Z. Zellforsch. 103, 192—217 (1970)

Fine Structure and Histochemistry of "Calcifying Globules" in Epiphyseal Cartilage*

ERMANNO BONUCCI
Istituto di Anatomia Patologica
University of Pisa — Pisa, Italy

Received October 9th, 1969

Summary. The cartilage matrix in which the early calcium salts are deposited has been studied in the tibial epiphyses and in the costo-chondral junctions of 30-day-old guinea pigs. The results may be summarized as follows:

(1) Structures of globular shape ("globules") are to be found throughout the entire epiphyseal plate. (2) They have a homogeneous matrix and are bounded by a membrane. (3) Early calcification occurs in globules. Calcification of collagen fibrils seems to occur later. (4) The earliest mineral deposited would seem to consist of tiny granules about 20 Å in diameter. Then apatite crystals are laid down, initially in small clusters and later filling the globules completely. (5) The globules are strongly osmiophilic. They seem to contain a fair amount of neutral polysaccharides, but no acid polysaccharides except a coating on their outer membrane. Hyaluronidase digestion does not affect globules. Papain digestion makes them more reactive to uranium and lead. (6) Globules are of cellular origin but they are almost certainly not pre-formed in the chondrocytes. Finally, the present paper advances the hypothesis that some globules derive from degenerating chondrocytes and others from the processes of normal chondrocytes.

Key-Words: Cartilage — Calcification — Calcifying globules.

Introduction

Considerable data have already been gathered about the mechanisms operative in cartilage calcification, but much uncertainty still remains as to their exact nature. Much of this data has been obtained with the electron microscope (Robinson and Cameron, 1956; Scott and Pease, 1956; Fitton Jackson, 1960; Takuma, 1960; Knese and Knoop, 1961a, b; Cameron, 1963; Anderson, 1964; Schenk *et al.*, 1967; Scherft, 1968). It has been found that apatite crystals are deposited in the epiphyseal plate at some distance from the metaphyseal capillaries in the longitudinal intercellular bars, where small crystal clusters of roundish shape are initially formed around hypertrophic chondrocytes. These crystal clusters increase in size without obvious reference to the orientation of the collagen fibrils. Moreover, they lay against an amorphous, dense background. As the metaphysis is approached, crystal clusters gradually coalesce and the longitudinal cartilage bars become completely calcified.

It has been suggested that the process of calcification that takes place in cartilage is the same as in bone, that is, in both these tissues calcium salts would seem to be nucleated by collagen fibrils. However, because there is no obvious

* The author is indebted to Mr. A. Benvenuti for his technical assistance. This work was supported by a grant from the Italian Research Council.

relationship in cartilage between crystals and collagen fibrils as there is in bone, a different mechanism of calcification has been considered possible (Cameron, 1963).

It has been reported (Bonucci, 1967) that the early deposition of calcium salts in the epiphyseal cartilage of guinea pigs and rats does not only involve collagen fibrils. The main structures involved are interfibrillary, osmiophilic, amorphous bodies of roundish shape. These bodies have a pericellular distribution but no direct connection with chondrocytes. It was suggested (Bonucci, 1967) that the characteristic pericellular distribution may be due to the fact that the osmiophilic bodies originate in the chondrocytes, and the hypothesis was advanced either that these bodies are formed in the cells and then excreted in the matrix such as they are, or that they are formed directly in the cartilage matrix under cellular control.

Calcification seems to start inside the osmiophilic bodies, which would seem to be gradually filled with crystals. The roundish shape of the crystal clusters would then be a direct result of the roundish shape of the osmiophilic bodies.

Thus, these bodies seem to be important structures in initiating cartilage calcification and more precise knowledge of their fine structure seems desirable. The investigation reported in this paper was carried out to find out more about the osmiophilic bodies, their fine structure, origin, evolution and fate, and how they calcify.

To simplify, the osmiophilic bodies will be called "globules" from now on.

Material and Methods

Costo-chondral junctions and upper tibial epiphyses of one-month-old guinea pigs were dissected under ether anesthesia, reduced to small fragments, and fixed at 4° C (a) in 1% osmium tetroxide, buffered to pH 7.2 with veronal acetate or phosphate buffer, (b) in 4% formalin, buffered to pH 7.2 with phosphate buffer, and (c) in 4% formalin followed by 1% osmium tetroxide. The specimens were dehydrated with acetone and then embedded in Araldite. To test what effect acetone dehydration had, other specimens were dehydrated with Durcupan and embedded in Araldite (Stäubli, 1963).

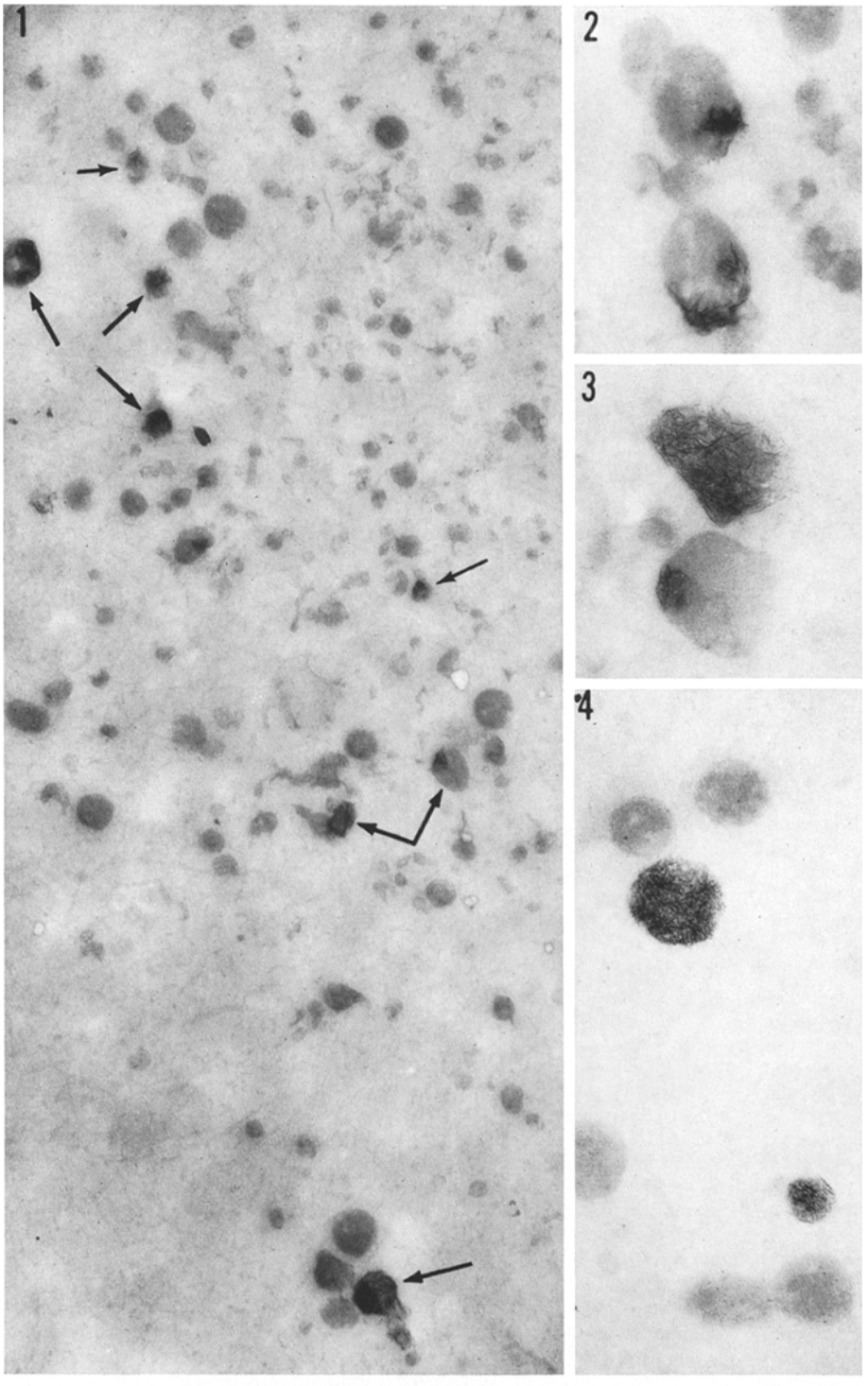
Other specimens were fixed in formalin and OsO_4, decalcified for 48 hours at 4° C with EDTA or 2% formic acid, repeatedly washed with distilled water and embedded in Araldite.

Other specimens were fixed in 4% formalin and digested at 37° C for 6 and 12 hours with hyaluronidase or with papain. Testicular hyaluronidase (Nutritional Biochemicals) was used in 0.1 N phosphate buffer (1 mg/1 ml) at pH 6.5. Papain (crude product, Nutritional Biochemicals) was used in 0.02 N acetate buffer (1 mg/1 ml) with KCN and EDTA, pH 5.4. Controls were left in the respective buffers for the same times and at the same temperature. Control and experimental specimens were dehydrated with acetone and embedded in Araldite. Some were postfixed with OsO_4.

Ultrathin sections cut with a Porter-Blum microtome were examined unstained or after uranyl acetate and/or lead citrate staining.

Other sections were treated with Mowry's colloidal iron method for acid polysaccharides, as suggested by Matukas *et al.* (1967), but we used free floating sections (Marinozzi, 1961). Neutral polysaccharides were stained with silver nitrate — methenamine after periodic acid oxidation (Marinozzi, 1961; Rambourg, 1967).

Sections 1 micron thick were routinely stained with Azure II and Methylene blue. Those obtained from specimens fixed in formalin, and those from specimens digested with hyaluronidase and with papain, were also stained with the periodic acid — Schiff method (PAS), Toluidine blue, pH 4.5, Alcian blue, pH 2.5, colloidal iron, and Sudan black B.



Figs. 1—4

Results

Fine Structure of the Globules

Globules *in unstained sections* from specimens fixed in osmium appear round or oval, and outside the cells (Fig. 1). They consist of a homogeneous, amorphous substance which is much more electron dense than the cartilage matrix. This electron density is due to osmiophilia, because no globules are visible in sections from specimens fixed in formalin.

Many globules contain needle-shaped crystals (Figs. 1—4). These crystals are like those found in fully calcified cartilage, although some of them, chiefly those found at the periphery of the globules, are curved. They produce hydroxyapatite electron diffraction and disappear after decalcification of the sections with EDTA or acid solutions.

Within the globules, crystals form small clusters, to be found at the periphery (Figs. 2, 3), over the outer border (Fig. 2), or near the center of the globules. Moreover, crystals can more or less fill the globules, completely masking their matrix (Figs. 3, 4). In this case, the crystal clusters have an almost perfectly round shape (Fig. 4), allowing them to be inductively identified as globules which have calcified.

Globules can also contain very small granules, about 20 Å in diameter. These granules are scattered at random within the globules (Figs. 2, 4). They are more electron dense than the matrix of the globules but less dense than the crystals. Electron diffractograms of globules containing these granules alone do not produce apatite diffraction rings. However, granules disappear after decalcification of the sections. Granules and crystals sometimes appear together, in the same globule (Fig. 2).

There is no relationship between the diameter of the globules and the presence of apatite crystals in them. Calcification does not begin simultaneously in adjacent globules either. Globules completely filled with crystals may be found near uncalcified globules; and the uncalcified ones may be seen near fully calcified trabeculae.

Where a number of calcifying and calcified globules are to be found, elongated tufts of crystals may be present too. They seem to be independent from the globules. They have a shape like that of calcified collagen fibrils.

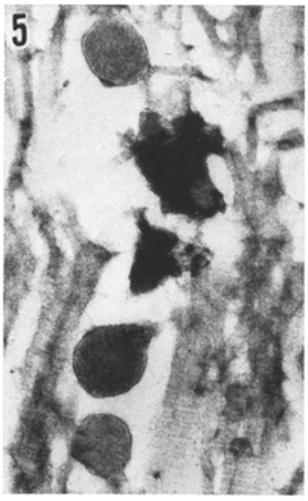
Globules in sections from osmium fixed specimens *stained with uranyl acetate and/or lead citrate* show greater electron density than globules in untreated sections.

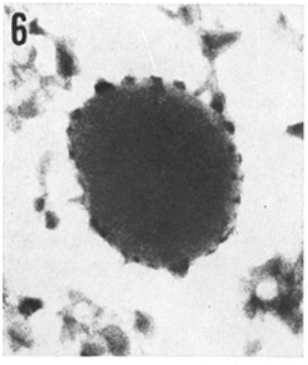
Fig. 1. Cluster of globules in an intercolumnar, longitudinal bar. Zone of maturing chondrocytes. Arrows point to globules containing apatite crystals. Unstained. ×35,000

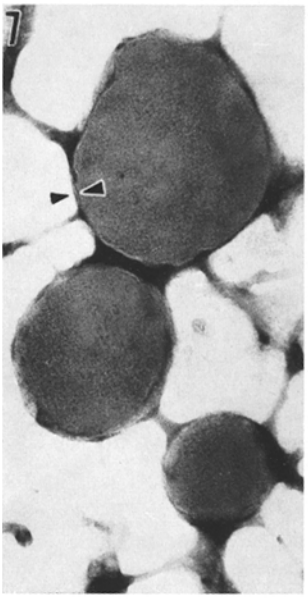
Fig. 2. Cluster of globules, two of which contain apatite crystals. These form small excentric clusters. The upper globule also contains very small granules. Unstained. ×68,000

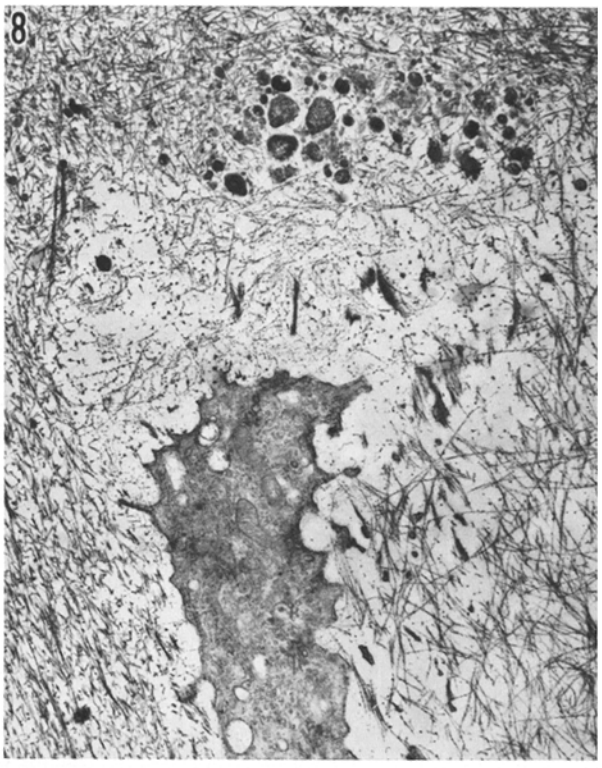
Fig. 3. Two globules, the upper one completely filled with crystals, the other in the early stages of calcification. Unstained. ×80,000

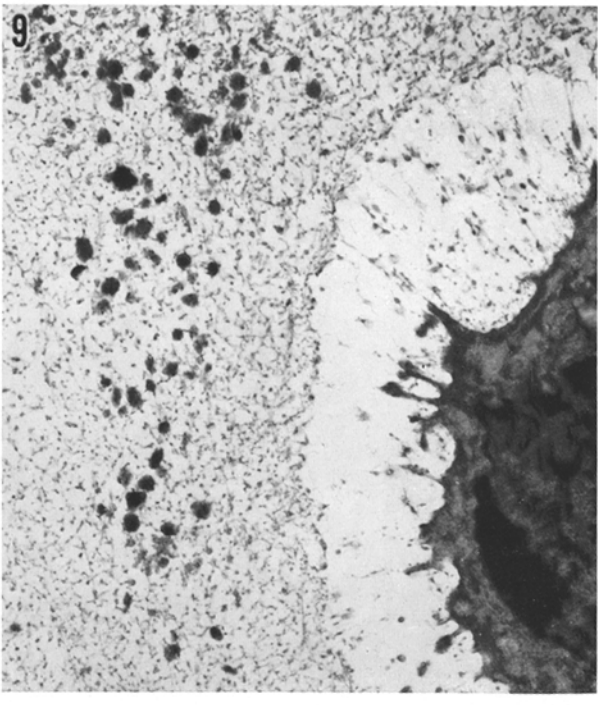
Fig. 4. A group of globules in the maturing zone. Two globules are completely filled with crystals and are recognizable as globules only from their roundish shape. The other globules are still uncalcified, but contain very small granules. Unstained. ×75,000











Figs. 5—9

Moreover, globules also become visible after this treatment in sections from formalin fixed specimens.

The homogeneous, amorphous substance of the globules is not greatly changed by staining. No substructures become evident in them (Figs. 5—7). However, staining reveals that all the globules are bounded by an electron dense border. When the globules are cut through their equatorial plane, a membrane can be detected in this outer border (Figs. 5, 7). It consists of an electron dense inner layer (about 30 Å thick), a clear intermediate space (about 30 Å thick), and an outer layer, which is rather variable in shape and thickness. It is sometimes thicker than 80 Å and often looks like a dotted line (Fig. 6). This is because of granular structures very similar to those sometimes present on the surface of the plasmamembrane in chondrocytes. However, it can be seen in many globules that this layer too is only 30 Å thick (Fig. 7), and when it seems more, this is because of an external coat which is often connected with thin fibrils in the cartilage matrix. These fibrils sometimes link globules together (Fig. 7) forming a kind of net in which the globules are like joining points between meshes of fibrils. When fibrils touch the outer layer of a globule they seem to spread around this unevenly, usually making it much larger and, often, irregular.

Distribution of the Globules in the Epiphyseal Plate

Globules, often collected in clusters, are present throughout the entire epiphyseal plate, although most of them are found in the maturing zone (for subdivision of the epiphyseal plate in zones see Ham, 1965). However, it must be considered that *the distribution of the globules is not uniform*, because at the same levels in the epiphyseal plate there are areas which contain many globules and other areas which do not contain any at all. The following description is based on the most frequently observed distribution of the globules.

In the *resting zone* globules are found both in the intercellular matrix and near the chondrocytes. In this case, they may either be separated from the cells by a sheath of cartilage matrix about 1—3 microns thick, in which only thin collagen fibrils are found (Fig. 8), or they may also be placed within the cell lacuna, intermingled with the cell processes.

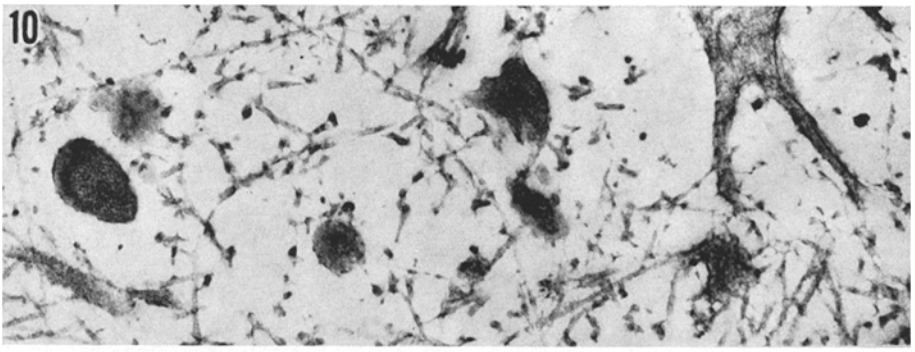
Fig. 5. A group of globules in the hypertrophic zone, stained with uranyl acetate and lead citrate. All the globules are bounded by a membrane and contain an amorphous substance. The two black areas correspond to two calcified globules. In this case the collagen fibrils have a clearly recognizable periodic banding. ×75,000

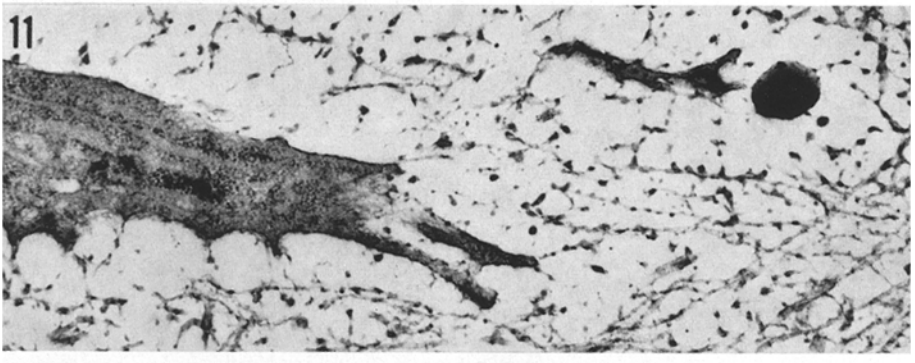
Fig. 6. A globule stained with uranyl acetate and lead citrate. Its outer border resembles a dotted line because of the presence of small granules. ×90,000

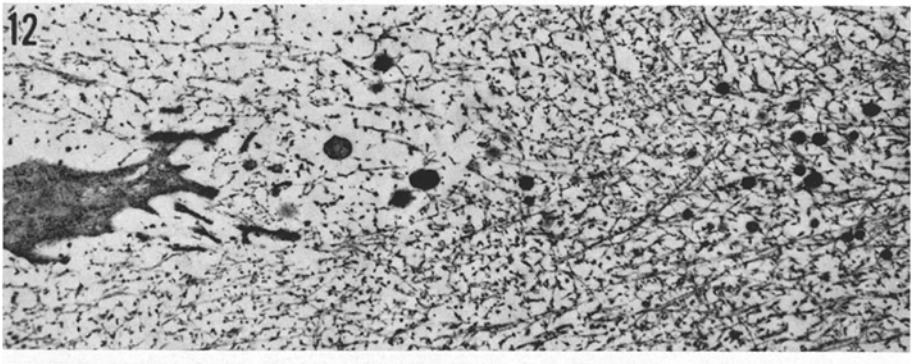
Fig. 7. A group of globules in the resting zone, stained with uranyl acetate and lead citrate. The globules are bounded by a membrane (indicated by the arrows), whose outer layer is thickened by an amorphous substance which is closely connected with thin fibrils. These link the globules together. The matrix of the globules is homogeneous and amorphous. ×75,000

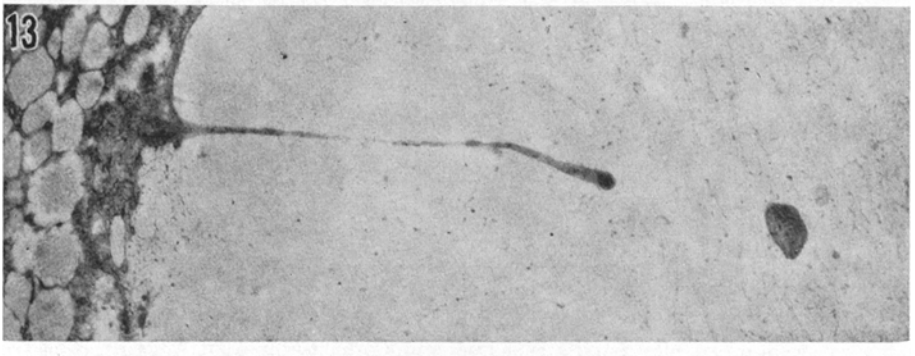
Fig. 8. A group of globules near the outer border of the lacuna of a chondrocyte in the resting zone. The electron density of the globules varies, the bigger ones showing a structure which looks like that of the chondrocyte cytoplasm. Uranyl acetate — lead citrate. ×10,000

Fig. 9. A group of globules round a chondrocyte in the maturing zone. A sheath of matrix is interposed between the globules and the cell. Uranyl acetate — lead citrate. ×10,000









Figs. 10—13

The globules found in the resting zone are not always homogeneous and amorphous. The structure of a few of them resembles that of the chondrocyte cytoplasm (Fig. 8) and sometimes these globules may contain small vesicles. Apatite crystals are never present.

In the *proliferating zone* globules are found only in the thick septa which separate the columns of flat chondrocytes, while they are not present in the thin, transverse sheaths of matrix interposed between the chondrocytes.

In this zone the globules may be found within the cell lacunae, often mingled with cell processes (Fig. 10), or at the end of a cell process (Fig. 11). However, most of the globules are outside the cell lacuna. They are characteristically collected at the two extremities of the flat chondrocytes, where they form elongated clusters which occupy the areas centering on the ideal continuation of the major axis of the cells (Fig. 12). The distribution of the globules is very irregular in this zone. They are totally absent near certain chondrocytes and very numerous near others. Globules are still uncalcified in this zone, although a few of them containing apatite crystals may occasionally be seen.

In the *maturing zone* globules are almost constantly pericellularly distributed (Fig. 9). However, they are generally absent from the side of the cells facing the transverse intercellular septa and occur mainly in the longitudinal, intercolumnar bars. Moreover, globules are separated from the border of the lacunae by a sheath of cartilage matrix about 1—3 microns thick. Globules may occasionally be found within the lacunae. In some cases, they are to be found at the tip or near the tip of a cell process (Fig. 13). Many globules in this zone contain apatite crystals. These increase in number as the hypertrophic, degenerating zone is approached.

In the *degenerating, hypertrophic zone* globules are not very numerous. At the limit between maturing and hypertrophic chondrocytes, where calcification of the matrix is still incomplete, they are mixed up with more or less roundish clusters of apatite crystals (Figs. 14, 15). The shape of these clusters may be almost perfectly round and their outline sharp, so that they look like the crystal clusters shown in Fig. 4. Other clusters of crystals have a radiating appearance and an irregular shape, and protruding crystals seem to spread from them into the adjacent cartilage matrix (Fig. 15).

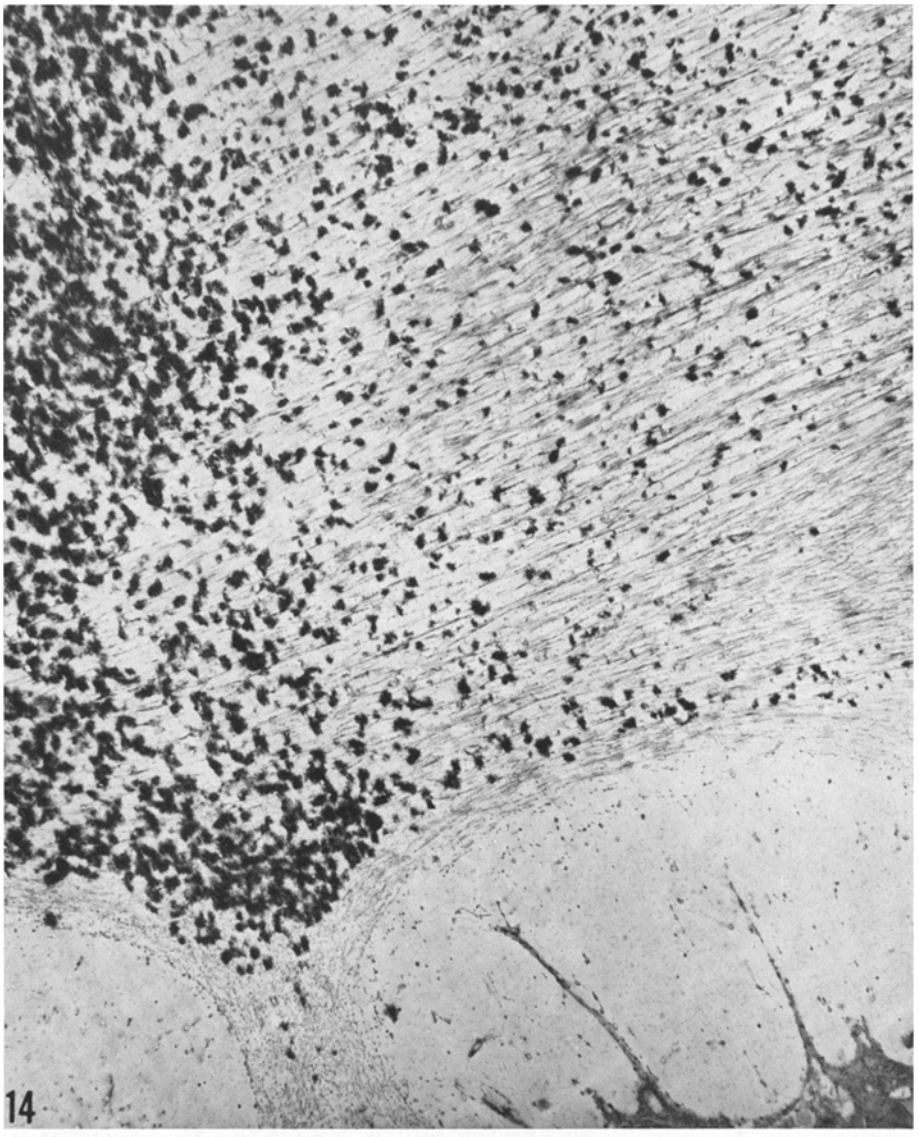
Globules further decrease in number as the more calcified zone is approached. Here, isolated globules can still be found, but the cartilage matrix is almost completely occupied by confluent clusters of apatite crystals.

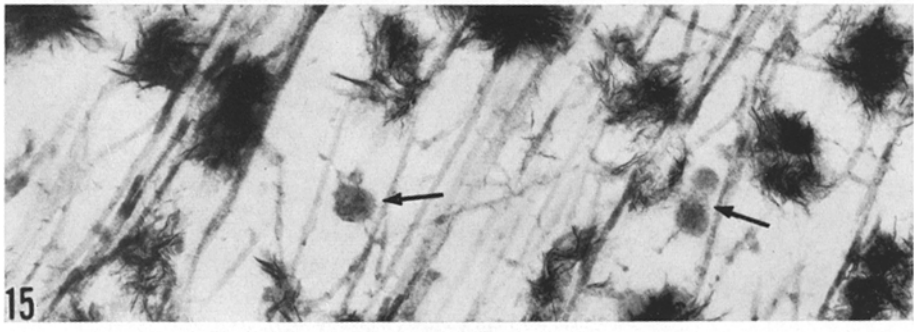
Fig. 10. Globules near the chondrocyte processes in the proliferating zone. The cell processes appear fragmented and serial sections show that in many cases this fragmentation is real. Globules are on the left, cell processes on the right. Uranyl acetate — lead citrate. ×45,000

Fig. 11. A globule near the tip of a cell process in the proliferating zone. The electron density of the globule is much greater than that of the cytoplasm. Uranyl acetate — lead citrate. ×30,000

Fig. 12. A group of globules in the proliferating zone. The globules are distributed along the continuation of the major axis of a flattened chondrocyte (partly visible on the left). Uranyl acetate — lead citrate. ×16,000

Fig. 13. A globule along the continuation of the major axis of a process belonging to a maturing chondrocyte. The process is very thin in the middle, swollen at the end. Uranyl acetate — lead citrate. ×16,000





Figs. 14 and 15

Relationship Between Globules and Chondrocytes

Globules are to be found chiefly around the chondrocytes but also in the intercellular matrix, so that no direct relationship between the globules and the chondrocytes is usually evident. However, globules may also be found within the cell lacunae, or they may have a more or less direct relationship with the cell processes.

The chondrocyte processes are sometimes very long (Figs. 17, 18). They may also cross the entire cell lacuna to end in the cartilage matrix (Fig. 16). More often, they are very short, thin and of irregular shape. Their structure is rather homogeneous, but ribosomes and small vesicles may be found in them. They are always very thin, but their tip may be greater than the other part of the process (Fig. 19). Sometimes, the process ends in a swollen, bulb-like tip, which resembles a globule (Fig. 13). In other cases, real globules are in contact with the tip of the processes (Figs. 21, 22) or are very near to it (Fig. 11). Moreover, in some cases the cell processes seem to be fragmented, because only short segments of them, ordered in the same radial direction, are present in the cell lacuna (Fig. 20). In these cases, the structure of the segments near the cell may still be cytoplasmic, while those farther away from the cell may have an amorphous structure similar to that of the globules.

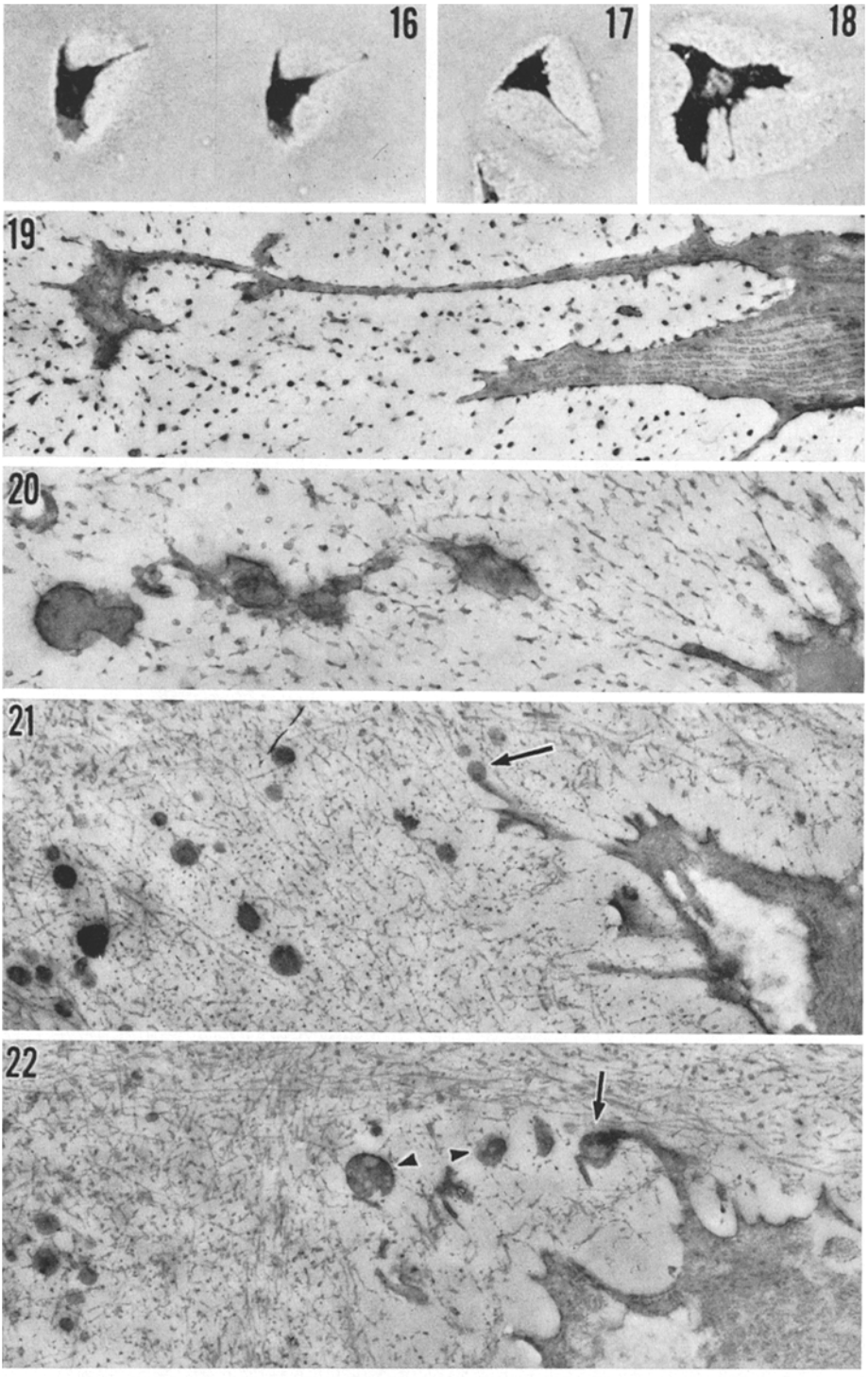
All these factors raise the doubt that the globules may be no more than cross sectioned cellular processes, although the former have a characteristic amorphous, homogeneous matrix which takes up much more uranium and/or lead than the cytoplasm of the chondrocytes and of the cellular processes.

In order to be able to differentiate accurately between globules and cross sectioned cellular processes, and in order to assess the relationship between them, they have been studied in serial sections. Intercellular globules, globules placed within cell lacunae, globules in contact with cell processes and globules near cell processes with a fragmented appearance have been studied in particular in this way.

Serial sections of intercellular clusters of globules (Figs. 23, 24) show that the globules are spherical or slightly ovoidal structures, having no direct relationship either with chondrocytes or with cellular processes. Their thickness is variable, ranging from about 300 to about 7,000 Å (mean value 1,200 Å). When a big globule is examined throughout a whole section series, it is easy to see that its diameter (and its electron density) increases from a minimum value to a maximum one, and then decreases again to a minimum value until the globule disappears. This behaviour, together with the relationship between the approximate thickness of the sections (calculated from their interference colour) and the maximum diameter of the globules, show beyond any doubt that these are true spherical bodies.

Fig. 14. Zone of hypertrophic, degenerating chondrocytes. Two cell lacunae and part of a chondrocyte are visible below. The intercolumnar bar is partly calcified, as shown by the numerous roundish clusters of apatite crystals. A few globules are present between the crystal clusters (see Fig. 15). Uranyl acetate — lead citrate. ×7,000

Fig. 15. Globules (indicated by the arrows) between roundish clusters of apatite crystals in a zone similar to that shown in Fig. 14. Uranyl acetate — lead citrate. ×55,000



Figs. 16—22

Serial sections of intralacunar globules (Fig. 25) show that these globules too are spherical or slightly ovoidal bodies. They are to be found in the neighbourhood of chondrocytes and their processes, but are not generally in contact with them. Their thickness is greater than that of the extralacunar globules, its mean value being 2,500 Å.

Serial sections of globules in contact with cell processes (Figs. 21, 22) again show that globules are spherical. They are situated at the tip of the processes, or near the tip but on one side of the processes. In some cases, globule and cell processes are joined by a thin filament.

Serial sections of globules placed near cell processes with a fragmented appearance still show that globules are spherical bodies. The fragmented appearance of the cell processes is often an artifact due to their irregular course (Fig. 26). In some cases however, the fragmentation is clearly real. In these cases the single fragments have a matrix like that of the globules, but an elongated or irregular shape similar to that of the cell processes. They may be in contact with the cell on the one hand and on the other with one or more globules. An example of this structure may be found in Fig. 25. These globule-like structures may form a chain, which begins from the cell, continues through a cell process and through one or more globule-like structure of irregular shape and ends with one or more globule.

Globules and Degenerating Chondrocytes

The presence of many globules around non-hypertrophic, degenerating chondrocytes is of particular interest (Fig. 27). These chondrocytes are to be found chiefly in the resting zone of the epiphyseal plate, but they may also occasionally be present in the maturing zone. They are adjacent to perfectly preserved chondrocytes, and sometimes degenerating and normal cells are almost touching.

Fig. 16. Two sections of a series, showing a chondrocyte in the resting zone with a long process which ends in the perilacunar matrix. Azure II — Methylene blue; ×1,000

Fig. 17. A chondrocyte in the resting zone, with a very long intralacunar process. Azure II — Methylene blue. ×1,000

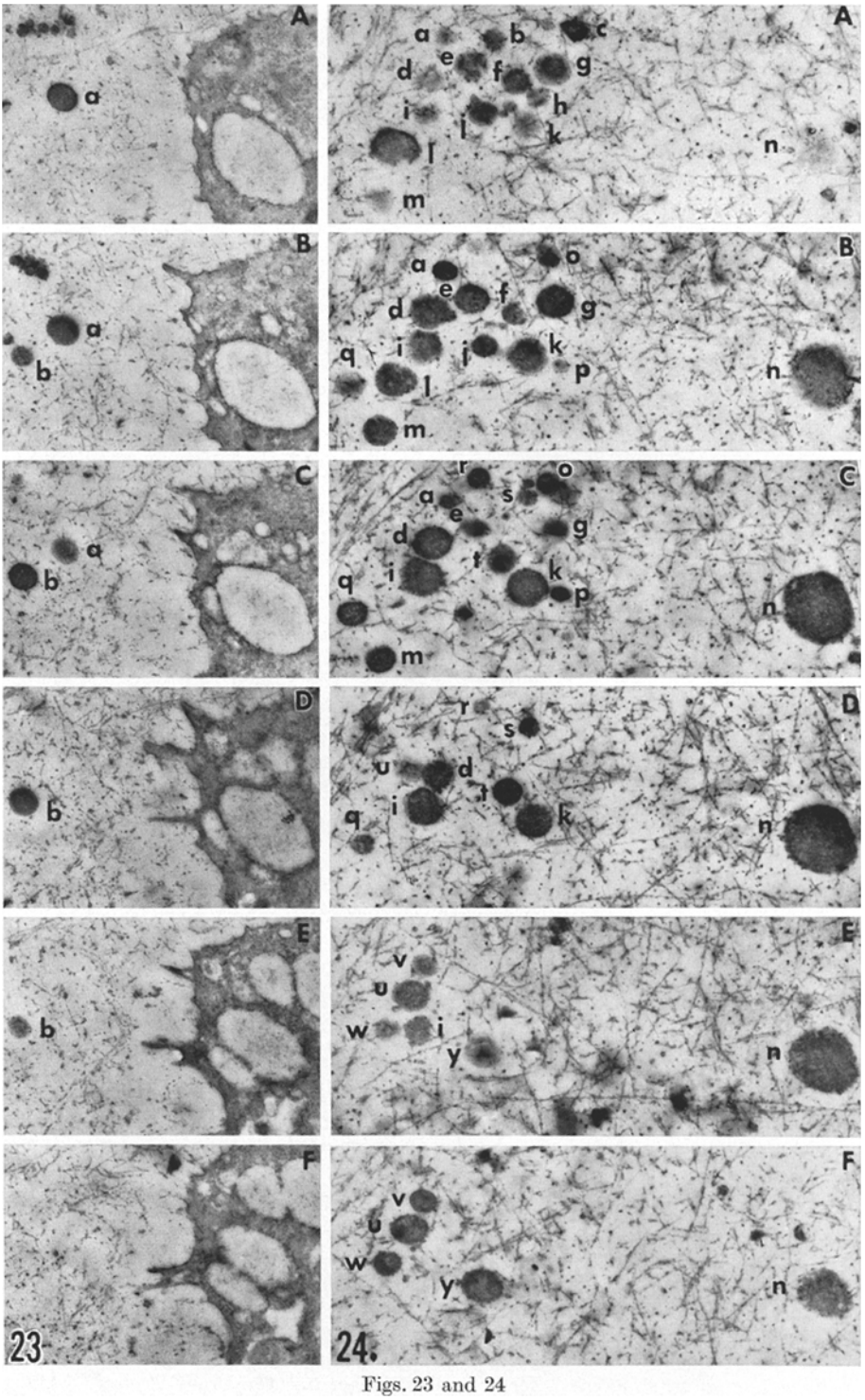
Fig. 18. A chondrocyte in the maturing zone, with a thin and very long process. Azure II — Methylene blue. ×1,000

Fig. 19. A chondrocyte process in the resting zone (like that shown in Fig. 17). The tip of the process is larger than the process itself. Uranyl acetate — lead citrate. ×30,000

Fig. 20. A fragmented cell process in the resting zone. The amorphous matrix and the roundish shape of the fragment looking like a globule are visible on the left. Uranyl acetate — lead citrate. ×34,000

Fig. 21. A section of a series of 5, showing a globule at the tip of a process (arrow). All the other globules have no direct relationship either with the chondrocyte or with its processes. The white zone in the cytoplasm (right) contains glycogen. Zone of proliferating chondrocytes. Uranyl acetate. ×18,000

Fig. 22. A section of a series of 7, showing a globule at the tip of a process (arrow). The other two intralacunar globules (small arrows) are also at the tip of other processes, as shown by the other sections of the series. The globules on the left have no relationship either with the chondrocyte or with its processes. Zone of maturing chondrocytes. Uranyl acetate. ×18,000



Figs. 23 and 24

The degree of degeneration is variable.Sometimes the general outline of the degenerating cell is clearly recognizable. The cytoplasm has however, a homogenized appearance, is rather granular, and does not contain ergastoplasmic cysternae. The nucleus may be shrunken and pyknotic. Degeneration may be more severe in other chondrocytes and in some cases is so advanced that only cell fragments are left (Fig. 28).

Globules are to be found around these cells, almost always intermingled with membrane bounded vesicles, shreds of cytoplasm and myelinic structures. In some cases, only a cluster of globules seems to be left in the place of a completely degenerated chondrocyte (Fig. 29).

Decalcified Cartilage

Globules are slightly modified in sections from specimens which had been decalcified before embedding. Their matrix is less electron dense than in untreated sections and they sometimes resemble empty vesicles bounded by a membrane.

In the maturing zone, where globules are mingled with roundish clusters of crystals, decalcification and staining reveal globules and, in place of the crystal clusters, roundish islands of thin fibrils. On the other hand no globules are visible after decalcification in the cartilage matrix which was previously fully calcified. After decalcification, this matrix appears to be made up of loose collagen fibrils, between which irregular clusters of a fibrillary material can be found.

Histochemistry: Optical Microscopy

Little histochemical data have been obtained from thick sections and optical microscopy. This is due on the one hand to the fact that most of the globules are so small that they are beyond or at the limit of the resolving power of the microscope; and on the other hand to the fact that it is not exactly known if Araldite can prevent or change histochemical reactions.

Globules stain Magenta red after PAS staining. They stain orthochromatically with Toluidine blue and do not stain at all with Alcian blue. No globules are visible either after staining with Sudan black B.

Fig. 23 A—F. Serial sections of a chondrocyte and two globules in the zone of maturing chondrocytes. Globule *b* is not present in section A; it appears in B, increases in thickness and in electron density from B to C, decreases from D to E and is no longer visible in F. Its thickness is 2,800 Å, which corresponds to a section thickness of about 700 Å. Uranyl acetate — lead citrate. ×14,000

Fig. 24 A—F. Serial sections of an intercellular cluster of globules. The big globule on the right (*n*) begins in section A and is present in all the following photographs, providing a useful point of reference. Its diameter, which, together with the electron density, increases from A to C-D and then decreases, is about 6,250 Å (measured in D), from which a section thickness of about 900 Å may be calculated, providing that as may be supposed the globule *n* disappears just after F. Note that globules *o* and *p* are present in sections B and C but not in sections A and D; globule *q* is present in B, C and D but not in A and E; globules *r*, *s*, *t* are present in C and D, but not in B and E. Globules *a*, *d*, *i*, *k*, *m* begin in A, increase in diameter and in electron density from A to B and than decrease to disappear in D. Globules *u*, *v*, *w*, *y* behave in a similar way. Uranyl acetate — lead citrate. ×15,000

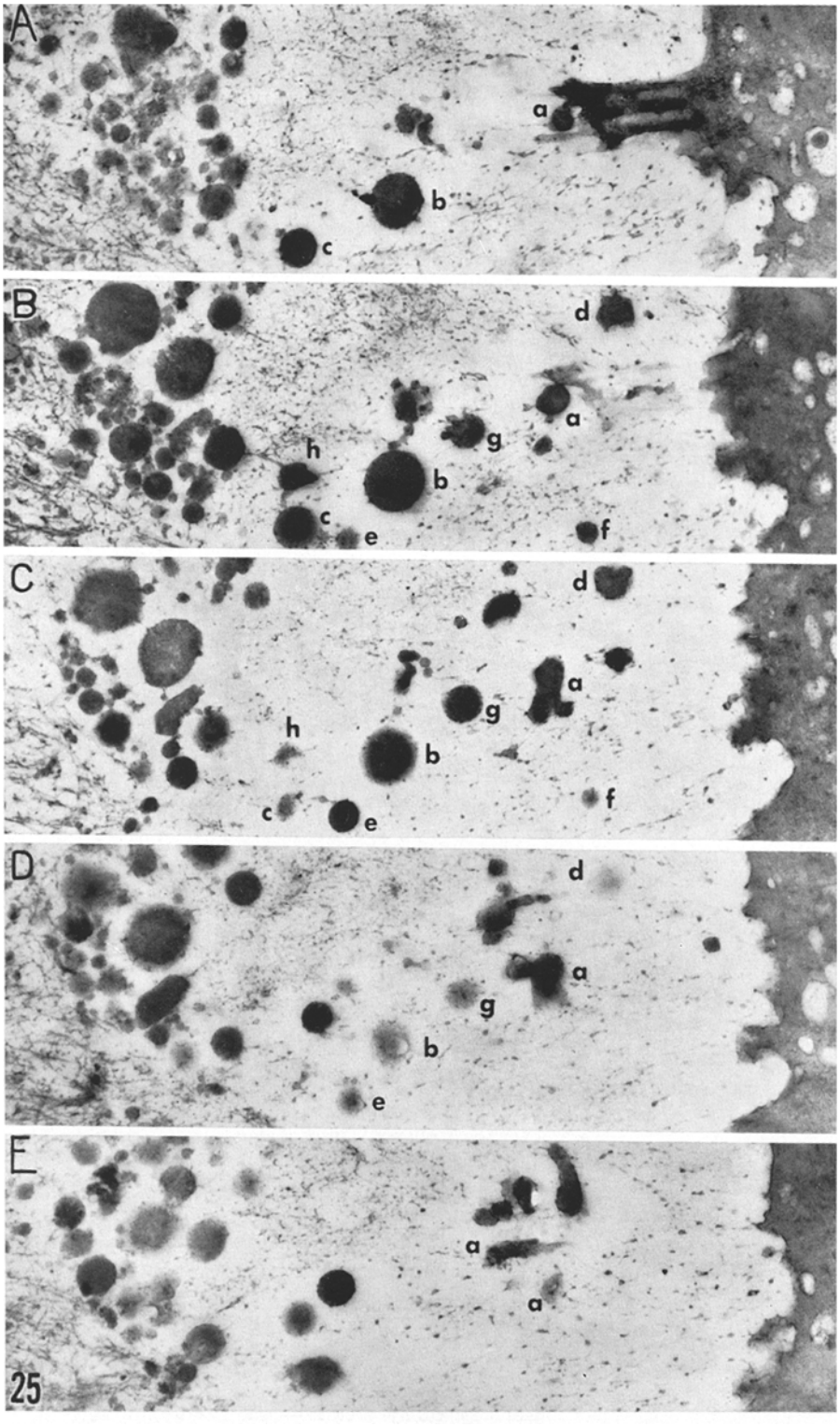


Fig. 25

Histochemistry: Electron Microscopy

Changing the buffer of the fixative and/or the dehydration method has no effect on the fine structure of the globules.

Globules in the sections from formalin fixed specimens treated with colloidal iron are not stained. Cells in the same sections are completely unstained, except for their outer surface and small granules in their lacunae. Very small clusters of iron granules are to be seen in the matrix. Between these clusters small rings of iron granules may be seen the same size as the globules. They seem to be the outer border of otherwise unstained globules.

The same staining method applied to sections from osmium fixed specimens shows the same structures as above. Here the globules are clearly recognizable, because their outer border is deeply stained by iron particles and their matrix is shown up by osmium tetroxide (Figs. 30, 31). The substance coating the outer border of the globules is stained in the same way as the outer membrane of the chondrocytes and like the rings found in sections from formalin fixed specimens. The matrix of the globules is made electron dense by its osmiophilia, but it is totally unstained by colloidal iron, except for a few scattered particles which are also to be found in the background.

When the silver nitrate-methenamine method is applied after 15′ of periodic acid oxidation, glycogen in cell cytoplasm and collagen fibrils in the matrix are stained very deeply, whereas globules are only slightly stained (Fig. 32).

Drastic digestion with papain does not make globules disappear. Chondrocytes are completely digested after 12 hours. Only residues of nuclei, myelinic figures and unidentified granular structures resembling glycogen are left in the cell lacunae. The cartilage matrix consists of "clean" collagen fibrils, forming a looser network than in control sections. Globules are easy to identify, because they are more electron dense than those in controls (Fig. 33). It is interesting to note that the calcified areas show an amorphous substance which, like the globules, is very deeply stained by uranyl acetate and lead citrate.

Hyaluronidase digestion does not change the fine structure of the globules. Colloidal iron staining of the matrix and of the border of the globules becomes negative.

Discussion

This investigation shows that the early calcium salts are deposited in the epiphyseal cartilage within pre-formed structures of round or ovoidal shape, called "globules" in the present paper. This is in agreement with previous research showing that apatite crystals are deposited not only on collagen fibrils, but also within "osmiophilic bodies" (Bonucci, 1967).

Fig. 25 A—E. Serial sections of a maturing chondrocyte and of its lacuna. Part of the chondrocyte may be seen on the right, the border of the lacuna on the left. Many globules are present in the lacuna. Globules *d*, *e*, *g* are visible in sections B, C, D but not in sections A and E; globules *f* and *h* are visible in B and C, but not in A and D; the diameter of the globules *b* and *c* gradually decreases from A to D and they are no longer visible in E. The structure marked *a* is made up of the same amorphous, electron dense substance as the globules but its shape resembles that of a cell process. Uranyl acetate — lead citrate. ×20,000

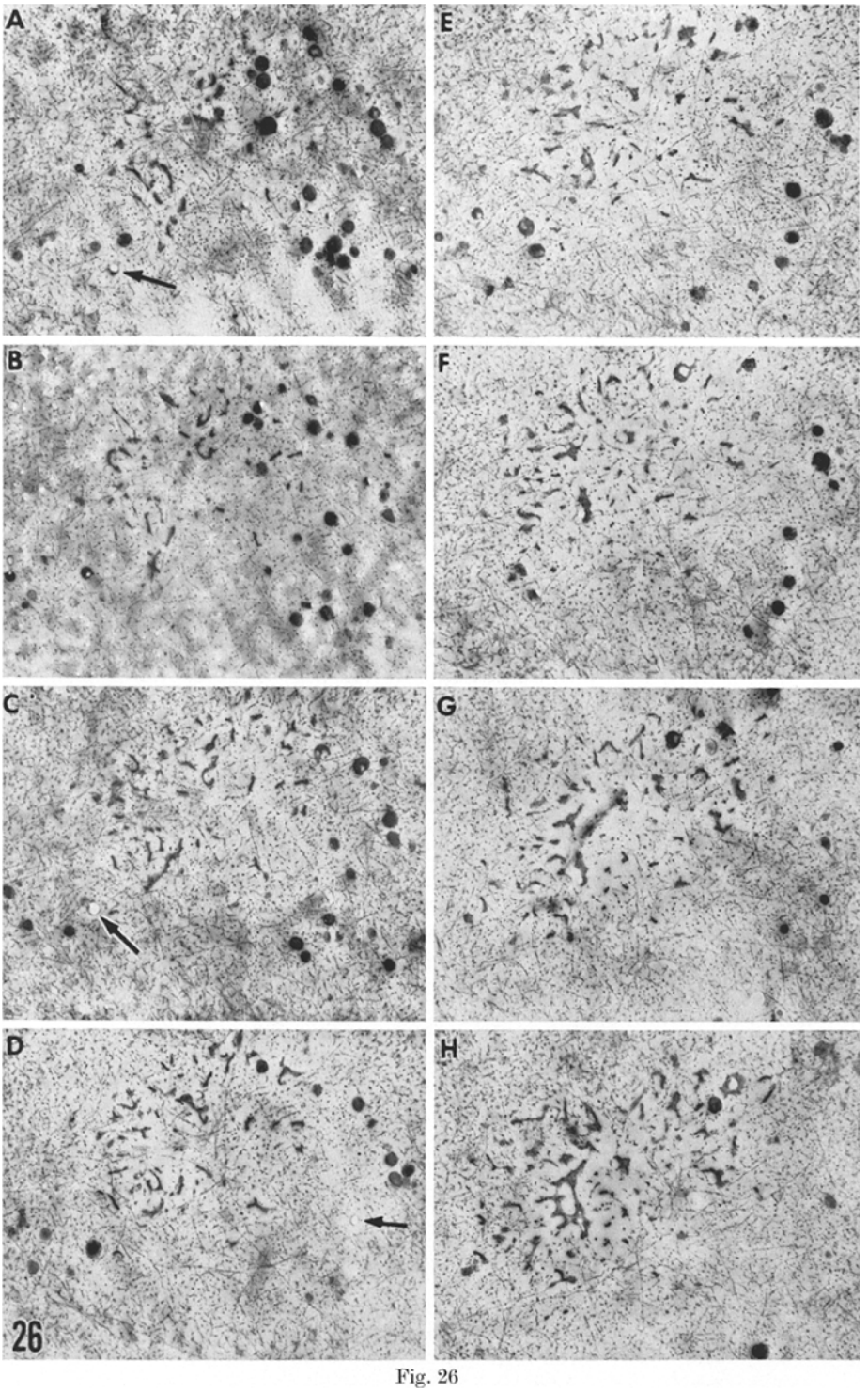


Fig. 26

Although the exact relationship between globules and collagen fibrils during the calcification process has not yet been established, calcification of collagen fibrils seems to begin later, when many globules are already mineralized. The coalescence of calcified globules and fibrils may result in fully mineralized cartilage.

Calcification seems to begin in the globules with the appearance of very small granules. Theoretically they might be cross-sectioned crystals, but this is not likely, because they are thinner and less electron dense than crystals. They are like, but smaller than, the earliest nuclei of calcium salt deposition found in collagen fibrils during bone calcification (Fitton Jackson, 1957). They do not however, give rings of electron diffraction, although they are solubilized by decalcifying solutions.

The fine structure of the globules is unchanged at this stage and it is still unchanged when the first needle-shaped crystals appear within them. But later, globules are filled with crystals and are no longer recognizable. At this point they have apparently undergone radical changes, because decalcification followed by staining, while it does not greatly change the fine structure of the previously uncalcified globules, does not reveal them in the zones previously fully calcified, where only collagen fibrils and clusters of thin fibrillary structures are recognizable (see also Bonucci, 1967, 1969).

Judging from these facts, it might be concluded that during calcification globules break off, which may perhaps cause the spreading of crystals in the adjacent matrix. It should be emphasized that the exact mechanism of crystal spreading from globules to the adjacent matrix is still to be clarified.

Since the matrix of the globules consists in a strongly calciphylactic substance, it would be very important to know, firstly the chemical structure of uncalcified globules, and secondly how this structure changes during calcification.

Globules contain a fair amount of neutral polysaccharides, as shown by the PAS reaction and by staining with silver nitrate after periodic acid oxidation. On the other hand globules do not contain acid polysaccharides. Toluidine blue, Alcian blue and colloidal iron are all negative. The lack of acid polysaccharides in the matrix of the globules is also shown by hyaluronidase digestion, which does not change the fine structure of the globules. Acid polysaccharides however, form a coat around their outer surface. This coat often appears as a dotted-line in sections stained with uranyl acetate and lead citrate and looks like the coating often found on the outer surface of the chondrocytes.

Digestion with papain does not greatly change the structure of the globules. Globules react more with uranium and lead. Probably this means that digestion

Fig. 26A—H. Serial sections through the peripherical part of a maturing chondrocyte, showing the commonest kind of spatial arrangement of the cell processes (the thin, elongated, irregular structures) and of the globules (the roundish bodies). Note that the thickness and the electron density of the globules are greater than those of the processes. The reconstruction of the serial sections shows that the cell processes are anastomized and form an intricate network, and that the globules are mainly outside it. Note that the cell lacuna is visible only in G and H, so that in the other sections (and chiefly in A) the cell processes are in the extralacunar matrix. Note also that during sectioning a few globules (indicated by the arrows) have been pulled off from the sections; this suggests that the consistency of their matrix is greater than that of the cartilage matrix. Uranyl acetate. ×9,000

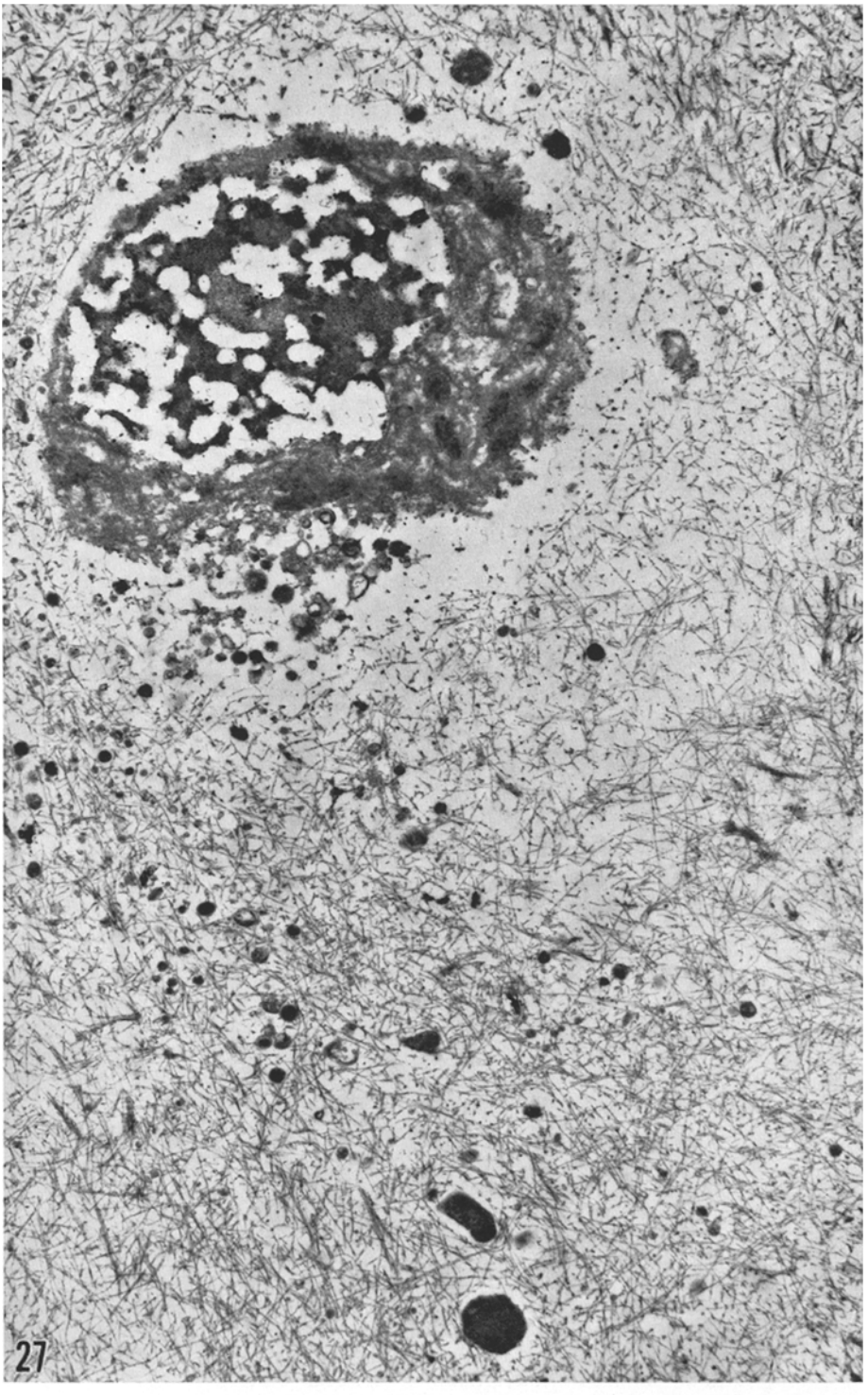


Fig. 27

of proteins does not disrupt the globules but makes many groups within globules free to react with uranium or lead.

All these findings do little to characterize the globules from a histochemical point of view. They only show that globules contain proteins and a fair amount of neutral polysaccharides and that they are surrounded by acid polysaccharides. Of course, the entire mechanism of globule change during calcification remains to be established.

On the other hand, more can be said about the origin of the globules. The presence both of a membrane around many globules, and of globules within the cell lacunae, shows that globules derive from chondrocytes. But these considerations and a further one, i.e. that globules are often adjacent or intermingled with cell processes, might be thought to suggest that globules are simply cross-sectioned cellular processes. Serial sections clearly show that they are not, globules being truly spherical or ovoidal bodies.

But if globules derive from chondrocytes, it is difficult to explain their presence in the matrix at some distance from cell lacunae. Globules are often found some way away from cells, usually from 1 to 3 microns from the lacunae, with the interposition of a sheath of matrix containing thin collagen fibrils alone. These findings, which seem to speak against globules originating directly from cells (Bonucci, 1967), will perhaps be explained only when the mechanism of globule formation is much better known.

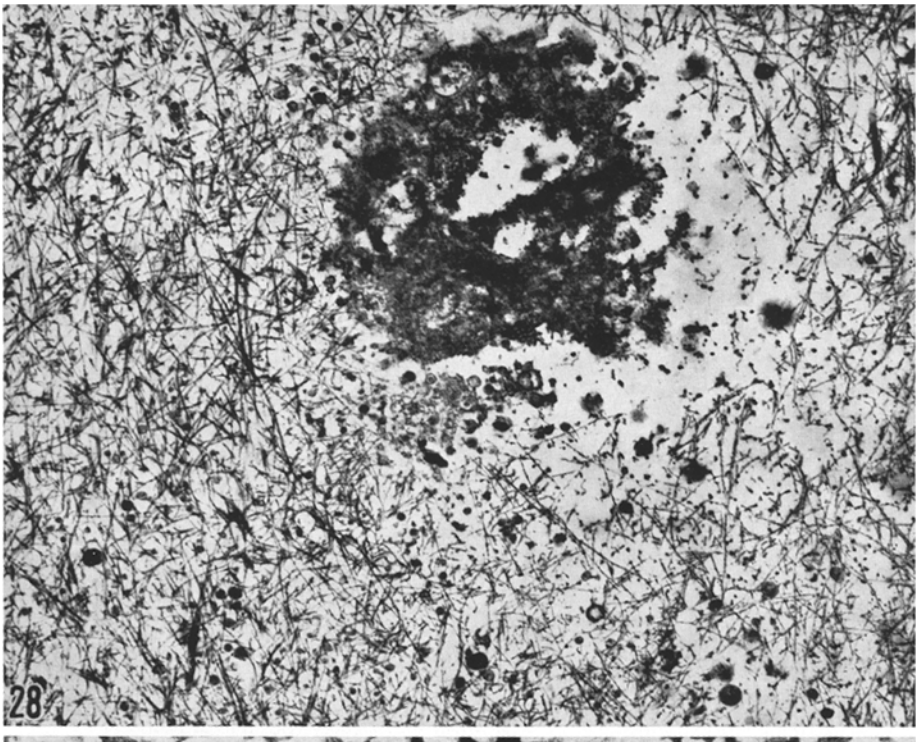
At present, at least part of the globules seems to derive from degenerating chondrocytes. Relatively little attention has been paid to these degenerating (non-hypertrophic) chondrocytes, although their presence in epiphyseal cartilage has long been known (see Schaffer, 1930).

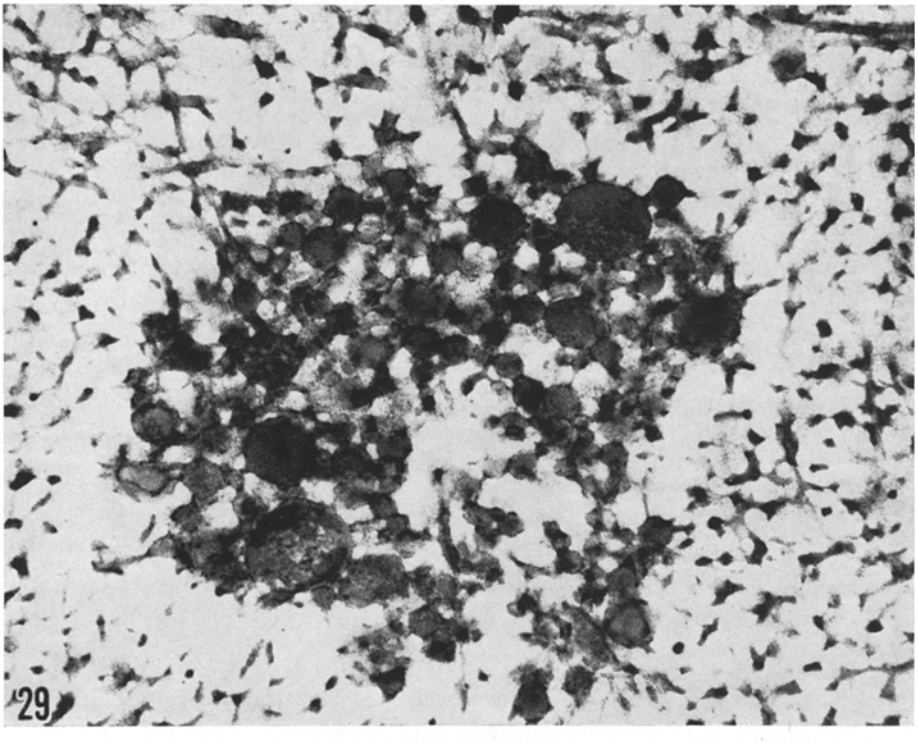
It was thought by Schaffer (1930) and others that these chondrocytes might change directly into cartilage matrix, because after their degeneration they are no longer visible and only a few granular structures are left in the matrix. The process was called "Verdämmern der Zellen", that is "vanishing of cells", and "chondromucoide Umwandlung", that is a degeneration which makes chondrocytes strongly basophilic and causes them to disappear, with formation of little drops which stain with mucicarmine (Pascher, 1923; Schaffer, 1930). Moreover, other changes in the matrix were described as "albumoid" degeneration ("albumoide Metamorphose"), characterized by the presence in the intercellular substance of small granules which contain proteins and stain like the elastic tissue (see Pascher, 1923; Amprino and Bairati, 1933).

These degenerative processes have also been described in human tracheal and costal cartilage during ageing (Amprino and Bairati, 1933). It is interesting to note that they are more frequent in those cartilage areas which will later calcify; and that the small drops and granules caused by chondromucoid and albumoid degeneration are pericellularly distributed like the globules and the first crystal clusters. More recently, degenerating chondrocytes have been described in the tibial cartilage of rat fetuses (Knese and Knoop, 1961b).

No substantial difference seems to exist between these processes of chondrocyte degeneration and those found during the present investigation. All seem to be characterized by progressive disintegration of the chondrocytes and by the formation in their place and/or around them of numerous granular and globular

Fig. 27. A degenerating chondrocyte in the resting zone. The nucleus is vacuolated, the cytoplasm contains no recognizable structures. Fragments of cytoplasm, empty vesicles and globules are all visible below the cell. Many globules are to be found around the degenerating chondrocyte and in the adjacent matrix. Uranyl acetate — lead citrate. ×7,000





Figs. 28 and 29

structures. It therefore seems possible that "Verdämmerung" and "chondromucoide Umwandlung" of the chondrocytes, and "albumoide Metamorphose" of the cartilage matrix are similar processes which lead to formation of the structures called "globules" in the present paper. Why non-hypertrophic chondrocytes degenerate is not at all clear. It is interesting to observe that degeneration often involves only one cell in a group of closely adjacent chondrocytes.

It seems obvious that the process of chondrocyte degeneration cannot explain the formation of all the globules found in the epiphyseal plate, because many of them surround perfectly preserved chondrocytes. Globules are moreover occasionally found in the lacunae of normal chondrocytes, where they are in more or less direct relationship with the cell processes.

Chondrocyte processes have not received great attention. However, on the basis of an electron microscope investigation (Tousimis and Follis, 1958), Follis (1960) stated that he was impressed by "the prominent extensions of the cytoplasm out into the surrounding matrix". The present investigation shows that the chondrocyte processes may be very long, that they may reach the adjacent matrix and form an intricate network around the chondrocytes.

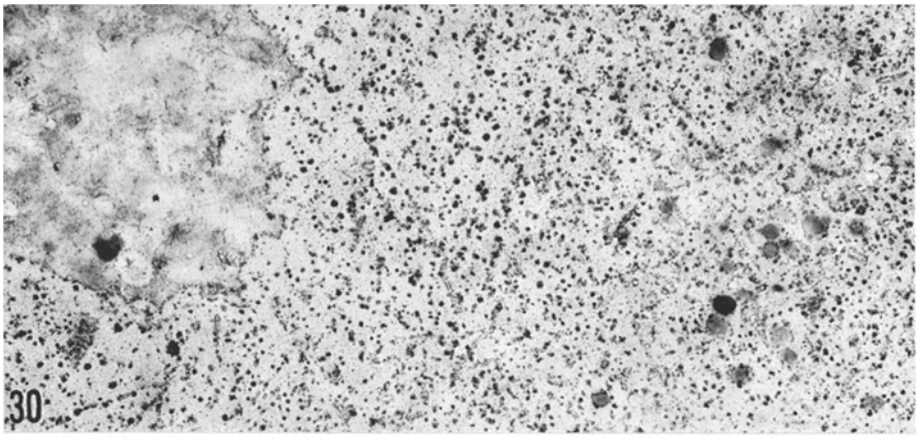
Globules may be found at the end of cell processes, or intermingled with them; the tip of the processes may moreover be swollen and of bulb-like shape. These patterns suggest that an intimate relationship exists between globules and cell processes. Besides, the occasional bulb-like appearance of the tip of the processes and the presence of globules near or in contact with the processes might be the morphological counterpart of the mechanism of globule formation, involving swelling of the tip of the processes and its successive detachment. This would explain the presence of globules along the line of the major axis of the cell processes and, in the proliferating zone, along the line of the major axis of the cells. This would also explain why globules are never to be found within the chondrocytes.

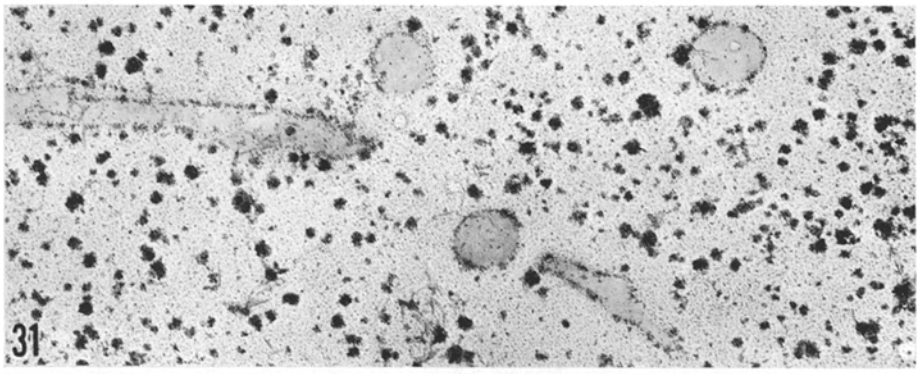
Structures of irregular shape, but with a globule-like matrix, are sometimes found within the lacunae. These structures are frequently placed between a chondrocyte or a chondrocyte process and one or more globules. Their shape resembles that of a segment of a cell process, their matrix that of a globule. These structures may possibly be true fragments of cell processes which break off from the cells, gradually acquire a homogeneous matrix like that of the globules, and in the end give rise to real globules.

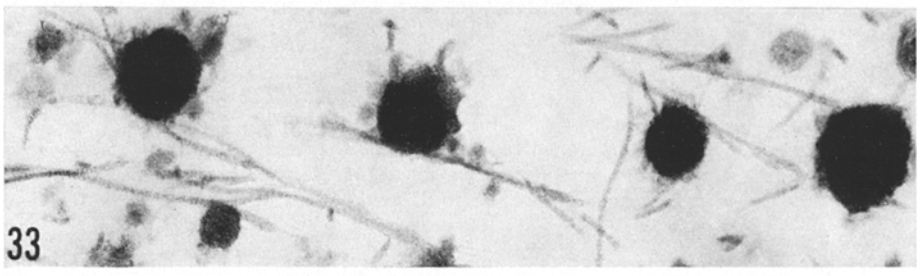
From these considerations, the hypothesis might be advanced that the globules are formed both from fragments of cytoplasm of degenerating chondrocytes, and from fragments of cell processes. However, the relative rarity of intralacunar globules and of globules in contact with the tip of the cell processes, and the difficulty of establishing with morphological, hence static, means the steps of a dynamic process, make further investigation into the origin of globules advisable,

Fig. 28. A degenerating chondrocyte in the resting zone. No recognizable structures are to be found in the cell, whose lacuna is still visible on the right. Many globules are present around the chondrocyte and in the adjacent matrix. Uranyl acetate — lead citrate. ×10,000

Fig. 29. A cluster of globules in the resting zone. This picture might show the last phase of a chondrocyte "Verdämmerung" (see the text). Uranyl acetate — lead citrate. ×56,000







Figs. 30—33

before one accepts unreservedly the above reported considerations. In any case, it seems established that globules are of cellular origin, as indicated above all by the presence of a membrane around them. This explains the pericellular distribution of the globules, although it remains to be ascertained why globules and chondrocytes are so often separated by a sheath of cartilage matrix. It may be suggested either that the cell processes penetrate the pericellular matrix, so that part of the globules may be formed directly outside the cell lacuna (as suggested for instance in Figs. 16 and 26), or that the globules which are formed within the lacunae remain between the preexisting matrix and the matrix secreted later.

Descriptions of intercellular structures resembling the globules reported in the present paper can be found in the literature. Palfrey and Davies (1966) seem to be talking about aggregates of globules when, in describing the fine structure of degenerating chondrocytes in the deep zone of femural articular cartilage, they mention dense bodies and vesicles. Ghadially *et al.* (1965), too, describe globule-like bodies, in this case near degenerating chondrocytes. Barnett *et al.* (1963) and Matukas and Krikos (1968) also found dense bodies near areas of calcifying cartilage.

The most interesting findings seem to be those reported by Anderson (1968, 1969) based on studies on the epiphyseal plate of mice. This author describes membrane bounded structures of variable shape which contain material of varying density sometimes but not often including ribosomes. These structures, called "vesicles" and considered to be of cellular origin, are to be found in juxtaposition to apatite crystals. Vesicles are considered to play a role in initiating calcification in the epiphyses.

It seems that the "globule" described here does not differ substantially from Anderson's "vesicle". Both contain apatite crystals; both are bounded by a membrane and are distributed in the intercolumnar longitudinal bars. Globules seem however to have a much more electron dense matrix than that of the vesicles. This difference may perhaps be due to different methods of tissue preparation. Moreover, vesicles would seem to contain cellular organelles at all levels in the epiphyseal plate, while globules show a cytoplasm-like structure only occasionally and limitedly to the resting zone. The possibility might be considered that the vesicles containing cellular organelles correspond to obliquely or cross sectioned cellular processes. Yet another difference exists between globules and vesicles, in as far as the latter are also found after

Fig. 30. Section stained with colloidal iron. Many small iron clusters are present in the matrix, but not in the cell (top left). To the right, a group of globules whose osmiophilic matrix is unstained. Iron deposition is visible around the globules and on the outer surface of the chondrocyte. ×14,000

Fig. 31. Section stained with colloidal iron. A chondrocyte process (left), three globules, and another cell process (bottom right) are shown. Small clusters of iron granules are present in the cartilage matrix. The outer border of the globules and of the cell processes is also stained, but no iron deposition is visible in the globule matrix nor in the cytoplasm, except for a few granules which are equally frequent in the background. ×40,000

Fig. 32. Section stained with silver nitrate after periodic acid oxidation. The black dots in the chondrocyte (left) are glycogen particles. Collagen fibrils are deeply stained. Globules (right) are stained, but less than glycogen and collagen fibrils. ×16,000

Fig. 33. Section digested with papain and stained with uranyl acetate and lead citrate. Globules are not destroyed, but are much more stainable than those in controls. ×70,000

decalcification in previously calcified zones, while globules are not. This point is rather intriguing, because both in this study and in previous ones (Bonucci, 1967, 1969) neither globules nor vesicles have been found in the *fully* calcified cartilage matrix after its decalcification. Nor are vesicle- or globule-like structures reported by Scherft (1968) in his investigation into the fine structure of decalcified cartilage. If we exclude the possibility of differences between the types of animals used in the experiments, it must be admitted either that globules and vesicles are different structures, or that the decalcified areas containing vesicles were previously not fully calcified, so that vesicles were present in the still uncalcified zones. It is in fact known that the calcified longitudinal bars of the epiphyseal cartilage may contain a central core of incompletely calcified matrix even after the beginning of ossification at the metaphyseal level (Cameron, 1963).

In any case, the previous (Bonucci, 1967) and the present investigation, and those of Anderson (1968, 1969) are in full agreement in considering that structures other than collagen fibrils are involved in initiating calcification in the epiphyseal cartilage.

References

Amprino, R., Bairati, A.: Studi sulle trasformazioni delle cartilagini dell'uomo nell'accrescimento e nella senescenza. Z. Zellforsch. **20**, 143—205 (1933).

Anderson, D. R.: The ultrastructure of elastic and hyaline cartilage of the rat. Amer. J. Anat. **114**, 403—433 (1964).

Anderson, H. C.: Vesicles in the matrix of epiphyseal cartilage: fine structure, distribution and association with calcification. In: Electron microscopy 1968 (D. S. Bocciarelli, ed.). Fourth Europ. Reg. Conf. Electr. Microsc. Rome: Tipografia Poliglotta Vaticana 1968.

— Vesicles associated with calcification in the matrix of epiphyseal cartilage. J. Cell Biol. **41**, 59—72 (1969).

Barnett, C. H., Cochrane, W., Palfrey, A. J.: Age changes in articular cartilage of rabbits. Ann. rheum. Dis. **22**, 389—400 (1963).

Bonucci, E.: Fine structure of early cartilage calcification. J. Ultrastruct. Res. **20**, 33—50 (1967).

— Further investigation on the organic/inorganic relationships in calcifying cartilage. Calc. Tiss. Res. **3**, 38—54 (1969).

Cameron, D. A.: The fine structure of bone and calcified cartilage. Clin. Orthop. **26**, 199—228 (1963).

Fitton Jackson, S.: The fine structure of developing bone in the embryonic fowl. Proc. roy. Soc. B. **146**, 270—280 (1957).

— Fibrogenesis and the formation of matrix. In: Bone as a tissue (K. Rodahl, J. T. Nicholson and E. M. Brown, eds.). New York: McGraw-Hill Book Co. 1960.

Follis, R. H., Jr.: Calcification of cartilage. In: Calcification in biological systems (R. F. Sognnaes, ed.). Washington: Amer. Ass. Adv. Sci. 1960.

Ghadially, F. N., Meachim, G., Collins, D. H.: Extra-cellular lipid in the matrix of human articular cartilage. Ann. rheum. Dis. **24**, 136—146 (1965).

Ham, A. W.: Histology. London and Philadelphia: Pitman Medical Publishing Co. and J. B. Lippincott Co. 1965.

Knese, K.-H., Knoop, A.-M.: Über den Ort der Bildung des Mukopolysaccharid-Proteinkomplexes im Knorpelgewebe. Elektronenmikroskopische und histochemische Untersuchungen. Z. Zellforsch. **53**, 201—258 (1961a).

— — Chondrogenese und Osteogenese. Elektronenmikroskopische und lichtmikroskopische Untersuchungen. Z. Zellforsch. **55**, 413—468 (1961b).

Marinozzi, V.: Silver impregnation of ultrathin sections for electron microscopy. J. biophys. biochem. Cytol. **9**, 121—133 (1961).

Matukas, V. J., Krikos, G. A.: Evidence for changes in protein polysaccharide associated with the onset of calcification in cartilage. J. Cell Biol. **39**, 43—48 (1968).

— Panner, B. J., Orbison, J. L.: Studies on ultrastructural identification and distribution of protein-polysaccharide in cartilage matrix. J. Cell Biol. **32**, 365—377 (1967).

Palfrey, A. J., Davies, D. V.: The fine structure of chondrocytes. J. Anat. (Lond.) **100**, 213—226 (1966).

Pascher, M.: Zur Kenntnis der Altersveränderungen in den menschlichen Kehlkopfknorpeln, insbesondere der körnigen Entartung der Knorpelgrundsubstanz, der Vascularisations-, Resorptions- und Verknöcherungsbefunde. Virchows Arch. path. Anat. **246**, 198—238 (1923).

Rambourg, A.: An improved silver methenamine technique for the detection of periodic acid — reactive complex carbohydrates with the electron microscope. J. Histochem. Cytochem. **15**, 409—412 (1967).

Robinson, R. A., Cameron, D. A.: Electron microscopy of cartilage and bone matrix at the distal epiphyseal line of the femur in the newborn infant. J. biophys. biochem. Cytol. **2**, 253—260 (1956).

Schaffer, J.: Die Stützgewebe. In: Handbuch der mikroskopischen Anatomie des Menschen (W. v. Möllendorff, ed.), Bd. II, H. 2. Berlin: Springer 1930.

Schenk, R. K., Spiro, D., Wiener, J.: Cartilage resorption in the tibial epiphyseal plate of growing rats. J. Cell Biol. **34**, 275—291 (1967).

Scherft, J. P.: The ultrastructure of the organic matrix of calcified cartilage and bone in embryonic mouse radii. J. Ultrastruct. Res. **23**, 333—343 (1968).

Scott, B. L., Pease, D. C.: Electron microscopy of the epiphyseal apparatus. Anat. Rec. **126**, 465—495 (1956).

Stäubli, W.: A new embedding technique for electron microscopy, combining a water-soluble epoxy resin (Durcupan) with water-insoluble Araldite. J. Cell Biol. **16**, 197—201 (1963).

Takuma, S.: Electron microscopy of the developing cartilagenous epiphysis. Arch. oral Biol. **2**, 111—119 (1960).

Tousimis, A. J., Follis, R. H., Jr.: Studies on the ultrastructure of rat epiphysial cartilage. Fed. Proc. **17**, 460 (1958).

Dr. Ermanno Bonucci
Istituto di Anatomia Patologica
Policlinico Umberto I
Viale Regina Elena 324
00161 Roma (Italia)