CHRONIC ACCELERATION AND OSTEOGENESIS

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In the last ten years, investigation into the effects of chronic acceleration on biological systems has provided much disparate data. One of the more consistent findings has been increases in body mass and bone deposition.

The effect of excess gravity on bone-forming elements of the growing perichondrial shaft of embryonic chicks was investigated through the use of the transmission electron microscope and various cytochemical techniques.

California Grey X chicks were obtained locally, placed in a centrifuge-incubator apparatus, and accelerated at a force of 5 G's throughout the course of their development. The acceleration was chronic except for short periods for the removal of experimental animals. Control animals were housed below the experimental animals in the centrifuge-incubator apparatus, and subjected to the same environment. Experimental and control animals were removed from the centrifuge on alternate days beginning with day 7 of incubation. The Tibio Tarsus long bone of the leg was removed from accelerated and control animals and prepared for electron microscopy and the various histologic procedures.

Acceleration produced significant differences in cell morphology and developmental sequence in five major areas: (1) An acceleration in the rate of development through the 17th day of incubation was characteristic of the experimental animals. The accelerated bone appeared to be approximately 18 to 36 hours more advanced chronologically than the control bone. (2) A scarcity of osteoclasts was observed with both the light microscope and the electron microscope. Osteoclasts were very rare in accelerated bone. (3) An increase in the numbers of active osteoblasts when compared to the numbers of inactive lining cells. (4) An obvious increase in the synthetic machinery and cellular organelles of the accelerated osteogenic cells. (5) A greater number of eccentrically shaped cell-inclusion bodies, which probably testifies to the workings of excess gravity on the fluid-colloid system of the cell.

The deficiency in accelerated bone of osteoclasts, coupled with the increase in numbers of active osteoblasts, is described as at least partially responsible for the increase in deposition of calcium hydroxy apatite.

CHRONIC ACCELERATION AND OSTEOGENESIS

THESIS

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CHAPTER I

INTRODUCTION

In the late 1960's and into the 1970's, a great many experiments were carried out to determine the effect of excess gravity upon biological materials and organisms. In most cases the impetus for such studies was the advances in Space Science and the inevitable prolonged periods of weightlessness to which the astronauts and space scientists had been subjected.

The inability to produce weightlessness in the laboratory leadsto the utilization of centrifugation and its effect on biological systems as an indirect method to study the effects of long term alterations in gravitational force, with hopeful application to weightlessness.

The physiological, metabolic, and morphological deviance noted in chronic acceleration studies present a rather confusing picture. At the present, this deviance still confuses investigators trying to amalgamate the data into any one coherent theory of gravitational effect on biological systems.

One of the most conspicuous differences noted by investigators was a general tendency for the chronically accelerated animal to demonstrate a loss in body mass. Oyama (34) ascribed this loss of body mass to inanition and reduced food consumption. This was correlated to significantly lower growth rates and final body weights in weanling rats who were subjected to prolonged centrifugation. Oyama and Platt (35), studying the effects of chronic centrifugation on reporduction and growth of rats and mice, again observed the growth rates to be significantly lower in accelerated animals. When an animal was returned to normal gravity, a transient increase in food and water consumption was a consistent observation. These studies strongly indicate decreases in body mass. Other experimentation under conditions of weightlessness and accleration present conflicting data.

Young and Tremor (47) subjected the developing eggs of Rana pipiens to prolonged weightlessness during the Biosatelite Two program, and found that the rate of development was unaffected. Redden (38) subjected chick embryos to chronic acceleration and noticed a drop in body mass in the centrifuged animals. The last two studies dealing with the effect of excess gravity or the lack of gravity on embryonic oviparous animals lend support to another parameter other than overt feeding behavior, with nourishment being less of a variable. In this regard, Wunder(45) demonstrated

a pronounced decrease in water intake, with a complex relation to increased urine output under increasing centrifugal field strength. This increase was also demonstrated not to be solely related to reduced food intake, and implied some kind of interruption of water balance physiology.

The differential growth and development of individual organs and systems has also been under scrutiny. Oyama (34) observed that prolonged centrifugation reduced the relative size of the adrenal glands, but, ironically, the relative size of the liver was increased in accelerated animals. Measurements were made of skeletal systems in several studies. Smith (40) found that the relative size of bones increased in adult chickens and turkeys subjected to chronic acceleration. Redden (38), using embryonic chicks as specimens, found significant increases (up to 34%) in the lengths and center shaft diameters of limb bones after chronic centrifugation at 5 G's.

Some authors have also inferred alterations in the endocrinological mechanisms due to chronic acceleration. Smith and Wingett (41) found inconsistencies in the differential mortality of adult chickens when compared to immature animals, all of which were subjected to chronic centrifugation. Several different pathologies were implicated, many involving alterations in endocrinology. Different mortality rates between males and females were also attributed to the

influence of sex hormones on adrenocortical function. Another of their findings strongly implicating endrocinological pathology was the delayed sexual maturity of birds exposed to chronic acceleration. Pitts, Bull, and Oyama (37) make reference to alterations in endocrinology and physiological regulatory mechanisms in their study of centrifugation on body composition in rats. In failing to correlate muscle hypertrophy to physical stress, much discussion was held of the probable interruption of these regulatory mechanisms.

The blood-vascular system has been subjected to acceleration experimentation, yet there is much disparity in the data published. Atherton and Ramm (1) found that with prolonged centrifugation there was no initial increase in erythrocyte count, and that decreases in erythrocytes were correlated with increasing field strengths. This was hypothesized to be due to the increasing force of gravity causing a decrease in arterial blood flow. The resulting decrease in blood pressure would therefore result in a decrease in circulation cells. Significant decreases in hemoglobin concentration were also shown to be a sensitive variable. Oyama (34) also demonstrated decreases in red blood cell counts and significant alterations in hematocrit values. No other associated histopathology could be found attributable to acceleration exposure or other experimental

treatment. In their study of accelerated chickens, Burton and Smith (14) observed that the erythrocyte number, plasma volume, and plasma proteins (globulins) increased, while erythrocyte size (mean corpuscular volume) and the plasma albumin-to-globulin fraction ratios were reduced.

Considering the wide range of changes, variance in the data, and conflicting theory, a true pattern of chronic centrifugation activity on any one particular system has failed to emerge. To date, all discussion of chronic acceleration phenomena has been limited to explanation at the gross physiological level. This has been exemplified in the "mechanical stress" theory of centrifugation-induced hypertrophy of muscle and skeletal tissue (13, 34, 35). There has been reason to doubt the overall application of such theories as suggested by Redden's work with embryonic chicks. In this case the embryo was in a "semiweightless condition" and did not exert any activity against the force vector, yet the center shaft diameter and overall length of long bones were shown to increase greatly over controls (38). Data to contradict the mechanical stress theory was also presented by Smith and Kelly (40). They found that the non-load-bearing wing bone (humerus) of accelerated chickens increased in size more than that of the load-bearing femur.

To date, no attempt to correlate a cellular approach to any of the investigated systems has been made. This would imply that many of the variations seen in acceleration studies might instead arise from alterations imposed by gravitational stress at the cell level. The primary fluid-colloid composition of cellular constituents would be particularly affected by even slight alterations in gravitational stress.

The present study was undertaken to examine the ultrastructural properties of calcium deposition and osteogenesis under centrifugal force in its relation to the histological and ultrastructural norm.

In addition to the well substantiated reports of a general loss in body mass, the greater size of bones taken from centrifuged animals seems to be a consistent finding in acceleration studies (13, 38, 41, 44). Chicken embryos were chosen as the subjects due to their property of being a "closed system", and less amenable to variation in nourishment, temperature, extraneous vibration, and frequent stops for feeding and container upkeep. (38).

It is the object of this study to attempt to show some morphological distinctions or alterations in the developmental chronology that may become visible through the use of the Transmission Electron Microscope and various cytochemical procedures. A search through the literature reveals

that such an ultrastructural approach has not been attempted. The information gained should lend greater insight into the process of extra calcium deposition due to chronic acceleration.

CHAPTER II

METHODS

Incubator Centrifuge

The incubator-centrifuge was constructed of 3/4-inch plywood. The incubator was large enough to contain the centrifuge head, belt drive, and motor, allowing all the experimental animals, both accelerated, and control, to be subjected to the same environment.

The incubator was electrically heated and thermostatically controlled to maintain a temperature of approximately 40 degrees Centigrade. Humidity was maintained by exposing an open tray of water next to a circulating fan which also helped maintain even temperatures and ventilation. A tray for the control eggs was mounted to a lever on the outside of the incubator to aid in "turning" the eggs daily without opening the incubator.

The centrifuge consisted of a motor-belt-driven circular head mounted inside the incubator with 30 foam-lined containers for eggs. The diameter of the head was 29 inches, and it was powered by a 1/8-horsepower motor with variable speed controls.

The centrifuge speed was calibrated with a hand tachometer to maintain a constant speed of 101 RPMs (this corresponded to a resultant force vector of approximately 4 times the force of gravity). The centrifuge was run continuously except for stops to remove experimental animals (38).

Animals

California Grey X fertile eggs were obtained locally in lots of 5 dozen. These eggs were placed in the incubator-centrifuge and allowed to develop for 48 hours before candling. This procedure allowed for the placement of fertile and developing eggs free from visible impairments into the centrifuge apparatus (38).

Experimental and control animals were removed every other day beginning on the seventh day of development and continuing through the 21st day. The actual chronological day of development was determined by Hamburger's method (18).

Immediately after removing the animal from the shell, the tibio-tarsus long bone of the leg was isolated by microdissection and immersed in a phosphate-buffered formaldehyde fixative (modified Millonigs) (2). The vials were numbered as to the chronological day of development, lot, and specimen number. These were set aside for the embedding procedure for electron microscopy.

Electron Microscopy

The bones were allowed to fix for up to 48 hours or more for better preservation of fine structure (2, 25). Mineral mitochondrial granules have also been shown to change spontaneously from an amorphic form to a more crystal or visible form if allowed to stand in the fixative for a suitable period of time (28, 29, 30).

Diced cross-sections were made through the center shaft (diaphysis) and post-fixed in phosphate-buffered osmium tetroxide for a period of 45 minutes prior to dehydration through ascending grades of ethyl alcohol (ETOH). EPON 812 was used as the embedding medium in the standard proportions of 3 parts "A" to 2 parts "B", except where the hardness of embryonic bone necessitated a harder EPON mixture. In brittle bone, 1 part of "A" to 2 parts "B" was used (15).

The individual diced cross-sections were flat-embedded allowing for the precise orientation of the cross-section. The specimen was later trimmed and viewed with the light microscope. The use of the microscope made it possible to glue the cross-section directly onto the head of a regular EPON cart mold and retain the cross-section perfectly during the cutting procedure.

Prior to thin-sectioning, thick sections (pink and green interference colors) were taken on glass knives, placed on

microscope slides, and stained with Paragon Polychrome stain (26). These were viewed with the light microscope to locate optimum areas for thin-sectioning.

Thin sections were taken on the Porter Blum M-2A Ultramicrotome. Sections were selected by the interference colors of light silver or gold. These were picked up on Formvar coated copper grids. The Formvar-coating was used to discourage "curling" of the specimens under high emission.

The grids were then stained in a solution of uranyl acetate saturated in 50% EtOH, for a period of approximately 2 minutes. This was followed by washing in 2 changes of distilled water (43). Lead citrate stain was prepared after a method by Reynolds (39), and the grids were immersed in this medium for approximately 45 seconds, followed by 4 rinses in distilled water.

Photography and viewing were performed on the RCA EMU 3F and Phillips Model 300 Electron microscopes.

Cytochemistry

The following cytochemical and histological procedures were carried out on Cryostat sections of the tibio-tarsus: alkaline phosphatase localization, glyoxal bis (2-hydroxanil) (CBHA) staining for calcium, and Pyronin-Methyl green stain for DNA and RNA localizations. Frozen sections were used exclusively and were cut on the American Optical Cryocut at approximately 10 microns thickness.

In addition to the Paragon sections, standard hemotoxylin and eosin procedure was carried out for morphology and to aid in the histological comparisons of fine structure.

Alkaline phosphatase histochemical localization was carried out, using the packaged reagent kit commercially available from Sigma Chemical Company. This employs the use of sodium naphthyl acid phosphate as a substrate. The product of the enzymatically cleaved substrate is coupled with the diazonium salt Fast Blue RR present in the incubation medium. This coupling reaction forms a colored and insoluble azo dye precipitate. This presumably localizes in the areas of alkaline phosphatase activity (48).

To help isolate accumulations of intracellular amorphic calcium, the Schiff base glyoxal bis (2-hydroxyanil) was used as a chromophore-chelate for unbound and labile calcium. Stock solutions of .04% NaOH diluted in 75% EtOH and a .08% NaOH diluted in 75% EtOH were prepared. Small portions of the stain were prepared by dissolving .02 grams and .05 grams of "GBHA" (obtained from Fisher Chemical Company) in 2 milliliters of the respective solvents. This was just enough to adequately stain affixed and frozen sections. The stain had to be prepared before use, as it degenerates quickly. Two changes of 95% EtOH were used as a rinse after sections were incubated under drops of the prepared stain for a period of 5 minutes, and then mounted in euparol (21, 22, 23, 24).

For qualitative comparisons between the cellular levels of nucleo-protein observed in control and accelerated tissues, the Pyronin-Methyl Green procedure for the cellular localization of RNA and DNA was performed. First the tissues were fixed in an alcohol-formalin fixative. The stain itself was prepared by mixing 10 ml of 5 % Methyl Green solution, 10 ml of 2% Pyronin Y solution, and 20 ml of a prepared Phenol-Glycerol mixture. This was prepared in advance in the proportions of 4 grams phenol to 40 ml glycerol, and diluted with 56 ml of distilled water (48).

Affixed frozen sections were incubated in the stain for a period of time up to 20 minutes, depending on the "take" of the stain. The sections were then washed in cold, recently boiled distilled water. The sections were then briefly dehydrated in acetone, and then absolute EtOH. Cover slips were mounted, using euparol mounting medium. In this procedure, DNA stains green, and RNA stains reddish. Viewing and microphotography were carried out on a Nikon Model microscope with photographic attachment, using Kodachrome 35-millimeter slide film.

CHAPTER III

RESULTS

A brief account of the present understanding of osteogenesis and calcification must precede the results of the present research. Enumerating some of the basic differences between the developmental biology of bone of embryonic fowl compared to the more classical mammalian osteogenesis also appears appropriate.

The first evidence of limb development is noticed at approximately 50 to 56 hours of incubation in the embryonic chicken. From this time on the "limb buds" are seen to extend outward and progressively lift off the main shank of the embryo. These earliest stages of limb development are characterized by blastemata of aggregated mesenchyme. This mesenchyme is mesodermal and in the case of the long bones is of scleratomal origin. It should be stated here that there are at least two major classifications of bone: that bone which forms from cartilage, and that bone which forms directly from membrane material with no cartilagenous precursor. The former is usually considered to be of scleratome

origin, and the latter, or "membrane bone", is of dermatome origin. Both of these are derived from primordial mesoderm or head mesenchyme (5, 11).

Chondroblasts also differentiate from this mesenchyme and later form mature chondrocytes or cartilage cells. The actual formation of cartilage (chondrification) is accomplished by the secretion of an optically homogenous matrix from these cells. The matrix is basically composed of collagenous fibrils and a ground or cementing substance. Actively secreting chondroblasts can be recognized by the greater amount of cellular organelles associated with active synthesis, such as endoplasmic reticulum, Golgi networks, and hypertrophied nuclei with prominent nucleoli.

The matrix accumulates in greater proportions and pushes the chondroyctes farther apart as the intracellular space becomes more dense with exudate and begins to calcify. The individual chondrocytes are enclosed in separate cavities or compartments called lacunae. As these cells divide, more matrix accumulates between them, and consequently the daughter cells are isolated also. The most peripheral chondrocytes are flattened out to form a boundary layer, the perichondrium. By at least the 7th day of development in the embryonic chick, the area that is to ossify and form bone has already been mapped out by this tubular cartilage template.

The cells that are primarily indicated in the deposition of calcium phosphate hydroxy apatite, or the main organic-inorganic mineral constituent of bone, are the osteoblasts. Ostoeblasts presumably take their origin from the mesenchyme and connective tissues that lie to the outside of the perichondrium (11, 12). The true primordial or presumptive cell that gives rise to the osteoblast is in dispute, as it has been shown that osteoblasts can arise from several precursors (8, 12). In terms of the long bones, it has been observed that these cells do arise from the periphery and replace the tubular perichondrium (11, 12). At this point ossification is in progress and the osteoblasts can be seen to line up in layers on the newly forming bone. Some of these are actively secreting and appear cuboidal in shape, while others are flattened and are believed to be in a transitory state, waiting to be called into action (27). The ring of osteoblasts and the more peripheral fibroblasts are now called the periosteum, as its appearance is simultaneous with the formation of bone nodules (11, 12).

Bone that is deposited by the periosteum is called perichondrial bone, because it forms a narrow ring around the chondrified central matrix. Primary centers of ossification can first be detected by the appearance of the chondrocytes which become hypertrophied. This is a prelude to the

appearance of osteoblasts in this area and the subsequent calcification of the partly calcified cartilage matrix. Secondary centers of ossification are more internal to the periosteum, and this phenomenon is called endochondrial ossification. These two layers of forming bone eventually fuse and cause a compartmentalization of bone matrix that later becomes infiltrated by blood vessels and nerve components (11). With constant remolding by osteoclasts and new bone deposition by osteoblasts, the systems of canals and compartments are packed in a layered fashion and are called lamellar bone, as contrasted to the more primitive unsymmetrical deposition of bone (called "woven bone" due, to the patchwork quilt network of newly deposited trabeculae or bone spicules). This network of canals and portals containing blood vessels, nerves, osteoblasts, and osteoclasts is called a "Haversian system", while each separate canal or portal is called an osteon (3, 7, 11, 12).

Growth of the bone in diameter is primarily accomplished by the deposition of bone nodules along the solidified and calcified cartilage matrix. This area is heavily infiltrated with fibrils of tropocollagen secreted by the osteoblasts and outlying fibroblasts, and is thought to represent pathways of nucleation for the apatite crystals, although this is disputed by some (7, 9, 12, 42). The narrow

collagenous area between the sites of bone nodule nucleation and the secreting osteoblasts is called the osteoid (11, 12).

At the same time as the bone is growing in diameter, it is also being reabsorbed from the inside, presumably by osteoclasts. In this way the presumptive marrow cavity enlarges as bone grows by apposition (10, 11, 12, 30). Osteocytes communicate with each other by long cytoplasmic extensions called caniculi. In this way nourishment is channeled from one cell to the next (11, 12).

In the mature osteon, a static state is never observed, as constant remodeling is being carried out by the action of osteoblasts and osteoclasts. For this reason it is incorrect to consider bone a static entity, for it is always in a dynamic state to meet the demands of the organism and the environment.

Growth in diameter throughout the length of the shaft (diaphysis) is basically different from growth in length. An area of proliferating cartilage (the epiphyseal plate) extends the length in both directions as it is replaced by an ossifying bone matrix (11, 12, 32). This plate never calcifies completely until a point in the developmental history of the organism is reached where growth in length stops (approximately 20 years in humans). For this reason the epiphyseal plate is often called the "growth plate" (11, 12, 32).

During these stages of growth, cartilage cells are still in abundance in the center of the bone shaft. As chondrocytes secrete a calcified matrix around themselves, they can be denoted by their abundant organelles, as mentioned earlier. Since cartilage is avascular and dependent on diffusion for the supply of nutrients to sustain activity, the gelling and mineralizing cartilage matrix prescribes cartilage death. These cells can be readily identified by their pyknotic nuclei, abundant lipid granules and vesicles, and a general state of dissolution when they are found in more heavily calcified areas (11, 12, 28, 29, 31, 32). Center shaft cartilage is either replaced by bone or penetrated by capillary buds which branch out and dissolve the cartilage matrix in front of them, forming the actual marrow cavity. Liberated cartilage cells that are still intact near eroded matrix cytolyze and disappear (11, 12).

In terms of the embryonic fowl, calcification does not necessarily precede the eroding away of the cartilage as it does in mammals. Only until the greater portions of the marrow cavity are constructed is this generally true (15). For this reason a diaphyseal section will display many types of cartilage in different stages of calcification, as well as good classical periosteum, perichondrial bone, and some endochondrial bone. In general, perichondrial ossification is demonstrated more extensively in avians than in mammals.

Endochondrial bone of chicks appears much later in development and is less extensive than mammalian bone.

Diaphyseal cross-sections are an excellent way to display a more panoramic view of the developing long bone in embryonic fowl. In a previous study by Redden (38), the center shaft dimensions were shown to greatly enlarge due to centrifugation, which also makes the diaphyseal cross-section the section of choice for this study.

The stages of osteogenesis in the chick are easily separated into three general stages: the prelamellar, lamellar, and postlamellar or remodeling stage (20).

The prelamellar stage is characterized by the formation of the cartilage template at about 7 to 8 days and the diaphyseal lamella at about 11 days of development. This coincides with the hypertrophy of chondrocytes near and in the template, as discussed earlier. At this point high levels of alkaline phosphatase are first noted in the osteogenic cells of the periosteum (12, 17).

Alkaline phosphatase is widely acknowledged to play an important part in the biochemistry of bone formation and reabsorption, yet its actual role is still obscure (12, 17, 20, 42).

Since high levels of glycogen are observed in highly active cells (which would also hold for osteoblasts), it has been hypothesized that alkaline phosphatase enzymatically cleaves hexose phosphate mono esters donated by the glycogen

catabolic pathways, in this way donating phosphate radicals to the synthesis of calcium phosphate hydroxy apatite—or to the precipitation of calcium phosphate at points of bone nodule nucleation (12, 17). This seems quite logical and has been borne out by some studies, yet the theory does not hold up well when all manners of organic calcification are surveyed. Alkaline phosphatase is found in all calcifying systems, yet glycogen is not (fracture callus studies; 12, 42). Other suitable substrates for alkaline phosphatase would include a variety of phospho-mono-esters (such as the serum glycerophosphate group). Another role for alkaline phosphatase has been suggested by Fleish and Neuman (17), by which the enzyme was shown to deactivate any one of several polyphosphates (ATP, AMP, ADP). These polyphosphates were shown to have an inhibitory effect on the process of calcification. In this way alkaline phosphatase was responsible for the deactivation of inhibitors, thereby allowing the process of ossification to commence (17).

The obtainable evidence links one or more of the isozymes of the alkaline phosphatase class intimately with bone formation. It has been clearly indicated that calcification appears to occur only when extracellular alkaline phosphatase is present (12, 17, 30, 42). In osteoblasts, the activity is highest during ossification and drops off sharply (sometimes completely)

when the cell is not actively involved with synthesis (12). For these reasons, alkaline phosphatase provides a suitable "marker" enzyme for studies of bone development.

The prelamellar stage can also be typified by ionic and labile calcium moving into the cell, and the probable incorporation within the mitochondria. This has been demonstrated with autoradiography, microincineration, and cytochemistry studies (28, 29, 30, 31, 32). Kashiwa (21, 22, 23, 24) has shown the Shiff base, glyoxal bis (2-hydroxinil), to be an effective cytochemical agent for the demonstration of this calcium flux.

The lamellar phase of development is described by intramembraneous ossification. After the primary lamellar bone has been laid down by the 11th day, new lamella is laid down in an intricate pattern which changes direction in the middle of the diaphysis (at the point of overlap). This bone, from day 12 or 13 to approximately 17, is then morphologically distinct from prelamellar bone (7, 8, 10, 11, 12).

When the lamellar matrix is first evident it is slightly acidophilic and becomes even more so. Finally this acidophilia gives rise to basophilia. At this point, the flux of calcium into the matrix is noted. This change to basophilia has been ascribed by some to the rise in extracellular phosphatase,

or mucopolysacharides and chondroitin sulphate. The basophilia disappears as the lamella becomes calcified (12, 20).

At day 17, the postlamellar stage is observed, and is defined by the resorption of the lamellar bone along with the appearance of endochondrial ossification. This resorption phase is also heralded by the first appearance of osteoclasts and the concomitant destruction of the lamella (20).

That lamella which is not destroyed continues to grow by apposition; therefore the osteoblasts and osteoclasts act in concert during the remodeling phase to remake the bone morphologically (11, 12, 20).

Normal Development

By the end of the 6th day of development, a thin band of perichondrial bone surrounds the cartilage and presumptive marrow cavity of the tibio-tarsus in the center shaft (diaphysis) section. This narrow band of bone separates the layers of chondrocytes found more centrally located from the periosteum edging its periphery. The periosteum is mainly comprised of osteoblasts and fibroblasts (Fig. 8 and 15). The osteoblasts can be seen to line up in close proximity to the developing bone, and are separated from it by a hydrated collagenous osteois (Figs. 1 and 2).

Osteoblasts

The osteoblasts as seen in Paragon sections and in the electron micrographs tend to fall into two general types. The first type may be packed together, appearing cuboidal individually, with prominent nuclei many times polarized towards the end of the cell farthest away from the bone matrix. These cells are typically heavy with endoplasmic reticulum, mitochondria, and Golgi complexes, and are the actively synthesizing cells responsible for the formation of new bone matrix. The second type of osteoblast is more elongate, and sparsely endowed with organelles. Many times these appear to be flattened or in a less active resting state, and are referred to as "lining cells". These osteoblasts are not thought to be responsible for active synthesis of bone matrix when in this state, yet they possess osteogenic competency, and when activated, are able to transform into "active" osteoblasts (12).

The osteoblastic layer can be several cell thicknesses, and the plasma membranes difficult to discern because tight junctions are not common to osteoblasts (27).

The fibroblastic layers are peripheral to the osteoblastic layers, and have more extracellular space than the latter. Cells with intermediate characteristics of both osteoblasts and fibroblasts can sometimes be found interspersed in the periosteum (Figs. 8 and 15).

The osteoblasts exhibit numerous cell processes that extend outwards toward the bone and to other cells in close proximity. These can be seen in cross-section close to the limiting membrane of any one osteoblast, with cellular processes of their own seen coursing out in all directions (Figs. 1 and 2). For this reason the osteoblast presents a very irregular cell outline.

The nucleus of the active osteoblast may be found towards the end of the cell farthest away from the developing bone, as stated, or it can be more centrally located in the cell. It is round or oval in appearance and typically demonstrates a prominent nucleolus. It is delimited from the cytoplasm by a double layered nuclear membrane (Fig. 1).

Cell Organelles

The endoplasmic reticulum is demonstrable in active osteoblasts and is extensive and well distributed throughout the cell. Many times fairly large cisternae testify to the active synthetic state of these cells (Fig. 4). Ribonucleoprotein particles (ribosomes) of about 110 or 125 Ångstroms can be seen adhering to the surface of the endoplasmic reticulum, justifying its common name of "rough surfaced endoplasmic reticulum".

Mitochondria are very numerous because of the active state of these cells, and are distributed near areas rich in

endoplasmic reticulum (Fig. 1). Mitochondria are not typically found near the Golgi complex which is usually juxtanuclear and very prominent. The Golgi apparatus is usually identifiable as an area rich in vesicles, secretion granules, and the densely packed lamella of the Golgi apparatus itself (Fig. 2).

Cell inclusions, consisting of lipid inclusion bodies, microbodies, and vacuoles of different types, are not in great abundance, but are found interspersed in the cytoplasm.

As early as 7 days of development in the embryonic chick, osteoblasts can be seen to become trapped in the calcifying matrix, and are then called osteocytes. Though this process is evident at this early stage of osteogenesis, it is more indicative of later periods of bone deposition.

Chondrocytes

The chondrocytes occupying the interior of the "tube" formed by the perichondrial bone can demonstrate many characteristics, depending on their age and the stage of calcification of the cartilaginous matrix. Chondrocytes are most conspicuous, due to their extremely irregular cellular appearance (Fig. 5). This is due to the great numbers of cellular processes that reach out from the cell into the cartilage matrix.

Most conspicuous are the fully mature chondrocytes that display prominent nuclei and subcellular organelles. These

are actively engaged in the production of tropocollagen, ground substance, and the accretion or organic matrix (5, 7). The large, sometimes lobed nucleus is bounded by a double-layered nuclear membrane (Figs. 7 and 12).

Active chondrocytes also display numerous and large vacuoles and vesicles, probably of a secretory nature. These are heavily lined with particles that appear to be exuded into the extracellular space. These particles dimish in size as they appear farther from the cell until they blend into the surrounding ground substance or matrix (Figs. 5, 9, and 10).

The physical properties of these particles are under investigation, but it is hypothesized that they are secretion granules or products of an organic or organo-mineral constituency. The numerous vacuoles that are opened to the extra-cellular space may account for the broken and irregular outline of these cells (Fig. 5).

In chondrocytes that are not fully matured from chondroblasts or are not in a state of active synthesis and accretion, fewer subcellular organelles are evident. The cells also appear to be more compressed, with a dense cytoplasm (Fig. 16).

The process of cartilage mineralization is necessary to the formation of endochondrial bone. As the chondrocytes become trapped in the mineralizing matrix that they are supplying as bone template, the nuclei become pyknotic and

the cell demonstrates a general appearance of dissolution and disintegration due to the restriction of nutrients diffusing into the cells (Fig. 9).

Development

From the 11th to the 13th day of development a constant trend can be noted; alternating spicules of bone begin to form in such a manner as to demonstrate the beginning of Haversian systems. This process begins with the appearance of secondary centers of ossification which interconnect and compartmentalize areas of bone proliferation into canal-like tunnels. These canals can be seen to be lined with active osteoblasts and to become penetrated by blood-vascular and nerve components at a later period of bone development. Giant multinucleated osteoclasts can be seen to occupy these lumina as early as the 10th day. Though these osteoclasts can be readily identified by their large size, many nuclei, and numerous mitochondria, they do not seem to display the activity of true bone resorption (the forming of Howeships lucanae) at this stage of osteogenesis.

The network of interdigitating osteocytic processes through their individual canaliculi is very evident as the thickness of the growing bone proportionately increases at the diaphysis.

Areas rich in collagen and bone nodules can be noted around osteoblasts that are walling themselves in before making the transition to osteocytes (Fig. 4). Many cross sections and longitudinal sections of collagen fibrils can be observed in and around these cells. The cross-striations of approximately 640 Angstroms periodicity are evident in these longitudinal sections (Figs. 2 and 11). The collagen that makes up the matrix on the cartilage side of the bone is not arranged in these large fibrils such as are found in the osteoid, but is more fibrous (5, 12).

From the 13th day the diameter steadily increases at the diaphysis. The general trend elaborated from the 11th day to this point accelerates, and rapid alterations are seen in the bone structure in cross section. Great accumulations of osteoclasts are seen at work, and a complete destruction of the central cartilage matrix is observed. In its place are seen great new masses of cell types, among which are many adipose and blood-vascular cells. Numerous small veins and capillaries break through this presumptive marrow, as great masses or accumulations of osmiophilic lipids and fats are observed. As the 21st day approaches, the bone itself takes on a different appearance; definite patterns of bone trabiculae formation become apparent, forming the more compact and striated-appearing lamellar bone type associated with the adult animal.

Accelerated Development

Osteoblasts.--Osteoblasts, both active and lining cells, displayed the characteristics described previously for days 7 through 21. When whole mounts or whole sections of bone were viewed with the light microscope (using Paragon-stained thick sections) it did appear that the accelerated sections contained a greater proportion of active cuboidal osteoblasts. In other words, the accelerated tissues do show a slightly smaller proportion of inactive osteoblasts or lining cells, when compared to the control tissues. It must be acknowledged that this can many times be influenced by the particular portion of the section viewed, yet many different sections and specimens in this study seem to bear this out. The slight preponderance of active cells, though not significant in numbers, should be considered as a possible distinction between the control and accelerated specimens.

Cell Organelles.--One apparent and obvious characteristic of the accelerated bone osteoblast is a great number of vacuoles and vesicles of a secretory nature. As mentioned earlier, vesicles, vacuoles, and inclusion bodies, though present, are not a dominant distinguishing feature of the normal osteoblast. The accelerated osteoblast presents a distinctly different appearance. The cytoplasm of these osteoblasts are heavily laden with vacuoles and vesicles of

many types. Many of these show the typical spheroid appearance of those found in the normal osteoblast, but many more have a more eccentric morphology. They form oblong and lobed shapes that are found in most cells of the accelerated periosteum (Fig. 8). Without exception these inclusions are found in conjunction with the Golgi complex, massive endoplasmic reticulum networks interspersed with mitochondria. Morphologically, this relationship would strengthen the assumption that these inclusions are primarily secretory in nature.

Another feature of the accelerated cells, that was not found to be peculiar only to osteoblasts, was a greater number of cell organelles in general. Though the ordinary osteoblast, synthesizing chondrocyte, or osteocyte are all very active cells with abundant organelles, those that were found in centrifuged tissues had very little room in their cytoplasm that was not crowded with either endoplasmic reticulum, mitochondria, Golgi networks, or the aforementioned secretory vesicles (Figs. 8, 12, 13, 14, and 15).

Chondrocytes.--Chondrocytes tend to be fairly heavily endowed with vacuoles and vesicles of different sizes in the normal state. Though no greater increase in these vacuoles were noted in the centrifuged cells, it was observed that they did demonstrate a greater number of cell organelles (Figs. 12 and 13).

Development.--Paragon whole mounts also demonstrate a trend for the accelerated tissues to show a general state of maturity 18 to 36 hours more advanced than that of the control animals. The earliest dated sections do not seem to demonstrate this to a great degree. Yet as development proceeds well into the 13th day, this tendency becomes more pronounced. It should also be noted that those deviances in the apparent chronology became less apparent as the hatch day approached; or, stated in another way, apparent differences in the maturational appearance of the cells and the bone became less obvious very late in the embryology of the individual, and therefore were more pronounced in the middle third of the developmental period (day 11 through 15).

Osteoclasts. Another unexpected observation was the comparative scarcity of osteoclasts in accelerated subjects. These large multinucleated cells were easily located with either the electron microscope or the light microscope in normal bone tissue. Osteoclasts were quite rare in the accelerated bone; less than 10 were counted in all sections observed, and these were limited to days 17 through 21.

Other than these five distinctions, the accelerated bone displayed much the same morphological appearance as the control or normal bone. The general size, shape, and intracellular relationships of all osteogenic and chondrocytic

cells were observed to be comparable and to demonstrate no more than a natural degree of variation.

Cytochemistry.--The cytochemistry failed to demonstrate any differences between the control and experimental animals. Since both sets of subjects yielded identical reaction results, one discussion of these results should suffice for both.

The alkaline phosphatase reaction was an intense one, since the diazonium salt Fast Blue RR was used. The cell membranes of the osteogenic cells all stained an intense blue. The reaction was more intense as the 13th day of development approached, as expected (17). It could be hypothesized that some degree of difference in the respective alkaline phosphatase reactions should have been noted—especially since the accelerated bone appears to mature more quickly. Tissues stained by this method showed no distinctions in the chronology or the degree of staining.

The GBHA technique proved to be excellent, and it might be noted that the .08% stain solution seemed to provide a more intense reaction (see Methods). Labile calcium stained a deep cherry red, while hydroxy apatite did not stain at all. Using this technique, it was determined that there was no visible difference in the total extracellular calcium between the accelerated and control animals.

The Pyronin-Methyl Green procedure yielded poor results due to several factors; one problem was that the original procedure was adapted from an older paraffin technique, and did not seem to give consistent results with frozen sections. Another problem was that the sections needed to be very thick to gain any staining capability; this in turn produced poorer sections for viewing. These thick sections were quite opaque and lost the distinction and contrast between cell and bone that thinner sections provide. Though this method had its limitations and inherent problems, a determination was made to the best limits of this procedure. No appreciable differences were noted in the general amount or distribution of ribonucleoproteins.

In summary, the cytochemistry became ancillary to the more in-depth electron microscope investigation. The histological procedures pointed out no true differences or distinctions between the accelerated and control bone, to the limit of resolution of each respective technique.

CHAPTER IV

DISCUSSION

Any one of the aforementioned observations taken by itself can lend some insight into the phenomenon of extra calcium deposition due to chronic centrifugation. However, many of the observations lead to new problems of interpretation, both in terms of the acceleration study at hand, and also concerning the general body of knowledge involved with the basic process of osteogenesis.

It should be stated at the outset that the basic differences noted between control and experimental animals are ones of degree or shades of differences in most cases, not in gross morphological distinctions. Even though these distinctions are by nature subtle, it is hoped that a basis for understanding this acceleration study can be gained by the appreciation that all of these forces acting in concert can drastically alter the developmental history of embryonic bone.

Before discussing the consequence of chronic acceleration on osteogenesis, it should be recognized that much is not known about the osteogenesis process, and many current theories are in heavy debate. The ambition of this study

was two-fold: first, to determine some basic similarities and dissimilarities between the accelerated bone and that of the normal bone; second, to learn new facts about the osteogenic process itself. The acceleration of tissues, both physically and physiologically, could lend some insight into the normal osteogenic sequence which may be interrupted in some way by chronic acceleration.

The first significant finding was an 18- to 36-hour increase in bone maturity, which corresponded to the greater increases in bone mass during the middle third of the developmental period. Less gain was apparent towards the day of hatching (21st day), and this also coincides with a falling off of bone deposition noted by Redden (38) after day 17. This acceleration in maturity could be due to alterations in the "stage" and "phase" specificity of the osteogenic sequence, which will be described later as a possible explanatory hypothesis.

An increase of active osteoblasts was noted in the accelerated tissue, and less of the flattened lining cells. This finding, taken by itself, would not explain the great amount of extra calcium deposited. However, if this fact is considered with the observations that osteoclasts are relatively rare in accelerated bone, and that osteoblasts themselves contain a much more abundant synthetic apparatus in accelerated bone, this does present

an enticing interpretation for the actual mechanism of extra nucleation of calcium hydroxy apatite.

There are two ways that osteoblasts could be involved in depositing more bone on a growing perichondrial shaft: an increase in osteoblastic activity by virtue of cellular output, and/or by an actual increase in numbers of active cells compared to the inactive cells. Neither of these phenomenon appears to vary to the degree needed to explain up to 30% increases in accelerated bone.

An additional way in which bone mass might be increased would be to decrease the activity of the osteoclast, allowing the growth of the perichondrial sheath to extend normally with a decreased resorption of bone from the interior by osteoclasts. This presents a nice hypothesis. The physical accumulation of more bone in the accelerated animal is resultant from the slightly greater numbers of active cells, a higher level of activity, and the lack of osteoclastic activity. This is even more understandable when one considers that the falling off of the accelerated bone mass from day 17 nicely coincides with the appearance of the few osteoclasts noted in days 17 through 21 in accelerated tissues. Yet this hypothesis is handicapped by the physical appearance of the osteoclasts themselves.

The osteoclast of the middle third of the developmental period observed in control animals did not morphologically

resemble the osteoclast of adult bone, or those described for the remodeling phase, where osteoclastic activity is high. The classical osteoclast of the literature is a large multinucleated cell with numerous mitochondria and little, if any, endoplasmic reticulum (27). The osteoclast found in the embryonic chick up to the 17th day of development are identical to this except that they possess moderate amounts of endoplasmic reticulum (Fig. 5). Also, the classical osteoclast actively engaged in calcium readsorption displays the fimbriated cell membrane in close proximity to the bone being absorbed. At this location there is a hollowed-out area known as "Howeship's lucuna". The osteoclasts observed from day 10 through day 17 do not demonstrate these fimbriated edges, implying a period of little or no activity.

Though the actual process of osteoclasis is not very well understood, it has been borne out experimentally that the "active"-appearing osteoclasts do incorporate calcium in their mitochondria, closest to the area of bone reabsorption, with little or no incorporation farther away from the site of activity (30).

Other experimentation has demonstrated that the collagen fibers of the osteoid must be in a certain biochemical formation to serve as a proper template or nidus for the nucleation of bone crystal. More than half of the proline

residues comprising the collagen fiber must be in the hydroxylated state (hydroxyproline) (6). Incompletely hydroxylated collagen is abnormally soluble and serves as a poor matrix for calcium hydroxyapatite deposition. Could these very early, apparently inactive osteoclasts indirectly influence the deposition of calcium through the action of a proline hydroxylase (6)? This would render the collagen matrix secreted by the osteoblasts unsuitable for nucleation, and would achieve the same function as active absorption by osteoclasts. This would also make the hypothesis of early osteoclasis tenable once more; however, a great deal of experimentation and observation needs to be done on these early multinucleated cell systems.

The presence of endoplasmic reticulum in the embryonic osteoclast and the absence of it in the classical model poses another interesting developmental question. It is commonly accepted that osteoclasts arise from the union of osteoblasts (12). Amalgamation of many osteoblasts forms giant multinucleated cells called osteoclasts. Curiously enough, no such mention is made to chick osteogenesis of osteoclasts prior to the remodeling stage or the 17th day of development (20). Yet one would hypothesize their existence or synthesis prior to this time.

It seems easy to accept that in early developmental stages, if osteoclasts do arise from the union of many

osteoblasts, they should probably be observed to contain the cellular remnants of their osteoblastic progenitors. In this case the presence of endoplasmic reticulum would denote the transitory state from osteoblasts which do contain an abundant endoplasmic reticulum.

It is curious why the literature does not reflect or speculate on these early multinucleated cells, so easily identifiable, except to ascribe to the osteoclast an untimely late appearance in the osteogenic process for reasons that are even more obscure (12, 20).

The apparent absence of osteoclasts in accelerated tissue needs some discussion. One possible explanation would be that the increased gravitational force exerted on the body tissues does not favor the formation and persistence of large, multinucleated cell systems, especially when the embryo is in a rather fluid and mesenchymous stage. It would be pure speculation, but it might be suspected that with the ongoing differentiation and accumulation of more body mass and density due to organogenesis, the more compact and dense tissues might allow the presence of multinucleated cell systems. There is little experimental data to fortify this, yet it would provide an interesting research project. The relative numbers of large multinucleated systems such as bone marrow megakaryocytes could provide another experimental subject.

The fact that this increased gravitational stress is acting on the primarily fluid and colloidal constituencies of the cell would be an easy logical assumption. It is also supported somewhat by the irregular inclusions and secretion granules observed in accelerated tissues (Fig. 7). Under normal circumstances these granules and inclusion bodies are elliptical in shape (Fig. 1). The widespread irregularity and curious nature of these bodies could be ascribed to the force vector and its influence on the colloidal particles of the cell.

Another observational difference between the accelerated and control bone was the great amount of organelles present in the accelerated osteogenic cells. This is not to imply that the normal osteoblast or chondrocyte is under-endowed, as these cells are by their nature very active and contain a large number of organelles, indicative of their activity. The accelerated cells contain by comparison a supranormal amount of cell inclusions and subcellular organelles. There is hardly any cytoplasm in these cells that is not heavily loaded with these bodies (Figs. 8, 12, 13, 14, and 15). The overall impression of these cells is one of a higher level of activity over that of the normal or control tissues. In addition to the great amount of Golgi apparatus, mitochondria, and endoplasmic reticulum, one can see extremely large and swollen cisternae, and the previously mentioned secretion

granules. These granules are located near the juxta-nuclear Golgi region, and, as mentioned, are great in number when compared to the control animals bone (Fig. 8).

Several hypotheses can be offered to explain in part the phenomenon of extra calcium deposition in accelerated animals. One hypothesis would be to suspect the cause of increased amount of bone to be a direct influence of the gravitational force vector operating on the individual bone cell or osteogenic cell and accelerating the normal synthetic activity to elevated levels of output. This would then explain the hyperactivity and morphological alterations induced by gravitational force from a purely cellular standpoint.

Another hypothesis would be to consider the problem as in interruption of the osteogenic process in terms of its phase and stage specificity. This would be a strictly developmental approach. The developing bone system must be appreciated in its developmental aspects like any other differentiated organ-tissue field. It must depend on a complex chain of tissue interactions, cell transformation, and possible inductive effects. The process of induction is inferred by several investigators attempting to explain the fibroblast-osteoblast transformation and the interactions during the nucleation of hydroxy apatite (17). Also, cellular transformation is a fundamental portion of osteogenic theory.

It is observed in fibroblast-osteoblast, osteoblast-osteoclast, and osteoblast-osteocyte transformations (11, 12, 15). The many morphological and synthetic changes in the chondrocyte types may also fall into this category. In all such developing systems, depending upon cellular transformations and inductive effects, the tissues must be in a proper "phase" of development. This refers to a special temporal arrangement of the cells in relation to the chronology of cells around them and the transformation of those that have preceded them (5).

"Stage" specificity refers to the actual physical arrangement of the cells undergoing change. Many times actual physical contact is needed to initiate an inductive effect on a developing field (5). The importance of this to the present experiment is that in most vertebrates studied, these parameters have been shown to depend a great deal on environmental factors acting through hormone systems (5). The most common example of this would be the effect of circadian rhythms (day and night cycles) on the hypothalamus, which can affect thyroid output, and therefore metamorphosis and organogenesis, in many vertebrates (5). Temperature has also been shown to have a similar effect in many cases (5).

The effect of gravity on developmental systems has no experimental precedent to rely upon. The chronological acceleration of the bone in centrifuged chicks and the virtual

absence of osteoclasts could indicate a possible role for gravity by alteration of the stage and phase specificity of the transforming cells.

A third hypothesis for the role played by gravity in this study would be that of a pure physiological effect; or an acceleration or strangulation of the blood vascular system and its components. This would be accomplished by a centrifugal effect upon the chick in the shell in relation to its blood vascular system, the direction of the circulation in terms of the force vector, as well as the entanglement or crimping of the major vitelline veins and arteries due to the shifting position of the chick as it increases in size. The beauty of this thesis comes in its ability to explain the falling off of bone deposition from the 17th day previously noted. This would come about due to the shifting of the enlarging embryo in relation to the yolk sac,which is dimishing in size. The vitelline veins could be easily strangulated, resulting in a restricted circulation. This potential for strangulation has been observed many times at approximately 17 days of development (38). Also implicit in this thesis is the concept that the normal flow of blood-borne hormones may either be retarded or obstructed from their target cells. It is well known that many blood-borne elements are of importance to the process of calcium deposition. The only way to properly deal with this experimentally

would be to bioassay for various levels of hormones and enzymes influential in the osteogenic process (parathyroid hormone, calcitonin, alkaline phosphatase, and possibly prolinehydroxylase to mention only a few). It has been shown that such a hypothesis could account for a falling off of calcium deposition, yet it is much more difficult to correlate a pure physiological approach such as this to the observed 30% increases in bone calciun in accelerated animals.

To explain such a complicated phenomenon as extra calcium deposition due to chronic acceleration in terms of only one of these hypotheses would at best be an incomplete explanation. When and if the elucidation of this problem is brought forth, it will probably be a composite of all three of these hypotheses or approaches instead of just one.

Finally, one should remember that during the evolution of organic life on this planet, the most constant environmental force acting on the evolving systems was probably gravity.

Gravity has remained constant, most likely, for the last 2.5 billion years of organic life, while other environmental forces acting on the young planet evoked their effects through gross climatic and geological change.

It should be of some interest to the biologist to even speculate if organic life as we know it should even possess

the ability to adapt to gross change in the gravitational force. While natural selection has circumvented problems that arise due to changes in environment, it has proceded metaphorically as if gravity was no appreciable parameter at all. If organisms and their component systems, either developing or adult, should be subjected to changes in gravitational stress for extended periods of time, it would seem that their ability to respond adaptively would be very suspect, due to their evolutionary heritage.

CHAPTER V

SUMMARY

The findings of the present study indicate that the reported supranormal deposition of calcium in accelerated animals can in part be explained by 1) the greater numbers of active osteoblasts found in accelerated animals, 2) the apparent absence of osteoclasts in accelerated tissues, 3) a greater number of secretion granules and a greater abundance of subcellular organelles in accelerated osteoblasts, osteocytes, and chondrocytes, 4) a general trend of accelerated development in terms of chronology (18 to 36 hours more advanced in centrifuged animals over controls), 5) a pecularity of the accelerated cells with the eccentric shapes of the secretion granules themselves, most probably testifying to the effects of gravitational force on the fluid-colloid system that is the cell.

Fig. 1--Osteoblast, 11-day control, with irregular cell outline due to cellular processes (CP) reaching into the osteoid (O). Note the double layered nuclear membrane (NM) and abundant endoplasmic reticulum (ER). The black strippling is bone (B). X 26,000

Fig. 2--Osteoblast, 13-day control, showing cross sectional and longitudinal views of the collagen fibers (C) making up the osteoid. A well developed Golgi complex (G) is juxtanuclear (N). X 24,000

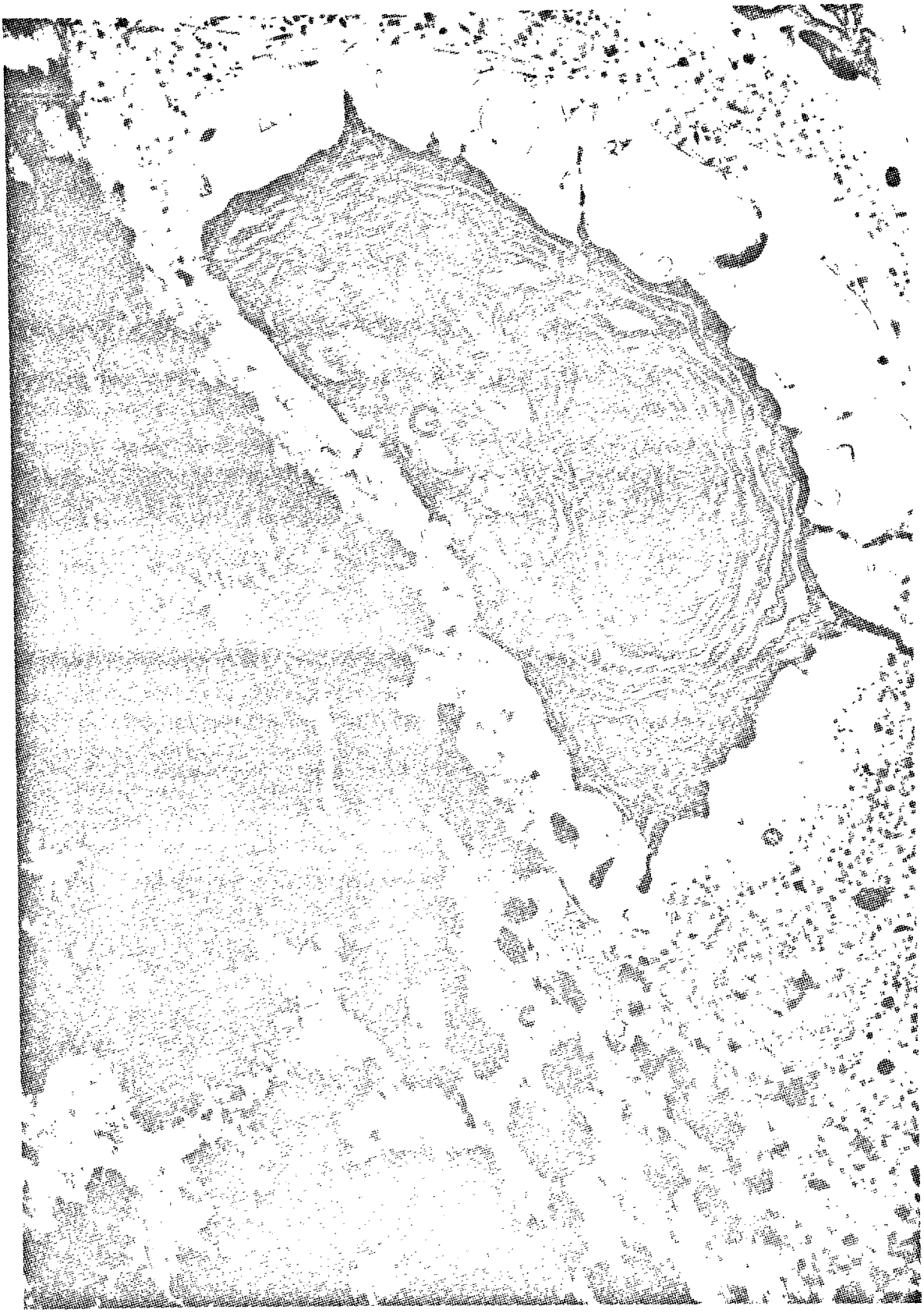


Fig. 3--Osteocyte, 13-day control, occupying it's lacunae (L) with interdigitating cell processes from adjacent osteocytes (CP). X 9,500

Fig. 4--Osteoblast, 11-day control, with an extensive collagenous osteoid (O) and very well developed cisternae (CI). X 37,000

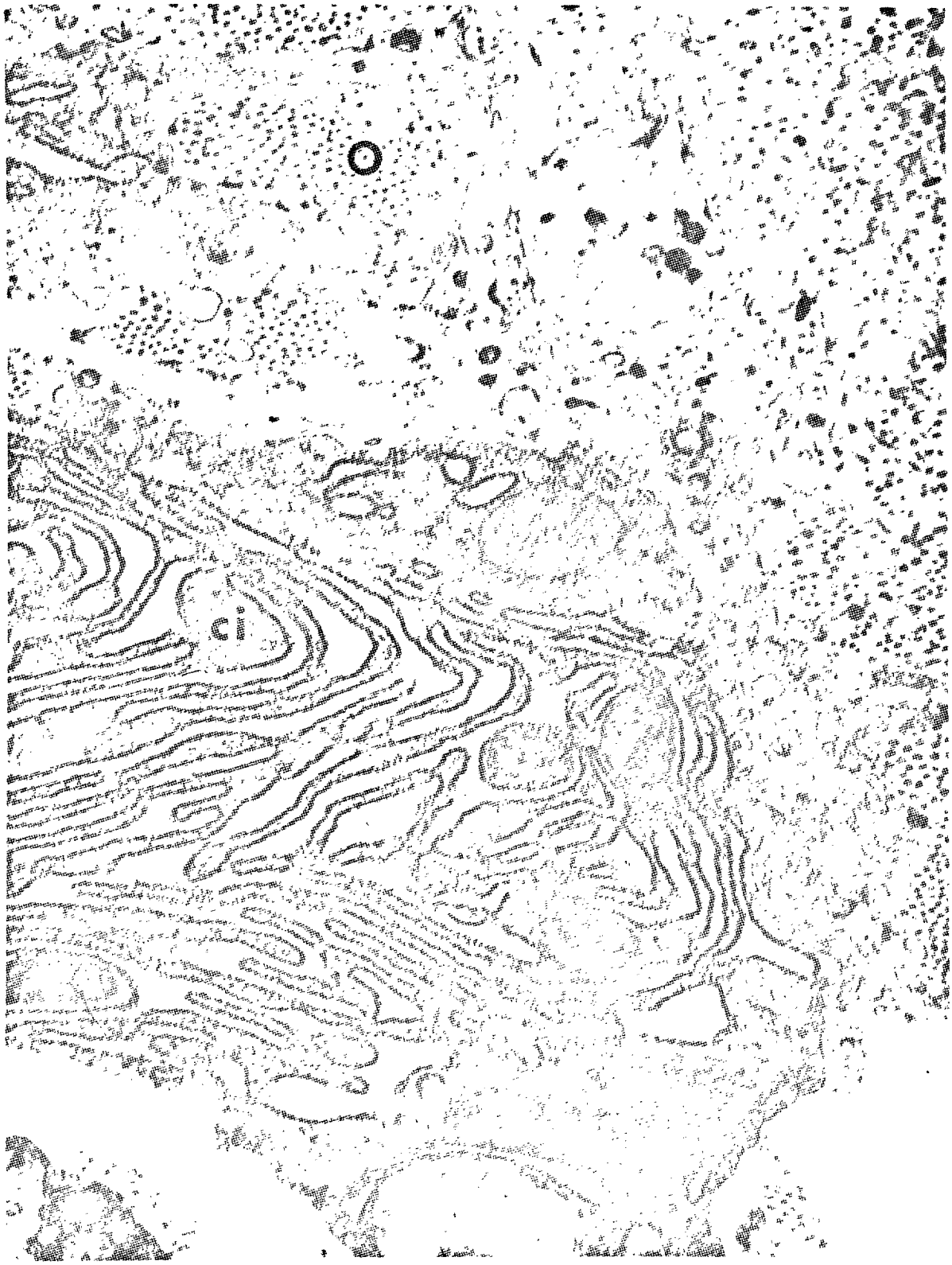


Fig. 5--Chondrocytes, 7-day control. Note the extremely irregular cell outline. X 3,060

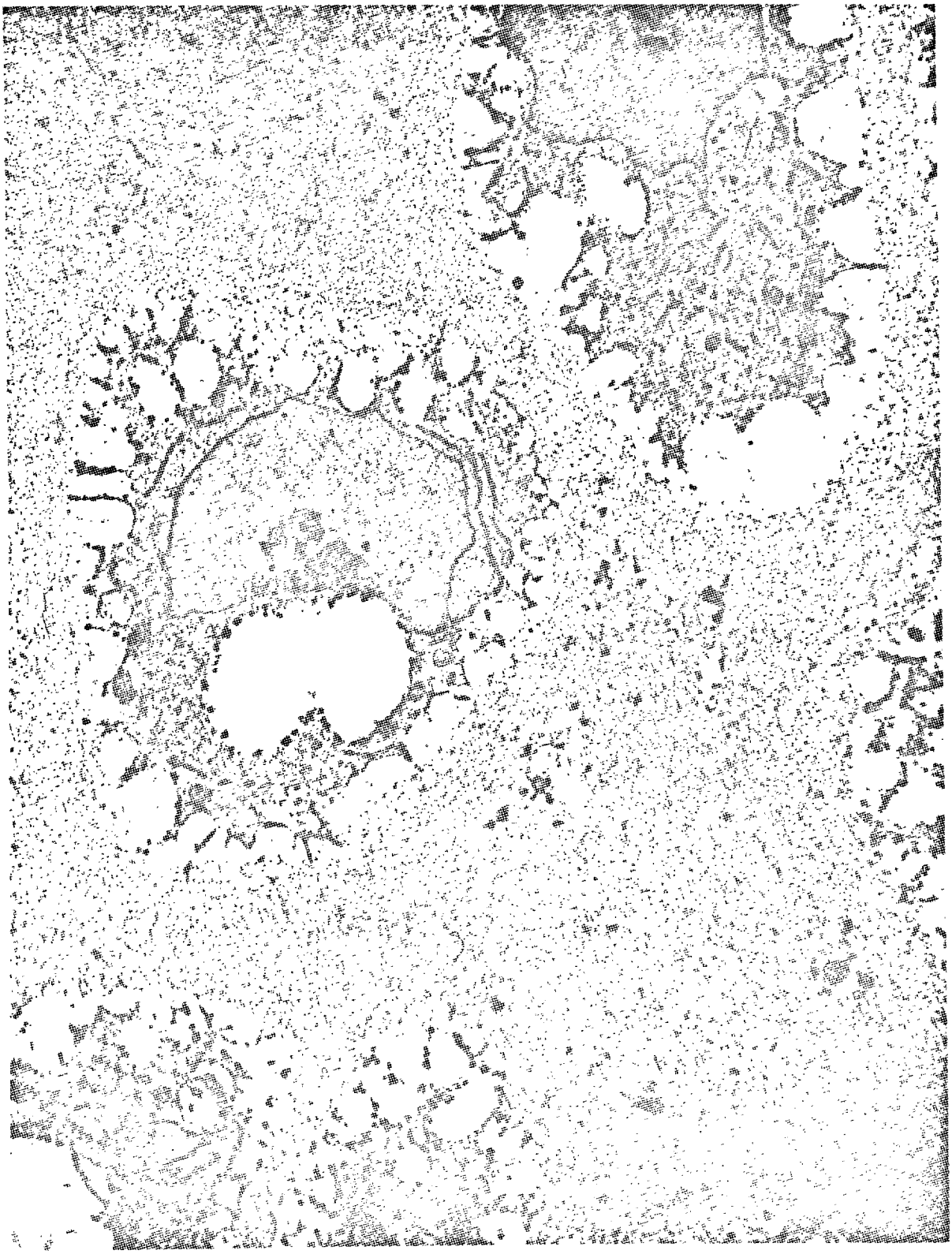


Fig. 6--Osteoclast, 13-day control, with 3 prominent nuclei (N), extensive endoplasmic reticulum (ER), and mitochondria (M). X 6,500

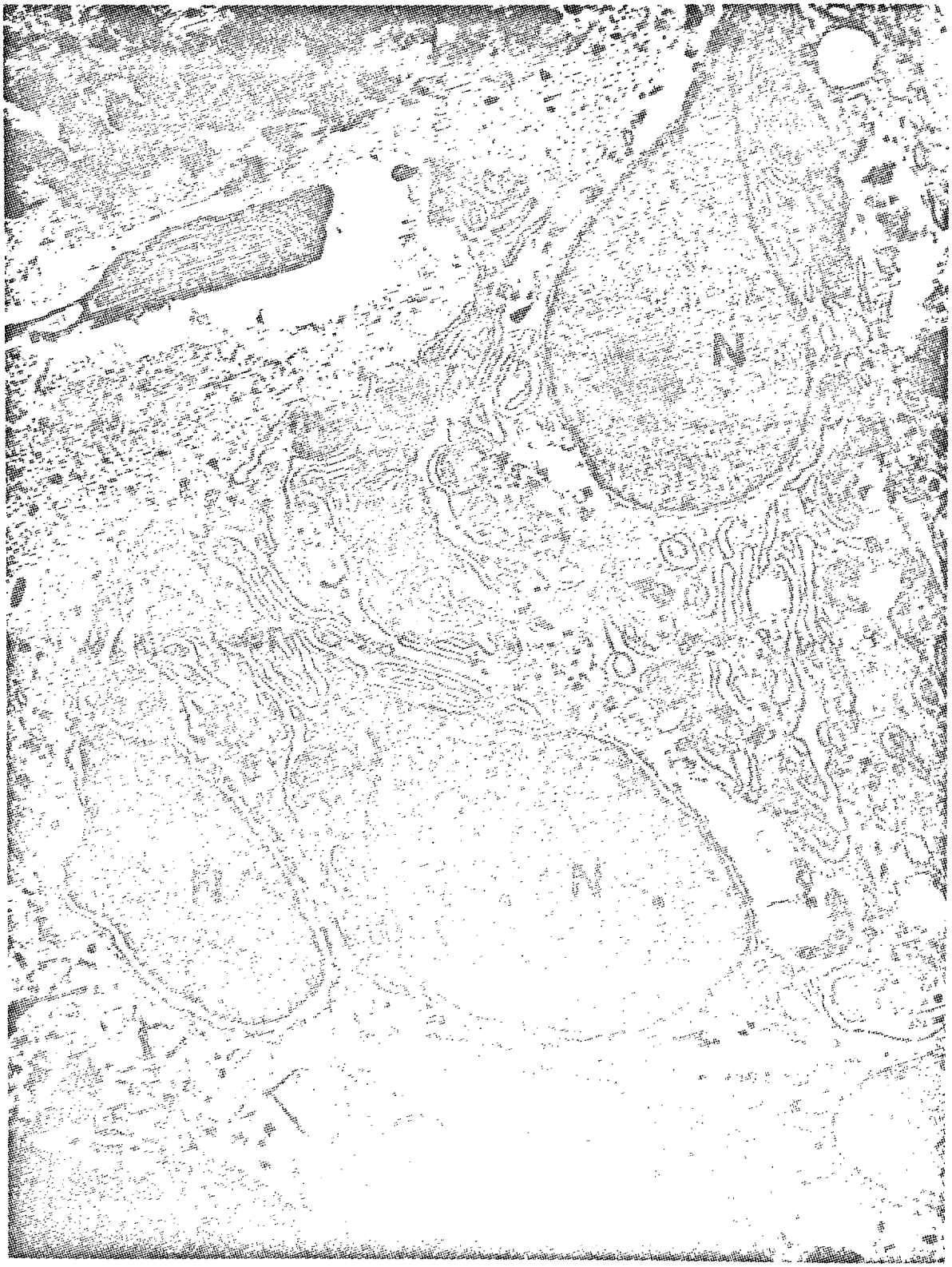


Fig. 7--Chondrocyte, 7-day accelerated. Note the large vacuoles (V), prominent nucleus (N) with a double layered nuclear membrane (NM), and golgi complex (G). X 22,000

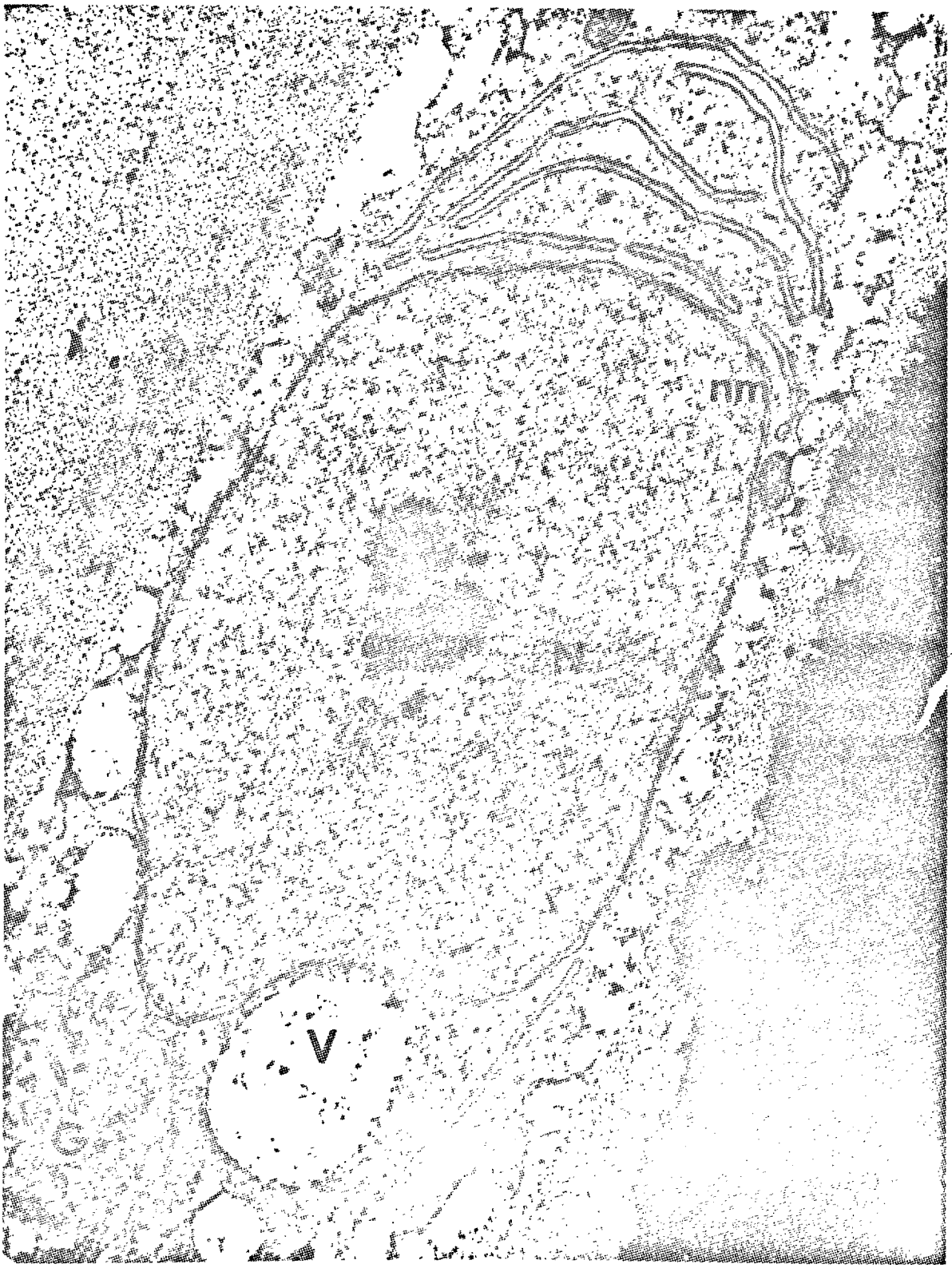

V

Fig. 8--Osteoblasts (Periosteum), 7-day accelerated with abundant organelles in the cytoplasm. Note the numerous eccentricly shaped inclusion bodies or vesicles (VE). X 6,000

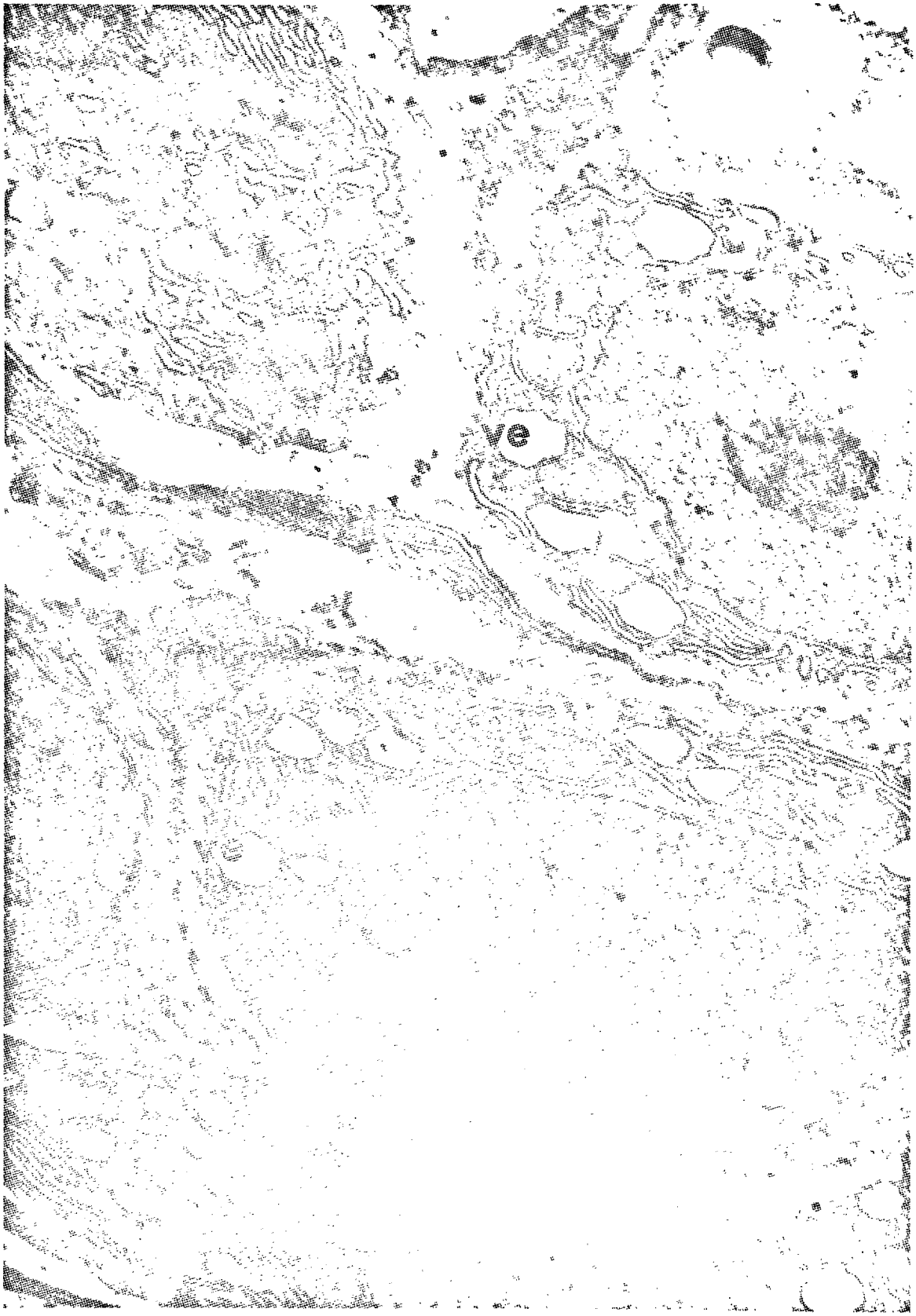

Ve

Fig. 9--Chondrocytes, 7-day accelerated, zone of dissolution. Note the disintegrating state of the cytoplasm and its organelles. X 6,000

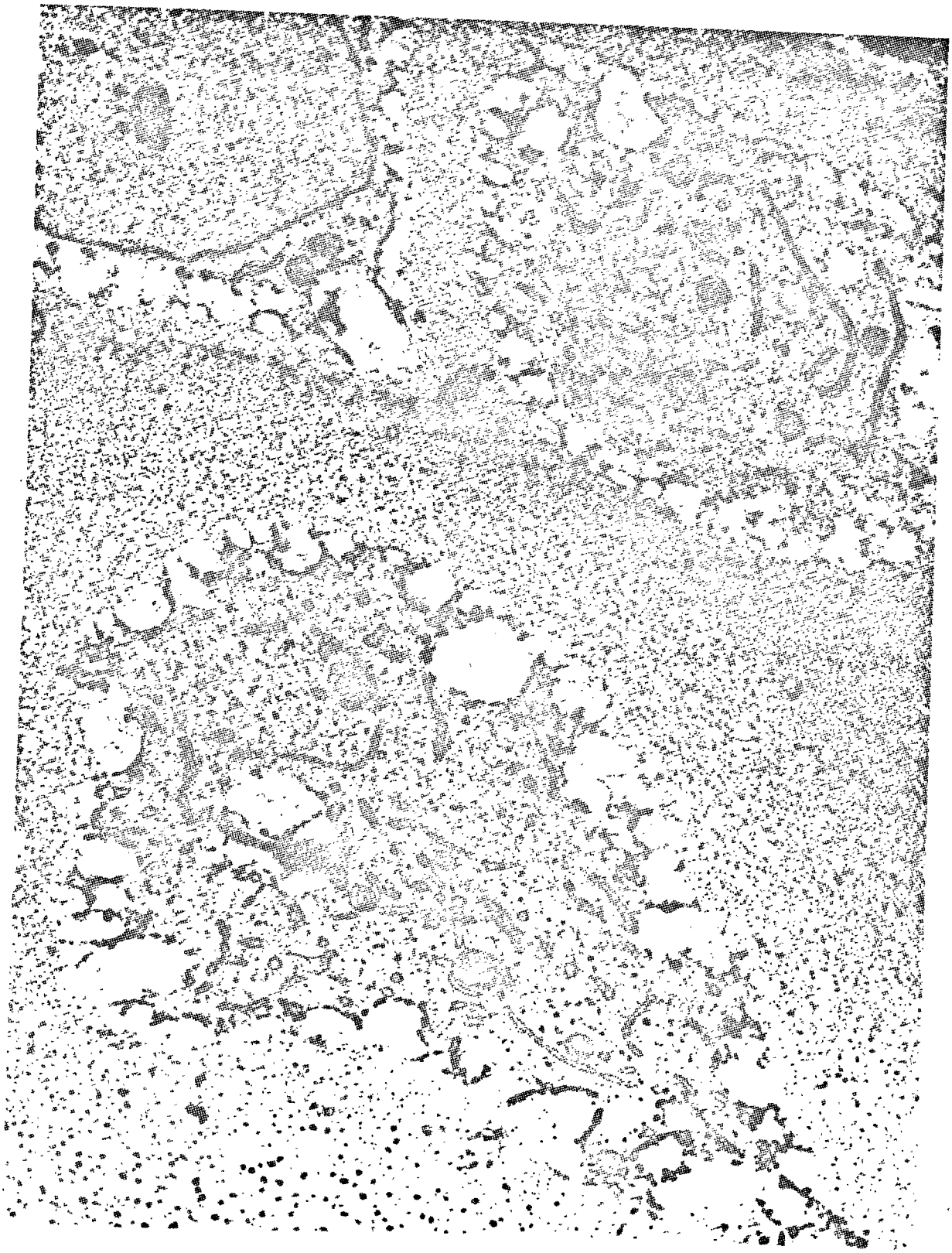


Fig. 10--Chondrocyte, 7-day accelerated, with large vacuoles (V) and abundant vesicles (VE). Particles (PA) seemingly exuded by way of the vacuoles appear to diminish in size as they work their way from the cell to become part of the matrix (MA). X 17,500

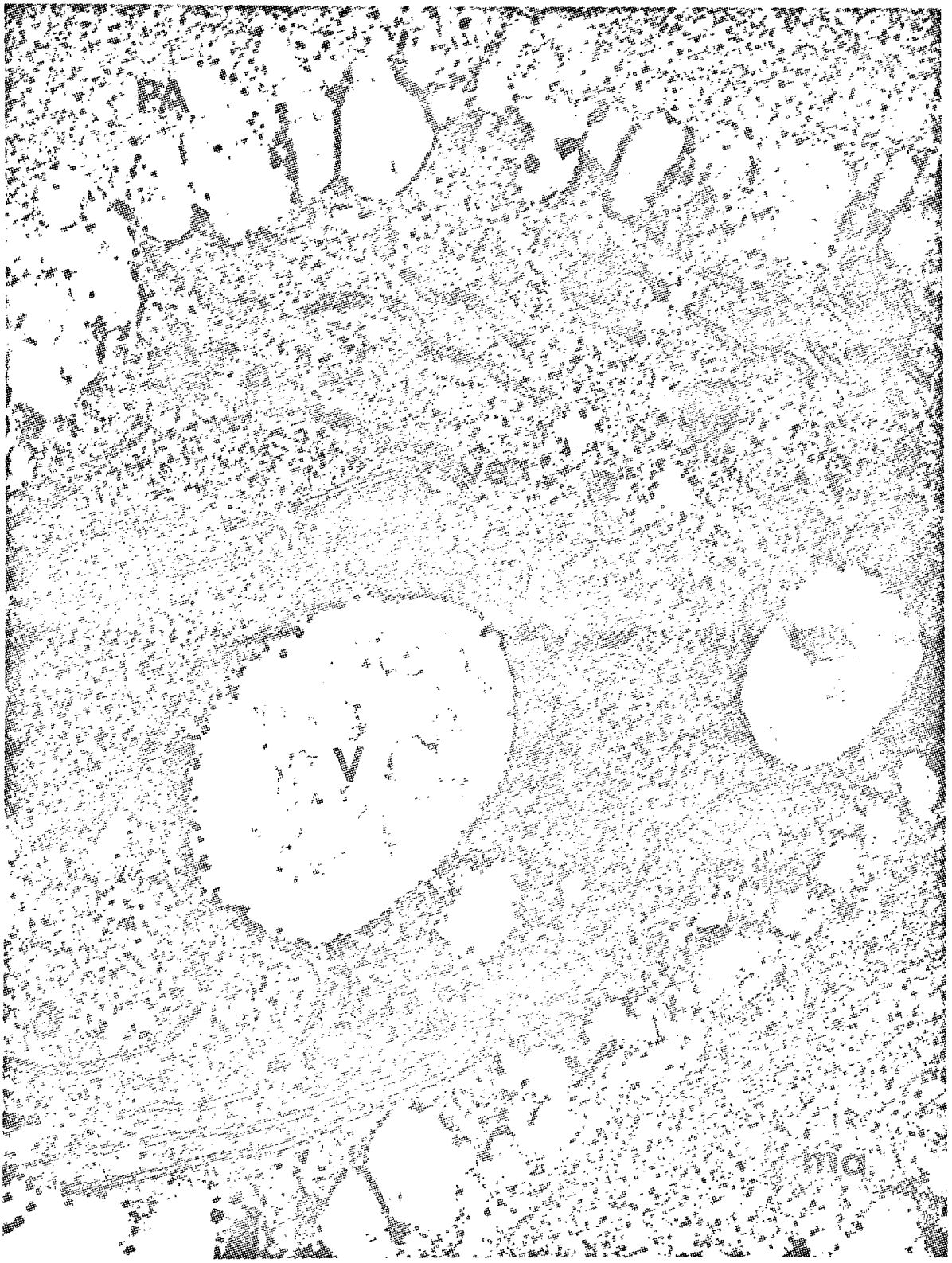


Fig. 11--Osteoid, 13-day accelerated. Note the 640 Å periodicity of cross striations on individual collagen fibers (C). X 30,000

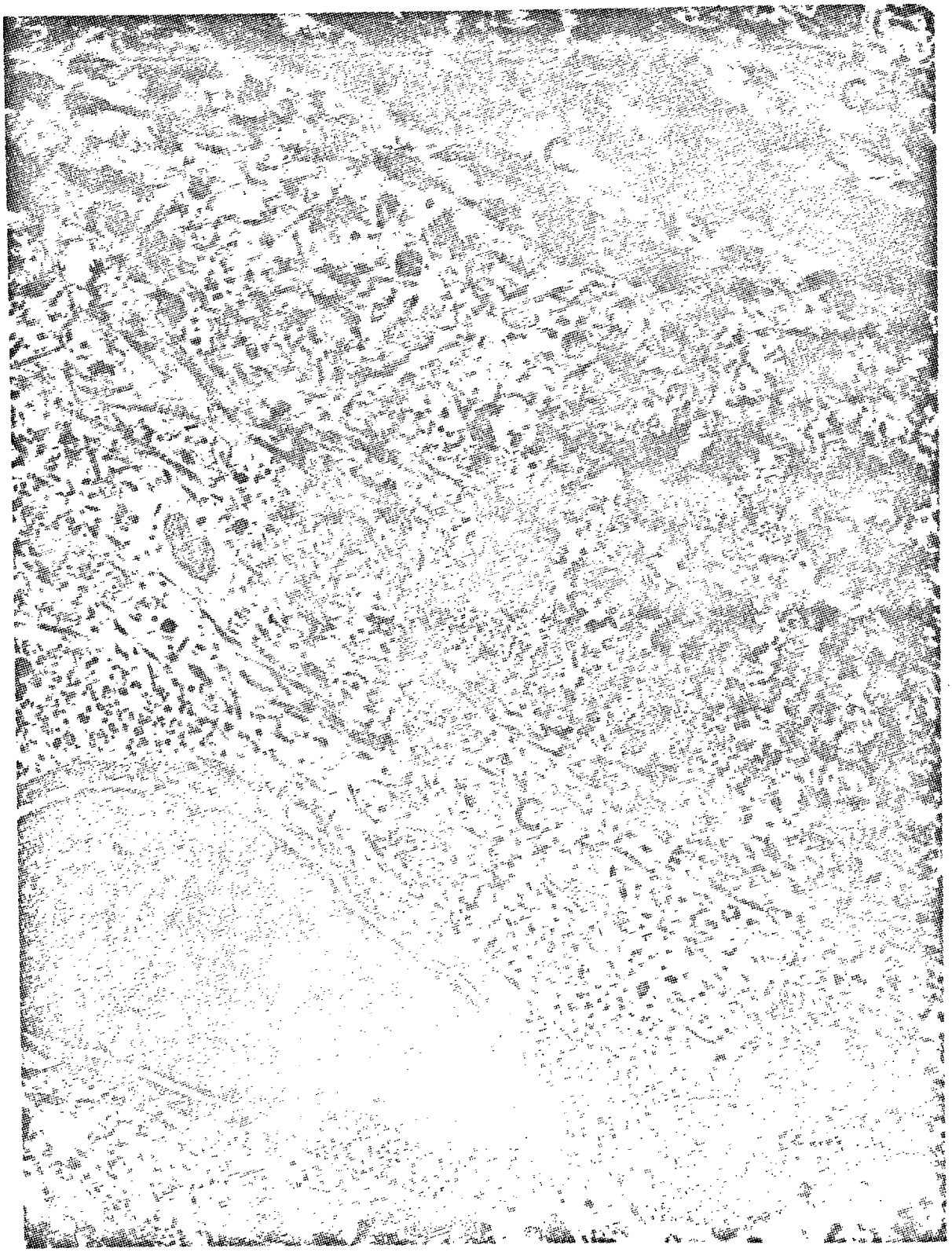


Fig. 12--Chondrocytes, 7-day accelerated, with large lobed nuclei (N) and extensive endoplasmic reticulum (ER) and mitochondria (M). X 12,500

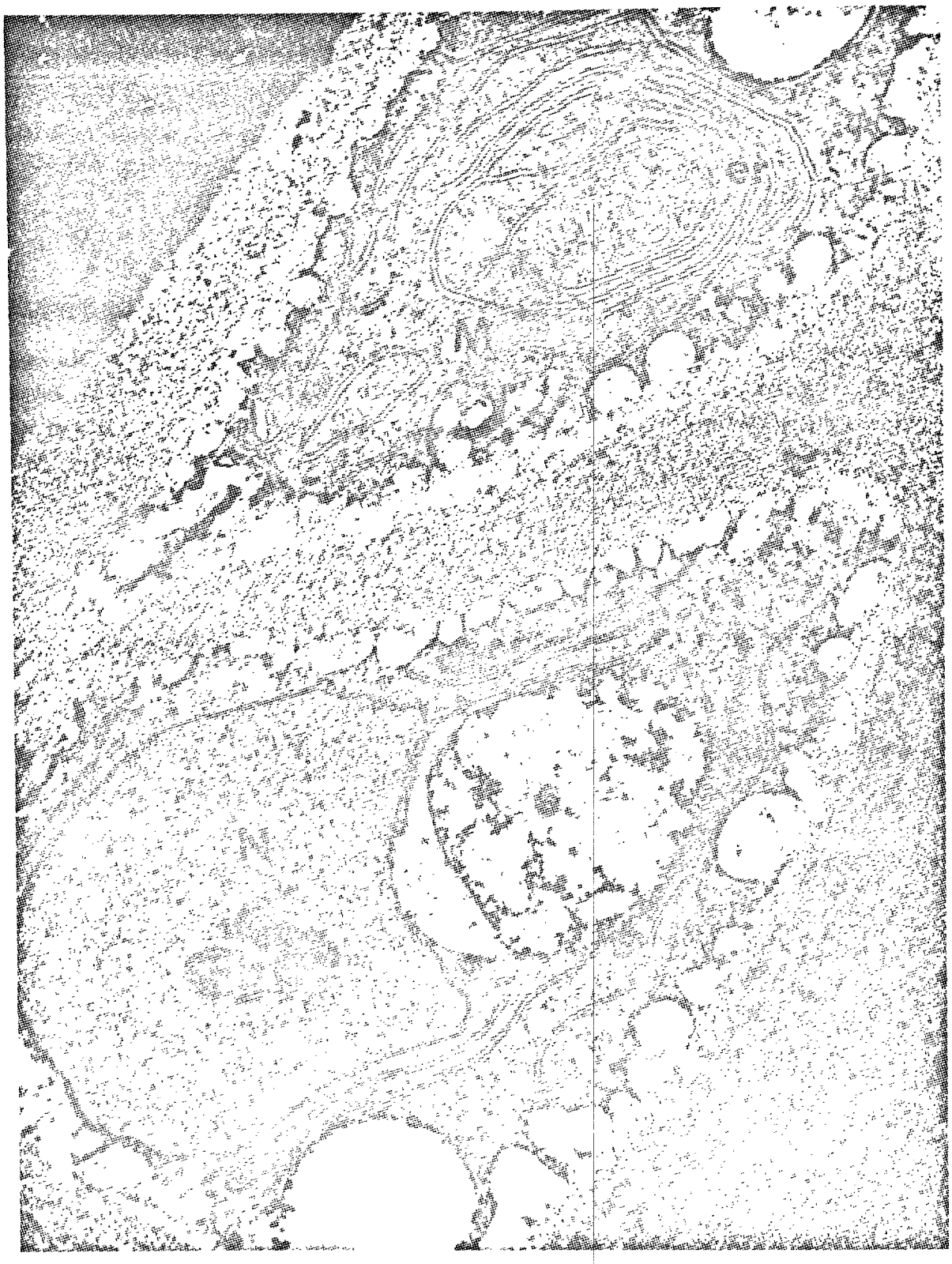


Fig. 13--Chondrocyte, 13-day accelerated, showing the extensive endoplasmic reticulum (ER) and mitochondria (M) found in accelerated cells. X 176,000

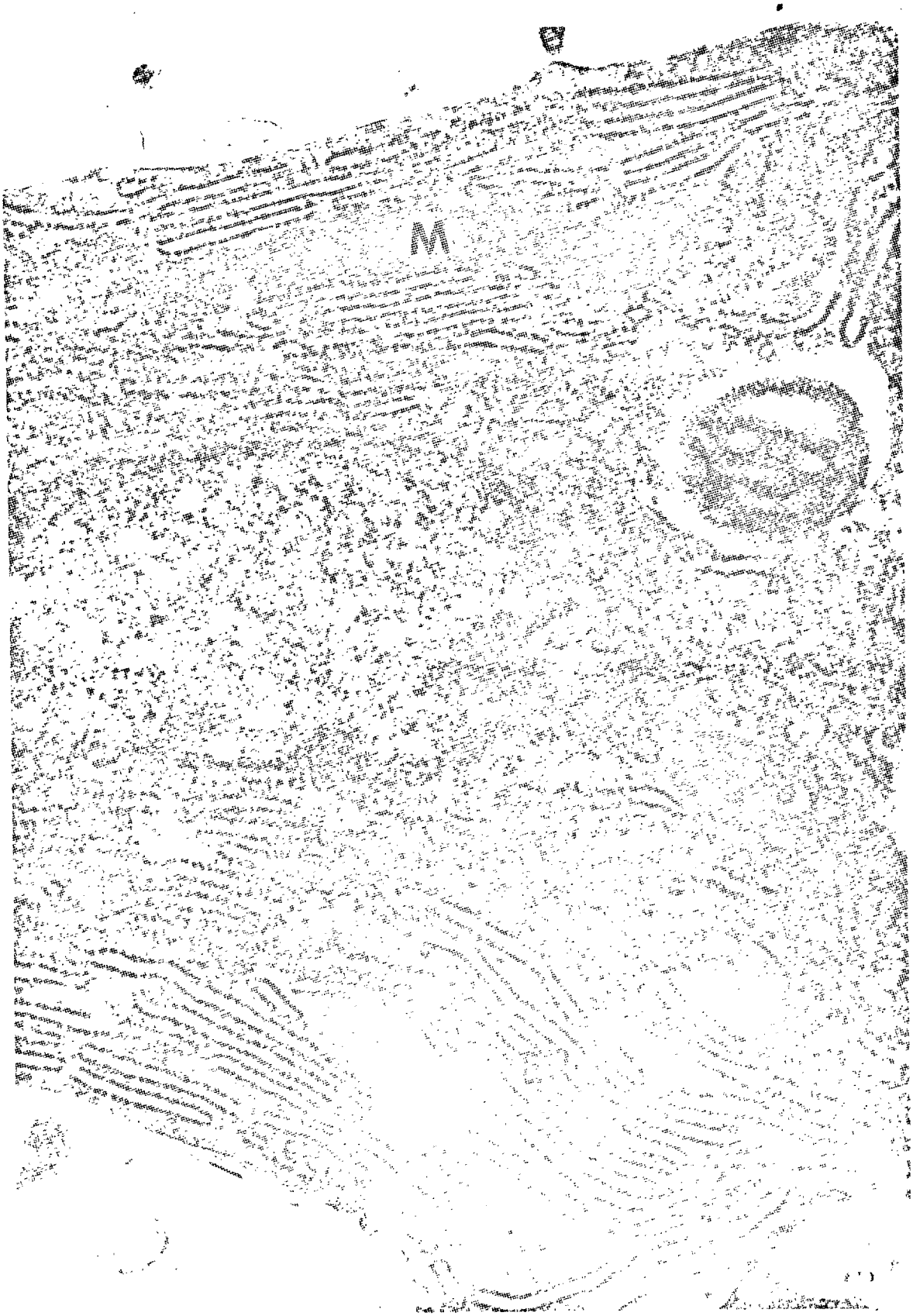


Fig. 14--Osteoblast, 7-day accelerated, with very extensive cytoplasmic organelles indicative of accelerated tissues. X 10,600

Fig. 15--Periosteum, 9-day accelerated, with numerous cell organelles. Note the erythroblast (E) seen in the intracellular space (IS) in advance of later blood vascular penetration. X 4,000

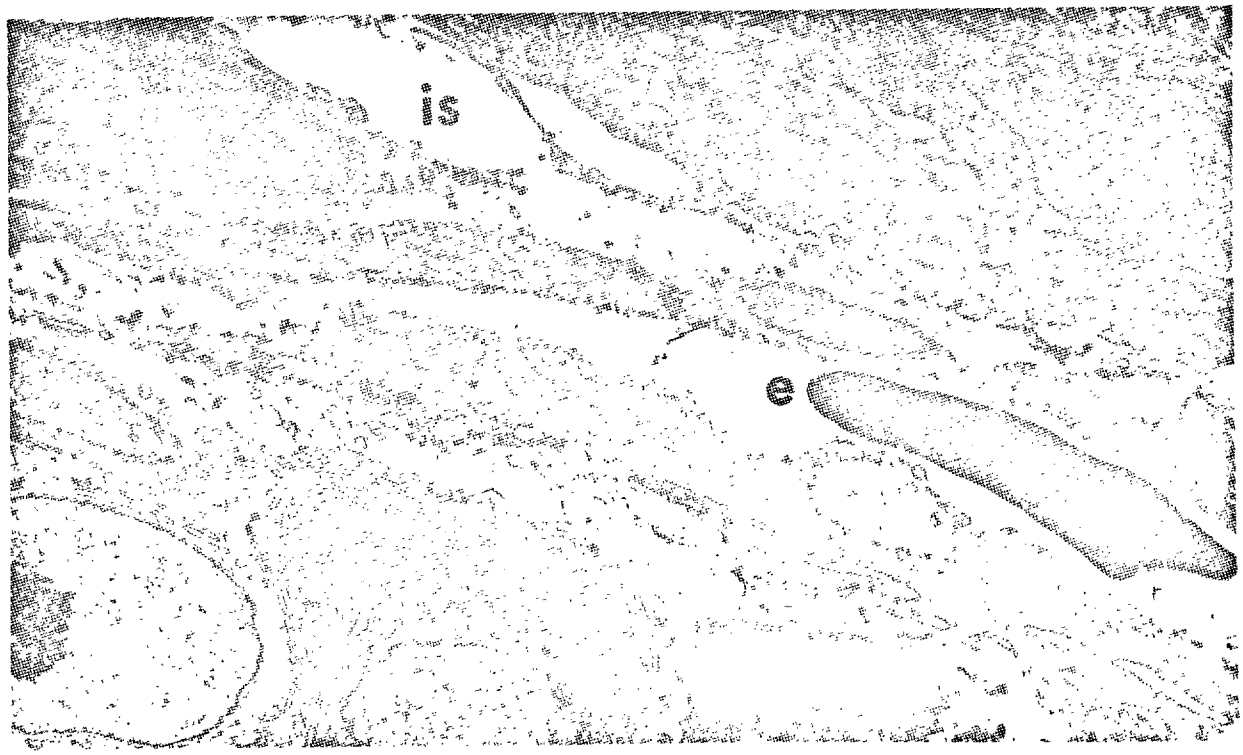
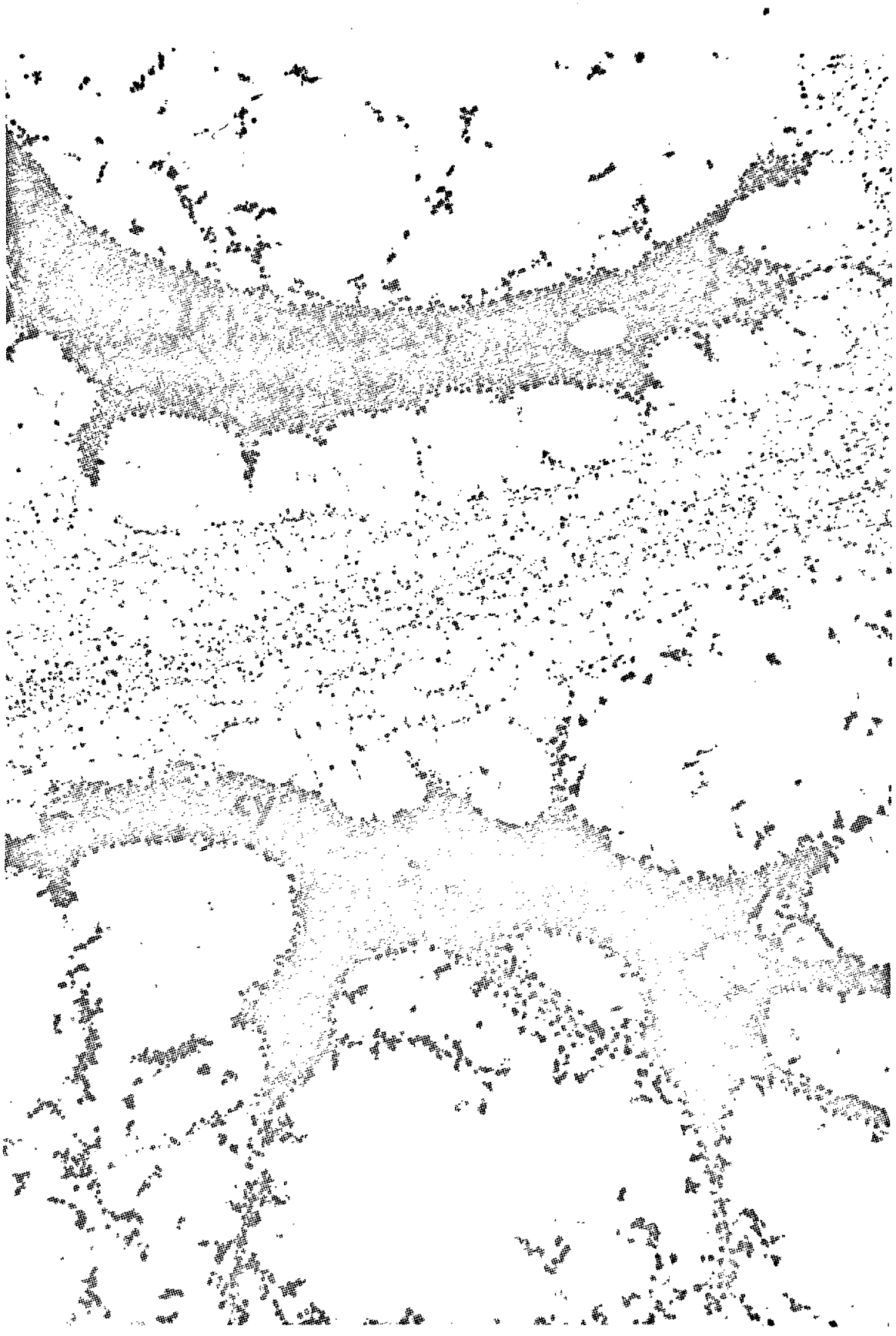


Fig. 16--Chondrocytes, 13-day accelerated, in the resting stage. Note the more compact cytoplasm (CY) and the scarcity of cellular organelles. X 16,900



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