their control mechanisms remain to be explored. Experimental studies in which damage is created in canine bone by cycling loading have demonstrated that remodeling sites associated with microcracks are more probable (four to six times) than expected by chance alone (site independent) (Burr *et al.*, 1985, 1993; Mori and Burr, 1993). It is estimated that remodeling events occur subsequent to microdamage and the local initiation of repair. One can thus infer that damage leads to remodeling. Bentolila *et al.* (1998) have noted an increase in the number of a typical appearing osteocytes following fatigue loading in rat bone.

Tanaka, Alam and Turner (2002) have shown that stochastic resonance enhances bone formation, by which nonlinear systems are able to amplify a response to a small periodic stimuli with the aid of noise (Gammaitoni, 1998). Stochastic resonance has been used to explain various phenomena in biological systems (Gammaitoni, 1998; Pierson and Moss, 1995), including the activity of cells under microgravity (Kondepudi, 1991). During exercise, as in

active sports, bone is subjected to quick variations in loading, which is equivalent to a noisy loading environment. Therefore, the possibility arises that bone cells are more responsive to fluid shear stress mediated by high impact activity compared to low impact activity. If so, the bone cell response to fluid shear stress might be nonlinear, that is, requiring an initial stress-kick. The initial stress-kick occurs during the quick transition from zero to a nonzero stress initiating a fluid shear stress stimulation of cells. NO production has been used as a parameter for bone cell activation since it is an early mediator of mechanical loading induced bone formation (Fox, Chambers and Chow, 1996) and it has been shown to be essential to adapt bone formation *in vivo* (Turner *et al.*, 1996).

One study (Bacabac *et al.*, 2005) suggests that the bone cell response to fluid shear stress is rate dependent, but that an initial stress-kick is required for the cells to respond. It has been reported that increasing either the shear stress amplitude or frequency, without increasing the average stress, enhanced NO production by bone cells *in vitro*. Bone cells respond similarly to similar rates of fluid shear stress, despite different frequencies (5 Hz, 21.99 Pa Hz or 9 Hz, 17.53 Pa Hz). Hence, the rate of fluid shear stress (i.e., $2\pi \times f \times \tau$) is proportional to the product of the fluid shear stress amplitude and frequency (Bacabac *et al.*, 2004).

Osteoblasts and osteocytes are mainly involved in the bone remodeling process (Frost, 1990). At the cellular level, bone remodeling can be traced to the combined action of the osteoblast and the osteoclast. Bone remodeling consists of a strict coupling of resorption and formation that continues throughout life and is necessary not only for skeletal growth but also in the maintenance of normal bone structure and function (Frost, 1964a; Harris and Heaney, 1969; Parfitt, 1982, 2002; Martin and Rodan, 2001; Eriksen, 1986). Throughout life, bone is continuously being remodeled with resorption of old bone (catabolic process) performed by osteoclasts and deposition of new bone (anabolic process) performed by osteoblasts. The activities of osteoblasts and osteoclasts are combined into defined anatomical spaces (BMUs) (Frost, 1983). It can be stated that bone remodeling takes place in focal remodeling units that consist of osteoblasts, osteoclasts and their precursors, in which resorption and formation are coupled. Bone resorption is likely the initial event that occurs in response to local mechanical stress signals. The sequence of a cycle of resorption \rightarrow formation on all surfaces appears to be valid for cortical bone remodeling as well as for trabecular bone. Hence, in densely compact bone the remodeling process must begin with bone resorption to produce surfaces for the apposition of new bone.

Several key components of the remodeling cycle are susceptible to systemic and local alterations. These, when perturbed, lead to a deleterious change in bone mass. The osteoblast–osteoclast interaction mechanism may have relevance for therapeutics as well as for understanding the dynamics of bone turnover. In particular, the activation of remodeling and the recruitment of osteoclasts represent the two most vulnerable sites in the cycle. The remodeling cycle clearly favors resorption over formation as the process of laying down new bone requires the combination of several processes. The osteoblast also functions to lay down collagen and regulate the mineralization of previously resorbed lacunae in the skeletal matrix.

The differentiation of mesenchymal stem cells become osteoblasts and rest on the surface of the remodeling space (Lian and Stein, 1999). Recruitment of the stem cells to osteoblasts, rather than adipocytes, is a critical step in bone formation and requires a series of factors that enhance differentiation. One of the most important components of this process is *cbfal*, a transcription factor that is essential in the early differentiative pathway of stem cells to bone and away from adipocytes (Ducy *et al.*, 1997). With the activation of resting osteoblasts,

osteoblastic cells begin to synthesize several types of collagen as well as elaborating a series of growth factors in the skeletal matrix, where they are stored in latent forms. These are then released during subsequent remodeling cycles.

In addition to endogenous (cellular) signals, external signals (e.g., PTH, GH, interleukin-1 and oestrogen deprivation) to resting osteoblasts and stromal cells cause these cells to release cytokines [interleukins such as IL-1, -6 and -11, as well as m-CSF, tumor necrosis factor (TNF) and TGF-6]. These enhance the recruitment and differentiate the function of multinucleated giant cells destined to become bone-resorbing cells (Lian and Stein, 1999; Lorenzo and Raisz, 1999; Udagawa *et al.*, 1999). It is now accepted that osteoproteoglycin (OPG) is a member of the TNF receptor super family, its role in bone remodeling being to act as a dummy receptor for the ligand now known as osteoprotegrin ligand (OPGL, TRANCE a RAKL). OPGL is a surface peptide that, when expressed on the osteoblast, can bind to the true OPG receptor on osteoclasts and initiate the cell-cell contact necessary for osteoclast activation and subsequent bone resorption (Suda, Takahashi and Martin, 1992).

The fundamental issue behind the choice of the stimulus signal is whether (re)modeling signals originate in the osteocytes themselves or whether they are an effect of increased flow through the osteocyte network. In the former case, they could be triggered by the osteocyte lacunar stress and strain concentrations, which can be much higher $15\times$ the tissue (continuum) level (Kufahl and Saha, 1990; Burger and Klein-Nulend, 1999; Nicolella and Lankford, 2002). In this case the time derivatives of SED, maximal principal strain (MPS) or volumetric strain (VS) could be relevant controlling parameters. The osteocytes may signal the osteoblasts and osteoclasts to change their bone remodeling activities (Burger and Klein-Nulend, 1999; Smith, Burger and Huyghe, 2002). Osteocyte signals are important in the mechanostat, the gravity sensing device that modulates bone formation.

The osteocytes within bone, together with the osteocytes lining external and internal surfaces and resting osteoblasts, form a continuous syncytium of cells lining all surfaces, including the larger trabecular spaces as well as lacunae and tiny canaliculi. The cellular continuum is organized into distinct functional subunits that do not always communicate directly with each other. The cells in each subunit may respond independently from neighboring units. Each of these pockets of cells may be termed as BMU, which can be considered to represent an organizational unit of metabolic activity that remains after a remodeling unit has completed its task. Rasmussen and Bordier (1974) have suggested that the term BMU be used during the remodeling phase.

BMU is most readily demonstrated in cortical bone and possibly extends to cancellous bone (Parfitt, 1994). The BMU exists and moves in three dimensions, excavating and refilling a tunnel through cortical bone or a trench across the surface of cancellous bone. A cortical BMU travels for about $400\ \mu\text{m}$ at about $20\ \mu\text{m}$ per day (Parfitt, 1994). A cancellous BMU travels about half this distance at about half the speed (Parfitt, 1996). It is suggested that one important local stimulus may be obtained from extracellular nucleotides. This can exist locally within the bone microenvironment to initiate intracellular signaling via P2Y receptors, which sensitizes cells to the action of PTH. This is one mechanism for integrating local and systemic responses in bone.

There is evidence that osteocyte apoptosis is an important factor in initiating new remodeling sites. The prevailing view is that an intact osteocyte-canalicular system inhibits the recruitment or activation of osteoclasts, and that disruption of the network by microcracks, for instance, releases the normal inhibition to resorption (Burger and Klein-Nulend, 1999). A positive

correlation has been shown between osteocyte apoptosis and bone resorption by osteoclasts in growing bone (Bronckers *et al.*, 1996) and between osteocyte apoptosis and increased activation frequency in estrogen-deficient osteoporosis (Noble *et al.*, 1997; Tomkinson *et al.*, 1997).

The quantum concept of remodeling in which discrete microscopic packets of bone are removed and replaced throughout the skeleton is now recognized as the predominant form of turnover in the adult skeleton. The primary event in remodeling is activation, during which lining cells retract from the bone surface to allow access of osteoclasts or their precursors to the underlying mineralized matrix. The focal nature of remodeling indicates that activation of this process is sensitive to local stimuli, including mechanical strain. In addition, systemic factors, in particular parathyroid hormone (PTH), are known to increase the rate of activation (Mundy, 1998). The visible bone damage is initiated at the molecular level and is then carried over to the submicroscopic level, proceeding and ending at the microscopic level (Parfitt, 2002). However, the signal pathways of the process and its detection threshold remain within the realm of investigation. Plasma calcium homeostasis requires rapid ion exchange (Parfitt, 1993), which is impeded by progression of secondary mineralization and loss of water from the surface mineral. It would be advantageous for hypermineralized surface bone to be preferentially replaced, but the existence of such targeted remodeling has not been established. A turnover rate greatly exceeding that required to maintain mechanical equilibrium could obviate the need for targeted remodeling. Such a need may, however, exist if turnover is at the low end of the normal range (Han and Mould, 1990). Thus, remodeling at foci is the combined action of systemic factors and local phenomena.

Both osteoblasts and osteoclasts are targets for nucleotide activation through multiple P2 receptor types (Arnett and King, 1997; Bowler *et al.*, 1995; Gallinaro, Reimer and Dixon, 1995; Kumagi, Sacktor and Filburn, 1991; Naemsch *et al.*, 1999; Reimer and Dixon, 1992; Weibe, Sims and Dixon, 1999). These receptors couple to multiple signal transduction cascades, including inositol triphosphate (IP₃)-mediated intracellular calcium release (P2Y receptors) or nonselective outward cation currents (P2X receptors). These signaling cascades, dependently and independently of mitogen-activated protein kinase activation, induce expression of genes such as *c-fos* (Bowler *et al.*, 1999), a transcription factor. The importance of this, in the early regulation of remodeling processes is reflected in the skeletal pathologies associated with its deletion (Wang *et al.*, 1992) or overexpression (Johnson, Spiegelman and Papaioannou, 1992). Nucleotides have been reported to modulate osteoblast induced bone formation (Jones *et al.*, 1997) and proliferation (Shimegi, 1996) and also modulate osteoclastogenesis and bone resorption (Arnett and King, 1997; Buckley *et al.*, 2000; Morrison *et al.*, 1998).

The ability of ATP (adenosine triphosphate) to effect neurotransmitter responses from certain nerve terminals has been proposed by Burnstock (1972). Since that time, a large family of receptors has been defined, both pharmacologically and molecularly, which transduce signals arising from nucleotide stimulation (Boarder *et al.*, 1994; Fredholm *et al.*, 1994). These P2 receptors have been subdivided into two classes: P2Y, which consists of five distinct receptors coupled to heteromeric G proteins, and P2X, which contains seven distinct receptors that function as cationic gated channels. Physiologically, P2X receptors are activated exclusively by adenine nucleotides, whereas P2Y receptors are responsive to adenine or uridine nucleotides or, in some cases, to both. Receptors are coupled to multiple specific cellular functions in processes as diverse as neurotransmission, wound healing, morphogenesis and apoptosis (Burnstock, 1993).

Some of the notable observations can be summarized thus:

- Cells of the osteoblast and osteoclast lineages express functional P2 receptors.
- Activation of P2 receptors results in the induction of *c-fos*, a transcription factor that plays a pivotal role in the regulation of bone remodeling.
- Extracellular nucleotides exhibit a potent synergy with PTH, enhancing intracellular calcium release ($[Ca^{2+}]_i$), downstream signaling and gene activation (Bowler *et al.*, 1999; Dixon and Sims, 2000; Jones *et al.*, 1997; Yu and Ferrier, 1993).
- Bone cells release ATP into the extracellular environment and this release is enhanced by mechanical strain and other combinations of regulatory parameters, leading to healing processes in bone.

For P2 receptors to modulate the activity of bone cells, ATP must exist at a sufficient concentration in the surrounding microenvironment to initiate receptor activation. All cells contain high intracellular ATP concentrations ($1\text{--}5\text{ mmol L}^{-1}$), as well as the capacity to release ATP following trauma. In addition, increased blood flow associated with the general inflammatory response could greatly increase the nucleotide concentration available upon platelet aggregation. Hence, at sites of tissue injury, wounding or fracture, high local ATP concentrations are present to activate P2 receptors. Nevertheless, it is obvious that nucleotides must exist transiently in the bone microenvironment without cell damage to be physiologically relevant regulators of bone remodeling. A real-time detection system has been used that relies on the high yield chemiluminescent reaction generated by luciferin and luciferase in the presence of ATP (Cobbold and Lee, 1991; Koop and Cobbold, 1993). This demonstrated that human osteoblasts constitutively release ATP into their extracellular environment in the nanomolar range. Released ATP probably reaches much higher concentrations near the membrane, because the presence of nucleotidases at the cell surface, membrane trapping and unstirred layer effects will all influence bulk phase measurement. This concept has been confirmed in a study in which membrane anchored luciferase revealed micromolar ATP concentrations at the surface of platelets (Beigi *et al.*, 1999).

In addition to receptors responsive to ATP, osteoblasts express P2Y (Albert *et al.*, 1997; Arnett and King, 1997) and P2Y receptors (Bowler, Bilbe and Gallagher, 1998; Maier *et al.*, 1997) are preferentially or selectively activated by uridine nucleotides. Some studies have demonstrated conclusively that cells release uridine triphosphate (UTP) into their extracellular environment, providing locally released agonist capable of activating these P2-receptor family members (Lazarowski *et al.*, 1997). Opinions differ as to why cells are so responsive to nucleotides. One hypothesis suggests that this may be a means of regulating ATP concentration at the plasma membrane, where it is required for the activation of ATP-dependent channels and enzymes (Schwiebert, 1999). Others propose that nucleotide release, through autocrine signaling, represents a universal key determinant in establishing the "set point" for activation of signal transduction pathways (Ostrom, Gregorian and Insel, 2000). It appears that nucleotide release is fundamental in defining basal cellular activity. Nucleotide-activated cellular activity is visualized as a wave of mobilized $[Ca^{2+}]_i$ spreading outward through the cell population from a single point of ATP/receptor contact (Osipchuk and Cahalan, 1992). This mechanism would seem to be important in cells and tissues that lack communication via gap junctions, such as chondrocytes and cartilage. In agreement with this model, chondrocytes abundantly express P2Y receptors (Leong, Russell and Caswell, 1994) and can release ATP (Figure 1.16) (Lloyd *et al.*, 1999).

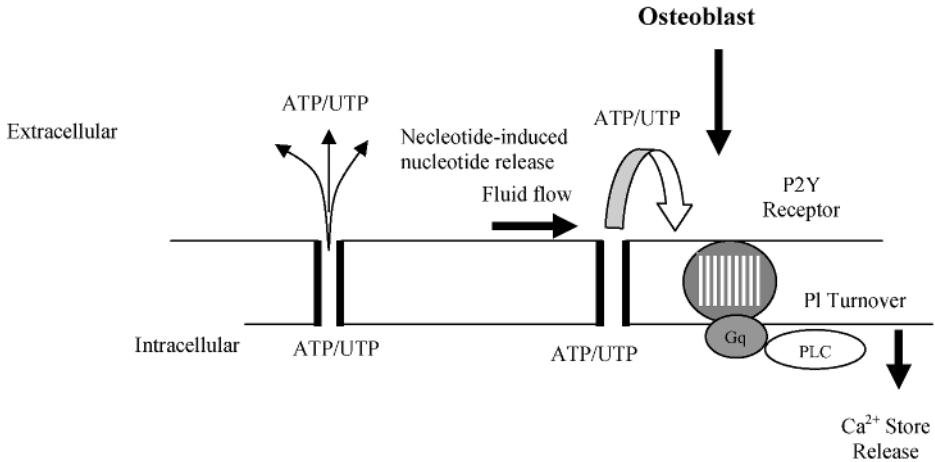


Figure 1.16 Mechanism of P2 receptors modulating bone cell activity. (Reproduced with permission from W.B. Bowler *et al.*, Extracellular nucleotide signaling: a mechanism for integrating local and systemic responses in the activation of bone remodeling, *Bone*, 28(5), 507–512 ©2001, Elsevier B.V.)

Further evidence for a distinct, nonlytic mechanism of nucleotide release lies in the observation that it can be regulated positively (and presumably negatively) (Figure 1.16) (Bodin, Bialy and Burnstock, 1991; Bowler *et al.*, 1998a, 1998b). In a positive feedback loop, UTP acts through P2Y (Albert *et al.*, 1997) receptors to upregulate ATP release from primary human osteoblasts (Bowler *et al.*, 1998a, 1998b). In addition, ATP and UTP release is modulated positively by fluid flow (Bowler, Bilbe and Gallagher, 1998; Lazarowski *et al.*, 1997). Indeed, recent evidence suggests that nucleotide release occurs as a consequence of fluid forces generated following cellular manipulation in almost all *in vitro* experimental systems (Lazarowski *et al.*, 1997; Ostrom, Gregorian and Insel, 2000). The importance of this finding becomes particularly apparent in the context of the bone microenvironment, where shear forces resulting from fluid flow within trabeculae and canicular spaces are thought to transmit mechanical forces (Hung *et al.*, 1995; Korenga *et al.*, 1994; Reich, Gay and Frangos, 1990; Turner *et al.*, 1994). These observations indicate that a cellular mechanism exists to release ATP into the extracellular environment at concentrations sufficient to activate cell surface P2 receptors and that this release can be modulated by mechanical and agonist stimulation.

Once released, ATP is rapidly broken down by ectonucleotidases that is bound on the extracellular side of osteoblast membranes (Caswell and Russell, 1988). The half-life for ATP released from human SaOS-2 osteosarcoma cells is estimated to be circa 50 s. This short half-life restricts the action of ATP to that of a localized signal. However, the ability of nucleotides to induce their own release provides a mechanism for sustaining and possibly propagating a more widespread response (Bodin and Burnstock, 1996; Bowler, Bilbe and Gallagher, 1998). ATP release and subsequent paracrine signaling in osteoblasts might provide a mechanism for cell-to-cell communication independently of gap junction (Jorgensen *et al.*, 1997). Elevated calcium levels can also activate various intracellular signaling systems in different cell types. One that is activated in osteoblasts upon P2Y receptor stimulation is the extracellular signal regulated (ERK) cascade.

The self-remodeling of long bone in child with midshaft fracture during angulation in response to mechanical stress is an example of the effects of environmental (epigenetic) factors on the genetically determined processes, development and tissue remodeling and may be explained with the help of Wolff's law (Figure 1.1). The causal relationship between bone modeling or remodeling and the mechanical stresses placed upon it is sufficiently well understood for clinical use to be made of it in the correction of congenital disorder of the skeleton (Frost, 1973; Pauwels, 1976). The maintenance of a continuous supply of reactive bone, housing the labile bone mineral, depends upon the continuity of remodeling of bone, which is believed to be determined by weight bearing and other forces acting externally on the skeleton as a whole, and is now seen to have important metabolic functions (McLean and Wrist, 1961). In orthodontics the ability to move teeth through bone in response to applied stress is well proven. However, the way in which the mechanical forces are mediated to the tissues at cellular level has not been well understood. The remodeling that occurs with the growth and in response to changing skeletal stress consists largely of resorption from one site to, and redeposition of bone on, another. The bone formation and resorption rates are closely linked and synchronized (Sissons, 1971; Lee, 1964; Manson and Walters, 1963; Harris, 1960) and have been found to be 10–15 μm weekly. Information on the rate of remodeling or turnover of the tissue has also been well documented (Lee, Marshall and Sisson, 1965; Marotti, 1963; Vander Hoeft, Kelly and Peterson, 1962; Frost, 1960). This may be of interest in understanding diseases of bone as well as its behavior under normal conditions.

1.10 Biochemical Markers of Bone and Collagen

An intermediate event in bone cell mechanotransduction is prostaglandin E_2 (PGE_2) synthesis and release. PGE_2 is released by osteoblastic cells in response to mechanical loading and has been implicated in regulating bone turnover *in vivo*. (Nolan *et al.*, 1983; Feyen *et al.*, 1984; Rodan *et al.*, 1986; Imamura *et al.*, 1990). Prostaglandins can regulate bone turnover by both stimulating bone formation and regulating bone resorption (Dietrich, Goodson and Raisz, 1975; Raisz and Fall, 1990; Jee *et al.*, 1991). Other studies have shown that bone cells respond to OFF with increase in PGE_2 release (Donahue *et al.*, 2003a; Saunders *et al.*, 2003).

Osteocalcin (OC) is a non-collagenous protein synthesized largely by the osteoblast; it contains three amino acids of γ -carboxyglutamic acid and is therefore known as bone Gla protein (Fraher, 1993). Osteocalcin is vitamin K dependent (Price, 1982) binds Ca and may be involved in the control of mineralization (Fraher, 1993; Lepage *et al.*, 1991). The Gla residues specific to OC are formed after translation of the protein occurs by a carboxylase enzyme system. In the presence of Ca, these Gla residues undergo conformational changes that allow OC to bind to hydroxyapatite and then accumulate in bone matrix (Lian and Gundberg, 1988). Furthermore, OC has a high affinity for hydroxyapatite (Price, 1982). The protein exists in a 1 : 1 ratio with collagen in bone and is proportional to hydroxyapatite (Lian and Gundberg, 1988). OC may regulate the size of the hydroxyapatite crystal. Osteocalcin is cleared through proteolysis and is cleared from circulation by the kidneys (Gomez *et al.*, 1995). Serum concentrations of OC are probably a product of diffusion into the circulatory system prior to binding to the hydroxyapatite (Price, 1982). Osteocalcin is mostly formed by osteoblast activity and thus is highly correlated with bone formation (Lian and Gundberg, 1988; Kannus *et al.*, 1996). Therefore, serum OC is invariably used as marker of bone synthesis.

It is reported that when animals are exposed to natural light OC undergoes a circadian rhythm (Lepage *et al.*, 1991). Osteocalcin concentrations in normal female standard bred horses were found to be consistent during the day from 0700 to 1900. The concentration then fell in the early evening hours (1900–2000) and increased at night until a peak at 0500 the next morning (Lepage *et al.*, 1991). These authors concluded that blood samples should be taken during daylight hours, since this period experienced the least fluctuation in OC concentrations. However, in another study, where horses were exposed to only fluorescent lighting, a circadian rhythm was not observed (Hope *et al.*, 1993).

Carboxy-terminal pyridinoline crosslinked telopeptides of type I collagen (ICTP) are a kDa portion of type I collagen that is released during resorption of bone (Hassager *et al.*, 1994) and can be quantitated in serum and urine (Risteli *et al.*, 1993). Once type I collagen is broken down into ICTP it can not be used again for synthesis of new collagen in bone and is cleared from the body in circulation through the kidneys (Tahtela and Tholix, 1996; Risteli *et al.*, 1993). Concentrations of ICTP decrease with the age of a horse, up until about four years of age, after which there are no significant differences (Price *et al.*, 1995). A diurnal cycle has also been reported with ICTP concentrations (Risteli *et al.*, 1993).

Finally, PYD and DPD are crosslinking amino acids residues of collagen released from bone matrix during turnover. An enzyme-linked immunoassay (ELISA) exists for PYD and DPD crosslinks in serum and urine. Since PYD is formed in cartilage and bone, PYD and DYP can be used as biochemical markers to analyze metabolic bone or cartilage diseases. Increased urinary PYD is seen in patients with osteoarthritis and rheumatoid arthritis (Robins *et al.*, 1986).

1.11 Summary

The concept of bone behavior consists of three parts. The first considers the solid state behavior of “*in vitro*” bone while the second is concerned with studying inherent bioelectricity under external stimuli with different possible combinations. This finally culminates (third part) onto its clinical behavior. Initially, however, there is need to understand basic bone properties such as piezoelectricity and its intermixing with stress generated potential. These two together define the physical aspects of total bone behavior.

The solid state behavior of bone appears to be one of the most baffling observations in solid state biology. It has been reported that bone has wide variety of properties ranging from semiconduction to piezoelectricity. Many semiconductor properties have been studied in detail in our laboratory (Behari and Andrabi, 1981; Andrabi *et al.*, 1980). In the present context the optical behavior is seen over a fairly wide band of frequencies. The choice of properties to be examined is dictated by the fact that it is important to characterize the solid state of the materials. It provides a clue as to the mechanism of charge transport, band gap and nature of the charge carrier besides unfolding possibilities for its use as an optical sensor. The surface morphological changes arising after UV exposure are studied by scanning electron microscopy to examine the changes at the microscopic level.

The above discussions show that bone growth and remodeling can be controlled by external stimulations. Though several successful attempts, mainly reported from Professor Bassett’s laboratory, are in clinical use, the search for optimizing the clinical usefulness of these is still on. It may be felt that this task will be considerably facilitated by knowledge of the basic mechanism at work. This task has been undertaken (Behari, 1991) in two ways. First, the

response of bone to electrical stimulations in wide range extending from DC to MHz frequencies regions, is scanned. So far only the DC and low frequencies stimulations have been examined.

The physiological state of bone under Ca and P deficiency is expected to provide data of considerable clinical importance. The model adopted is to stimulate the pathological conditions in a colony of rats extending over both sexes. The physical state of bone is characterized by measurement of various parameters, using, for example, X-ray, infrared spectra, transmission electron microscopy, fluorescence, spectrochemical analysis, and with various biochemical messengers. Accompanying biological changes are measured by an estimation of blood parameters, for example, Hb cell counts, P_H , P_{CO_2} , P_{O_2} , Ca and P. Various biophysical parameters used to characterize bone osteoporosis are elaborated in Chapter 4. In addition, various established techniques of measuring bone osteoporosis are discussed.