ABSTRACT

Carol Zalewski Lunney. OSSIFICATION OF THE STAPEDIAL CRUS OF THE RAT: AN ELECTRON MICROSCOPIC STUDY. (Under the direction of Dr. Everett Simpson) Department of Biology, December, 1971.

The stapedial crus undergoes extensive remodeling from a cartilagenous column at birth to a crescent-shaped osseous structure at adulthood. This study investigates the fine structural changes that occur as the cartilagenous crus undergoes ossification. The first appearance of calcification is seen at day 8. By day 14 the newly formed calcified cartilage is being resorbed by osteoclasts. Typical brush borders are seen. The final osseous crescent shape is determined by day 18, after which time little bone formation or resorption occurs.

OSSIFICATION OF THE STAPEDIAL CRUS OF THE RAT: AN ELECTRON MICROSCOPIC STUDY

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DEDICATION

This thesis is dedicated to my parents; to my husband, David; and to my very best friend, Sam.

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ABBREVIATIONS

AC- anterior crus

ACr- apatite crystals

AM- amorphous matrix

Bas- basal body

BB- brush border

CaM- calcified matrix

Can- canaliculus

Cbl- chandroblast

Ccy- chondrocyte

Cem, CL- cement line

Cen- centriole

Cil- cilium

col- collagen

CP- cytoplasmic processes

CV- coated vesicles

DC- degenerating cell

F- fibrils

Fbl- fibroblast

FP- footplate

GG- golgi

Gly- glycogen

Gr- granule

H- head

Lip- lipid

M- matrix

Mi- mitochondria

Mf- microfilaments

MPcy- micropinocytosis

MT- microtubules

N- nucleus

Nk- neck

nu- nucleolus

Obl- osteoblast

Ocl- osteoclast

Ocy- osteocyte

OF- obturator foramen

Opr- osteoprogentitor cell

PC- posterior crus

Pch- perichondrium

Pcy- phagocyte

PM- particulate matter

Prib- polyribosomes

PV- pinocytotic vesicles

RBC- red blood cell

RC- reticular cell

RE- ruffled edge

RER- rough endoplasmic reticulum

Rib- ribosomes

S- satellite

Sc- spider cell

Vac- vacuole

Ves- vesicle

INTRODUCTION

The small amount of work reported in the literature on the ultrastructure of the stapes (Dass, 1966; Reydon, 1968; Chevance, 1969) has been limited to human tissue obtained from cadavers or by surgical stapedectomy. An extensive literature search resulted in little information concerning comparative studies of laboratory animals. From the point of view of an osteogenic study, the small and slender crus of the stapes is an inviting specimen to an electron microscopist. Bone is cut for electron microscopy with the greatest difficulty. Therefore, a specimen that is small enough to be sectioned intact and without decalcification is valuable in an osteogenic study. The stapedial crus fulfills all of these requirements.

In a previous study using this material, Tyndall (thesis, 1970) found that the rat stapes, which is preformed in cartilage, undergoes a tremendous morphological change from birth to adulthood. The material observed by Tyndall included specimens taken at birth, day 21, day 52 and adulthood. The greatest change in morphology and cell structure appeared to occur between birth and day 21. Tyndall hypothesized that these extreme morphological changes were caused by much bone deposition and resorption; however, her specimens were not taken at close enough intervals to observe these processes.

The purpose of this study, then, was to elucidate the ultrastructural changes that occur as the stapes undergoes extensive remodeling from a cartilagenous structure at birth to an ossified structure at adulthood. Special attention was given to the periods between birth and day 10 when bone is initially deposited and day 14 to day 18 during which time the most remodeling occurs.

REVIEW OF LITERATURE

The stapes is shaped as its name suggests, in the form of a stirrup, and is the last in the series of the three ear ossicles which extend from the tympanic membrane to the oval window of the inner ear. Structurally it consists of a head, neck, two lateral extensions (the anterior and posterior crura), and the base or footplate (Plate 1). This ossicle received its name in 1546 when it along with the oval and round windows were described by the Italian anatomist Ingrassin (Shambough, 1967).

In the human stapes, the anterior crus (crus rectilineum) is generally the slenderer and straighter of the two crura. The posterior crus (crus curvilineum) is curved. The stapedius muscle is attached to the neck in seventy-five per cent of the cases; in the others it is located at the shoulder of the posterior crus (Bast and Anson, 1949).

Development of the human stapes in many ways parallels that of the rat stapes although there do appear to be a few differences. The human stapes is fully ossified several weeks before birth (345 mm fetus; 38 weeks) (Bast and Anson, 1949) but the newborn rat approximates a 120-140 mm human fetus in development (Marovitz, 1968). Strong (1925) observed three small irregular ossifications in one-day rats. He stated that these appeared to be centers for the ear ossicles, which were clearly defined at eight days. A more recent study disclosed ossification in the rat auditory ossicles at day 4 (Marovitz, 1971).

Information relating to the type and formation of bone comprising the auditory ossicles is almost non-existent. Linck et al. (1967) and Shambough (1967), state that the ossicles of the adult human are unique in that the endochondral bone derived from cartilage remains unchanged from the time of ossification during fetal life throughout the life of the individual. Ham (1969) contends that the ossicles are atypical long bones. Besides these observations, an osteogenic study of the fine cellular changes that occur as the ossicles undergo development is lacking.

I. Evolutionary Development of the Stapes

The evolutionary development of the hearing mechanism, including the auditory ossicles, has been the subject of much debate. Goodrich (1930), Torrey (1962), Ballard (1964), Hopson (1966), Thomson (1966), Webster (1966), and Shambough (1967) have all contributed reviews on this subject. What follows here is a summary of these reviews.

The most primitive hearing organ consisted of a simple fluid-filled sac which contained sensory cells in contact with a dense but mobile membrane. This type of hearing mechanism is still evident in present-day crustaceans and some fishes. However, as animals moved onto land, the hearing organ underwent tremendous change. The two major divisions of the hearing mechanism in present-day mammals come from different anlagen: the sound-perceiving neurosensory apparatus of the inner ear from the ectodermal otocyst; and the sound-conducting mechanism from the branchial or gill structure. The first gill cleft becomes the

external auditory meatus and tympanic cavity with an intervening tympanic membrane. The cartilages of the first and second branchial arches on either side of the first gill cleft were modified to form a lever-like ossicular chain from the tympanic membrane to the small oval window. This middle ear pressure transformer matches the difference in acoustic impedance between air and water (Shambough, 1967).

Mammals are the only vertebrates to have a middle ear chamber containing a three-ossicle chain. The malleus and incus, derived respectively from the mandibular and palatoquadrate cartilages of the mandibular segment, first appeared in mammalian species (Thomson, 1966). The evolutionary origin of the stapes, however, can be traced to the upper part of the hyomandibular element. Comparative anatomical and embryological evidence suggests that the ear drum-stapes originated in ancestral fish. The connection of the operculum to the hyomandibular in fishes is homologous with the tympanic process of the tetrapod stapes (Goodrich, 1930; Ballard, 1964; Thomson, 1966).

In embryos of various tetrapod species, the upper skeletal rudiment of the hyoid arch forks at its superior end into a medial prong which differentiates into an ear ossicle, the columella, while the lateral prong becomes lost or appears as the stapedial ligament in mammals.

In dipnoi (lungfishes) and sometimes in amphibia, the columella fuses with the ear capsule and is lost as an individual structure. In the rest of tetrapods it remains free, filling the fenestra vestibuli of the otic capsule (Ballard, 1964).

The rod-like stapes of the reptile becomes considerably shortened in mammals. It is practically confined to the fenestra ovalis and may correspond only to the proximal portion of the reptilian stapes (Torrey, 1962). At one end of the expanded footplate fits into the oval window and at the other end the mammalian stapes forms a joint with the incus. With few exceptions the stapes contains a tiny perforation, the pathway of an embryonic artery which dries up and disappears during development as other arteries invade its territory. In the few mammalian species (rats, bats, cetaceans) in which this stapedial artery persists to adult stage, it still pierces the stapes, just as it runs through its precartilagenous anlage (Thomson, 1966; Webster, 1966).

Since mammals are the only vertebrates with three ossicles, it has been hypothesized that the elaborate ossicular chain adds protection to the cochlea. This arrangement gives the advantage of having two muscles instead of one available to damp loud sounds (Shambough, 1967).

II. Types of Bone Formation

Before discussing the embryological origins of the stapes, the various types of ossification should be reviewed.

Embryologically, all bones develop from mesoderm. However, differences in differentiation of the primitive mesenchyma make it possible to distinguish two main types of bone: 1) membrane bone, like the flat bones of the face and skull, and 2) cartilage bone, like the long bones. This latter type is also characteristic of the otic capsule and auditory ossicles (Bast and Anson, 1949; Weinmann, 1955).

Membrane bone, the development of which is termed intramembranous ossification, is formed directly from the mesenchymal blastema. In this case the mesenchymal cells give rise directly to the osteoblastic cells. In cartilage bone, however, the mesenchyme differentiates into precartilage and true hyaline cartilage before ossification begins. The development of this type of bone is termed intracartilagenous ossification, and may be divided into three types: perichondrial, endochondral, and intrachondrial bone. Each future bone in this case is preceded by a bar of cartilage which is surrounded by a layer of connective tissue, termed the perichondrium. The outer edge of this perichondrium consists of compact mesenchyma while the inner cells develop into true chondrocytes. When a portion of this cartilage is about to develop into bone, the chondrocytes enlarge and the matrix becomes impregnated with calcareous salts. At this time the deep layer of the perichondrium takes on the characteristics of an epitheloid membrane and becomes the osteogenic layer, giving rise to osteoblasts. In this manner bone is deposited on the underlying calcified cartilage and is termed perichondrial bone. From this point on, the outer layer of cells is called the periosteum.

In this change from cartilage to bone, vascular osteogenic buds develop and penetrate the newly-formed perichondrial bone. They enter the lacunae of the enlarged chondrocytes and remove the necrotic cartilage cells. Osteoblasts develop from the osteogenic bud and deposit fibrous tissue and calcareous salts within these excavated areas while the areas not filled with bone develop into primary bone marrow. The bone formed in these areas is known as endochondral or replacement bone.

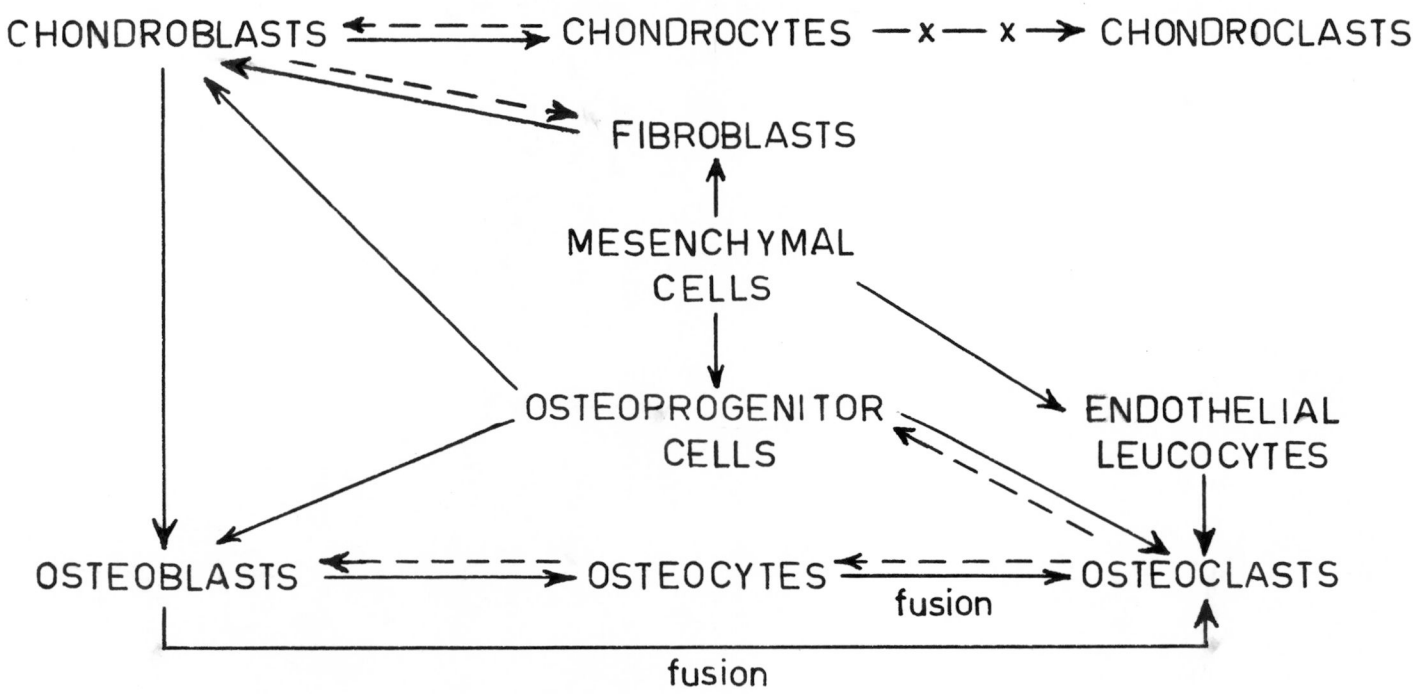
When the osteogenic buds do not remove the calcified cartilage, as they do in endochondral development, the resulting calcified cartilage with true bone filling the lacunae is referred to as intrachondrial bone (Bast and Anson, 1949). This mosaic tissue is held to be characteristic of the bone of the otic capsule and the auditory ossicles (Weinmann, 1955).

In summary, perichondrial and endochondral bone develop, as do membrane bones, in membranous connective tissue; however, they differ from membrane bones in one very important respect: the first deposit of calcareous material occurs on the surface of a remnant of calcified cartilage which is eventually removed.

III. Cell Types Involved in Ossification

As mentioned previously all bones develop from mesoderm with the stem cell in both types of ossification being mesenchymal. Factors involved in further differentiation of this cell into cartilage or bone are not well understood. Diagram 1 outlines the cell types that may be found in bone that is undergoing either intramembranous or intracartilagenous ossification. Arrows show the direction in which the cells may differentiate. As is evident from the diagram, the process of ossification is a dynamic system with many cell types having the ability to dedifferentiate under certain conditions.

Mesenchymal cells are embryonic connective tissue cells with an outstanding capacity for proliferation and further differentiation. Besides developing into cartilage and bone, these cells may also differentiate into phagocytes (Ham, 1969).



——→ Normal differentiation

– – → Dedifferentiation

—x→ Proposed in 1930, but unsubstantiated by recent observation

Diagram 1. Cell Types Involved in Ossification.

Osteoprogenitor cells, also called "reticular" or "spindle" cells, retain both osteogenic and hemopoietic capabilities. They are identified by their location, morphology, and direct origin from mesenchymal cells (Young, 1963). Ham (1969) suggests that when osteogenic cells differentiate in the presence of capillaries, they differentiate into osteoblasts. If they proliferate and differentiate in a non-vascular environment, where it is assumed the oxygen content is low, they differentiate into chondroblasts and form cartilage.

Fibroblasts are elongate cells that generally have little surface contact with other cells and are frequently separated by a collagenous matrix. The cytoplasm contains prominent RER (rough endoplasmic reticulum), golgi, mitochondria, and a large prominent nucleus. The plasma membrane shows some undulations and occasional microvillous projections (Anderson, 1967; Gould, 1968). This cell type functions in the production of collagen. It is believed that amino acids enter the cell membrane, join with ribosomes on RER, and are incorporated into various proteins that are stored in the RER cisternae. From here the material moves to the plasma membrane to be released into the matrix (Gould, 1968).

Chondroblasts are round to oval cartilage cells having much RER, golgi, large amounts of glycogen, and large cytoplasmic lakes that might be reservoirs of secretory material (Ross, 1968). Vesicular golgi may be seen containing small granules and fibrils about 80 Å in diameter. These are probably sulfated mucopolysaccharides, precollagen, or both (Anderson, 1967).

As the cartilage cell matures to become a chondrocyte, the nucleus becomes centrally located within the cell. There may be a decrease in the amount of glycogen present and the surface of the cell is deeply scalloped. The granular and fine non-periodic filaments seen in the chondroblast may also be found in the chondrocyte, possibly in larger quantities. Also, fat droplets are a normal feature of chondrocytes (Sokoloff, 1969).

In 1930 Weidenreich coined the term "chondroclast" to mean multi-nucleated cells which resorb dead tissue resulting from the ossification of calcified cartilage (Bourne, 1961). However, this term is not present in recent literature. Weidenreich's chondroclast may very well be the multi-nucleated macrophages mentioned by another (Weinmann, 1955).

Osteoblasts are the surface cells of growing or developing bone (Frost, 1963; Robinson, 1964). They are united by intercellular bridges and may lengthen and develop anastomosing processes. The nucleus is always near the surface that is not in contact with the bone; golgi and mitochondria are present. A vacuole near the nucleus may contain an attraction sphere and centrioles (Weinmann, 1955).

If bone formation is at a high level, osteoblasts are irregularly cuboidal (Weinmann, 1955; Frost, 1963) or pear-shaped and contain many mitochondria but no glycogen (Bourne, 1956). If bone formation is slow, the osteoblasts appear squamous (Weinmann, 1955; Pritchard, 1956).

The chief function of the osteoblast is to form collagen (Frost, 1963). During deposition of bony matrix osteoblasts contain large amounts of granular glycoproteins which may be utilized in the production

of the ground substance (Bourne, 1961). It has been suggested that RER cisternae of osteoblasts may occasionally break open through the plasma membrane and liberate their contents (Frost, 1963).

When an osteoblast has become completely surrounded by calcified matrix, it is then termed as osteocyte. It always retains contact with the surface cells through its anastomosing processes; the pathway for this connection is termed a canaliculus. In humans and canines, no osteocyte is more than .3 mm from a blood vessel (Robinson, 1964). Osteocytes are rich in glycogen, but their RER is not as profuse as in the osteoblast (Frost, 1963). The cytoplasm is granulated and slightly basophilic; they may contain fat droplets; they have mitochondria and golgi but undergo no mitosis (Weinmann, 1955). If an osteocyte escapes the matrix, it may become an active osteoblast again, and then become embedded in new matrix once more (Robinson, 1964). Also, osteocytes may arise directly from cartilage cells or freed cartilage cells in endochondral bone formation may become osteoblasts and give rise to osteocytes indirectly (Pritchard, 1956).

Kolliker in 1873 first used the term "osteoklast" for multinucleated cells of bone and associated them with resorptive processes. He identified the brush border and assumed that the cell had a phagocytic function (Woessner, 1968). There are many suggestions as to how the osteoclast forms. One theory is that they result from a coalescence of wandering endothelial cells (endothelial leucocytes) (Cameron, 1961) which may be regarded to be histiocytes (Hancox, 1956). Another theory

suggests that osteoclasts are derived from the fusion of osteocytes that were liberated from bone (Frost, 1963) or by the fusion of several osteoblasts. Although the fusion has never been seen, the RER typical of osteoblasts is often seen in osteoclasts (Robinson, 1964).

Although it was believed for a time that osteoclasts were degenerative cells, ultrastructural studies prove differently. The cells are typically multinucleated (they may have 1-100 nuclei), contain numerous mitochondria (which may contain dense bodies between the cristae) (Chu'an, 1931; Robinson, 1964), much endoplasmic reticulum and small and numerous golgi. The cytoplasm appears foamy and the cell may have extensile processes (Frost, 1963).

Scott and Pease (1956) were among the first electron microscopists to examine bone resorption, which is the most important function of the osteoclast. They concluded that the "ruffled border" (infolding of plasmalemma) functions in phagocytosis and extracellular digestion of collagen.

Osteoclasts may be found in shallow grooves called Howship's lacunae (Weinmann, 1955) but in any case they must operate on a free surface (Harris, 1970). Cinematography shows an "active sweeping motion of the osteoclast with a bubbling, boiling activity at the surface and in the cytoplasm as the bone "melts" away. Some of the breakdown products are taken into the cell (Goldhaber, 1963; Hancox, 1961).

During the life span of an osteoclast (about 48 hours) it resorbs six to eight times the volume of bone that may be deposited by an osteoblast in its life span of several weeks (Hancox, 1956; Johnson, 1966; Harris, 1970).

The fate of osteoclasts is another point of discussion. They may undergo local degeneration, followed by phagocytosis of their remains. Some believe they may revert to osteoblasts or reticular cells. In this form, they may find their way through vessels and enter the circulation (Hancox, 1956).

Recently, much information has been reported concerning the theory of bone resorption. Entire reviews have dealt solely with this aspect of ossification. In summary, however, it will be mentioned that bone resorption may be caused, inhibited, or accelerated by any one, or all, of the following factors: enzyme production (Harris, 1970), change in pH at the resorption site (Vaes, 1968; Harris, 1970), circulatory disturbance in the bone nutritive tissue (Weinmann, 1955), change in Ca^{++} ions in the blood serum as controlled by the parathyroids (Cameron, 1967) and the presence or absence of pyrophosphate (Owen, 1965; Woessner, 1968; Harris, 1970; Guyton, 1971).

IV. Embryological Development of the Stapes

In 1949, Bast and Anson published a book entitled "The Temporal Bone and the Ear" in which a detailed description of the development and ossification of the human auditory ossicles is given. Most of the general concepts described at that time have remained undisputed. The following is a summary of the development of the human stapes from embryo to adulthood, as described by Bast and Anson.

In the human, the malleus and incus both form from the first branchial or mandibular arch but the stapes is formed from two sources: Reichert's cartilage of the second branchial (hyoid) arch and cleft and the wall of the otic capsule. At 16 weeks (fetal) the ossicles are fully formed in cartilage. Ossification of the stapes begins at a solitary center on the obturator aspect of the base and then spreads along each crus toward the head of the ossicle. In a 160 mm fetus erosion of this newly formed bone is well under way. This erosion renders the ossicle foraminous on the obturator surface and the bone in this area is removed by osteoclasis. At 210 mm, the internal periosteal wall of the crura, base and head are entirely removed, together with all bone and associated marrow; the base becomes a deeply sculptured plate, the crura become C-shaped members and the neck an excavated cylinder. Once destroyed, the bone and associated tissues are never restored and the resulting form remains similar through adulthood.

Since the current study specifically involves the ossification process of the rat stapedial crus, a brief summary of bone development of the human crus may be in order.

Each crus in the 100 mm fetus is a cartilageous column. The hyaline cartilage is then altered and eroded by invasive buds of osteogenic tissue which causes the cartilage to be dissolved away while undergoing calcification. Externally the formation of an osseous shell keeps pace with the progress of internal dissolution, so that a solid cartilagenous cylinder is converted into a hollowed bony tube whose cavity contains a primitive marrow but almost no endochondral bone. Up

to this stage in morphogenesis, the regular processes of intracartilagenous ossification are at work. However, after this, the periosteal shell of each crus undergoes extensive erosion on its inner surface which is rendered extremely foraminous. Bone of endochondral type is so slight in amount that the crura are almost completely periosteal in derivation, and their cavities contain no cancellous bone. The periosteum ceases to be active as soon as the osseous shell is formed so the crural tube does not increase in circumference. Neither end has an epiphyseal plate; therefore, there is no increase in length. Terminally, each crus resembles a miniature tibia halved longitudinally. After the marrow cavity is laid open by erosion, mucous membrane, with submucosal tissue, replaces the embryonal marrow (Bast and Anson, 1949).

V. Osteogenesis of the Otic Capsule

The formation and development of the otic capsule is similar to that of the ossicles in that they are all preformed in cartilage. Since much research on the otic capsule has been reported, its study may shed light on the development of the auditory ossicles.

The otic capsule consists of a thin layer of compact bone and the inner surface is lined by a thin periosteum. It is preformed in cartilage and gradually replaced by bone. The cartilage removal involves an edemic degeneration (not hypertrophy) of the chondrocytes and calcification of the intercellular substance. Only then is the intercellular substance removed by the activity of osteoclast-like cells, while the chondrocytes, whose capsules are opened, necrotize; their remnants are then removed by macrophages (Weinmann, 1955).

During growth of the cartilagenous otic capsule the destruction of cartilage is by direct mucoid degeneration, and then by necrosis of the hyaline cartilage without preceding enlargement of chondrocytes or calcification of the intercellular substance. Weinmann (1955) calls these degenerating chondrocytes "spider cells". Necrotic debris of cartilage is removed by macrophages.

Perichondrial bone forms an outer and inner "periosteal" layer in the development of the bony otic capsule. Only the middle layer develops by replacement of cartilage.

In this process, enlargement of the chondrocytes by intranuclear and intracellular edema and calcification of the intercellular substance precede the resorption of the cartilage and the invasion of the excavated spaces by proliferating connective tissue. The young connective tissue invades the cartilage and the opened cartilage capsules are frequently filled with bone tissues. One or more osteocytes can then be seen in the area formerly occupied by an edematous chondrocyte. The irregular paths of invasion cause the more prominent appearance of what has been previously described as intrachondrial bone; that is, bone tissue entirely filling empty lacunae of the cartilage.

Once the otic capsule has fully developed, any signs of internal reconstruction by balanced resorption and apposition seem to be almost negligible (Weinmann, 1955).

METHODS AND MATERIALS

All animals used in this study were albino rats, Ratus norvegicus, Holtzman strain. Their diet consisted of Purina Laboratory Chow and tap water.

Three fifty-five day old females were bred and both they and their offspring provided the material for the study. Offspring of both sexes were randomly taken at birth, 4, 6, 8, 10, 14, 18, 21, and 52 days. The original females were sacrificed at approximately one hundred and twenty-five days.

The animals were sacrificed by cervical dislocation under light ether anesthesia.

Access to the stapes required removing the lower jaw first and then entering the tympanic or middle ear cavity through the external auditory meatus. The malleus and incus were removed, the stapedial ligament was severed and then the stapes was gently removed from its seat at the oval window.

Stapes from the right and left ears of the animals were immediately fixed for two hours at 4°C in 3% glutaraldehyde (Fisher) buffered with .1M sodium cacodylate at pH 7.4. This pH and temperature were maintained throughout fixation and dehydration. Specimens were washed several times in .1 M sodium cacodylate buffer, post-fixed for two hours in 2% osmium tetroxide buffered with .1M sodium cacodylate, and dehydrated for thirty minutes each in 30%, 50%, 70%, 95% and 100% ethanol. Two changes of 15 minutes each in propylene oxide insured complete dehydration.

Infiltration of the material with resin was accomplished by placing the specimens in vials containing a mixture of 1 part propylene oxide to 1 part Araldite 6005 resin (Fullam) for 1 hour under a vacuum of 15 in. Hg. After this time an equal volume of resin was added to the vials and the material was placed under vacuum overnight. The following day the material was placed for 2 hours in fresh resin containing 2% accelerator. The stapes were then individually oriented and embedded in flat silicone molds (Ladd) and polymerized at 55°C for 48-72 hours.

Both thick (5000A) and thin (600A) sections were cut on a Sorvall Porter-Blum MT2-B ultramicrotome using glass and DuPont diamond knives. To prevent demineralization of the bone the water on which the sections were floated was maintained at pH 8.0 (Boothroyd, 1964). Sections were picked up on either uncoated, 200 mesh or parlodian-coated, carbon-stabilized 100 mesh grids.

Sections for light microscopy were stained one minute with toluidine blue or saffarin 0, or boht, and studied and photographed using a Nikon L-Ke or Zeiss WL Research microscope equipped with a Microflex Nikon AFM camera attachment.

Thin sections were double-stained for 5 minutes each with lead citrate (Reynolds, 1963) and 2% aqueous uranyl acetate. The specimen studied with scanning electron microscopy was first shadowed with carbon, then gold-palladium, and viewed with a Jeolco SM-3 scanning electron microscope. A Hitachi HS-8 was used for all transmission electron microscopy observations. Photographs were taken using Kodak LR film and prints were made with a Durst Laborator 138S enlarger with a "Multipoint" high resolution light source.

RESULTS AND DISCUSSION

1. Gross Morphology

The human stapes is fully ossified several weeks before birth; this is not the case in the laboratory rat. In rats, stapes removed at birth are entirely cartilagenous in nature. Although this cartilage model appears dense in the central regions of the stapes, the tissue lining the obturator foramen is very loose. The quantity of this loose tissue decreases noticeably in later stages and by day 8 is not present around the stapes. By day 10 and day 14 the crura take on a definite stirrup shape and scattered pitting can be seen on the obturator surface. By day 18, erosion of the obturator surface becomes evident, causing the crus to assume a C-shape which by day 21 is clearly defined (Plate 1, Fig. A). The crura retain this crescent morphology to adulthood. Some erosion of the head and neck region also occurs (Plate 1, Fig. B).

In the development of the human stapes, the stapedial artery pierces the obturator foramen in the early stages but is usurped by other arteries by the time the stapes is fully ossified. However, in all stages from birth to adulthood the stapedial artery of the rat stapes continues to pierce the obturator foramen.

The anterior crus is readily identified in humans because it is the slenderer of the two crura. Differences in the rat crura are less pronounced and therefore it is difficult to distinguish the two.

2. Light Microscopy

A series of cross sections through the rat crura from birth to adulthood (that is, Birth, 6, 8, 10, 14, 18, 21 and 52 days and Adult) show evident progressive morphological changes (Plate 2, Figs. A - I). The size differences seen can be attributed to individual variation of animals as well as difficulty involved in sectioning the same area in each specimen. At birth the crus consists of hyaline cartilage (Plate 2, Fig. A). The chondrocytes are the largest in the center and are contained within lacunae which are widely separated by an amorphous, hyaline matrix. Chondroblasts are seen bordering the inner margin of the perichondrium. Light microscopy shows little change in cellular detail from Birth to day 6 (Plate 2, Fig. B) although electron microscopy discloses significant change which will be discussed later. By day 6, a rounding and slight hypertrophy of the chondrocytes is visible. In both newborn and 6 day old specimens, a large amount of loose connective tissue surrounds the perichondrium. This type of tissue becomes scanty by day 8 (Plate 2, Fig. C) and is not present in older specimens (Plate 2, Figs. D - I). The most significant difference between days 6 and 8 is the appearance of calcified material in the matrix of the later stage. During this interval the chondrocytes hypertrophy and undergo edemic degeneration. Several cells clump together forming a "cell nest" within lacunae (Ham, 1969). The matrix is impregnated with calcium salts and isolates the cell nests from each other. Some areas of the matrix show invasion by other cell types.

These may be the macrophages described by Weinmann (1955) or the chondroclasts of Weidenreich (Bourne, 1961).

By day 10 (Plate 2, Fig. D) the center of the crus is almost devoid of its previously calcified matrix but an outer shell of perichondrial bone is present. The central cells appear to be degenerating; chondrocytes are no longer recognizable and are replaced by reticular cells. Some of the cells seen at this stage may be precursors of the forthcoming vascular channels. By day 14 (Plate 2, Fig. E), two large blood vessels have penetrated the center of the crus and there is a scarcity of calcified matrix on the obturator surface. At the edge of the calcified matrix, cells are frequently seen in depressions called Howship's lacunae. These cells are possibly osteoclasts, which are involved in resorption of the matrix. Although the obturator surface is still composed of reticular cells, the lateral surface is covered by a prominent periosteum.

A C-shaped crus is first evident in day 18 specimens (Plate 2, Fig. F). A large amount of loose tissue still clings to the obturator surface but no calcified matrix other than that of the newly formed crescent is present. A prominent blood vessel still courses its way along the obturator edge.

The rat stapes reaches definitive shape and size by day 21 (Plate 2, Fig. G). Day 52 and Adult crura show insignificant changes (Plate 2, Figs H - I). In the three oldest specimens, osteocytes appear scattered throughout the bony matrix. The periosteum is very thin, and a fair amount of loose tissue is still seen in days 21 and 52 along the obturator surface.

In humans and canines an osteocyte is never found to be more than .3 mm from a blood vessel (Robinson, 1964). Since the maximum width between the two ends of the mature crus is .2mm and the thickness ranges from 25 to 50 microns, there is probably no need for vascularization within the crus itself.

3. Electron Microscopy

Birth: The perichondrium at this stage consists of long, slender cells with oval nuclei which are probably fibroblasts (Plate 3). At the inner surface the cells increase in size although they are still somewhat flattened. These are young cartilage cells, or chondroblasts, which exhibit a large nucleus and profuse RER, but only a few mitochondria. The center-most cells are the mature chondrocytes. These cells, as well as their nuclei, appear round and contain much RER. Large vacuoles in close proximity to the RER are scattered throughout the cytoplasm. Phagocytosis may be observed at this stage. In such cases there is a dark outer cell containing a dense nucleus and ER, and an inner, lighter cell containing large vacuoles and some RER of its own. It appears that the outer cell engulfs and phagocytizes the inner one (Plate 3). Tyndall (1971) previously reported a similar process to be autolysis. The matrix at this stage of development is mostly amorphous material with a few scattered fibers. In the fibroblast area where the cells are liberating matrix rapidly, the matrix appears more dense than it does between the mature cartilage cells. Cross and longitudinal sections of collagen fibers (exhibiting periodicity) are present in the area of loose connective tissue but no periodicity is recognizable in the fibers scattered throughout the cartilage matrix.

Day 4: The greatest ultrastructural change noticed between birth and day 4 (Plate 4) is the relative abundance of lipid granules in cells of the later stage. These lipid droplets are noticeable in both the

cells of the perichondrium and maturing cell areas. (Since lipid substances are found at sites that are in the process of calcifying, they are proposed to act as seeding sites in the initiation of calcification (Richelle and Dallemagne, 1965). Both the chondroblasts and chondrocytes exhibit abundant RER and the matrix contains more fibrous material at this stage than it did at birth. Ciliary bases are observed in several of the cell types present at this stage (i.e. fibroblasts, chondrocytes). Further remarks on the presence of these organelles will be made later.

Day 6: On first glancing at the cross-section of a day 6 specimen (Plate 5) one would suppose that the material is improperly fixed. However, on closer observation, it is realized that the muddy appearance is due to the presence of much particulate matter in the matrix of both the cartilage and perichondrium. The granular effect in the cells is the result of the presence of large quantities of glycogen. This phenomenon of glycogen accumulation in hypertrophic and/or senescent chondrocytes has long been recognized and a role in matrix calcification is proposed (Harris, 1932).

Compared to a day 4 specimen, the chondrocytes at day 6 do not increase much in size although the nuclear cytoplasmic ratio appears greater. RER is still present but appears slightly edemic. There are many vesicular bodies. It has been suggested that discharge of intracellular materials in the vesicles into the matrix may play a role in calcification (Cameron, 1963; McLean & Urist, 1968). A definite

delineation is seen between cartilage cells and the perichondrium (arrows). This boundary consists of fibrous material running parallel to the perichondrial border. This is probably collagen, because collagen is thought to be a necessary foundation for the deposition of the calcified material (Glimcher & Krane, 1968).

Scattered throughout the crus are cells that appear to have undergone shrinkage and condensation (Plate 6, Fig. A). These cells have been called "spider" chondrocytes (Anderson, 1967) probably because the cytoplasm is so vacuolated that it looks like a spider's web.

Other chondrocytes at day 6 appear to elaborate matrix. These cells contain many vesicles (Plate 6, Fig. B) which, when observed at higher magnification (Plate 6, Fig. C) are seen to contain both fibrous and particulate material. It is thought that the vesicle, when filled with material, moves to the periphery of the cell and ruptures. This discharge of intracellular materials into the matrix may play a role in calcification (Cameron, 1963; McLeon & Urist, 1968).

Day 8: The first evidence of true bone deposition is seen in a day 8 specimen (Plate 7). At this time dense, amorphous material is deposited in the matrix between clusters of cartilage cells. Cells at the center of the crus undergo enormous edemic changes. This cannot be called hypertrophy because the cytoplasmic content does not increase but the cells fill with fluid; hence, the term edemic. The nuclei are pycnotic, the scant endoplasmic reticulum is dilated, the cytoplasm is tenuous and contains many large vacuoles. Few mitochondria remain. The cellular picture in this area of the crus is one of dissolution.

At the same time that this process occurs in the center of the crus, the outside edge undergoes dramatic changes of another sort (Plate 8). The inner surface of the perichondrium of day 6 begins to undergo calcification. Possibly, some of the fibroblasts of the day 6 perichondrium become spindle-shaped osteoprogenitor cells. At this stage (day 8) a cell that appears to have been a chondroblast might now be surrounded by bone to become an osteocyte.

At this time, then, two types of bone are evident. In the center, calcified cartilage exists while the bone formed at the edge appears to be of the perichondrial type.

The ultrastructural changes that occur from birth to day 8 are summarized in Plate 9.

Day 10: After the center of the crus is impregnated with calcified matrix (calcified cartilage) and its periphery consists of perichondrial bone, the cell nests containing the chondrocytes are shut off from any blood supply (Plate 10). At this time the obturator surface consists of a layer of fibroblast and reticular cells. What happens to the isolated chondrocytes is debatable. There are at least two possibilities: 1) the chondrocytes lose the excess fluid that was present in their edemic condition and assume osteogenic potencies, (intrachondrial bone formation), or 2) they necrotize leaving lacunae that are filled by reticular cells that have osteogenic or hemopoetic potencies, (endochondral bone formation). In either case by day 10 cells are seen

isolated by calcified material, singly or in groups. These cells have eccentric nuclei, and highly vacuolated cytoplasm. Their cell surfaces rarely touch the edge of lacunae in which they are found. Although both collagen and bone crystals are seen to protrude into these lacunae, no definite resorptive sites can be recognized.

Day 14: In corroberation of light microscopy, electron microscopy shows that extensive erosion and vascularization of existing tissues in the crus occur about day 14. At this time resorption sites called "brush borders" are numerous at the obturator surface (Plates 11 - 16). At high magnifications the brush border is seen to consist of free crystals approximately 500 Å long at the edge of the bone surface (Plate 14). This size is in agreement with previous findings (Anderson, 1964). Associated with these crystals are multinucleated cells containing much RER and many mitochondria (Plate 15). These are called osteoclasts; they function in removal of the fine crystals. At the bone surface their cell membrane appears "ruffled" due to an extensive infolding of the plasmalemma. The crystals are trapped in these folds and through pinocytosis are taken into the cell. Deeper in the osteoclast, mitochondria may also be seen to contain crystals (Plate 14, Fig. 13). How the crystals enter the mitochondria is questionable. To my knowledge, micropinocytosis has not been seen at the mitochondrial surface. However, it has been suggested that calcium does accumulate within the mitochondria and when it reaches certain levels, will precipitate as apatite crystals within the organelles (Gonzales and Karnovsky, 1961; Roodyn, 1967; Shapiro and Greenspan, 1969).

In summary, calcification of the cartilagenous crus begins at day 8, is complete at day 10, and resorption begins by day 14.

Day 18: Resorption of the calcified cartilage from its central area gives the crus its crescent shape. By day 18 resorption is at a minimum and no typical brush borders are seen. A layer of periosteal tissue surrounds the entire crural surface (Plate 2).

The cell types involved in intramembranous ossification can be recognized at day 18. Osteoblasts and osteocytes are seen at the periosteal border (Plate 16, Fig. A). The osteoblasts exhibit cytoplasmic extensions. It is through these extensions that the cells retain their contact with the periosteum after they are surrounded by bone and become osteocytes. In a cross-section of the crus at day 18 small openings containing cytoplasmic material are seen. These are called canaliculi; they are the small canals formed as the calcified material surrounds the cytoplasmic extensions (Plate 20, Fig. B). Both the osteoblasts and osteocytes exhibit large nuclei. At high magnifications, an osteoblast may be seen to have micropinocytotic vesicles at the surface of the bone. RER is abundant and centrioles are sometimes found (Plate 17). Other cell types are mingled with the osteoblasts at the obturator surface. Several granulated cells with polymorphic nuclei can be seen (Plate 18). The cells may function in the secretion of mucous, since the stapes is covered with submucosal tissue in the adult stage (Bast and Anson, 1949). However, the cells also resemble mucrophages, granulated leucocytes or mast cells, all of which would be reasonable assumptions since blood vessels still traverse this area.

Days 21 and 52: Since Tyndall (1971) discussed extensively both day 21 and day 52 specimens, only brief mention will be made here of these stages. After the crus obtains its crescent shape, little bone deposition or resorption is noticeable. Flattened cells with oval nuclei and many mitochondria surround the crus (Plate 20, Fig. A). Canaliculi provide a connection of these cells to the osteocytes within the bone (Plate 20, Fig. B). In several instances a cement line may be seen (Plate 21). This is a dense line formed when bone resorption ceases and bone deposition once again begins, as stimulated by the needs of the organism. When such a need arises, cells may become osteocytes or osteoclasts and bone deposition or resorption once again begins; hence, the reason for calling bone a dynamic tissue.

4. Specialized Structures

Cilia: Cilia have been reported in all tissues of birds and mammals except bone and blood (Scherft, and Daems, 1967; Rash, 1969; Wheatley, 1969; Boquist, 1970; Dubois and Giroud, 1970). Therefore, it is not surprising to find these organelles in the hyaline cartilage of the young crus. They are observed in almost all developmental stages treated in this study and are especially prominent in material taken at Birth, day 4, and day 6. Both cross and longitudinal sections through cilia are observed (Plate 22). Each cilium consists typically of a basal body and a shaft. The basal body consists of a circle of nine triplets of microtubules with the center of the body having no definite

tubular arrangement. The microtubular arrangement of the shaft is one of nine doublets. The lack of a centrally located pair of tubules indicates that the cilia observed in this study do not function in motility (Sleigh, 1962).

Although, as mentioned above, cilia have not previously been found in bone or blood, an interesting cell with a cilium was found in an 18 day old crus that was completely ossified (Plate 23). In fine structure this cell resembles the osteocytes described by Jande and Belanger (1971); that is, it is surrounded by collagen and calcified matrix, has extensile processes, and exhibits a polymorphic nucleus, numerous vacuoles and much ribosomal material. The tubular arrangement of the cilium seen in this cell is unknown since it was sectioned tangentially.

The possible function of a cilium in an osteocyte is unknown; however, ciliary structures found in other tissues are thought to have any of the following functions; secretory, chemoreceptor, sensory, motile or rudimentary, depending on their location and internal structure (Boquist, 1970). Rash et al. (1969) stated that "the abrupt transformation from mitotic replicative tissue to nonmitotic structuring tissue is correlated with the disappearance of centrioles and the formation of cilia." A later study by Fonte et al. (1971) found no such correlation; cilia were found in both dividing and non-dividing cells.

Coated vesicles: "Bristle-coated" or "dense-rimmed" vesicles were observed in several of the cell types involved in the ossification process. Fiend and Farquhar (1967) have distinguished two different types:

1) Large coated vesicles (about 1000 A in diameter) located in the apical cytoplasm and that have 18-25 bristles. 2) Small coated vesicles (about 750 A in diameter) found mostly in the Golgi region have 8-13 bristles. The larger vesicles are believed to act as heterophagosomes (cells that take up and degrade large molecules by incorporation of protein within membrane pockets followed by segregation and digestion within lysosomes) for the transportation of absorbed proteins to the lysosomes. Their origin is the cell surface by pinocytic invaginations of the apical cell membrane. From this point they move inward and fuse with multivesicular bodies. The content of the smaller vesicle is generally more granular than the larger ones and are thought to serve as primary lysosomes by transporting hydrolytic enzymes from their source at the Golgi complex to their site of action, the multivesicular bodies.

Although the larger vesicles were not found during this study, many of the smaller (750 A) coated vesicles were observed, (Plate 24). They are similar to those mentioned by Friend and Farquhar (1967) in both size and the number of radial bristles (8-13). Although they appear at times to be free in the cytoplasm, they are also seen in close proximity to the Golgi apparatus. In several instances, it appears that the vesicles might actually be pinching off from the Golgi cisternae (Plate 24, Figs. B and C). Since the coated vesicles in this study were found primarily in osteoblasts or osteocytes, they may be functioning in the transport of mucopolysaccharides for the production of collagen.

CONCLUSION

In the present study ossification of the rat stapedial crus was not observed until day 8. This is inconsistent with previous findings by Strong (1925) who observed ossification centers in one-day old rat stapes and Marovitz (1971) who found evidence of ossification in stapes of four-day old rats. These discrepancies have probably resulted from the type of study made in each case. Dass and Makhni (1966) state that ossification centers can be visualized earlier in "cleared specimens" than in histologically stained sections. Strong (1968) used cleared material. The studies made by Marovitz (1971) involved detection of acid mucopolysaccharides as a determining factor for ossification. Since this substance is probably present in large amounts before obvious calcification occurs, there would be an earlier detection time. At day 8 the crus consists of an inner core of calcified cartilage and an outer shell of perichondrial bone. By day 10 invasion of the obturator surface by reticular cells is evident. At this time intrachondrial bone appears to be present in the largest quantities. However, by day 14 extensive erosion and vascularization of the crus occurs. This is accomplished by osteoclasts which resorb the intrachondrial bone at sites called "brush borders." At the same time that resorption occurs on the obturator surface, periosteal bone is deposited at the lateral surface. By day 18 all of the temporary bone is removed and the crus is crescent-shaped. Cells that are typical of intramembranous ossification (osteoblasts, osteocytes) are recognized

at the lateral surface and several unidentified cells containing granules are seen lining the obturator surface. Although these may be granular leucocytes, mast cells, or macrophage cells, it is strongly believed that they could be mucous secreting cells since the stapes is covered with submucosal tissue in the adult specimen. From day 21, only insignificant changes in cellular detail are seen. The periosteum is thin and consists of flattened cells. These are probably inactive osteoprogenitor cells. When the organism is stimulated in some manner to produce or resorb bone, these cells may then become respectively osteoblasts or osteoclasts.

Two specialized structures are noted in this study. They are cilia and coated vesicles. Single cilia are found in many cells at all stages studied, but were most numerous in cartilage tissue from Birth to day 6. This was not surprising since Scherft and Daems (1967) previously discovered single cilia in chondrocytes. However, an 18 day old specimen was found to exhibit an osteocyte containing a single cilium. Further work will be necessary to support this finding.

"Bristle-coated" vesicles of the small size (750 A diameter) described by Friend and Farquhar (1967) are seen in many of the cells involved in ossification. What appeared to be the actual formation of coated vesicles from the Golgi apparatus was recognized in several cases. Since the Golgi apparatus is involved in the formation of collagen (Gould, 1968) it is probable that these small coated vesicles contain mucopolysaccharides.

PLATE 1, A: A light micrograph of a 21 day rat stapes. The arrow shows the point of attachment of the stapedial ligament. 40 X.

PLATE 1, B: A scanning electron micrograph of an adult rat stapes. Note the crescent shape of the crura as well as the well defined rim of the footplate. 40 X.

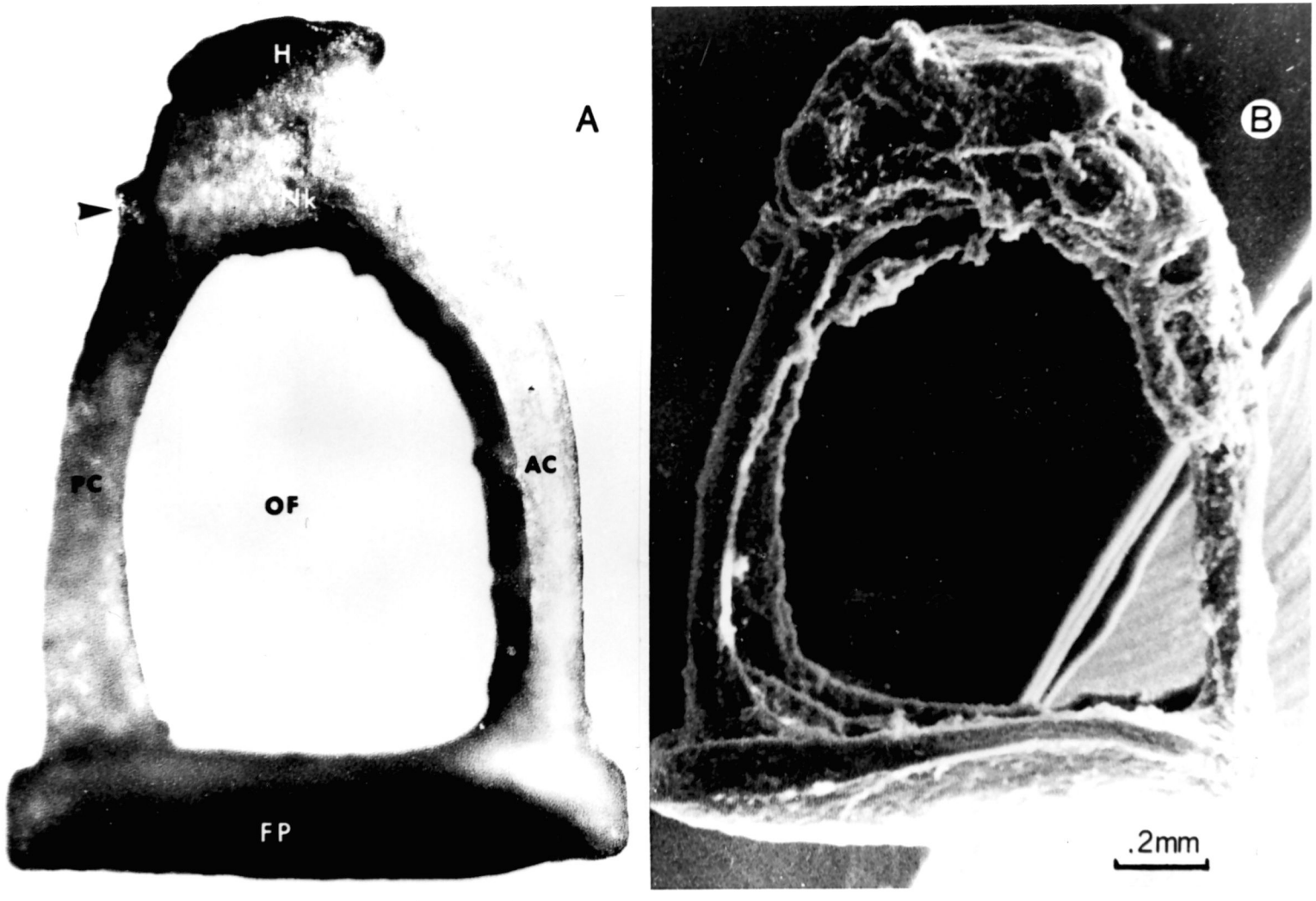


PLATE 1

PLATE 2, A - I: A series of light micrographs showing cross sections of crura from birth to adulthood. At birth (A) the crus consists of typical hyaline cartilage with mature chondrocytes (center) surrounded by a perichondrium. By day 6 (B) the chondrocytes become slightly edemic. Calcified material (black areas) is first evident at day 8 (C). The center cells enlarge tremendously and clump together forming "cell nests." By day 10 (D) an outer shell of calcified material is recognizable. Some of the central cells are reticular and may be precursors to the vascular system. Two prominent blood vessels pierce the crus of the day 14 specimen (E). At this time an outer shell of bone is prominent at the lateral surface (right upper portion). The obturator surface shows signs of erosion. The C-shape is first seen at day 18 (F). This specimen is surrounded by much loose tissue. By day 21 (G) the crus has reached its definitive size and shape. Little erosion occurs here or in day 52 (H) or adult (I) specimens. Note the thin periosteum in the adult (I) crus. 200 X.

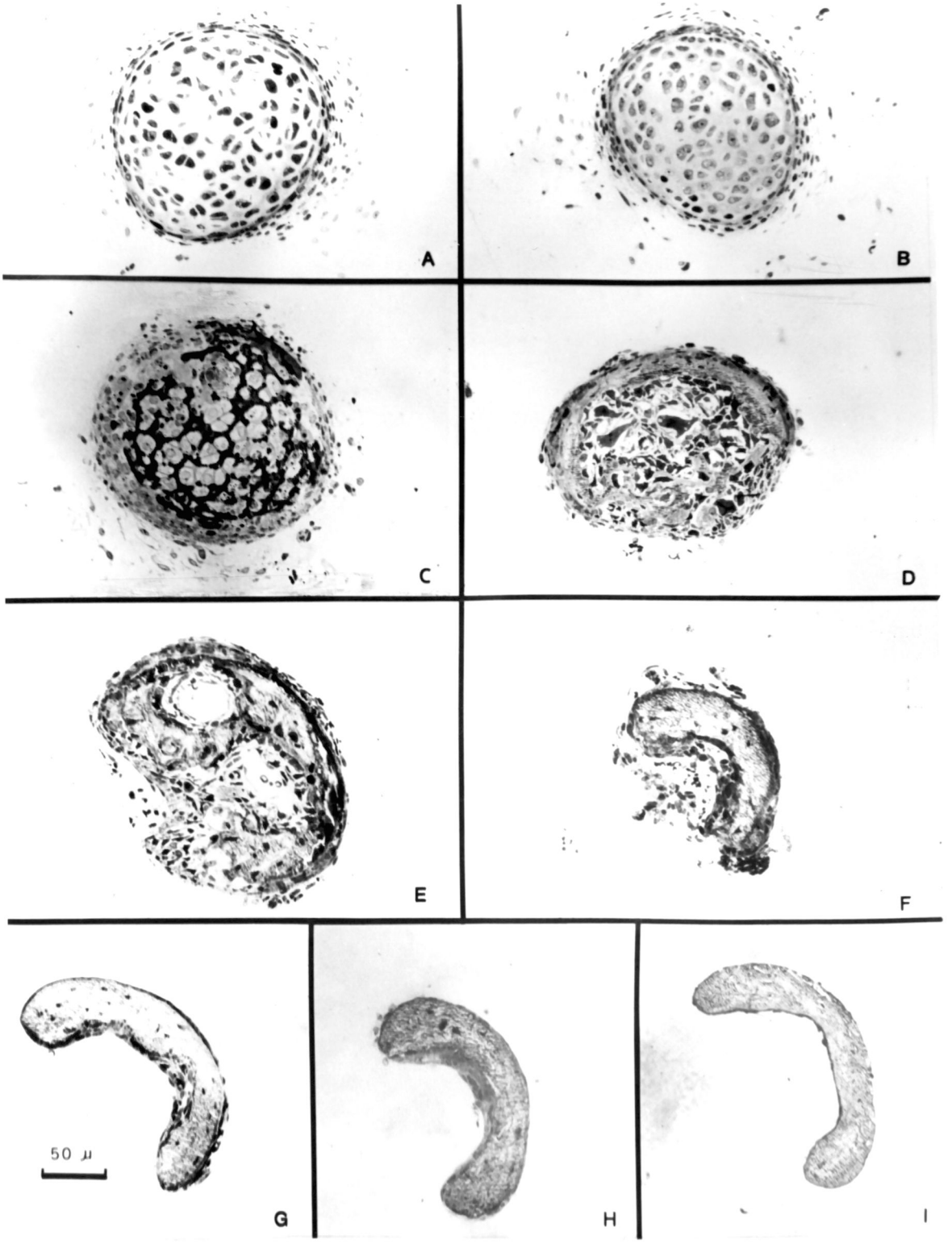


PLATE 2

PLATE 3. An electron micrograph mosaic of a crural cross section at birth showing both chondroblasts and chondrocytes surrounded by an amorphous matrix. A thick periosteum contains fibroblasts and collagen fibers. An outer dark cell (right side) containing a dense nucleus and ER appears to be engulfing the inner, lighter cell which contains many vacuoles and some ER of its own. 5000 X.

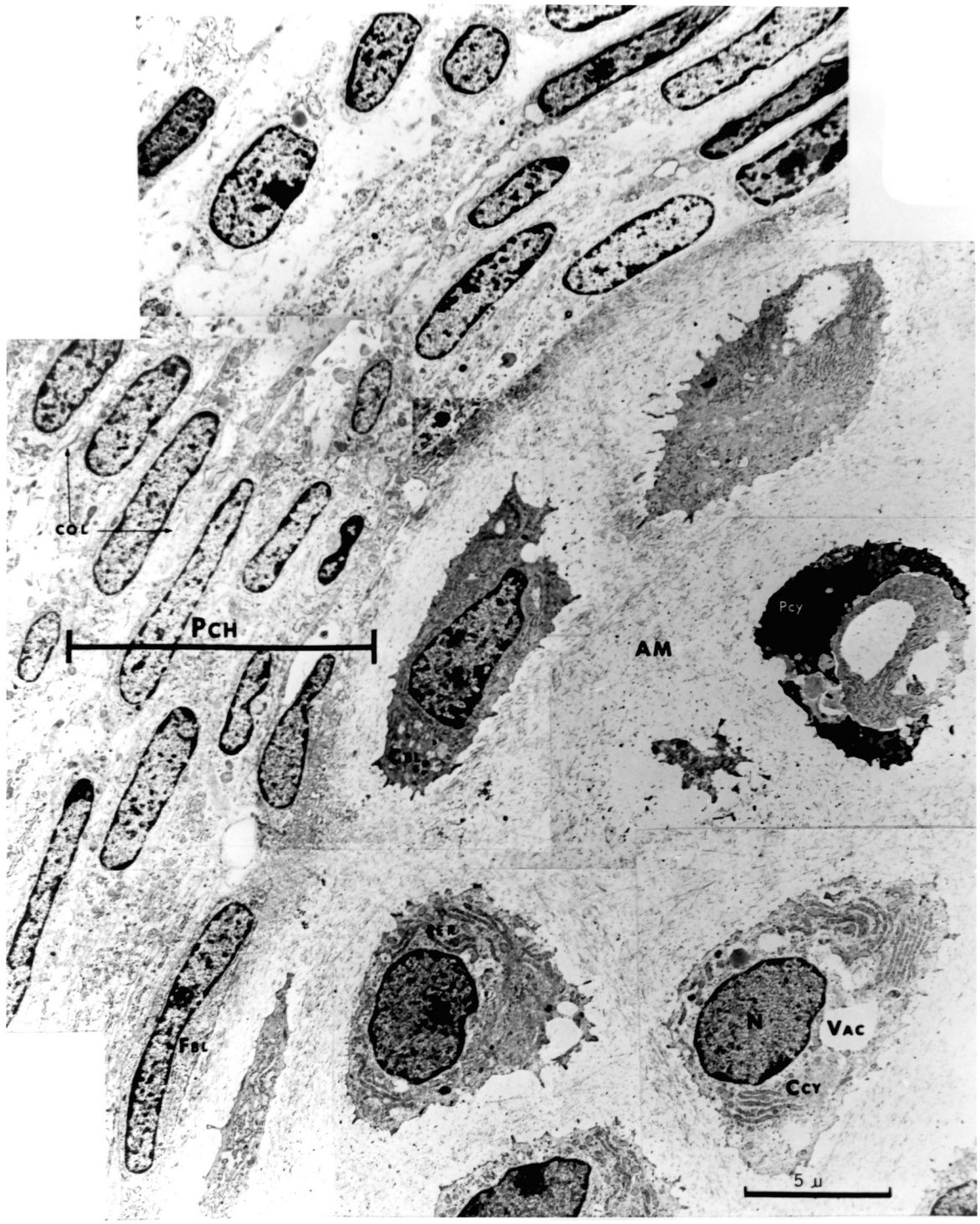


PLATE 3

PLATE 4. An electron micrograph mosaic of a day 4 specimen. Note the presence of much lipid in all the cell types. At this time the matrix surrounding the cartilage cells contains many small fibrils. Ciliary bases (arrows) are seen in both a chondrocyte and fibroblast. 6000 X.

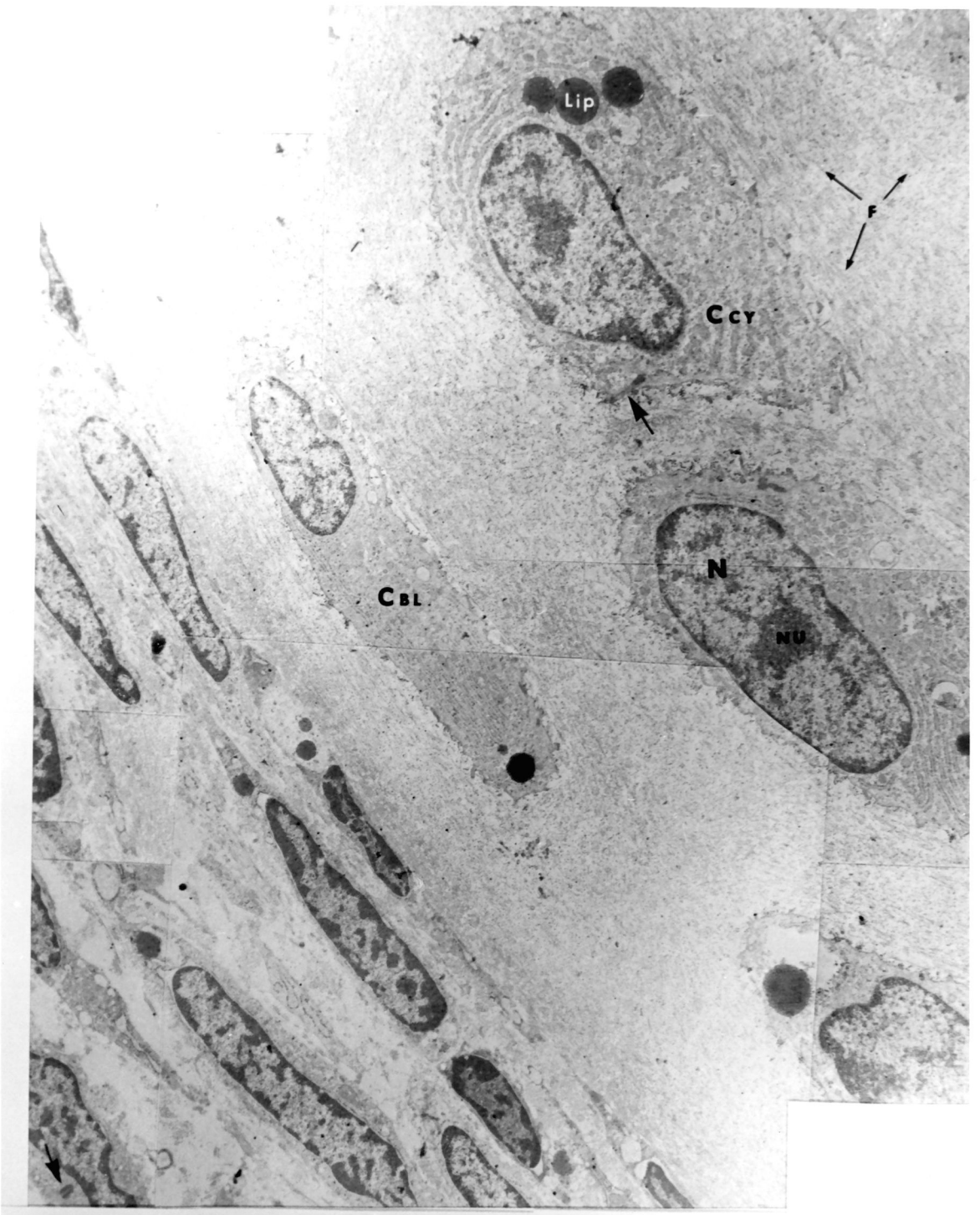


PLATE 4

PLATE 5. An electron micrograph mosaic of a day 6 specimen. Note the vesicular nature of the chondrocytes and the fibrillar appearance of the matrix. Arrows show the delineation of the changing periosteum. 4500 X.

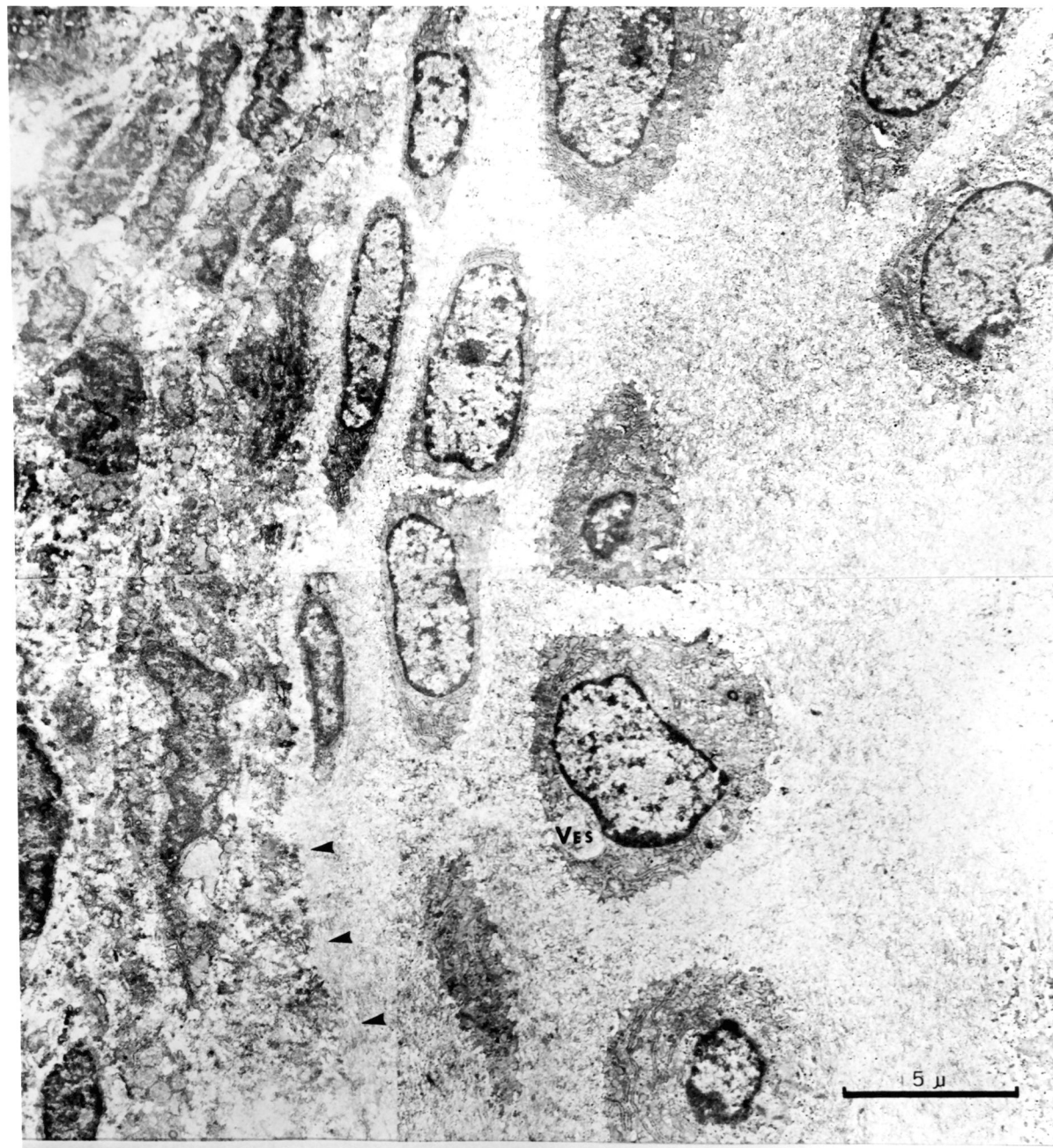


PLATE 5

PLATE 6, A. A senescent chondrocyte of a day 6 specimen, often called a "spider cell" because of the web-like appearance of the ER. Large empty spaces between the ER are suggestive of degeneration. An adjacent chondrocyte, however, appears normal with its extensive RER. Particulate matter is seen at the cell membrane in both cells. 10,000 X.

PLATE 6, B. A typical chondrocyte of a day 6 specimen showing the scalloped edges, a vesicular nature and much glycogen. 10,000 X.

PLATE 6, C. An enlargement of Fig. B. The large vesicles contain fibrous and particulate matter. Possibly these are precursors of collagen. Also much glycogen is seen within the cell. 50,000 X.

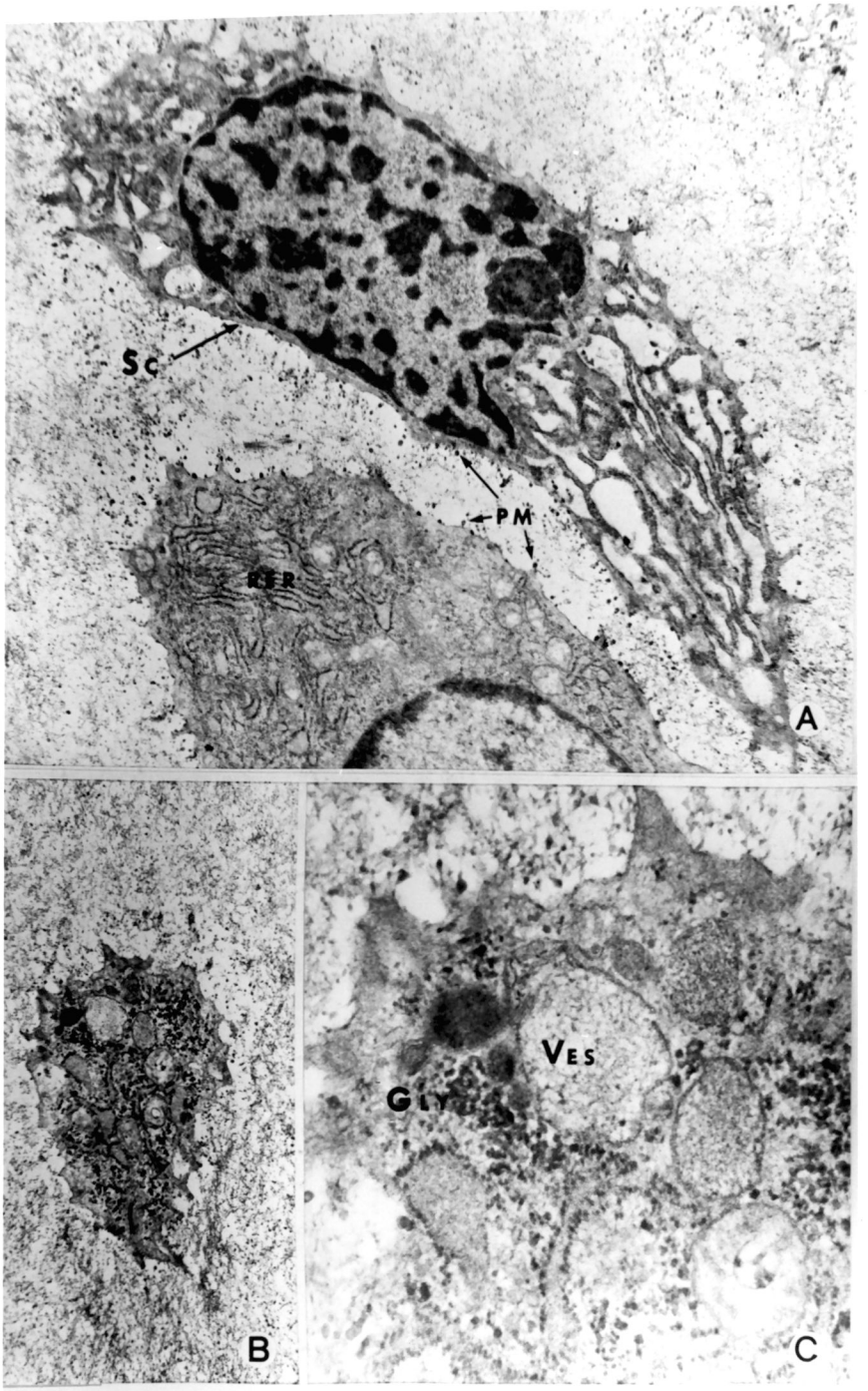


PLATE 6

PLATE 7. This electron micrograph mosaic is taken from the center of a cross section of a day 8 crus. The first appearance of calcareous salts is seen in this day 8 specimen. White areas represent the heaviest deposits of calcium in the matrix. The chondrocytes have undergone edemic degeneration. Their cytoplasm is thin and vacuolated and the nuclei are pycnotic. The matrix is calcified in such a way that the chondrocytes are surrounded singly, or in groups, by the tempory bone. 6300 X.

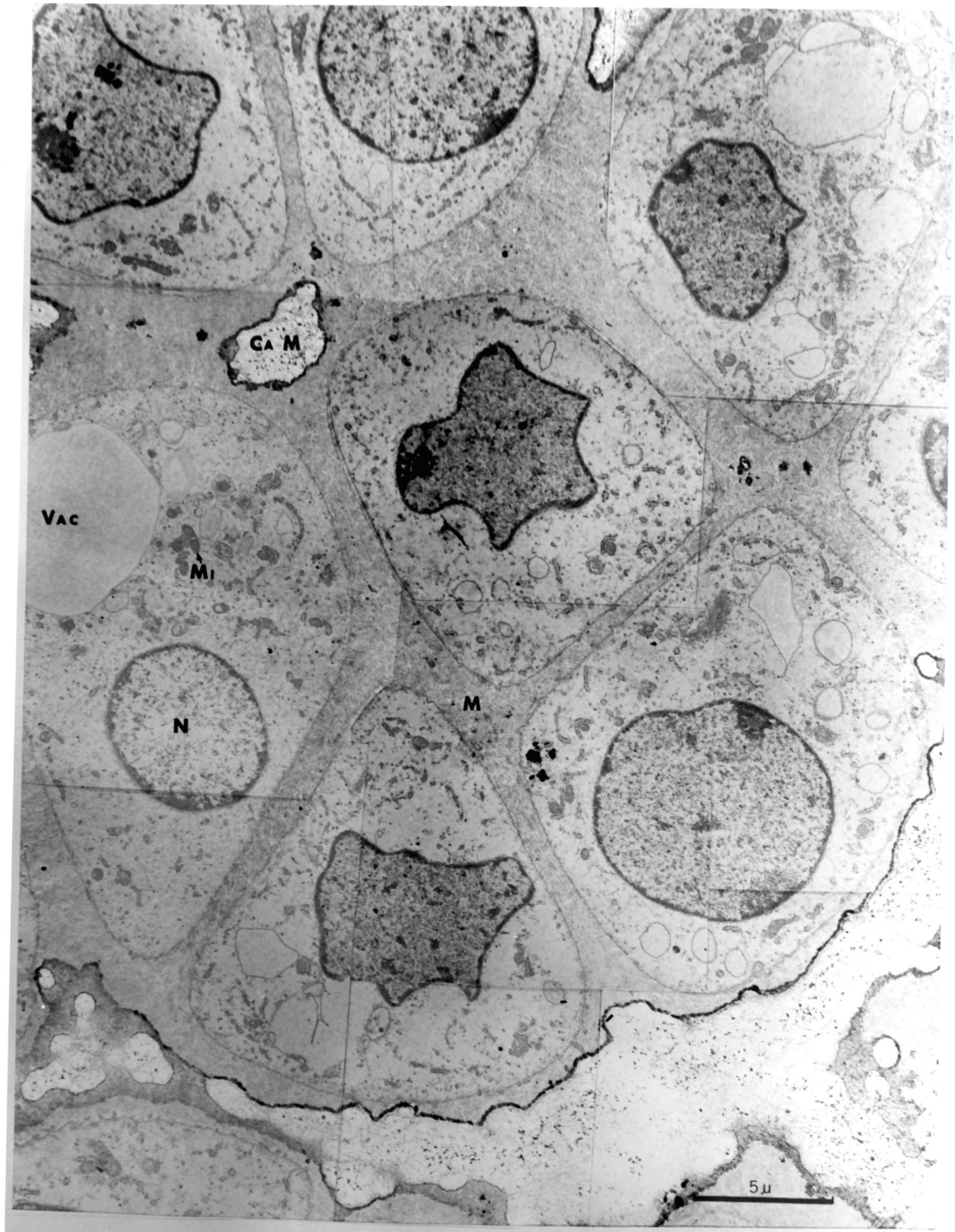


PLATE 7

PLATE 8. The outer edge of a day 8 specimen showing calcified matrix (black areas). Note the large amount of collagen present at this time. The perichondrium of day 6 has given way to osteoprogenitor cells. A cell that was probably a chondroblast at day 6 now appears to be an osteoblast. The bone seen in this mosaic could probably be termed perichondrial. 4200 X.

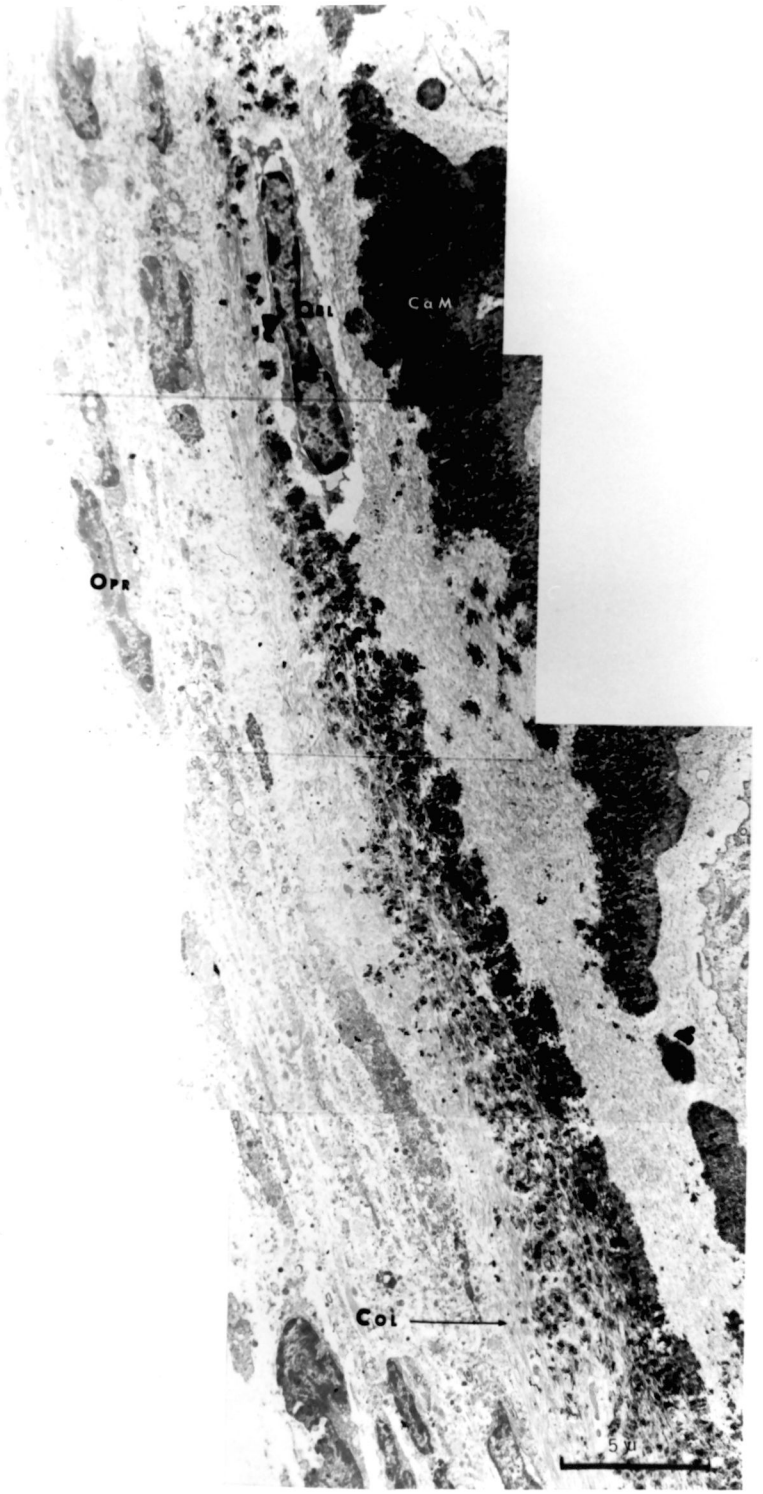


PLATE 8

PLATE 9. A summary of cell and matrix changes from birth to day 8. At birth (Plate 9, Figs A & B) the centermost cells attain their maximum size. The matrix is thin, amorphous and contains a few scattered fibers. Day 4 (Plate 9, Figs C & D) shows the presence of much lipid; the matrix becomes very fibrillar in nature. About day 6 (Plate 9, E & F) the tissue takes on a granular appearance. This material seen in the cells is glycogen. The matrix, at this stage, contains fibrillar material upon which dense particles rest. These particles are also seen in large quantities along the cell border. They are possibly seeding sites for future calcification. By day 8 (Plate 9, G & H) both the cells and their nuclei become edemic. The matrix contains five fibers that appear to have some periodicity. Upon these fibers the first calcified material rests. From this time, the tissue is termed calcified cartilage, a temporary type of bone. A, C, E, G. 3500 X; B, D, F, H. 16000 X.

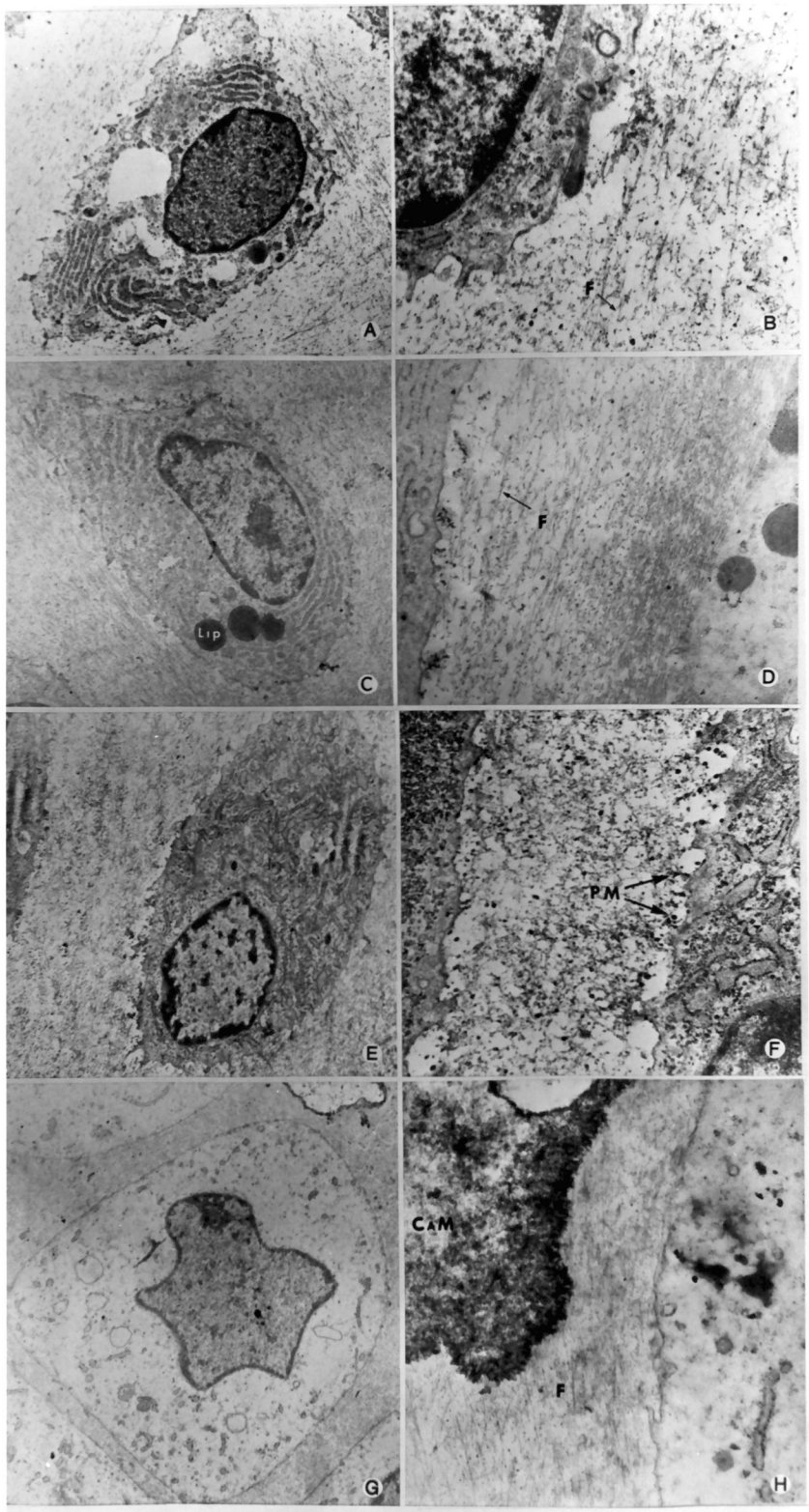


PLATE 9

PLATE 10. An electron micrograph mosaic of a day 10 specimen from the obturator edge (left) to the inner portion of the crus (right). The cell (?) surrounded by calcified matrix is probably a chondrocyte. 1,700 X.

PLATE 10

PLATE 11, A: Day 14. A brush border and an osteoclast containing two nuclei are seen. 5600 X.

PLATE 11, B. An enlargement of Plate 11, A showing the free apatite crystals at the brush border. 25,000 X.

PLATE 11, C. At the edge of a day 14 specimen an osteocyte that is surrounded by calcified matrix is seen in close proximity to a blood vessel. 5600 X.

PLATE 11, D. Day 14. A cell that appears to be degenerating is surrounded by calcified matrix. 5600 X.

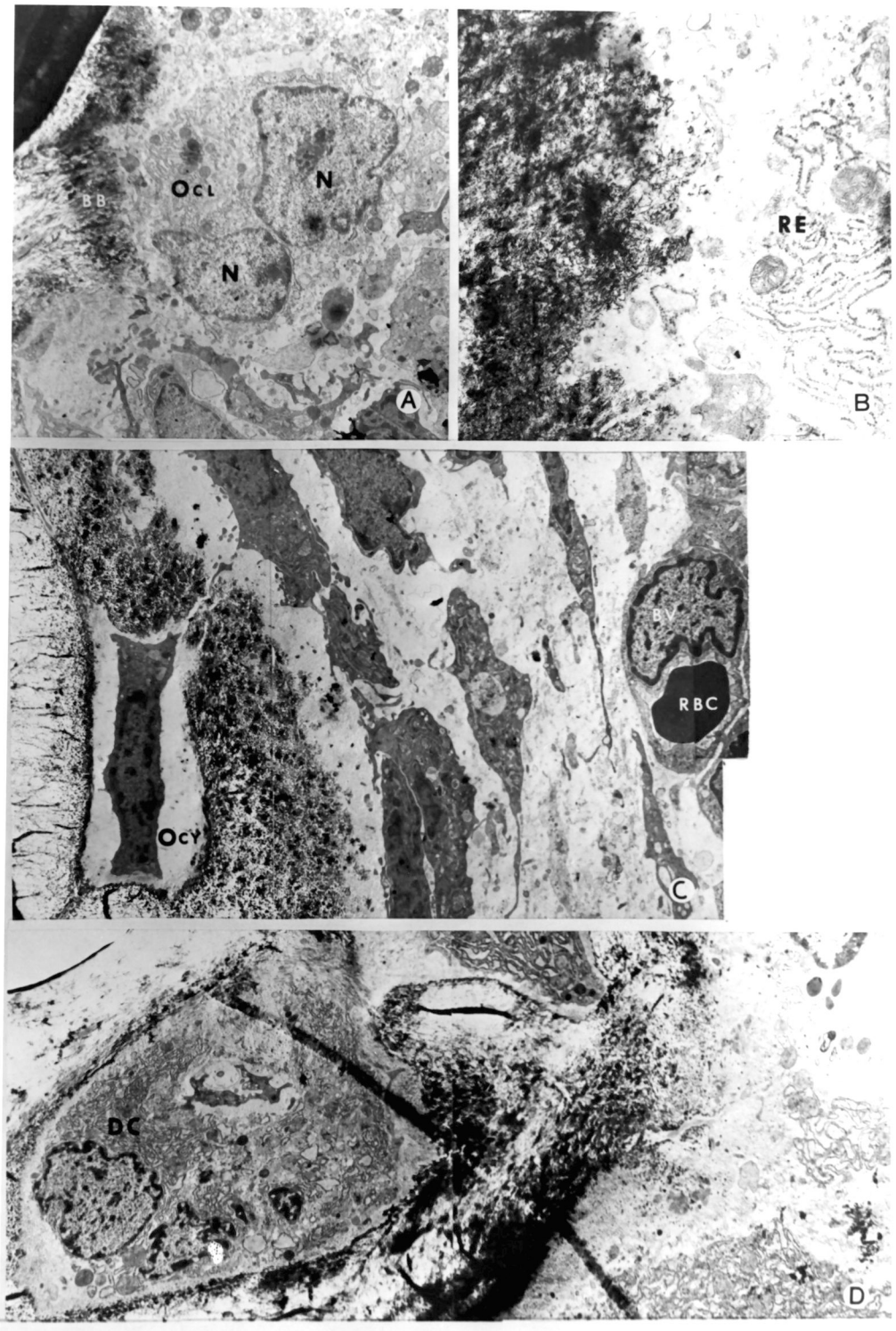


PLATE 11

PLATE 12, A. A chondrocyte surrounded by calcified matrix that will be eventually resorbed. 10,000 X.

PLATE 12, B. An enlargement of Plate 12, A showing the collagen periodicity and much RER. 30,000 X.

PLATE 12, C. Another enlargement of Plate 12, A which shows dilated RER and mitochondria that appear to contain apatite crystals. 30,000 X.

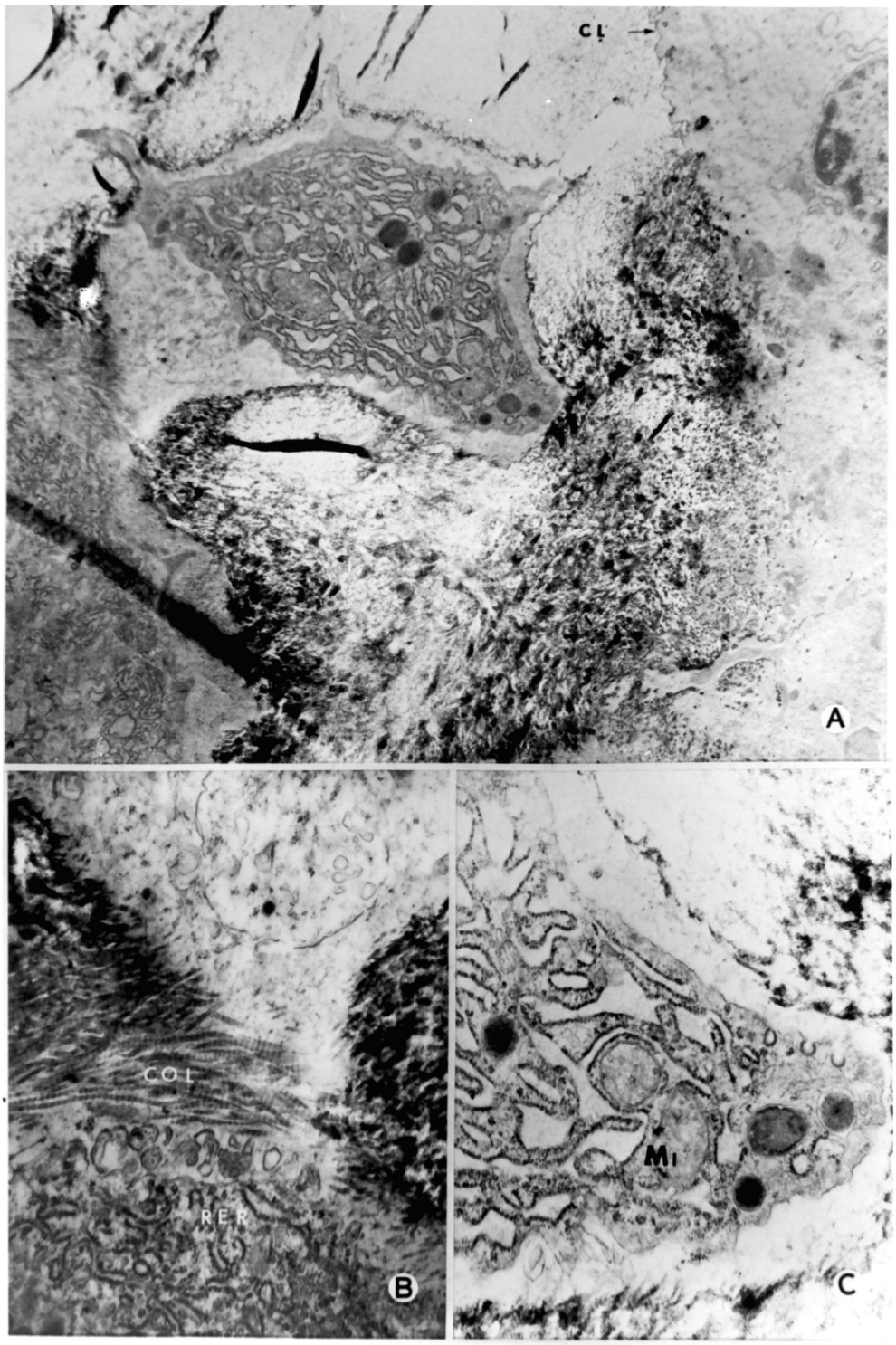


PLATE 12

PLATE 13. An electron micrograph mosaic of a day 14 specimen that shows several brush borders. Two cells are numbered to show the sequence of bone removal. Cell No. 1 is still surrounded by calcified matrix. The matrix surrounding cell No. 2, however, is being resorbed by the giant cell (osteoclast) at the right. It is felt that cell No. 2 will soon be incorporated into this giant cell. 5000 X.

PLATE 13

PLATE 14, A. An enlargement of the brush border marked with an asterisk on Plate 13. Note the fine crystals at the edge of the brush border (arrows). These crystals will be trapped in the folds of the plasmalemma (ruffled edge) and transported deeper into the cell. 27,000 X.

PLATE 14, B. A high magnification micrograph of apatite crystals within the mitochondria of an osteoclast in a day 14 specimen. 40,000 X.

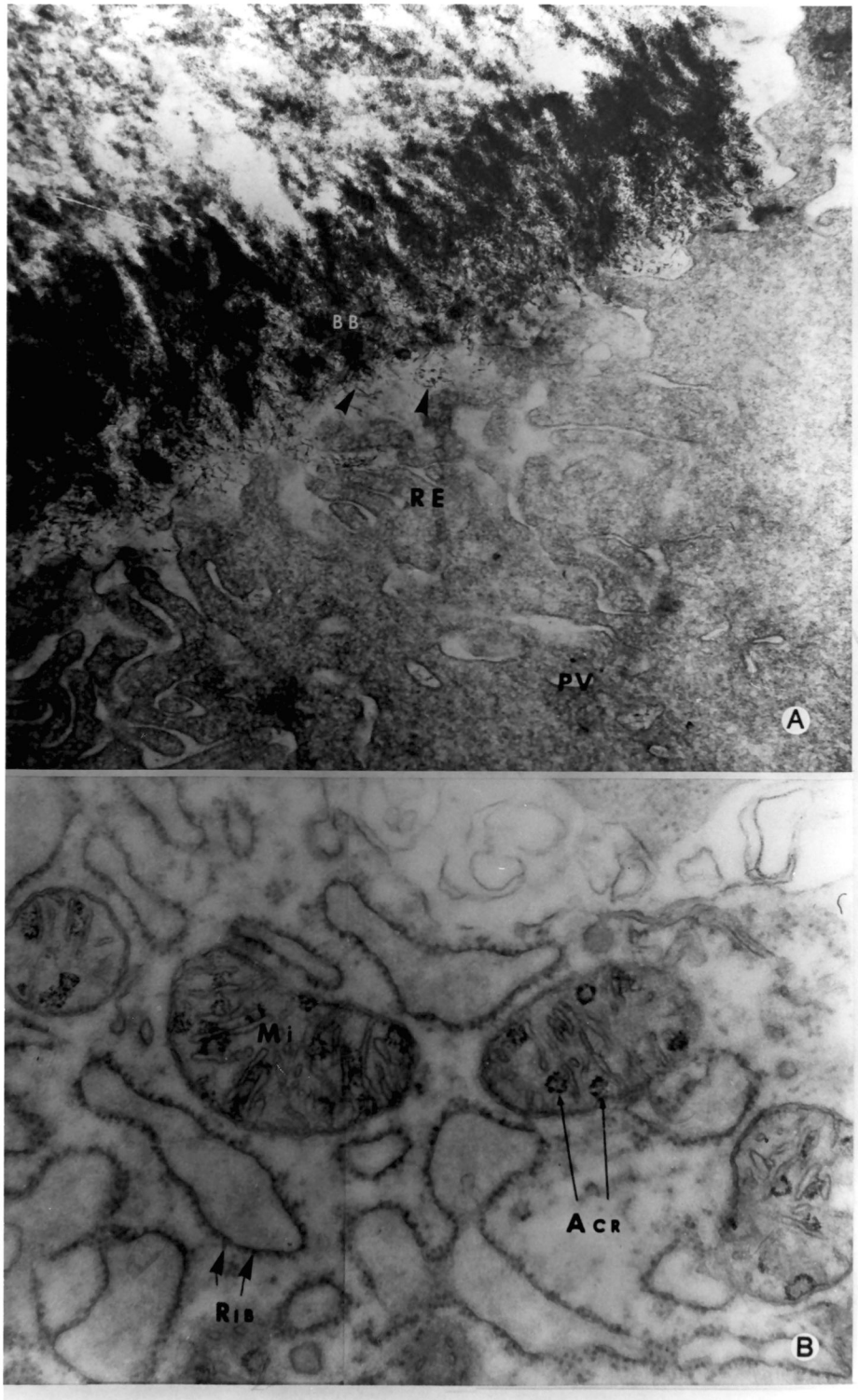


PLATE 14

PLATE 15, A. Another brush border and multi-nucleated osteoclast of a day 14 specimen. 7500 X.

PLATE 15, B. An enlargement of the asterisk area in Plate 15, A. Note the polyribosomes near the brush border. The arrows show areas where the membrane appears dense and possibly coated with bristles that are extended radially into the vacuoles. These may be sites of "bristle-coated" vesicle formation. Also note the crystals in the vacuoles near this area. 27,000 X.

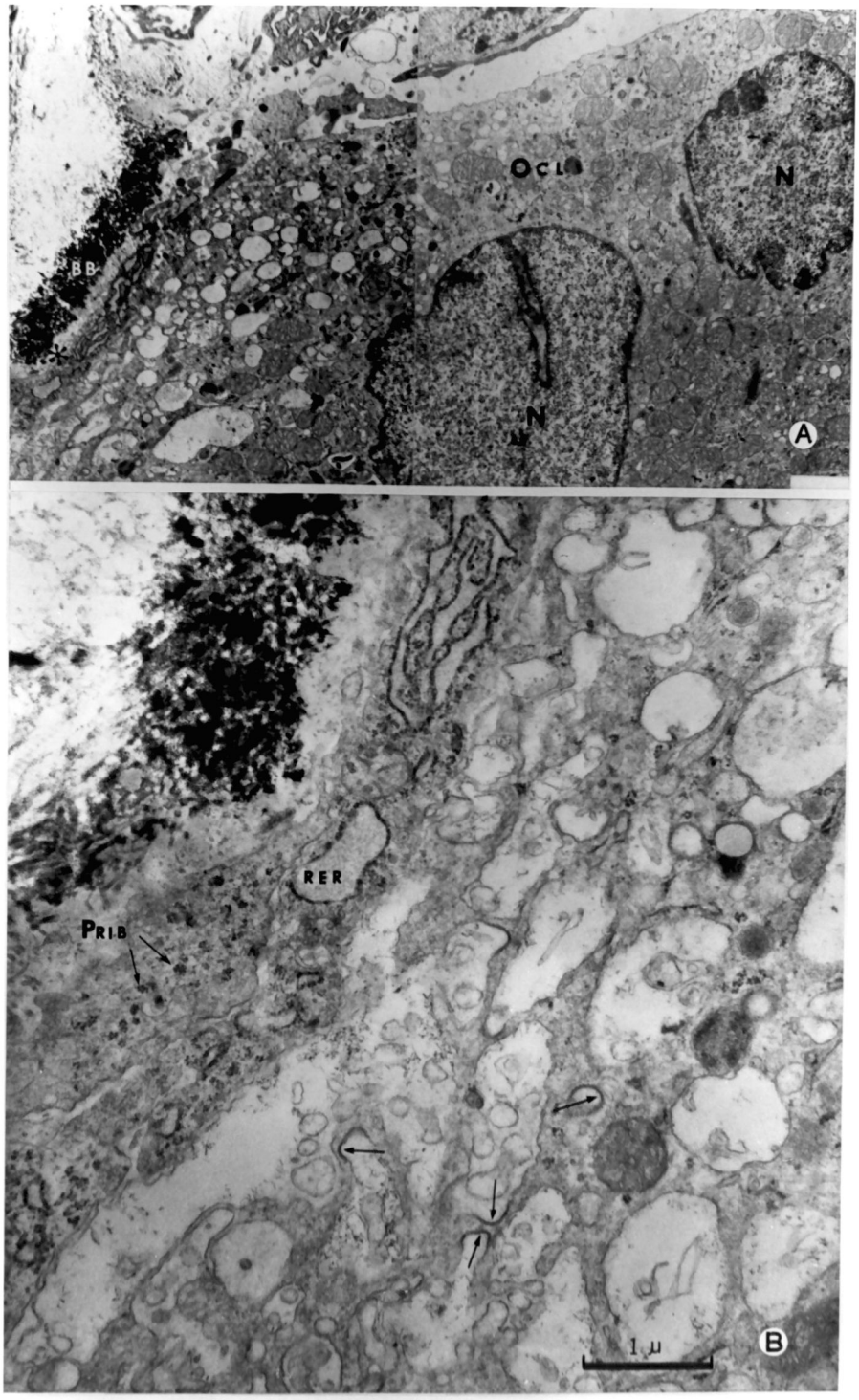


PLATE 15

PLATE 16, A. An area of bone deposition in a day 18 specimen. The numbers signify the stages of osteocyte formation. Cell No. 1 is a typical osteoblast. Cell No. 1 is becoming surrounded by calcified matrix but would probably still be termed an osteoblast. Cell No. 3 is totally surrounded by bone and is an osteocyte. 5200 X.

PLATE 16, B. Day 18. This shows the outer edge of bone (right) and an osteocyte embedded in the matrix (left). Note the many canaliculi (arrows). 5200 X.

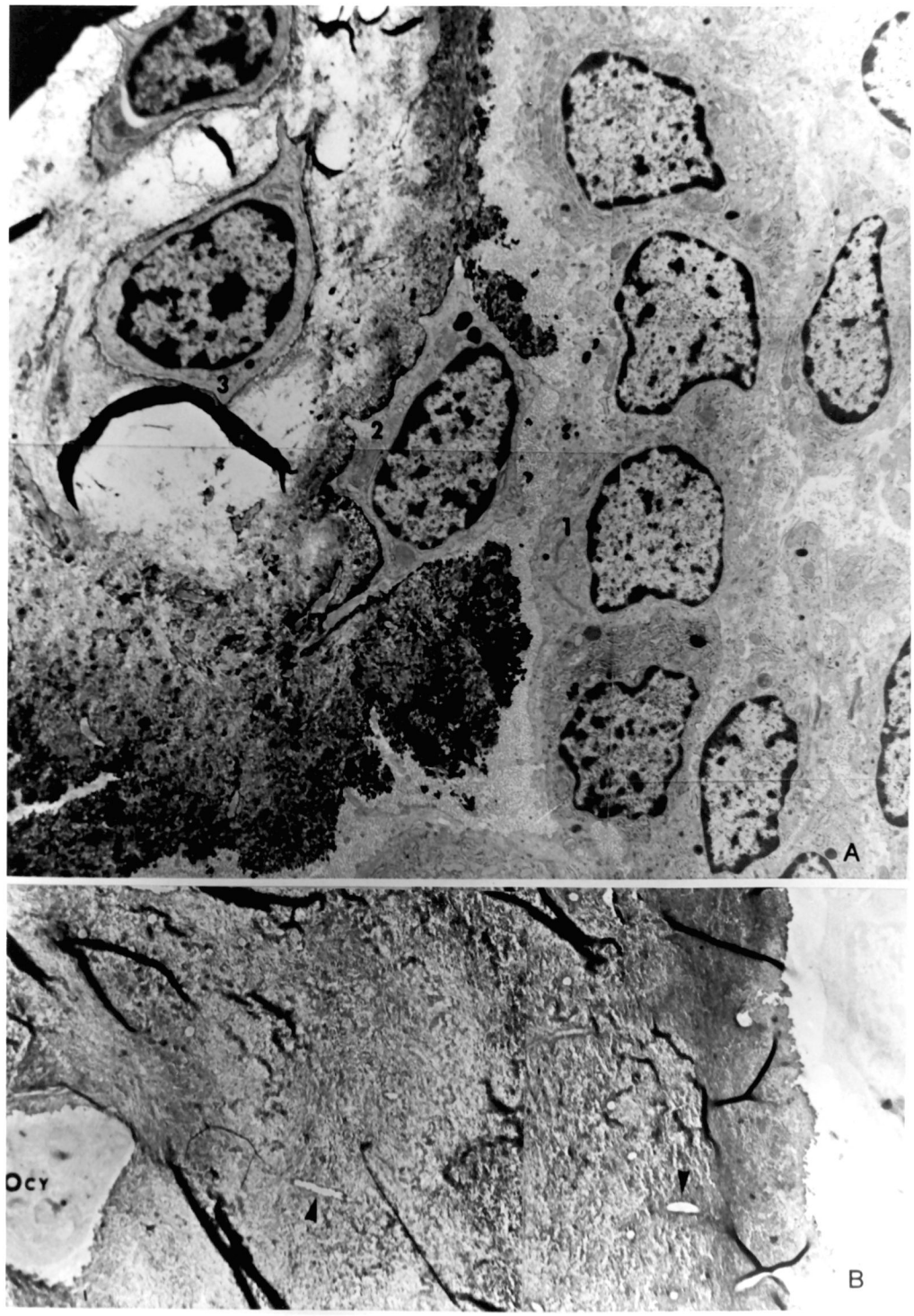


PLATE 16

PLATE 17, A. Day 18. An edge of the crus undergoing bone formation. 5600 X.

PLATE 17, B. An enlargement of Plate 17, A. Note the process of micropinocytosis at the edge of the cell bordering the calcified matrix. A centriole is noticed. 25,000 X.

PLATE 17, C. Day 18. At some previous time bone deposition must have ceased and then continued because a cement line is noticeable. Cross-sections through collagen are numerous. 24,000 X.

PLATE 17, D. Day 18. A small area of bone resorption which is a natural occurance in mature bone. 24,000 X.

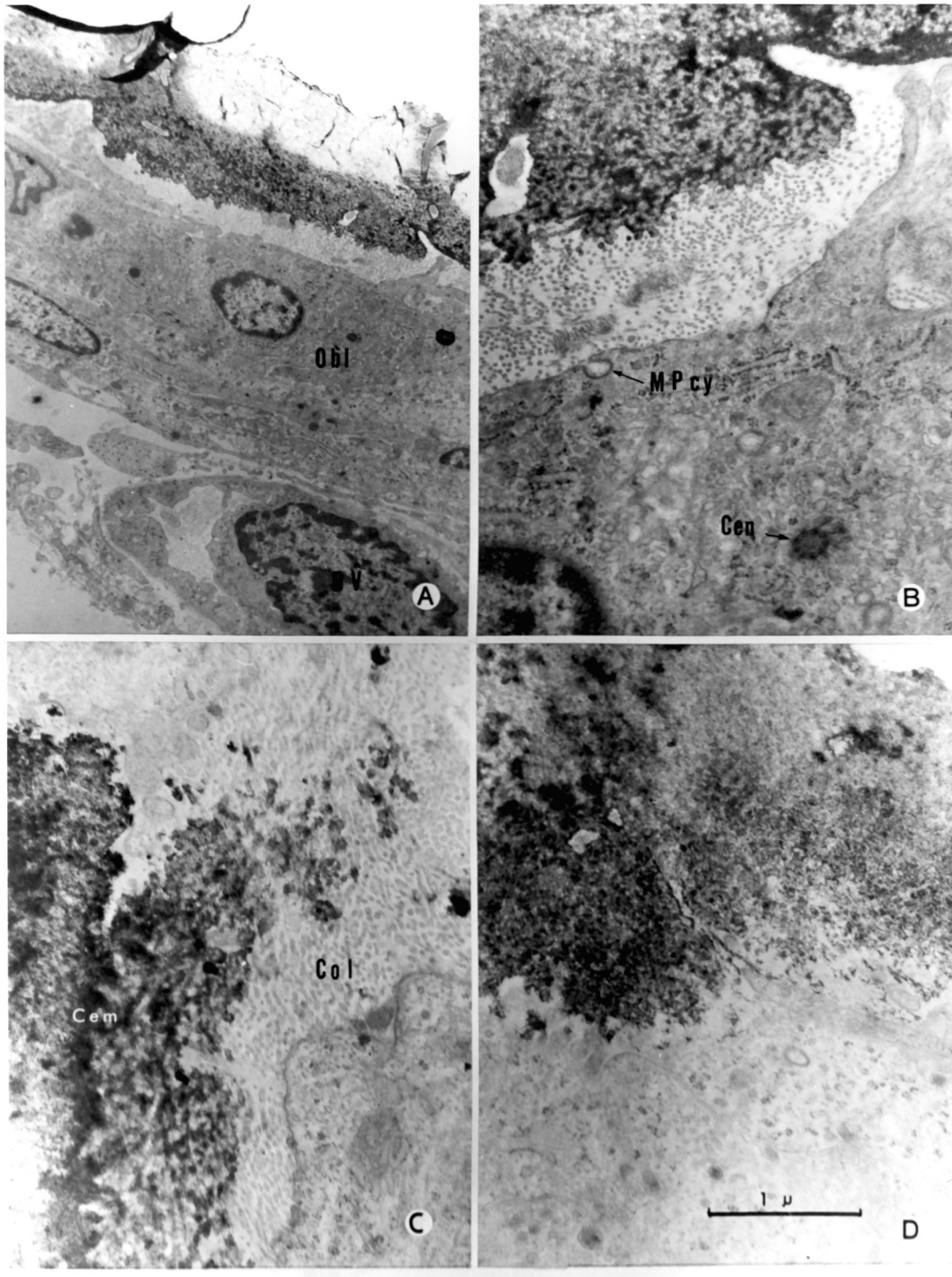


PLATE 17

PLATE 18. A section taken at the obturator surface of a day 18 specimen. The identity of cells 1 and 2 is unknown. Possibly they are mucous secreting cells or granular leucocytes. 6000 X.

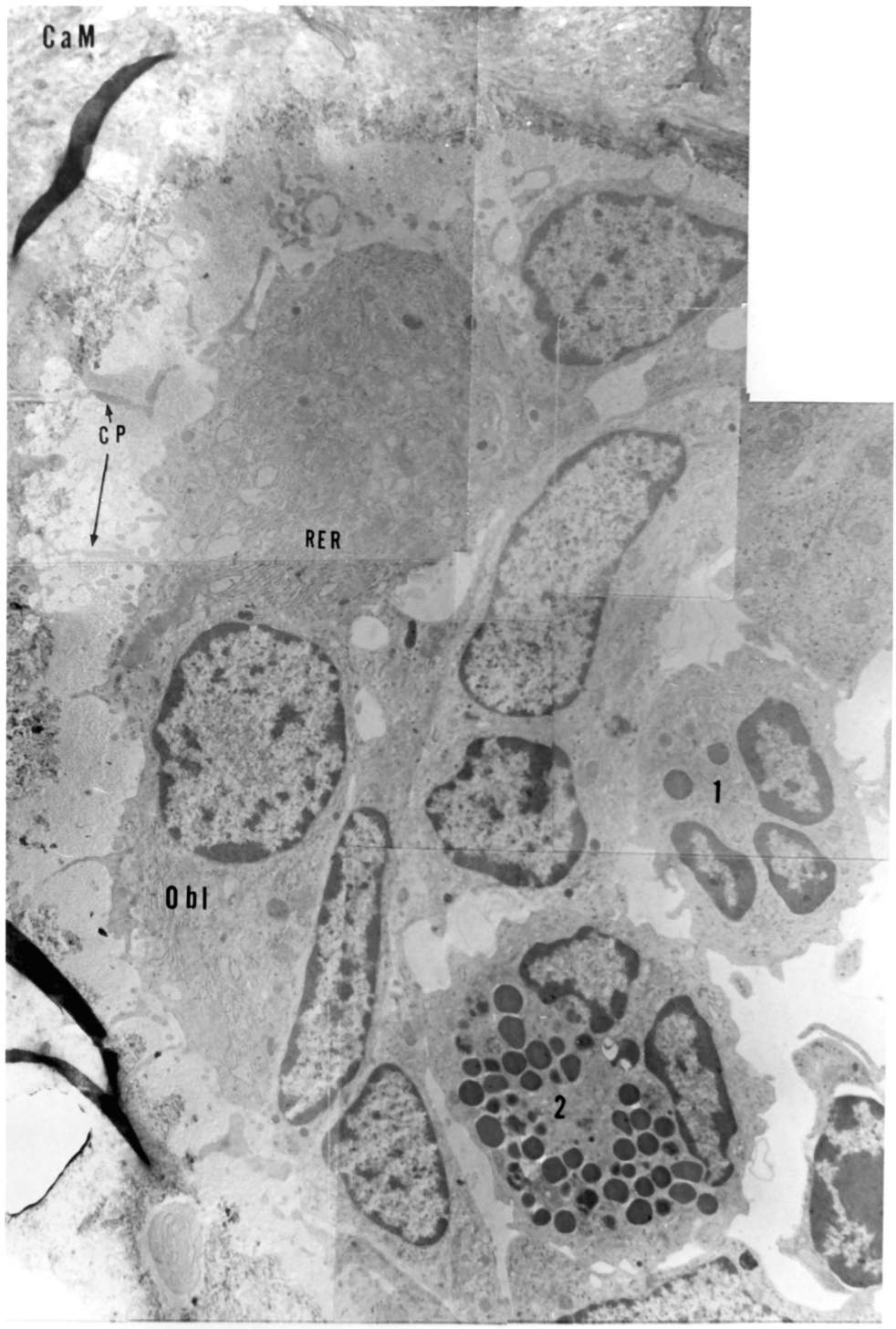


PLATE 18

PLATE 19, A. An enlargement of one of the granulated cells seen in Plate 18. Note the polymorphic nucleus, coated vesicles, prominent golgi, and multivesicular bodies. 26,000 X.

PLATE 19, B. The same cell as seen in Plate 19, A but sectioned at another area. Note that in the dense area (arrow) in Plate 19, A there now appears a centriole. The lobes of the nuclei also appear closer together. 18,000 X.

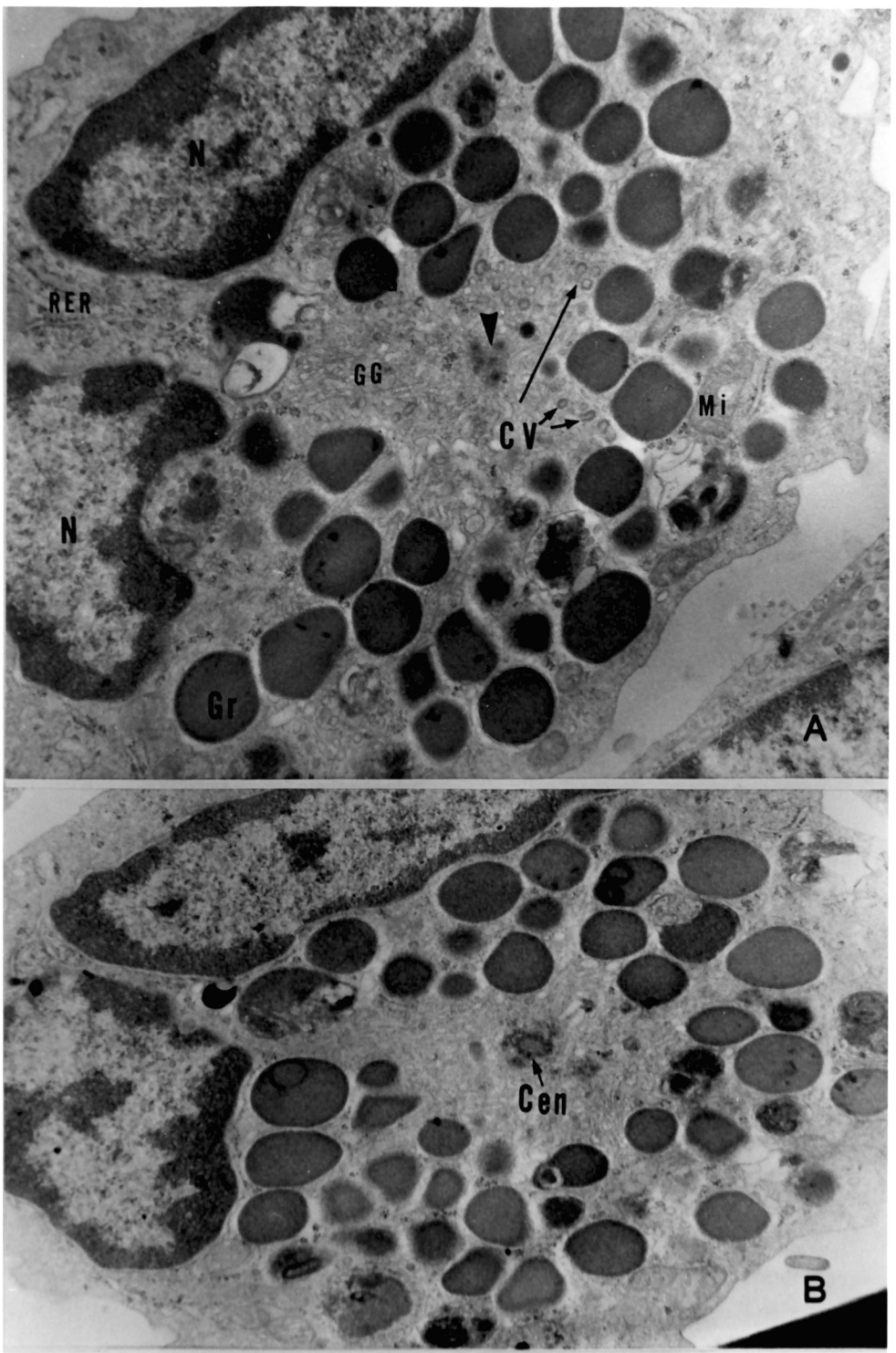


PLATE 19

PLATE 20, A. An edge of a day 21 specimen showing cells that are elongated with oval nuclei and prominent mitochondria. 6000 X.

PLATE 20, B. A canaliculus in bone of a day 21 specimen. 6000 X.

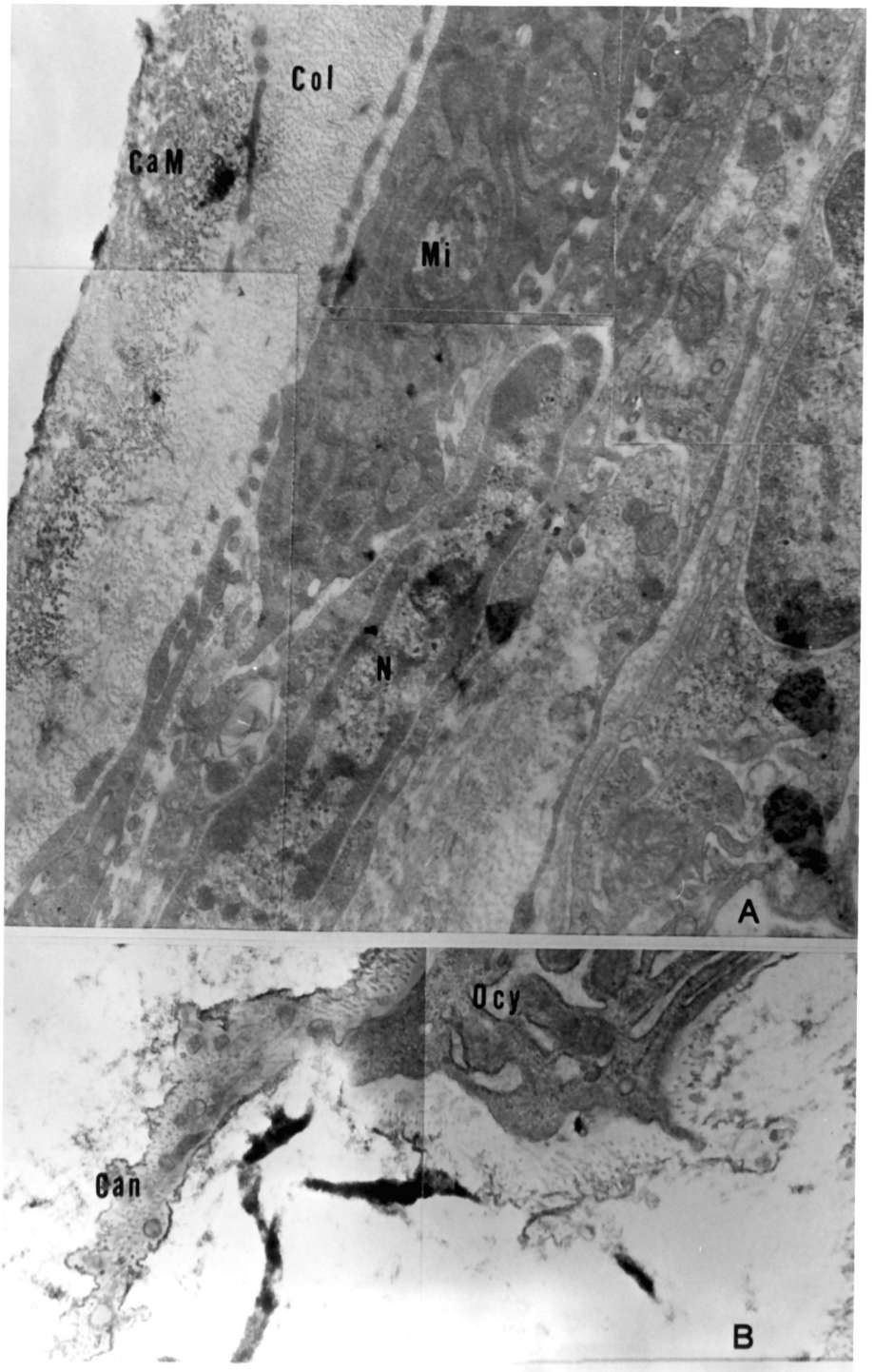


PLATE 20

PLATE 21, Top. Day 52. An osteoblast and osteocyte in close proximity to a blood vessel. The black areas are calcified matrix. Also note the presence of a cement line. 7000 X.

PLATE 21, Bottom. Day 52. An osteoblast seen here exhibits a centriole (left) and golgi (right). 24,000 X.

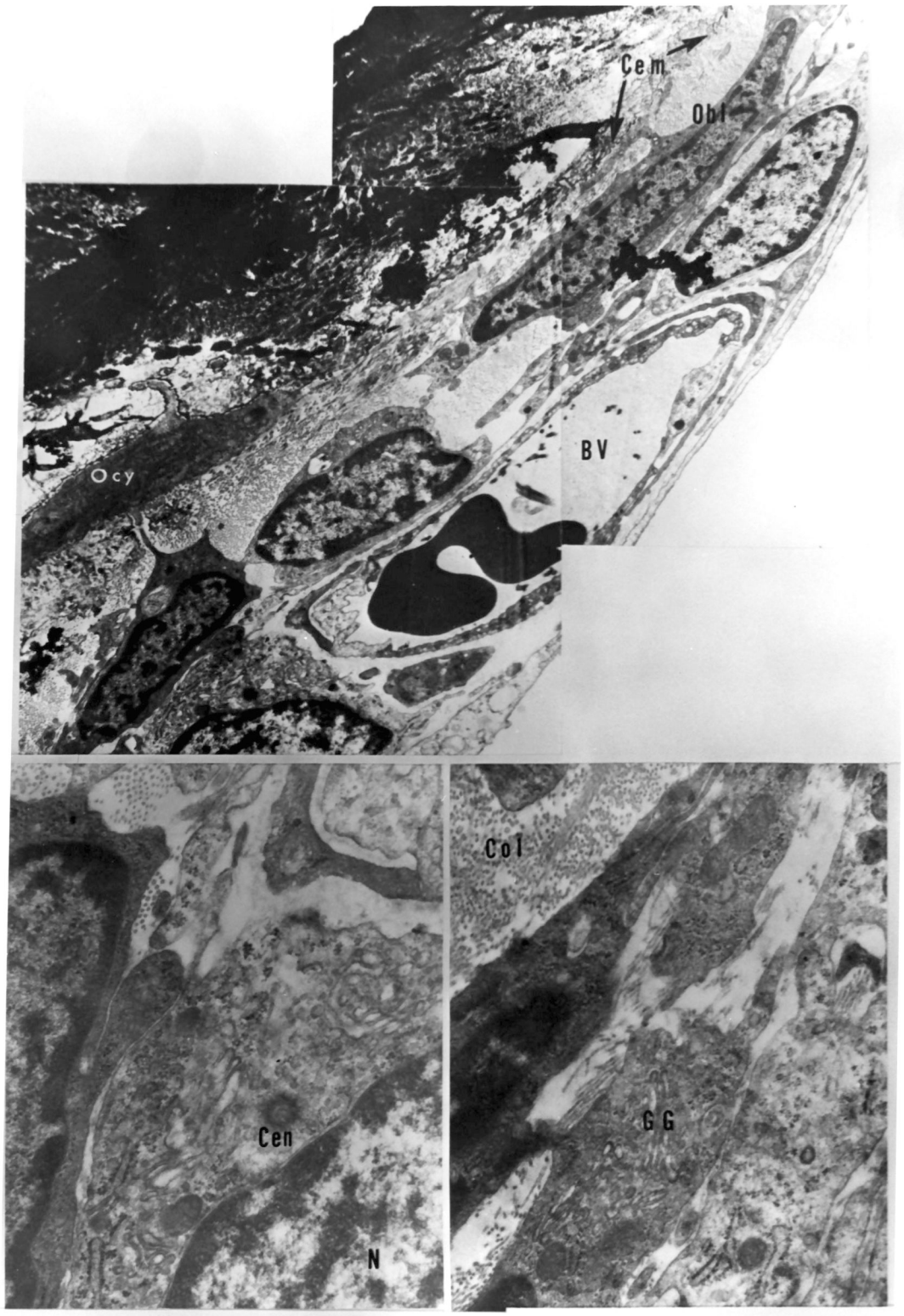


PLATE 21

PLATE 22, A and B. Two cilia found in chondrocytes of a day 6 specimen. Note their close proximity to the nuclei. Also a basal body and golgi are seen nearby. 27,000 X.

PLATE 22, C. A basal body found in an osteoprogenitor cell at day 14. Satellite bodies and microfilaments are also seen here. 50,000 X.

PLATE 22, D. An enlargement of the basal body seen in Plate 22, C. Note the 9 + 0 turbular arrangement consisting of nine triplets. 95,000 X.

PLATE 22, C. A cross section through a ciliary shaft. Note the 9 + 0 arrangement consisting of nine doublets. 66,000 X.

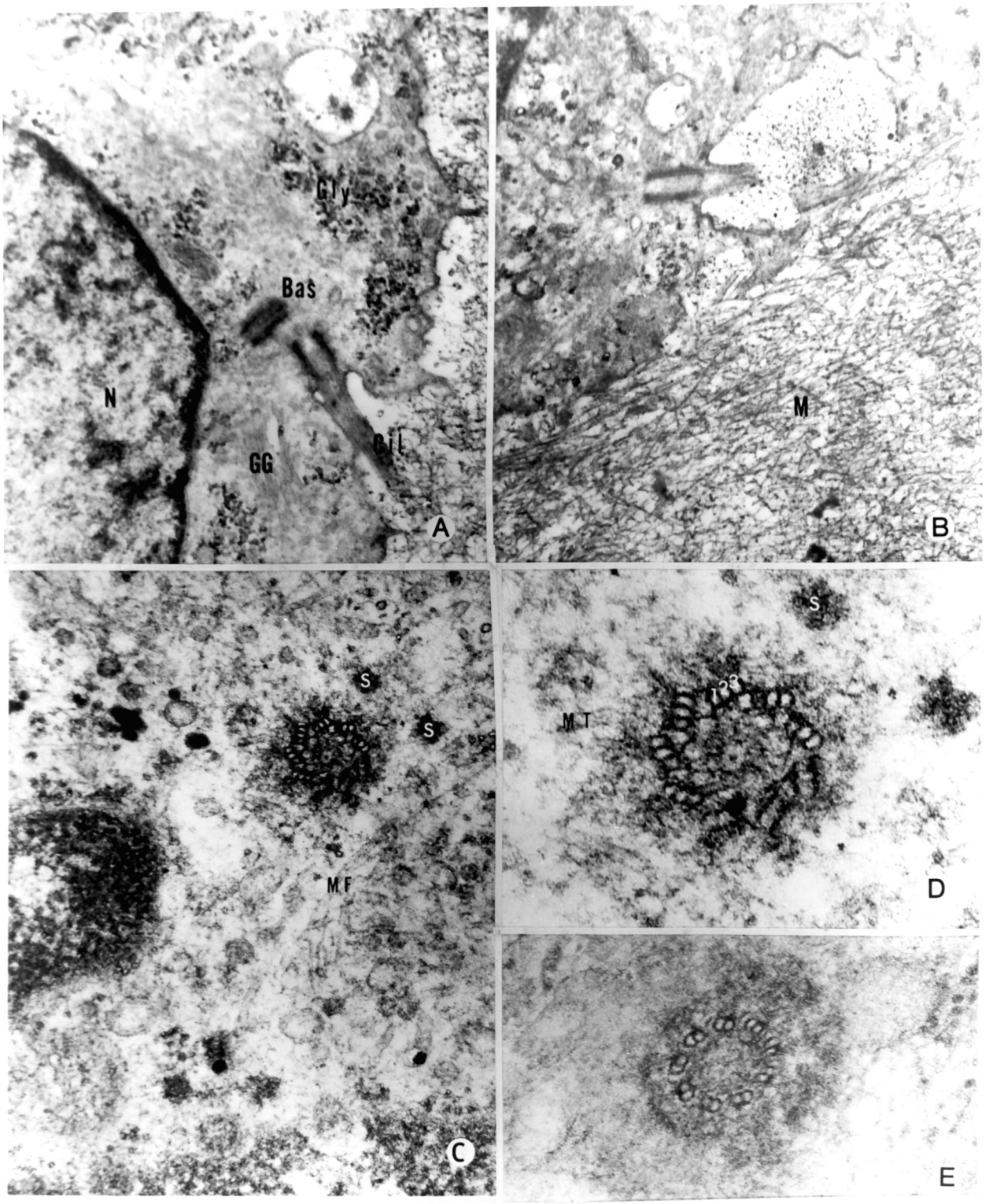


PLATE 22

PLATE 23, A. A ciliated osteocyte found in a day 18 specimen. The cell is surrounded by collagen and calcified matrix. 18,000 X.

PLATE 23, B. An enlargement of Plate 23, A. Note the polymorphic nucleus, golgi and vacuolated cytoplasm. The tip of a cilium is also seen. 27,000 X.

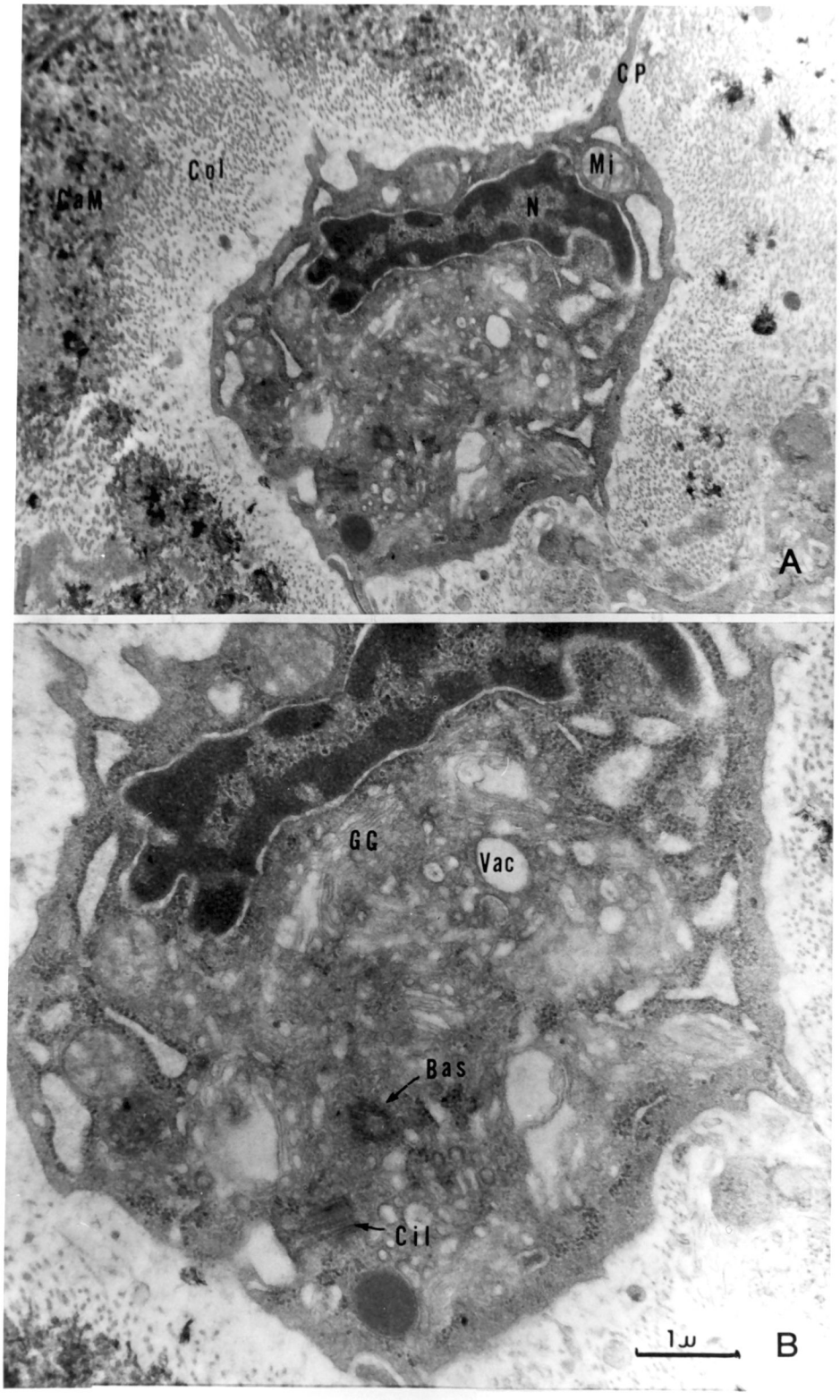


PLATE 23

PLATE 24, A. Coated vesicles were noticed in several osteoblasts. This specimen showed one coated vesicle that appeared to be pinching off from the golgi. 22,000 X.

PLATE 24, B and C. An enlargement of Plate 24, A. The numbers signify the possible steps in coated vesicle formation. 40,000 X.

PLATE 24, D. A cilium surrounded by several coated vesicles. 38,000 X.

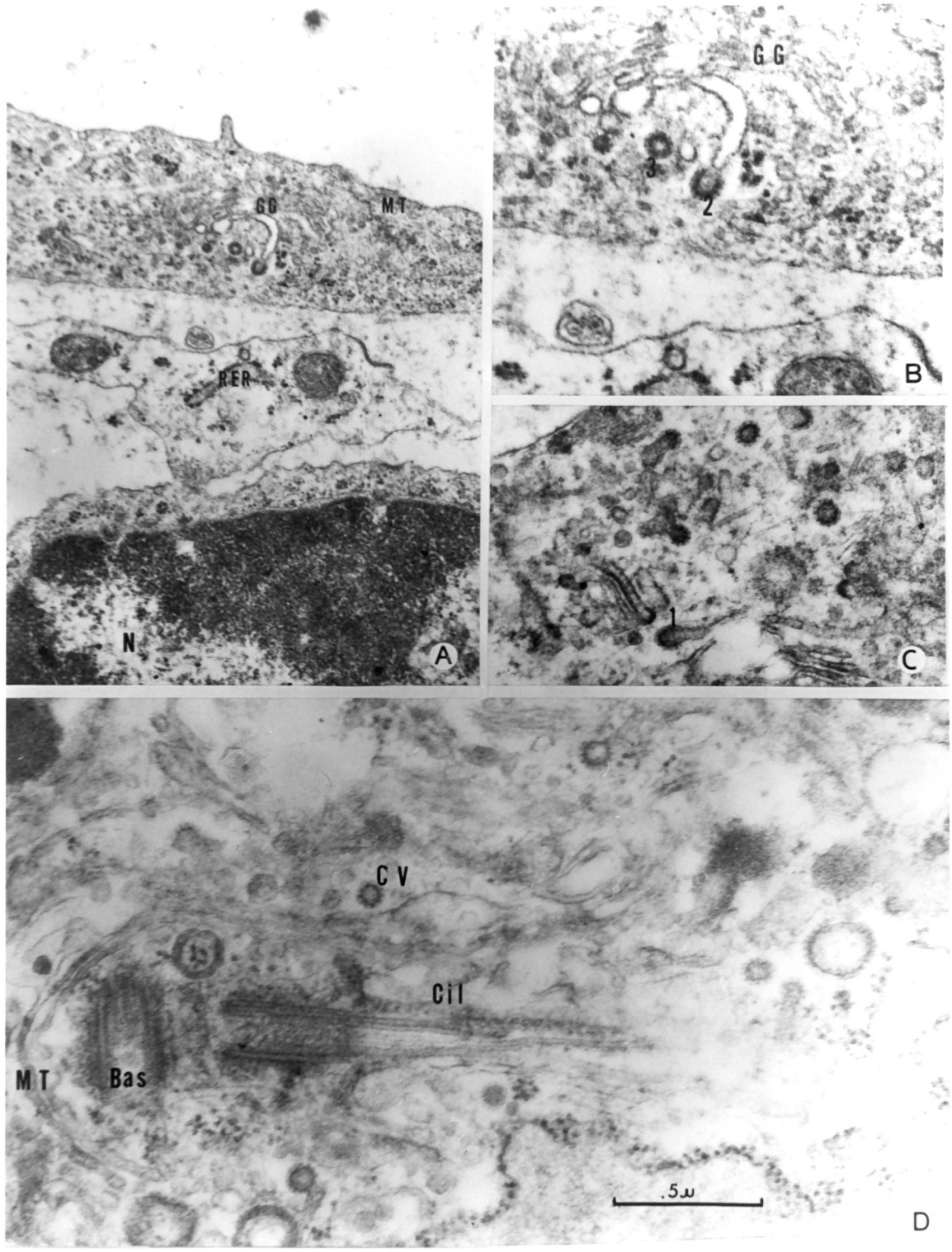


PLATE 24

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