The Fine Structure of Blastema Cells and Differentiating Cartilage Cells in Regenerating Limbs of *Amblystoma* Larvae*

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ABSTRACT

Regenerating forelimbs of larval salamanders, *Amblystoma punctatum*, were fixed in OsO_4 at various intervals after amputation and were sectioned for study with the electron microscope. The dedifferentiated cells comprising the early blastema were found to have a fine structure similar to that of other undifferentiated cells and to have lost all of the identifying morphological features of their tissues of origin. The cytoplasm of such cells is characterized by numerous free ribonucleoprotein granules and a discontinuous vesicular endoplasmic reticulum. The cells have more abundant cytoplasm and are in closer contact with each other than was previously realized. The layer of condensed ground substance investing most differentiated cell types is lacking. After a period of rapid cell division, the morphology of the blastema cell changes. Cytoplasm is now sparse and contains a high concentration of free ribonucleoprotein granules, but little endoplasmic reticulum. The differentiating cartilage cell, however, develops an extensive, highly organized endoplasmic reticulum and the Golgi apparatus also appears to become more highly differentiated and more extensive at this time. Small vesicles appear throughout the cytoplasm at the time the new cisternae originate and may contribute to their formation. These and other changes in the cytoplasmic organelles are discussed.

INTRODUCTION

The electron microscope has made it possible to extend the investigation of classical problems in growth and differentiation to the level of the macromolecular organization of the cells involved. It has revealed that the cytoplasm of differentiated cells exhibits a complexity of organization not appreciated heretofore in studies with the light microscope and, furthermore, by means of correlated biochemical studies, it has added greatly to our knowledge of the functions of some of the cytoplasmic organelles depicted. Of particular interest to students of growth has been the recent work (1–3) demonstrating the particulate nature of the basophilic component of the cytoplasm and its relation to protein synthesis. Small particles of ribonucleoprotein are found either dispersed throughout the cytoplasm or closely associated with the surfaces of the membrane-limited system of vesicles and tubules now commonly called the endoplasmic reticulum (4, 5). The form of this system or organelle and the abundance of the cytoplasmic nucleoprotein granules have been shown to vary widely in different cells. Variations in the fine structure of mitochondria and of the Golgi apparatus in different functional states have also been reported. The present study of the regenerating salamander limb was undertaken with the object of describing the behavior of these cytoplasmic organelles in a biological system in which dedifferentiation, proliferative growth, and redifferentiation occur.

The results of this study confirm and extend previous descriptions, based on light microscopy, of the histology of the regenerating limb of *Amblystoma*. The cells of the early blastema

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appear undifferentiated in all features of their submicroscopic morphology and a characteristic sequence of changes in the cytoplasmic organelles during the growth and differentiation of the regenerate has been demonstrated with the electron microscope. The observations reported here are mainly concerned with the cytomorphosis of the cartilage cells. A future report will deal with the more complex changes that occur in muscle during regeneration of the salamander limb.

Materials and Methods

Amblystoma punctatum larvae, raised from eggs collected near Baltimore, were used in the present study. The animals were kept in finger-bowls (3 per bowl) at 23°C. and were fed *Tubifex* worms. When taken for study, larvae were about 18 mm. in length and the forelimbs possessed the fourth finger-bud. Limbs were amputated through the middle of the humerus and regenerating limbs were fixed at daily intervals to the 10th postoperative day and every other day thereafter to the 20th day after amputation. After being fixed for 2 hours in 1 per cent OsO_4 buffered to pH 7.4 (6), the limbs were washed in distilled water, dehydrated in alcohol or acetone and embedded in *N*-butyl methacrylate, according to the techniques described by Borysko (7, 8). Longitudinal sections (0.05 μ or less in thickness) of whole limbs were cut with a Servall microtome (9) and examined with an RCA EMU 2E or 3B electron microscope. Forty limbs were sectioned. Micrographs were taken at original magnifications of 1500 to 8000 and enlarged photographically as desired.

Thicker (2 μ) sections from the same blocks were cut with the same microtome and examined with a phase contrast microscope. Some of these sections were stained, without removing the plastic, with thionine or by the periodic acid–Schiff reaction.

OBSERVATIONS

Course of Regeneration as Revealed by Light Microscopy.—The histology of the regenerating limb of the larval *Amblystoma punctatum* has been described in detail by Butler (10), Thornton (11, 12) and others and is summarized briefly here as a background for the electron microscope observations. After amputation through the humerus, epithelium migrates over the wound surface, covering it completely within about 4 hours. Beginning 2 to 3 days after amputation and continuing until about the 6th or 7th day, there is extensive dedifferentiation of the muscle, the cartilaginous humerus and other tissues of the stump (11, 12). This results in the accumulation at the tip of the limb of a group of undifferentiated cells which will be referred to here as the *early blastema*. The next phase of regeneration is one of rapid proliferation of blastema cells, bringing about an increase in the length of the regenerate. The first signs of cartilage and muscle redifferentiation appear on about the 10th day. Two finger-buds are formed, soon to be followed by a third and fourth. The process of regeneration is usually completed 16 to 20 days after amputation.

The electron microscope studies to be reported here will deal with the appearance of (*a*) the dedifferentiated cells of the early blastema, (*b*) the dividing and interphase cells of the proliferating blastema, and (*c*) the differentiating cartilage cells. Although the major portion of the description will be concerned with the appearance of the cytoplasmic organelles, reference to the general features of these cells will also be made.

Fine Structure of the Dedifferentiated Cells of the Early Blastema.—The cells that accumulate in the early blastema, 3 to 6 days after amputation, are strikingly different from the cells in their tissues of origin. Dedifferentiating cartilage and muscle cells lose all of the morphological features which characterized them in their previous state of differentiation. As the cartilage cells are released into the blastema by the dissolution of the cartilage matrix, the character of their cytoplasm changes markedly. The relatively abundant cytoplasm of the early blastema cell has an appreciable density compared to the extremely low electron scattering properties of the cytoplasm in the mature cartilage cell. The cytoplasmic organelles change in appearance in a manner that will be described below. Nuclei have a fine granular karyoplasm which is less dense than that of the differentiated cell types. There is little evidence of organized extracellular material in the early blastema. The cells are in close contact with each other and are remarkably uniform in appearance (Figs. 1 and 4). This intimate association of blastema cells has not been recognized heretofore in studies with the light microscope.

The cytoplasmic components that show the greatest change during cell dedifferentiation are the endoplasmic reticulum and its associated ribonucleoprotein granules. In the differentiated muscle or cartilage cell, the endoplasmic reticulum is in the form of a highly organized, continuous network of membrane-bounded cavities. In the dedifferentiated cell of the early blastema, how-

ever, it becomes discontinuous, appearing in electron micrographs as round or oval vesicles and occasional elongate profiles (Figs. 1 and 4). These vesicles are often rather large and dilated in appearance and their contents are of lower density than the surrounding cytoplasm. The membranes of the endoplasmic reticulum are sparsely covered with delicate granules, which are seen best in tangential sections (*g*1, Figs. 3 and 4). Free granules of similar size (100 A) and density increase in number in the cytoplasm during dedifferentiation (Figs. 1, 4, and 8). Inasmuch as the cytoplasm of these cells is basophilic when appropriately stained for light microscopy, it seems reasonable to assume that these small granules seen in electron micrographs are ribonucleoprotein and correspond to the particulate component of the cytoplasm described by Palade (1).

Mitochondria in cells of the early blastema range from 300 to 500 mμ in diameter, are elongate in shape, and have a light matrix and irregularly oriented cristae. A striking feature of this stage is the close association between endoplasmic reticulum and mitochondria (Fig. 4). The membranes of the endoplasmic reticulum often appear to be closely adherent to the limiting membranes of the mitochondria and a thin layer of increased density can sometimes be seen between the membranes of the two organelles in these areas of close apposition (Fig. 2). In older blastema cells (Fig. 7), the endoplasmic reticulum is at times similarly associated with mitochondria, but the relationship appears to be more intimate in the early blastema than at any other stage.

The Golgi apparatus, like the endoplasmic reticulum, assumes a predominantly vesicular form in the early blastema cell. It consists of aggregates of small (40 to 100 mμ) vesicles which are juxtanuclear in position (Fig. 8). The smooth surface and regularity of these vesicles, as well as their occurrence in clusters, distinguishes them from the larger, more irregular vesicles of the endoplasmic reticulum. Flattened Golgi vesicles, which are a rather constant feature of differentiated cells, have not been seen in cells of this stage. A common structure associated with the Golgi apparatus at all stages is a sizeable membrane-bounded vacuole containing numerous small vesicles (*x*, Figs. 8 and 9). The significance of this structure is unknown, but vacuoles of similar appearance have been reported in a variety of cells, including basophil leucocytes (13), certain tumor cells (14), rat and spider oocytes (15), and a green alga (16).

In early blastema cells the nuclear envelope, with its inner and outer membranes, contains many pores (*p*, Fig. 4), whereas in differentiated cartilage cells nuclear pores are uncommon. Continuity of the cisternae of the endoplasmic reticulum with the perinuclear cisterna, on the other hand, is frequent in the older cells and rarely seen in early blastema cells. Nucleoli are prominent in the young cells and consist of inhomogeneous aggregates of dense granules (Fig. 4). They are located towards the center of the nucleus and have not been observed in contact with the nuclear envelope at any time.

Fine Structure of the Dividing Blastema Cell.—At the end of the period of blastema formation (6 to 7 days after amputation), the cells begin to proliferate rapidly. At the beginning of the stage of proliferation the cells have relatively abundant, granular cytoplasm, but the amount of cytoplasm decreases as cell division proceeds. Dividing cells tend to be closely aggregated in groups at the tip of the limb in the older blastema, whereas in the younger blastema (7 to 10 days) they are more uniformly distributed. Chromosomes of dividing cells are made up of dense, granular material and are similar in morphology to those described in other somatic cell types by Porter (17).

Perhaps the most striking feature of the undifferentiated cell in mitosis is the granularity of its cytoplasm (Fig. 5). In addition to the greatly increased concentration of free granules, there is a notable variation in granule size and density. Numerous small (~ 100 A) particles of low density (*g*, Fig. 5) impart an over-all grayish background to the cytoplasm in electron micrographs. Interspersed with these particles are larger, darker granules whose size ranges from 200 to 250 A (*g'*, Fig. 5). These larger granules are of about the same size as the granules seen in the non-chromosomal portion of the prophase nucleus and probably have been released from the nucleus after dissolution of the nuclear membrane.

Both the Golgi apparatus and the endoplasmic reticulum of the dividing blastema cell are broken up into isolated vesicles. Similar fragmentation of these organelles has been described in dividing mammalian cells (17). The vesicular elements of both organelles are small and tend to be located in the peripheral cytoplasm, where they are often arranged in rows like the links in a chain (Fig. 5). Paired tubuled structures of the kind described by

Porter (17, 18) as "spindle filaments" have occasionally been observed in dividing blastema cells.

The mitochondria of cells in mitosis also tend to be disposed at the periphery (Fig. 5). They vary from 250 to 400 mμ in diameter, are elongate or round in sections, and have irregularly arranged cristae. There are also, in the dividing cell, smaller (100 to 200 mμ), round bodies which resemble the mitochondria in density. They have a limiting membrane and a suggestion of internal membrane structure (*m'*, Fig. 5). These bodies resemble the "ultrachondriome" described by Oberling *et al.* (19) and Selby (20) and the "growth granules" seen in mitotic cells (21), which Porter (17) suggests may be small mitochondria.

Fine Structure of the Older Blastema Cell.—The amount of cytoplasm in the older blastema cell (10 to 16 days) is considerably less than that of the early blastema cell. The intercellular space is increased and extracellular fibrils are more prominent. Fibrils and/or condensed extracellular ground substance may be closely associated with the outer surface of the cell membrane (*E*, Fig. 7). The nucleus, which is about the same size as in the early blastema cell, is darker and no pores have been identified in the nuclear envelope of these older cells.

The cytoplasmic organelles of older blastema cells differ in several respects from those of earlier stages. The mitochondrial matrix becomes progressively more dense (Fig. 7). Elements of the endoplasmic reticulum are not abundant but do occur as elongate profiles which have granules attached to their surfaces. The free granules of the cytoplasm are numerous and closely packed. The Golgi apparatus consists primarily of small vesicles.

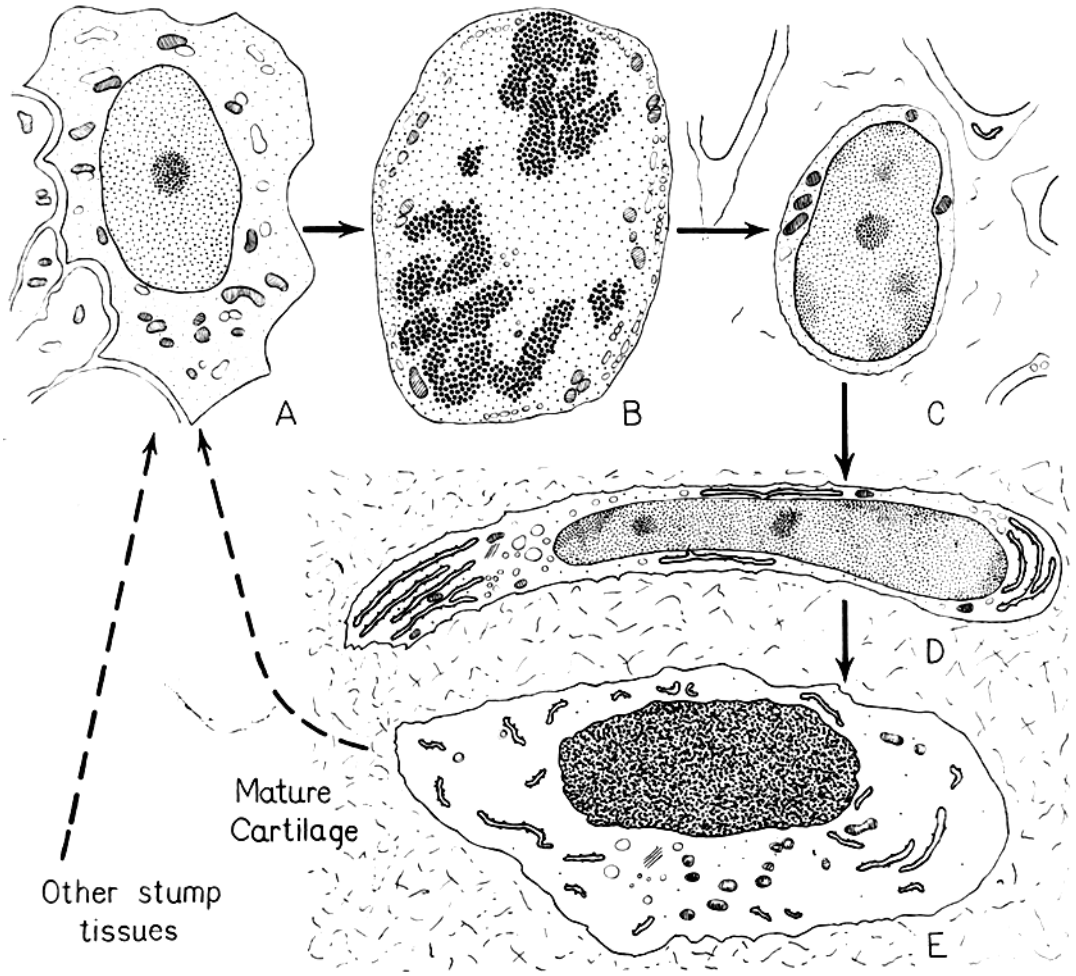
Fine Structure of the Differentiating Cartilage Cell.—The first visible sign of cartilage differentiation in the regenerate is a lining up of cells in the center of the regenerate. In sections studied with the light microscope these cells appear to be close together, but the electron microscope reveals that they are separated by considerable intercellular space. Such cells at first have very little cytoplasm but as cartilage differentiation proceeds, the cytoplasm increases greatly in amount and the cells gradually become more elongate in form (Figs. 9 and 10). The nucleus elongates and increases in density, but remains about the same size (*N*, Fig. 10). The cell membrane may have delicate fibrils attached to its outer surface. The fibrils of the early cartilage matrix are similar in size (100 A) and appearance to the intercellular fibrils found throughout the blastema, but are far more numerous (*F*, Fig. 10). They closely resemble the fibrils described in mammalian cartilage (22, 23) and, like them, lack cross-striations but occasionally, in older cartilage, they may have a beaded appearance.

The most striking change in the cell organelles in the course of cartilage cell differentiation is the great increase in endoplasmic reticulum. Numerous cisternae are formed and these tend to be arranged in parallel rows. In elongated cartilage cells, the prevailing direction of orientation of the endoplasmic reticulum is from the nucleus outward towards the tips of the cell processes (Fig. 9), whereas in cells which are less elongated, the cisternal profiles tend to run parallel to the nuclear envelope (Figs. 10 and 11). The appearance of the elongate profiles in sections leaves little doubt that they represent a continuous system of membrane-limited flat cavities or *cisternae*, as originally suggested by Palade and Porter (24). The cisternae show much branching and anastomosing (Figs. 10 to 13) and communications with the nuclear envelope or so called perinuclear cisterna (25) are very common (*x*, Figs. 10 and 12). The distance (50 mμ) between the limiting membranes of the cisternae is more constant in cartilage cells than in some cell types and, moreover, remains constant as the cytoplasm hydrates in the older cartilage cell (Figs. 14 to 16). Furthermore, the content of the cavities, which is homogeneous and moderately dense, does not change in appearance in spite of the loss of density of the cytoplasmic ground substance in older cartilage cells (Figs. 14 to 16). These features demonstrate that the material contained between the limiting membranes of the cisternae is quite different from the surrounding cytoplasmic ground substance.

The membranes of the cisternae have dense granules attached to their outer surfaces and a moderate number of free ribonucleoprotein granules are present in the cytoplasm of the differentiating cartilage cell (Figs. 9, 12, and 13).

While the cartilage matrix is being formed, the Golgi apparatus in the cartilage cell is very well developed. Similar hypertrophy of the Golgi apparatus during chondrogenesis has also been noted in mammals with light microscopy (26). In the elongating young cartilage cell in the regenerate, it may occupy a third to a half of the cytoplasm at either end of the nucleus. It consists

of flattened vesicles arranged in parallel array, numerous empty appearing vacuoles and many smaller, round vesicles (Fig. 9). The small vesicles (approximately 40 mμ in diameter) extend into the surrounding cytoplasm and may be clustered about the membranes of the endoplasmic reticulum in a manner suggesting that they are fusing with the cisternae (*v*, Figs. 13 and 15). The con-



TEXT-FIG. 1. Diagrammatic representation of the major changes in the fine structure of cells during cartilage dedifferentiation and redifferentiation. The cellular outlines, and those of the nucleus and mitochondria, were drawn from tracings of actual micrographs. For the sake of simplicity, however, it was necessary to omit much of the endoplasmic reticulum and to enlarge the size of and leave out many of the ribonucleoprotein granules of the cytoplasm. The stippling in the nuclei is not intended to portray the relative size of the granules contained therein, but only to indicate the increasing density of the nucleus as the cell matures. (*A*) The early blastema cell, derived (broken lined arrows) from dedifferentiating cartilage and other tissues of the amputated stump; (*B*) the dividing blastema cell; the dark aggregations of granules represent chromosomes; (*C*) the older blastema cell, which has considerably less cytoplasm than the early blastema cell and lies farther away from neighboring cells; (*D*) the differentiating cartilage cell; although such cells are symmetrical, the portion of this cell that would extend to the right was not included in the section and this apparent asymmetry has been utilized in the drawing to indicate, diagrammatically, different types of orientation of the cisternae of the endoplasmic reticulum in cartilage cells; (*E*) the mature cartilage cell; the short lines surrounding this and the preceding cell represent the fibrils of the cartilage matrix. See text for further description.

tent of the small vesicles is slightly more dense than the surrounding cytoplasmic ground substance.

The mitochondria of the differentiating cartilage cell are similar to those of older blastema cells. The matrix is dark and may contain dense particles (Fig. 9). The cristae, although still irregular, are more often oriented transversely than was the case earlier in differentiation.

Fine Structure of the Mature Cartilage Cell.—The cartilaginous skeleton of the limb is reconstituted by the 16th to 20th day and, thereafter, new cartilage is added mainly by appositional growth. The cells making up the bulk of this older cartilage are quite different in appearance from the younger cells. The shape of the cell changes from elongate (Fig. 14) to oval or round (Fig. 16). The nucleus does not change appreciably in size, but becomes more dense. The cytoplasm, on the other hand, increases in volume and loses density, a change which may reflect increasing hydration (22). The cytoplasmic organelles are decreased in number or, at least, are fewer in relation to the volume of cytoplasm. The cisternae of the endoplasmic reticulum lose their parallel orientation and break up into shorter segments (Figs. 14 to 16). The granules attached to their surfaces are less prominent and the number of free granules in the cytoplasm is greatly diminished (Figs. 15 and 16). The small vesicles previously scattered throughout the cytoplasm diminish in number (Fig. 15) and finally disappear (Fig. 16). During the period when they are disappearing, they are frequently clustered about the cisternae of the endoplasmic reticulum (*v*, Fig. 15). The Golgi apparatus also seems to diminish in volume but continues to be composed of flattened vesicles in parallel array, small circular vesicles, and a few larger vacuoles. The mitochondria of mature cartilage cells are similar to those in developing cartilage cells, except for the presence of scattered areas of low density in their matrix (Figs. 15 and 16). These do not appear to be artefacts of specimen preparation.

The mature cartilage cell, like the younger one, completely fills its enlarged lacuna. The cartilage matrix is not significantly different in appearance in the two stages compared here, but, eventually, a thin layer of calcium is deposited beneath the perichondrium. This does not occur, however, until much later in the development of the limb. It is evident that these mature cartilage cells, which closely resemble the hypertrophied cartilage cells described in the mammalian epiphysis by Scott and Pease (22), live for a considerable period of time and undoubtedly play a role in maintaining the cartilage matrix. Thus, in spite of a light microscope appearance which might suggest impending deterioration (27), it does not seem appropriate to consider these cells as degenerate.

Text-fig. 1 is a diagrammatic summary of the major changes occurring in the nucleus and cytoplasm of a blastema cell as it dedifferentiates, divides, and later differentiates into cartilage.

DISCUSSION

The observations recorded here permit us to consider in the discussion some of the general features of dedifferentiated cells of the early blastema and to speculate concerning the possible functional significance of the structural changes that take place in the organelles during the course of differentiation of cartilage cells in the regenerating limb.

In the formation of the blastema, muscle fibers appear to break up into cellular units and cartilage cells are liberated from their lacunae by dissolution of the hyaline matrix. Myofibrils and other formed elements or characteristic cytological features which served to distinguish the separate cell types in their previous state of differentiation are lost. Heretofore, the possibility could not be excluded that cells retained separate identities and that among blastema cells of diverse origin, morphological differences persisted that were beyond the reach of the light microscope. It can now be stated with considerable assurance that, within the limits of resolution attainable at present with the electron microscope, no such differences exist and that the cells do indeed appear to revert to a single, simplified cell type.

The fragmentation of the endoplasmic reticulum into isolated vesicles during cellular dedifferentiation is a particularly striking feature of this initial stage of regeneration. The endoplasmic reticulum does not become highly organized again until cellular differentiation begins. A somewhat similar sequence of events in developing liver led Howatson and Ham (28, page 67) to state, "... it seems reasonable to suggest that the organized ergastoplasmic structures are related to the differentiated state." Indeed, an unorganized, vesicular form of the endoplasmic reticulum has been described in such undifferentiated cell types as oocytes (29) and spermatogonia (13, 30, 31)

and the system is only sparsely represented in myeloblasts (32), lymphocytes (13), and other relatively undifferentiated cells. However, organized parallel cisternae have been reported in certain spermatocytes (33) and in the yolk nuclei of oocytes (29, 34). It would seem necessary, therefore to define the situation more clearly and to speak of the organized ergastoplasm as a specific differentiation of the endoplasmic reticulum closely related to specialized cellular functions, but not necessarily characteristic of the "differentiated state" as implied by Howatson and Ham. Parallel arrays of cisternae are typical of many secretory cell types, such as pancreatic and parotid acinar cells (35), thyroid cells (36), and liver parenchymal cells (37) and it has been suggested that this specialization of the endoplasmic reticulum in related to the synthesis of proteins intended for secretion (24). Thus, the appearance of parallel cisternae in the cartilage cell at the time of matrix formation may well indicate that the cell is involved in the production of some protein component of the extracellular hyaline ground substance. This interpretation is strengthened by the fact that after the cartilage matrix is fully formed, the endoplasmic reticulum of the chondrocytes becomes less highly organized in appearance.

The Golgi apparatus also undergoes a series of changes during the growth and differentiation of the regenerate. During cartilage matrix formation, it assumes the complex organization of lamellae, vacuoles, and vesicles described by Dalton and Felix (38), whereas in dedifferentiated and dividing cells it is predominantly in the form of vesicles. Here, too, it may be permissible to speculate that the organized Golgi apparatus of the cartilage cell is a highly differentiated form of this organelle, which plays some role in the elaboration of cartilage matrix, whereas the aggregation of vesicles which occur in blastema cells represents a less specialized phase of the organelle.

Mitochondria do not show as much variation during regeneration as the other organelles. The mitochondrial matrix appears less dense and the cristae more irregular in early blastema cells, a feature which has been noted in certain other "immature" cells (39, 40). The close association between mitochondria and the endoplasmic reticulum in early blastema cells is a particularly striking phenomenon. A close association of mitochondria and cytoplasmic membranes has been reported in regenerating liver by Bernhard and Rouiller (41), who interpreted their observations as indicating that mitochondria play a role in the elaboration of "ergastoplasm" (endoplasmic reticulum). In our material, the elements of the endoplasmic reticulum are *decreasing* at the time when their relationship to mitochondria is the most intimate. It seems unlikely, therefore, that the close association between these two organelles indicates anything more than a transfer of energy or metabolic products.

Some of our observations do, however, have a bearing upon the problem of the origin of endoplasmic reticulum (ergastoplasm). At the time of formation of the extensive cisternae that arise in differentiating cartilage cells, numerous small vesicles appear in the cytoplasm and these establish relationships with the profiles of the endoplasmic reticulum which suggest that they may coalesce with them to form new cisternae. In an earlier investigation, the belief was expressed that these small vesicles arose *de novo* in the cytoplasm by association of the small granules in rows which then became transformed into a membrane bounding a small cavity (42). Further observations now lead us to consider it more likely that these vesicles originate from preexisting membranous elements in the Golgi region of the cell. Other investigators have described the formation of lamellar systems of membranes by coalescence of vesicles. Studying livers of rats refed after fasting, Fawcett (37) was of the opinion that smooth surfaced vesicles arising in the peripheral cytoplasm may fuse to form rough surfaced cisternae. The genesis of the membranous discs of chloroplasts (43) and of the outer segment of the retinal rod (44) have also been attributed to coalescence of a population of small vesicles and recent evidence (45) suggests that lamellar systems of membranes may arise in testicular cells *in vitro* by a similar mechanism.

One of the most interesting features of the blastema cell is its acquisition of large numbers of free ribonucleoprotein granules in its cytoplasm. Since these granules have been shown by the recent work of Siekevitz and Palade (3) to be directly involved in protein synthesis, their appearance in the cytoplasm of dedifferentiated blastema cells would suggest that active protein synthesis is beginning at this time. Recent autoradiographic studies with labelled methionine by Bodemer (46) support such a concept. Inasmuch as a predominance of free ribonucleoprotein granules in the cytoplasm appears to be associated

with cells actively engaged in the synthetic processes required for growth (1, 28, 29, 35), it seems reasonable to conclude that the dedifferentiating blastema cell is preparing for the rapid cell division soon to ensue. Indeed, the fragmentation of the endoplasmic reticulum and Golgi apparatus and a number of the other changes occurring in the cells as they enter the early blastema might also be interpreted as preparatory to a phase of active synthesis of new cells.

Finally, attention should be called to the changes in the extracellular components of the limb during blastema formation and subsequent redifferentiation. In the early blastema, extracellular fibrils are reduced in number and the layer of condensed extracellular material ("basement membrane") which invests most differentiated cell types is lacking. The dedifferentiated cells are in intimate contact with one another whereas later, during the phase of differentiation, extracellular material can be found surrounding the various cell types. Thus it would appear that at both the cellular and extracellular levels, a remarkable dedifferentiation of structural organization occurs during the formation of early regeneration blastema. These features are undoubtedly of significance for the ensuing rapid proliferation of a large mass of cells which later in regeneration will be capable of differentiating into a new and complete limb.

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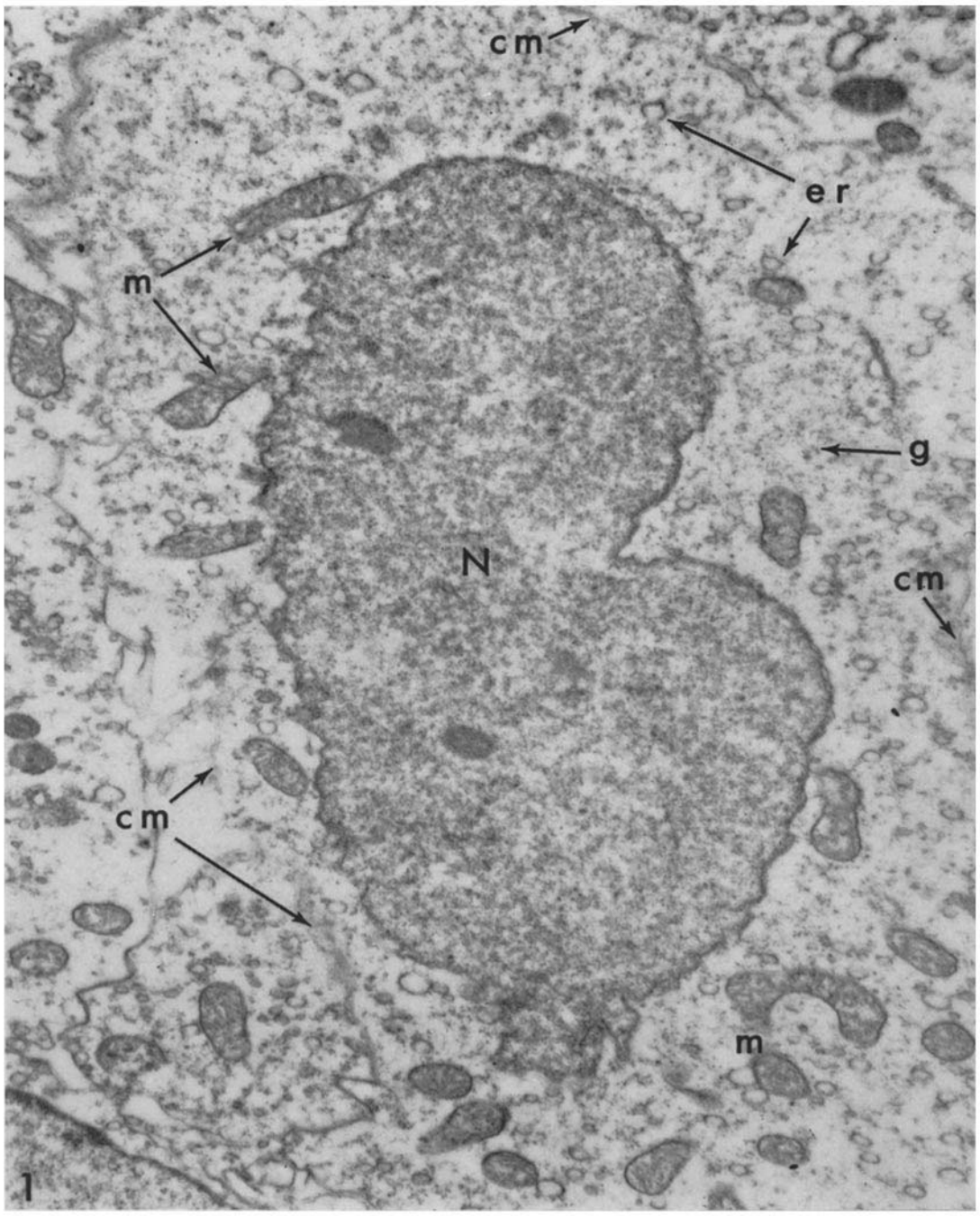
Explanation of Plates

Plate 275

Fig. 1. Electron micrograph of a section of an early blastema cell. The nucleus (*N*) is composed of numerous fine granules and is less dense in appearance at this stage than in more differentiated cell types. Cytoplasm is relatively abundant as compared with the older blastema cell (Fig. 7) and the cells are closely packed and in intimate contact with each other. The cell membrane (*cm*) is cut obliquely and is broken on the left. Parts of two adjoining blastema cells appear in the left portion of the picture and part of another cell on the upper right. No extracellular ground substance appears to be interposed between the closely associated early blastema cells. The cytoplasm is characterized by numerous free ribonucleoprotein granules (*g*). The endoplasmic reticulum (*er*) consists of vesicles which show no suggestion of continuity. Mitochondria (*m*) have a relatively light matrix and irregular cristae. In the lower right corner, a mitochondrion (*m*) can be seen which has two vesicles of the endoplasmic reticulum associated with it. 6 days. × 12,000.



(Hay: Blastema and cartilage cells)

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FIG. 2. A smaller portion of the electron micrograph shown in Fig. 4 at higher magnification to illustrate the nature of the relation of endoplasmic reticulum (*er*) to mitochondria (*m*). The cell membranes (*cm*) of the two cells are labeled. The membranes of the endoplasmic reticulum are closely associated with the outer mitochondrial membranes. Occasionally, a dense material (arrow) can be seen between endoplasmic reticulum and mitochondrion in these areas of close apposition. × 40,000.

FIG. 3. Enlargement of a portion of the electron micrograph shown in Fig. 4 to illustrate the very fine granules (*g 1*) attached to the membranes of the endoplasmic reticulum. These granules are best shown in a tangential section such as this one. In differentiated cartilage cells, the granules attached to the membranes of the endoplasmic reticulum are more prominent than they are at this stage. 6 days. × 40,000.

FIG. 4. Electron micrograph of portions of the cytoplasm of two early blastema cells. The nucleus (*N'*) of one cell appears on the left and the nucleus (*N*) of the second cell on the right. The cell membranes (*cm'* and *cm*) of the two cells are in close apposition and can be traced from the upper left downward towards the center of the micrograph. The endoplasmic reticulum (*er*) occurs primarily in the form of vesicles of varying size, the membranes of which are sparsely covered with delicate ribonucleoprotein granules (*g 1*) that can be seen best in tangential sections of the surface of the membrane (see enlargement in Fig. 3). Free granules (*g 2*) of similar type are widely dispersed throughout the cytoplasm. The cytoplasmic ground substance has a slight electron density, but the content of the endoplasmic reticulum has a very little density at this stage. Mitochondria (*m*) have irregular cristae and the mitochondrial matrix is not as dense as it is in older cells. Almost every mitochondrion in this micrograph has a vesicle of the endoplasmic reticulum associated with it. Such close association of these organelles is more marked at this stage than at any other.

Nuclei (*N*, *N'*) contain fine granules which vary somewhat in size and density. The nuclear envelope consists of an inner membrane (*nm 1*) next to the nucleus and an outer membrane facing the cytoplasm. These membranes, together with the cavity they enclose, will be referred to in the legends that follow as the perinuclear cisterna. Frequent pores (*p*) are present in the nuclear membrane at this stage. The nucleolus (*n*) is composed of dense granules aggregated in an irregular pattern. 6 days. × 28,000.

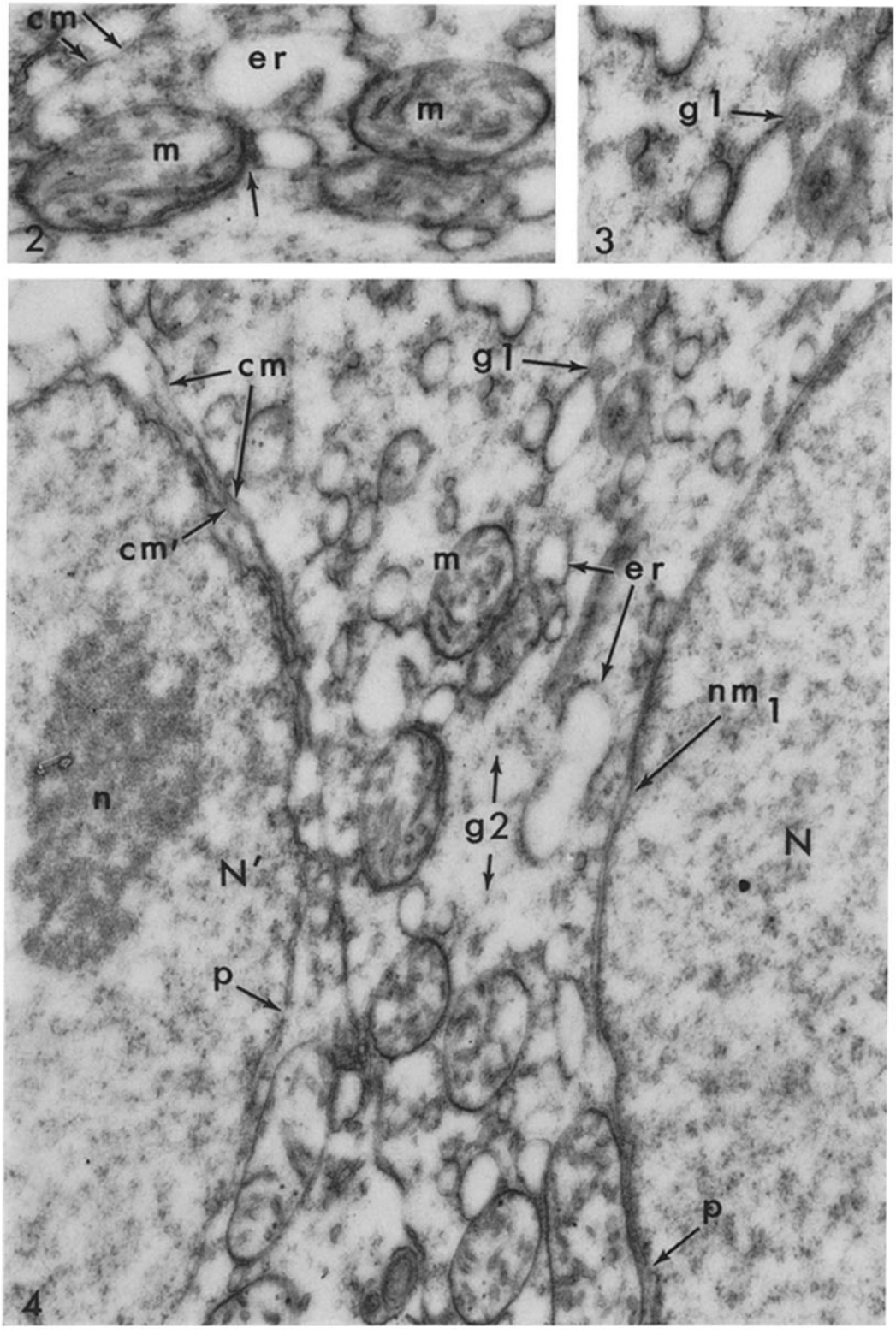

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FIG. 5. Electron micrograph of a portion of the dividing blastema cell shown at lower magnification in Fig. 6. Chromosomes (*ch*) appear as dense granular condensations with irregular, ill defined borders. Free cytoplasmic granules are a prominent feature of the mitotic cell and show considerably more variation in size (100 to 250 A) and density than in the interphase cell. Larger, dense granules (*g'*) are interspersed with numerous small granules (*g*) which impart an over-all greyish background to the cytoplasm. The endoplasmic reticulum consists primarily of small vesicles, most of which are disposed to the periphery of the cell (*er*), although a few are dispersed more centrally (*er'*). The elongate form (*e*) of the reticulum is rare. Smaller vesicles (*v*) with a more dense content than that of the endoplasmic reticulum also appear at the periphery of the cell and probably represent the Golgi apparatus. Mitochondria (*m*) vary widely in size and small bodies (*m'*) of density similar to that of mitochondria and possessing a suggestion of internal membranous structure are common during mitosis. The cell membrane (*cm*) is on the right of the picture. 16 days. × 25,000.

FIG. 6. Same electron micrograph as Fig. 5, reduced to show the entire section of the cell. The portion of this dividing cell shown in Fig. 5 is indicated by the enclosed area. The dark granular condensations labelled *ch* are chromosomes. × 2,800.

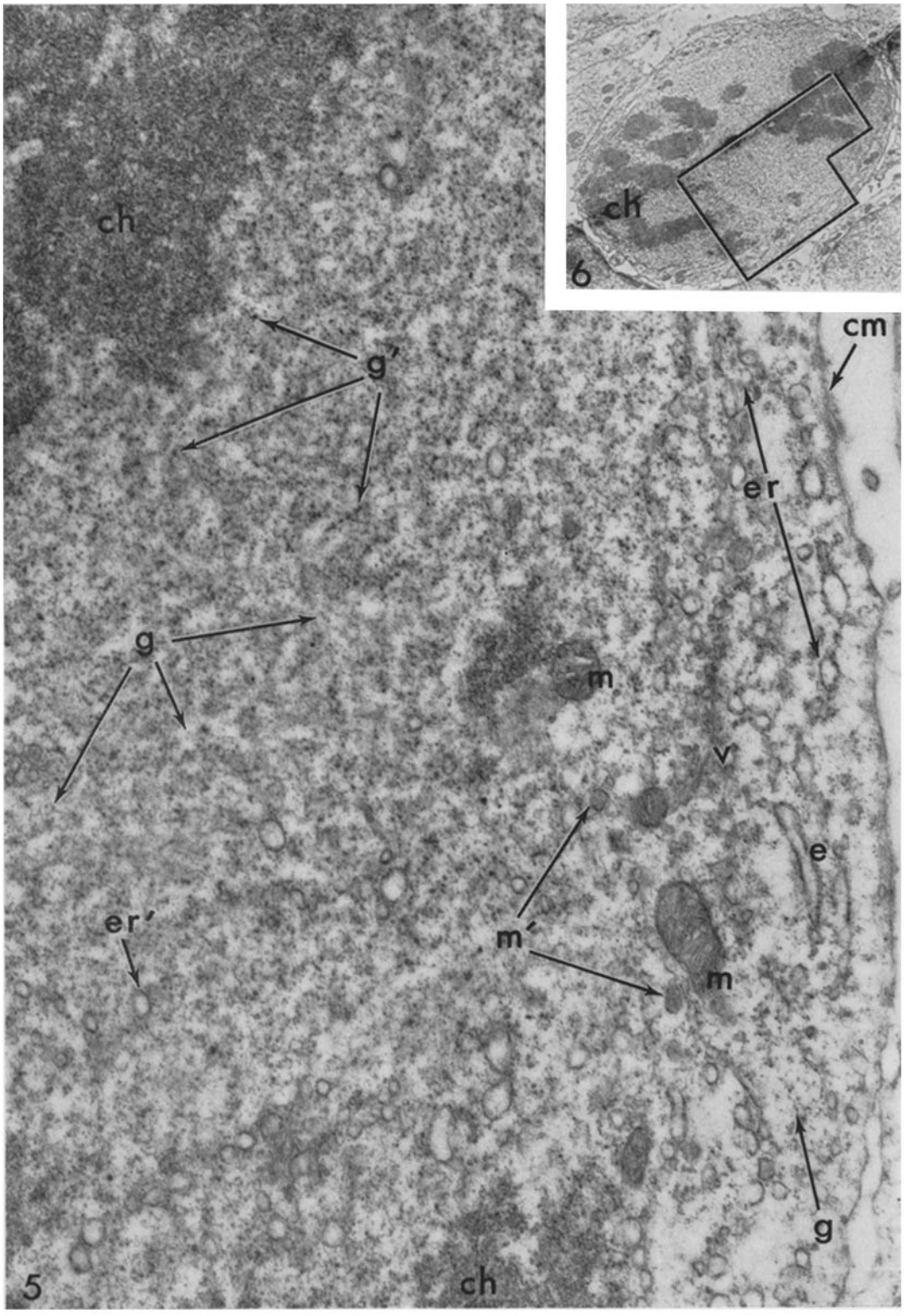

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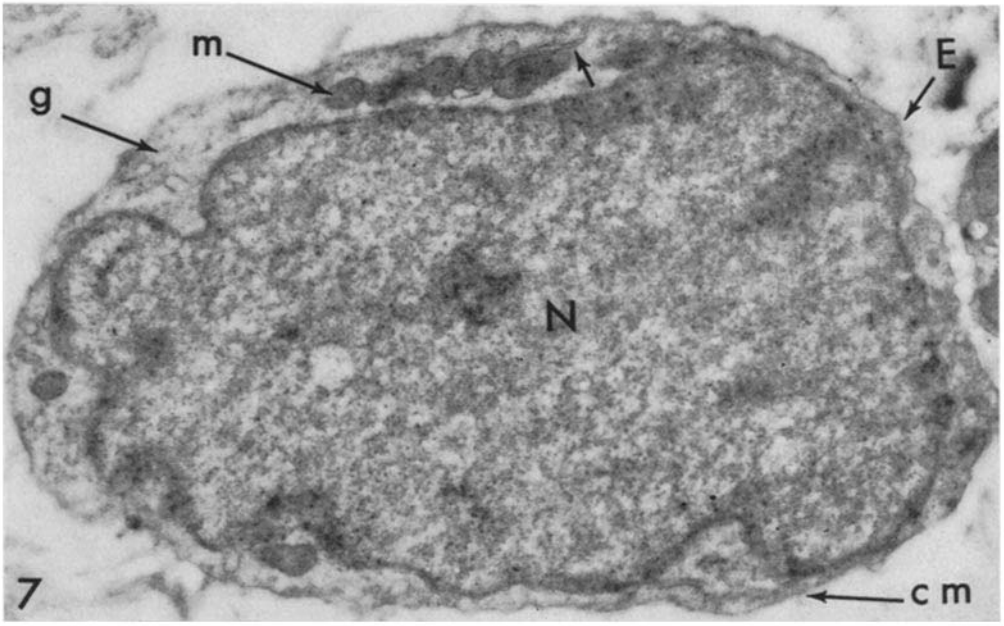
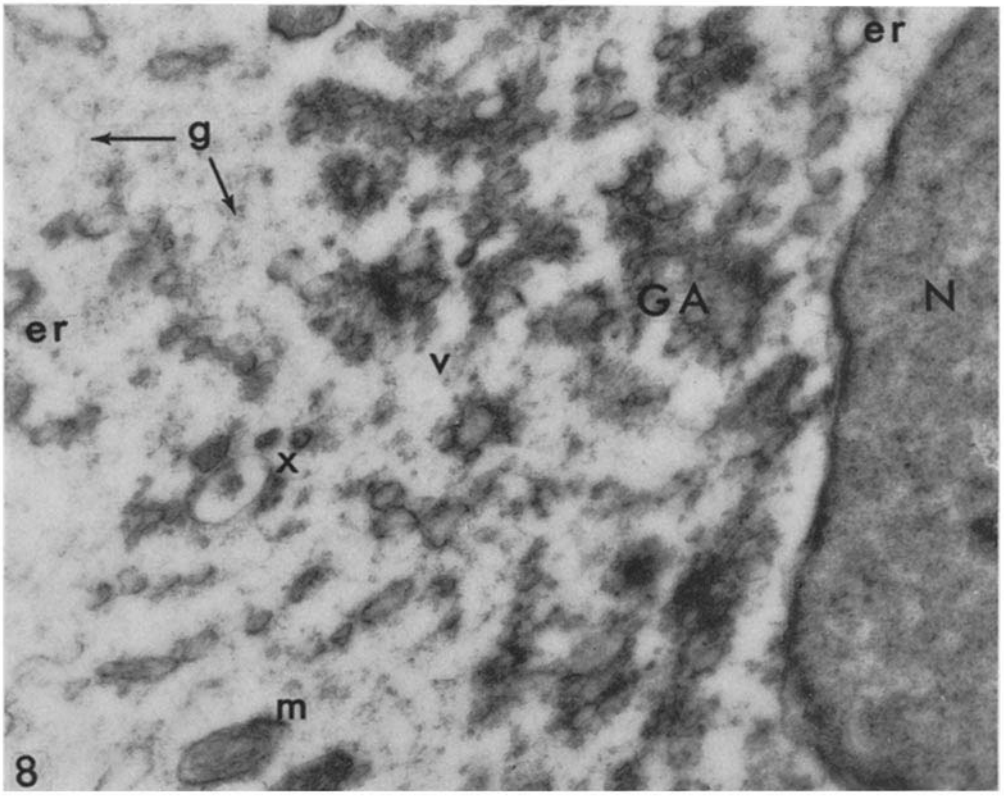
FIG. 7. Electron micrograph of a section of an older blastema cell. Such cells have less cytoplasm than the cells of the early blastema (Fig. 1) and are separated by considerable intercellular space. A variable amount of extracellular material (*E*) is closely associated with the surface of the cell membrane (*cm*). The nucleus (*N*) is dense and contains areas of granular condensations. Mitochondria (one of which is labelled *m*) have a dense matrix. The endoplasmic reticulum occurs as a few oval or elongate profiles, the membranes of which are covered with small granules. Although the reticulum may at times be in close proximity to mitochondria (small arrow), this relationship is not as intimate as it was earlier (Fig. 4). Free ribonucleoprotein granules (*g*) are abundant in the cytoplasm. 16 days. × 11,000.

FIG. 8. Electron micrograph of a portion of an early blastema cell to show the Golgi apparatus (*GA*). The nucleus (*N*) is to the right. The Golgi apparatus of blastema cells is made up primarily of small vesicles (*v*) and does not contain the larger vacuoles and lamellae which are present in cartilage cells (Fig. 9). An associated component which occurs in both cartilage and blastema cells is a membrane-limited vacuole containing small vesicles (*v*). The characteristic vesicular endoplasmic reticulum (*er*), delicate cytoplasmic granules (*g*), and light mitochondrial matrix (*m*) of the early blastema cell are well shown in this micrograph. Some idea of the relative abundance of cytoplasm in the early blastema cell as contrasted with the older blastema cell can be gained by comparing this micrograph with the one above (Fig. 7). 3 days. × 20,000.





(Hay: Blastema and cartilage cells)

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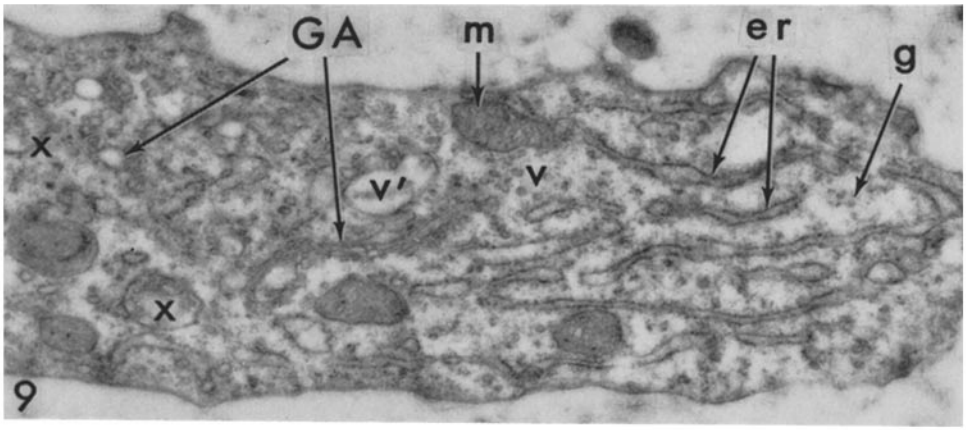
FIG. 9. Electron micrograph of part of the cytoplasm of a young cartilage cell. This particular cell is elongate in shape and is surrounded by newly formed cartilage matrix. The nucleus, which is to the left, does not appear in the figure. The Golgi apparatus (*GA*) is larger and more highly organized in cartilage cells than in blastema cells. In addition to the small free vesicles (*v*) and membrane-bounded collections of vesicles (*x*) present earlier (Fig. 8), it now consists of flat vesicles in parallel array and large, empty appearing vacuoles (*v'*). Peripheral to the Golgi apparatus, the granule-covered membranes of the endoplasmic reticulum make up a prominent part of the cytoplasm. These are now arranged as numerous cisternae (*er*) which tend to run parallel to one another. A few free cytoplasmic granules (*g*) occur between the cisternae. The cristae of the mitochondria (*m*) seem to be more regularly arranged and the matrix is more dense than in early blastema cells. 16 days. × 20,000.

FIG. 10. Low magnification electron micrograph of a section of another cartilage cell. The prominent cisternae of the endoplasmic reticulum (*er*) are arranged in rows which are parallel to the nucleus in some areas of the cytoplasm. Frequent anastomoses between the cisternae themselves and between the cisternae and the perinuclear cisterna (*x*) are apparent. The area enclosed in the small square is enlarged in Fig. 12 to demonstrate these connections more clearly. At numerous points (small arrows), the cisternae approach the cell membrane, but actual communications with the extracellular space have not been seen. Mitochondria (*m*) are variable in size and shape and are randomly oriented in the cytoplasm. The nucleus (*N*) is more dense than in undifferentiated cells. The cells are now separated by newly formed cartilage matrix (*matrix*) which contains many fine fibrils (*F*). 16 days. × 8,800.



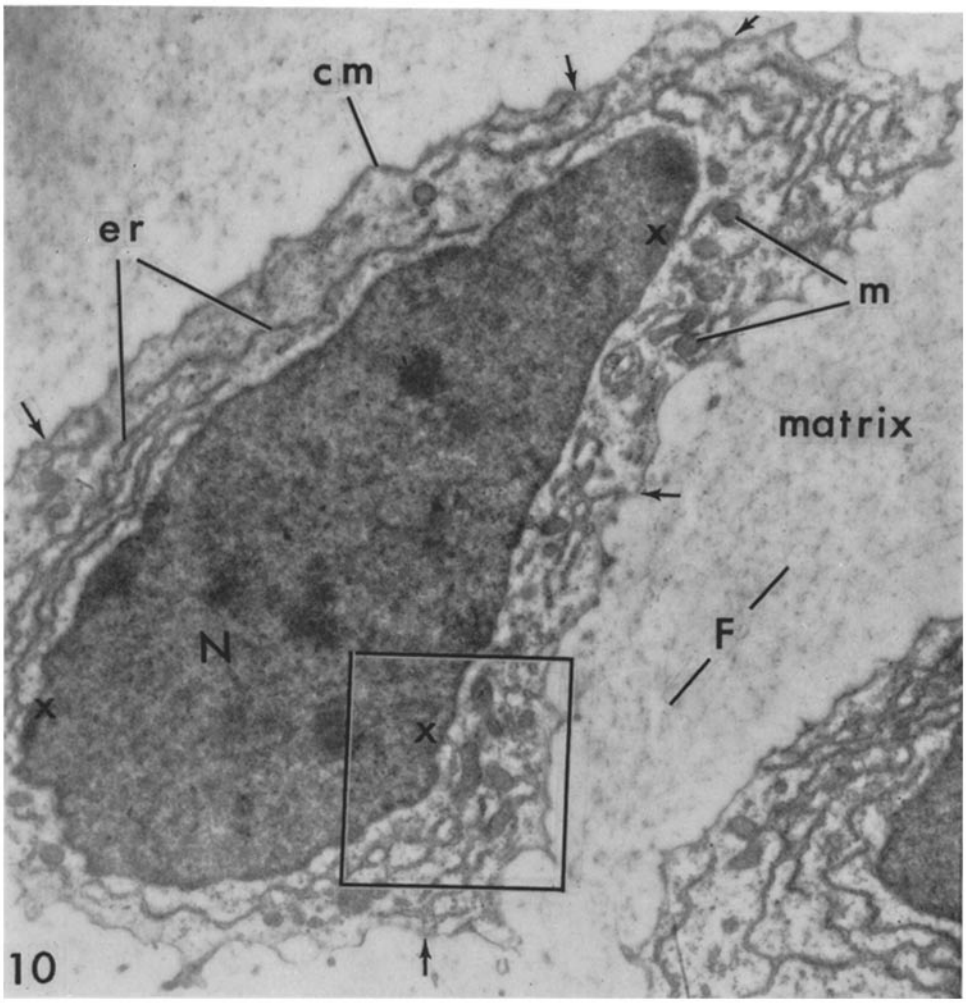


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FIG. 11. Low magnification electron micrograph of a section of a young cartilage cell. The branching of the cisternae of the endoplasmic reticulum (*er*) is particularly well shown in the area enclosed in the square, which is reproduced at higher magnification in Fig. 13. The small arrows indicate points at which the cisternae approach the cell membrane (*cm*). At times, a series of vesicles seems to intercede between the cisternae and the cell membrane at such points (small arrow on the right side of the figure). The basic arrangement of endoplasmic reticulum in differentiating cartilage cells consists of cisternae in parallel array, but the exact orientation of the profiles with respect to the nucleus and cell membrane varies from cell to cell (*cf.* Figs. 9 to 11). A small portion of the Golgi apparatus (*GA*) of this cell appears below the nucleus (*N*), which is sectioned tangentially. One ot the numerous mitochondria occurring in the cytoplasm is labelled (*m*). The cell is surrounded by newly formed cartilage matrix (*matrix*). 16 days. × 8,800.

FIG. 12. Higher magnification of a portion of the cartilage cell in Fig. 10 to show a connection (*x*) of a cisterna of the endoplasmic reticulum with the perinuclear cisterna. The nucleus (*N*) is to the left and the cell membrane (*cm*) to the right. The endoplasmic reticulum shows much branching and anastomosing (*er*). At the point labelled *x*, it is sectioned perpendicular to the limiting membrane and the dense granules attached to its surface are apparent. It cannot be said with certainty whether these granules continue along the cytoplasmic surface of the outer nuclear membrane, but there is considerable condensation of granular nuclear material along the nuclear surface of the inner nuclear membrane (nm_1). The small arrow indicates an area where the endoplasmic reticulum approaches the cell membrane. The numerous free vesicles (*v*) in the cytoplasm appear to emanate from the Golgi region of the cell. Mitochondria (*m*) have a dark matrix and transverse cristae. × 20,000.

FIG. 13. Higher magnification of a portion of the cartilage cell in Fig. 11 to show the branching of the cisternae of the endoplasmic reticulum (*er*). The nucleus (*N*) is at the bottom of the figure and the cell membrane in the upper right. It is common to see a slight dilatation of the cavity of the cisternae in these areas of branching. Ribonucleoprotein granules (*g*) occur throughout the cytoplasm and are especially prominent on the membranes of the reticulum. Small vesicles (*v*) of the type usually associated with the Golgi apparatus may be seen clustered about the edges of the cisternae. One of the membrane-limited aggregations of vesicles (*x*) shown in Figs. 8 and 9 appears between two of the cisternae pictured here. A mitochondrion (*m*) appears in the upper portion of the micrograph. × 20,000.

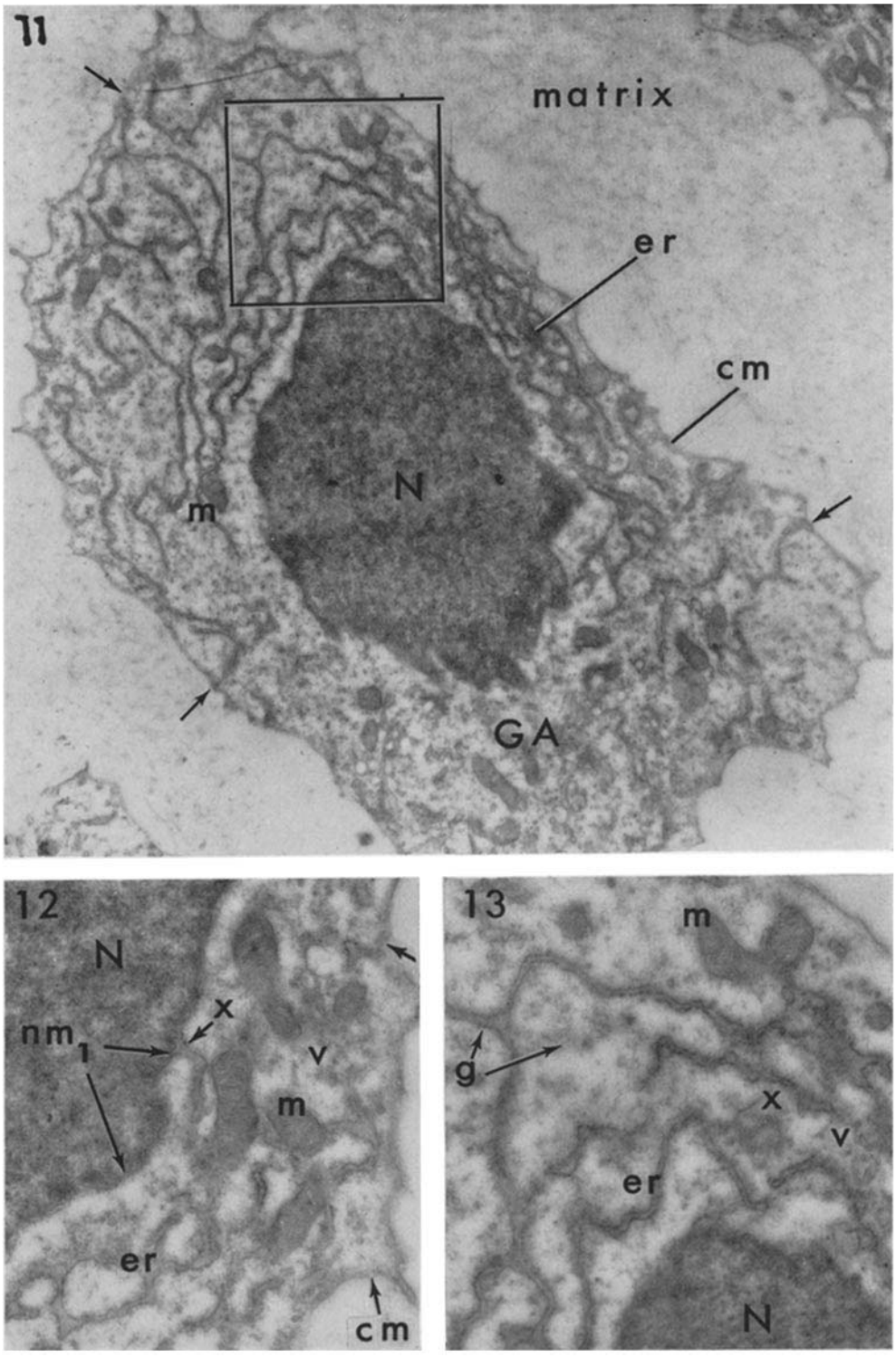

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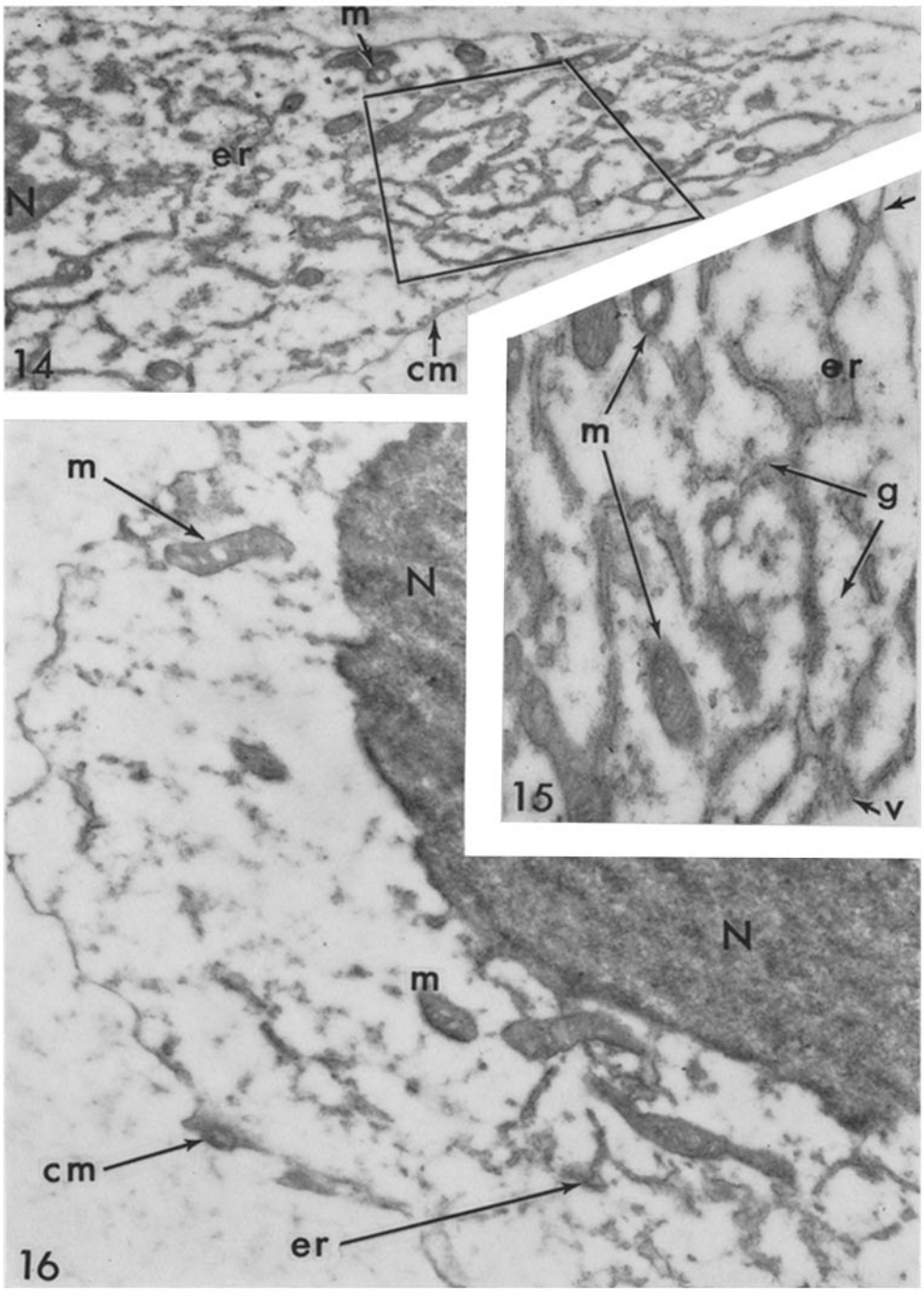
FIG. 14. Electron micrograph at low magnification of a portion of a more mature cartilage cell. A small part of the nucleus (*N*) appears at the left. The loss of density of the cytoplasmic ground substance and concomitant increase in the amount of cytoplasm are particularly notable features of the older cartilage cell. The cisternae of the endoplasmic reticulum (*er*) are not as highly organized as they were in younger cartilage cells and they appear to have fragmented into shorter segments. The mitochondrial matrix now contains areas of low density (*m*). The cell membrane (*cm*) is labelled in the lower portion of the micrograph. The enclosed area is shown at higher magnification in Fig. 15. 16 days. × 8,800.

FIG. 15. Higher magnification of part of the cytoplasm of the cartilage cell shown in Fig. 14. The content of the endoplasmic reticulum (*er*), although showing about the same density as previously, now stands out in better contrast due to the decreased density of the cytoplasmic ground substance. The small arrow indicates a point at which a cisterna approaches the cell membrane. The small vesicles (*v*) which were so prominent in the cytoplasm of young cartilage cells are decreasing in number and give the appearance of fusing with the cisternae. The granules (*g*) in the cytoplasm and covering the membranes of the reticulum are less prominent than they were earlier. As noted above, areas of low density can be seen in the mitochondria (*m*). × 20,000.

FIG. 16. Electron micrograph of a mature cartilage cell. The changes beginning in the cell pictured in Figs. 14 and 15 have advanced in this cell. The cisternae of the endoplasmic reticulum (*er*) are reduced in number and unorganized in appearance. Small vesicles are rarely seen, except in the immediate region of the Golgi apparatus (not shown here). The localized areas of decrease in the density of the mitochondria matrix (*m*) so typical of this stage are quite apparent in this micrograph. The nucleus (*N*) is dense, but the cytoplasmic ground substance has little density and contains few, if any, free ribonucleoprotein granules. The cell membrane (*cm*) appears on the left side of the picture. The morphology illustrated here is typical of the older cartilage cell after the period of active hyaline matrix formation. 16 days. × 14,000.



(Hay: Blastema and cartilage cells)