THE FINE STRUCTURE OF OSTEOBLASTS IN THE METAPHYSIS OF THE TIBIA OF THE YOUNG RAT

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ABSTRACT

The appearance of osteoblasts after fixation with OsO_4 is described in this paper. They have the basic structures found in other types of cells. The most striking feature is the array of rough-surfaced membranes of the endoplasmic reticulum; this feature is in keeping with the osteoblast's function of producing collagen as the bone grows. The sacs formed by these membranes probably represent the protein-containing granules described by other workers using the light microscope. They contain fine fibrillary material, and similar fibrils are to be found free in the cytoplasm. These fibrils could be tropocollagen units, although fibrils recognizable as collagen by their structure are found only outside the cell. The arrangement of the cell organelles does not seem to be related to the formation of collagen, but correlation of the fine structures of the cells with the histochemical and cytochemical findings in these cells reported by other workers leaves no doubt that they are directly concerned in the production of the organic matrix. It has not been possible to show that osteoblasts influence the passage of calcium or phosphate ions from the blood to the bone matrix.

INTRODUCTION

The growing end of a long bone is an excellent site for the study of osteoblasts. A considerable number of these cells are engaged there in the rapid formation of new tissue and are in an orderly array as they follow the capillaries which invade the epiphysis. The appearance of the cells in this and other areas of postfetal osteogenesis and in the embryo in a number of species is well documented (Fell, 1925; Heller *et al.*, 1950; Pritchard and Ruzicka, 1950; Pritchard, 1952, 1956). Similarities between the cells in these various sites, in both appearance and behaviour, have led to the placing of all osteoblasts in one group and regarding them as belonging to a specialized branch of the larger family of fibrogenic connective tissue cells (Pritchard, 1956).

The fine structure of bone has been recorded by a number of authors, beginning with Wolpers in 1949, but many of them have dealt with the bone matrix rather than with the cells involved in its formation (Robinson, 1952; Robinson and Watson, 1952; Robinson and Cameron, 1957; Molnar, 1959). A general description of the process of lengthening of a long bone has been reported (Robinson and Cameron, 1956, 1958), with only passing reference to the cells. Information about the fine structure of osteoblasts in the metaphysis is confined to the papers of Scott and Pease (1956), Sheldon and Robinson (1957), and Durning (1958). Only Fitton Jackson (1957) has described osteoblasts in any other site of bone formation.

This report describes osteoblasts in the metaphysis, where probably all these cells are mature and the cell population is stable as indicated by the fact that dividing cells have not been found.

MATERIALS AND METHODS

The material was obtained from albino rats 4 to 8 weeks old, weighing 35 to 70 gm., which were killed

by a blow on the neck. The tibiae were removed, the proximal ends cut through in a coronal plane with a razor blade, and buffered osmium tetroxide (Caulfield, 1957) was applied to the exposed surface immediately. A slice about 0.5 mm. thick was then transferred to a welled slide containing fixative at 0°C.; more fixative was applied to the freshly exposed surface and another slice cut. The slices were trimmed while still in the welled slide and then fixed in small bottles at 0°C. for 1 to 2 hours. Every effort was made to expose the tissue to fixative as soon as possible, but up to 5 minutes might elapse between killing the animal and immersing the last piece of tissue in the fixative. The material was not decalcified. After fixation, the tissue was washed briefly in water and dehydrated in increasing concentrations of acetone in water at 0°C. Final dehydration was carried out in acetone alone at room temperature. The tissue was transferred to a mixture of one part acetone and one part methacrylate monomer, then into monomer alone, and finally into a partly polymerized mixture (Borysko, 1956), where it was left overnight. The embedding mixture was made of 1 part methyl methacrylate and 9 parts butyl methacrylate to which 0.5 per cent benzoyl peroxide had been added. A modification of the flat-embedding technique (Borysko and Sapranauskas, 1954) was used and the methacrylate polymerized at 60°C. (Borysko, 1956) for 48 hours. Sections were cut on either a Farrant and Powell (1957) or a Porter-Blum microtome using diamond knives (Fernández-Morán, 1953). They were picked up on carbon-coated grids, stained (except for that shown in Fig. 4) with lead hydroxide (Watson, 1958; Peachey, 1959), and examined with a Philips EM 100B electron microscope at 60 or 80 kv.

OBSERVATIONS

The osteoblast has a variable shape which may be modified by its position (Figs. 1 to 4). It has

FIGURE 1

Osteoblasts closely packed near the edge of a spicule of bone. The cell on the left is separated from the calcified tissue by a layer of loosely arranged collagen fibrils (*A*). The zone of smooth-surfaced membranes of the endoplasmic reticulum (*B*) is clearly distinguishable from the rough-surfaced variety. The latter are in a spiral pattern at *C*, and there are many dilated sacs at *D*. A number of mitochondria (*E*) are at the border of the smooth-surfaced endoplasmic reticulum. Dense secretory granules can be seen at *F*. The smooth-surfaced membranes lie near the nucleus in the cell on the right. Small cytoplasmic processes (*G*) lie between the cells. The narrow zone (*H*) at the periphery of the cell is free of organelles. × 7,000.

FIGURE 2

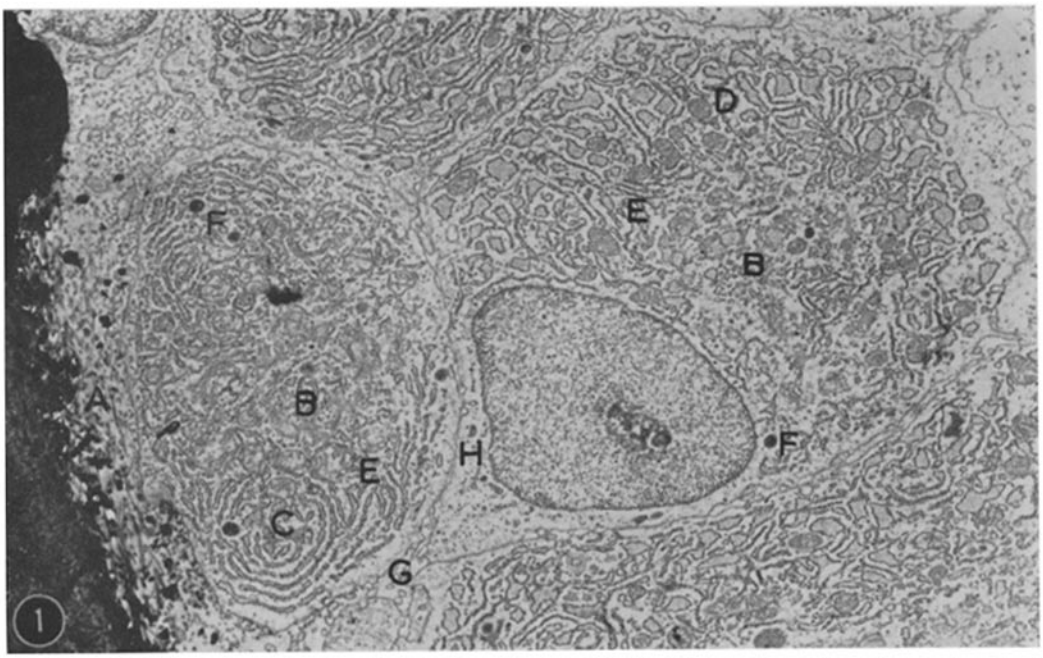
A fusiform osteoblast with a laminated system of flattened rough-surfaced sacs (*A*) at one pole. The zone of smooth-surfaced membranes (*B*) again lies near the nucleus, and there are a number of mitochondria surrounding it. Cytoplasmic processes lie both between the cells (*C*) and among the collagen fibrils (*D*) at the edge of the calcified tissue. The nucleus has varying concentrations of dense material inside its limiting membrane, and the nucleolus is prominent. The narrow zone (*E*) at the periphery of the cell is free of organelles. × 7,000.

FIGURE 3

The elongated appearance, pseudopodia, and degree of separation of all the cells lying between the capillary endothelium (*A*) and the bone (*B*) could be interpreted as demonstrating movement. There is also very little collagen between the cells and the calcified matrix. × 3,000.

FIGURE 4

This figure further illustrates the great variability in the shape of osteoblasts. This cell has one surface closely applied to the underlying spicule of bone at the left and has an extension lying under a neighbouring cell. The other surface is relatively free and the whole appearance is one of movement. The section represented in this figure was not stained. × 4,000.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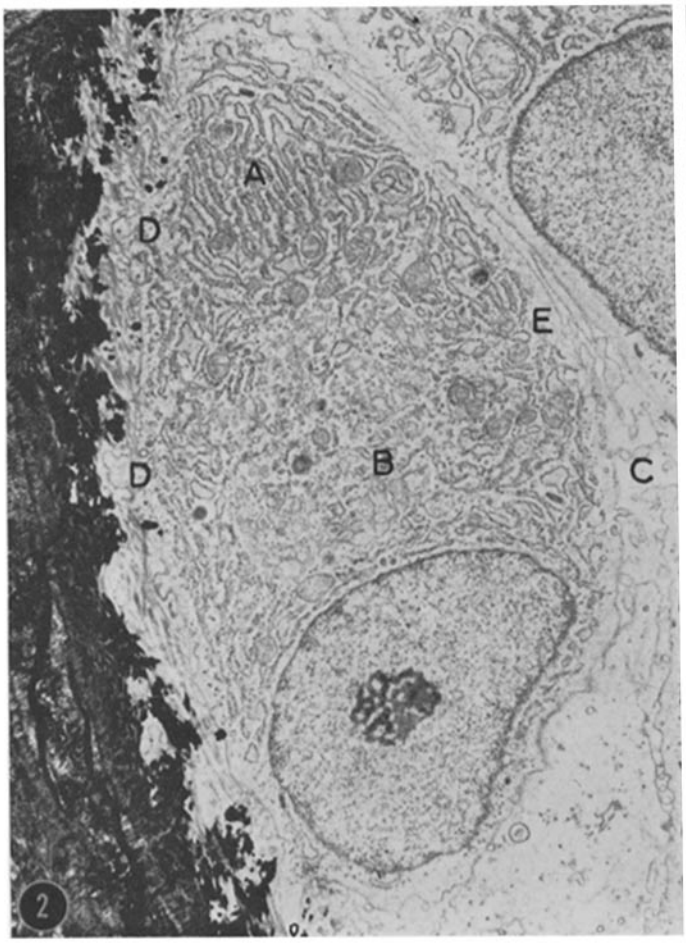
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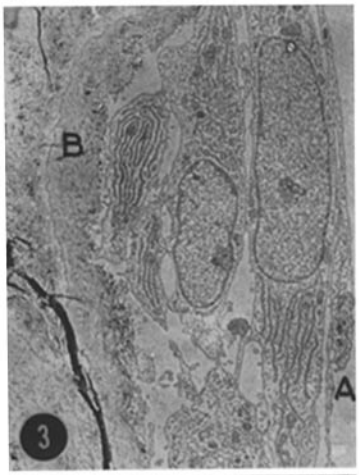
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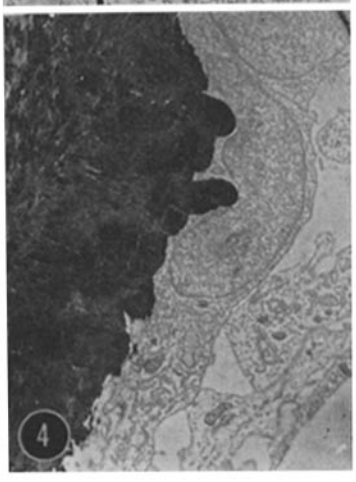
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the usual components which have been described in other cells, and its endoplasmic reticulum is well organized. Its rough-surfaced membranes are fairly well separated from the smooth-surfaced variety which are found in a zone near the nucleus. Mitochondria are found mainly among the rough-surfaced membranes; in some cells they may be arrayed at the margin of the area of the smooth-surfaced membranes.

The nucleus tends to be eccentric in the cell, and the nucleolus is usually prominent.

Cell Surface Membrane

The cell surface membrane is a continuous membrane which, in common with other membranes in the cell, can be shown in appropriate sections to be composed of two closely applied dense lines (Fig. 6). It is separated from the endoplasmic reticulum by a cortical zone up to 0.5 micron in width (Figs. 1 and 2) which may contain very fine fibrils (Figs. 8 and 21). Many osteoblasts have cytoplasmic processes, some of which may mingle with those of a neighbouring cell (Figs. 1 and 2) or be partly embedded in the forming osteoid (Fig. 2). In some areas the extensions resemble pseudopodia and may contain organelles (Fig. 7). No specialized areas of the cell surface, such as desmosomes, were seen.

The Endoplasmic Reticulum

The *rough-surfaced membranes* are the most striking components of the cytoplasm (Figs. 1, 2, 11, and 12). Limiting membranes (Figs. 8 and 9), studded on their outer surface with small particles, enclose part of the cytoplasm; in any one section this compartment is seen as a collection of flattened or dilated cisternae. The cisternae or sacs, however, can be shown in serial sections to be continuous with one another throughout much of the cytoplasm.

FIGURE 5

Part of an osteoblast showing a mitochondrion and rough-surfaced membranes of the endoplasmic reticulum. Collagen fibrils cut in cross-section lie on the left of the cell surface membrane. × 56,000.

FIGURE 6

a, cell surface membrane; *b*, a membrane of the endoplasmic reticulum; *c* and *d*, mitochondrial membranes. In each case there is a suggestion that the membranes have two components (arrows). × 140,000.

FIGURE 7

Parts of two osteoblasts. *A*, pseudopodia; *B*, small cytoplasmic processes; *C*, dilated sacs and *D*, flattened sacs formed by the rough-surfaced membranes. *E*, closely packed granules in the nucleolus. The calcified matrix lies on the left of the figure. × 13,000.

FIGURE 8

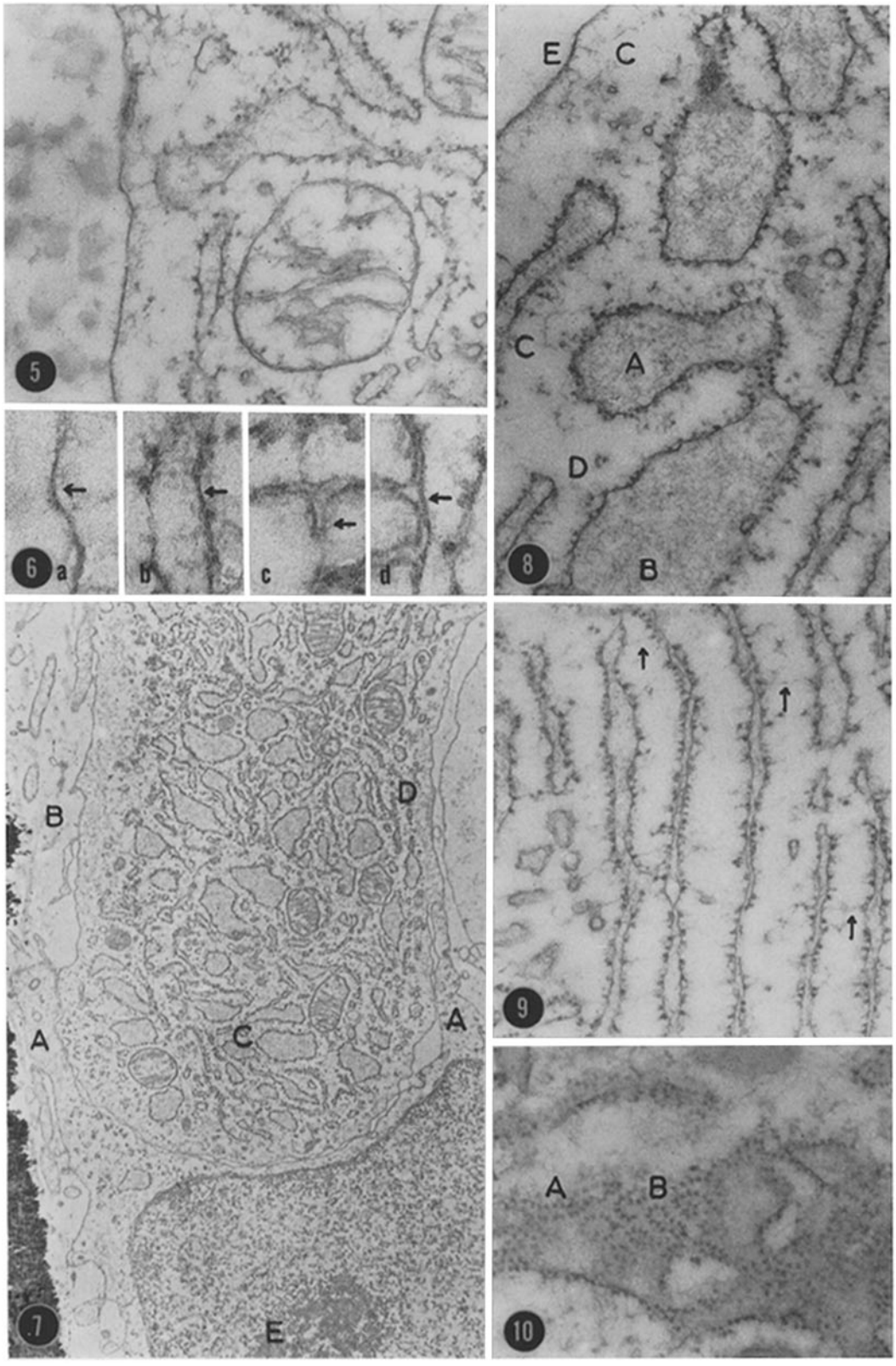
Part of an osteoblast. Ribonucleoprotein (RNP) particles are located on the outer surface of the membranes forming the rough-surfaced sacs of the endoplasmic reticulum. The sacs contain fine fibrils (*A*) and small, faintly stained round particles (*B*). There are also fine fibrils (*C*) and isolated groups of particles (*D*) in the rest of the cytoplasm. The cell membrane is at *E*. × 56,000.

FIGURE 9

Flattened sacs formed by the rough-surfaced membranes in parallel array. Fibrils run between the sacs, some apparently between the RNP particles (arrows). × 56,000.

FIGURE 10

RNP particles in chains (*A*) and spirals (*B*) on the outer surface of a sac. Fibrillary material lies both inside and outside the sac. × 56,000.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The arrangement of the sacs varies in different parts of the cell. In some places they appear in a laminated array of parallel sheets (Figs. 2, 9, and 11) or are sometimes disposed in a spiral fashion (Fig. 1). In other sites many of them are dilated into irregular shapes (Figs. 1 and 7) and may be segregated towards the periphery of the cell (Fig. 12). There is no definite evidence that they are connected with the extracellular space, although occasionally a sac appears to be in contact with the cell surface membrane (Fig. 12).

Within the sacs there are both small granules and fibrillary material (Fig. 8). These granules are about the same size as, but less dense than, the ribonucleoprotein (RNP) particles which are profusely distributed in chains and spirals on the outer surface of each membrane (Fig. 10). Most of the sacs are well separated from one another; the intervening space may contain isolated groups of RNP particles (Fig. 21). Sometimes narrow fibrils connect the adjacent sacs (Fig. 9) or lie between them (Fig. 13).

The *smooth-surfaced membranes* stand out quite clearly in a circumscribed area (Figs. 1 and 2) usually near the nucleus. The main components appear as cross-sections of tubes of varying diameter (Figs. 14 and 15), but there are also collapsed sacs which are sometimes arranged close together in a laminated fashion. They may be associated with small dilated sacs or vesicles (Fig. 14). Between the membranes there are occasional clusters of small particles (Fig. 15), fine fibrils (Fig. 15), some rough-surfaced sacs, and large, round dense structures (Fig. 14). The last are also to be seen in other parts of the cytoplasm (Figs. 1 and 11) and may have a finely granular pattern or be composed of closely packed alternating layers of dense and less dense material (Figs. 16 and 17). Rarely are they found impinging on the cell surface membrane.

FIGURE 11

One end of an elongated osteoblast showing a parallel array of flattened rough-surfaced sacs on the left, and part of the zone of smooth-surfaced membranes on the right. Secretory granules at *A*. The nucleus lies beyond the right side of the figure, and the edge of the calcified tissue is at *B*. × 13,000.

FIGURE 12

Flattened and dilated rough-surfaced sacs. The dilated sacs are segregated towards the periphery of the cell and one of them is in contact with the cell membrane at *A*. × 13,000.

FIGURE 13

Fibrils (arrows) in the cytoplasm outside the sacs. × 56,000.

FIGURE 14

The zone of smooth-surfaced membranes. The main component is in the form of tubes (*A*). Flattened membranes (*B*) and vesicles (*C*) probably represent the Golgi complex. Secretory granule at *D*. Rough-surfaced membranes at *E*. × 28,000.

FIGURE 15

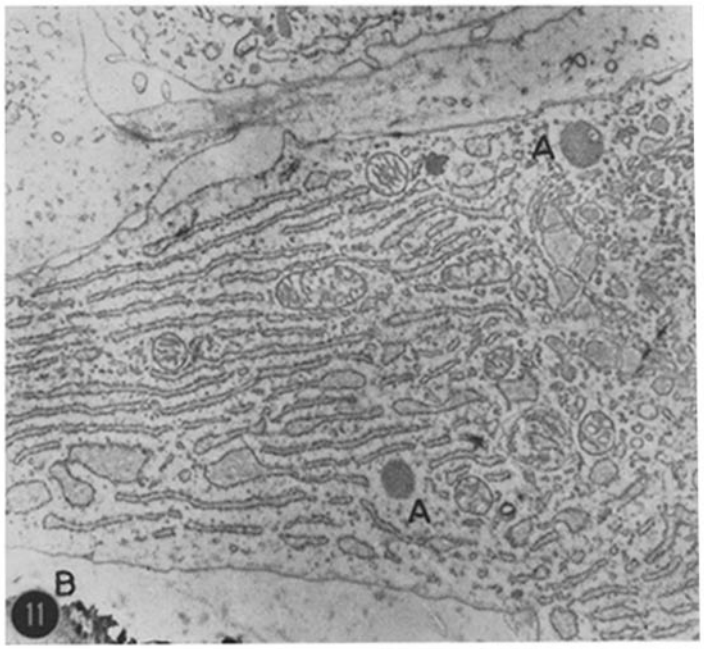
The zone of smooth-surfaced membranes. *A*, small particles in groups. *B*, fine fibrils. *C*, tubes. *D*, flattened sacs. *E*, dilated sacs. *F*, rough-surfaced membranes. × 28,000.

FIGURE 16

Granule, possibly secretory, among the rough-surfaced membranes. × 56,000.

FIGURE 17

Granule with a different appearance from that in Fig. 16. The upper left segment is finely laminated. × 42,000.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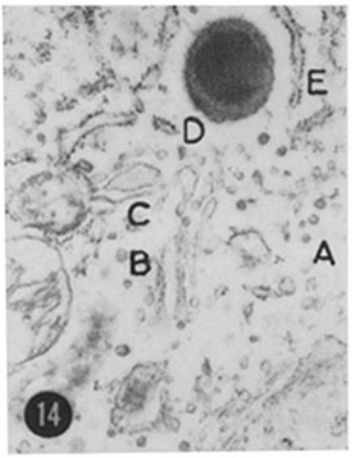
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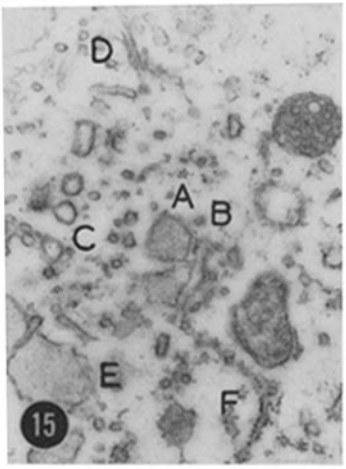
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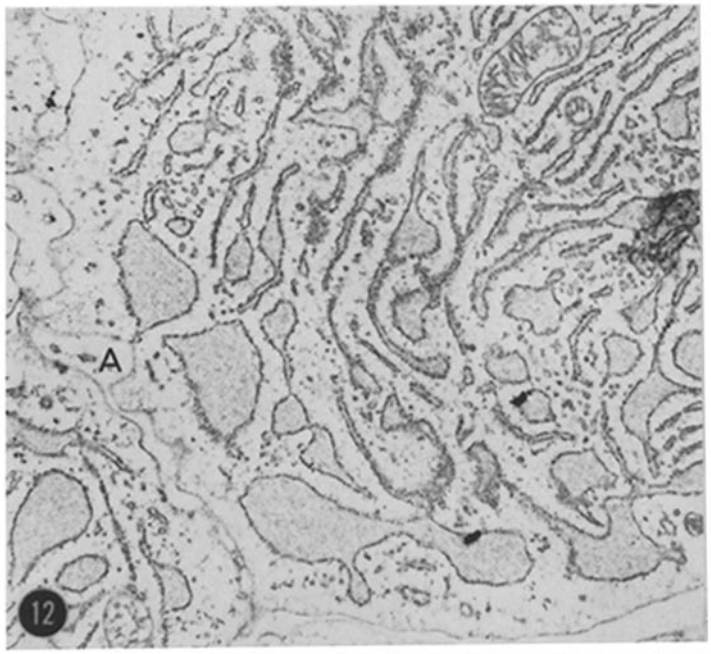
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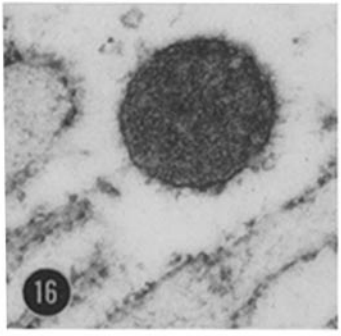
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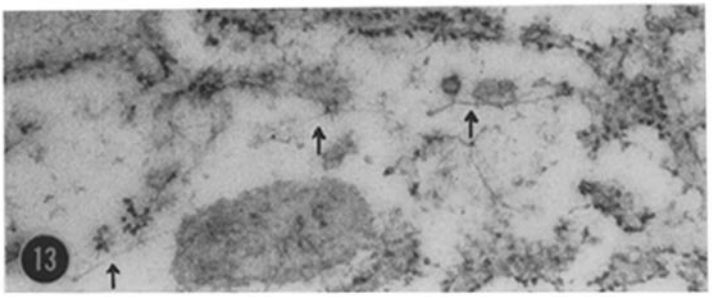
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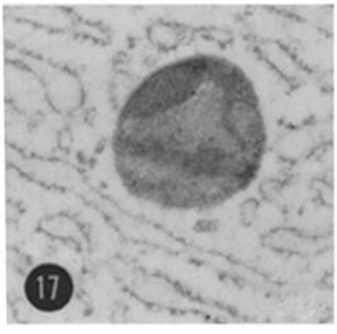
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The Mitochondria

The mitochondria usually appear round or oval in shape (Figs. 5, 11, and 18), but may be elongated and branched. They are bounded by an outer and an inner membrane separated by a less dense zone about 80 A in width (Fig. 5). The cristae are similarly formed and may or may not be continuous with the inner limiting membrane.

The Nucleus

The nucleus is surrounded by two membranes (Figs. 18 and 19). Nuclear pores can only occasionally be seen (Figs. 18, 19, and 20), but the alternating concentrations of nuclear material which flank the pores (Watson, 1959) are frequently present. The outer membrane may be covered with RNP particles (Figs. 18 and 19). A nucleolus appears as an irregular pattern of closely packed particles (Fig. 7); the remainder of the nucleus contains loosely arranged particles and fibrils (Fig. 18) rather like those in the cytoplasm.

DISCUSSION

In the developing metaphysis, osteoblasts can most simply be defined as those cells adjacent to forming bone. They lie between the bone matrix and the endothelial cells of a capillary and can b distinguished from the latter mainly by their extremely well developed endoplasmic reticulum. The cells may form a closely knit mass (Fig. 1) and may be in almost continuous contact with one another. Where the line of contact is broken, there are often small processes extending into the intervening space (Figs. 1 and 2). In some regions the cells are more widely separated and their outline and pseudopodia suggest movement (Figs. 3 and 4). Since the capillaries are growing into the epiphysis, it appears that osteoblasts must follow unless they are formed by differentiation from endothelial cells or by division of osteoblasts at the site. However, no evidence of cells intermediate between the two types or of mitosis has been found.

Although the shape of the cell varies, the cytoplasmic contents seem to show little change that can be related definitely to the state of the cell, *i.e.* whether it is apparently moving or stationary, or whether it is at a site where collagen is being produced or not. Sometimes when the cell is elongated, the rough-surfaced endoplasmic reticulum is layered in parallel sheets at one pole (Figs. 2 and 11); however, there may equally well be a collection of flattened and dilated sacs arranged in no recognizable pattern (Figs. 1 and 7). The membranes may become aligned as the

FIGURE 18

Mitochondrion near the nucleus. The outer nuclear membrane is studded with RNP particles. A nuclear pore is suggested at *A*. The nucleus contains fine granules and small particles. × 56,000.

FIGURE 19

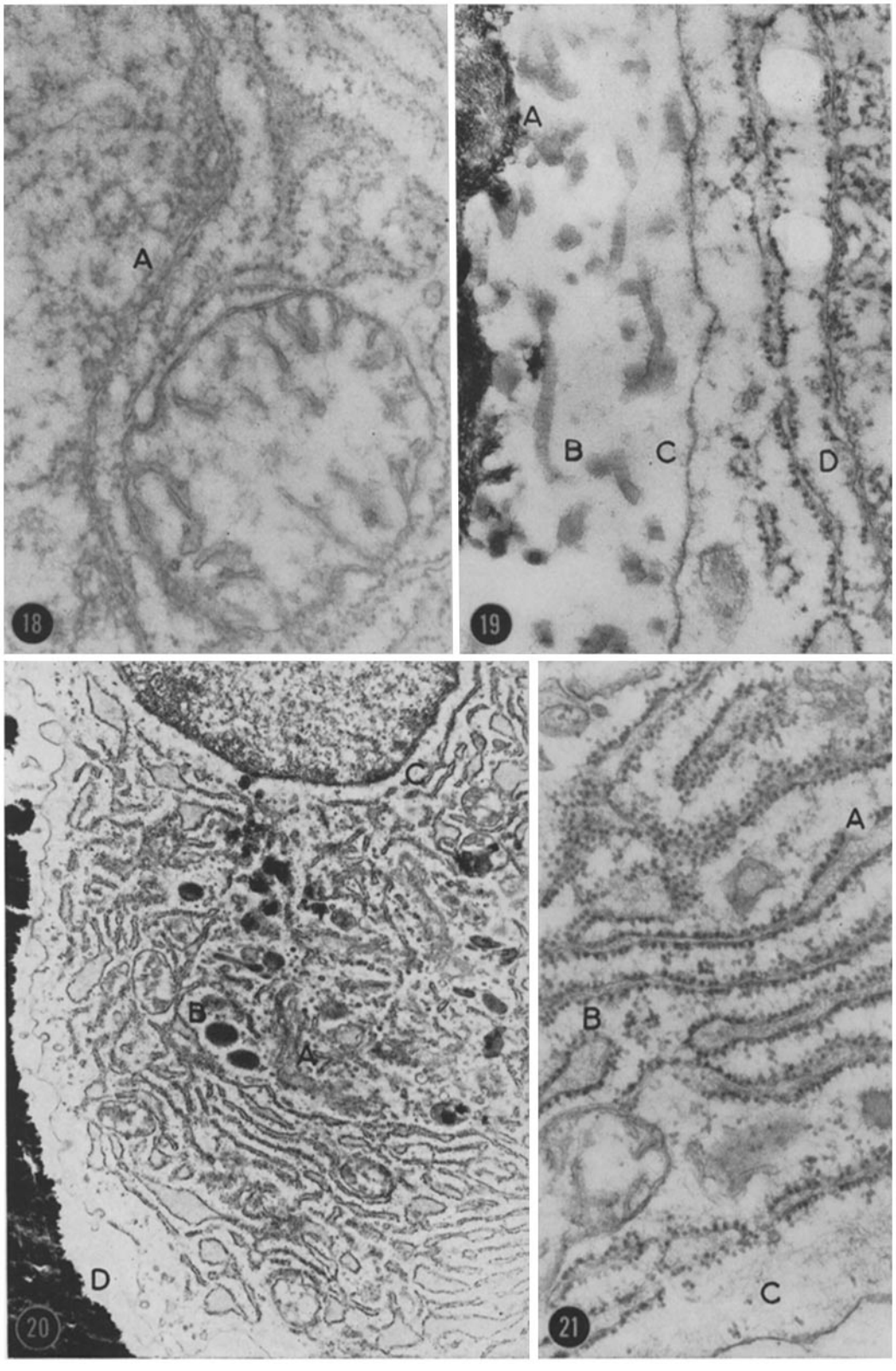
Part of a cell close to calcified tissue. *A*, apatite crystals. *B*, collagen fibrils. *C*, cell surface membrane. A nuclear pore can be seen at *D*. × 56,000.

FIGURE 20

Smooth-surfaced and rough-surfaced membranes. Most of the rough-surfaced sacs are flattened and some lie among the smooth-surfaced membranes. *A*, Golgi complex. *B*, secretory granules. *C*, nuclear pore. There are many small processes extending from the cell surface membrane. Very little collagen is evident between the cell and the calcified tissue (*D*). The irregular black objects represent surface contamination during lead staining. × 13,000.

FIGURE 21

Fibrillary materials in the rough-surfaced sacs (*A*), between them (*B*), and near the cell surface membrane (*C*). There are many small particles between the sacs and separated from the membranes. × 56,000.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result of forces set up inside the cell as it moves, although in fixed cells in other organs the same layering may be seen. Variations in the arrangement of membranes may be an artifact of fixation and dehydration.[1]

Sheldon and Robinson (1957) have noted the dilated sacs of the rough-surfaced endoplasmic reticulum, and similar sacs can be seen in the illustrations in the papers by Scott and Pease (1956) and Fitton Jackson (1957). Sheldon and Robinson suggested that the increased distance between the membranes may be associated with a particular phase of secretion. These dilatations are also to be found in fibroblasts (Fitton Jackson, 1956; Porter and Pappas, 1959) and in other cells (Bernhard and Rouiller, 1956; Palade, 1956). Since the average distribution of the RNP particles over a length of membrane seems constant, and since there is no marked difference in their numbers whether the sacs are flattened or dilated, it is probable that the sacs are not formed by a stretching of the membranes, but by a change in their outline. A change in outline could be the result of the accumulation of additional material within the sacs at particular sites, although it could also be due to rearrangement of the cytoplasmic contents in response to changes in the cellular environment; *e.g.*, cell movement, accumulation of extracellular matrix, changes in pressure as the capillary grows into the epiphysis, uneven or delayed penetration of the fixative (Haguenau, 1958). Whatever the reason, the sacs do not appear to take any special shape in relation to matrix formation.

Fitton Jackson and Randall (1956) and Sheldon (1959) described PAS-positive granules in osteoblasts. The former interpreted this observation as indicating the presence of an aminopolysaccharide such as hyaluronic acid; Sheldon suggested that these granules may be involved in protein formation, and he believed them to correspond to the dilated sacs of the rough-surfaced endoplasmic reticulum. Fitton Jackson (1960) also demonstrated protein in the granules and by differential centrifugation detected bound hydroxyproline in the fractions with large cytoplasmic particles. By examining this material with the electron microscope she showed that the large particles had a varied internal structure. Mitochondria have not been found responsible for binding hydroxyproline. From a detailed examination of osteoblasts in the metaphysis, it seems likely that the dilated sacs (Figs. 1 and 7) are the only other cytoplasmic organelles which are of the right size and sufficiently numerous to account for the number of large granules described by Fitton Jackson.

The RNP particles of osteoblasts are very prominent and their arrangement in spirals and chains is clearly visible when a section passes obliquely through or tangential to the membranes on which they lie (Fig. 10). In cultured osteoblasts Fitton Jackson (1960) showed that radioactive proline was incorporated into small particles isolated by centrifuging, and it is most likely that these were RNP particles.

The region of smooth-surfaced membranes shown in Figs. 1, 2, and 11, which is the site of the juxtanuclear vacuole in hematoxylin- and eosin-stained sections, is almost identical with that in the periosteal osteoblast illustrated by Fitton Jackson (1957). This quite large region in the cells is clearly demarcated from the remaining cytoplasm. This region has not been described in other fibrogenic cells. It has been noted by Sheldon and Robinson (1957) and can be seen in the illustrations in the paper by Scott and Pease (1956). The presence of occasional rough-surfaced membranes among the other components of this region suggests that all the membranes of the cytoplasm are parts of a continuum (Figs. 14 and

[1] A rather different picture is seen when the tissue is frozen and dried. Durning (1958) did not observe the endoplasmic reticulum which is usually found after osmium fixation, but described a system of vacuoles occupying most of the cytoplasm. The only other cells examined after such treatment have been from the pancreas (Sjöstrand and Baker, 1958; Hanzon *et al.*, 1959), and the appearance of the cytoplasm of these cells does not resemble that in the osteoblasts described by Durning. This discrepancy may be accounted for by the difference in cell type, but variations in the technique of freeze-drying used by the different authors may also be responsible for it. There is no doubt that mechanical damage during the removal of bony slices may disrupt the cells and introduce artifacts before fixation. Freezing and drying may ensure that these and subsequent distortions cannot occur. However, at present it is not possible to see any relationship in the appearances of the cytoplasm obtained by the different methods (particularly as regards the osteoblast), and this problem awaits further study. In the meantime it must be admitted that the freeze-drying technique may give a more accurate picture of the osteoblast, though a less satisfactory one in terms of the information which has been derived from tissues fixed in osmium tetroxide.

15). The Golgi complex is probably represented by the laminated membranes and the associated vesicles (Figs. 14 and 20), but it is not so well developed as that illustrated in fibroblasts by Fitton Jackson (1956) and Porter and Pappas (1959). The large granules (Figs. 1, 11, 14, 16, and 17) found in this region and in other parts of the cell may be secretory. They occasionally impinge on the surface membrane of the cell as if about to pass through it. The importance of the array of mitochondria (Figs. 1 and 2) about the area is not known.

The part played by osteoblasts in organic matrix formation now seems well established. The recognition of the significance of their cytoplasmic basophilia, which is common to all cells manufacturing proteins, helped to resolve the argument existing between authors who considered that osteoblasts were directly involved in bone formation and authors who considered them to be bystanders (Pritchard, 1956). Further evidence came from the isolation of radioactive proline from particulates of cultured osteoblasts after it had been introduced into the culture medium. Correlation of this work of Fitton Jackson and the studies of the fine structure of the cells suggests that the site of assembly of collagen precursors is the rough-surfaced membranes of the endoplasmic reticulum which are also responsible for cytoplasmic basophilia.

Recently Carneiro and Leblond (1959) have shown that labelled glycine passes through the cell and later appears in the matrix, but the actual stepping stones are still obscure. Assuming that the tropocollagen units are aligned on the RNP of the endoplasmic reticulum, it is possible that the material in some way accumulates in the sacs. The fibrillary matrix within the sacs (Figs. 8, 10, and 21) could be composed of macromolecules of collagen. Similar fibrils are to be found diffusely in the cytoplasm (Figs. 8 to 10) and sometimes concentrated near the cell surface membrane (Fig. 21). Whether or not they are tropocollagen fibrils, they do not aggregate into larger ones inside the cell. This finding is in agreement with that of Porter and Pappas (1959), who considered that monomeric collagen from the sacs of fibroblasts is discharged into the cells' environment to polymerize either at the cell surface or in association with already formed collagen fibrils. The same process probably occurs in osteoblasts. One route by which the small fibrils could reach the cell surface would be via the cytoplasmic matrix. They might also be discharged from the sacs directly through the cell surface membrane, although connections between these two structures are rare. Though it would be attractive to presume that this intracellular material is tropocollagen, it could simply be another cytoplasmic material which is in solution in the living cell but becomes aggregated as a result of fixation and embedding.

This study throws no light on the relation of the osteoblast to the deposition of mineral. Apatite crystals are readily seen outside the cells (Fig. 19), but there is no sign of any accumulation of mineral within them, as suggested by Bélanger (1959). There do not appear to be in the literature any other reports giving evidence of the direct involvement of osteoblasts in the transfer of calcium and phosphate from the blood to the bone matrix. Nor does mineralization seem to be associated with any rearrangement or variation in the organelles of the cells. In fact, mineral appears in the cartilage of the epiphysis beyond the invading capillaries at a distance from any cell.

The observations and discussion presented here and in other studies allow the following conclusions to be drawn concerning the function of osteoblasts. There is evidence that collagen precursors are formed in relation to the rough-surfaced membranes of the endoplasmic reticulum and that these precursors eventually leave the cells to become a component of the organic matrix of bone. Small fibrils of the size of tropocollagen units occur within the cells, but the first fibrils recognizable as collagen appear outside the cells. The collagen becomes calcified, but it has not been possible to demonstrate that osteoblasts are responsible for this.

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