C H A P T E R I

INTRODUCTION

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The skeleton constitutes 16% of whole body weight (Forbes, Cooper and Mitchell, 1953; Forbes, Mitchell and Cooper, 1956) and accounts for 99% of body calcium (Mitchell, Hamilton, Steggerda and Bean, 1945) and 80% of body phosphorus (Maynard and Loosli, 1956). Apart from providing support for the body, the skeleton acts as a reservoir of minerals, particularly, calcium and phosphorus, and makes it possible for their concentrations in blood and soft tissues to be finely regulated (Owen, 1952). Phylogenetic evolution goes hand in hand with the evolution of the skeleton which is a major determinant of the shape, size and coordinated movements of higher animals.

Technically, the skeleton is tissue formed by the thickening and hardening of certain parts of the body so as to provide support to the whole body. In some invertebrates, the thickened epithelium becomes a calcified shell to form an exoskeleton. The higher animals have a more complex and versatile endoskeleton. Bone combines great rigidity with some degree of flexibility and has half the tensile strength of steel. This is made possible by the fact that bone is formed by the mineralization of a fibrous network of organic matter and is subject to constant renewal.

Bone is a highly specialized type of connective tissue, composed of three types of cells, osteoblasts, osteocytes and

osteoclasts. The osteoblasts are concerned with the formation of bone, osteocytes with the maintenance of bone as a living tissue, and the osteoclasts with the resorption of bone. All these cells are closely inter-related. Bone cells originate from mesenchymal cells which are capable of differentiating into fibroblasts, bone or cartilaginous cells. The development of any particular type depends upon the physiological state of the animal. Thus in the healing of fractures, cartilage is frequently found in the callus of new bone and in damaged muscle in fibrous tissue form (Draper and Chalmers, 1968).

The osteoblasts located on the surface of growing or developing bone synthesize collagen. During their active phase osteoblasts contain alkaline phosphatase which assists in a way, not fully understood, in the process of mineralization. During the active growth of bone, they appear to be in a continuous layer. When bone formation ceases osteoblasts become spindle shaped and resemble fibroblasts or reticular cells. (Draper and Chalmers, 1968).

As the bone matrix is elaborated by the osteoblasts, a number of these cells become trapped in the new bone tissue and they are referred to as osteocytes. These cells are enclosed within lacunae or gaps and are connected with both each other and to the bone surface by a large number of canaliculi which provide a route for metabolic exchange between the bone and the tissue fluids (Draper and Chalmers, 1968).

Osteoclasts are giant cells with a large number of nuclei, often as many as 15 to 20. They lie on the bone surface at the sites of bone resorption. Osteoclasts are the only cells known to be capable of bone resorption. They are also found at sites of bone remodelling (Draper and Chalmers, 1968).

There are certain characteristics that differentiate bone from other forms of connective tissue, the most important being that it is hard. It is perhaps the only tissue that heals after injury without a scar. In the formation of bone, the bundles of collagen fibres formed by the osteoblasts are cemented together to form plates of uncalcified bone matrix or osteoid consisting of ground substance composed largely of mucopolysaccharides. During calcification, minute crystals of calcium phosphate are deposited on this osteoid.

According to Nichols, Hirschmann and Rogers (1971) calcium phosphate packets are formed within the bone cell and they are then released at the bone surface to form a calcification front. In the same manner, the circulating levels of calcium and phosphorus are maintained by the same packets releasing their contents into a solubilizing environment near the capillaries. The studies of Martin and Matthews (1969, 1970) also support this view. They showed that the mitochondria of the bone cells contain granules of calcium phosphate that persist after ashing (incineration at 500° for 15 minutes). The concentration of these mitochondrial granules varies with the type of cell,

the same being less numerous in the zone of provisional calcification and more numerous in the hypertrophic zone. It would appear that the calcium accumulated by the mitochondria of bone cells as well as that in the newly mineralizing bone is amorphous and that it undergoes a transition to the crystalline type on the bone surface (Wasserman, 1972).

Long bones are formed from cartilage by a process known as endochondral ossification. In the cartilage collagen fibres are immersed in a firm amorphous gel, which contains large amounts of chondroitin sulphate. The cartilage cells or chondrocytes are situated in lacunae within this matrix. Except in the surfaces of the joints, all cartilage is surrounded by a membrane called perichondrium, having an outer layer of fibrous tissue and an inner layer of flattened chondroblasts. (Yust, 1957).

New cartilage is formed in two ways - interstitial growth, which is achieved by division of cells within the lacunae, and oppositional growth, by proliferation of new cells from the perichondrium. During the development of the vertebrates the skeleton is first laid down as hyaline cartilage and is gradually removed and replaced by bone. In the adult, hyaline cartilage is found in the articular and costal cartilages. All forms of cartilage are without an integral blood supply, all nutrients and waste materials

travel to and from the cells by diffusion from vessels in the perichondrium or fluid in the joint space.(Yust, 1957).

Ossification begins at the middle of the cartilage. A thin layer of uncalcified matrix or osteoid is laid down between the perichondrial membrane and the portion of the shaft containing hypertrophied cartilage. This osteoid extends around the shaft and forms a ring or collar which is quickly calcified to form periosteal bone. This bone is directly in contact with cartilage (Fig. 1). The inner cellular layer of the perichondrium starts differentiating into bone forming cells, namely osteoblasts. These cells lay down a thin layer of membrane bone. The perichondrium within which the periosteal collar of the bone has developed is called periosteum. Once the primary collar is well established it is penetrated at several points by small cellular masses called periosteal buds, derived from periosteum. The periosteal bud is essentially a large vascular cellular connective tissue. Its cells have a wide range of developmental potentiality and can give rise to osteoblasts, osteoclasts, bone marrow precursors and blood vessels. (Scothorne, 1968).

The cartilage cells are rapidly destroyed and replaced by proliferating and differentiating cells, which are derived from the periosteal bud. As soon as cartilage cells are replaced by differentiating cells, some of the undifferentiated cells of the

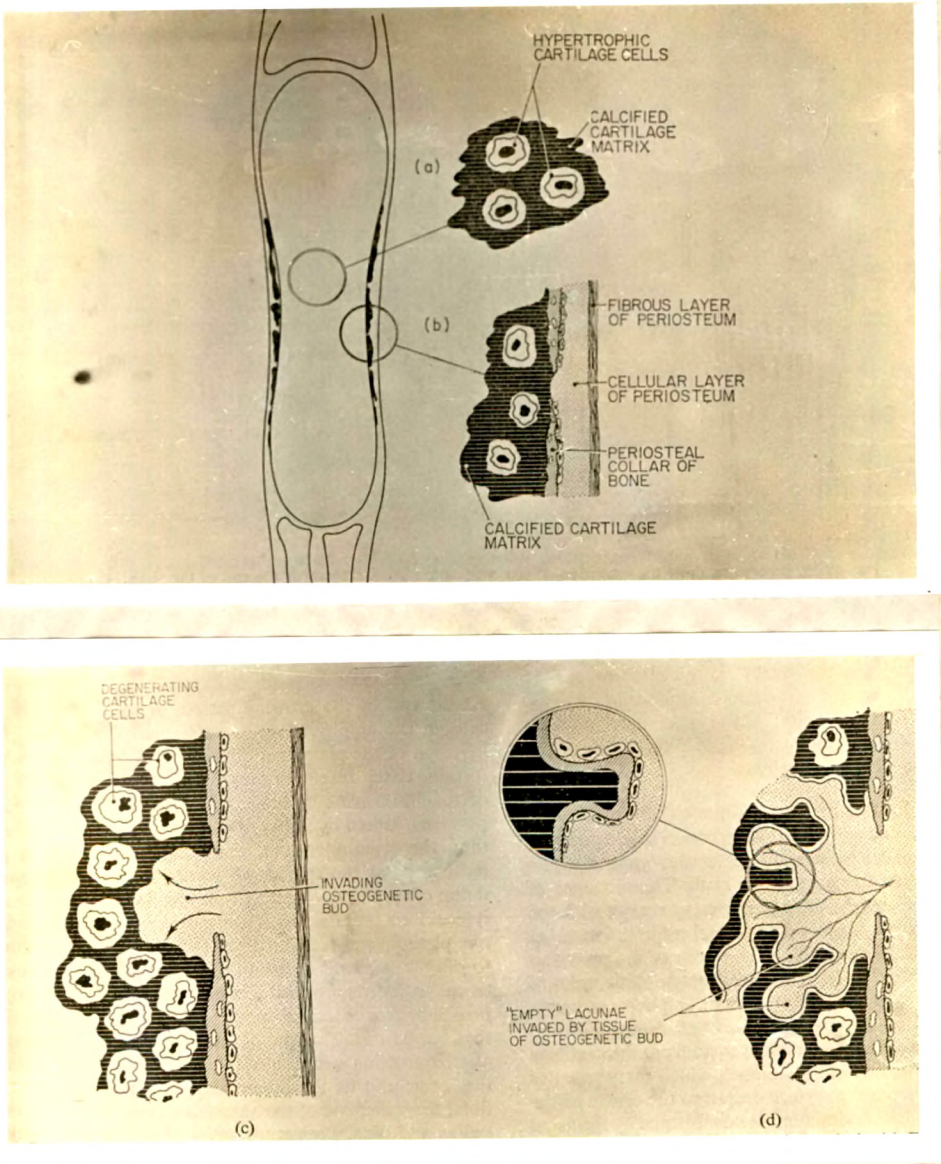

Fig. 1 : Four stages in the establishment of the primary ossification centre in the middle of the shaft. (a) Enlargement and degeneration of cartilage cells and calcification of the cartilage matrix, (b) the formation of the periosteal collar of bone, (c) and (d) the invasion of the empty lacunae by the periosteal (osteogenetic) bud, consisting of bone-forming and bone-destroying cells, and developing blood vessels and bone marrow elements.

Reproduced from Draper and Chalmers (1968).

periosteal bud develop blood vessels and circulation is quickly established to supply nutrients. Some cells of the periosteal bud differentiate into osteoblasts which are responsible for the bone formation (Scothorne, 1968).

The process of ossification extends towards each end of the bone resulting in increase in length. Increase in diameter is accomplished by continuous resorption at the periphery of the epiphyseal plate and its deposition to the external part of the shaft and by the deposition of bone mineral under the periosteum and the simultaneous resorption of the same from the inside (Scothorne, 1968).

The different parts of the bone are shown in Figure 2.

There are two main forms of bone - lamellar bone and woven (fibre) bone. The lamellar bone is made up of a series of layers in each of which the collagen fibres have a distinct orientation which differs from the adjoining layers. This arrangement also contributes to the strength of the bone. Again, lamellar bones have two different structural forms, namely, cancellous or spongy bone and cortical or compact bone. The cortex (periphery) of bone is commonly compact osseous tissue, while cancellous or spongy bone is found in the medulla. The compact bone is made up of a large number of rod-like units known as osteones or Haversian systems, having laminated walls. The walls of the canals have a composition

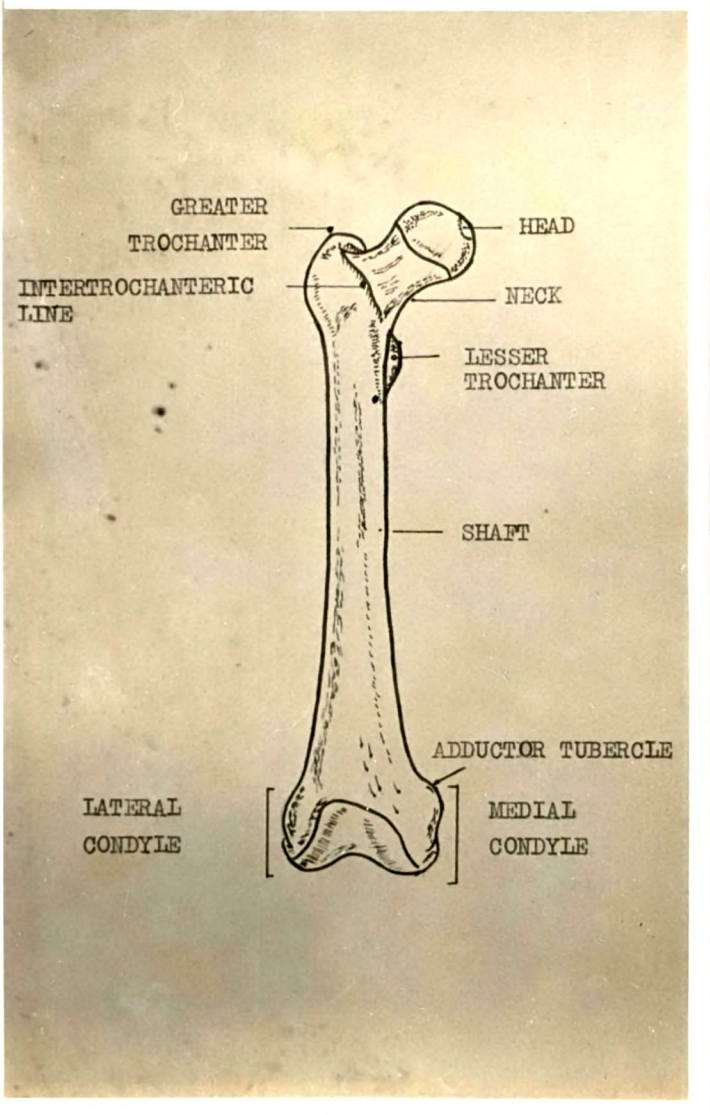

Fig. 2 : Anterior view of the right femur. Reproduced from Draper and Chalmers (1968).

and structure identical to the trabeculae. These osteones are inter-woven with each other. Cancellous bone is found at the expanded ends of the long bones and forms most of the irregular bones of the skeleton. It is arranged in the form of trabeculae with an orderly pattern. These trabeculae are made up of a varying number of closely adjoining bone plates or lamellae. Within the interstitial substance are small spaces or lacunae within which lie the osteocytes. The lacunae are connected with each other through a network of canaliculi (fine channels). The canaliculi of marginal lacunae extend to the surface of the trabeculae. Small trabeculae do not have blood vessels in them. They are bathed in the tissue fluid of the surrounding marrow from which the osteocytes in the lacunae derive their nutrients. The canalicular system can only carry fluid at an adequate rate over a relatively short distance. Hence the thickness which nonvascular trabeculae attain is limited. Trabeculae thicker than 0.2 mm have blood vessels in special canals and the lacunae canaliculi communicate with vascular spaces. Bone can develop a new pattern of trabeculae in response to mechanical stress. For example, if a joint is surgically fused, new trabecular patterns develop in the participating bones. The mechanism by which bone adapts to stress is still not clear. (Draper and Chalmers, 1968).

Woven bone occurs mainly during embryonic life or during the phase of rapid bone formation as for instance, at zones of endochondral ossification. This type also appears as early callus in the healing of fracture. Mechanically, woven bone is inefficient as structural material and is normally succeeded rapidly by the formation of lamellar bone. The transition of cartilage to true bone is incomplete in some rare diseases and the affected infants have fragile skeletons. Woven bone consists of a loose framework of delicate bone trabeculae which resemble loosely woven material and have large and numerous osteocytes (Draper and Chalmers, 1968).

A long bone consists chiefly of diaphysis (or shaft), the metaphysis (the extremity of shaft) and epiphysis, The epiphysis is separated at first from the diaphysis by a layer of cartilage cells but unites with the same at a certain stage of development. This epiphyseal fusion occurs in different bones at different ages. Thus, endochondral ossification occurring generally at the ends of long bones, involves different stages, namely multiplication of cartilage, maturation and degeneration of these cells in the epiphyseal plate, the vascularization of the degenerated cartilage by blood vessels, the formation of a net work of bone trabeculae by growing connective tissue cells, and finally, the remodelling of this bony tissue. Under normal circumstances all these processes proceed in equilibrium which results in

continuous extension of the metaphyseal bone trabeculae thus extending the length of the bone shaft. Increase in diameter is achieved by the continuous erosion of the inner layer and the addition of an outer layer (Sissons, 1971).

Some biochemical differences are found between the three types of bone cells. Some of them are summarized in Table 1. Both the osteoblasts and osteoclasts show greater metabolic activity than osteocytes. It is interesting to note that whereas osteoblasts show a high activity of alkaline phosphatase, the osteoclasts show a high activity of acid phosphatase. Phosphatase activity is essential for the release of phosphate needed for bone mineralization which is facilitated by an alkaline pH. Phosphatase activity is also needed for the resorption of bone for which an acid medium would perhaps be more favourable. Similarly, the difference between osteoblasts and osteoclasts with regard to glycogen and phosphorylase are also of interest.

The inorganic and organic constituents may vary in proportion from one part of the skeleton or a bone to another. The intercellular portion of bone is a calcified collagenous substance which makes up the great mass of bone. The interstitial substances include the organic framework or matrix and the inorganic part consisting of minerals and water. The adult mammalian bone contains 45% moisture, 25% ash, 20% protein and 10% fat. Bone ash contains approximately

Table 1 : Biochemical differences in bone cells*.

	Osteoblast	Osteocyte	Osteoclast
Glycogen	++++**	0 or +	0
RNA	++++	+	++++
Mucoprotein	+	±	+++
Alkaline phosphatase	++++	0	0
Acid phosphatase	0 or ±	0	++++
Phosphorylase	+++	0	0
β-glucuronidase	+++	0	++++
Succinic dehydrogenase	+	0	++++
Cytochrome oxidase	+	0	++++

* On the basis of histochemical observations (Simkiss, 1967).

** Concentrations assessed visually and scored on an arbitrary scale of 0 to ++++ (after Cabrini, 1961).

36% calcium, 17% phosphorus and 0.8% magnesium. Other minerals are present in much smaller amounts (Maynard and Loosli, 1956).

Collagen has a low content of aromatic amino acids and a high content of proline, hydroxyproline, glycine and glutamic acid (Eastoe, 1955). Bone collagen differs from that of other connective tissues in that it can accumulate minerals in an ordered fashion during bone formation (Draper and Chalmers, 1968).

Ground substances are the extracellular and inter fibrillar components of all connective tissues. The mucopolysaccharides present in the same are composed of hyaluronic acid, chondroitin sulphate and some proteins.

The water content of bone varies with following factors : (1) species (2) age (3) nutritional state and (4) the nature of the bone tissue under examination. A most important concept regarding bone water is that the organic matter of normal bone remains relatively constant to its volume and calcification of bone occurs by replacement of water by crystals of bone mineral. As the crystals get deposited by displacement of water, the space between crystals becomes smaller and smaller until a maximal mineralization is achieved.

The principal lipid depot of the bone is in the marrow and the quantity varies with the state of nutrition (Zobrisky, 1969).

The bone of the neonate contains more water and less mineral (Widdowson and McCance, 1960). The maturation of the bone is associated with a decrease in water, an increase in ash and a greater cohesion of bone mineral.

The nitrogen content of bone also increases with age during the period of growth but remains relatively constant during adult life (Widdowson and McCance, 1960). However, the increase in the mineral component is greater than that in nitrogen so that the ratio of Ca/N also increases with age (Table 2). This is reflected in ash or A:R ratio which is defined as the ratio of the mineral mass to the fat-free organic mass.

Table 2 : Changes in chemical composition with age in the whole femur in the rat*.

age (days)	calcium	total N	Ca/N ratio
	g per 100 g. fat-free bone		
0	2.24	2.56	0.88
7	2.25	2.16	1.04
14	4.38	2.56	1.71
33	7.54	2.96	2.54
56	13.0	3.36	3.87
108	18.3	3.46	5.30
158	19.3	3.38	5.71

* Values taken from Widdowson and McCance (1960).

Not much information is available on variation in mucopolysaccharide content with age. In bovine nasal cartilage total mucopolysaccharide is not found to show much variation with age but the ratio of keratin sulphate to chondroitin sulphate is found to increase (Tin-Wai Goh and Lowther, 1966).

As the formation of bone involves that of the organic matrix, ground substance and bone crystals, the same presupposes the availability of the nutrients needed for the synthesis of each. The formation of collagen which is a protein containing hydroxylysine and hydroxyproline, may be expected to be influenced by the protein and vitamin C status of the animal as the latter is involved in hydroxylation reactions. Vitamin A is needed for the synthesis of mucopolysaccharides. The bone minerals are composed of calcium and phosphorus the availability and utilization of which depend on an adequate supply of vitamin D. A deficiency of any of these nutrients as well as the general nutritional state of the animal can therefore be expected to affect the growth, renewal and composition of the skeleton.

When the overall supply of nutrients is affected because of undernutrition skeletal growth is also affected as might be expected. Studies on swine have shown a slower rate of bone growth and decreased width of the shaft (Pratt and McCance, 1965). However, the retardation in skeletal growth

is much less than that of whole body growth and bone length is less affected than its weight or composition (Widdowson and McCance, 1960).

Dietary protein is one of the most important factors involved in normal bone development. Frandsen, Nelson, Sulon, Becks and Evans(1954) investigated the effects of protein deficiency by maintaining young rats for 6-7 weeks on diets containing 24, 6, 3 and 0 per cent casein as the sole protein source. In the protein deficient animals the growth of the long bones was retarded. Histologically the bones showed a decreased width of the epiphyseal plate, diminution in the number and size of cartilage cells and an increase in the amount of cartilaginous ground substance.

A decrease in the ash content of bone, an increase in fat, and an altered ratio of ash to matrix were found by Platt and Stewart (1962) in young pigs subjected to protein deficiency. Addition of calcium to the diet of these pigs did not improve either the growth or the radiographic appearance of the bones (Stewart and Platt, 1961).

In adult animals maintained on a protein deficient diet, epiphyseal changes are not evident but there is a progressive loss of both organic and inorganic constituents. However, the ash to matrix ratio may not be appreciably affected (Fontaine, Mandel and Girise, 1950) although an altered

ratio of mineral to matrix has been found by Gontea, Dumitrache, and Goontia (1960). El Maraghi and Stewart (1964) fed adult rats high and low protein diets at two different levels of calcium intake and found that radiographically the bones of animals fed the high protein diet were denser than those of the protein deficient animals. Ash content was decreased in those fed the low protein diet. On the other hand only minor changes were brought about by differences in calcium intake.) Range of Ca

In both young and adult animals the effects of a low protein diet could not be prevented by increasing the calcium content of the diet. In fact a high calcium diet affected adversely the bone of the low protein animal (El Maraghi, Platt and Stewart, 1965). In unpublished studies in this department animals fed a 5% protein diet are found to require less calcium in the diet (100 mg per 100 g) than those fed a 20% protein diet (400 mg per 100 g) for maximum bone mineralization. Incidentally, low protein animals are found to form urinary calculi unless the mineral content of the diet is reduced (Theophilus, 1961).

The role of vitamin A in the synthesis of mucopolysaccharides which are major constituents of ground substance is well known (Fell and Mellanby, 1953; Fell, Mellanby and Pelc, 1954). In the growing animal vitamin A controls the shape of the bone and specially its fine moulding, by

influencing the position and the activity of osteoblasts and osteoclasts. When there is a deficiency of this vitamin, the bone does not stop growing but a controlling influence on its growth is lost. The size of the bone continues to increase but some are malformed and contain excessive amounts of cancellous tissue whose spaces are often full of fatty marrow. This overgrowth of cancellous bone may be accompanied by reduction in compact bone (Mellanby, 1943; 1947). Fragile long bones are observed with excessive amounts of vitamin A. The fragility may be due to the proteolytic activity of acid hydrolase, released from lysosomes on the bone matrix constituents (Fell, 1964).

Vitamin D is concerned with several phases of mineral metabolism. It is essential for the transport of calcium from the intestine, the activity of the parathyroid hormone and regulation of the urinary excretion of phosphorus. It is also believed to activate bone calcification although the physiological mechanism by which this is accomplished is unknown (Hartles, 1970). Vitamin D is found to facilitate the activity of intestinal phytase which is of great importance in increasing the availability of both calcium and phosphorus from diets based on cereals rich in phytate (Steenbock, Krieger, Wiest and Pileggi, 1953). Bone contains calcium and phosphorus approximately in the ratio 2:1, that in the whole body being 1.8:1. When the diet contains these two minerals in unbalanced proportions their

utilization is affected (Korenschevsky, 1922). The effects of this imbalance are found to be prevented or appreciably reduced by vitamin D (Zucker, Hall and Young, 1941; Copp, Chance and Duffy, 1947; Underwood, Fisch and Hodge, 1951).

The bones of animals suffering from experimental rickets contain low ash with higher amounts of uncalcified matrix and consequently a low ash : matrix (A:R) ratio (Mellanby, 1921; 1925; Pappenheimer, 1922; Shohl and Wolbach, 1936).

It is not surprising that water soluble vitamins which are essential for general metabolism have also been found to influence skeletal development. Bone abnormalities in new born rats were observed when mothers were fed on diets deficient in either vitamin B_2 or B_{12}, the incidence being higher when both were omitted (Grainger, O'Dell and Hogan, 1954). A characteristic feature was a high Ca:P ratio suggesting an impaired utilization. This suggestion is supported by the observation that bone alkaline phosphatase activity as well as the incorporation of labelled phosphorus are reduced in these animals.

The role of vitamin C in the formation of connective tissue is fairly well established. This could well be because of the role of vitamin C in the hydroxylation of amino acids as collagen contains hydroxylysine and

hydroxyproline. The abnormalities found in vitamin C deficiency includes the formation of an abnormal bone matrix (Wolbach and Howe, 1926) which is not calcifiable and decreased bone phosphatase activity (Bourne, 1956). "Scurvy lines" on the tibia and femur are common features associated with vitamin C deficiency (Zobrisky, 1969).

As the bone is largely made of minerals, particularly, calcium and phosphorus, it is needless to point out the importance of an adequate supply of both for normal bone development. Animals with an adequate calcium supply have long and dense metaphyses indicating maximum calcium storage and relatively diminished osteoblastic and osteoclastic activity which shows minimum calcium turnover and maximum storage of bone minerals (Zobrisky, 1969).

In growing rats an inadequate calcium intake results in relatively short metaphyses indicative of a short supply of calcium. The osteoblasts and osteoclasts are very active suggesting the rapid turnover of calcium for metabolic purposes. The bones of these animals are porous and look similar to those of very young animals (Sherman, 1947).

When a diet containing calcium and phosphorus in an abnormal proportion (Ca:P::4 to 5:1) and no vitamin D is given to growing rats they develop rickets. Rats developed rickets even in the presence of small amounts of vitamin D if a diet

with very low concentrations of phosphorus (about 0.017% or less) is given (Follis, Day and McCollum, 1940) but vitamin D if present in larger quantities prevented the condition (Zucker et al., 1941).

The bones of animals given diets deficient only in phosphorus contained lower amounts of ash than those of control animals both in absolute terms and as percentages of fat-free dry bone. This condition can probably be linked to mineral osteoporosis due to calcium deficiency (Stewart, 1965).

The mineral elements present in animal tissue include chiefly Ca, Cl, Mg, K, P, S, Na and Fe. At least six other elements namely, Co, Cu, I, Mn, Se and Zn are required in trace quantities. Sodium apparently is incorporated in a stable crystal fraction of bone, from which it is not removed until released by bone resorption. The ability of bone to release sodium ion is therefore quite limited, the sodium in bone being relatively insoluble and inert (Zobrisky, 1969).

Severe zinc deficiency in laying hens resulted in skeletal malformation of the embryo such as smallness of limbs and absence of vertebral development (Zobrisky, 1969). In birds zinc deficiency results in a shortening and thickening of the long bones of the legs, low osteoblastic activity and narrow epiphyseal cartilage with a reduced rate of cell division and maturation (O'Dell, Newberne and Savage,1958).

Although manganese and copper are present in bone only in trace amounts, deficiencies of both have been implicated in abnormalities of bone development in chicks (Wolbach and Hegsted, 1953). In manganese deficiency in rats the bones of the deficient animals are shorter, less dense, have a lower tensile strength and contain less phosphorus (Amdur, Morris and Heuser, 1945). These changes are associated with a suppression of the proliferation of epiphyseal cartilage cells, a decline in bone matrix formation, failure of growth and general decrease in bone strength (Zobrisky, 1969).

A deficiency of copper is associated with a reduced activity of both osteoblasts and osteoclasts, but growth and calcification are unaffected (Baxter, Van Wyk and Follis, 1953).

An altered ratio of Ca:P is observed in the bones of magnesium deficient rats. Calcium content is increased and magnesium content decreased without any major change in phosphorus concentration (Orent, Kruse and McCollum, 1932; Watchorn and McCance, 1937). Histological changes in the bones are minimal, none being found in the epiphyseal cartilages (Watchorn and McCance, 1937; Blaxter, 1956).

The body of adult man is estimated to contain about 1200 g of calcium and 670 g of phosphorus according to Leitch and Aitken (1959) but others have reported slightly different

values, 1194 g of calcium and 613 g of phosphorus for man (Forbes et al., 1953; 1956; Forbes and Lewis, 1956) and 856 g of calcium and 445 g of phosphorus for woman (Widdowson, McCance and Spray, 1951). These variations are not surprising in view of the normal range of biological variations. The body of the full term infant contains 25-28 g calcium. Most of the calcium and phosphorus in the body is in the skeleton, that in soft tissues and extracellular fluids being 5-8 g in the case of calcium and 65-80 g in the case of phosphorus. About half of the latter is to be found in striated muscle (Neuman and Neuman, 1960; Leitch, 1964).

In the young animal almost the entire skeleton is in a sensitive state of exchange equilibrium with the blood. In the adult animal this is true of some part or other of the skeleton, about a third of the same being in a similarly sensitive state at any one moment. This sensitive state enables a rapid mobilization of calcium from the bone when the blood level of the same falls, and deposition, when the same rises (Leitch, 1964).

The serum contains about 10 mg of calcium per 100 ml (normal limits 9-11 mg). In plasma, calcium is distributed in three phases; it may be found either in combination with protein or in a free and ionized form or in combination with other inorganic elements. The concentration of calcium in the blood plasma is maintained within rather narrow limits

by a number of regulatory factors. As ionic calcium is involved in the regulation of cell permeability and irritability any alteration in the same is associated with marked effects (Bell, Davidson and Scarborough, 1970).

The effect of calcium on cardiac muscle including striated muscle, nerve, intestine and kidney function may be due to its action on permeability. The action of calcium is interrelated with that of sodium, potassium and magnesium. The distribution of these minerals in the intra and extra cellular fluid varies (Table 3).

Table 3 : Concentrations of cations in intracellular fluid and extracellular fluid* (in milliequiv.per litre).

cations	extracellular fluid	intracellular fluid
Na^{+}	145	10
K^{+}	5	150
Ca^{++}	2	2
Mg^{++}	2	15
Total	154	177

* Values taken from Bell et al. (1970).

Ionic concentration of calcium in serum is of importance in muscle excitation and coupling. Smooth muscle excitability is highly sensitive to calcium ion, the general effect of

which is to increase or maintain contraction (Leitch, 1964). This effect might be through a "relaxation factor", present in muscle, which is inhibited by calcium ion (Mommaerts, Brady and Abbott, 1961). It has been suggested that a regulatory protein complex is probably present in the actin filaments, and that in the absence of calcium, this regulatory complex prevents action from activating the myosin ATPase. This inhibition is removed when calcium is supplied (Huxley, 1972).

The effects on heart muscle appear to depend on both the absolute and relative concentrations of calcium and potassium in the perfusing fluid. With a very high concentration of only the former, the perfused heart finally stops in systole. With a moderately high concentration the contraction is prolonged, the relaxation retarded, and the amplitude of contraction altered (Leitch, 1964).

The excitability of peripheral nerve is also closely related to the concentration of calcium in the solution bathing the nerve cells. Deprivation of calcium ions lowers the threshold to stimulation with raised excitability of the peripheral nerve (Brink, 1954; Frank and Fuortes, 1961). On the other hand a raised concentration of calcium ion reduces and ultimately destroys the excitability of motor nerves (Leitch, 1964).

Calcium also plays a role in the regulation of kidney function. A state of hypercalcemia results in the failure of kidney to concentrate urine. Polyuria and low osmotic pressure of urine are associated with failure of sodium and water reabsorption. Such a state may arise with excessive doses of vitamin D and hyperactivity of parathyroid gland (Gill and Bartter, 1961; Wirz, 1961).

Calcium also has a role in the clotting of blood. Fibrin, necessary for the clotting of blood, is derived from fibrinogen and the thrombin needed for this conversion is formed from the prothrombin complex which includes calcium (Leitch, 1964).

In contrast, a reduction in the level of protein bound calcium, as is sometimes found in protein deficiency in association with lowered levels of serum albumin, does not produce any serious metabolic disturbance (Bell et al., 1970).

In contrast to calcium, ionized phosphorus has little or no pharmacodynamic action of its own. It is a part of enzymes and many other important substances in the body which accounts for its relatively greater presence in the soft tissues.

The absorption and utilization of calcium are affected by several factors such as the age of the animal (Draper, 1963) the composition of the diet (Leitch, 1964) and the

nutritional state of the animal (Wasserman,1963) as well as physiological states such as pregnancy (Hummel, Sternberger, Hunscher and Macy, 1936) and pathological states such as steatorrhea and hyperparathyrodism (Cantarow and Trumper, 1968).

Regulation of calcium balance unlike that of sodium and potassium balance is achieved mainly by that of absorption from the intestine and the calcium in urine represents mostly endogenous calcium. Consequently these minerals show a different pattern of excretion (Table 4).

Table 4 : Sodium, potassium and calcium balance of adult male (mg/day).

	intake	output		% of total output	
		urine	faeces	urine	faeces
Sodium*	3105	2990	115	96	4
Potassium*	2340	2145	390	85	15
Calcium**	1008	135	858	14	86

Values taken from :
* Passmore, Meiklejohn, Dewar and Thow (1955).
** Walker, Fox and Irving (1948).

In man, the amount of calcium in the body increases from about 25-28 g at birth to 1100-1200 g in the adult. A similar increase is found in the animals. This increase involves daily accretions of calcium in the growing skeleton

and it is not surprising that the absorption of calcium is more efficient in the younger animal which also shows relatively smaller endogenous losses (Table 5). Similar observations were made earlier by Henry and Kon (1953). However, a somewhat greater absorption in aged rats as compared to mature rats has been reported by Hironaka, Draper and Kastelie (1960) who also found greater endogenous losses in former. In this connection, not much variation in calcium absorption with age was found in men ranging in age from 20-69 years (Malm, 1958).

Table 5 : Absorption of dietary calcium by rats of various ages*.

Age (weeks)	% of calcium intake		Endogenous fecal calcium		Maintenance requirement (mg)	
	absorbed**	Re-excreted	mg/day	mg/kg body wt.	per rat	per 100 g body wt.
4	98	0.7	1.2	30	1.2	3.0
12	57	2	7	39	12	6.6
24	46	6	12	39	26	8.4
48-72	41	7	18	40	44	9.8
106	24	6	22	49	92	20.0

* Data taken from Hansard and Crowder (1957).

** Corrected for endogenous fecal calcium.

Although calcium is absorbed from all over the intestinal tract, the rate of transfer varies in different segments and appears to be maximum in the duodenum, perhaps because of a relatively abundant supply of mucosal tissue in the same. Using everted gut sacs, active transport is most easily demonstrated in this part of the small intestine in several species, the golden hamster being an exception (Schachter, 1963).

As calcium is absorbed only in ionisable form, the availability of calcium depends on the type of food consumed. Milk calcium is easily liberated and absorbed (Ling, Kon and Porter, 1961). Also milk is the richest source of calcium in the diet. Further, the presence in the same of high quality protein, lactose etc. which are believed to facilitate calcium absorption are points in its favour.

Calcium in plant foods is believed to be less readily liberated because of the decreased digestibility of plant proteins due to the presence of cellulose (Leitch, 1964). Calcium in animal tissues is easily available but animal foods are not particularly rich sources of calcium (Table 6).

As the utilization of calcium in foods is believed to be limited by the presence of phytate and oxalate, the amounts of the same, particularly oxalate, have also to be taken into account while considering the calcium value of foods. Ragi

Table 6 : Different foods as sources of calcium.

foodstuff	calcium* (mg/100 g)	approximate amount (g) providing 100 mg of calcium*
rice and maize	10	1000
wheat, jowar and bajra	25-40	250-400
dals (dehusked legumes)	67	150
whole legumes including soybean	200	50
groundnut	50	200
leafy vegetables	250	40
root vegetables	20	500
other vegetables	40	250
milk (buffalo)	200-250	40-50
milk (cow)	118	85
egg	50	200
fish	10-13	750-1000
meat	10	1000
Selected sources		
ragi	33	30
sesame seed	1000	10
agathi leaves	1000	10
elephant yam	50	200
sitaphal (custard apple)	400	25
wood apple	133	75

* On the basis of values given by Watt and Merrill (1963) for fish and meat and those given by Gopalan, Ramasastri and Balasubramanian (1971) in other cases.

(Eleusine coracana) contains a high amount of phytate and sesame a high amount of oxalate. Leafy vegetables have variable amounts of oxalate, leafy vegetables such as fenugreek containing practically none (Table 7).

Table 7 : Oxalic acid, phytin and calcium content of ragi, sesame seeds and leafy vegetables*.

	per 100 g edible portion		
	calcium (mg)	oxalic acid (mg)	phytin (mg)
Ragi (Eleusine coracana)	344	0	209
Sesame seeds (Sesamum indicum)	1450	1700	-
Amaranth (Amarantus tricolor)	397	772	2
Curry leaves (Murraya koenigii)	830	132	35
Spinach (Spinacea oleracea)	73	658	0
Fenugreek (Trigonella foenum graecum)	360	13	0

* Based on data given by Gopalan et al. (1971).

Absorption of calcium also depends on previous dietary history, being more efficient in subjects adapted to low levels of intake and less efficient in those adapted to high

intakes. Nicolaysen, Eeg-Larsen and Malm, (1953) observed that rats fed low calcium diets showed a greater efficiency of absorption. A retention of as much as 89% has been found in children (Nicholls and Nimalasuria, 1939).

Vitamin D is concerned with several aspects of mineral metabolism including intestinal absorption of calcium, tubular reabsorption of phosphates from the kidney, deposition of bone and tooth mineral, resorption of bone, accumulation of citrate in bone and functioning of parathyroid hormone (Hartles, 1970).

It is now established that vitamin D is converted to 25 hydroxycholecalciferol or 25 HCC in the liver (DeLuca, 1969) which is further hydroxylated to 1, 25 dihydroxycholecalciferol (1,25 DHCC) in the kidney (Fraser and Kodicek, 1970). It has been suggested that in the kidney 25 HCC induces the formation of an enzyme, which is involved in its further hydroxylation (Tanaka and DeLuca, 1971). 25 HCC is the major form of vitamin D present in circulating plasma and is much more effective than cholecalciferol in curing rickets. 1, 25 DHCC seems to be metabolically the most active form as judged by its initiation of intestinal transport and bone-mineral mobilization (Haussler, Boyce, Littledike and Rasmussen, 1971). The formation of calcium binding protein as well as a calcium dependent ATPase seems to be induced or facilitated by vitamin D (Melancon and DeLuca, 1970; Wasserman, 1970).

As vitamin D is synthesized in the body, a dietary supply of vitamin D is not essential. However, when such synthesis is deficient, as is often the case in very young children not exposed to adequate sunshine, administration of vitamin D is found to facilitate the absorption and utilization of calcium (Jeans and Stearns, 1937; 1938). In experimental animals, the effects of highly unbalanced proportions of calcium and phosphorus are corrected by administration of vitamin (Zucker et al., 1941; Copp et al., 1947; Underwood et al., 1951).

A high efficiency of calcium absorption during pregnancy is suggested by studies on both man and experimental animals (Beaton, 1961). In studies using semipurified diets apparent absorption in rats was found to increase from 45% in the pre-pregnant state to 90% during pregnancy (Rajalakshmi and Khanam, unpublished). A retention of as much as 46 g was found in an 18 year old primipara during the last two months of pregnancy although the subject had a poor nutritional status (Hummel, Hunscher, Bates, Bronner and Macy, 1937). A better retention of calcium during pregnancy is also suggested by studies at Hyderabad (1968) which describes calcium balance studies in women during pregnancy. According to these studies, apparent absorption of calcium increases during the second and third trimesters with an average of 53% and 58% (Table 8). Two subjects who showed a negative

balance during the first trimester showed 43% and 62% absorption during the last trimester although the intakes remained at the level of about 450 mg. The net retention would have been of the order of 250 mg during the latter half of pregnancy.

Table 8 : Utilization of calcium during pregnancy*.

subject	trimester of pregnancy		
	I	II	III
	per cent retained		
1	negative	42.8	42.5
2	62.2	68.1	72.5
3	54.6	67.5	not done
4	56.1	57.2	not done
5	negative	37.1	62.0
6	negative	not done	58.8
7	41.7	53.3	not done
8	34.0	38.2	not done
9	negative	not done	not done
10	63.3	57.4	55.9

* Values taken from Annual Report of nutrition research laboratories, Hyderabad (1968).

Amounts would increase the value of the table

The dietary factors implicated in the inhibition of calcium absorption include phytate (Leitch and Aitken, 1959), oxalate (Leitch, 1964), amino acids such as glutamic acid (Raven, Lengemann and Wasserman, 1960) and excessive amount of fat (French, 1942; Calverley and Kennedy, 1949).

It is now fairly well-accepted that the role of phytate in calcium absorption has been grossly overestimated (Walker et al., 1948; Leitch, 1964; Rajalakshmi and Ramakrishnan, 1969a; Rajalakshmi, 1972). In animal studies in this laboratory phytate is not found to have any effect (Varkey, 1967). Similar observations have been made in human subjects by Walker et al. (1948) and Cruickshank, Duckworth, Kosterlitz and Warnock (1945) although McCance and Widdowson (1942) and Krieger, Bunkfeldt, Thompson and Steenbock (1941) found an adverse effect. The conflicting findings may be partly because of the use of sodium phytate in some studies rather than natural phytates (McCance and Widdowson, 1942; Cruickshank et al., 1945; Walker et al., 1948; Robert and Yudkin, 1961) and also differences in the customary diets of the people. The fact that people deriving a major portion of their calcium from cereals do not show the expected incidence of calcium deficiency suggests that calcium is well utilized even in the presence of phytate.

Regarding glutamic acid Wasserman, Comar and Nold (1956) found the same to be inhibitory when administered along with Ca^{45} to fasted rats. However, it does not seem that glutamic

acid would have similar role in natural diets. Cereal proteins are rich in glutamic acid (Naik and Das, 1972) and yet cereal calcium is not all that poorly absorbed. Further, milk is an excellent source of calcium although casein is rich in glutamic acid.

The findings with regard to sugar and fat have also not been consistent. Impaired utilization of calcium and/or abnormalities in bone formation have been found with diets containing excessive amounts of fat (Bunkfeldt and Steenbock, 1943) or sucrose (Outhouse, Smith, Merritt and White, 1937). However, in studies in this laboratory animals fed high fat and high sugar diets were not found to show changes in bone composition (unpublished data). The type of carbohydrate used has also been implicated (Mitchell, Hamilton and Beadles, 1937).

The factors believed to facilitate calcium absorption include lysine, lactose and citrate. The favourable effects attributed for lysine rest on the observations of Wasserman et al. (1956) who found enhanced absorption of Ca^{45} when administered along with lysine to fasted rats. The effects are presumed to be on absorption as they were not found with intraperitoneal administration of Ca^{45}. Similar effects were found for other amino acids such as L-arginine and L-tryptophan. The effects of lysine supplementation to a

basal diet however seem to depend on the adequacy of the diet with regard to lysine. Beneficial effects were observed when a diet lacking in lysine was supplemented with the same (Likins, Bavetta and Posner, 1957) but not when a casein diet was supplemented (Raven et al., 1960; Prasanna Kumari, 1966). Wasserman et al. (1956) attribute these negative effects to the high amounts in casein of glutamic acid but this ignores the fact that calcium is well utilized from milk and casein diets. Generally, beneficial effects of lysine supplementation to diets poor in this amino acid and the lack of such effect when the diet is adequate suggest that the former may be mainly due to an improvement in protein quality. The effects of the administration of lysine and other amino acids along with Ca^{45} to fasted rats are found under artificial conditions and may have no relevance for natural diets.

Beneficial effects for lactose have been found both when administered to fasted rats along with Ca^{45} (Wasserman et al., 1956) and when added to a basal diet. Similar effects have been found for a number of carbohydrates including mannose, cellobiose, raffinose, D and L-xylose, D and L-arabinose and polyalcohols such as manitol, sorbitol and inositol (Kline, Keenan, Elvehjem and Hart, 1932). Wasserman and Taylor (1969) attribute these beneficial effects to the

capacity of these sugars to act as 'carriers' by chelating with calcium, or possibly a non-specific effect on the metabolic machinery of the mucosal cell. They also suggest that the ineffectiveness of sugars such as glucose and sucrose may be due to their very rapid absorption so that they are not available at the time of calcium absorption.

From the point of view of practical nutrition, however, lactose does not seem to be of much significance in calcium absorption. In studies in this laboratory, bone composition was not different in rats fed a mixture of wheat and legumes or the same with skim milk powder. Both the diets contained adequate amounts of calcium (Ramachandran, 1968).

The chief disorders of calcium metabolism related to diet in man are skeletal retardation and rickets in young children and osteomalacia in adolescents and adults. Osteoporosis must also perhaps be added to this list as it is sometimes but not generally associated with dietary deficiencies.

Although frank rickets is relatively rare in this country, skeletal retardation in young children is fairly widespread (Table 9). The children look clinically normal and no skeletal abnormalities are evident. But radiological examination shows a delayed appearance of ossification centres, a deficit in bone age as compared to chronological age and

decreased thickness of the cortex in the long bones. The diets are deficient in a number of nutrients including energy, protein, calcium and vitamin A (Table 10). The skeletal retardation observed could therefore be due to a deficiency of calcium and other nutrients. In studies conducted in this laboratory the administration of a food supplement made of wheat and bengal gram and not providing much of calcium was found to be associated with some improvement in skeletal status although greater improvement was found with further additions of leafy vegetables and lime (Rajalakshmi, Sail, Shah and Ambady, 1973). This suggests that protein calorie malnutrition may be an important factor in the etiology of skeletal retardation in children.

Table 9 : Incidence and extent of skeletal retardation in different age groups*.

Age (yrs)	No. of subjects		No. with retardation**		Mean retardation*** (months)	
	M	F	M	F	M	F
1	7	5	0 (0)	1 (20)	-	0.5
1 - 5	30	38	27 (90)	35 (92)	12	15
5 - 10	45	38	41 (91)	34 (90)	29	19
10 - 13	14	21	13 (93)	16 (76)	27	10
13 - 16	10	14	8 (80)	9 (64)	22	13

* Values taken from Rajalakshmi (1973).

** Percentage incidence given in parentheses.

*** In the case of those showing retardation.

M, male; F, female.

Table 10 : Nutrients provided by the diet in rural Baroda.*

	age (years)		
	3-6	10-13	13-17
calories	900	1300	1600
protein (g)	22	36	45
calcium (mg) - in milk	200	160	160
- other sources	100	128	182
phosphorus (mg) - total	650	1085	1379
- phytate	330	625	700
vitamin A (mcg)	210	257	227

* Values taken from Gandhi (1972).

This suggestion is strengthened by the observation that in some studies, supplementation with only calcium and without an overall improvement of the diet was without effect (Gandhi, 1972). These studies underline the need for more systematic studies on the effects of specific deficiencies of nutrients such as food energy, protein, vitamin A, calcium etc. on skeletal development.

The present studies were undertaken in this context on the effects of undernutrition and protein deficiency on the growth and composition of the bone in rats at different ages. Additional studies were undertaken on the skeletal status of animals fed diets based on different combinations of cereals, legumes and leafy vegetables. Skeletal development was judged by the length, weight and composition of the femur with regard to moisture, fat, ash and calcium contents. These studies are described in this thesis.