

## Electron Microscopic Analysis of Vascular Cartilage Canals in the Humeral Epiphysis of Hatchling Leatherback Turtles, *Dermochelys coriacea*

JOHANNES A.G. RHODIN<sup>1</sup>, ANDERS G.J. RHODIN<sup>2,4</sup>, AND JAMES R. SPOTILA<sup>3</sup>

<sup>1</sup>Department of Anatomy, College of Medicine, University of South Florida, Tampa, Florida 33612 USA  
[Fax: 813-974-2058; E-mail: jrhodin@golgi.panat.med.usf.edu];

<sup>2</sup>Chelonian Research Foundation, 168 Goodrich Street, Lunenburg, Massachusetts 01462 USA  
[Fax: 508-840-8184; E-mail: RhodinCRF@aol.com];

<sup>3</sup>Department of Bioscience and Biotechnology, Drexel University, Philadelphia, Pennsylvania 19104 USA;

<sup>4</sup>Corresponding Author for Reprint Requests

**ABSTRACT.** – Cartilage canals and growth cones in the humeral epiphysis of leatherback sea turtle hatchlings, *Dermochelys coriacea*, were investigated using a combination of light microscopy and transmission electron microscopy. Canals contained large interconnected vascular sinusoids near the leading growth cone tip, supplied by several feeding arterioles and drained by multiple venules. Individual capillary sprouts were not found. Metachromatic staining of the surrounding cartilage matrix was reduced in a narrow marginal zone in front of and on the sides of the canals. Epiphyseal matrix was not calcified in front of and near the leading tip of the growth cone, but many chondrocytes in these regions showed signs of cell death and nuclear pyknosis, a condition apparently unique to the leatherback and not previously reported in other vertebrates. Many fibroblastic cells, rich in granular endoplasmic reticulum, as well as macrophages, monocytes, and occasional multinucleated chondroclasts, occupied interstitial space in the growth cone tip of the cartilage canal between sinusoids and cartilage matrix. Near the base of the canal, surrounding cartilage matrix was calcified and chondrocytes hypertrophic and disintegrating. Involvement of the sinusoids and the cells near the growth cone tip of the canal in the process of cartilage resorption and canal formation is discussed in relation to the very rapid growth of leatherback turtles.

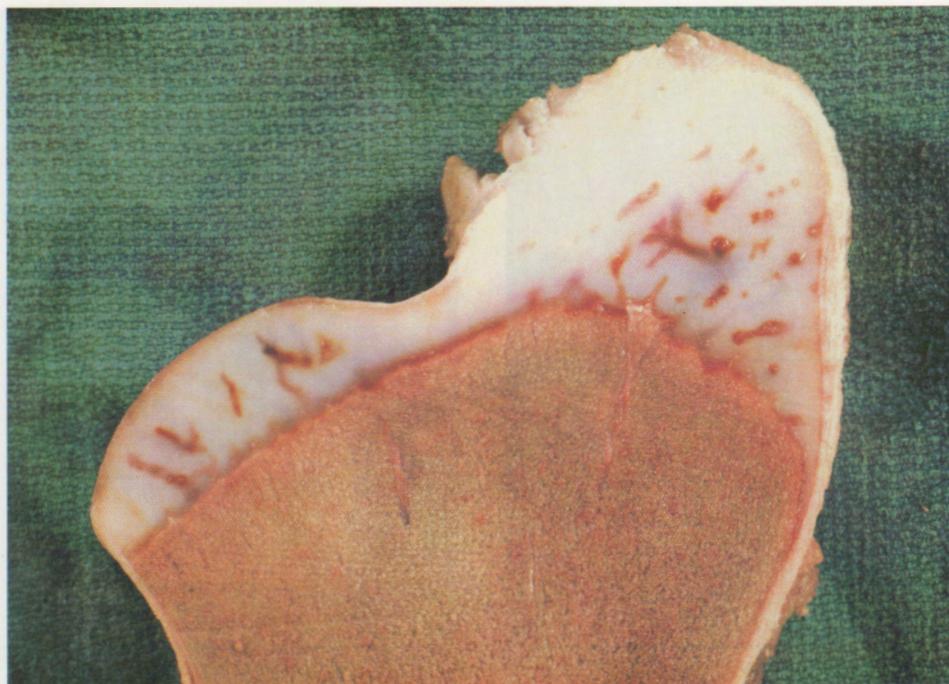
**KEY WORDS.** – Reptilia; Testudines; Dermochelyidae; *Dermochelys coriacea*; sea turtle; anatomy; histology; ultrastructure; cartilage canals; growth; cartilage; bone; vascular system; capillaries; epiphysis; chondrolysis; chondrocytes; chondroclasts

The leatherback sea turtle, *Dermochelys coriacea* (Testudines: Cryptodira: Dermochelyidae), is the world's largest living chelonian. Though somewhat closely related to the hard-shelled sea turtles (Cheloniidae) in a number of primitive plesiomorphic features, the leatherback has developed an array of unique derived features which render it without a doubt the most remarkably specialized turtle in the world.

Unique among sea turtles in its nearly exclusively pelagic habitat, the leatherback migrates regularly into frigid Arctic oceanic waters where it feeds almost exclusively on jellyfish (Lazell, 1980; Lutcavage and Lutz, 1986). It undertakes frequent dives of up to 1200 m, much deeper than other turtles or marine mammals (Eckert et al., 1986, 1989; Eckert, 1992). It is well adapted for deep and lengthy dives, with its blood oxygen carrying capacity, hemoglobin concentration, and muscular myoglobin content all significantly greater than in other sea turtles and more similar to marine mammals (Lutcavage et al., 1990). It regularly maintains its body temperature well above cold surrounding water temperatures, a result of gigantothermy: the ability to use large body size, relatively low metabolic rate, peripheral tissue insulation, and circulatory changes to maintain high body temperatures under varying environmental temperatures (Frair et al., 1972; Paladino et al., 1990). It has developed heat retention mechanisms of thickened subcutaneous fibrous

adipose tissue combined with counter-current heat exchangers in intertwined multiple arterial and venous vascular bundles in its flippers so as to avoid heat loss in cold waters (Greer et al., 1973). Its body is covered with a corselet of dramatically and uniquely reduced carapacial and plastral shell bones that are reinforced with a continuous layer of small irregular intercalated carapacial dermal bones that appear to have their origin in the cutaneous skin layer (Gervais, 1872; Deraniyagala, 1936). Its mitochondrial DNA pattern is ancient and different from all other sea turtles (Bowen et al., 1993). The skeleton is characterized by an unusually high degree of neotenic retention of well-developed thick cartilages, which are even further specialized through the development of cartilaginous vascularization, a condition unlike all other living turtles that instead retain primitive, thin, and totally avascular cartilages (Rhodin, 1985).

Leatherbacks can reach a body weight of up to 900 kg and carapace lengths of up to 180 cm, with sexual maturity usually reached at about 250 kg and 135 cm (Pritchard, 1971; Fretey, 1978; Eckert and Luginbuhl, 1988). A hatchling leatherback weighs only about 30 g, and to reach sexual maturity therefore means an approximately 8000 fold increase in body weight. The rate at which that growth is achieved may be extremely rapid, perhaps even approaching mammalian growth rates. No studies have yet succeeded in



**Figure 1.** Longitudinal cross-section of proximal humerus in a freshly dead adult leatherback turtle, showing red vascular endochondral bone in the metaphysis, white cartilage in the humeral articular epiphysis to the left and the greater tuberosity apophysis to the right, with numerous transphyseal and perichondral vascular cartilage canals penetrating the cartilage.

verifying the age at sexual maturity in leatherback turtles, but evidence from limited captive studies and histologic evaluation of skeletal growth of its appendicular bones has led to the hypothesis that maturity might be reached in as short a time as 3 to 6 years (Rhodin, 1985). Recently, Zug and Parham (1996), analyzing growth rings in scleral ossicles using skeletochronology, have predicted that average sexual maturity occurs at approximately 13–14 years for females and that maturity may be reached in a minimum time of 5–6 years. They felt that for conservation and management purposes, age at maturity for leatherbacks should be considered as 9 years, which would make leatherback growth (about 76 g/day on the average to reach 250 kg) as fast as many mammals and more rapid than any other known reptile (Case, 1978; Rhodin, 1985).

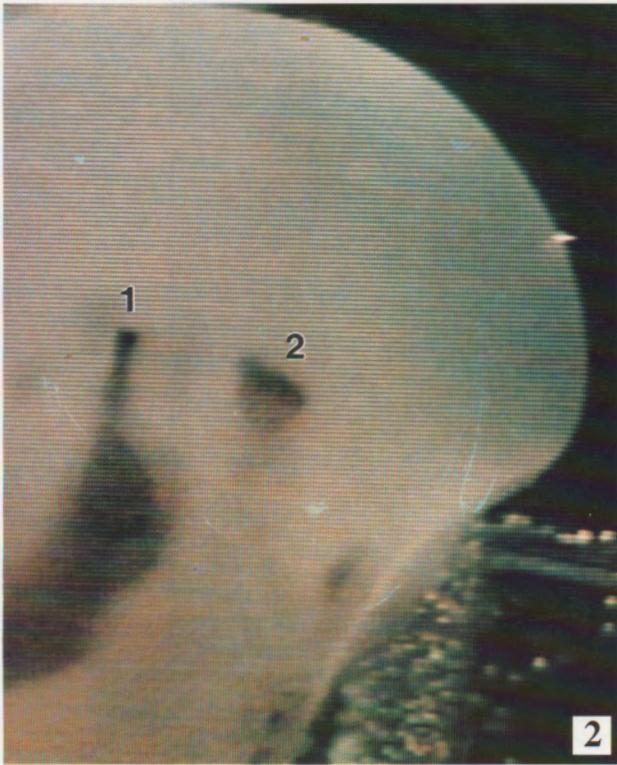
The leatherback's pattern of bone and cartilage growth, or chondro-osseous development, is unique among living chelonians and unlike any other living vertebrates, in some ways resembling marine mammals more than other turtles or reptiles (Rhodin et al., 1981; Rhodin, 1985). Alone among all living chelonians, the leatherback vascularizes its cartilaginous epiphyses through the formation of vascularized cartilage canals (Fig. 1). Unlike other extant vertebrates that also vascularize their epiphyses, including most mammals and some lizards and birds (Haines, 1938, 1969; Hogg, 1980), the leatherback does not form secondary ossification centers as a result of this vascular ingrowth into epiphyseal cartilage, but retains vascularized chondroepiphyses into adult life.

In previous descriptions and discussions of the comparative chondro-osseous development and growth of marine

turtles, the unique patterns of vascular ingrowth and the formation of cartilage canals in the leatherback have been analyzed by osteological studies, gross anatomy, and light microscopy (Rhodin et al., 1980, 1981; Rhodin, 1985). We now further elucidate the ultrastructural characteristics of these processes by means of transmission electron microscopic analysis.

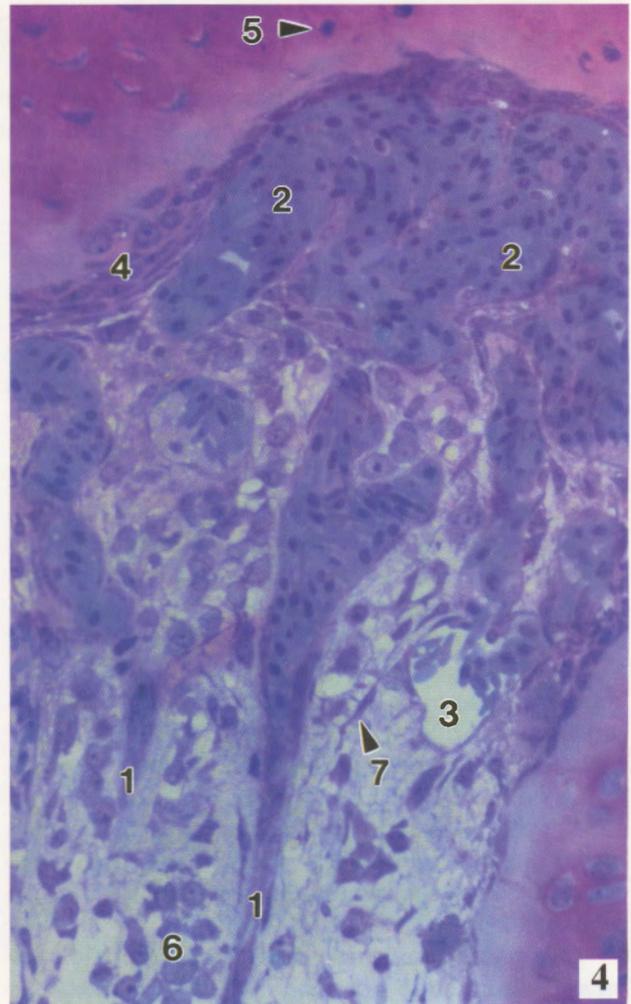
#### MATERIALS AND METHODS

Leatherback sea turtle (*Dermochelys coriacea*) eggs were obtained at Tortuguero, Costa Rica, incubated and hatched at Drexel University (Philadelphia), and two hatchlings shipped to University of South Florida (Tampa). At the age of 16 days, weighing 50 g, and with a carapace length (CL) of 63 mm, one hatchling (CRF 1862) was anesthetized in a MS222 (Tricaine-Methane-Sulfonate) solution, 5 g per gallon, and complete anesthesia achieved after 3 hours. At that point, the plastron was removed and a solution of 2% glutaraldehyde was perfused for 5 min via a cannula inserted into the heart ventricle, with the fixative returning through cuts in both atria. Following death, both humeri were dissected and freed from muscles, cut into several pieces and after about an hour submerged in a 1% solution of osmium tetroxide for 2 hours. A second hatchling (CRF 1863, 65 mm CL, weight 55 g) was not perfused, but the humeri were removed, cut into several pieces, and submerged in a solution of 2% glutaraldehyde for 2 hours (Fig. 2), followed by submersion in 1% solution of osmium tetroxide for 2 hours. All bone pieces were then decalcified in a 10% solution of EDTA (Ethylene-Diamine-Tetra-

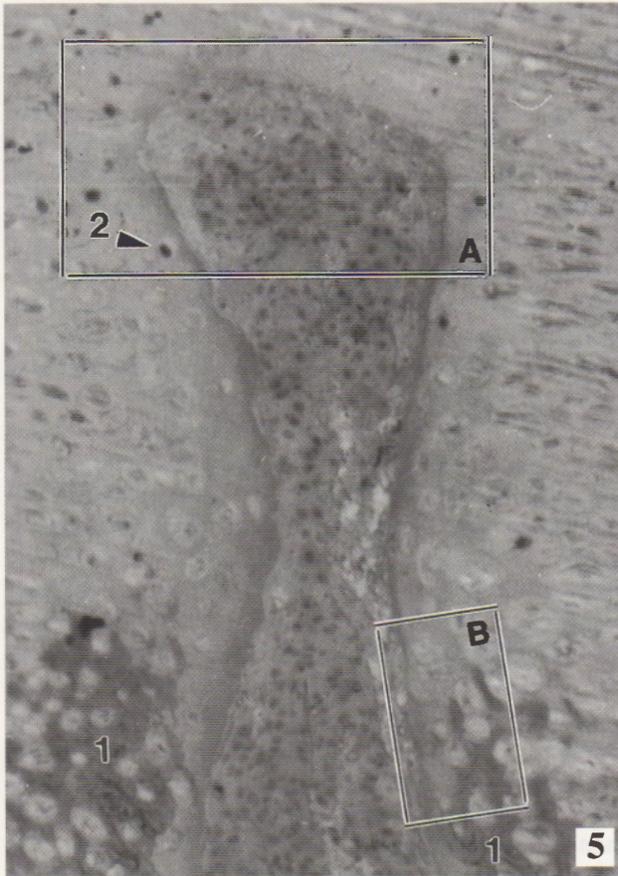


**Figure 2.** Epiphysis and metaphysis of a halved proximal humerus of a two-week old leatherback hatchling, submerged in glutaraldehyde solution before fixation in osmic acid. Several cartilage canals enter the epiphysis from the diaphysis and the periphery of the metaphysis. Since the blood vessels in the growth cones of the cartilage canals contain red blood cells, they are readily visible in the semitransparent bluish hyaline cartilage. The leading tips of the growth cones may be pointed (1) or have a broad front (2). The growth cone identified as (1) was sectioned longitudinally and photographed in the light microscope (Fig. 5) and in the transmission electron microscope (Fig. 6). Mag. 5X. Photographed from video monitor.

**Figure 3.** Longitudinal section of a cartilage canal and growth cone similar to (1) in Fig. 2. From a leatherback hatchling proximal humerus fixed by submersion in glutaraldehyde and osmic acid, embedded in JB-4 Plus and stained with toluidine blue. The cartilage canal contains bone marrow cells and blood vessels with anastomosing sinusoidal capillaries concentrated at the leading tip of the growth cone. Metachromatic staining of cartilage matrix is slightly reduced in a narrow zone (1) around the leading tip of the growth cone, probably due to proteoglycan removal. Lateral chondrocytes (2) are hypertrophic and matrix lacunae vacuolized, since the cartilage matrix (3) surrounding them is becoming mineralized. Most of the chondrocytes (4) near the front of the growth cone look normal, but some (5) are extremely dense and pyknotic. Mag. 170X. 10 mm = 5.8  $\mu$ m.

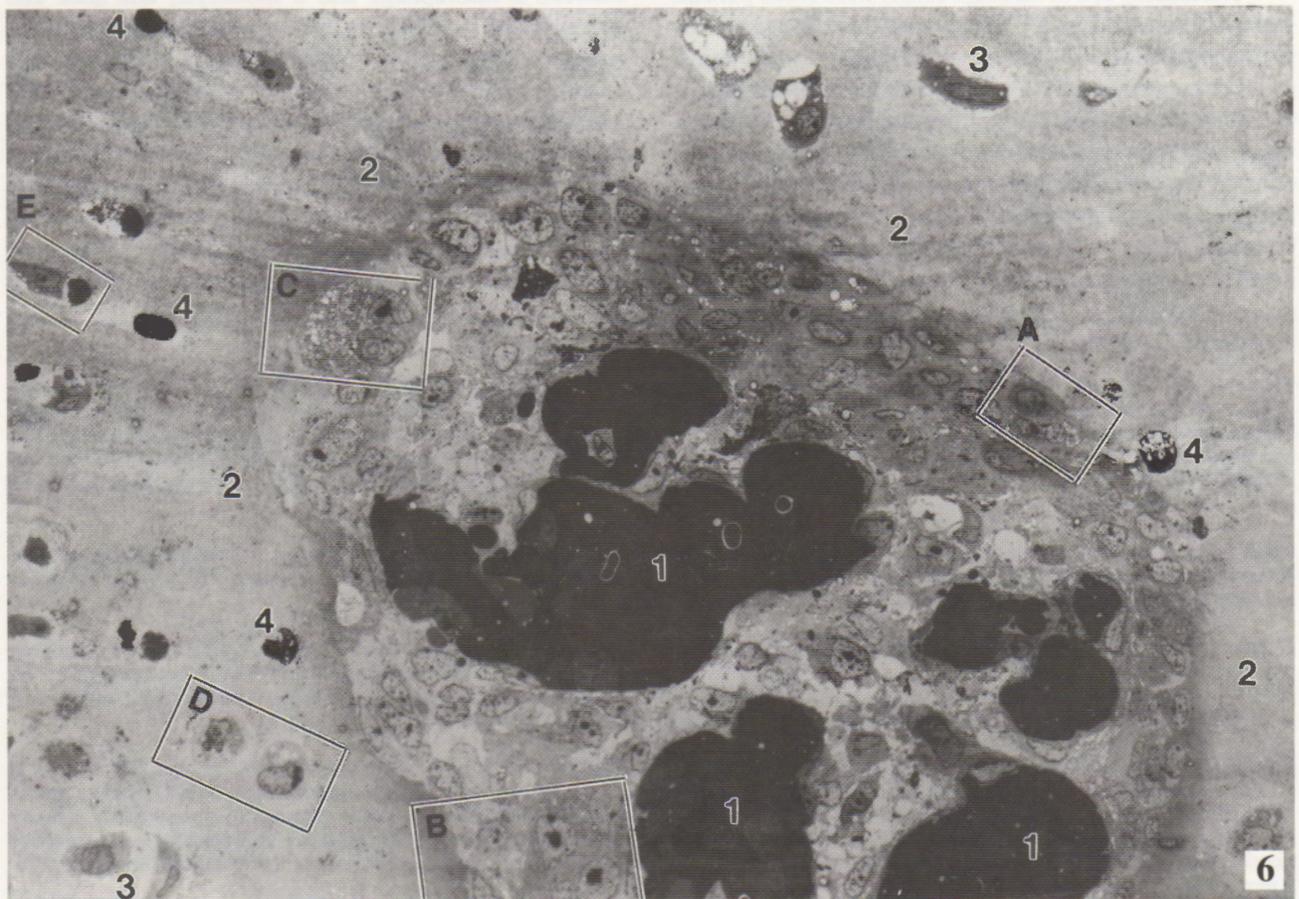


**Figure 4.** Enlarged detail of Fig. 3. The core of the cartilage canal contains at least two constricted feeder arterioles (1) which give rise to a glomerulus of anastomosing, large sinusoidal capillaries (2). These are drained by venules (3). A large number of nucleated erythrocytes fill sinusoidal capillaries, since this specimen was not fixed by vascular perfusion. Between capillaries and the cartilage matrix (4) are layers of small, flat, and cuboidal fibroblastic cells and macrophages. One pyknotic chondrocyte is seen at (5). The connective tissue core of the canal contains marrow cells (6) and fibroblasts (7). Mag. 360X. 1 mm = 2.8  $\mu$ m.



**Figure 5.** Longitudinal section of the cartilage canal and growth cone marked (1) in Fig. 2. In this preparation, calcified cartilage matrix (1) around the diaphyseal part of the canal is clearly seen, as well as several pyknotic chondrocytes (2) around the leading tip of the canal. However, this section does not show the narrow zone of cartilage matrix which has reduced metachromatic staining reaction in Fig. 3. Area (A) is enlarged in Fig. 6, and area (B) is enlarged in Fig. 13. Mag. 80X. 10 mm = 12.5  $\mu$ m.

**Figure 6.** Low magnification transmission electron micrograph of a section from the same specimen seen in area (A) in Fig. 5. It demonstrates the advantage of examining the same object, first in the light microscope and then in the electron microscope, making it possible to identify the same area and the same cells in both sections. This is the leading tip of the cartilage canal and the growth cone surrounded by the cartilage matrix and chondrocytes of the epiphysis. Sinusoidal capillaries stand out with their content of darkly stained erythrocytes (1). At this magnification, it is difficult to positively classify the various cells, but those present within the rectangles have been examined at higher magnifications and identified as follows. Cells which border on the cartilage matrix (2) are: (A) fibroblastic cells and macrophages (see Fig. 7); (B) multinucleate chondroclast (see Fig. 8); and (C) vesiculated chondroclast (see Fig. 9). Other cells in the core of the canal are endothelial cells, pericytes, monocytes, and some leukocytes. Cells present in the cartilage matrix (2) are: (D) hypertrophic chondrocytes (see Fig. 11); and (E) cartilage lacuna with two chondrocytes, one normal and one pyknotic (see Fig. 12). There are other normal (3) and pyknotic chondrocytes (4) present. Mag. 780X. 1 mm = 1.3  $\mu$ m.



Acetic acid) for 48 hours, dehydrated in a graded series of ethanol alcohol, submerged in propylene oxide, and embedded in EM-bed-812, a plastic resin. Some bone pieces were embedded in JB-4 Plus, a water-soluble plastic resin, producing a clear, transparent cast. Transparency of the embedding media allowed for easy identification of length and width of vascular ingrowths and selection of specific growth cones and cartilage canals. These were then sectioned serially, either parallel to or across the long axis of growth cones, beginning near the leading tip of the cartilage canal. Thick sections for light microscopy (Figs. 3–5) were alternated with thin sections for electron microscopy (Figs. 6–15). Thick sections were stained with toluidine blue and thin sections with uranylacetate and lead citrate. Ultrastructural analysis was carried out in a Philips 301 transmission electron microscope.

## RESULTS

### Cartilage Canals and Growth Cones

**Light Microscopy.** — In the humeral epiphysis and metaphysis cartilage canals originated from the perichondrial-osteal cuff. Several stages of these canals were observed; some extremely short, mere bulges into the hyaline cartilage matrix, others extremely long, extending into the center of the cartilage and the epiphysis. This report deals only with the long cartilage canals.

The leading tips of cartilage canals were either pointed or broad-fronted (Fig. 2). Canals contained bone marrow cells and blood vessels with anastomosing sinusoidal capillaries concentrated at the leading tip. These cells and vessels concentrated in the advancing tip of the canal are defined as the growth cone, whose function it is to actively penetrate the surrounding cartilage matrix, creating a cartilage canal. The core of the canal contained at least two feeder arterioles which gave rise to a spray of anastomosing large sinusoidal capillaries. Individual capillary sprouts were not found.

Several postcapillary venules with large lumens drained the sinusoids, returning blood to the perichondrial vasculature (Figs. 3–4).

At the growth cone tip of the cartilage canal, between the sinusoidal capillaries and cartilage matrix, were layers of small, flat, and cuboidal mononuclear cells (Fig. 4), as well as large multinuclear cells. We identified these cells as monocytes, macrophages, fibroblastic cells, and chondroclasts (Fig. 6). Sinusoids were lined by endothelial cells and displayed an occasional pericyte. Marrow cells observed were mostly developmental stages of leukocytes. Nucleated erythrocytes and thrombocytes were present in the blood vessels.

**Electron Microscopy.** — Multinuclear cells in the canal were chondroclasts. Some had a smooth and unruffled cell membrane in very close contact with the epiphyseal cartilage matrix, to the extent that it was difficult to resolve the membrane (Fig. 9). Others had an extremely ruffled cell surface with numerous microvilli (Fig. 10). The cytoplasm contained many small mitochondria and vesicles, a fair amount of free ribosomes, several small granules (presumably lysosomes), and phagocytic vacuoles, some of which contained traces of cartilage matrix with hydroxyapatite crystals.

Macrophages were mononucleated and contained primary lysosomes and phagolysosomes (Fig. 7). We identified monocytes as such based on their very small number of lysosomes (Fig. 8). At times it was difficult to differentiate between a macrophage and a monocyte, and their cell structures led us to believe that they could represent mononuclear precursor cells of chondroclasts. Fibroblastic cells contained many profiles of granular endoplasmic reticulum and numerous free ribosomes, some mitochondria, and an electron dense cytoplasmic matrix (Fig. 7). These cells were often in close contact with the cartilage matrix and displayed large bulbous cytoplasmic protrusions which penetrated into the cartilage matrix. Endothelial cells and pericytes of the sinusoids displayed a structure reminiscent of and very

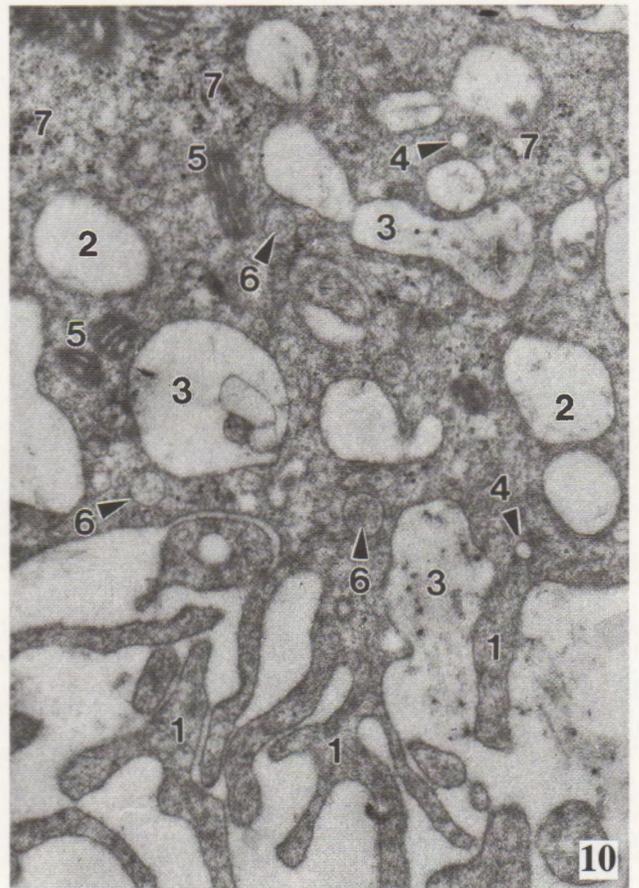
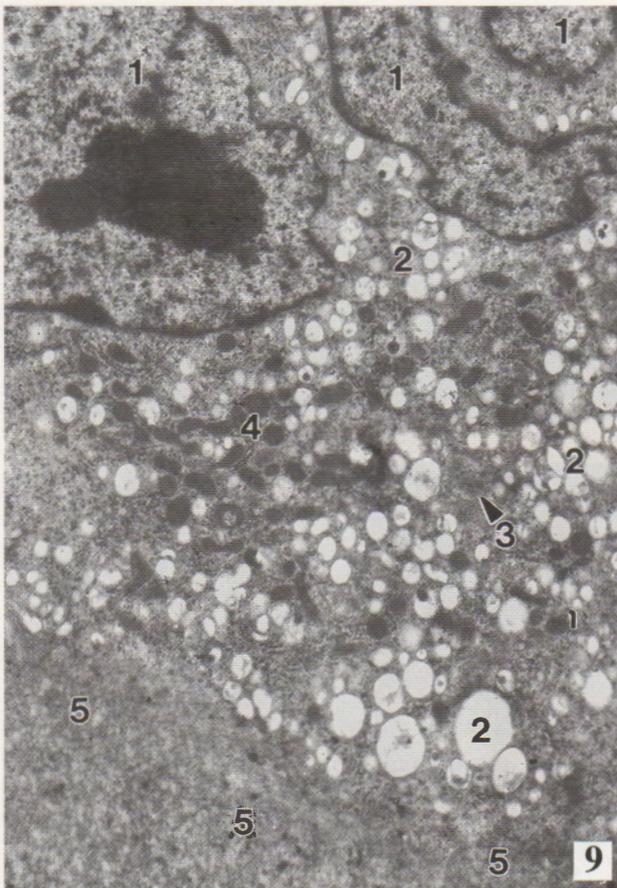
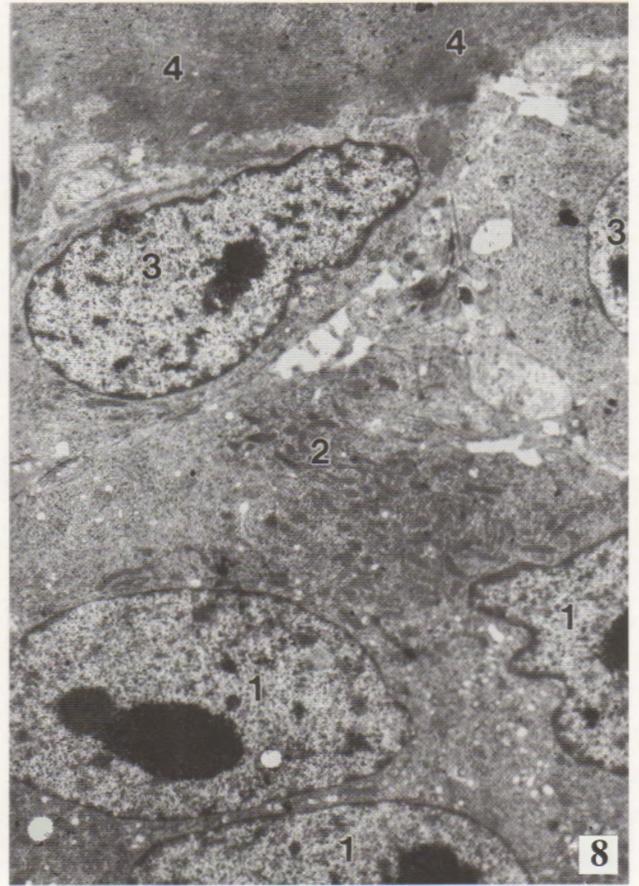
(Figures on facing page)

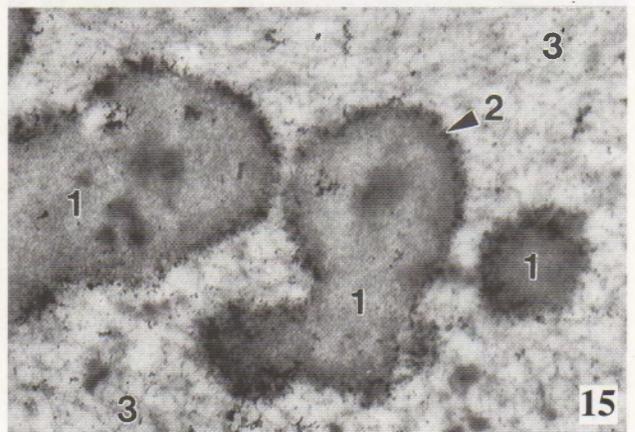
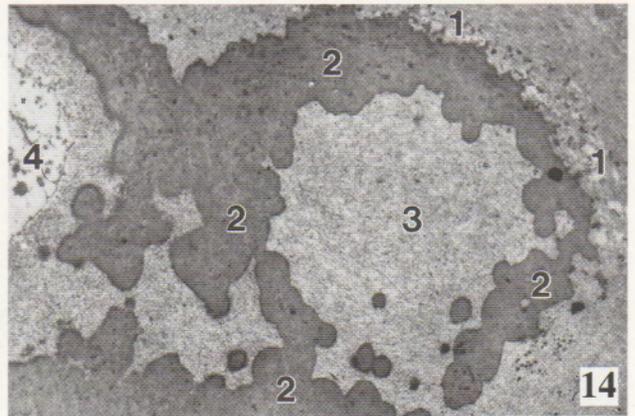
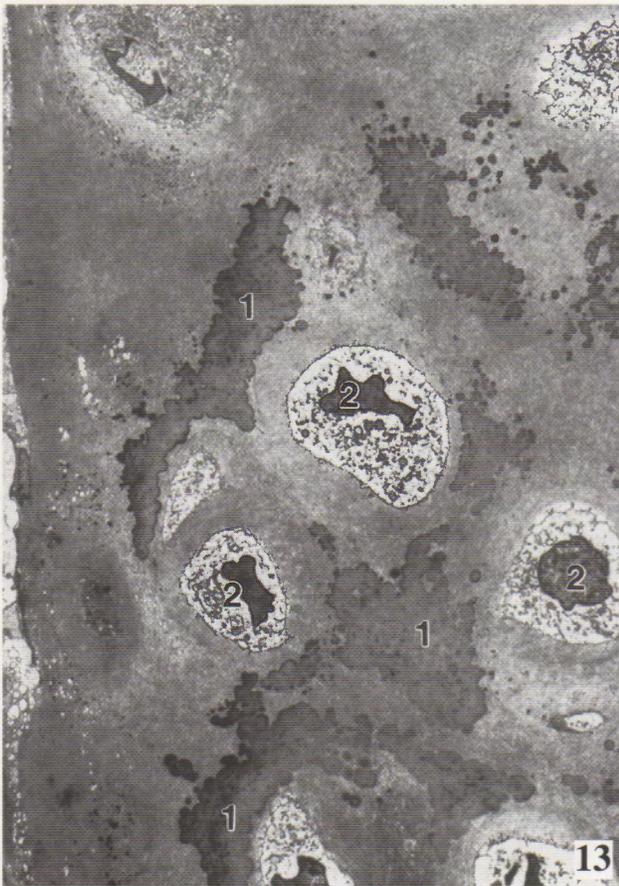
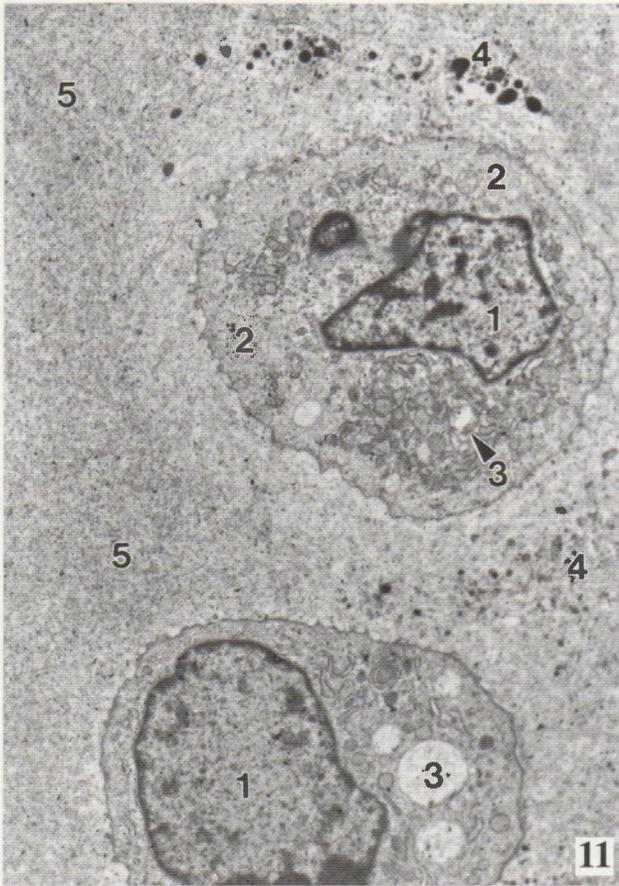
**Figure 7.** Enlargement of Area (A) in Fig. 6. Two cells border directly on the cartilage matrix (1). The fibroblastic cell has a lobulated nucleus (2), an electron dense cytoplasm with many profiles of granular endoplasmic reticulum (3), and some mitochondria. A large, bulbous cytoplasmic process (4) penetrates the cartilage matrix. We assume that this cell actively synthesizes chondrolytic enzymes. The second cell has an oval nucleus (5) and is a macrophage with several phagolysosomes (6). Its cytoplasm is less electron dense than that of the fibroblastic cell, and has fewer profiles of granular endoplasmic reticulum. We assume that the macrophage removes cartilage matrix. Mag. 9700X. 1 mm = 0.1  $\mu$ m.

**Figure 8.** Enlargement of Area (B) in Fig. 6. Nuclei (1) are part of a multinucleate chondroclast, the cytoplasm of which contains numerous small mitochondria (2) and ribosomes. Cells with the oval nuclei (3) are in direct contact with the cartilage matrix (4) and are either monocytes or macrophages. Mag. 5,000X. 1 mm = 0.2  $\mu$ m.

**Figure 9.** Enlargement of Area (C) in Fig. 6. Nuclei (1) belong to a multinucleate chondroclast with many vesicles in the cytoplasm representing phagolysosomes (2) among primary lysosomes (3) and mitochondria (4). The chondroclast is in very close contact with the cartilage matrix (5) to the extent that it is not possible to resolve its cell membrane at this magnification. We assume that this chondroclast is very active in cartilage matrix breakdown and phagocytosis. Mag. 8,600X. 1 mm = 0.11  $\mu$ m.

**Figure 10.** Detail of an active chondroclast at high magnification. This cell was found near mineralized cartilage matrix, but was not in direct contact with the matrix. It displays a ruffled border (1), many phagocytic vacuoles (2), some containing traces of cartilage matrix with calcium crystals (3), many small vesicles (4), and mitochondria (5). There are several small granules (6), presumably primary lysosomes, and a small number of ribosomes (7). We assume that a chondroclast with ruffled border is also actively phagocytosing cartilage matrix. However, since this particular cell contains calcium crystals, its ultrastructure is very similar to that of an osteoclast. Mag. 31,500X. 1 mm = 0.032  $\mu$ m = 32 nm = 320 Å.





similar to these cells in the capillaries of mammals and frog tadpoles, *Xenopus laevis* (Rhodin and Lametschwandter, 1993). There was no basal lamina underlying the endothelium of the sinusoids.

### Epiphyseal Cartilage

*Light Microscopy.*—Epiphyseal cartilage displayed, in general, the typical pattern of interstitial cartilage growth with chondrocytes in their lacunae, many of which were undergoing mitotic divisions in the continued process of cartilage growth (Figs. 3–5). The cartilage matrix was homogeneous without calcifications. Metachromatic staining of cartilage matrix was reduced in a narrow 10–20  $\mu\text{m}$  marginal zone in front of and on the sides of the leading tip of cartilage canals (Figs. 3–4).

There was a distinct difference in chondrocyte appearance in front of the cartilage canal and on its sides. In a narrow zone in front of and surrounding the leading tip of the vascular canal, many chondrocytes were undergoing a process of cell death, indicated by their highly pyknotic nuclei (Figs. 3, 5–6). On the other hand, chondrocytes located more peripherally and below the leading growth tip showed processes typical for stages preceding endochondral bone formation: proliferation, hypertrophy, and degeneration, concomitantly with calcification of the cartilaginous matrix (Fig. 5).

*Electron Microscopy.*—There was active interstitial chondrocyte division among cells in front of the leading tip of the cartilage canal. However, perilacunar cartilage matrix in places began to show an accumulation of matrix vesicles or dense bodies with a high electron dense core, probably an indication of calcium phosphate (Fig. 11). Vesicles presumably serve as nucleation sites for mineral deposition. In lacunae with active cell division, one or both of the daughter chondrocytes showed various degrees of nuclear pyknosis and cell death (Fig. 12), as judged by disintegration of cytoplasm and vesiculation of cytoplasmic organelles.

In the cartilage zone peripherally and below the leading growth tip, chondrocytes showed signs of hypertrophy (Fig. 11). Their cytoplasm had a bloated appearance, cisternae of the granular endoplasmic reticulum were dilated, and mitochondria were vesiculated and swollen. There were many electron dense core matrix vesicles present in the matrix surrounding hypertrophic chondrocytes. The matrix, consisting of delicate collagenous fibrils without detectable bandings, increasingly became more mineralized distally (Figs. 13–14), with broad electron dense areas, containing minute calcium crystals (Fig. 15).

### DISCUSSION

Leatherback sea turtles have thick vascularized cartilages, unlike all other extant turtles that have thin avascular cartilages. The vascular cartilage canals in the leatherback are apparently similar to the same structures in mammals and birds that also vascularize their cartilages. Based on extensive work on the structure and function of cartilage canals in mammals and birds (primarily chickens), it appears that cartilage canals are: 1) nutritive to cartilage too thick or growing too fast to derive all of its required nutrition by direct diffusion from the synovial fluid in contact with the joint surface; 2) a source of prechondral mesenchymal cells for continued interstitial cartilage expansion in rapidly growing cartilage; 3) structurally supportive in thick cartilage, especially when surrounded by narrow sleeves of hypertrophying and calcifying cartilage, and; 4) usually accompanied by the eventual formation of a secondary ossification center within the penetrated cartilage (Levene, 1964; Lutfi, 1970a; Wilsman and Van Sickle, 1970, 1972; Kugler et al., 1979; Ogden, 1980; Kuettner and Pauli, 1983).

Our investigation on the ultrastructure of chondro-osseous development in leatherback sea turtle hatchlings concentrated on the growth cone tip regions of long cartilage canals in the humeral epiphysis, utilizing a combination of

(Figures on facing page)

**Figure 11.** Enlargement of Area (D) in Fig. 6. Nuclei (1) of two hypertrophic chondrocytes. The cells are considered hypertrophic, based on their enlarged and rounded appearance, compared to chondrocytes undergoing proliferation, when the shape is flatter and spindle shaped. The cytoplasm (2) is slightly bloated, and some cell organelles are vesiculated and dilated (3). Matrix vesicles and dense bodies (4) are present in the perilacunar cartilage matrix (5). Mag. 5800X. 1 mm = 0.17  $\mu\text{m}$ .

**Figure 12.** Enlargement of Area (E) in Fig. 6. Two chondrocytes in the same lacuna have just completed cell division. One (1) has the typical normal shape of chondrocytes undergoing proliferation, the second (2) has a pyknotic nucleus and its cytoplasm is condensed and disintegrating. This cell is considered dead. Mag. 8700X. 1 mm = 0.11  $\mu\text{m}$ .

**Figure 13.** Enlargement of Area (B) in Fig. 5. In this part of the cartilage, there is a pronounced degree of cartilage matrix calcification, as judged by the electron dense areas (1) which almost completely surround the lacunae. Chondrocytes (2) have pyknotic nuclei and pronounced disintegration of the cytoplasm. The cells are dead. Part of the cartilage canal is seen to the extreme left. Mag. 1500X. 1 mm = 0.67  $\mu\text{m}$ .

**Figure 14.** Enlarged detail of one electron dense area in the cartilage matrix, similar to those seen in Fig. 13. There is a mixture of matrix vesicles and dense matrix granules (1) at the periphery of the dense area (2), which almost entirely surrounds a cartilage lacuna (3), situated below the level of the plane of this section. Part of a disintegrating chondrocyte is also seen (4). Mag. 3300X. 1 mm = 0.3  $\mu\text{m}$ .

**Figure 15.** Electron dense matrix area at higher magnification. It consists of an electron dense, amorphous core (1) and many minute, highly electron dense granules (2), mostly at the periphery. The cartilage matrix (3) consists of a loose network of delicate collagen fibrils. Mag. 25,000X. 1 mm = 0.04  $\mu\text{m}$  = 40 nm = 400  $\text{\AA}$ .

light microscopy and transmission electron microscopy. Some of our findings are similar to investigations of cartilage canals and resorption in avian long bones, but other findings are new and perhaps unique to the leatherback. Cellular details of cartilage resorption by the growth cone at the tip of the cartilage canal are remarkably similar in the leatherback hatchling and embryonic chick (Sorrell and Weiss, 1980).

It is reasonably well established (Stump, 1925) that formation of cartilage canals is the result of an active ingrowth of blood vessels with resorption of cartilage matrix. Another theory proposed by Haines (1934) suggested that the canals are formed by passive inclusion during rapid synthesis of cartilage matrix around perichondrial vessels. This theory, however, has not gained wide acceptance, and is not supported by most observations.

Some investigators (Cameron, 1961; Schenk et al., 1967) have suggested that the advancing capillaries themselves accomplished the erosion of the cartilage matrix by a mechanism of resorption, but the majority of investigators have concluded that both blood vessels and perivascular and connective tissue cells are involved (Lutfi, 1970b; Silvestrini et al., 1979; Howlett, 1979, 1980; Sorrell and Weiss, 1980, 1982; Cole and Wezeman, 1985; Fukushima et al., 1991). Cells that have been described and suggested as being involved in the erosion process are monocytes, macrophages, chondroclasts, fibroblastic cells, and pericytes.

In the present material, the growth cone tips of cartilage canals in the leatherback humerus contain all of the cells mentioned, in addition to large, interconnected vascular sinusoids lined by thin endothelial cells and a small number of pericytes. It is uncertain how these sinusoids elongate, since the typical individual blind-ended capillary sprouts described in the rat mesentery (Rhodin and Fujita, 1989) and the tadpole tail-fin (Rhodin and Lametschwandtner, 1993) were not found near the tip of the cartilage canal. In both of these other species, the endothelial vascular sprouts, solid at first, seek out their counterparts, merge, and a sprout lumen opens up and blood flow starts. In the very narrow cartilage canals of the leatherback epiphysis, relatively large blood volumes and possibly high blood pressures may exist as judged by the numerous erythrocytes in the vessels, and this possible high intraluminal pressure may be the driving force in elongating the sinusoids and creating anastomoses. In support of this hypothesis is the fact that endothelial cell mitoses are not observed near the tip of the canal but only closer to the feeding arterioles. From a functional point of view, the possible high blood pressure in the narrow canal probably increases the effectiveness of the absorptive and removal processes which bring about the formation of the canal itself. This situation is similar to the resorption cavity formed by the cutting cone of the remodeling unit of cortical bone in mammals, where blood vessels and osteoclasts in the cutting cone create a space for future osteons (Parfitt, 1976).

In terms of the cellular elements present in the growth cone tip of the cartilage canal, there is no doubt that mac-

rophages and chondroclasts are involved in the removal of cartilage matrix and debris from disintegrating chondrocytes. However, the cells that are in direct marginal contact with the surrounding cartilage matrix and provided with bulbous cytoplasmic extensions into the cartilage, are the fibroblastic cells. We tend to agree with Sorrell and Weiss (1980), based on their study of embryonic chick long bones, that these cells, which have a high content of granular endoplasmic reticulum, represent protein synthesizing cells, probably secreting chondrolytic enzymes. Reduced metachromatic staining of a narrow marginal matrix zone near the tip of the canal is an indication of removal of proteoglycans, a first step in making collagen more susceptible to the proteolytic enzymes secreted by macrophages (Takemura and Werb, 1984). Conversely, Sorrell and Weiss (1982) demonstrated by autoradiography that the fibroblastic cells were not engaged in either synthesis of sulfated proteoglycans or the production and secretion of collagen.

In our opinion, chondrolytic enzymes probably secreted by marginal fibroblastic cells along the leading growth cone tip of cartilage canals may also have a toxic effect on chondrocytes in proximity to the canal tip, since we observed marked cell death in this region, as judged by the pyknotic nuclei of the chondrocytes. This finding has not previously been reported in other vertebrate species, and may be unique to the leatherback. It probably facilitates more rapid matrix resorption as the canal advances, since the dying chondrocytes cannot maintain the proper biochemical milieu of the cartilage matrix in advance of the canal tip, and allows for a more rapid penetration of the cartilage canal as it grows into the epiphyseal cartilage of the rapidly growing leatherback hatchling. This finding provides additional support for the hypothesis that leatherbacks have developed cartilaginous vascularization as a specialization related to their very rapid growth to a large body size (Rhodin, 1985).

One problem that remains to be solved is the origin of the fibroblastic cells, as well as the mechanism whereby chondroclasts are formed. Fibroblastic cells are reminiscent of pericytes (Schulz et al., 1977), although the latter are enveloped by an external lamina, have abundant pinocytotic vesicles, and much fewer profiles of granular endoplasmic reticulum. Since pericytes are almost always present in association with capillaries and venules (Rhodin, 1968) it is tempting to suggest that pericytes give rise to fibroblastic cells, but immunohistochemical investigations would be required to demonstrate this point. Complicating the hypothesis that pericytes could be precursors of fibroblastic cells is the opposite finding in rat mesentery that the precursors of capillary sprout pericytes are fibroblasts which become incorporated into endothelial sprouts and enveloped by an external lamina (Rhodin and Fujita, 1989).

It is much more likely that blood-borne monocytes represent the precursor cells not only for fibroblastic cells, but also for macrophages and chondroclasts. In our material, monocytes and macrophages are often difficult to differen-

tiate, and fibroblastic cells and macrophages differ only with regard to their content of granular endoplasmic reticulum. Furthermore, the ultrastructure of chondroclasts is reminiscent of macrophages, except that the former are multinucleate. In their studies of regenerating newt limbs, Fischman and Hay (1962) demonstrated that mononuclear leukocytes were precursor cells of osteoclasts, although intraskeletal progenitor cells have also been suggested (Hall, 1975). There is basically little structural difference between an osteoclast and a chondroclast and it can be assumed that chondroclasts are also formed by a fusion of monocytes.

As far as the structure of leatherback chondroclasts is concerned, we identified at least two types. Type I chondroclasts had a smooth and unruffled cell membrane that was in very close contact with the cartilage matrix. Type II cells displayed a surface that was extremely ruffled and was most often associated with the more proximal end of the cartilage canal where the surrounding matrix was calcified. In the chick tibia, Howlett (1980) also described two types of chondroclasts, both located near calcified cartilage, and very similar to our two types. On the other hand, Fukushima et al. (1991) described two types of osteoclast cells in the chick tibia that were described as the opposite of what we report here. They claimed that active osteoclasts had a ruffled border with many small concentrated cytoplasmic vesicles, whereas resting osteoclasts lacked a ruffled border and had evenly distributed cytoplasmic vesicles. In our material, intermediate stages were encountered between the smooth and the ruffled cell surface types, and it is likely that this reflects a very active chondroclast cell surface similar to the moving cell projections of a macrophage that can be observed and recorded by intravital video recording during phagocytosis. These contradictory findings will require further studies utilizing immunohistochemical methods whereby one can positively mark and identify cells, their precursors, and their derivatives.

Cartilage matrix peripheral to and below the leading growth cone tip underwent calcification and the chondrocytes showed the typical stages of hypertrophy, bloating of the cytoplasm, and degeneration. This process appears similar to endochondral ossification in mammals (Anderson and Parker, 1966, 1968; Bonucci, 1967) and has also been described in birds (Howlett, 1979).

In conclusion, the leatherback turtle has developed the ultrastructural cytoarchitecture and cellular mechanisms for generating very rapid penetration of vascular canals into the rapidly growing cartilaginous matrix of its bones. Unlike all other living turtles, and more like mammals and some birds, the leatherback appears to have successfully developed the requisite physiologic and anatomic specializations that allow it to support very rapid growth to a large body size. Many of these specializations appear unique among extant vertebrates. The leatherback's specialized biology and marvelous adaptations are fertile ground for further studies, and continuing efforts to increase our understanding of this unique and endangered species should prove rewarding.

### Acknowledgments

We thank Richard A. Byles, U.S. Fish and Wildlife Service, for facilitating the permitting process of obtaining otherwise doomed leatherback eggs from Costa Rica. Eggs were imported under CITES and U.S. Endangered Species permits to J.R. Spotila, with subpermits for histological preparation by J.A.G. Rhodin. Laboratory work at the University of South Florida benefitted from help by Josef Stingl, with treatment of animals approved by the animal care committee. Specimens are stored at Chelonian Research Foundation (CRF) and University of South Florida Department of Anatomy. We thank the following colleagues for insightful and helpful reviews of this manuscript: Scott Eckert, Bruce Rothschild, John Ogden, George Zug, John Behler, and Peter Pritchard.

### LITERATURE CITED

- ANDERSON, C.E., AND PARKER, J. 1966. Invasion and resorption in endochondral ossification. *J. Bone Joint Surg.* 48A:899-914.
- ANDERSON, C.E., AND PARKER, J. 1968. Electron microscopy of the epiphyseal cartilage plate. A critical review of electron microscopic observations on endochondral ossification. *Clin. Orthop.* 58:225-241.
- BONUCCI, E. 1967. Fine structure of early cartilage calcification. *J. Ultrastruct. Res.* 20:33-50.
- BOWEN, B.W., NELSON, W.S., AND AVISE, J.C. 1993. A molecular phylogeny for marine turtles: trait mapping, rate assessment, and conservation relevance. *Proc. Natl. Acad. Sci. USA* 90:5574-5577.
- CAMERON, D.A. 1961. Erosion of the epiphysis of the rat tibia by capillaries. *J. Bone Joint Surg.* 43B:590-594.
- CASE, T.J. 1978. On the evolution and adaptive significance of postnatal growth rates in terrestrial vertebrates. *Quart. Rev. Biol.* 53:243-282.
- COLE, A.A., AND WEZEMAN, F.H. 1985. Perivascular cells in cartilage canals of the developing mouse epiphysis. *Amer. J. Anat.* 174:119-129.
- DERANIYAGALA, P.E.P. 1936. Some postnatal changes in the leathery turtle *Dermochelys coriacea*. *Ceylon J. Sci. B.* 19:225-239.
- ECKERT, K.L., AND LUGINBUHL, C. 1988. Death of a giant. *Mar. Turt. Newsl.* 43:2-3.
- ECKERT, S.A. 1992. Bound for deep water. Leatherback turtles can pursue their prey half a mile straight down. *Natural History* 92(3):28-35.
- ECKERT, S.A., ECKERT, K.L., PONGANIS, P., AND KOOYMAN, G.L. 1989. Diving and foraging behavior of leatherback sea turtles (*Dermochelys coriacea*). *Can. J. Zool.* 67:2834-2840.
- ECKERT, S.A., NELLIS, D.W., ECKERT, K.A., AND KOOYMAN, G.L. 1986. Diving patterns of two leatherback sea turtles (*Dermochelys coriacea*) during interesting intervals at Sandy Point, St. Croix, U.S. Virgin Islands. *Herpetologica* 42:381-388.
- FISCHMAN, D.A., AND HAY, E.D. 1962. Origin of osteoclasts from mononuclear leukocytes in regenerating newt limbs. *Anat. Rec.* 143:329-338.
- FRAIR, W., ACKMAN, R.G., AND MROSOVSKY, N. 1972. Body temperature of *Dermochelys coriacea*: warm turtle from cold water. *Science* 177(4051):791-793.
- FRETEY, J. 1978. Mensurations de tortues luths femelles adultes, *Dermochelys coriacea* (Linné), en Guyane française. *Bull. Soc. Zool. France* 103:518-523.
- FUKUSHIMA, O., BEKKER, P.J., AND GAY, C.V. 1991. Characterization

- of the functional stages of osteoclasts by enzyme histochemistry and electron microscopy. *Anat. Rec.* 231:298-315.
- GERVAIS, P. 1872. Ostéologie du *Sphargis luth* (*Sphargis coriacea*). *Mem. Nouv. Arch. Mus. Nat. Hist. Nat. Paris* 8:199-228.
- GREER, A.E., LAZELL, J.D., JR., AND WRIGHT, R.M. 1973. Anatomical evidence for a counter-current heat exchanger in the leatherback turtle (*Dermodochelys coriacea*). *Nature* 244:181.
- HAINES, R.W. 1934. Cartilage canals. *J. Anat.* 68:45-64.
- HAINES, R.W. 1938. The primitive form of epiphysis in the long bones of tetrapods. *J. Anat.* 72:323-343.
- HAINES, R.W. 1969. Epiphyses and sesamoids. In: Gans, C., Bellairs, A.d'A., and Parsons, T.S. (Eds.). *Biology of the Reptilia*. Vol. 1. Morphology A. New York: Academic Press, pp. 81-115.
- HALL, B.K. 1975. The origin and fate of osteoclasts. A review. *Anat. Rec.* 183:1-12.
- HOGG, D.A. 1980. A re-investigation of the centres of ossification in the avian skeleton at and after hatching. *J. Anat.* 130:725-743.
- HOWLETT, C.R. 1979. The fine structure of the proximal growth plate of the avian tibia. *J. Anat.* 128:377-399.
- HOWLETT, C.R. 1980. The fine structure of the proximal growth plate and metaphysis of the avian tibia: endochondral osteogenesis. *J. Anat.* 130:745-768.
- KUETTNER, K.E., AND PAULI, B.U. 1983. Vascularity of cartilage. In: Hall, B.K. (Ed.). *Cartilage*. Volume 1. Structure, Function, and Biochemistry. New York: Academic Press, pp. 281-312.
- KUGLER, J.H., TOMLINSON, A., WAGSTAFF, A., AND WARD, S.M. 1979. The role of cartilage canals in the formation of secondary centres of ossification. *J. Anat.* 129:493-506.
- LAZELL, J.D., JR. 1980. New England waters: critical habitat for marine turtles. *Copeia* 1980(2):290-295.
- LEVENE, C. 1964. The patterns of cartilage canals. *J. Anat.* 98:515-538.
- LUTC AVAGE, M.E., BUSHNELL, P.G., AND JONES, D.R. 1990. Oxygen transport in the leatherback sea turtle *Dermodochelys coriacea*. *Physiol. Zool.* 63(5):1012-1024.
- LUTC AVAGE, M., AND LUTZ, P.L. 1986. Metabolic rate and food energy requirements of the leatherback sea turtle, *Dermodochelys coriacea*. *Copeia* 1986(3):796-798.
- LUTFI, A.M. 1970a. Mode of growth, fate and functions of cartilage canals. *J. Anat.* 106:135-145.
- LUTFI, A.M. 1970b. Study of cell multiplication in the cartilaginous upper end of the tibia of the domestic fowl by tritiated thymidine autoradiography. *Acta Anat.* 76:454-463.
- OGDEN, J.A. 1980. Chondro-osseous development and growth. In: Urist, M.R. (Ed.). *Fundamental and Clinical Bone Physiology*. Philadelphia: J.B. Lippincott, pp. 108-171.
- PALADINO, F.V., O'CONNOR, M.P., AND SPOTILA, J.R. 1990. Metabolism of leatherback turtles, gigantothermy, and thermoregulation of dinosaurs. *Nature* 344:858-860.
- PARFITT, A.M. 1976. The action of parathyroid hormone on bone. Relation of bone remodeling and turnover, calcium homeostasis, and metabolic bone disease. Part I. Mechanisms of calcium transfer between blood and bone and their cellular basis: morphological and kinetic approaches to bone turnover. *Metabolism* 25:809-844.
- PRITCHARD, P.C.H. 1971. The leatherback or leathery turtle *Dermodochelys coriacea*. IUCN Monogr. 1:1-39.
- RHODIN, A.G.J. 1985. Comparative chondro-osseous development and growth of marine turtles. *Copeia* 1985(3):752-771.
- RHODIN, A.G.J., OGDEN, J.A., AND CONLOGUE, G.J. 1980. Preliminary studies on skeletal morphology of the leatherback turtle. *Marine Turtle Newsl.* 16:7-9.
- RHODIN, A.G.J., OGDEN, J.A., AND CONLOGUE, G.J. 1981. Chondro-osseous morphology of *Dermodochelys coriacea*, a marine reptile with mammalian skeletal features. *Nature* 290:244-246.
- RHODIN, J.A.G. 1968. The ultrastructure of mammalian venous capillaries, venules and collecting veins. *J. Ultrastruct. Res.* 25:452-500.
- RHODIN, J.A.G., AND FUJITA, H. 1989. Capillary growth in the mesentery of normal young rats. Intravital video and electron microscope analyses. *J. Submicrosc. Cytol. Pathol.* 21:1-34.
- RHODIN, J.A.G., AND LAMETSCHWANDTNER, A. 1993. Circulatory pattern and structure in the tail and tail fins of *Xenopus laevis* tadpoles. *J. Submicrosc. Cytol. Pathol.* 25:297-318.
- SCHENK, R.K., SPIRO, D., AND WIENER, J. 1967. Cartilage resorption in the tibial epiphyseal plate of growing rats. *J. Cell Biol.* 34:275-283.
- SCHULZ, A., DELLING, G., RINGE, J.D., AND ZIEGLER, R. 1977. Morbus Paget des Knochens. Untersuchungen Ultrastruktur des Osteoclasten und ihrer Cytopathogenese. *Virchows Arch. Pathol. Anat.* 376:309-328.
- SILVESTRI, G., RICORDI, M.E., AND BONUCCI, E. 1979. Resorption of uncalcified cartilage in the diaphysis of the chick embryo tibia. *Cell Tissue Res.* 196:221-235.
- SORRELL, J.M., AND WEISS, L. 1980. A light and electron microscopic study of the region of cartilage resorption in the embryonic chick femur. *Anat. Rec.* 198:513-530.
- SORRELL, J.M., AND WEISS, L. 1982. The cellular organization of fibroblastic cells and macrophages at regions of uncalcified cartilage resorption in the embryonic chick femur as revealed by alkaline and acid phosphatase histochemistry. *Anat. Rec.* 202:491-499.
- STUMP, C.W. 1925. The histogenesis of bone. *J. Anat.* 59:136-154.
- TAKEMURA, R., AND WERB, Z. 1984. Secretory products of macrophages and their physiological functions. *Am. J. Physiol.* 246:C1-C9.
- WILSMAN, N.J., AND VAN SICKLE, D.C. 1970. The relationship of cartilage canals to the initial osteogenesis of secondary centers for ossification. *Anat. Rec.* 168:381-392.
- WILSMAN, N.J., AND VAN SICKLE, D.C. 1972. Cartilage canals, their morphology and distribution. *Anat. Rec.* 173:79-94.
- ZUG, G.R., AND PARHAM, J.F. 1996. Age and growth in leatherback turtles, *Dermodochelys coriacea* (Testudines: Dermochelyidae): a skeletochronological analysis. *Chelonian Conservation and Biology* 2(2):244-249.

Received: 24 March 1994. Accepted: 18 October 1995.