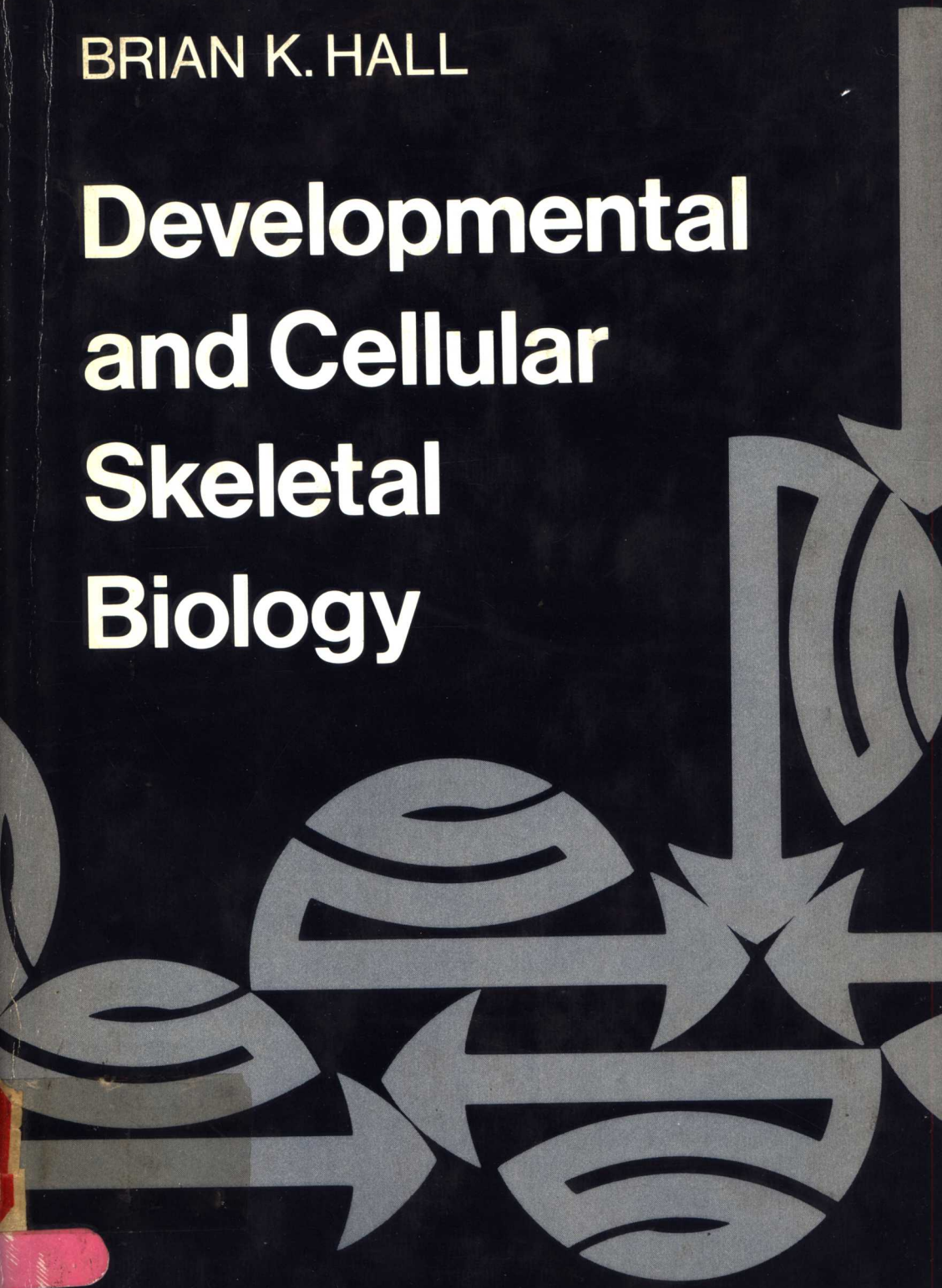
BRIAN K. HALL

Developmental and Cellular Skeletal Biology



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Preface

The skeleton has fascinated man ever since it was realized that, aside from one or several sets of genes, bare bones are his only bequest to posterity. But the skeleton is more than an articulated set of bare bones: Its three-dimensional conformation establishes the basis of our physical appearance; its formation and rate of differentiation determine our shape and size at birth; its postnatal growth orders us among our contemporaries and sets our final stature, while its decline in later life is among the primary causes for loss of the swiftness and agility of youth. Not surprisingly, the skeleton is a central focus of many biomedical disciplines and investigations.

For the developmental or cell biologist, the skeleton provides an excellent model for studies in cell differentiation, morphogenesis, polarized growth, epithelial–mesenchymal interactions, programmed cell death, and the role of the extracellular matrix. The skeleton supplies the geneticist with a permanent record of the vicissitudes of its growth, whereby the phenotypic expression of genetic abnormalities can be studied. The orthopedic surgeon earns a livelihood from the correction of these abnormalities, while the orthodontist corrects the position of teeth displaced consequent to alveolar bone dysfunction. Physiologists, biochemists, and nutritionists all are concerned with the skeleton's store of calcium and phosphorus and its response to vitamins and hormones; the hematologist, on the other hand, finds that the skeleton houses the progenitors of the blood cells. Pathologists endeavor to understand the disease states that result from abnormalities in skeletal cellular differentiation or function; surgeons want to prevent formation of skeletal tissues in the wounds that bear witness to their work. Veterinarians, physical anthropologists, radiographers, forensic scientists—indeed, the list could go on.

All of these individuals work in specialized fields, each with its own literature, jargon, and mode of operation. And while they share a common interest in the skeleton as an *organ,* practitioners in a given area are rarely exposed to the viewpoints and advances in the other fields. Working from the premise that knowledge of the cell biology, development, and growth of the skeleton is basic to *all* these specialties, this book is intended as a review of what is known of how the skeleton arises, differentiates, and grows. It is my hope that this overview of the status of these aspects of skeletal biology will appeal to all those specialist groups whose members claim the skeleton as "their organ," to research workers entering the fascinating and diverse field of skeletal biology, and to graduate and senior undergraduate students interested in cell biology, development, and/or growth. To this end, the present work utilizes examples and literature from several of these fields. Of course, venturing outside one's proscribed specialty in this way is hazardous, and errors of omission and of commission are almost inevitable. Although I have profited greatly from discussions and correspondence with colleagues, especially Drs. W. A. Beresford, A. I. Caplan, W. A. Elmer, A. W. Ham, M. A. Hardy Fallding, A. Y. Friedenstein, R. J. Goss, E. J. Kollar, R. A. Kosher, C. S. LeLièvre, P. F. A. Maderson, A. H. Melcher, W. J. Moore, M. L. Moss, P. Person, R. L. Searls, G. Strudel, P. V. Thorogood and M. S. Tyler, I take full responsibility for any errors that remain.

My own interest in the skeleton was kindled by the late P. D. F. Murray, whose 1936 monograph *Bones, A Study of the Development and Structure of the Vertebrate Skeleton* still remains one of the most lucid, and paradoxically, one of the most modern treatments of the developing skeleton. My own research and that of my honors and graduate students have been supported by the National Research Council of Canada (grant no. A5056) and by the Research Development Fund of Dalhousie University. I am indebted to Sharon Brunt, who has provided expert technical assistance for this research, created an unfailingly cheerful atmosphere in the laboratory, and spent many hours proofreading and correcting the present manuscript. Finally, I owe an immeasurable debt to my wife June, who spent countless hours with Derek and Imogen so that I could lose myself in the intricacies of the developing skeleton. If this monograph were to have a dedication it would be "In spite of Derek and Imogen."

Brian K. Hall

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1

Types of Skeletal Tissues

I. INTRODUCTION

Of the four classes of mineralized tissues, namely, bone, cartilage, enamel, and dentine, the first and second are also skeletal tissues. For completeness, a fifth "catchall" category could also be added. This category would include the tissues which, on the basis of one criterion or another, are intermediate between two of the above tissues. Examples of these would be tissues intermediate between bone and cartilage or between enamel and dentine. The usefulness of these tissues in understanding the development of skeletal tissues will become apparent as our discussion progresses.

These four broad classes have been further subdivided. The various subdivisions proposed reflect the interests of the skeletal biologist and the scope of his or her field. For example, the embryologist wants to subdivide on the basis of developmental process; the anatomist on the basis of structure; the pathologist on the basis of deviation from the norm, and so on. A brief review of the four classes of mineralized tissues follows, with special attention paid to the two skeletal tissues.

II. BONE

Bone is a vascularized, supporting skeletal tissue (although it may arise ectopically outside the skeleton), which is deposited by osteoblasts and by osteocytes, and removed, and hence remodeled, by osteoclasts and by osteocytes. Glycosaminoglycans and collagen of type $[\alpha 1(I)]_2\alpha 2$ (type I collagen) comprise its extra-

cellular matrix, which is permeated by canals and impregnated with hydroxyapatite. Bone functions to support the body; it acts as a site for attachment of ligaments and muscles, as a storehouse for calcium and phosphorus as well as for the hemopoietic tissues of the adult, and as a major site for the metabolic regulation of mineral homeostasis. Bone is found only in vertebrates.

A. Cellular Bone

Bone is classified on the basis of developmental processes as either endochondral (developing by the replacement of a cartilaginous model) or intramembranous (developing by the replacement of a fibrous or fibrocellular model). Although these terms primarily apply to the process of ossification, often they are used to specify the bones that result from these processes. Cellular vs. acellular bone; cancellous (woven) vs. lamellar bone; coarse-fiber vs. fine-fiber bone—these are the classifications of the histologist. [The reader is referred to H. M. Smith (1947), Ørvig (1967), Gardner (1971), Hancox (1972a), Pritchard (1972a), Ham (1974), and Patterson (1977) for in-depth discussions of bone type and bone development.] To illustrate the locations within the body where particular types of bone might be expected, the following list is provided. (a) Bone with coarse bundles of parallel fibers: at the sites of attachment of tendons and ligaments in both birds and mammals and in the ossified tendons of birds; (b) bone with coarse bundles of woven fibers: in the fetal mammal and in the early stages of fracture repair; (c) bone with fine bundles of parallel fibers: in the long bones of birds and young mammals, and around blood vessels in ossified tendons; (d) bone with fine, lamellated fibers: in the adult mammal; and (e) bone with both coarse and fine fibrous bundles: near attachment sites of tendons and ligaments and where coarse bundles are removed and replaced.

The processes that produce these various types of bone differ. Coarse, woven, bone is deposited rapidly, and as a result, the osteocytes and fibers are haphazardly arranged. Replacement by lamellar bone is considerably more orderly and predictably progressive, with the formation of primary and then secondary osteons. The primary osteon has a central canal of diameter less than 100 μ; it lacks a cement line, while having two or more central blood vessels. Interstitial lamellae are absent. The secondary osteon has a larger central canal, is limited externally by a cement line, has one central blood vessel, and is wedged between interstitial lamellae. The lamellae may be concentrically arranged, as in a haversian system; they may be circumferential, as in near-surface bone, or interstitial, as in remnants of old osteons. The life span of osteons, and the time required to produce them, vary from species to species. In a 2-year-old cat, it takes 50 days to make an osteon, while in a 45-year-old man, the process takes 100 days. And, since the average life span of that osteon will be 15 years, only 0.05% of the skeleton is turned over per day. Nor is the rate at which an osteon

mineralizes uniform. While 70% of the mineralization occurs within one to two days of deposition of the uncalcified osteoid, the remainder can take many months. These rate differences become important when assessing pathological states, particularly in metabolic bone diseases, as has been emphasized by Bordier *et al.* (1969), Baylink *et al.* (1972), and by Fornasier (1977).

The standard histology textbook gives the impression that all mammalian bone is fine lamellar bone containing numerous secondary osteons. In fact, this picture is true only for human bone. Enlow and Brown (1958), Enlow (1966a), and Singh *et al.* (1974) have provided surveys of mammalian bone, with the latter authors attempting a quantification based on the number and size of primary longitudinal canals, and on the number of lacunae and empty lacunae. Necrotic, acellular areas, avascular areas, and areas lacking primary or secondary osteons, are all common and normal; these, moreover, can vary from bone to bone within an individual, with age for a given bone, and between individuals. One side of a bone may be highly vascularized and the other side may be avascular. A highly sophisticated knowledge of the microenvironment in which bone and bones develop is necessary before we can interpret this diversity of expression of the differentiated state of the osteocyte. One of the aims of this book is to marshal and analyze some of that evidence.

In the past, it has been argued that the specialized histology of bone represents either an adaptation to the mechanical stresses placed upon it, or an adaptation to the metabolic requirements for calcium and/or phosphorus. Recently, de Ricqlès (1973, 1974a,b) has related bone histology to the pattern of growth and general metabolism exhibited by the particular species or bone. These notions have achieved some prominence in the discussions on the possible warm-bloodedness of the dinosaurs (Bennett and Dalzell, 1973; Desmond, 1976). De Ricqlès contrasts the bones of the slow-growing salamander, which might achieve a body weight of 20 g after four years, with those of many mammals that achieve weights of several hundred kg after two years. He has correlated types of periosteal bone (which comprises most of the bulk of the long bones) with vascularity, species growth rate, rate of mineralization, fiber organization (a function of their rate of deposition), and periodicity of bone deposition. His studies merit the close attention of all those interested in the functional significance of bone histology.

The most recently deposited, unmineralized, metabolically active bone (*osteoid,* a term and concept developed by Virchow, 1853) is found adjacent to either periosteal or endosteal bone surfaces. These surfaces are lined by formative cells (osteoblasts), by resorptive cells (osteoclasts), and by precursor cells (osteoprogenitor cells). These bone surfaces are of prime importance in metabolic function, in reaction to vitamins and hormones, and in initiation of pathological change (Neuman, 1969; Ramp, 1975). Owen (1970, 1971) has studied the dynamics of the cells on these surfaces. Recent ultrastructural evi-

dence indicates that junctions exist between adjacent osteocytes, between osteocytes and osteoblasts, and between osteoblasts (Holtrop and Weinger, 1971; Furseth, 1973; Weinger and Holtrop, 1974; Stanka, 1975). As a result, rapid transport throughout the bone can be achieved—one need only witness the appearance of radioisotope within cortical bone just minutes after intraperitoneal injection. Endosteal surfaces are especially important in bones that exhibit secondary remodeling. In 40 cm^3 of the human pelvis there is 80 cm^2 of periosteal surface, but 1600 cm^2 of endosteal surface. How these active cell layers are generated and maintained will be discussed in Chapters 6 and 7.

B. Acellular Bone

When bone is found to be acellular, it is usually regarded as degenerated, pathological, or the artifactual result of poor histological processing. [In fact, Stinson (1975a,b) has gone so far as to resurrect the medieval notion that *all* bone is dead and that osteocytes are mythical artifacts!] In compression-induced arthritis in rabbits, acellular areas occur in both the articular cartilage and the underlying subchondral bone (Gritzka *et al.*, 1973). Caisson disease of bone represents the development of avascular necrosis as a result of diving or tunneling under increased pressure, the degree of necrosis being closely correlated with diving time, depth of dive, etc. (Jaffe, 1972; Ohta and Matsunaga, 1974; Walder, 1976). These examples of acellularity obviously result from abnormal circumstances. However, the comparative histological studies mentioned in Section A indicate that large areas of acellular or avascular bone can be part of the normal histology of mammalian bone; hence, the sampling problem and the difficulty in assigning causation encountered by the paleontologist, forensic scientist, and anthropologist [see Enlow (1966a,b) and Wells (1973) for discussions].

There are, however, two major groups of vertebrates in which bone acellularity is the rule and not the exception. These are the modern teleost fishes and the Agnatha, the jawless vertebrates of the Ordovician period. The existence of this acellular bone is not well known. Because of this, and because it provides a link between evolutionary and developmental studies on the one hand, and contrasts normality with pathology on the other, acellular bone will be discussed at some length.

It was Kölliker (1859) who first described acellular bone in the teleosts. He called it *osteoid* (a term now reserved for recently deposited, unmineralized, cellular bone) and postulated that the ancestors of the teleosts possessed cellular bone, i.e., that acellularity was a secondary condition. I shall return to this evolutionary question in Chapter 2.

Moss (1961a) and Enlow and Brown (1956, 1957, 1958) provide the bulk of the histological studies available on acellular bone in the teleosts. Because acellu-

lar bone is found in various orders of higher teleosts that inhabit both fresh and salt water, it has been postulated that the development of acellular bone is not correlated with retention of calcium or phosphorus, since neither of these elements is in limited supply in aquatic environments (Moss, 1961a, 1963, 1965). However, the availability of the calcium and phosphorus stored in acellular bone varies from species to species (Simmons, 1971). When Simmons and Marshall (1970), examining ^{45}Ca uptake in the acellular bone of the toadfish (*Opsanus tau*), found little, they therefore concluded that little osteogenic activity was taking place. However, when administered intraperitoneally to the pike (*Esox lucius*), tetracycline is rapidly incorporated (within 3 hr) into the acellular bone, a finding that indicates both osteogenesis and metabolic activity (Meunier and Boivin, 1974). In the killifish (*Fundulus kansae*), 80% of the calcium is stored in the acellular bone in diffusible form and can be mobilized in response to seasonal needs or under conditions of induced stress, such as hypophysectomy (Brehe and Fleming, 1976). Calcium from skeletons of species having cellular bone, e.g., the American eel (*Anguilla rostrata*), is similarly mobile (Fenwick, 1974). The cells respond to exogenous calcitonin or pituitary extract by diminished bone resorption, as do the osteoblasts and osteocytes of mammalian bone (Lopez, 1970a,b; Lopez and Martelly-Bagot, 1971; Lopez and Deville, 1973). The availability of the calcium stored within the acellular bone might be explained by the presence of vascular canals, whose perivascular connective tissue cells might mobilize the calcium (Moss, 1963). However, the basis of calcium utilization remains to be elucidated, and might well provide useful information for the treatment or prevention of pathological necrosis of cellular bone.

Although it is especially interesting, the development of acellular bone during ontogeny has received little study. It was noted that the teleosts, which now have acellular bone, arose from ancestors that possessed cellular bone. Similarly, during teleost ontogeny, acellular bone arises secondarily from cellular tissues. Again, Moss (1964a,b) provides the little information that is available. He has shown that acellular bone may arise by osteogenesis within the osteoprogenitor cells of the periosteum, by osteogenesis within cells of tendons (the so-called tendinous osteogenesis), or by metaplastic transformation of cartilage into bone. In each case—and these various types of osteogenesis may be found within one individual at different skeletal sites—the extracellular matrix calcifies, either trapping the osteocytes that become pyknotic, or leaving the osteocytes on the surface. The entrapped pyknotic osteocytes calcify [much as in mineralization of invertebrate cartilages or in lignification of plants (Person and Philpott, 1963)] and an acellular tissue results (Moss, 1961b, 1963). A far more detailed analysis of the development of such bones still is necessary.

Another approach to the study of the development and physiology of acellular bone has been the exploration of its ability to repair fractures. A comparison of

the responses to a fracture stimulus by cellular and acellular bone was investigated by Moss (1962a). The fractured acellular opercular bones and lower jaws of the cichlid (*Tilapia macrocephala*) produced a callus of calcified cartilage and bone. Fibroblasts from periskeletal connective tissue modulated to form osteo- and chondroprogenitor cells, with these cells forming the cartilage and bone of the callus. Under acalcemic conditions, the ability to form this callus was diminished, whereas under similar conditions species with cellular bone, such as the goldfish (*Carassius auratus*), were capable of initiating fracture repair. The loss of bone cells during ontogeny is not, then, an impediment to the repair of fractures of those same bones, for an ability to modulate adjacent cells into the skeletogenic series has been developed. (The concept of races of cells with skeletogenic potential will be explored in Chapter 3.) Once again, the study of acellular bone might provide valuable insights into this basic developmental question.

Moss (1962b) also utilized implantation of acellular and cellular bones to ectopic sites as a means of assessing the varying potentials of the two bone types. Bones were implanted either subcutaneously or into defects in the femora or crania of adult rats. In the subcutaneous sites, both cellular and acellular bone produced an immune response, and were resorbed and removed by host cells. In the intraskeletal sites, both bone types were initially incorporated into the host bones, and were then slowly removed by resorption. The extracellular matrix of the acellular bone possesses a species specificity, which can elicit a rejection response.

One finds acellular bone in the oddest places. In sharks, the basal tissue supporting the teeth is acellular bone (Moss, 1970; Kemp, 1977), even though elasmobranchii are traditionally classified as having a purely cartilaginous skeleton. The fact that they are capable of producing bone is a good example of the ability of the progenitor cells of the skeleton to retain, over very long periods of evolutionary time, the ability to modulate to either cartilage or bone. Moss (1977) has discussed possible inhibition of osteoblast activity by calcified cartilage in other parts of the shark skeleton.

Acellular bone [so-called aspidin(e)] is one of the skeletal tissues found in the earliest fossil vertebrates (see Chapter 2). The oft-debated views on the relationship between cellular and acellular bone during evolution take on an element of rationality when viewed in light of the knowledge of the ontogenetic development of acellular bone from cellular tissues. It is difficult to imagine how the acellular bone of the Agnatha could have arisen other than by development from a cellular tissue. The important question linking the development and evolution of this tissue is why the osteoblasts and osteocytes do not persist in the tissue as they develop. Moss (1968a–c), Hall (1975a), Maderson (1975), Schaeffer (1977), and Patterson (1977) have addressed this interplay between the knowledge of developmental processes and evolutionary mechanisms.

III. CARTILAGE

Cartilage is an avascular, supporting, and articular skeletal tissue (although like bone, it may arise ectopically outside the skeleton), deposited by both chondroblasts and by chondrocytes, and removed by chondroclasts. Its extracellular matrix, primarily composed of glycosaminoglycans, contains a smaller collagen component of type $[\alpha 1(II)]_3$ (type II collagen). Cartilage may or may not exist as a mineralized tissue. Cartilage functions as the primary embryonic skeletal tissue in many parts of the embryo and as the articular tissue at joints on both endochondral and membrane bones (in the latter case, the cartilage is known as secondary cartilage). Cartilage is found in both vertebrates and invertebrates.

A. Vertebrate Cartilage

Although cartilage is subdivided into types primarily according to histological criteria, the cartilage that provides the model for endochondral bones can be classed as primary cartilage, and that which arises on membrane bones as secondary cartilage. Most cartilage develops from mesoderm, but some types, notably Meckel's cartilage, parts of the chondrocranium, and the visceral cartilages, are of neural crest (ectomesenchymal) origin.

On histological grounds, cartilage is considered to be *hyaline* if the extracellular matrix is composed of predominantly glycosaminoglycan; it is termed *elastic* if elastic fibers occur in the extracellular matrix, or *fibrous* (fibrocartilage) if there is an increased collagenous fiber content in the matrix. Hyaline cartilage is found in the embryonic models of endochondral bones and in the larynx; elastic cartilage in the pinna, larynx, and epiglottis; and fibrous cartilage where ligaments and tendons attach to bone, in the intraarticular discs of the joints, and as articular cartilage at joint surfaces. While cartilage is normally avascular, nonchondrified channels, which may carry blood vessels some distance into the body of the cartilage, may be present within the cartilaginous matrix (Novak, 1964; Moss-Salentijn, 1975). And, in contrast to the solely appositional growth of bone, the growth of cartilage is both interstitial and appositional. It is thought that this feature explains its success as an embryonic skeletal tissue. The resistance of cartilage to compression enhances its usefulness in growing organisms and makes it an ideal tissue for joint surfaces. For basic references on cartilage, the reader is referred to Fell (1925) and Gardner (1971) for the cytology of chondrogenesis in avian and human embryos, respectively; to Ham (1974) for the histology of cartilage; to Godman and Porter (1960) for the classic ultrastructural analysis of cartilage, and to Serafini-Fracassini and Smith (1974) for the biochemistry and physical structure of cartilage.

Historically, the distinction between cartilage and bone has been known at least since the time of Aristotle (384–322 B.C.), who recognized and separated

the Chondrichthyes from the Osteichthyes on the basis of the presence of a cartilaginous or an osseous skeleton. In fact, up until the 18th century, it was thought that cartilage transformed into bone. In 1736, Robert Nesbitt set himself to "shew the ancient and common notion of all bones being originally cartilaginous to be a vulgar error," and that the "bony particles in foetuses begin to be deposited or to shoot either between membranes or within cartilages." In 1848, W. S. Sharpey refined this, proposing his Theory of Substitution, viz., that bone replaces cartilage in endochondral bones.

Today, the difficulty of distinguishing cartilage from bone arises in at least three contexts. The task of the paleohistologist is the identification of these tissues, and to determine their relationships during the evolution of the vertebrates. The pathologist, when examining the skeleton, often finds skeletal tissues that are not classifiable into any one category, and there are classes of tissues in the nonpathological skeleton that are apparently intermediate between cartilage and bone.

When discussing bone, I used acellular bone to illustrate the usefulness of studies of those classes of skeletal tissues that are not often discussed in textbooks, but that yield valuable insights into developmental processes. I shall do the same with cartilage, using some of the invertebrate cartilages as examples.

B. Invertebrate Cartilages

Interest in invertebrate cartilage has been rekindled over the last eighteen years, primarily through the work of Person. Historically, the existence of invertebrate cartilage has been known since the early 1800's. The recognition that many invertebrates possessed a cartilagelike (chondroid, chordoid, mucoid) supporting tissue structurally similar to the supporting and parenchymal tissues of plants was one of the generalities that led Schwann and Schleiden to the formulation of the Cell Theory in 1838–1839. The studies of the 19th and early 20th centuries have been exhaustively summarized and reviewed by Schaffer (1930), who, however, did not regard these tissues as true cartilages. Their "immature" histology, combined with a scant extracellular matrix, the inability to detect glycosaminoglycans and collagen in their matrices—both of which are diagnostic of vertebrate cartilage—and their failure to mineralize or to calcify, led Schaffer to regard them as chondroid tissues. By "chondroid" he meant cartilagelike, and he classified such tissues into grades of greater or lesser resemblance to cartilage (without implying any phylogenetic trends or affiliations).

Schaffer's view prevailed, and skeletal biologists subsequently turned to other problems. Following his 1930 review, no research publications on invertebrate cartilages appeared until 1959. However, since 1959, information on the light and electron microscopy, the biochemical characterization of the extracellular