CHONDROGENESIS, STUDIED WITH THE ELECTRON MICROSCOPE

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ABSTRACT

The role of the cells in the fabrication of a connective tissue matrix, and the structural modifications which accompany cytodifferentiation have been investigated in developing epiphyseal cartilage of fetal rat by means of electron microscopy. Differentiation of the prechondral mesenchymal cells to chondroblasts is marked by the acquisition of an extensive endoplasmic reticulum, enlargement and concentration of the Golgi apparatus, the appearance of membrane-bounded cytoplasmic inclusions, and the formation of specialized foci of increased density in the cell cortex. These modifications are related to the secretion of the cartilage matrix. The matrix of young hyaline cartilage consists of groups of relatively short, straight, banded collagen fibrils of 10 to 20 mμ and a dense granular component embedded in an amorphous ground substance of moderate electron density. It is postulated that the first phase of fibrillogenesis takes place at the cell cortex in dense bands or striae within the ectoplasm subjacent to the cell membrane. These can be resolved into sheaves of "primary" fibrils of about 7 to 10 mμ. They are supposedly shed (by excorticationation) into the matrix space between the separating chondroblasts, where they may serve as "cores" of the definitive matrix fibrils. The diameter of the fibrils may subsequently increase up to threefold, presumably by incorporation of "soluble" or tropocollagen units from the ground substance. The chondroblast also discharges into the matrix the electron-dense amorphous or granular contents of vesicles derived from the Golgi apparatus, and the mixed contents of large vacuoles or blebs bounded by distinctive double membranes. Small vesicles with amorphous homogeneous contents of moderate density are expelled *in toto* from the chondroblasts. In their subsequent evolution to chondrocytes, both nucleus and cytoplasm of the chondroblasts undergo striking condensation. Those moving toward the osteogenic plate accumulate increasingly large stores of glycogen. In the chondrocyte, the enlarged fused Golgi vesicles with dense contents, massed in the juxtanuclear zone, are the most prominent feature of the cytoplasm. Many of these make their way to the surface to discharge their contents. The hypertrophied chondrocytes of the epiphyseal plate ultimately yield up their entire contents to the matrix.

INTRODUCTION

It was from his microscopical observations on developing cartilage, which seemed to bear a close resemblance to plant tissue, that Schwann (1) was led to the idea of the cell as the basis of correspondence between the structures of animals and plants, and so to the generalized doctrine of the cell state. When, at the same time he put forward his theory of the generation of cells from the

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"formless blastema," the nature of the relationships between cells and the intercellular substance became an issue of fundamental importance in biology. Indeed, ever since the promulgation of this cell theory, and the subsequent recognition of the cellularity of all the connective tissues by Virchow (2), the problem of the mutual relations between cell and matrix has continued to occupy the attention of investigators, among whom were some of the most noted biologists of the last century. The history of the ideas germinated during a century of observation on the nature and origin of the intercellular materials of the connective tissues has been reviewed in the summaries of Lewis (3), Stearns (4), Wassermann (5, 6), Studnička (7), Klemperer (8, 9), and Cameron (10) and in the remarks of Porter (11), and need not be recapitulated here. Although much that was confused has been elucidated, the way in which the cells take part in the formation of both fibrils and ground substance of the connective tissue matrices has yet to be described with the precision enabled by modern methods. There is now no reasonable doubt that the connective tissue cells elaborate collagen, and both tissue culture experiments (12–14) and radioautographic studies (15, 16) have established that such cells synthesize and secrete at least the acid mucopolysaccharides characteristic of connective tissue ground substance. Three main ideas relict from the last century, but modified in detail, continue to influence conceptions about how the cell fabricates the matrix materials, and the manner in which these are organized. These are that: (*a*) the intercellular substances are "lifeless" products of secretion (2, 17, 18, *inter alia*); (*b*) they are derivatives of protoplasm resulting from transformation of the cytoplasm, ectoplasm, or cell surface ("umbildungslehre") (5, 6, 11, 19, 20); (*c*) they are depositions or new formations within the fluid extracellular environment laid down apart from the cell, but probably under cellular "influence" (21, 22, *inter alia*).

Cartilage, as the connective tissue with the most abundant and least soluble ground substance, would appear to be the most favorable object in which to study, at more adequate resolutions, cellular phenomena related to the formation of intercellular matrix, especially during the period of its most active deposition in the embryo. It should be added that the fine structure of matrix of mature hyaline cartilage, although described as it appears in thin sections (23–30), has not hitherto been examined in the course of its development, in which certain features of its construction are more manifest. Since developing hyaline cartilage undergoes the whole course of maturation and senescence from mesenchyme to terminal chondrocyte telescoped, as it were, within a relatively short space and time, an opportunity is afforded, moreover, to study the structural changes in the electron microscope of cells and matrix concomitant with normal differentiation, function, and decay. With the object in view, then, of defining the role of the chondroblast in the biogenesis of extracellular matrix materials, of describing the structural maturation of this series of mesenchymal cells, and of adumbrating our information about the organization of cartilage matrix, a study was undertaken of the embryonic rat epiphysis, from the first appearance of the cartilaginous anlage in the mesenchyme until the development of the definitive epiphyseal apparatus.

MATERIALS AND METHODS

The embryonic cartilage for these studies was obtained from rats of the Sprague-Dawley strain. At estimated gestational ages between 12 to 16 days, gravid females were anesthetized with ether, the uterus mobilized, and each fetus delivered into the operative field without disturbing the placenta or cord. The best fixation resulted from injection of the chilled fixative directly into the hind limb rudiment from a syringe fitted with a 27-gauge needle. The limb was then immediately excised, immersed in a drop of fresh fixative in which it was trimmed, and the tibial and femoral cartilage or precartilage cut into blocks of about 1 cubic mm. The fragments were then transferred to weighing bottles containing 5 to 10 ml. of fixative at about 4°C for 1 hour. Tissues from 25 embryos were studied.

Mature cartilage was fixed by injecting chilled fixative beneath the perichrondrium of the exposed proximal tibial-distal femoral epiphyses of anesthetized newborn rats. The specimens were then excised at once and subsequently handled like the embryonic cartilage.

The fixative that gave the most satisfactory results was 1 per cent oxmium tetroxide solution buffered at pH 8.0 to 8.2 with veronal, with or without added sucrose, to which 0.2 to 0.4 mg./ml. of $CaCl_2$ had been added. Following their fixation the specimens were rinsed either in cold buffer solution or distilled water. Some were then postfixed for several hours in 5 per cent formalin, either buffered at neutrality with M/15 phosphate, or containing 0.5 per cent cetyltri-

methylammonium bromide (cetavolon)[1] in an attempt to preserve better the matrix ground substance.

All tissues were dehydrated rapidly in ethanol, and embedded at 45° or 60°C. in a prepolymerized 1:9 mixture of methyl and butyl methacrylate containing 0.075 per cent uranyl nitrate.

Thin sections, cut with the Porter-Blum microtome, were mounted on copper grids previously coated with a carbon film and examined unstained, or after staining with 2 per cent uranyl acetate for 20 minutes, saturated "lead hydroxide" for 2 minutes (32), saturated ammonium molybdate for 30 minutes, or 0.25 per cent ruthenium oxychloride for various times. Other sections were picked up on grids after exposure to 2 per cent phosphotungstic acid for 5 to 15 minutes and to 10 per cent ferric chloride solutions for variable times.

Electron micrographs were taken with either an RCA EMU-2C or with a Siemens Elmiskop I at original magnifications of from 5,000 to 11,000 diameters. Thick sections from each block were examined with the phase microscope for topographic orientation.

OBSERVATIONS

Topography of Developing Cartilage

Prechondral Mesenchyme and Precartilage: The cells of the massed mesenchyme which constitute the centers of chondrification, share with other mesenchyme cells such characteristics as a high nucleocytoplasmic ratio, large nucleoli, relatively small mitochondria, underdeveloped endoplasmic membrane systems, numerous free ribonucleoprotein particles, and a watery or empty appearance of the hyaloplasm. These embryonic cells are more difficult to fix adequately than corresponding mature tissues of the adult.

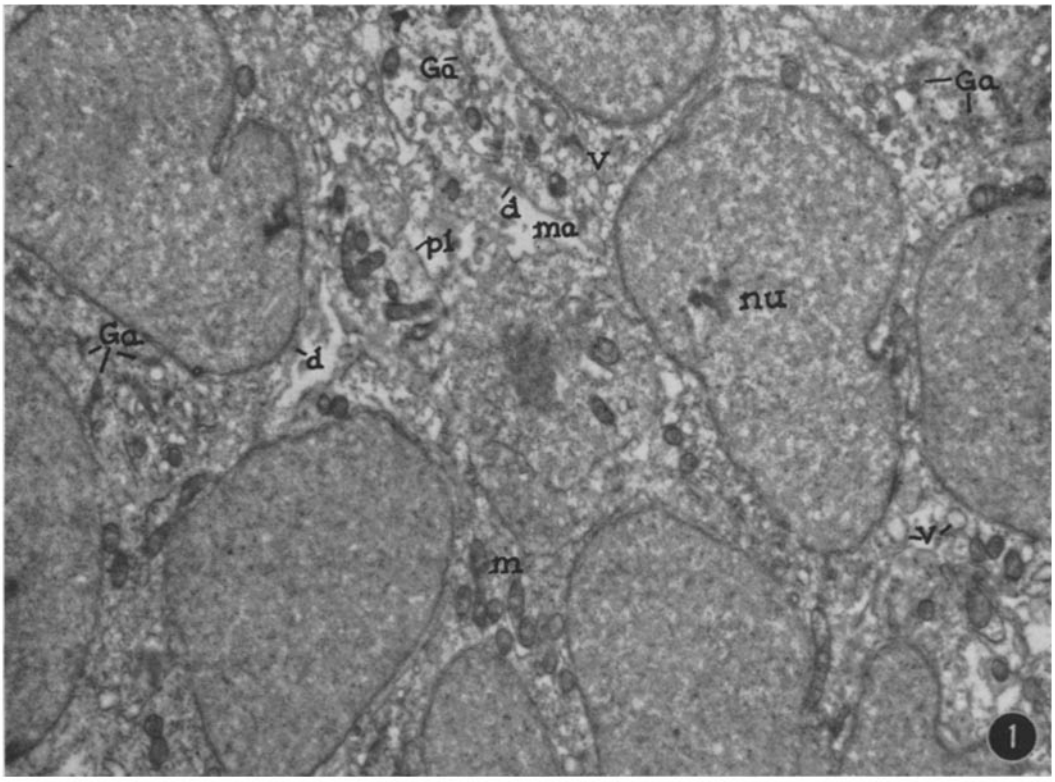
The organization of the packed epithelioid mass into precartilage is heralded by the occurrence of intercellular clefts, lacunae, and fissures which separate each cell from contact with its neighbors (intussusceptional or expansive growth). In the fixed preparations these spaces at first contain little or no electron-dense material. The separation begins at the center of the cartilaginous anlage, an event which coincides with structural transformations in the cells indicative of enhanced physiological activity. Such tissue is shown in Fig. 1. The clefts and spaces in a somewhat later stage (Fig. 3) contain some homogeneous material of very low density, as can be seen by comparing the grey of these areas with the white of nearby empty or extracted artifactitious foci in the same photograph. On closer inspection short lengths and sections of fine filaments, about 100 A in diameter (range 70 to 120 A), are seen sparsely and randomly scattered throughout the homogeneous material that occupies the spaces. This material also contains small scattered granules of much greater electron density. Thus, at the earliest recognizable stage in the transformation of precartilaginous mesenchyme to primitive cartilage, the 3 elements composing the matrix of cartilage are represented (Figs. 1, 3, and 4).

Embryonal and Young Cartilage: As continued deposition of matrix material between the cells and further separation of the chondroblasts occurs, the tissue assumes the typical histological appearance of young cartilage (Fig. 2). The chondroblasts become cells of irregular stellate shape with slender processes enclosing bays and inlets which account for the typically scalloped outlines. Many are found in mitosis (Fig. 2, *k*). Even at low magnifications the enlargement of mitochondria and acquisition of membranous structures and vacuoles can be appreciated.

The components of the matrix at this intermediate stage of development are more abundant, larger, and more clearly defined. The fibrils are short (mean length in sections 0.6 μ), relatively straight, sometimes kinked or curved, and range from 100 to 180 A in diameter.

In unstained sections of the fibrils no fine structure is usually visible, but a periodicity is sometimes suggested, especially in metal-shadowed preparations. The periodic cross-striations illustrated in Figs. 12 and 21 become clearly demonstrable only in sections which have been treated with phosphotungstic acid solutions or in very thin sections judiciously stained with uranyl acetate. In preparations stained with lead or barium hydroxide, or ammonium molybdate the fibrils are generally less well represented. The usual periodicity shown consists of alternate narrow dark (adielectronic) lines and light (dielectronic) bands, the interband length ranging approximately between 70 and 90 A (average and mode $\simeq$ 80 A). However, considerable variation is encountered. In certain locations the narrow (dark) adielectronic lines occur in closely spaced doublets whose dense bands are separated by a less dense band of 50 A; the doublets are spaced apart at longer intervals of 100 A by dielectronic (light) bands; the period from doublet to doublet is about 210 A. (These periodicities, while different from those of typical collagen, are however integrals of 640.) The fibrils tend to lie mostly in loose bundles, sheaves, or heaps which

[1] The use of cetavolon, a basic detergent and precipitant of polyanions, for histological fixation of acid mucopolysaccharides was suggested by Williams and Jackson (31).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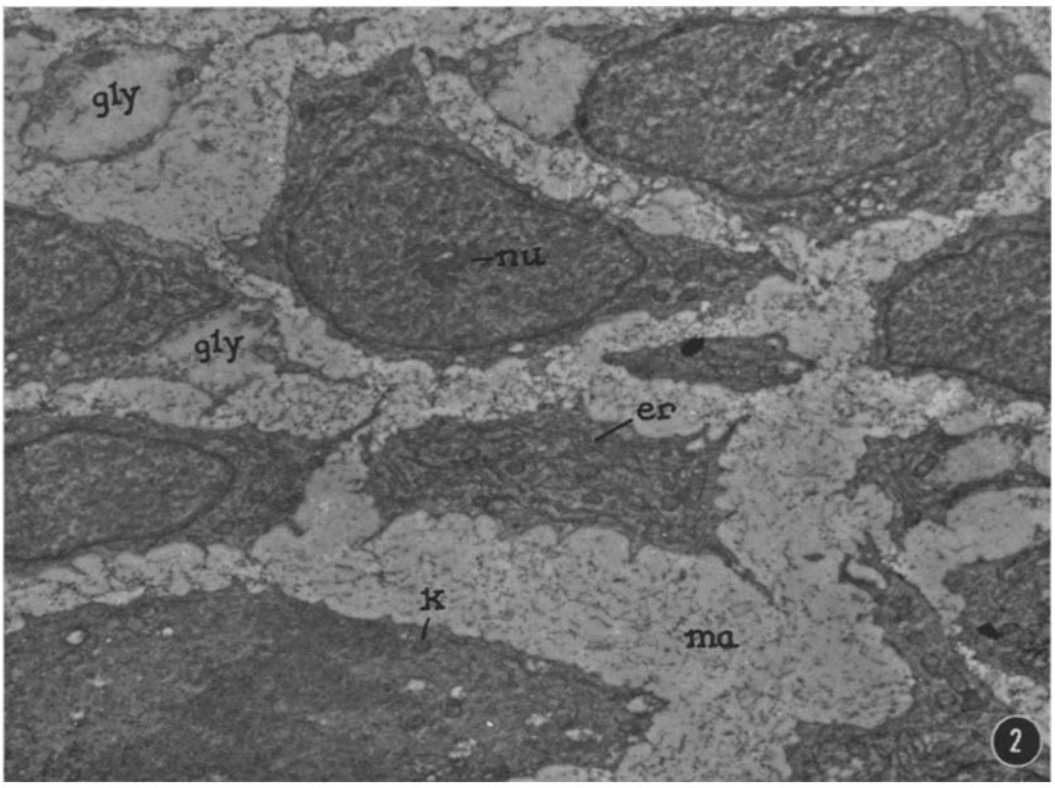
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appear to be distributed randomly throughout the intercellular space and generally exhibit no preferred orientation (Figs. 2, 6-8, 20). In unextracted sections (Fig. 8) the bundles or groups are seen to be embedded in vaguely limited interconnected clouds of amorphous homogeneous material of moderate density which appear to be condensations of the ground substance. The dense matrix granules (Fig. 6, *mg*), which are of fairly regular size, averaging 120 A (range 100 to 140 A), are distinguishable by their much greater density from cross-sections of fibrils and from the larger ribonucleoprotein granules of the cytoplasm. They may lie either free in the ground substance or upon or within the fibrils. The nature of these is uncertain, but they probably represent mineral deposits.

Young Mature Cartilage: In the matrix of young hyaline cartilage (Fig. 6) the fibrils are thicker and longer. In mature matrix they may average 250 A in thickness, but the range (about 150 to 500) is greater. The amorphous ground substance is denser, and its distribution about the fibrils is therefore more evident.

The cells, which may now be designated chondrocytes, are further separated by wide plates of matrix. They have undergone extensive development; both their cytoplasm and nuclei are characteristically very dense, and elaborate systems of membranous channels and vacuoles as well as inclusions of low to moderate electron-scattering capacity can be discerned in the cytoplasm. The cells of the chondrification center in the periphery of the expanding cartilage nodule still retain certain of the characteristics of chondroblasts; those at the circumference, in the perichrondrium just outside the matrix, are usually elongated or stretched parallel to the surface where bundles com-

Legend for Figures

Chondroblast—*ch*
Nucleus—*n*
Nucleolus—*nu*
Nuclear membrane—*nm*
Cell membrane—*pl*
Mitochondria—*m*
Dense particles or granules—*p*
Endoplasmic reticulum—*er*
Dilated cisternae—*dl*
Densities in cortex or at cell surface—*d*
Glycogen deposits—*gly*
"Rarefactions"—*r*
Golgi area—*Ga*
Vacuoles or vesicles—*v*
Pool or lake limited by double membrane—*l*
Double membrane—*dm*
Stoma—*st*
Matrix—*ma*
Dense granules of matrix—*mg*
Fibrils—*f*
Ground substance—*gs*
Cell in mitosis—*k*

FIGURE 1

Precartilage. Some early separation of the massed mesenchymal cells constituting het precartilage by the primitive matrix spaces (*ma*) has occurred. The soluble contents of the spaces or clefts have mostly been extracted in preparative procedures. The cell membranes (*pl*) cannot be continuously followed as distinct lines about all cells. At the cell surfaces facing some of the matrix spaces, patches of dense material (*d*) are discernible. The membrane-limited systems of the cytoplasm, the endoplasmic reticulum, are represented chiefly by small dispersed vesicles (*v*), only a few of which are studded with dense particles. Very small, widely scattered aggregations of smooth vesicles appear to constitute the dispersed Golgi apparatus (*Ga*) of these cells. The mitochondria (*m*) are relatively small and dense. Nucleolus is indicated at *nu*. Uranyl acetate stain; × 8,000.

FIGURE 2

Embryonal hyaline cartilage. Each chondroblast is now an island in a continuous matrix phase (*ma*), which is seen to consist of short rather straight fibrils embedded in a ground substance of moderate electron density. The chondroblasts typically present irregular scalloped outlines and multiple processes. As compared with the less differentiated younger cells of Fig. 1, cytoplasmic and nuclear condensation, nucleolar (*nu*) enlargement and marked development of endoplasmic reticulum (*er*) has occurred. Some chondroblast contain deposits of glycogen (*gly*). PTA stain; × 6,000.

posed of the more typical collagen fibrils of 640 A periodicity are found. Continued maturation or aging of the cartilage produces the tissue which has been described in older or adult animals (23–30).

In the newborn rat, occasional long, slender, wavy collagen fibrils, usually in bundles, can be found within the matrix, whose more usual fibrillar component consists of shorter, somewhat straighter fibrils between 180 to 500 A thick, which exhibit the shorter periodicity. The random bundles of these tend to intermesh. The chondrocytes found near the center of such cartilage, and especially the hypertrophic chondrocytes aligned near the epiphyseal line exhibit striking changes, most marked of which are due to regressive alterations and the accumulation in them of large deposits of materials.

The Cells

Cells of the Prechondral Anlage: Mesenchyme, Prechondroblast, and Early Chondroblast: The cells of the prechondral anlage (Fig. 1) are more difficult to preserve intact than other embryonal cells, their cell membranes being especially liable to disruption.

The interphase nucleus of the prechondroblast is large relative to the cytoplasmic mass, and is usually seen notched or indented; its chromatin is shown as a reticulum of floccules composed of fine threads and granules of less than 70 A, separated by areas of low density. Chromatin material in denser masses is usually apposed against the inner aspect of the nuclear envelope. The one or two nucleoli consist of tight coils of large dense strands.

The cytoplasm has a characteristic clear or extracted appearance because the relatively abundant cytoplasmic matrix or hyaloplasm, except for some floccules, has hardly any electron-scattering substance, and also because the numerous small cytoplasmic vacuoles and vesicles are mostly "empty."

These membrane-bounded vesicles, usually of round or oval outline, range from 20 to nearly 100 mμ in diameter. They may occur in clusters or chains, but only occasionally as small narrow elongate cisternal profiles scattered throughout the cytoplasm. These and some of the smaller vesicles studded with small, dense (ribonucleoprotein?) particles evidently constitute a discontinuous form of the endoplasmic reticulum. Unattached particles of the same size and density as those attached to the vesicles are also found, in small numbers, free in the cytoplasm and these together with the attached particles are taken to be the ribonucleoprotein granules of Palade (33) and must contribute to the diffuse basophilia of the cytoplasm of these cells. Most of the cytoplasmic vacuoles, however, are agranular. Such scattered vacuoles, and a few small compact collections of contiguous flattened membranes in short stacks of 2 or 3, set within denser areas of the ground cytoplasm, represent the widely dispersed Golgi complex of these cells.

The mitochondria are typically small, dark rodlets about 0.3 μ in diameter, with rather irregular transverse cristae. Their relatively great electron-scattering power as compared with mitochondria of differentiated cells appears to be due not only to their compact form, but also to the somewhat higher density of the matrix in the inner chamber. Intramitochondrial granules are but rarely encountered. The mitochondria tend to occur in groups having no constant location in the endoplasmic cytoplasm; they are, however, often found in contact with the nuclear envelope. While vesicles of the endoplasmic reticulum are also frequently seen in contact with mitochondria (Fig. 1, *m*), this does not seem to be a regular relationship.

In preparations otherwise adequately preserved (Fig. 1) the delicate plasma membrane of each individual mesenchymal cell is difficult to trace, so that the boundaries of approximated cells are often undefined and the tissue presents the aspect of a syncytium. In some sectors the line of the cell membrane at the cell surface abutting on the spaces opened by separation of the cells is replaced by a poorly delimited, dense material.

As the cells of the procartilage begin to separate (Fig. 3), they acquire a greater volume of cytoplasm in which structural differentiation becomes obvious. These *early chondroblasts* (Fig. 4), which now lie isolated in the gathering matrix, are multiprocessed cells of markedly indented, scalloped, or irregularly fluted outline. Their interphase nuclei are somewhat denser than those of the mesenchymal cells: the more coarsely granular chromatin is disposed into more compact masses and the relative volume of intervening karyoplasm is somewhat reduced.

Distinct membrane-bounded channels and cisternae of the endoplasmic reticulum, with their associated ribonucleoprotein particles are now quite numerous. Expanded segments of cisterna, some of relatively large size (0.3 μ) are interposed between narrower segments; both are

filled with substance denser than that of either the ground cytoplasm (hyaloplasm) or the extracellular matrix. Collections of small particles, apparently unattached, are also found in the cytoplasm. The Golgi apparatus is represented by scattered focal collections of vesicles and a few (usually 3) flattened double membranes in short contiguous compact stacks larger than those of the prechondroblast. Dispersed throughout the cytoplasm are variable numbers of the non-granular vesicles, singly or in chains and seemingly devoid of electron-scattering contents.

The mitochondria have enlarged considerably, and are distributed at random in the endoplasm chiefly as elongate cylindrical forms up to 3 microns in length and about 1.25 microns in width. The double membranes of wall and cristae are clearly visible and the central space is expanded by a matrix of moderate density.

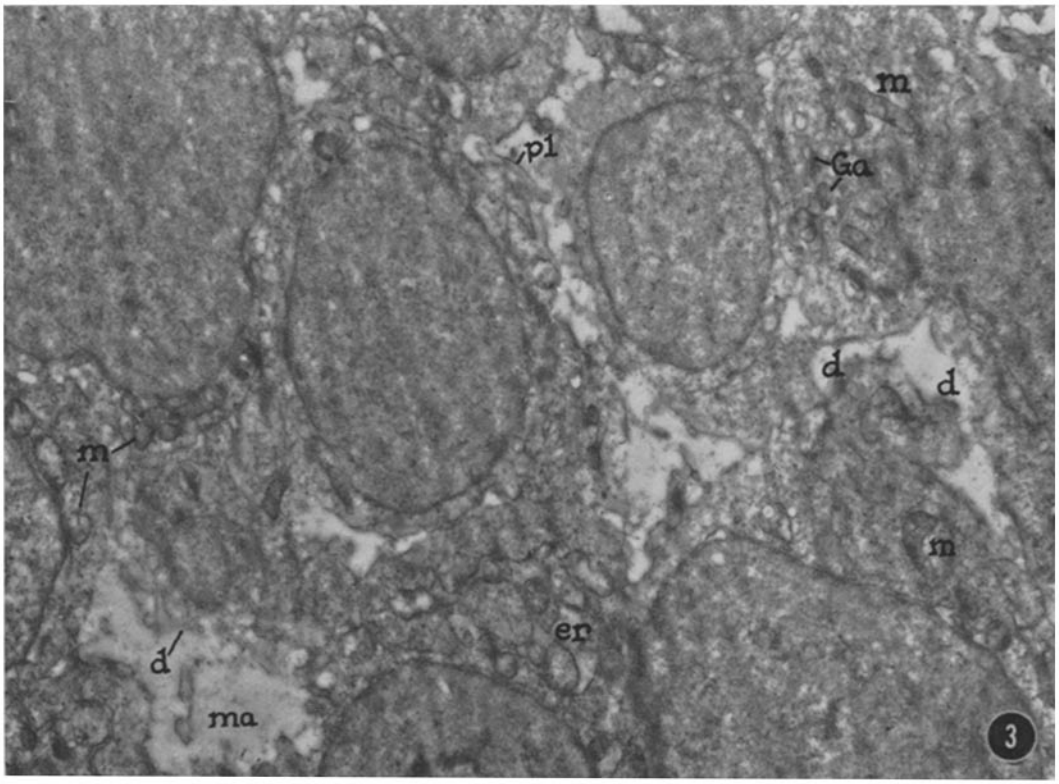
At the surface of the cell, a definitive membrane line (in favorable images seen as a 3-layered structure), or its tangentially sectioned aspect, can be traced. The cell membrane in this and later stages of development is seen as a sharp line only along parts of the cell perimeter because of the many angulated cytoplasmic processes, bays, and declivities. At some sectors, particularly at the bases of some of the excavations or bays, the membrane is not identifiable and is often seemingly replaced by a narrow band of some dense material at the surface (Figs. 3, 9, 10, 17, 19). In other locations such densities are seen in the ectoplasm or cell cortex immediately subjacent to and contiguous with the intact cell membrane (Figs. 13, 20, 30). Patches of dense, finely fibrillar material may also be seen deposited upon the plasma membrane; *i.e.* overlying the cell surface (Figs. 4, 11). Foci of "empty" appearance, to be subsequently described as rarefactions, are sometimes seen at this early stage of development in the subcortical endoplasm beneath the densities (Fig. 4, *r*).

Mitoses are frequently encountered in this population of cells. During mitotic division scattered mitochondria and collections of small agranular vesicles, as well as short segments of endoplasmic reticulum, are found only in the peripheral cytoplasm. The impression is gained that in mitosis all of the membrane-bounded channels are both fragmented and considerably reduced in amount as compared with the interphase cells, as noted also by Porter (34). The peripheral cytoplasm of these dividing cells is further remarkable for the large numbers of free granules resident there. This population, pointed out by Hay (35) in the dividing blastema cell, consists in the rat chondroblast of smaller (100 A), and much denser granules.

The Developed Chondroblast: With progressive differentiation there is continued augmentation of cytoplasm and increase and redistribution of its contained membranous channels (Fig. 5). The granular endoplasmic reticulum becomes especially well developed, its intercalated dilatations sometimes attaining 0.5 μ in size; the filled-out channels are traceable for lengths exceeding 5 μ. As the endoplasmic reticulum proliferates it may form roughly parallel arrays or concentric whorls, which occupy a considerable share of the endoplasm. These cisternae make up the intercommunicating system described by Palade and Porter (36), whose continuity with the perinuclear cisterna is particularly well shown in Fig. 17. The contents of the endoplasmic reticulum remain slightly denser than the hyaloplasm. Some clusters of apparently free granules having the same characteristics as the attached ribonucleoprotein particles, are still present in the cytoplasm.

The vesicles and membranes of the Golgi complex are now also markedly increased in size and number, and have gathered into a rather extensive juxtanuclear zone which may be referred to as the Golgi area (Figs. 5, 30, 31, *Ga*). Here are found extended lamellar stacks of from 4 to 8 approximated flattened cisternae set in a denser ground cytoplasm, and closely associated swarms of agranular vesicles or vacuoles of varying size usually ranging from 30 mμ to about 0.25 μ in diameter. The smaller vacuoles enclose slightly to moderately adielectronic contents (Fig. 5, arrow); the largest ones may show contracted central plugs of such material, or may appear quite empty. The cisternal piles may be variously oriented with relation to one another even in the same thin section, and the vesicles are interspersed between and in the area adjacent to them. Small isolated vesicles may be distributed at a considerable distance from the main juxtanuclear Golgi zone. Connections between the flattened and expanded agranular forms are frequently seen but images of transitions to rough membranes, encrusted with ribonucleoprotein particles, are far less frequent (Fig. 31, arrow).

At this stage, deposits or lakes of stored substances become the most striking inclusions in the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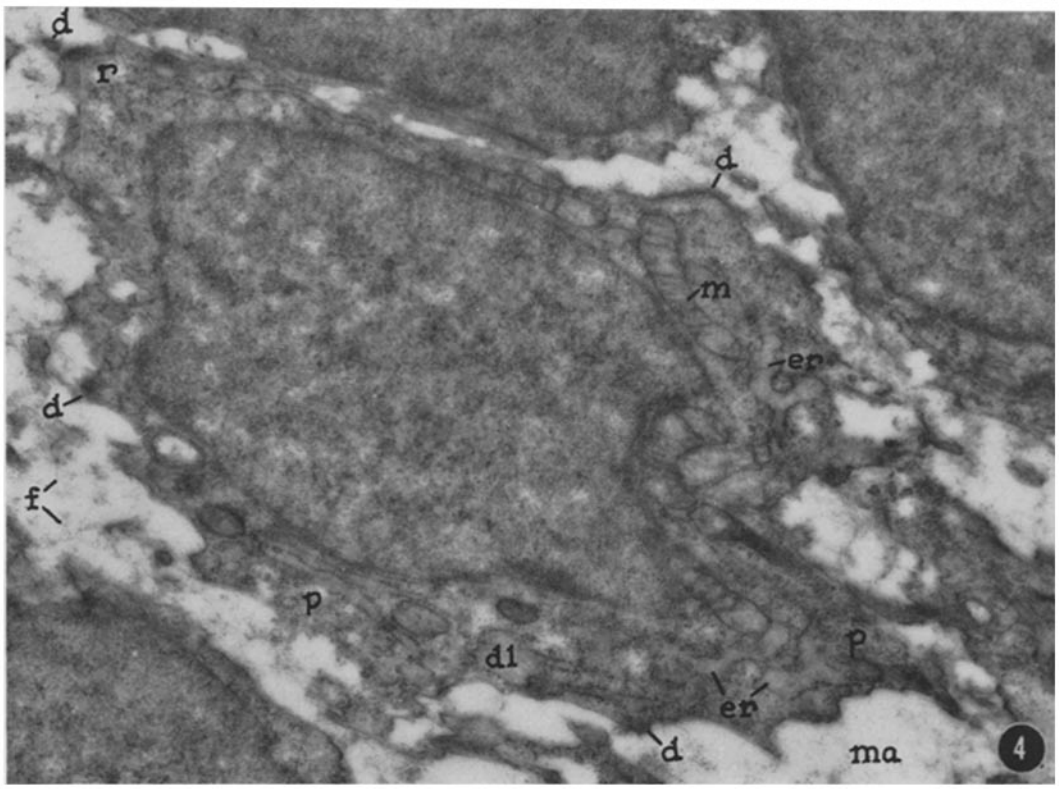
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cytoplasm. These are of two kinds: the first is a coarse flocculent or feathery reticulum of moderately dense homogeneous material within which sometimes there are scattered fine granular elements of greater density, occasionally arranged in circlets. These irregular agglomerations, which may be quite extensive, appear to lie free in the cytoplasm and are not limited by any membrane line or other formed structures (Figs. 2, 25, *gly*). They have the histochemical (*q.v.*) and morphological characteristics of glycogen. The contents of the second type of lake or accumulation are more variable in appearance (Figs. 24, 25, 28, *l*). In all there is a constant material of moderate electron density greater than that of the unbounded inclusions, which, after staining with lead hydroxide, has a finely granular or filamentous texture, with units smaller than 35 A. Added to this there may occur small dense particles ranging from about 80 to 200 A. (Ribonucleoprotein particles of the endoplasmic reticulum in the same cells average about 200 A.) Small vesicles of from 20 to 70 mμ, with smooth membranes, are also sometimes included (Figs. 20, 28) as are small fragments of cytoplasm (Fig. 27). These contents are always enclosed in a definite membranous envelope. This limiting structure is unique in that it always appears as two lines separated by a variable space which averages 100 A or more (Figs. 24, 28, 29, *dm*). In certain sections (Fig. 28, *v*) this doubled membrane is broken up into chaplets or strings of contiguous, small, sometimes flattened vesicles having about the same dimensions as the original double membrane. These lakes bounded by double membranes are usually already about ¼ or more of a micron in diameter when first recognized, and they evidently may grow to attain quite large size. Their origin and fate will be considered subsequently.

The cell surface is the site of more of the special dense areas involving the cortex and cell membrane. There are also larger overlying heaps of attached fine fibrils on the cell surface. The cortical ectoplasmic densities are evidently in the form of narrow elongate bands (Figs. 13, 15) which are most usually seen in oblique or transverse section (*q.v.*).

In the peripheral cytoplasm of the cortex and subcortex there are also found the irregular, ill defined zones of reduced density which have been referred to above as "rarefactions" (*q.v.*). These attain a greater development in the older cell.

The Chondrocytes: Further maturation is characterized by increasing condensation of both nucleus and cytoplasm of the cell, which comes to present the aspect shown in Figs. 7 and 8. These cells, designated chondrocytes, are placed more centrally in the embryonal cartilage nodule, and

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FIGURE 3

Very early cartilage. This is a more mature stage than that shown in Fig. 1. Sparsely distributed, very fine (about 100 A) fibrils and a ground substance of low electron-scattering capacity occupy the matrix spaces (*ma*). Numerous dense patches of finely fibrous material on cell surfaces are indicated at *d*. In the chondroblast cytoplasm a few definite channels of the endoplasmic reticulum (*er*) with local dilatations of the cisternae are present. These and the membranes of many vesicles are studded with dense particles. The mitochondria (*m*) are larger than those of the prochondroblasts. Uranyl acetate stain; × 7,500.

FIGURE 4

Early chondroblast. This is an electron micrograph at higher magnification of a chondroblast like those shown in Fig. 3. The fine fibrillar components (*f*) of the young matrix (*ma*) are shown. The excavated and scalloped cell outline is characteristic of chondroblast. Focal densities (*d*) are seen at the cell surface. Cisternae or tubules of the endoplasmic reticulum (*er*), with their intercalated dilations and incrusting dense particles, are increased in size and number. Some dense particles (*p*) are also seen lying free in the hyaloplasm. Vaguely delimited foci of reduced density (*r*) are scattered throughout the cytoplasm, but are principally in the subcortex. Other organelles are indicated as before. Uranyl acetate stain; × 15,000.

although still in possession of all of the organelles associated with functional activity, show signs both of altered secretion and diminished growth. Mitoses are not usually found among them.

Their endoplasmic reticulum is elaborately developed, and is most often disposed as closely spaced series of long, particle-studded channels in an ordered, parallel array, thus deserving the name ergastoplasm. Their contents remain moderately dense. The Golgi complex is now more completely concentrated in the juxtanuclear area where its elements are increased in size and number. Of these, the most arresting are numerous rather large vacuoles, some up to 0.25 μ, many of which contain a central plug of a material of moderate to marked density, the extremely dense component being as adielectronic as lipid. In addition, small (less than 50 A) particles of very high density are often contained in the plugs (Fig. 7, *v*). A few vacuoles stream out from the juxtanuclear Golgi area, and come to lodge in greatest concentration in the subcortical or ectoplasmic region of the cell where they may underlie the plasma membrane or some of the cortical densities previously mentioned. This position at the cell periphery they share with some superficial deposits of glycogen (Figs. 7 and 25, *gly*) and with the irregular empty or "rarefied" spaces of very low density having ragged outlines and no apparent limiting membranes (Fig. 8, *r*). On closer inspection most of these spaces or rarefactions appear to be fused vacuoles emigrated from the Golgi complex, which have lost their membranes, since remnants of membranes may sometimes enclose sectors of these spaces. Some, however, simply appear as irregular uncircumscribed foci of reduced electron density, containing no formed elements, and giving no clue as to their origin. They are invariably found in chondrocytes and usually also in younger cells and cannot be regarded as mere artifacts. In places (Fig. 8, *d*) overlying dense bands of cortical ectoplasm may be arched or lifted away from the rest of the cytoplasm by the space below, and occasionally, breaks in these bands may partly detach them from the cell. The densities in the cortical ectoplasm of the chondrocytes are broader and longer than those of earlier stages. Indeed, much of the cortex appears ultimately to be transformed into the dense material. Most of the chondrocytes of the columns of the proximal part of the epiphyseal plate, no longer possess extensive glycogen deposits, and the pools of material bounded by double membranes are infrequent. These cells, which are usually present in the interior of the new cartilaginous nodule, become surrounded by increasingly thicker matrix walls; they undergo but little change in form save for a still greater increase in nuclear and cytoplasmic density, enlargement, and confluence of the Golgi vacuoles, and occasionally the acquisition of some lipid droplets.

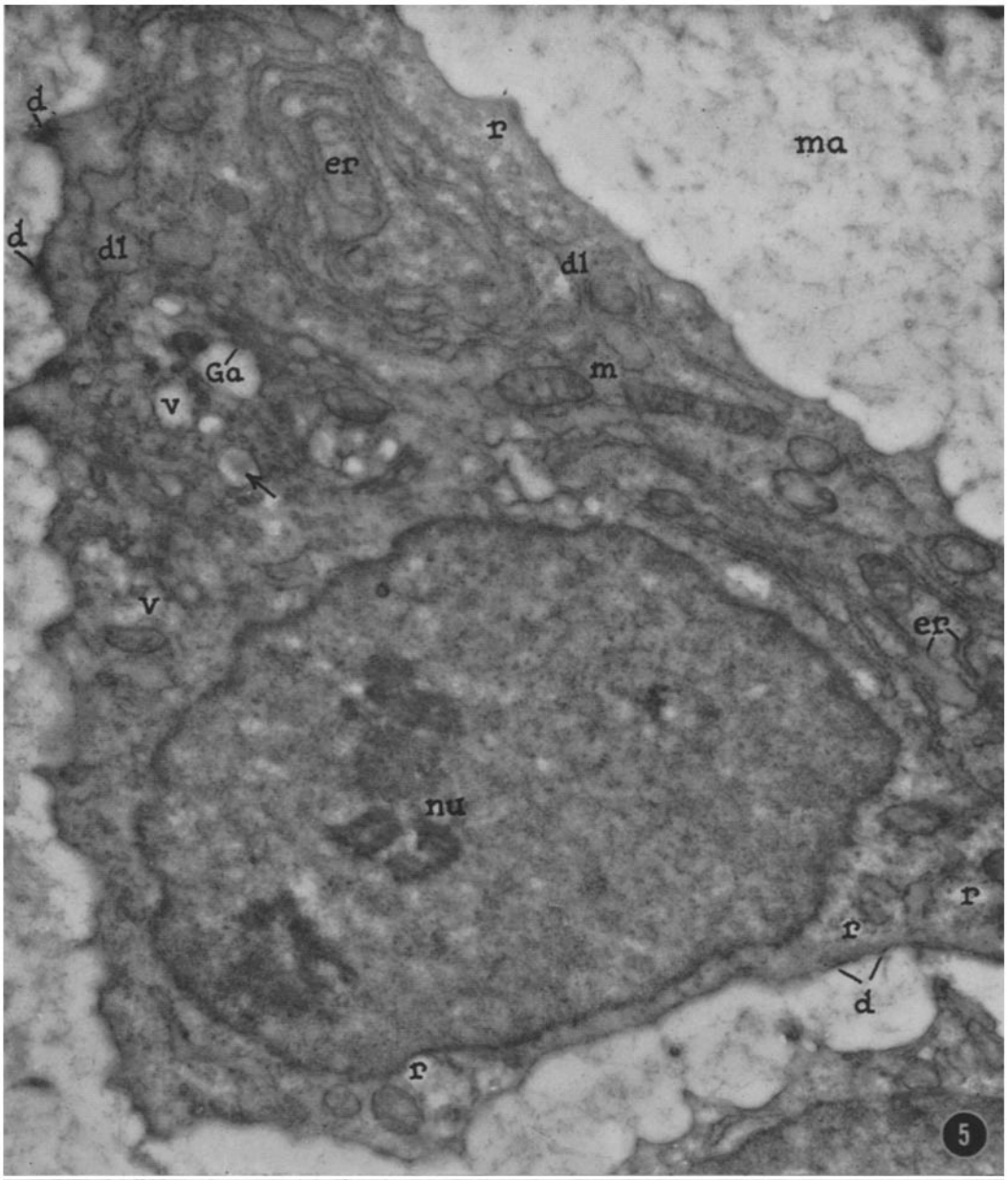
The Hypertrophied Chondrocytes: However, those chondrocytes destined to enter the osteogenic plate, *i.e.* those closer to the metaphysial line of the epiphysis, especially the more distal chon-

FIGURE 5

Developed chondroblast. The cytoplasmic volume is increased in this more differentiated chondroblast. The more numerous cisternae and cisternal dilatations (*dl*) of the endoplasmic reticulum (*er*) are now more closely spaced and constitute an ergastoplasm. The cisternal contents are more dense than the extracellular matrix or the hyaloplasm. In the latter, there are numerous poorly delimited areas of low density referred to as "rarefactions" (*r*). The Golgi apparatus, (*Ga*) with its stacks of flattened agranular cisternae and swarms of vesicles and vacuoles is concentrated in one area of the cell. Some of the vacuoles enclose moderately dense contents (arrow). Cortical densities at the cell surface are indicated at *d*. × 15,000.

FIGURE 6

Matrix of embryonal cartilage. The tip of a chondroblast (*ch*) is shown. Fibrils ranging in thickness from 180 A near the cell to an extreme of about 500 A in the interterritorial matrix (lower left) are shown. These are relatively short, straight or kinked fibrils tending to lie in heaps which are more or less randomly oriented. In addition, small dense granules (*mg*) averaging 120 A are present in the matrix. PTA stain; × 15,000.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drocytes arranged in columns, undergo further striking changes that are chiefly regressive (Fig. 7). There is further condensation, *i.e.* increased electron density of the hyaloplasm, which now contains a higher concentration of dense particles, both free and associated with small elements of the endoplasmic reticulum. The cavities of the latter are partly collapsed and no longer have ballooned-out segments. There is a noticeable concentration of cytoplasmic organelles and also greater compaction of the nuclear components. The Golgi complex is represented almost entirely by enlarged vesicles, many containing dense material which has seemingly shrunk away from the inner aspect of the membrane. These are frequently seen to approach the cell surface (Fig. 7, *v*) where open communication may be established between their lumens and the extracellular space through disappearance of a sector of the fused overlying membrane. The vacuoles of the Golgi area, because of their large number, are crowded into contiguity; coalescence and pooling, after breakdown of the intervening membranes, appear to be frequent. In late stages this process results in the formation of small pools. Similar union of the subcortical rarefied or empty zones results in the rather long areas which separate the cell cortex from the endoplasm (Fig. 7, *v*). The cortex, as noted, is more and more replaced by the dense bands previously described.

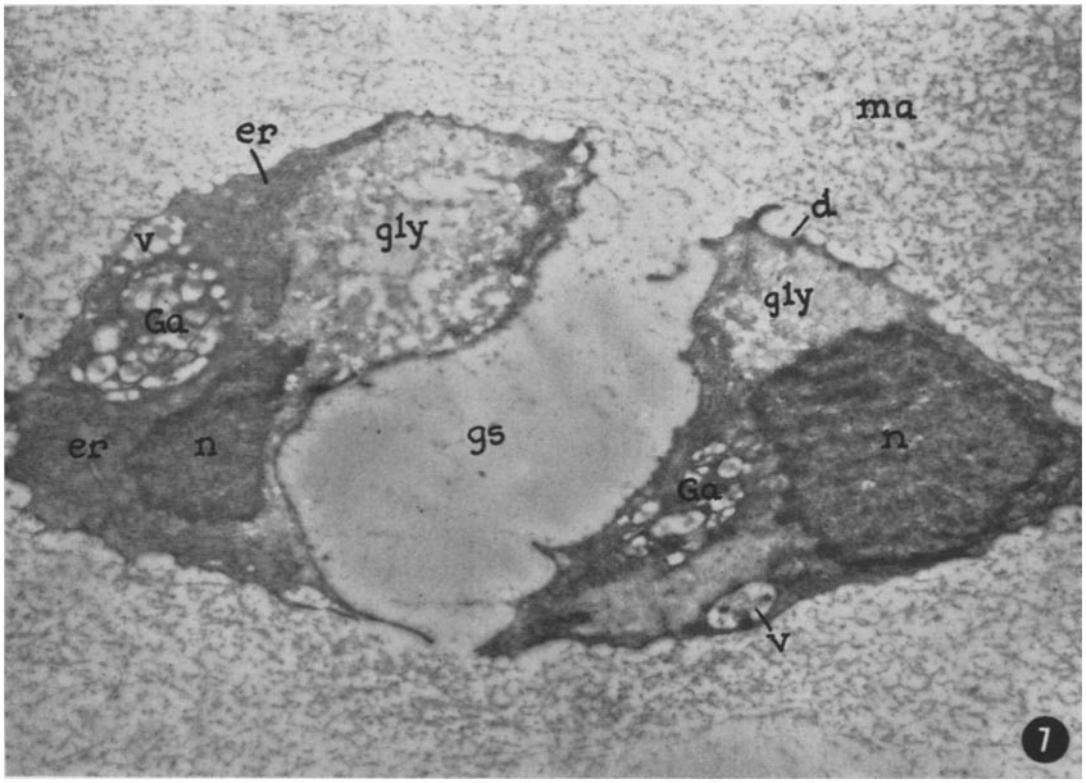
The greater part of the endoplasm of these cells comes to be occupied by massive lakes of glycogen. These burgeon out, compressing the channels of the endoplasmic reticulum further together and pushing them to the periphery of the cell. The Golgi center maintains its original position longer. The fairly dense contents of the lumens of the endoplasmic reticulum are evident, but like those of the other organelles stand out with diminished contrast against the darker condensed hyaloplasm. Mitochondria are of the same enlarged form as described above but are distinctly fewer in number, owing in some part to the inclusion of a smaller area of cytoplasm in each section, but probably more to actual loss, for evidences of degeneration can be seen in those which persist. Such chondrocytes are sometimes surrounded by a narrow band (usually not more than 1.0 μ) poor in fibrils, filled with a homogeneous substance of low electron scattering power; this represents the

FIGURE 7

Isogenous chondrocytes in hypertrophic zone near future ossification center. These are the terminally differentiated chondrocytes. Large unbounded deposits of glycogen (*gly*) occupy most of the cytoplasmic area; both nucleus and the cytoplasm proper are condensed. The proliferated and hypertrophied Golgi apparatus (*Ga*) takes up a large juxtanuclear zone. It consists mainly of large vacuoles, many of which are forming pools by coalescence, which contain materials of varying density. Some vacuoles (*v*) approach the cell surface, presumably to discharge their contents. Some of these have fused to form spaces underlying the cortex. The ergastoplasm (*er*) with its relatively dense contents is compressed into the remaining cytoplasm. In the concavity between the chondrocytes there is an area of afibrillar ground substance (*gs*) of moderate density (the territorial matrix). Stained with uranyl acetate; × 6,000.

FIGURE 8

Chondrocytes, detail. In the chondrocyte at the lower right there is a collection of vacuo es of different sizes. These, together with a few small, agranular cisternae make up thle Golgi apparatus (*Ga*). Most have moderate to markedly dense contents. Some are coalescent. A few appear in the subcortex of the cell. The proliferated channels of the ergastoplasm (*er*) with their dense contents are crowded into the remaining cytoplasm. Mitochondria (*m*) are visible in several places. The hyaloplasm is condensed, and some cortical densities (*d*) are evident. In the cell at the upper left, a coalescent series of low densities are seen underlying an extensive zone of the cortex in which densities are present. These become subcortical "rarefactions" (*r*). Some dilated cisternae of the granular endoplasmic reticulum are shown. The fibril groups and their associated ground substance are evident in the matrix (*ma*). Nuclei are indicated at *n*. Stained with uranyl acetate; × 12,000.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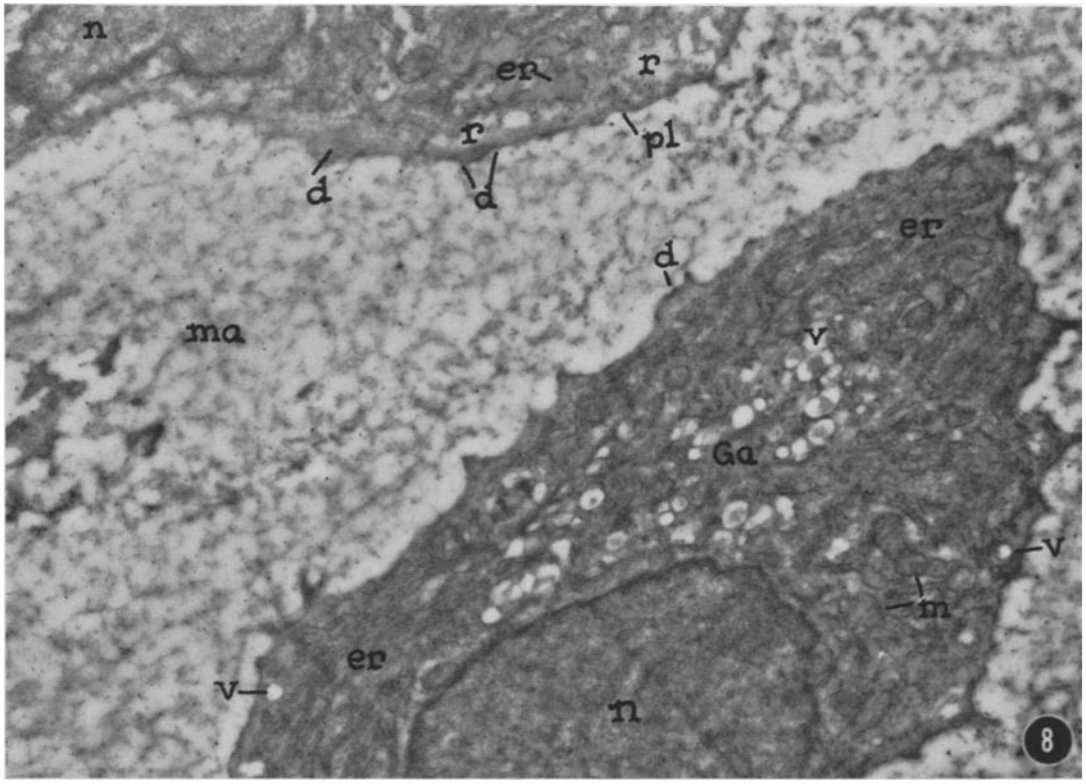
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"capsule" or "moat." Frequently, hypertrophied chondrocytes are seen adjacent to large lakes of a uniformly textured homogeneous material of low to moderate electron-scattering capacity; such lakes interposed between their concave surfaces often separate pairs of isogenous cells (Fig. 7, *gs*). These in effect are enlarged moats. In later stages, even more of the cytoplasm is taken up by the material of the Golgi vesicles and by the massive deposits of glycogen, compressing between them narrow bridges of cytoplasm.

Fibrillogenesis

The close attachment of tufts, brushes, or fasciated bundles of fibrils, or of dense fibrillar material, to certain foci on the cell membrane is evident during all stages of maturation (Figs. 17 to 19, 23, 30, *f*), and can hardly represent merely fortuitous adhesion. Higher resolution reveals that these clumps of fibrillar material at the cell surface are usually related to the dense areas of the cortical ectoplasm, which they tend to overlie. At favorable angles of section, the line of the cell membrane is sometimes seen to intervene between the cortical densities and the overlying fibrils (Fig. 11). In some instances, however, a direct continuity is traceable between the substance of the densities and tufts or tassels of fine fibrils extending out into the extracellular matrix (Figs. 17, 18). The fibrils of the matrix everywhere tend to lie in roughly parallel array in heaps or bundles (Figs. 20, 30, *f*). These appearances suggest that the fine matrix fibrils may originate from elements preformed within the dense bands of the cortical ectoplasm of the chondroblast. In very thin (40 mμ) sections stained with uranyl acetate, but particularly in those treated with solutions of phosphotungstic acid, closer examination discloses that the cortical densities are indeed constructed of oriented arrays of fine, straight, often discontinuous fibrillae in nearly parallel order, each of which measures in the range of 60 to 100 A in width (Figs. 9, 10, 13 to 16). There is sometimes a suggestion that each of these fibrils (designated primary fibrils) is actually formed by adlineation of a row or series of discontinuous elements (Fig. 14, *f*, arrow). The sheaves or fasces of primary fibrils which in thicker, unextracted sections would appear as dense bands in the cortical ectoplasm without defined internal order, are visible in Figs. 14 to 16. Such bundles are several microns long, and up to 0.5 μ in width, forming rods or ridges which may represent the fibroglia or stress fibers of classical histology. Oblique or transverse sections are therefore more numerous than coronal or parasaggital views of them.

Since at certain points these cortical densities or

FIGURE 9

Chondroblast, with process. A tangential and oblique section of a dense stria (*d*) in the form of an arc, is situated mainly in the cortical ectoplasm of the cell. Where the densities appear to abut on the surface no definitive plasma membrane line is visible owing probably to the obliquity of the section. Some vesicles near the cell surface are seen at *v*. Other structures are indicated as before. PTA stain; × 30,000.

FIGURE 10

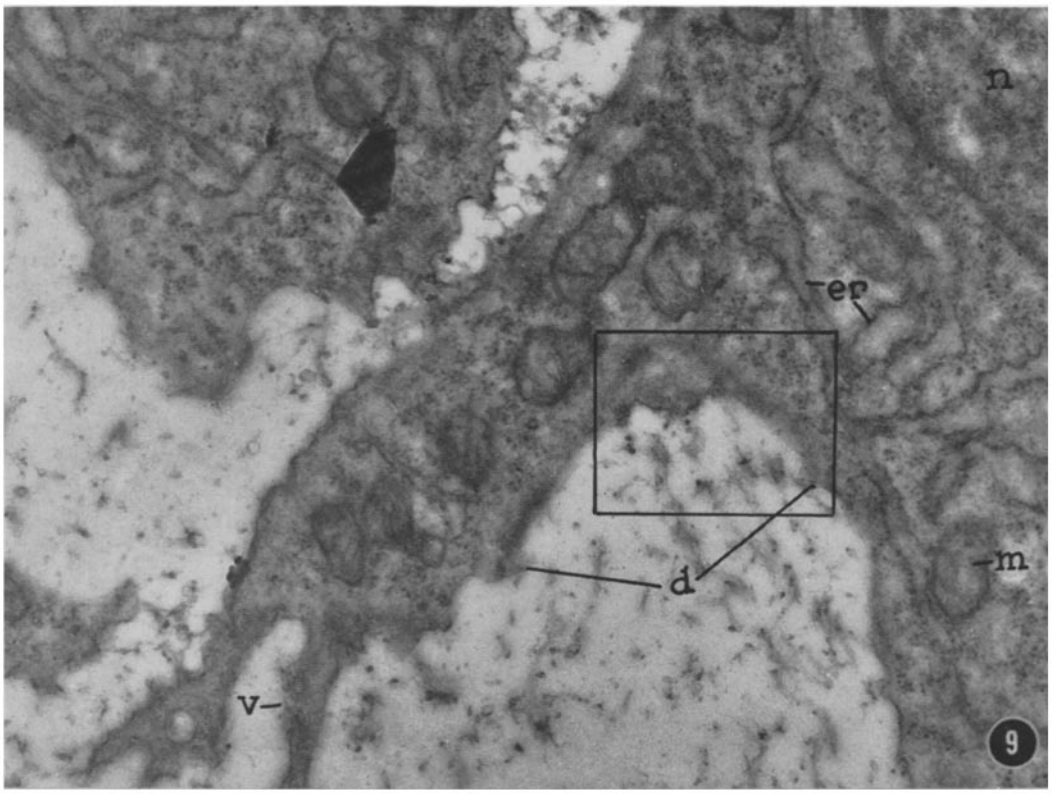
Detail comprising inset of Fig. 8. The dense areas appear to show some oriented structure of asymmetric or fibrillar elements arranged in roughly parallel array. PTA stain; × 68,000.

FIGURE 11

Detail of cell surface of a chondroblast showing a cortical density. The plasma membrane can be followed as a distinct line, (*pl*). Dense material in some places fibrillar appears to overlie the surface and to underlay the membrane as though in the cortical ectoplasm. See text for discussion of topography. × 74,000.

FIGURES 12*a* and 12*b*

Detail of matrix fibrils of about 180 A, to show periodic striation. The average interband period is 80 A. PTA stain; (*a*) × 47,000, (*b*) × 108,000.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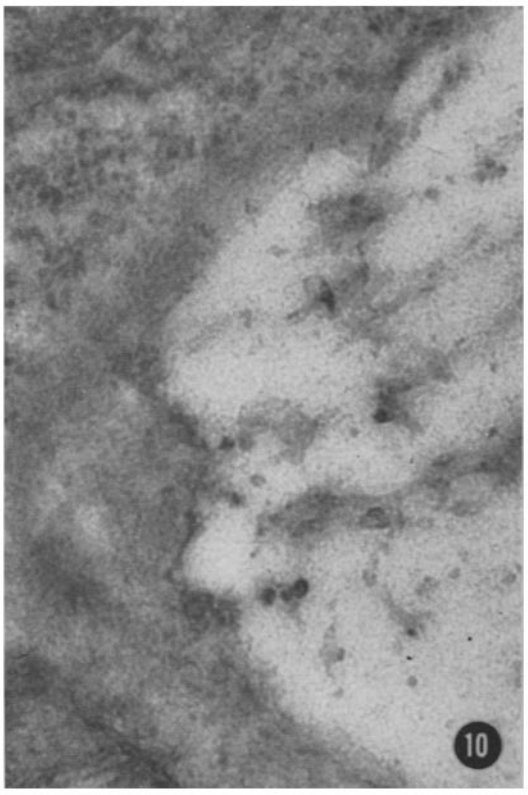
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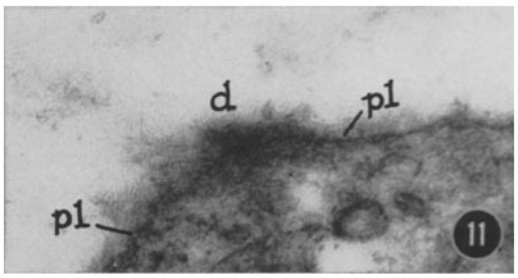
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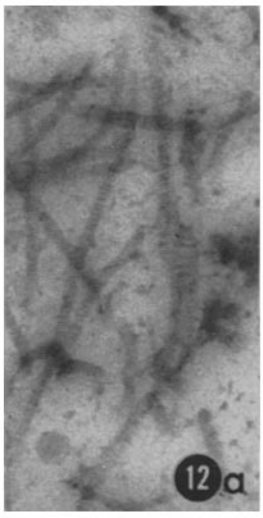
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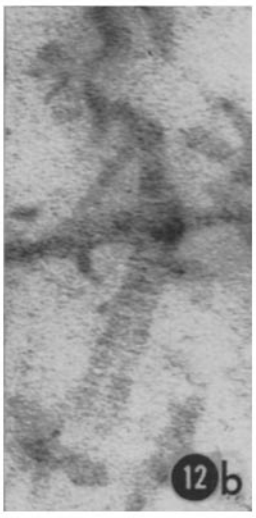
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bundles appear to be continuous with matrix fibrils (Figs. 17 and 18), it seems possible that sheaves of "primary" fibrils constituting the cortical densities might be detached, sloughed or shucked off from them, and that these become the original elements of new matrix fibrils. The apparent separation of cortical fibril bundles (densities) from the endoplasm by subjacent "rarefactions" or fused vacuoles, and the subsequent communication with the exterior (as it were by bursting) of these "rarefactions" also suggests that parts of the cortical densities overlying the "rarefactions" may be lifted off in the process, folded back, and partly detached from the cell.

The primary fibrils in the area of the cortical ectoplasm which are thus supposedly shed from the cell into the matrix do not exceed 100 A in thickness. Fibrils are almost always still in parallel bundles, heaps, or brushes of rather short lengths when they lodge at the cell surface, and mostly remain in sheaves, albeit looser, when they are seen further in the depths of the young matrix. It has already been noted that in the matrix the interstices and surroundings of the bundle are of a denser material than the ambient ground substance. The modal diameter of the fibrils in the early matrix is 120 A, with a range from 100 to 160 A. As the matrix matures, as in older embryos, or in the areas furthest removed from the cells and in those closer to the epiphyseal plate, fibril thickness and length increase; the diameter ranges between 180 to 300 A in such locations, and in embryos of 16 days' gestation (Fig. 6). In the hyaline cartilage of 18-day embryo to newborn the range of thickness is between 180 and 500 A, and the average length of fibril traceable in section is greater than in younger matrix. There is then a 3- to 5-fold increase in mean fibril size during maturation. If, as the evidence suggests, it

FIGURE 13

Chondroblast surfaces, exhibiting cortical densities (*d*). In the upper cell, the cell membrane (*pl*) can be traced as a definite line. The section is oriented normal to the cell surface. Underlying is a probably parasagittal section through a density in the cortical ectoplasm in the form of a stria, ridge, or band. A paralleled ordering of linear elements appears to compose the poorly delimited dense area. A subjacent focus of reduced density in the hyaloplasm ("rarefaction") is shown. × 50,000.

FIGURE 14

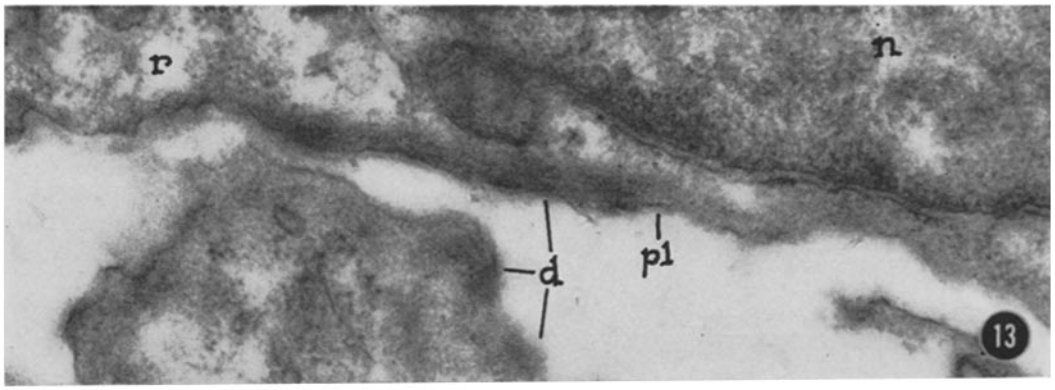
Chondroblast surface, thin section. Close inspection of the dense bands in the cortical ectoplasm reveals that they consist of arrays of linear filaments (*f*) (primary fibrils) which measure from 60 to 100 A; in some of these (arrow) a periodic alternation with an average interval of 80 A is discernible in the original. The cell membrane is sectioned mostly normal to its surface and appears as a definite line, (*pl*). Subcortical "rarefactions" are marked *r*. PTA stain; × 52,000.

FIGURE 15

Tangential section of a chondroblast near a surface illustrates that the cortical density (*d*) is in the form of a stria or ridge composed of a close bundle of fine filaments in parallel array. Dilated cisternae (*dl*) are prominent. Other parts are indicated as before. PTA stain; × 25,000.

FIGURE 16

Chondroblast, surface, thin section detail. A closely packed sheaf of fine filaments (primary fibrils), each of which measures 80 to 90 A, is shown at *f*. Together they would constitute a cortical or surface density as seen in thicker sections. They appear to lie both within and without the cell, as though extending across the definitive line of the normally sectioned cell membrane (*pl*). Some possible interpretations are given in text. PTA stain; × 50,000.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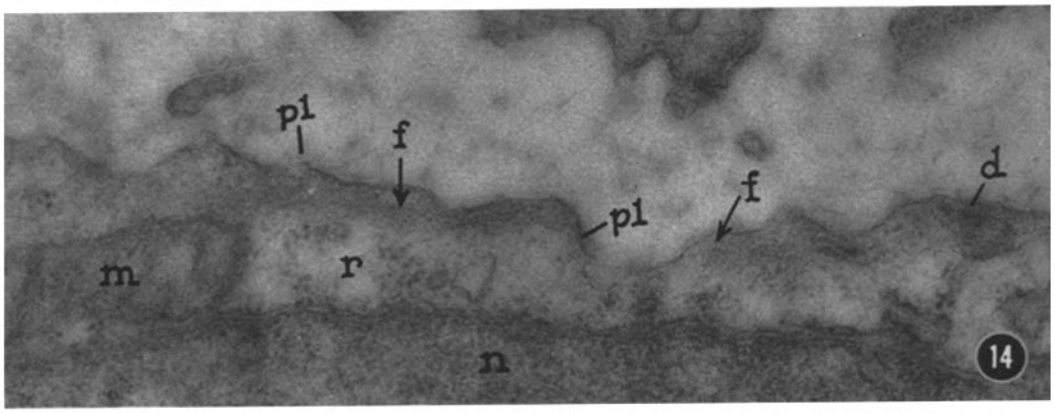
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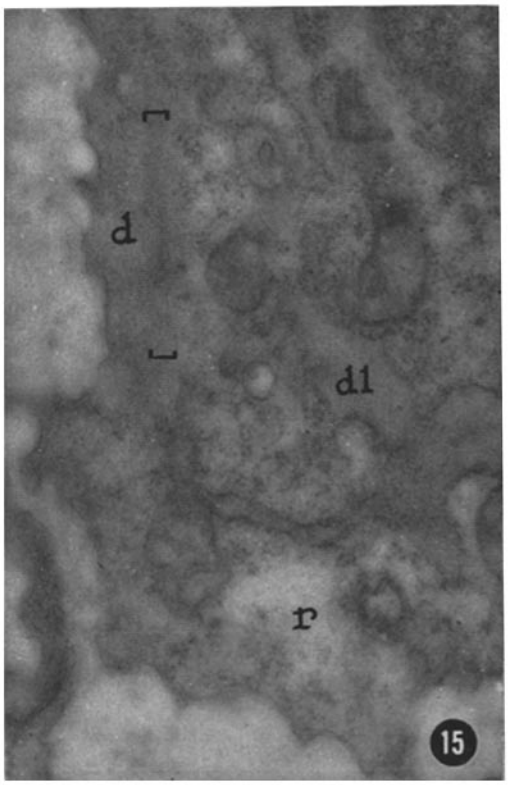
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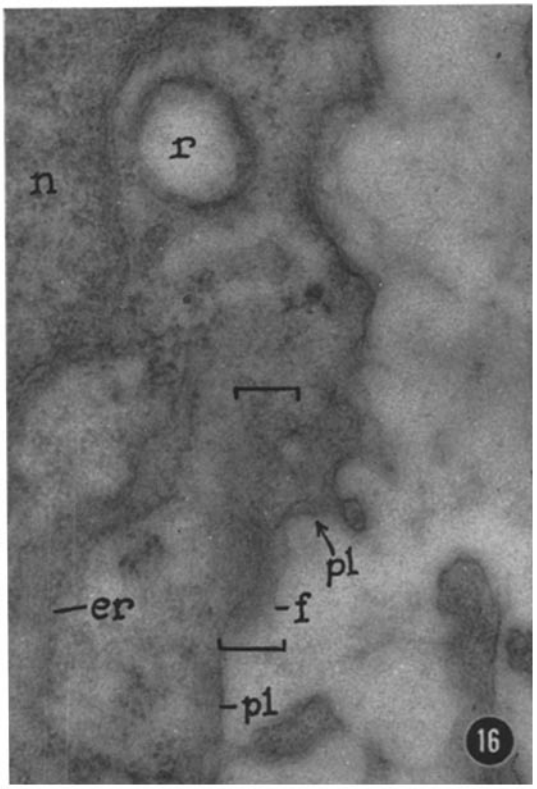
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is supposed that the primary fibrils are continually shed at the cell surface, then it is obvious that further growth, both diametric and linear, takes place during their residence in the matrix. Such growth can occur only by the accretion upon or to the 100 A primary fibril of a like substance derived from a soluble precursor in the ground substance.

An alternative interpretation of the electron micrographs which must be considered is the possibility that the fibrillar densities, apparently ectoplasmic or cortical, are actually bundles of extracellular or surface-adherent fibrils which, at various angles of section, oblique or tangential to the cell surface, are projected onto the area of the ectoplasm. Wassermann (38) has dealt in detail with some of the geometrical problems of determining in electron micrographs of thin sections whether fibrils are on the surface or in the cytoplasm of cells. Clearly, unless very thin (50 mμ or less) sections are employed, and unless the cell is cut somewhere normal to its surface (thus depicting the plasma membrane, if one is there), such topological distinction may be hazardous. Figs. 13, 14, and 20 illustrate cortical densities apparently lying well embedded in the ectoplasm beneath an intact plasma membrane which has seemingly been sectioned perpendicular to the cell surface. The only situation in which fibers actually lying on the cell surface may appear in the photographic projection to lie within the ectoplasm beneath the cell membrane, seemingly sectioned normal to the surface, is illustrated in Fig. 22. The membrane must be represented as a single discrete line of not more than 140 to 150 A. This interpretation of Figs. 13 to 16, and 20 requires the assumption that sharp, steep, straight declivities of the cell's surface, such as would be visualized on end as a line, do occur often along considerable stretches of the chondroblast surface. It would also demand that the membrane of the perpendicular edge be free of attached fibrils. As a corollary to this hypothesis the membrane line, having less depth, would appear a

FIGURE 17

Surfaces of two chondroblasts, detail. Two densities somewhat tangentially sectioned at each cell surface, and apparently situated in the cortical ectoplasm are shown at *d*. One is in direct continuity with a tassel of fibrils (*f*) extending into the matrix. At the arrow, an infundibular connection is shown between a dilated channel of the endoplasmic reticulum (*er*) and the perinuclear space. Nuclear membrane is indicated at *nm*. × 49,000.

FIGURE 18

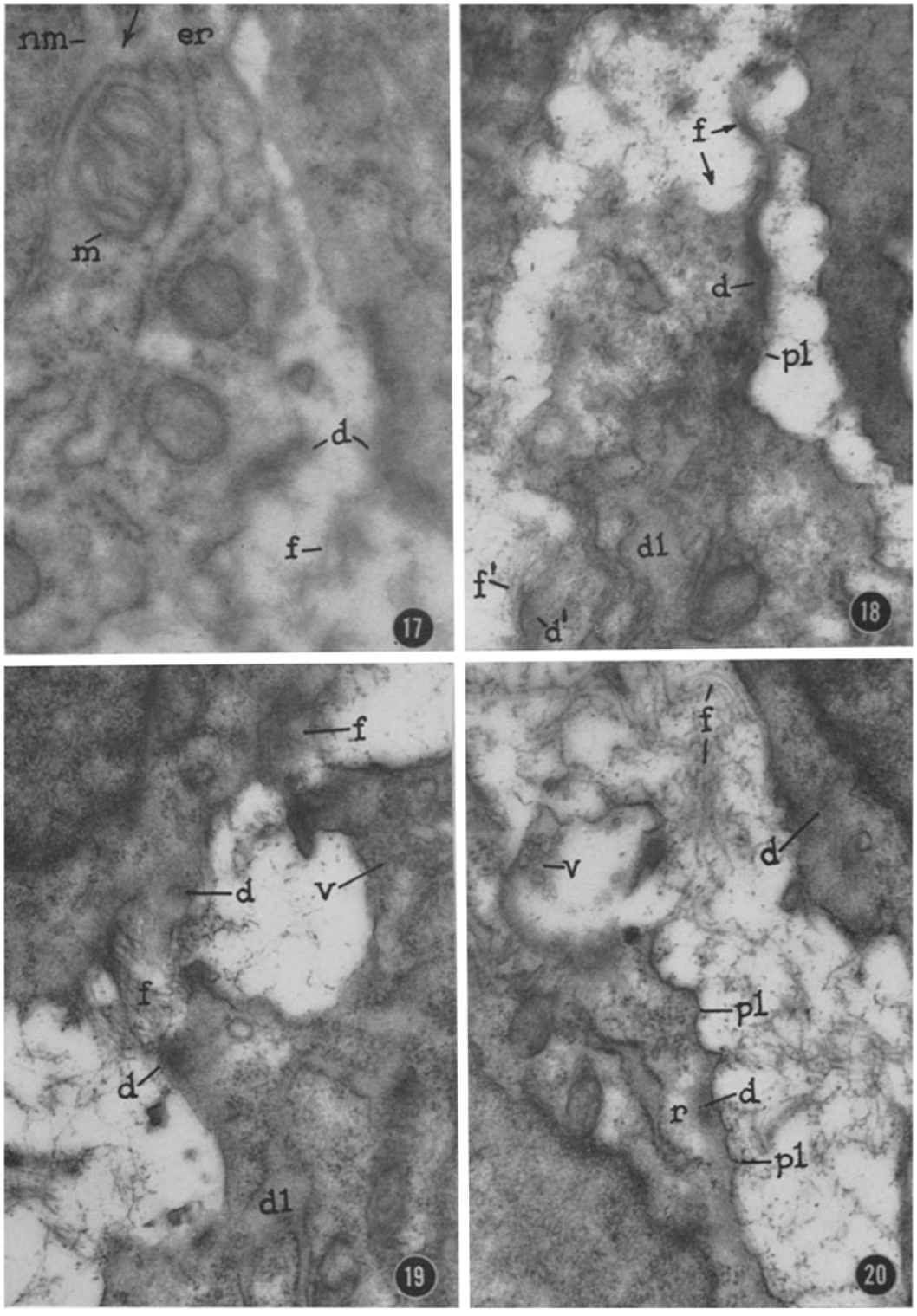
Chondroblast process, detail. The cell membrane is shown at *pl* as a distinct line. Underlying is a dense area (*d*), apparently in the cortex, consisting of fine fibrils which are continuous with the tassel of fibrils extending into the matrix. The tip of the cell (at arrow) has the form of a previously ruptured bleb. Dilated cisternae of the endoplasmic reticulum with their dense contents are at *dl*. Another density (*d'*) with an overlying sheaf of fibrils (*f'*) is shown at the lower left. Lead hydroxide stain; × 32,000.

FIGURE 19

Chondroblast surfaces, detail. Close adherence of tufts of fibrils to the cell surface is shown at *f*. Cortical densities (*d*), some small vesicles (*v*) and dilated cisternae (*dl*) are also indicated. Lead hydroxide stain; × 30,000.

FIGURE 20

Chondroblast surfaces. A large vacuole containing smaller vesicles (*v*) appears to have formed a surface bleb on the cell. Cortical dense areas (*d*) are shown underlying the distinct line of the cell membrane (*pl*). Subjacent poorly delimited areas of hyaloplasm of reduced electron scattering capacity ("rarefactions") are shown at (*r*). Some sheaves of fibrils (*f*) in roughly parallel order are shown in the cartilage matrix. Lead hydroxide stain; × 30,000.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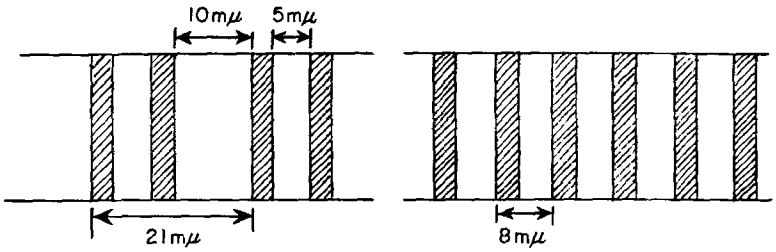


FIGURE 21

Diagram of the two patterns of spacing of cross-bands observed in the fibrils of hyaline cartilage after treatment of sections with 2 per cent phosphotungstic acid.

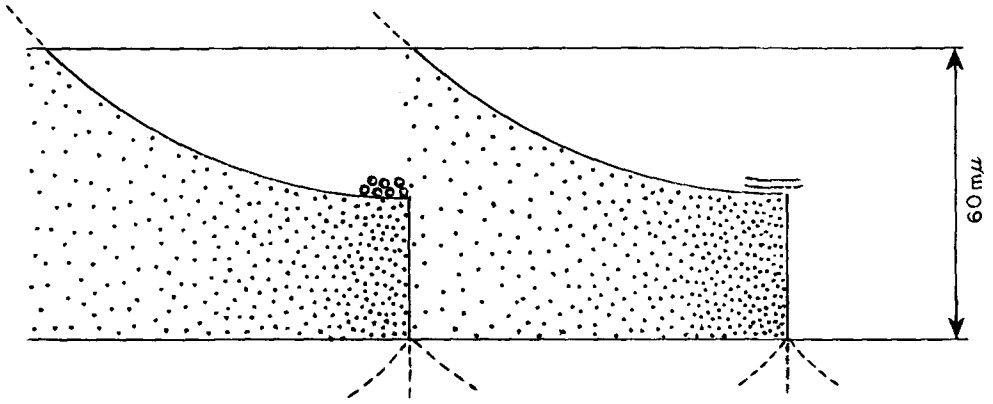


FIGURE 22

Schematic cross-sectional representation of a circumstance in which extracellular fibrils actually upon the cell membrane, may seem in projection (*i.e.* when viewed from above) to lie in the ectoplasm beneath the plasma membrane. See text.

less dense in such areas. While this interpretation can neither be defended nor excluded by the study of single micrographs, it remains unlikely that formations like those illustrated in Fig. 22 would occur fortuitously with the regularity and frequency of the cortical densities, nor would cross-sections of densities be seen so often. The plasma membrane lines in question are not noticeably less dense. Moreover, the primary or unit fibrils which make up the cortical densities are often about half as wide as those usually found on the cell surface. For these reasons, the intracytoplasmic localization of the cortical densities in the ectoplasm is thought to be more probable. Sometimes, however, in sections normal to the surface of the cell, when the cell membrane can be followed as a continuous single line, the individual primary fibrils of a bundle, apparently lying in the cortical ectoplasm, can be traced uninterrupted across the membrane, protruding into the intercellular matrix (Fig. 16). The interpretation of such images is difficult but they might possibly be explained by a formation like that of Fig. 22.

Intracellular Stores and Visible Contributions of the Cells to the Ground Substance

The chondroblasts have been seen to contain stores or pools of substances of different appearance enclosed in the cytoplasm These are all ultimately discharged to the exterior where they become a part of the mixture composing the amorphous ground substance.

The most striking inclusions of the active chondroblasts are the regular spherical vacuolar pools bounded by distinct doubled membranes and containing very finely fibrillar or "amorphous" matrices and, usually, dense granules (Figs. 24 to 29, *l*). That these vacuoles may some-

times grow to the enormous size of several microns is shown in Figs. 26 to 28. In Fig. 28 it can also be observed that after the pool membrane establishes contact with the cell membrane a stoma may form by the disappearance of a sector of the junction area, thus establishing direct contact between the lumen of the vacuole and the cartilage matrix. At the margins of the stoma (Fig. 28, *st*) the membranes of the vacuole and the cell are continuous. An egress is thus provided for the mixed contents of the vacuole. More frequently, such remarkably expanded and turgid vacuoles burgeon out as convex blebs on the cell surface (Figs. 20, 26, 27). Limiting their inner aspect and separating their contents from the cytoplasm, is the double membrane; externally, on the extracellular side, this membrane fuses to form a single line, which in most instances is seen as broken or interrupted. In these instances a stoma may not form, but the contents of the bleb are probably released through attenuation and rupture of the external or convex limiting membrane; the concave membrane applied to the cell would, of course, become cell membrane. The chemical nature of the amorphous and granular contents of these vacuoles will be the subject of another communication, but morphologically the double membranes obviously enclose a mixture of components, some of which are cytoplasmic particles or organelles such as mitochondria (Fig. 27) or vesicles (Figs. 20, 28) which have become entrapped. The granules, when present, are in the same range of size and density as the ribonucleoprotein particles associated with the endoplasmic reticulum. The mitochondria entrapped in these expanding pools, as shown in Fig. 27, are expelled with the other contents of the bleb into the matrix, where they may appear for a time as shriveled or collapsed structures.

The mode of origin of these large vacuoles and their doubled limiting membranes is not revealed in the electron micrographs, but any hypothesis must account for the frequent inclusion within them of formed cytoplasmic structures. The agranular vesicles derived from and associated with the Golgi apparatus differ from these large vacuoles in their smaller size, in being bounded by a single membrane line, and, when they do not appear empty, in having denser contents of quite different character. The supposed processes of eversion or of blebbing affecting the large vacuoles with doubled membranes occur only in chondroblasts.

Among the various contents enclosed within the doubled membranes are small (20 to 70 mμ) vesicles with smooth membranes represented by single lines, enveloping homogeneous contents of low to moderate density (Figs. 20, 28, 29). These too are expelled with the other materials. They may also be found in small rows or groups in the peripheral cytoplasm of active chondroblasts, and in some cells are piled into large staphyloid clusters at the surface of the cell from which, in the absence of a distinct overlying cell membrane, they are apparently released into the matrix (Fig. 32). Within the endoplasm, vesicles of these dimensions are of course most numerous in the zones of the Golgi apparatus, where they are members of a larger population of vacuoles of diverse size ranging from 20 to 200 or more mμ. In the chondroblast the larger of these vacuoles, which usually seem to be empty, do not as frequently attain the peripheral position of the small vesicles here described. It is possible that the small vesicles with contents of low density originate as a part of the Golgi complex. It may also be that they are budded more directly from the endoplasmic reticulum (see Fig. 29, *v*), whose contents resemble those of the vesicles in density and homogeneity. Channels of the endoplasmic reticulum are very rarely seen to abut directly on the cell surface; those which approach the surface are separated from the exterior by a membrane line. In any case such vesicles can be expelled intact from the cell.

The Golgi apparatus, as has been noted, undergoes a marked development as the chondroblast matures. Its large vesicles or vacuoles increase, seemingly at the expense of both smaller vesicles and stacks of membrane, and accumulate the materials of moderate to very high electron density which have been described. The denser contents may be the older, since they are more frequent in the more peripheral vesicles and in the more differentiated chondrocytes. The extremely dense contents of some of these vacuoles must be altered or diffused on discharge to the matrix, since comparably large and dense bodies are not found there.

It has been noted that through breakdown of contiguous membranes in the approximated vacuoles in the Golgi region, small pools of moderately dense material may arise in the chondrocyte cytoplasm through mingling of their

contents. These, like the great lakes of glycogen, must await the dissolution or sloughing of a part of the cytoplasm in which they lie to reach the exterior, a process which occurs especially in the late hypertrophied chondrocyte.

DISCUSSION

Electron microscopy of developing epiphyseal cartilage affords some new insights into the structure of cells during their growth, differentiation, and senescence, in the processes of formation, storage, and secretion of extracellular substances, and in the elaboration of fibers (Figs. 34–37).

The Cells

It is not surprising that the approximated cells of the precartilaginous mesenchyme should have been thought by some histologists (20) to be fused into a symplasm with a common cytoplasm, for even with the electron microscope definitive plasmalemmal lines cannot be traced for every cell in well fixed preparations. Since, however, at least incomplete borders are discernible in most cells, it is probable that Fell (17) was correct in assuming that each mesenchymal cell was structurally independent and that the syncytial appearance was artifactitious.

The simplified structure of the mesenchyme cell describes its primitive state. In these as in certain other undifferentiated cell types such as the early blastema (35), developing liver (39), gonial cells (33, 40), embryonic fibroblasts (41) the system of membrane-bounded channels which in maturer cells constitutes the endoplasmic reticulum and Golgi is represented only by small, dispersed, relatively unorganized, mostly smooth vesicles (Fig. 34). The most obvious characteristic of the cytoplasm of the mesenchymal cell, the emptiness or low electron density of its hyaloplasm is undoubtedly an extraction effect resulting from a higher water content than that of differentiated cells, and a lower concentration of constitutive or structural protein. The greater hydration of embryonic as compared with mature tissues is well known: in the case of the cartilage cells, it has been calculated that 83 per cent of the cell mass of articular cartilage of young puppies (before 16 weeks) is water (42). In embryonic cells this value must be considerably higher. Water content of the cellular part falls to 74 per cent in 17- to 25-week-old animals (42) and in adult animals chondrocyte water is 68 per cent (43). The presence of many free granules, presumably ribonucleoprotein, in the cytoplasm of these cells is characteristic also of other growing and dividing cell populations (33, 44), and is evidently associated with augmentation of endocellular protein. As structural differentiation proceeds beyond the prechondroblast stage, the number of free granules diminishes except during mitosis. In differentiated cells the small granules are membrane bound.

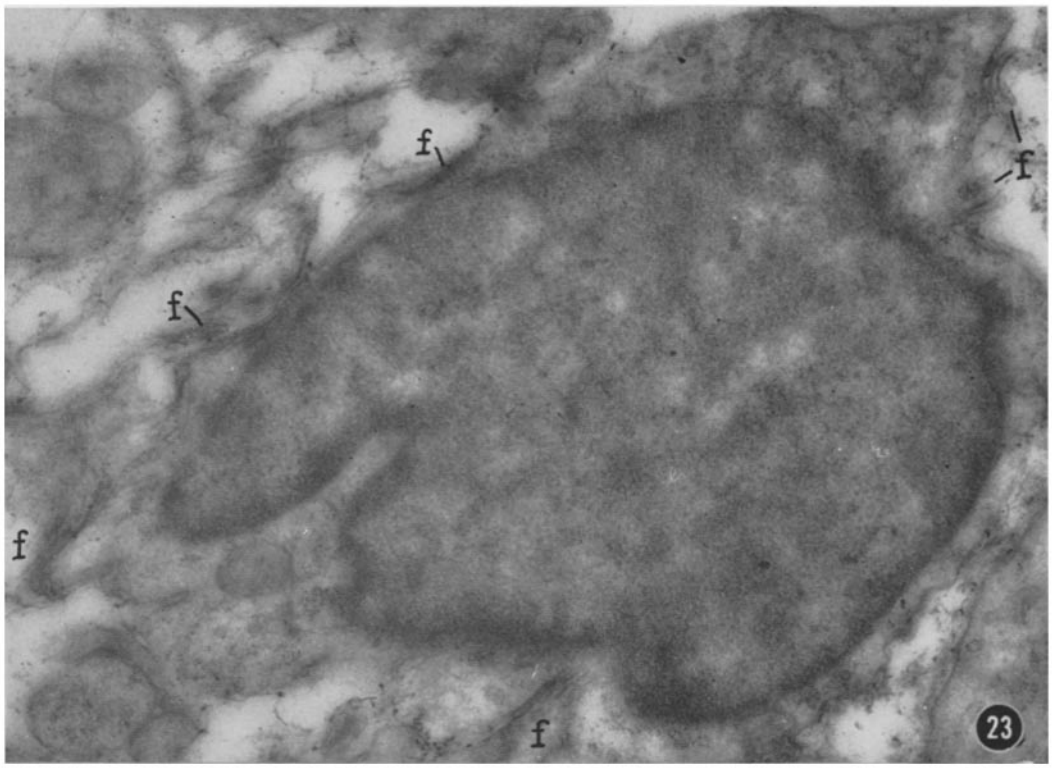
Differentiation, *i.e.* the assumption of specialized function as expressed in the formation of matrix, is first heralded in cells of this kind by structural modifications of both the cell's surface and cortex, and the vacuolar systems of the cytoplasm (Fig. 35). Organization of the latter may occur through the alignment of minute vesicles and their fusion into narrow elongate cisternal profiles which very soon become associated with granules to constitute the (primitive) endoplasmic reticulum. Adlineation and coalescence of small vesicles to form the membrane-bounded reticulum also appears to be the most probable morphogenesis of the latter in other differentiating cells (35, 40). Throughout the remainder of its career the endoplasmic reticulum of the differentiating chondroblast undergoes a marked development in size, amount, and organization (Fig. 36).

FIGURE 23

Perichondrial fibroblast or chondroblast. Parallel arrays of collagen fibrils (*f*) lie at the surface in intimate relation to the cell membrane in this relatively thick section. Approximately × 20,000.

FIGURE 24

Chondroblasts. The dispersed stacks of agranular membranes together with scattered swarms of vesicles and vacuoles comprise the Golgi apparatus (*Ga*) of these cells. Small pools of a relatively homogeneous material of low to moderate density, containing numerous dense granules and enclosed by a distinct double membrane are shown at *1* and *1'*. These approximate 0.4 and 0.8 μ in diameter respectively. Lead hydroxide stain, × 22,000.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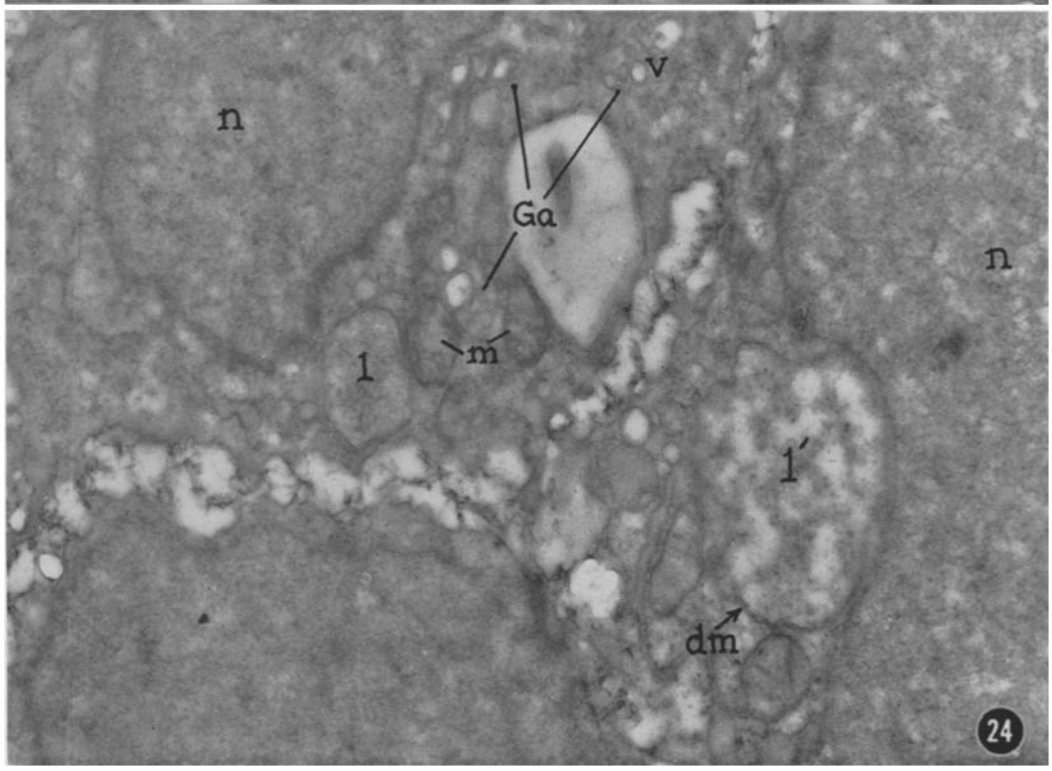
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The relationship between the granular endoplasmic reticulum provided with cisternae and the synthesis of protein for export is by now sufficiently established to make the identification of such ergastoplasmic structures an acceptable criterion for protein secretion by any cell (36, 37, 44–48). Indeed, the active differentiating chondroblast, in addition to the synthesis of constitutive cell protein and enzymes (such as those concerned with polysaccharide synthesis), presumably also produces for export collagen (in some form) and at least part of the protein of the chondromucoprotein, and probably neutral mucoprotein fractions. The continued elaboration of organized ergastoplasm in later stages, and its persistence in the late chondrocytes and hypertrophied chondrocytes would imply not only continued protein synthesis but also segregation and storage, since with the disintegration of hypertrophic chondrocytes, the dense contents of their dilated cisternae must be liberated. The manner in which the contents of cisternae are normally used or expelled is not readily apparent; direct openings to the exterior, if they occur at all, are infrequent, although reportedly these do sometimes occur in other cells (41, 49). The cavities and membranes of the granular endoplasmic reticulum are also known to be continuous with at least some of those of agranular or smooth reticulum which are part of the Golgi complex, the whole of this vacuolar system being dynamically interconnected (46, 50).

It is presumably *via* this other division of the membranous vacuolar system that the contents of the endoplasmic reticulum are dispersed to the outside. The Golgi complex also undergoes striking transformation in the course of cytodifferentiation. From its representation in the mesenchymal cells of the chondrous anlage as small, scattered, agranular vesicles (Fig. 34), the definitive Golgi apparatus first appears as grouped elements, small but of typical form in the early chondroblast (Fig. 35). In these cells the units consisting of lamellar stacks of closed membrane and associated vesicles are widely dispersed, but in more mature, probably more active chondroblasts, these have mostly gathered together as a structure of impressive dimensions, at first largely composed of membrane stacks. In its further history the great numbers of vesicles proliferated from the Golgi complex are devoted to encompassing, segregating, and transporting dense materials. The Golgi complex achieves its maximal development in the chondrocyte (Fig. 37), in which cell it has been a favorite object of study by cytologists beginning with classical papers of Pensa (51) and including those of Laguesse (52), Dubreuil (53), Parat and Godin (54), and Fell (17). Sheehan (55) is the most recent observer to

FIGURE 25

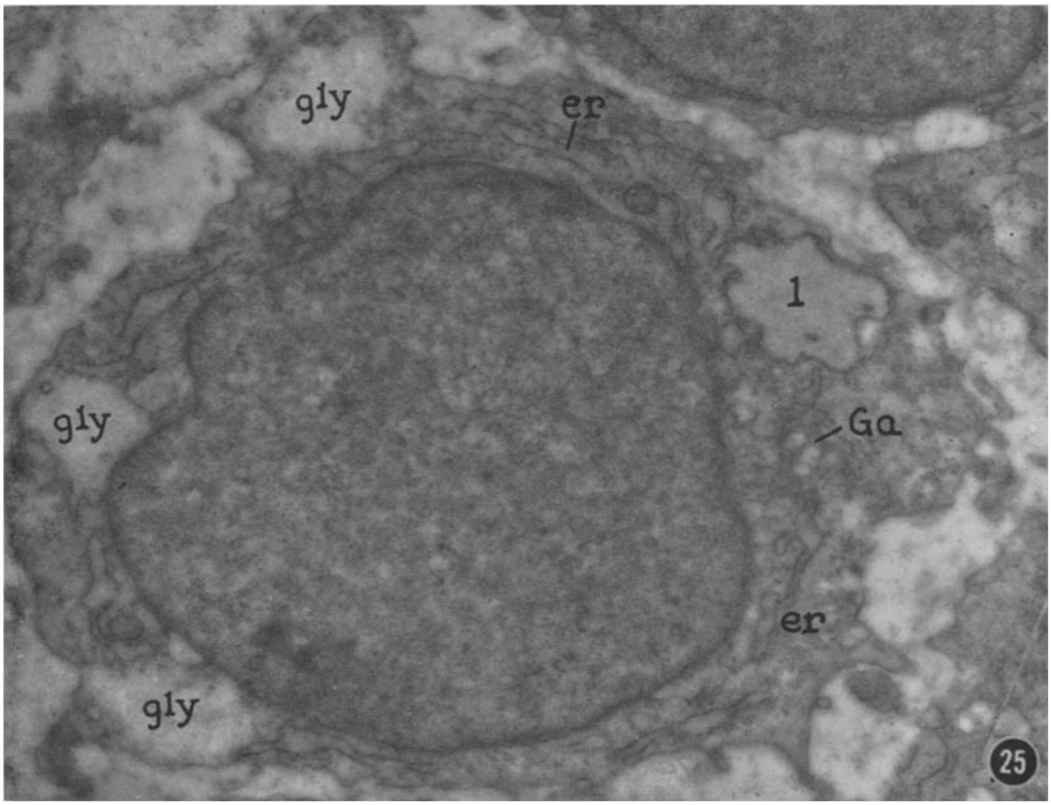
General view of chondroblast to show the disposition of organelles and cytoplasmic inclusions. Attention is directed especially to the glycogen deposits (*gly*) which have no distinct boundaries, to the concentrated Golgi apparatus (*Ga*) and to the pool of moderately dense material delimited by a doubled membrane (*l*). × 15,000.

FIGURE 26

Chondroblast. A large pool or lake with granular and homogeneous contents has formed a huge bleb at the cell surface, distending the cell membrane which appears to have ruptured at one section (arrow). At higher magnifications a double membrane can be seen to separate the contents of the pool from the cytoplasm (see Fig. 28). Lead hydroxide stain; × 8,000.

FIGURE 27

Chondroblast. A similar bleb is seen at the surface of this chondroblast. Its contents include a relatively homogeneous material of low to moderate density, some dense granules, and two intact mitochondria which have evidently become entrapped. The membrane (*dm*) which separates the contents of the pool from the cytoplasm is doubled. The stretched external membrane is seemingly broken at several points. Lead hydroxide stain, × 19,000.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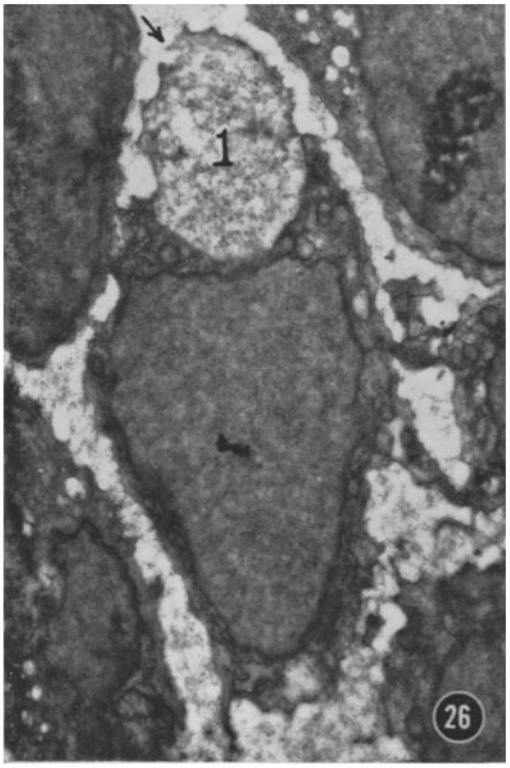
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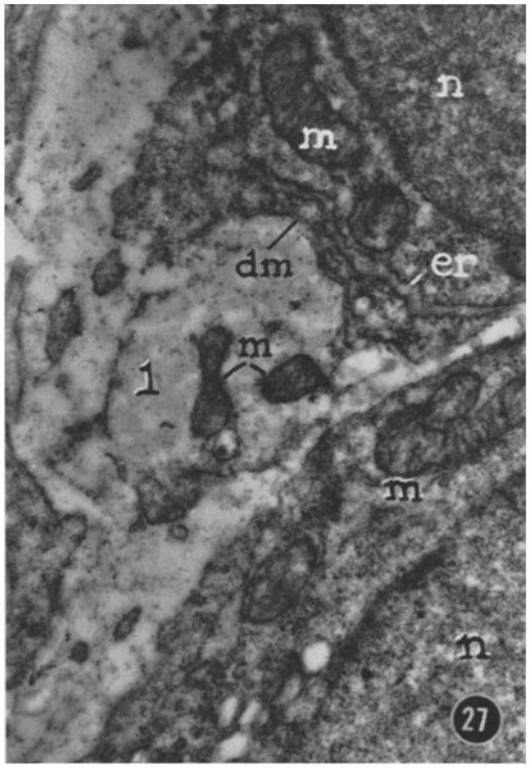
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have described the Golgi apparatus of developing chondrocytes from impregnated and from supravitally stained preparations. The so called neutral red segregation vacuoles or vacuome were regarded by Sheehan (55) as bodies formed *de novo*, in contrast to Parat and Godin (54) who would derive the network appearance from artifactual union of vacuoles which, in most preparations, supravitally dyed, occurred as rings and crescents. All these observers pictured a definitive compact grouping of bodies in the juxtanuclear region whose structures varied with the methods employed to reveal them, but which can be interpreted retrospectively with the information afforded by the electron microscope (50, 56–58). The outer osmiophilic nets or caps presumably represent metallic deposits on and between adjacent vacuolar or cisternal membranes, and the central osmiophobic areas, probably the vacuolar contents.

Collections of vacuoles as visualized with the electron microscope were described in chondrocytes by Scott and Pease (24). Although Zelander (30) also saw such juxtanuclear vacuoles in electron micrographs of chondrocytes he denied that they were Golgi components because he saw no flattened membranes. The integral relationship of these vacuoles with the more typical formations of the Golgi complex can be traced in a continuous series in the present study (see Fig. 31) and hardly seems questionable.

The mitochondria undergo a noteworthy increase in size (Figs. 34–36), which bespeaks an increased oxidative activity (59, 60). From histochemical examination it is known that chondrocytes possess cytochrome oxidase activity and several dehydrogenase systems (28, 61).

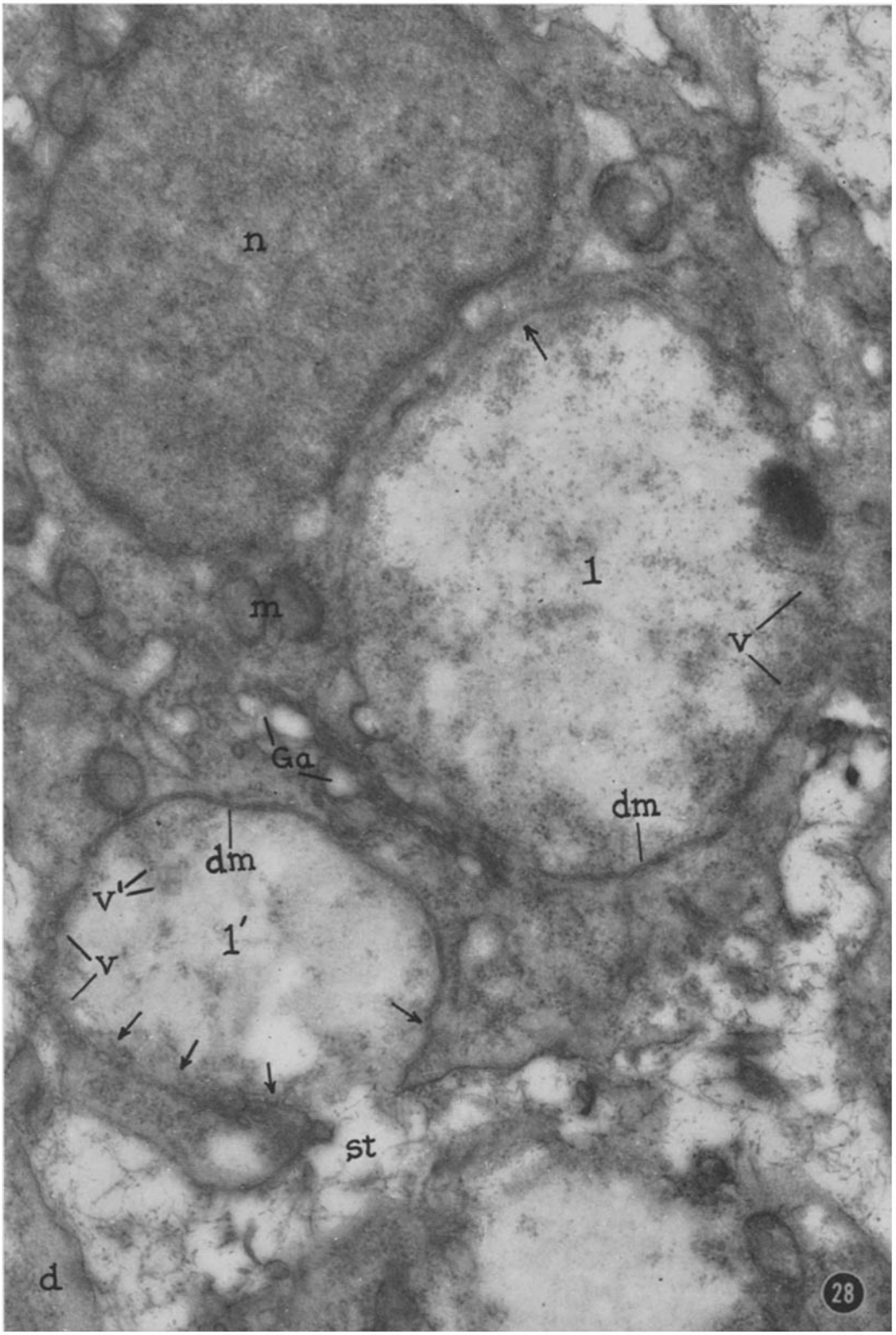
The progressive increase in density of most of the hyaloplasm in the course of chondroblastic differentiation is an arresting feature. In the chondrocyte the compression of the ground cytoplasm between the cisternae of ergastoplasm and Golgi apparatus and the various inclusions could conceivably contribute somewhat to its density, but like the similar changes occurring simultaneously in the nucleus, must chiefly reflect marked changes in composition, probably entailing dehydration and protein concentration. Cytochemical studies which include interferometric measurements should provide accurate and quantitative information. Most of the electron micrographs of cells of the cartilage hitherto published (24–30) have been of mature chondrocytes and hypertrophied chondrocytes. These cells at the terminally differentiated stage, exhibit structural features characteristic of the late secretory function and probably diminished synthesizing capacities. Moreover, their density makes it more difficult to observe intracellular detail. For the morphological correlations of active cell growth, and formation and secretion of products, study of earlier stages and the sequence of development have proven most useful.

Intracellular lipid droplets, conspicuous in some types of chondrocytes (20, 62–64), were only occasionally observed in young chondroblasts and some chondrocytes, more rarely in mature chondroblasts, and did not regularly accompany chondrocyte hypertrophy or degeneration. More novel inclusions in the cytoplasm are those reported in the chondrocytes of rabbit ear cartilage, by Sheldon and Robinson (62) as a feltwork of dense material, believed to be elastin or precursor. Such material would appear to be peculiar to elastic cartilages, and was not observed in cells or matrices of the material of this study, nor reported by others studying hyaline cartilage (24–30).

The pocked and excavated surface of the chondroblast testifies to participation of the plasma membrane and cortical ectoplasm in the elabora-

FIGURE 28

Chondroblast. The pools (*l*) bounded by double membranes (*dm*) have grown to very large dimensions in this chondroblast. At the section marked *V* the definitive double lines of the membrane are replaced by clusters of vesicles. In other sectors (arrows) the wall appears to consist of chains of approximated flattened vesicles. The contents of the pool at lower left (*l'*) are in communication with the extracellular (or matrix) space through a large stoma (*st*) probably formed by fusion and focal retraction of cellular and vacuolar membranes. The pools contain homogeneous material of low to moderate density, dense granules and a few vesicles (*v'*). The Golgi apparatus is shown at *Ga*. Uranyl acetate; × 29,000.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tion or discharge of materials to the exterior, through blebbing and bursting of vacuoles and through the shedding of new fibrils (Figs. 36–38). The pits or bays are especially prominent in the chondrocyte after the shrinkage due to preparative procedures, and have drawn the attention of light microscopists since Virchow (2); they are well displayed in the electron micrographs of Scott and Pease (24), Follis and Tousimis (27, 28), and Zelander (30) *inter alia*.

The Matrix

The extracellular matrix of hyaline cartilage, the connective tissue under longest chemical scrutiny, is composed of both formed and amorphous protein and polysaccharide materials. The presence of collagen in mature cartilage was signalized long ago by Schwann, who noted that gelatin is extracted after boiling. Indeed, about 16 to 18 per cent of the dry weight of normal rat epiphyseal cartilage is collagen (65, 66) of which some fraction is probably soluble in neutral salt solutions ("tropocollagen"). The acidic mucopolysaccharide, chrondroitin sulfate A (and a minor fraction of chondroitin sulfate C), makes up about from 15 to 20 per cent of the dry weight of hyaline cartilage (67, 68), at least a third of which is present as a non-collagenous, soluble chondromucoprotein (68, 69). It has been postulated that aggregates of this mucoprotein (69), and at least some of the chondroitin sulfate present in other forms are linked in some manner to collagen (68, 69). An additional acid mucopolysaccharide, keratosulfate, has recently been discovered in human hyaline cartilage by Meyer (67). These sulfated compounds are, of course, the chromotropes responsible for the metachromasia of cartilage. The neutral mucopolysaccharides of cartilage constitute an important, insufficiently characterized fraction of its ground substance, in which they are present in combination with protein, possibly collagen (70). Their content of neutral sugars (mannose, fucose, galactose, glucose) and the galactose of keratosulfate, yielding vicinal aldehydes on periodic acid oxidation, is responsible for the coloration of cartilage with the PAS method (71).

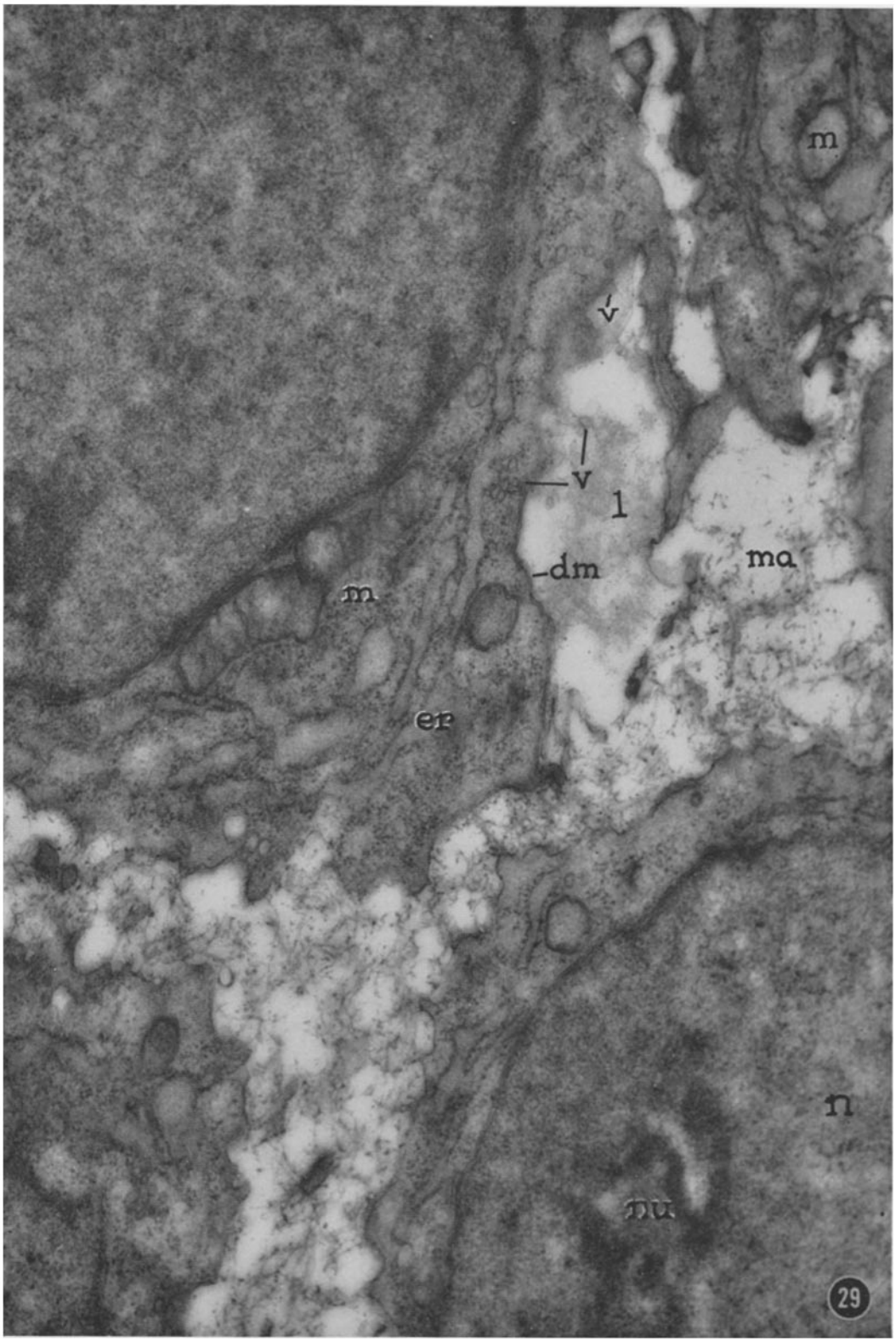
Rat epiphyseal cartilage has a total water content as high as 87 per cent according to Follis (28), and its mineral ash, some of which is intracellular, is reported as high as 25 per cent of the total dry weight (28).

The organization of these materials and their structural interrelations remain the object of continuing investigations. The fibrillar arrangement of hyaline cartilage has been known since the last century, and is demonstrable in the light microscope with polarized light, special staining or silver impregnation methods, or maceration procedures (72, 73).

Meshes of fine fibrils were recognized in shadowed or disintegrated specimens in first electron microscopic observations of hyaline cartilage matrix (23, 74) and were subsequently described in various species. In thin sections, the fibril of hyaline cartilage reportedly ranges in thickness from 100 to 250 A in human newborn epiphysis (25), about 100 A in kitten epiphysis (24) and amblystoma limb (35), 60 to 200 A in rat (27), and 85 to 550 A in mouse (30). None of these authors was able to discover any clear transverse banding of the small fibrils. However, Follis and Tousimis (65) in some fibrils of 180 to 220 A obtained from homogenized and dried preparations of rat hyaline cartilage, found an indication of periods of 100 to 135 A and of 180 to 200 A. In developing and in more mature cartilage examined in the present study, fibril width varied with age, and consequently therefore in more developed specimens, with distance from the chondroblast. Fibrils of all sizes exhibited periodicity but only in some fibrils could banding be seen. The fact that these striations were brought

FIGURE 29

Parts of three chondroblasts. The most conspicuous feature of the chondroblast at the upper left, is the large lake (*l*) which appears as a partly collapsed cyst. Its external limiting membrane, seen as a single line, is convoluted. Its contents are separated from the cell cytoplasm over at least a part of its perimeter by a double membrane (*dm*). The boundary zone of the remaining sector is represented by a band of increased density containing many vesicles (*v*), some of which are also present in the lumen of the partially empty cyst. Large mitochondria (*m*) and prominent channels of the endoplasmic reticulum (*er*) with moderately dense contents are present in the cytoplasm, as are groups of vesicles (*v*). Lead hydroxide stain, × 31,000.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to visibility best after exposure of sections to solutions which both extract and stain, suggests that failure to discover them is due to masking or coating of the fibrils by amorphous soluble substances. This explanation has also been invoked by van den Hooff (75) and Dische *et al.* (70) to explain the apparent aperiodicity of the thin fibrils in all mucoid-rich connective tissues, and might explain the occurrence of banding in some washed isolated preparations (65). There can be little doubt that the fine fibrils of cartilage are a form of collagen possessing unusual periodicity.

No consistent orientation of fibril heaps with reference to the cells was discerned. It is understandable that a more or less random distribution of bunches of relatively short thin fibrils throughout a gelated matrix would constitute a resilient and shock absorbing tissue, to which the bundles of more typical collagen in the perichondrial zone must add tensile strength. The afibrillar pericellular moats, chiefly of the hypertrophic zone, are not artifacts or shrinkage spaces, as has sometimes been claimed, but are rather areas more or less entirely occupied by ground substance.

The amorphous ground substance, represented in the electron micrographs by clouds of moderate density, are in close relationship to the fibril bundles, which they interpenetrate and about which they are concentrated. While proof of the composition of these floccules of ground substance must await utilization of more refined methods, it is reasonable to assume, tentatively, that the chondromucoprotein, neutral mucopolysaccharide-protein complexes, chondroitin sulfate and keratosulfate, and soluble collagen may reside there.

This ground substance changes with maturation, becoming progressively denser and increasing in amount. Its importance in maintenance of the tissue turgor is well illustrated by the collapse of cartilages in the rabbit as a result of the action of a protease (76), presumably upon its chrondromucoprotein (77).

Development and maturation of cartilage matrix entail both quantitative and qualitative changes in composition. Water content falls as solids (fibrils, ground substance) and minerals accumulate (78, 79). The difference in collagen content between procartilage and hyaline cartilage was signalized by Schwann over a century ago, when he could not obtain gelatin by boiling very young cartilage. The ground substance of pro-

FIGURE 30

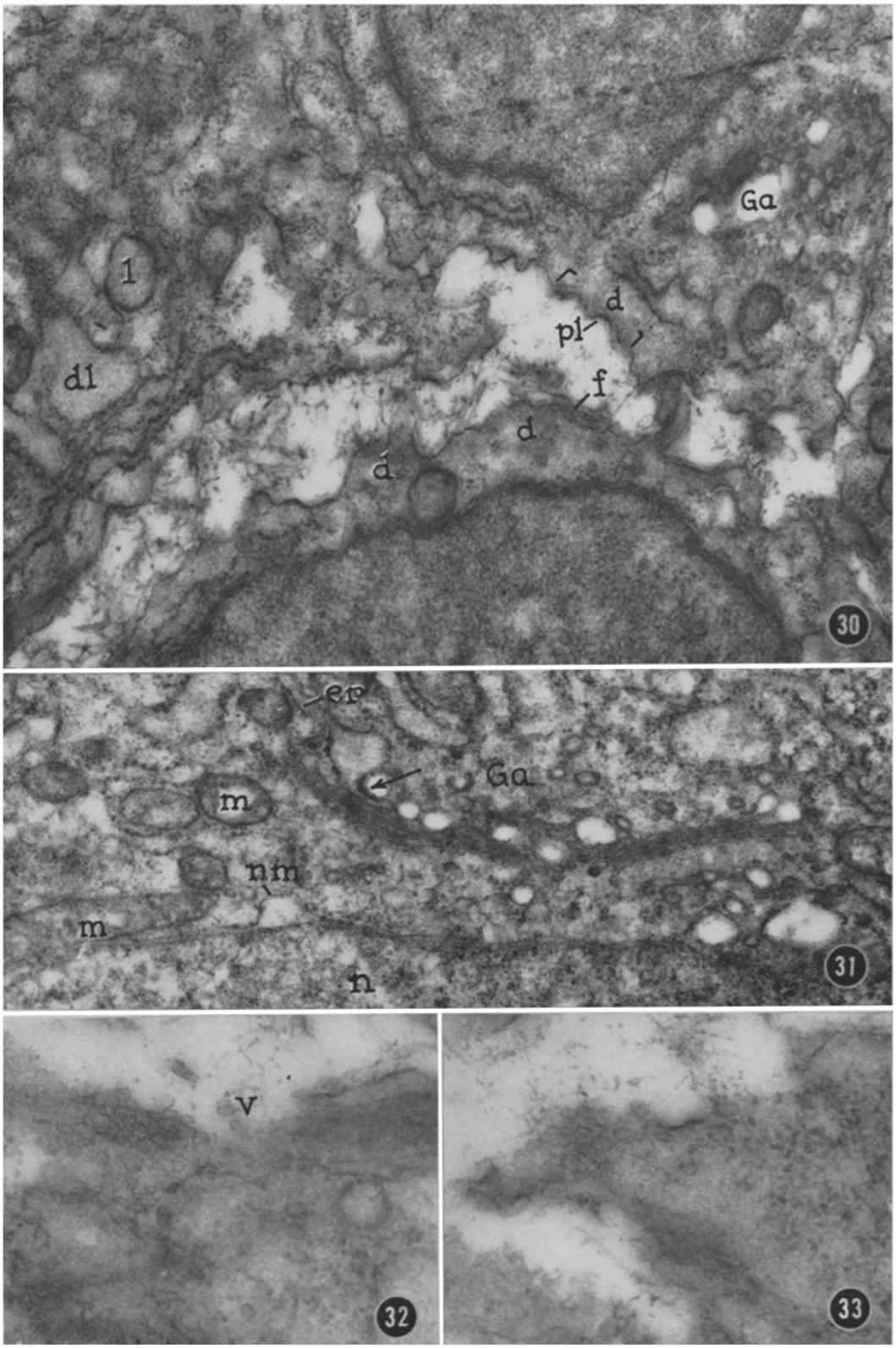
Sectors of three chondroblasts. The extensively developed Golgi apparatus consisting of stacks of smooth, flattened cisternae and swarms of vesicles, concentrated in a juxtanuclear area is shown at *Ga*. Dense areas or bands of the cortical ectoplasm apparently subjacent to the cell membrane are marked *d*. A sheaf of parallel fibers at the cell surface is shown at *f*. Dilated cisternae of the endoplasmic reticulum are at *dl*, and a small (0.32 μ) pool limited by a doubled membrane is at *l*. × 30,000.

FIGURE 31

A detailed view of the Golgi apparatus (*Ga*) of a chondroblast, probably before a phase of maximal activity, is shown in a zone near the nucleus (*n*). Approximated smooth membranes of the flattened cisternae, lying in stacks of 5 or 6, are the predominant component, about which many vesicles and vacuoles are clustered. At the *arrow*, a cisterna of the endoplasmic reticulum (*er*), encrusted with dense particles, appears in direct continuity with a flattened, smooth walled cisterna of the Golgi apparatus. An outpocketing of the external granular membrane (*nm*) of the nuclear envelope reveals the perinuclear space. Lead hydroxide. × 30,000.

FIGURES 32 and 33

Clusters of vesicles are shown at the chondroblast surface. These have moderately dense contents, enclosed by "single" membranes. They range from 30 to 100 mμ in size. Some small vesicles (*v*) lie in the extracellular space. × 48,000.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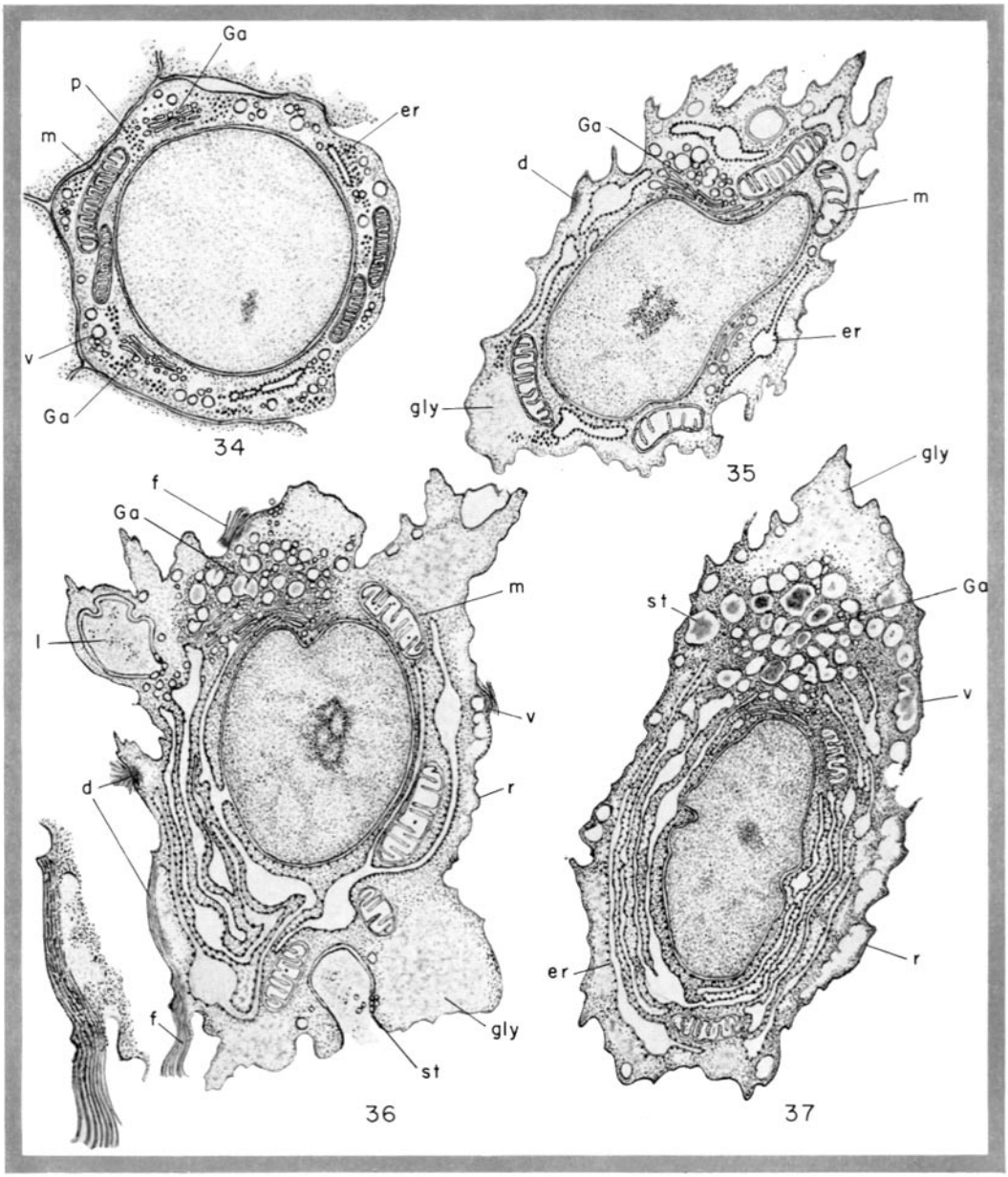

A schematic representation of the successive stages in the differentiation of the chondroblast from mesenchymal cell to chondrocyte, illustrating the development of organelles involved in the secretion of cartilage matrix, is shown in Figs. 34 to 37.

FIGURE 34

The cell of the prechondroblastic mesenchyme (prechondroblast), exhibits features typical of other undifferentiated mesenchymal cells. It has a relatively high nucleocytoplasmic ratio; the cytoplasmic hyaloplasm, which is of low density, contains congeries of small smooth walled vesicles (*v*) and scattered clusters of apparently free ribonucleoprotein granules (*p*). The mitochondria (*m*) are small, compact, and dense. Only occasional, very small channels of the endoplasmic reticulum (*er*) may be discerned, and the Golgi areas (*Ga*) are represented only by several scattered, very small groups of flattened cisterns with smooth membranes, and minute vesicles.

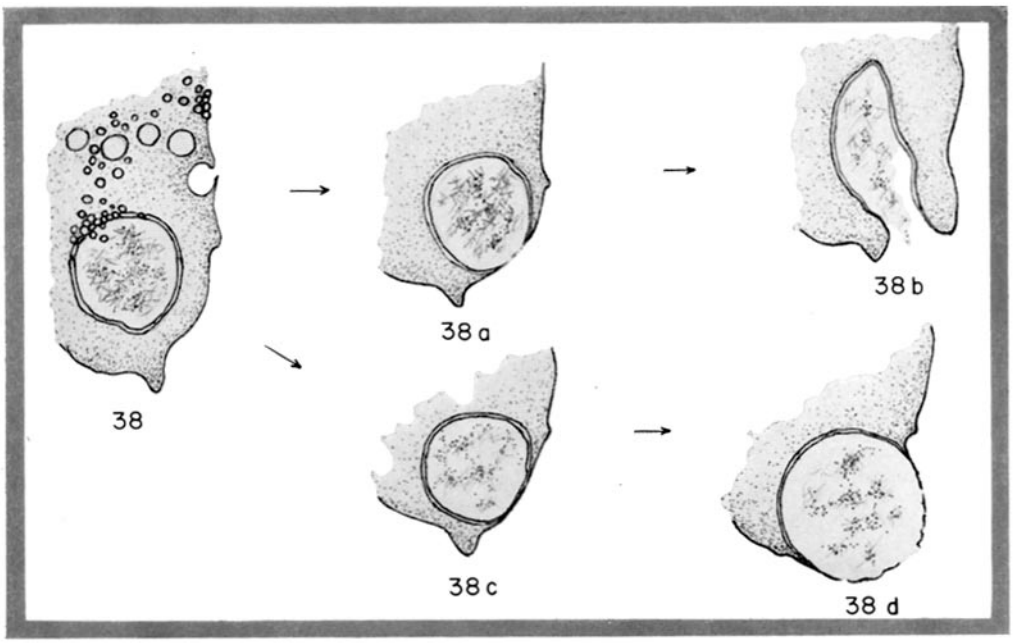

FIGURE 35

The beginning of secretory function and the next step in differentiation is heralded by the separation of each cell from its neighbors by the primitive matrix. The early chondroblast acquires a more abundant cytoplasm, in which the developing organelles concerned in the secretory process are emerging. The outline of the isolated chondroblast is typically scalloped or bayed, and there are numerous cytoplasmic processes. A few channels of the endoplasmic reticulum (*er*), some of which have expanded or sacculated segments, can be seen, and the ribonucleoprotein granules are almost exclusively associated with its membranes. The Golgi area (*Ga*), now well defined, is composed of many lamellar stacks of membranes together with some vacuoles. The mitochondria (*m*) have enlarged and appear to be expanded by their contents. The nucleolus is larger and denser. Small deposits of glycogen (*gly*) represented in the diagram by coarse floccules may sometimes be found free in the cytoplasm. A few smooth walled vacuoles are also evident. At the cell surface, in the cortical ectoplasm, foci of increased density (*d*) are discernible, very often in association with bundles of collagen fibrils which overlie them.

FIGURE 36

The fully developed, actively secreting chondroblast lies in a well formed matrix of sheaves of short straight collagen fibrils, and an amorphous ground substance. It is a cell of ragged outline with plentiful cytoplasm containing numerous inclusions. The endoplasmic reticulum (*er*) has undergone great development, and is seen as an extensive convoluted system of rather wide cisternae with numerous focal expansions or reservoirs; its contents are always denser than the hyaloplasm. The Golgi area has also undergone a similar enlargement. The flattened cisternae and the numerous small vesicles and larger vacuoles (*v*) which compose it are gathered chiefly in the juxtanuclear area from which center individual vacuoles appear to migrate peripherally to the cell surface. Here, some of the vacuoles lodge at the cortex under the cell membrane; they may lose their membranes in this situation (*r*). Connections between the endoplasmic reticulum and the smooth walled cisterns of the Golgi area are sometimes seen, and it is surmised that the contents of the endoplasmic reticulum may contribute to those in the Golgi vacuoles. In chondroblasts of this stage, large accumulations of granular or finely fibrillar, and amorphous material, enclosed by characteristic doubled membranes are often found. The contents of these are expelled into the extracellular space by a process illustrated in Fig. 38.

cartilage is evidently hydrated, quite soluble, and rather scant, in contrast to that of older cartilage. Concomitantly the materials contributed to the matrix from the Golgi vacuoles of the chondrocyte are denser and more variegated, and probably differ qualitatively from the less dense, homogenous contents of the vesicles and blebs of the earlier chondroblast. The tissue continues to differentiate in later life, and the aging of cartilage is accompanied by progressive changes in mucopolysaccharide composition, with a decline in the concentration of chondroitin sulfate, a rising keratosulfate fraction (80), and possibly an increased content of neutral mucopolysaccharide (81, 82, but see 80). Rather drastic changes in qualitative composition probably accompany differentiation and maturation of most or all connective tissue ground substances. They have been best documented in the shifting ratios of hyaluronate to chondroitin sulfate in skin during embryogenesis (83), and there is reason to believe that the primitive ground substance of most

FIGURE 36 (*continued*)

At the surface of the cell, the densities of the cortical ectoplasm are discernible as definite striae, such as that shown on the lower left side of the cell at its surface. They are believed to participate in fibrillogenesis. The inset to the left side of the cell shows this stria at higher magnification. It consists of parallel arrays of fine filaments ("primary fibrils"). These are pictured as peeling off into the matrix (excortication) and in continuity with a sheaf of collagen fibrils which extends into the extracellular space (*cf.* Fig. 18). (It is hypothesized that the filaments or primary fibrils formed in the cortical ectoplasm are dispensed into the extracellular space and that these become the cores or centers upon which definitive collagen fibrils are assembled by the addition of tropocollagen secreted by the chondroblast.) Cortical ectoplasm with its striae is frequently separated from the endoplasmic cytoplasm by rows of fused vacuoles: these may be instrumental in lifting off the overlying cortical ectoplasm with its dense bands. Deposits of glycogen are usually found in the active chondroblast.

FIGURE 37

The product of further differentiation, the late chondroblast or early chondrocyte, exhibits a remarkable increase of both glycogen and the Golgi components. Glycogen is present in large unbounded masses throughout the cytoplasm and between the channels of the endoplasmic reticulum. The latter appears to be compressed by the masses of glycogen: its channels, usually close together, are narrow and have fewer dilatations. The intervening cytoplasm, and indeed the nuclear contents, have become dense and compact. The Golgi area is at its greatest development. It now consists mostly of larger vacuoles, many of which have dense contents. These make their way peripherally to the surface, as shown in the sketch, and after fusion of vacuole and cell membranes and establishment of a stoma discharge their contents (upper left side of cell). Others lodge in rows under the cortical rim of the cell where they may fuse and lose their membranes, constituting areas of reduced density, (*r*), at the right side of the cell. These may function to detach the cortical ectoplasm from the rest of the cytoplasm. Large vacuoles with double membranes are no longer found at this stage.

FIGURE 38

Diagram illustrating the formation and fate of cytoplasmic vacuoles of the chondroblast. The small vesicles and larger vacuoles contained by a single membrane arise in the Golgi area, migrate to the cell surface, and their contents are there exteriorized. Small vesicles are apparently sometimes expelled entire, with their envelopes.

The contents of the large vacuoles with double membranes are thought to arise by transformation of the hyaloplasm. The pool of material formed in this process is believed to be secondarily enveloped by a double membrane, possibly derived from vesicles originating in the Golgi area. (Clusters of such vesicles often constitute sectors of the wall of such vacuoles.) In Figs. 38*a* and *b*, the vacuole is seen to approach the cell membrane and to fuse with it. A stoma is formed at the point of contact, and the contents expelled, collapsing the sac. The outer membrane of the vacuole becomes the new cell membrane. In Figs. 38 *c* and *d* the vacuole is shown to approach the cell membrane and to burgeon out as a bleb, as though expanded by the turgor of its contents. Subsequent rupture of the fused outer membrane permits the contents to escape. The nature of these contents is unknown.

mesenchymal tissues of the embryo, whatever their prospective fate and final composition, is largely hyaluronic acid.

Fibrillogenesis

Even after the electron microscope, in resolving the collagen microfibrils (the primary (38) or unit (11) fibril), had further defined the problem to a consideration of intial origin of these elements, the three dominant concepts bequeathed from earlier decades found support by contemporary investigators. These are that: (*a*) fibrils are formed in the milieu of the intercellular spaces by the aggregation of molecular (monomeric) units synthesized in fibroblasts (or perhaps other cells) (22, 41, 84); (*b*) fibrils are formed intracellularly and shed (3, 6, 38); (*c*) fibrils are formed, at least initially, at the cell surface or interphase by a fibrillar transformation of some of the surface ectoplasm (85). Current opinions are based on evidence from chemical extraction of collagen and its reconstitution *in vitro* (86) and from observations on fiber-forming cells in electron microscope. Gross (22) and Schmitt, Gross, and Highberger (84), following a series of studies on extractable collagens, incline to the view that a monomeric precursor of collagen synthesized by the connective tissue cells is aggregated into fibrils in the extracellular space under the influence of inducing agents resident there. Porter (11) and Porter and Pappas (85), cognizant of the high degree of structured orientation of the collagen fibers in many tissues, directed attention to the role of the cell surface where they observed that the unit fibril is initially formed and, after being shed, continues to grow. Both Wassermann (6, 38) and Fitton-Jackson (18, 87) gave support to this thesis. Wassermann (38) was of the opinion that fibrils are principally formed within the exoplasmic cytoplasm from which they are dispensed, while Fitton-Jackson (87), although admitting the presence of cytoplasmic fibrils, tended more to the view that the principal site of fibril formation as well as fibril growth was nevertheless extracellular. The visual evidence presented by these authors was not unequivocal, however, and Wassermann (38) was especially aware of the topographical difficulties in attempting to localize the fibrils of the pericellular mantle to the intra- or extracellular areas. More recently, Kajikawa, Tanii, and Hirono (41) have again put the issue in doubt by their inability to find fibrils in any part of the fibroblast cytoplasm, the cortex included. They, like Gross, consider that collagen fibrils are formed in the extracellular space, under the influence of the physicochemical conditions prevailing there, from precursors synthesized in the endoplasmic reticulum and discharged from the cell.

It is generally agreed that fibroblasts and chondroblasts are but modulated forms of the same cell family, and there is no reason to believe that the basic events of fibrillogenesis are different in essence, if not in detail, in any of the fibrogenic connective tissue cells. Indeed, the production of fibrous sheaths by all animal and plant cells generally exhibits certain fundamental similarities. This is not to say, however, that different aspects of fibrillogenesis, for instance cortical transformation, may not be more emphasized in some types of fibroblastic cells than others, for example secretion of tropocollagen, at least during some stages, or in different environments.

Porter (11) and Wassermann (6) have expressed similar opinions. The evidence of the present study is interpreted as showing that unit or primary fibrils are assembled within the ectoplasm of the cells (along striae possibly corresponding to the fibroglia of light microscopy) and that such fibrils may be continuously dispensed in groups into the intercellular ground substance in which they grow or mature by the orderly addition to them of monomeric units from soluble tropocollagen secreted by the chondroblasts. In the process it is supposed that the ectoplasm is continuously being shed (Fig. 36).

This idea of fibrillogenesis, substantiated by the morphological investigations of this decade, has been given credence by chemical evidences marshalled by D. S. Jackson and Bentley (88). In their view also the primary fibril may be regarded as a template core upon which new molecules are added, the outer layers always the more loosely aggregated, hence more soluble and isotopically most active. The evidence for this view of fibrillogenesis does not exclude the possibility that fibrils might also be built from tropocollagen units in the matrix.

Secretion of the Ground Substance

The presence of mucoid ground substance has of course been repeatedly linked with the formation of fibers and fibrils in whose organization and composition the mucopolysaccharides are sup-

posed by some to take part (70, 75, 86, 89, *inter alia*), and upon which fibrillogenesis was thought to be at least partly dependent. In this connection it is important to distinguish, as Porter and Pappas (85) and Wassermann (6) have done, between formation of the primary or unit fibril and its subsequent growth and aggregation leading to fibers or bundles. Most contemporary authors agree that neutral salt-soluble, presumably monomeric or tropocollagen, is involved in the growth of collagen fibrils and that this is also secreted by the fibroblast or its analogue (22, 88). The soluble collagens thus form a part of the proper ground substance (22, 84, 87, 88). But the function of the mucopolysaccharides in fibril growth or fiber formation remains unclear.

With respect to the role of the cells in production of the other protein and the polysaccharide components of the ground substance, the position was essentially the same as that previously assumed for fibrillogenesis, namely: (*a*) that these substances are formed in the cell and secreted (13, 17); (*b*) that they arise through a "transformation of the cytoplasm," in particular the ectoplasm and its processes (mesostroma) (19) (a view adopted to explain chondrogenesis by the transmutation of the cytoplasm into ground substance (20, 90)); (*c*) that these or some of these substances, perhaps formed elsewhere and contributed by the plasma, are merely deposited *in situ*, probably as a result of some activity of the fibroblastic cell (21).

While inquiry into the nature and source of the connective tissue ground substance has had as long a history as that relating to the fibers, speculations as to its mode of origin remained uninformed until more of its chemical composition had been revealed. Cartilage, of all the mature connective tissues, is the richest in ground substance, which, moreover, is present in gelated form and readily demonstrable histologically. Studies of its development, increase, and metaplasia, and the formation of cartilage matrix in tissue cultures (17, 91, 92) have suggested to most students that matrix ground substance and therefore also its chromotropic material is a product of the chondroblast. More definitive tissue culture experiments have since shown that hyaluronic acid and chondroitin sulfate C are synthesized and secreted *in vitro*, even in simplified media, by divers cells of mesenchymal origin (12). Equally conclusive data have come from radioautography after $S^{35}O_4$ administration, which has, in addition, provided valuable topographic information. Dziewiatkowski (93), Bostrom and Odeblad (94), Bélanger (95), and Amprino (96) were among the first to show that radiosulfate enters the cartilage and other tissues almost exclusively as sulfomucopolysaccharide, probably mostly chondroitin sulfate. The serial radioautograms of Bélanger (95), Pelc and Glucksmann (97), and Amprino (96) illustrated quite clearly that this uptake of radiosulfate is a metabolic function of the cartilage cells, in which it first accumulates within 2 hours after administration, and thereafter begins to appear in the matrix. The capacity of different cartilage cells to accumulate radiosulfate is directly proportional to the amount of cytoplasm and the quantity of stored glycogen.

Electrons are emitted from S^{35} with an energy yielding radioautographic tracks of excellent resolution for histology, but barely adequate for intracytoplasmic localization, at least in sectioned material. In the extended cytoplasm of cultured chick cells Mancini *et al.* (98) reported that radiosulfate first appeared in the perinuclear cytoplasm and subsequently diffused into all the cytoplasm including the peripheral cell processes and prolongations. The conjoined use of specific or selective stains with radioautography may augment information by increasing resolution. Passage of radiosulfate into the matrix is generally paralled by its increased metachromasia, but no direct relationship between fixation of S^{35} and PAS-reactivity of the tissue is observable (99). Metachromatic granules have sometimes, although by no means consistently, been localized in the cytoplasm of the chondrocyte (64) and fibroblast (18); diastase-resistant, PAS-stained granules have been found, without apparent constant location in the cytoplasm of chondrocytes (28), osteoblasts (100), and fibroblasts (18).

It is impossible on the basis of presently available topological information definitely to localize sulfomucopolysaccharide to a particular organelle. The observations of Mancini *et al.* (98), if they are true of the chondroblast, are suggestive of accumulation in the region of Golgi apparatus and subsequent dissemination to the periphery. Additional information respecting the functions of specific cytoplasmic organelles in mucopolysaccharide production may come from attempting to correlate the degree of radiosulfate uptake with cytoplasmic fine structure during the progression of changes which take place during differentiation. It is noteworthy that the mesenchyma of the

undifferentiated precartilage already shows a relatively higher radiosulfate uptake than other mesenchymal areas not destined to form cartilage. Radiosulfur incorporation increases as differentiation progresses (15) and is greatest in the adlineated and the hypertrophied chondrocytes at the metaphyseal aspect of the epiphyseal plate (16). It is more moderate in the proliferating cells of the central zone and less in the perichondrium. This distribution closely parallels the extent of development of the Golgi apparatus (Figs. 34–37), which, it may be inferred, is in some way concerned with the secretion of sulfated material.

Cytologically, secretion implies formation and sequestration of a special product in form of granules. The Golgi apparatus has been implicated in this process since the classic researches epitomized in the papers of Bowen (101) and Hirsch (102) The extensive development, even hypertrophy, of the Golgi complex in the chondrocyte had long been regarded as evidence of its secretory activity by Parat and Godin (54) and Fell (17) *inter alia*. Electron microscopy adumbrates this view and raises the question as to the nature of the structurally heterogeneous materials being sequestered in the multifarious lumens of this organelle. Typically, secretory granules, wherever identified in other cells, are made up of an internal substance of some density contained within a single, thin, smooth membrane (50). In the differentiating chondroblast, vacuoles delimited by single membranes, either empty or enclosing contents of low to moderate density, exist in a wide range of sizes; in the course of maturation, as the Golgi apparatus becomes enlarged and concentrated, the vacuolar contents change markedly in form and density. It is probable that the kinds of material being sequestered change during differentiation. At least some of these (almost surely the proteins) must be formed in the granular endoplasmic reticulum, which has been described as being continuous with the Golgi complex. Together they are thought to comprise a ramified membranous system of intercellular channels bringing the ergastoplasm, the Golgi complex, and the plasmalemma into dynamic continuity (46, 50). It may therefore be supposed that materials are transmitted from the channels of the endoplasmic reticulum to the spaces of the Golgi vacuoles. Since these vesicles or vacuoles are conveyed to the cell surface and discharged from the cell, either as whole microvesicles retaining their membranous coats (Figs. 20, 28, 29, 32) or after fusion of plasma and vacuolar membranes, *via* temporary stomata (Fig. 28), this process may be regarded as a form of merocrine secretion.

The origin of the mixture of materials ("lakes" or "pools") enveloped in the double membranes is not yet known. The inclusion within them of particles which have the size and form of ribonucleoprotein granules, of small vesicles, and occasionally of whole mitochondria, suggests that the contents are pooled in the "ground substance" of the cytoplasm, entrapping adjacent organelles, and are secondarily encapsulated. The frequent occurrence of microvesicles in and around these pools, and the fact that collections of such vesicles sometimes appear to comprise sections of their walls (Fig. 28) suggests that the enclosing double membrane might possibly be formed by coalescence of the membranes of many flattened vesicles or cisternae, which had come to surround the pools (Fig. 38).

An alternative hypothesis of the origin of the double membrane which has been entertained, that they might be mitochondrial, is less likely, and seeming transitional stages represented by swollen mitochondria (Fig. 31) must be interpreted with due caution. The fate of these pools, and the disgorgement of their contents have been illustrated (Fig. 38); insofar as actual cytoplasmic constituents are liberated from the cell, the process may be likened to apocrine secretion. In a typical example of apocrine secretion such as that of the intestinal goblet cell, the ergastoplasm and subsequently the proliferated Golgi complex are clearly involved in formation and continence of the product, which is enclosed in vesicles delimited by the usual single membrane before pooling occurs (50).

In the postulated sloughing of the fibril-forming striae of the cell cortex, in the formation and discharge of the contents of the big double membrane-covered pools, in the release of single membrane-covered vesicles, and in the accumulation and dumping of glycogen into the matrix, the cell appears to be yielding up specialized areas of its cytoplasm in the process of matrix formation. At the terminal stages of these processes in the osteogenic areas, particularly when great masses of glycogen and the coalescent contents of pools and vacuoles have reduced the intervening cytoplasm to a lacework of narrow bands, it may appear in the light microscope that the matrix is actually coextensive with the cellular contents, and that the cell, as such, is disappearing and is

hardly separate from its matrix. This has led to the interpretation in classical histology of the conversion of cytoplasm, hence the transformation of whole chondrocytes to matrix (19, 20, 90).

Glycogen and the Hypertrophic Cells

The presence of glycogen in cartilage cells has been known for a century. Its special accumulation in the hypertrophic cells of the osteogenic plate and in the cartilages about to ossify, was made known in 1885 (103), and the speculations excited by this circumstantial relationship to the process of osteogenesis (104), have since remained lively (105). Glycogen is found in increasing amounts as cartilage cells mature (106). Specifically, some developed chondroblasts, chondrocytes within the cartilage nodule (near the future ossification center of the epiphysis), and especially hypertrophic chondrocytes accumulate glycogen; adlineated chondrocytes of the proliferating zone of the epiphyseal plate, not yet hypertrophic, have little glycogen (107). The discovery of alkaline phosphatase in developing bone and its apparent association with glycogen in the chondrocyte made the theories of the role of glycolysis in endochondral ossification yet more intriguing.

Fell (17), in her classic study of the histogenesis of bone and cartilage, regarded the development of the chondroblast series in the epiphysis from mesenchyme to hypertrophied chondrocyte as a specific differentiation producing "more or less specialized elements whose sole function is the secretion of cartilage matrix." Harris (106), on the other hand, equated these cellular changes and the accumulation of glycogen with a process of senescence and degeneration, a formulation which evoked a subsequent controversy (108). The changes in fine structure of these cells before hypertrophy occurs are those of continued specific differentiation; "hypertrophy" is an exaggerated continuation of the intracellular storage of pooled materials, especially glycogen but also the material within the Golgi apparatus. Simultaneously, the densification of both nucleus and cytoplasm which must mean loss of water and/or hydrophilic protein, and the continued shedding of visible cell substance can reasonably be interpreted as a process of senescence and ultimately of cell death, with contribution of its inclusions to the matrix.

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