A CYTOLOGICAL AND HISTOCHEMICAL STUDY OF BONE AND CARTILAGE FORMATION IN THE RAT

By J. J. PRITCHARD

*St Mary's Hospital Medical School, London, W.*1

INTRODUCTION

Recent advances in histochemical and cytological techniques have given a fresh impetus to the study of cell morphology in relation to functional activity. So far, however, comparatively little attention has been given to the bone-forming cells in this respect, and it was therefore decided to investigate certain aspects of the cytology of these cells in early centres of ossification in the hope of throwing light on the significance of the morphological changes which accompany the differentiation of osteoblasts from their mesenchymal precursors, and to attempt to correlate these changes with stages in the deposition of bone matrix. Similar, but less detailed, investigations were also carried out on the chondroblastic series of cells.

The investigation has been made on material from rat foetuses, and special attention was given to the distribution of alkaline phosphatase, glycogen, nucleoproteins, mucopolysaccharides, mitochondria and Golgi elements.

The association of alkaline phosphatase with the process of calcification in bone, cartilage and tooth has been confirmed by numerous workers since Robison (1923) first suggested it. Since Gomori (1939) published his well-known histochemical method a number of investigators have studied phosphatase activity in these tissues at the cytological level, and it is now established that osteoblasts, hypertrophic cartilage cells and odontoblasts show a high degree of enzyme activity. Variations in phosphatase activity during the differentiation of osteoblasts, and at different stages in matrix deposition, however, do not appear to have been studied, so that it is an open question whether phosphatase activity is solely, or even principally, associated with calcification, or whether it is in some way related also to the processes of cell differentiation and the deposition of the organic matrix.

The presence of glycogen in calcifying tissues has long been known, but received little attention until Harris (1932) first suggested that it might be the chemical precursor of the hexosephosphate esters postulated by Robison as the substrate for alkaline phosphatase in the calcification process. The discovery by Gutman & Gutman (1941) of a phosphorylase in calcifying cartilage which could convert glycogen into hexosephosphate, and other biochemical studies of recent years, have tended to support Harris's hypothesis. Our knowledge of the precise distribution of glycogen in bone and cartilage, however, is meagre, and as in the case of alkaline phosphatase, it is not known whether glycogen storage in these tissues is solely related to the process of calcification, or whether it has other roles.

The investigations of Brachet (1947, 1950), Caspersson (1947) and others in recent years have resulted in the identification of basophilic substances in many types of cell with nucleic acids, and the hypothesis that these are concerned in the manufacture of proteins is strongly supported. In view of this it seemed desirable to

study afresh the well-known cytoplasmic basophilia of the osteoblast, for protein manufacture may well be an important function of this cell in connexion with the deposition of the organic components of the bone matrix. The chondroblast, likewise, seems implicated in the deposition of cartilage matrix, and its basophilia has also been investigated.

Young connective tissues show metachromasia to basic dyes, and recolour Schiff's reagent after periodic acid, owing to their content of mucopolysaccharides. The well-known metachromasia of cartilage matrix is also attributable to its mucopolysaccharide content. The importance of these substances in connective tissue economy is now being realized (Gersh & Catchpole, 1949), and it was therefore decided to investigate their distribution in developing bone matrix.

Finally, it is generally agreed that the osteoblast possesses a large Golgi element (Fell, 1925; Hill 1936) and numerous mitochondria (Dubreuil, 1913), but little is known about their behaviour during the differentiation of the cell and at different phases of its functional activity. In view of recent advances in our knowledge of the structure and chemical composition of these organelles in other cells, it seemed desirable to re-investigate them in the osteogenic series of cells.

MATERIALS AND METHODS

Norwegian rat foetuses varying in age from 12 to 20 days were obtained from animals whose pregnancies were timed from the day of finding spermatazoa in the vaginal smear.

Generally the foetuses were decapitated and the heads and trunks fixed separately, partly because most of the observations were to be made on the head, and partly because decapitation assisted the penetration of the fixatives.

A variety of fixatives was used according to the staining methods subsequently to be employed, including Bouin's and Helly's fluids, Regaud's and Aoyama's fixatives, and absolute alcohol. The specimens were not decalcified.

All embedding was done in paraffin, and sections were cut 2–7 μ thick according to the type of observation to be made on them.

Glycogen was usually demonstrated after fixation by absolute alcohol; Bouin's and Helly's fluids were also tried, but were found to be unsatisfactory owing to partial loss and diffusion of the carbohydrate. Sections 5–7 μ thick were flattened on 70 % alcohol and dried with a minimum of heat. After removal of wax they were treated with a thin (0·5 %) solution of celloidin in alcohol-ether. A number of staining methods were used. Best's carmine, and iodine, were found to be unreliable, so most sections were stained by either Hotchkiss's (1948) periodic acid-Schiff (P.A.S.) method, or by the author's chromic acid-diammino silver technique (Pritchard, 1949). These two methods gave comparable results, except that the smaller particles of glycogen were more sharply defined by the silver method. A saliva control test was performed on each batch of sections.

Alkaline phosphatase activity was investigated after absolute alcohol fixation by Gomori's method, using a veronal buffer. In order to reduce diffusion of the enzyme and its products to a minimum, sections were treated as for glycogen above, and the incubation times were kept as short as possible. Thus incubation times of 10 min., 30 min., 1 hr., and 2 hr. were employed as a routine on each batch of sections, and

most of the significant observations were made after the shorter periods. Controls were obtained by omitting the glycerophosphate from the incubating solution; these showed the distribution of preformed inorganic phosphates in the bone matrix. In some cases another type of control was employed. Sections were dipped into either 5 % trichloracetic acid or hot (80° C.) water for 5–10 min. before incubating in the usual way. The enzyme was destroyed by these procedures.

Cytoplasmic basophilia was usually demonstrated by pyronin-methyl green staining. In correct proportions this combination stained the basophilic material of the cytoplasm, and the centre of the true nucleolus (plasmosome), red and that of the nucleus, including the periphery of the nucleolus, bluish green. The proportions needed had to be found by trial, as they varied according to the fixative. It was found that the clearest-cut results were obtained after absolute alcohol fixation, although Helly's and Regaud's fluids gave reasonably satisfactory pictures. Other basic dyes such as methylene blue, toluidine blue, thionin and saffranin were also used, but these did not differentiate between cytoplasmic and nuclear basophilia so clearly as did the pyronin-methyl green mixture. In some cases control sections were digested with a ribonuclease preparation in order to determine how far the cytoplasmic basophilia shown by pyronin was due to ribonucleic acids.

The *desoxyribonucleic acids* of the nucleus were stained by Feulgen's method, after fixation by absolute alcohol, Helly's or Regaud's fluids. *Mucopolysaccharides* were identified by their metachromatic reaction towards basic dyes, especially toluidine blue, and by their ability to recolour Schiff's reagent after periodic acid (P.A.S. test), it being generally agreed that few substances apart from mucopolysaccharides give both these reactions.

For the study of *mitochondria* the tissues were fixed in Regaud's or Helly's fluid, post-chromed, and stained with either Heidenhain's iron haematoxylin, Altmann's aniline-fuchsin-methyl green, Sudan black, or by the author's silver-reduction method (Pritchard, 1952). This last method consists essentially in placing sections, from distilled water, for 10–20 sec. in diluted Wilder's silver diammino hydroxide solution, and without washing plunging them with agitation into a large volume of very dilute formalin (0·1 %) for 20 sec., and finally washing in tap water. Usually some nuclear staining occurred, but this could be removed by the cautious application of 2 % potassium ferricyanide solution, differentiation being controlled under the microscope. The mitochondria were left sharply and densely stained against a clear background, except for calcified bone matrix which was also densely stained. It was usually desirable to counterstain with a red dye such as saffranin, acid fuchsin or eosin. Mitochondria were also demonstrated supravitally in smears of periosteal cells scraped from the surface of the foetal skull vault and stained with Janus green B (1/10,000).

The *Golgi element* was shown by Aoyama's cadmium-silver method. In osteoblasts, but not in other cells, the element was also often clearly visible in preparations stained for reticular fibres by Wilder's method after Bouin fixation. In some cases, again, the author's silver method for mitochondria after Helly's fixation showed the Golgi element clearly in a number of cell types, including the osteoblast.

Besides these more or less specific staining methods, the general histology of centres of ossification was studied in preparations stained by Delafield's, Weigert's

and Heidenhain's haematoxylin, with eosin, Van Gieson, and Ponceau fuchsin-light green as counterstains.

In order to correlate the results obtained by different staining methods, wherever possible adjacent sections from a series were stained by different methods. Thus after alcohol fixation, glycogen, alkaline phosphatase, cytoplasmic basophilia and mucopolysaccharides were studied in successive sections: and after Helly's and Regaud's fixatives, mitochondria, basophilia, the Feulgen reaction and the general morphology were studied in a similar manner. The use of counterstains such as saffranin, toluidine blue and methylene blue, which give an overall picture of cytoplasmic and nuclear basophilia as well as matrix metachromasia, in preparations designed primarily to show glycogen, alkaline phosphatase, the Golgi element or the mitochondria, helped greatly in the task of correlating the various cytological characteristics one with another, and also with the stages in matrix deposition.

PRELIMINARY OBSERVATIONS

(Including terminology)

The observations on the cytology of the osteogenic series of cells described in this paper were made at sites of intra-membranous ossification in the skull vault, the facial skeleton and the periosteum of long bones. Endochondral ossification was not investigated. The cytology of the cartilage series of cells was studied in the chondrocranium, including Meckel's cartilage, and the cartilage models of the limb bones, ribs and vertebrae.

Preliminary observations by routine histological methods for the demonstration of cells and fibres were made on foetal rat, mouse, rabbit and human material with the object of providing a uniform terminology to serve as a basis for the subsequent cytological observations.

These observations showed that once the osseous territories had become defined by the differentiation of a fibrous periosteum, the periosteal surfaces of all actively growing bones, whatever their origin, presented a similar pattern of cell and fibre lamination (Pl. 1, figs. 1, 2, and 4). From without inwards the following zones could be identified: (1) a zone of *periosteal fibroblasts* and tangentially orientated coarse collagen fibres; (2) a zone of proliferating and differentiating *pre-osteoblasts* in a network of fine collagen fibres; (3) a zone of *definitive periosteal osteoblasts* arranged along radially directed bundles of coarse collagen fibres which merged into trabeculae of uncalcified bone matrix (osteoid) and then into calcified bone; and (4) a network of primary cancellous bone whose cavities were lined with *medullary osteoblasts*. In the earlier stages of osseous development all these zones were readily identifiable in transverse or longitudinal sections, but at later stages, owing to a reduction in the thickness of the inner zones, they were shown best in oblique tangential sections which exaggerated their thickness (cf. Stump, 1925).

Although the definitive periosteum of all the growing bones presented this uniform appearance, the method of arriving at this condition differed somewhat as between the bones of the cranial vault, the facial skeleton, and the bones of the axial skeleton and limbs. In the cranial vault the fibrous periosteum was differentiated simultaneously with the appearance of pre-osteoblasts at sites of imminent

osteogenesis. In the facial skeleton (Pl. 1, fig. 5) condensations of pre-osteoblasts and the maturation of the more centrally placed of these into definitive osteoblasts, preceded the formation of the fibrous periosteum; while in the rest of the skeleton the fibrous perichondrium acted as a ready made fibrous periosteum once the underlying chondroblasts had disappeared and the pre-chondroblasts had been transformed into pre-osteoblasts.

The definitive periosteal osteoblasts (Pl. 1, fig. 3) were large pyriform or ovoid cells with an eccentrically placed hypochromatic nucleus containing one to three conspicuous nucleoli. Their cytoplasm appeared granular and was markedly basophilic except for a large clear area next to the nucleus termed the *juxta-nuclear vacuole*. Cytoplasmic processes united adjacent cells.

The *pre-osteoblasts* included an outer zone of small round cells with a centrally placed, moderately chromatic nucleus, small nucleoli and only slight cytoplasmic basophilia; and an inner zone of cells with morphological characteristics intermediate between those of the outer zone and definitive periosteal osteoblasts. Mitotic figures were frequent among the pre-osteoblasts, especially in the outer zone.

The *medullary osteoblasts* showed all transitions between cells with characteristics identical with those of the periosteal osteoblasts, and flattened inconspicuous resting cells lining inactive bony surfaces

Centres of chondrification also showed a gradation of cell and matrix types from without inwards (Pl. 1, fig. 6). The outermost zone of *perichondrial fibroblasts* and tangentially running coarse collagen fibres was followed by a zone of small round *pre-chondroblasts* in a network of fine collagen fibres, and then by a zone of *chondroblasts* in which the fibrous matrix was progressively masked by a deposit of hyaline cartilage matrix. This zone in turn merged into definitive cartilage containing *chondrocytes* and an abundance of hyaline matrix.

In certain cartilages, and at a later stage, hypertrophy of the chondrocytes was accompanied by a partial loss of matrix and the calcification of the remainder. This was followed by cellular degeneration and osteoblastic invasion preliminary to endochondral ossification.

In some situations, e.g. the ramus of the mandible, cartilage islands were formed within areas of membrane bone formation. This secondary cartilage (De Beer, 1937) appeared to arise by the differentiation of pre-osteoblasts into chondroblasts. Such cartilage was either completely resorbed, converted directly into osteoid, or else formed a growth cartilage to be replaced gradually by endochondral bone.

HISTOCHEMICAL AND CYTOLOGICAL OBSERVATIONS

Alkaline phosphatase (Pl. 3)

Bone formation

With prolonged incubation almost all nuclei will show enzyme activity, part of which is genuine and part of which is an artefact due to diffusion. For the purposes of this paper only phosphatase activity which is demonstrable in under 1 hr. is considered significant. Many of the observations were made after only 10 min. incubation in order to reduce artefacts due to diffusion to a minimum, and also to show up sites of maximal activity preferentially.

Enzyme activity appears coincidentally with the emergence of condensations of pre-osteoblastic mesenchyme, i.e. on the 14th day of pregnancy for the mandible, clavicle and maxilla, which are the earliest centres to appear. At this stage the nuclei of the pre-osteoblasts are sharply stained with the cobalt sulphide precipitate in contrast with the nuclei of the general mesenchyme which are unstained (Pl. 3, fig. 21). The cytoplasm and the intercellular fibrillar matrix are unstained at this stage. As the pre-osteoblasts *begin* to differentiate into definitive osteoblasts enzyme activity is greatly augmented as shown by the heavier precipitation (Pl. 3, fig. 22). Not only the nuclei, but the cytoplasm and intercellular fibres (Pl. 3, fig. 23) as well show intense activity. The definitive osteoblasts, however, while continuing to show intense activity, exhibit rather less activity than the layer of pre-osteoblasts which immediately surrounds them, their cytoplasm in particular being less heavily stained (Pl. 3, figs. 24, 25). The osteogenetic fibres between the cells are at first intensely active but they lose their activity as they become incorporated within the bone matrix. Similar extracellular phosphatase activity is always found where pre-osseous collagen fibres are being formed, whether it be in the periosteum, or in the medullary spaces.

The fibroblastic layer of the periosteum shows no activity except after prolonged incubation, when the *nuclei* begin to stain patchily near sites of intense activity in the deeper layers of the periosteum. This appearance must be interpreted as a diffusion artefact. The nuclear staining of the pre-osteoblasts, however, is unlikely to be an artefact, because it appears in the earliest mesenchymal condensations before activity has appeared in any other cells (Pl. 3, fig. 21). Similarly, the nuclear activity of the osteoblasts is probably genuine, for it is evident after very short periods of incubation and is unrelated to the intensity of staining of the neighbouring cytoplasm. After 2 hr. incubation, however, diffusion artefacts in the osteoblastic layer are very obvious in some sections, for the nuclei become intensely stained out of proportion to the increased staining of the cytoplasm.

With short periods of incubation, then, developing membrane bones show the following regional differences in enzyme activity (Pl. 3, figs. 23–25). The fibroblastic zone is inactive; the pre-osteoblastic zone shows at first only nuclear activity, followed by intense nuclear, cytoplasmic and extracellular activity; the periosteal osteoblasts show intense nuclear, less intense cytoplasmic and a diminished extracellular activity; while the medullary osteoblasts show similar features to the periosteal though less intensely, and the extracellular activity is confined to the surface of newly formed osteoid lamellae.

Similar differences in enzyme activity are found in the periosteum of the cartilage bones at the onset of ossification (Pl. 3, figs. 26, 27, 29). The differences are naturally most readily observed where growth is particularly rapid, and the cellular zones are thickest.

The phosphatase-positive osteogenetic tissue and the phosphatase-negative fibroblastic tissue are very clearly delineated at the sutural junctions (Pl. 3, fig. 28), where differentiation of osteoblasts is seen to be taking place on either side of a neutral non-osteogenetic zone.

The association of phosphatase activity with calcification is shown by comparing adjacent sections stained for phosphatase and for pre-formed bone salt, from which

it is evident that phosphatase activity is intense in areas undergoing calcification. However, the greatest intensity is found in the region of pre-osteoblasts and pre-osseous collagen fibres just outside the region in which bone salt is being deposited.

Cartilage formation

Alkaline phosphatase activity is not found in association with the formation of cartilage unless hypertrophic changes supervene. Cartilages which do not ossify, such as some of the nasal cartilages and the greater part of Meckel's cartilage, therefore show no activity at any time.

The onset of the hypertrophic changes is marked by the appearance of nuclear phosphatase activity in the cells which are about to enlarge (Pl. 3, fig. 30). As the cells hypertrophy nuclear activity becomes much more intense and then the cytoplasm and finally the extra-cellular matrix show intense activity as well. The appearance of extracellular activity is associated topographically with the deposition of inorganic salts in the residual matrix. Like the inorganic salt, extracellular phosphatase activity first appears in the capsular zones surrounding the cells, and then spreads throughout the matrix. Eventually, as the hypertrophic cartilage cells degenerate, enzyme activity diminishes and finally disappears.

Glycogen (Pl. 2)

Bone formation

The pre-osteoblastic cells of the anlagen of the membrane bones show uniform and intense glycogen storage in their cytoplasm (Pl. 2, fig. 11). This is in sharp contrast with the surrounding non-osteogenetic mesenchyme which shows little or no glycogen except where cartilages and muscles are about to be formed. Comparison of adjacent sections stained for glycogen and phosphatase shows that the areas of intense glycogen storage correspond very well with the areas of nuclear phosphatase activity, though if anything the glycogen area is a little larger, indicating that glycogen storage slightly precedes phosphatase activity in the differentiation of pre-osteoblastic cells from the mesenchyme. Later the more centrally placed pre-osteoblasts show diminished glycogen storage as phosphatase activity becomes maximal (Pl. 2, fig. 12). Fully mature periosteal osteoblasts are characterized by their low glycogen content; in many places they appear to contain no glycogen at all (Pl. 2, figs. 13, 14 and 16). The medullary osteoblasts, on the other hand, usually contain some glycogen, but never as much as the pre-osteoblastic cells of the periosteum.

Glycogen is also found in the reticular cells of the bone marrow, and in some of the developing blood cells, but conditions were not favourable for identifying the cell types concerned.

This pattern of glycogen storage, viz. heavy deposition in the earlier pre-osteoblasts, dropping to little or none in the periosteal osteoblasts, with a reappearance of glycogen in the medullary osteoblasts, was a constant feature of all the membrane and cartilage bones studied. The fibroblastic zone of the periosteum, however, showed little or no glycogen storage at any time (in contrast to Bevelander & Johnson's (1950) findings). This was very apparent at the cranial sutures, where the

sutural fibroblasts were clearly demarcated from the osteogenetic cells on either side by the difference in glycogen content (Pl. 2, figs. 19, 20), as they were by differences in alkaline phosphatase activity (Pl. 3, fig. 28).

Cartilage formation

In developing cartilage the pre-chondroblasts showed fairly intense glycogen storage, but no glycogen was found in the chondroblastic series of cells (Pl. 2, fig. 15). Only with the enlargement and approaching maturity of the cells and matrix did glycogen reappear (Pl. 2, fig. 17). The definitive chondrocytes invariably showed intense cytoplasmic storage. Like the fibrous periosteum, the fibrous perichondrium was glycogen-free. Glycogen storage remained intense in the early stages of cell hypertrophy prior to endochondral ossification, but the glycogen was lost as the matrix began to calcify and the cells degenerated (Pl. 2, fig. 18). Permanent hyaline cartilages retained intense glycogen storage indefinitely.

Both the bone and the cartilage-forming cells therefore agree in showing a sharp drop in glycogen storage at the onset of matrix deposition, but whereas in the bone-forming series of cells storage was highest at the pre-osteoblastic stage, in cartilage it was highest at the definitive chondrocyte change. The loss of glycogen from the cartilage cells as the matrix calcified was very striking. By contrast, in the bone-forming cells glycogen reappeared as the matrix became calcified.

In the course of their embryonic development many tissues exhibit either glycogen storage or alkaline phosphatase activity. With very few exceptions, however, the simultaneous presence of alkaline phosphatase activity *and* glycogen storage in the same cell is confined to the calcifying tissues (cf. Horowitz, 1942) though, as has been shown, in these tissues changes in glycogen storage and phosphatase activity do not run parallel courses.

Feulgen reaction for desoxyribose nucleic acids (Pl. 4, figs. 31, 32)

The nuclei of the pre-osteoblastic cells stained with about the same intensity as those of the general mesenchyme. As the nuclei enlarged and definitive osteoblasts appeared there was a progressive loss of staining intensity which persisted in the medullary osteoblasts (Pl. 4, fig. 32). The nuclei of osteoclasts and reticular cells, however, showed fairly intense staining, while those of the haemopoietic cells were very heavily stained. It was therefore an easy matter to distinguish osteoblastic nuclei from other nuclei in Feulgen preparations from a consideration of their staining intensity alone.

Mitotic figures, of course, show up clearly in Feulgen preparations, and it was at once evident that the majority of them lay in the outer pre-osteoblastic zone. Mitoses were not found in mature osteoblasts. This observation is in agreement with the widely held view that cell division is incompatible with mature functional activity.

The increase in nuclear size as osteoblasts were differentiated was accompanied by an increase in the size of the nucleoli also. The increase was apparently chiefly due to the increase in the ribonucleic acid component, for the larger nucleoli showed only a thin outer rim of Feulgen-positive material, the central part being unstained. The large nucleoli of the osteoclasts had a similar structure.

In the cartilage series of cells a similar, but even more striking, loss of Feulgen staining intensity was regularly observed (Pl. 4, fig. 31). The pre-chondroblasts stained with about the same intensity as the general run of mesenchymal cells, but the chondroblasts and chondrocytes showed very little staining at all.

These observations suggest a loss of desoxyribose nucleic acid from the nucleus during the differentiation of osteoblasts and chondroblasts, but the loss may be only an apparent one, a reflexion of the dilution of a constant amount of material as the nucleus enlarges, as has been maintained for other cells (Vendrely & Vendrely, 1949; Mirsky & Ris, 1947, 1949). The low staining intensity of the osteoblasts might be explained in this way, but it is difficult to believe that dilution alone accounts for the very dramatic loss of Feulgen intensity in the differentiating cartilage cells.

Cytoplasmic basophilia and ribonucleic acids

The intense cytoplasmic basophilia of the mature osteoblast has long been recognized. With any of the usual basic stains osteoblasts are always conspicuous because of their cytoplasmic basophilia (Pl. 4, fig. 34). Characteristically, the juxta-nuclear vacuole remains clear (Pl. 1, fig. 3). With the pyronin-methyl green staining combination the osteoblast cytoplasm stained red and the nuclear membrane and chromatin granules blue-green. The nucleoli (plasmosomes), however, showed a central pyroninophil area and a peripheral rim of methyl-green stained material. The cytoplasmic basophilia of the osteoblasts (and indeed of all embryonic cells) was easily destroyed by hydrolysis with 1 % HCl at 60° C. for 2 min., and by the action of dilute alkalis in the cold, both procedures leaving the nuclear basophilia (except for the centre of the nucleolus) unaltered. Ribonuclease digestion also removed the basophilia of the cytoplasm and of the nucleolar centre.

Taking the above evidence, and that of the Feulgen studies, into consideration, there can be little doubt that the cytoplasmic and central nucleolar basophilia of the osteoblast is associated with its ribonucleic acid content. The basophilia was not uniform throughout the non-vacuolar cytoplasm, but appeared to be related to a granular material. Whether this was an artefact of fixation, or whether the ribo-nucleic acid was contained in preformed granular components of the cytoplasm, perhaps mitochondria and microsomes (cf. Brachet, 1947), could not be determined. The cytoplasm of the pre-osteoblasts showed a variable degree of basophilia, depending upon the stage of differentiation, but it was never so marked as in the definitive osteoblasts (Pl. 4, fig. 33).

The medullary osteoblasts showed less intense basophilia than the periosteal osteoblasts, the intensity varying with the size, and therefore presumably with the functional activity, of the cells. The osteocytes showed comparatively little baso-philia, while the staining of the osteoclasts was very variable, in some the whole or part of the cytoplasm was intensely basophilic, in others the cytoplasm was eosinophilic.

The development of cytoplasmic basophilia in the cartilage series of cells ran an approximately parallel course to that of the osteoblastic series, increasing as the chondroblasts matured, and decreasing again in the mature chondrocytes. With the onset of hypertrophic changes cytoplasmic basophilia was lost. No cell of the cartilage series, however, ever showed such conspicuous cytoplasmic basophilia as did

the periosteal osteoblasts, whose staining intensity was equalled only by the spinal ganglion cells and some of the epithelial cells of the developing skin, glands and alimentary tract.

In the osteogenetic series of cells maximum cytoplasmic basophilia, and therefore presumably maximum ribonucleic acid content, was associated with the sites of most rapid deposition of bone matrix. In the chondroblastic series also this relationship between basophilia and matrix deposition was observed, but in a less striking manner.

Mucopolysaccharides

Metachromasia towards toluidine blue and methylene blue was exhibited by the fibres and ground substance of the pre-osseous matrix surrounding the pre-osteoblasts of the periosteum, and by the newly deposited osteoid matrix adjacent to the definitive osteoblasts (cf. Follis & Berthrong, 1949; Bevelander & Johnson, 1950; Follis, 1951). Calcified bone matrix exhibited metachromasia only after treatment with dilute hydrochloric acid (cf. Howard, 1951*a*, *b*). With the periodic acid-Schiff test the pre-osseous fibres, osteoid and calcified matrix gave an increasingly strong positive reaction in that order.

Uncalcified cartilage matrix was of course intensely metachromatic but as in the case of bone, calcified cartilage matrix was only metachromatic after decalcification. Both calcified and uncalcified matrix gave a moderately strong positive reaction with the P.A.S. test.

This co-existence of metachromasia and a positive P.A.S. reaction in bone and cartilage matrices is presumptive evidence for the presence of mucopolysaccharides. It is not clear why the metachromatic reaction should be inhibited by calcium salts, but the appearance of the reaction after decalcification disposes of Sylvén's (1947*a*, *b*) contention that mucopolysaccharides disappear as calcification begins.

Mitochondria

Living osteoblasts scraped from the surface of the parietal bone of foetal rats and examined supravitally with 1/10,000 Janus green on a warm stage showed numerous filamentous mitochondria stained bright blue-green against a clear background. As the cells died the mitochondria fragmented into small granules and the nucleus and finally the general cytoplasm took up the stain. It would appear therefore that the mitochondria in the living osteoblast are filamentous in form, and that the granular forms sometimes found in stained sections are artefacts due to imperfect fixation (cf. Fischer, 1948). This was borne out in the specimens stained for mitochondria by the classical aniline-fuchsin, methyl-green technique, by iron haematoxylin and by the author's silver-reduction method, where, in the best-fixed preparations, and near the surface of specimens exhibiting poor fixation centrally, the mitochondria were uniformly filamentous or rod-like (Pl. 4, fig. 38), whereas in poorly fixed areas they were in the form of strings of granules, isolated granules or amorphous clumps. Mitochondria were most numerous in the large periosteal osteoblasts, in which they occupied every part of the cytoplasm except the juxtanuclear vacuole, and generally tended to be orientated in the long axis of the cell. A concentration of mitochondria around the vacuolar region was sometimes observed, but was never general (cf. Hill, 1936). Mitochondria in the fibroblastic

and pre-osteoblastic zones of the periosteum were much less numerous (Pl. 4, fig. 36). In fact, under the lower powers of the microscope, the osteoblasts were easily identifiable by their mitochondrial content alone. The mitochondria of the medullary osteoblasts (Pl. 4, fig. 35), varied in numbers according to the size of the cell, but in general were less numerous than in the periosteal cells. They were filamentous in form in the best fixed preparations.

The osteoclasts were always striking in mitochondrial preparations because their cytoplasm was invariably packed with coarse granules and short rods (Pl. 4, fig. 37); filamentous forms were never observed so that it is unlikely that these appearances were artefacts.

In developing cartilage mitochondria were most numerous in the perichondrial cells. The mature cells usually contained granular forms rather than filaments (cf. Sheehan, 1948), but as the cartilage matrix is necessarily a barrier to rapid fixation, it is possible that the mitochondria are filamentous in the living cell.

In general, however, it may be said that in both the osteoblastic and chondroblastic series of cells, the mitochondria were most numerous where the rate of deposition of intercellular matrix was greatest.

The Golgi element

The true morphology of this organelle in living cells is still uncertain, but it is generally agreed that the structures seen in silver- and osmium-impregnated tissues are artefacts (myelin figures) produced during the precipitation of the lipoidal components. Nevertheless, the overall size and position of the precipitation artefact is a general guide to the size and position of the element in life, and these features can be regarded as affording evidence concerning the secretory activity of the cell.

In successful Aoyama preparations the periosteal cells show a localized deposit of silver next to the nucleus (Pl. 1, fig. 7) occupying the site of the juxta-nuclear vacuole seen in ordinary preparations (cf. Fell, 1925). The silver deposit is in the form of discrete granules in some cells (Pl. 1, fig. 8), in the form of a continuous reticulum in others (Pl. 1, fig. 9), but no significance can be attached to these morphological differences owing to the artificial character of the silver deposit. The size of the silver deposits increased steadily as the osteoblasts matured, those of the large periosteal osteoblasts having a diameter two to three times those of the early pre-osteoblastic cells (Pl. 1, fig. 10). which were in turn clearly larger than those of the periosteal fibroblasts, and the general mesenchyme.

With Aoyama's technique the cytoplasm of the medullary osteoblasts usually showed a uniformly heavy precipitate of silver throughout, and so the size and position of the Golgi element in these cells remained in doubt. It was found by accident, however, that after Bouin's fixation, Wilder's 'on-the-slide' silver method for demonstrating reticular fibres often showed a deposit of silver in the periosteal and medullary osteoblasts, strictly localized to the vacuolar area of the cytoplasm. No other cells showed this phenomenon. Also, after Helly's fixation, the author's silver method for mitochondria often showed discrete silver precipitation in the Golgi region of the cytoplasm of most of the cells in the embryo, including the osteogenetic series of cells. It therefore seems likely that the generalized argento-

philia of the cytoplasm of the medullary osteoblasts shown by the Aoyama preparations does not give a true indication of the size of the Golgi element in these cells. More probably the Golgi element remains a discrete juxta-nuclear body throughout the life of the osteoblast.

Attempts were made to demonstrate the Golgi element by means of Sudan black B, both in paraffin sections after Helly fixation, and in frozen sections of material fixed with formol-calcium, but without convincing results. The osteoblasts, however, were conspicuous in such preparations because of the diffuse, finely granular, sudanophilia of their cytoplasm, while the osteoclasts showed numerous heavily coloured particles like those seen in the mitochondrial preparations already described.

The Golgi element of the chondroblastic series of cells was usually readily impregnated by Aoyama's technique. It took the form of a crescentic area of silver precipitation capping the nucleus. Unlike the osteogenetic series of cells, changes in the size of the element during the maturation of the chondroblasts were not found. There appeared to be a disintegration of the element at the hypertrophic stage.

DISCUSSION

The cytological and histochemical evidence presented in this paper amply justifies the classification of periosteal cells into three main groups, viz. fibroblasts, pre-osteoblasts and definitive osteoblasts.

The *definitive periosteal osteoblasts* are large pyriform or ovoid cells with an eccentric nucleus and a large juxta-nuclear vacuole. They tend to be arranged in columns and rows adjacent to newly formed bone matrix. They exhibit no mitoses, contain little or no glycogen, and their Feulgen-staining intensity is low. The cytoplasm of these osteoblasts is intensely basophilic, and both nucleus and cytoplasm show considerable alkaline phosphatase activity. The Golgi element is very large and there are numerous filamentous mitochondria. The associated intercellular matrix consists of bundles of collagen fibres which exhibit metachromasia to basic dyes and a high phosphatase activity, merging into trabeculae of osteoid bone matrix which also exhibit metachromasia, but no phosphatase activity, and then into trabeculae of definitive calcified matrix which only exhibit metachromasia after decalcification. Pre-osseous fibres, osteoid and calcified matrix give an increasingly intense positive reaction with the periodic acid-Schiff test.

The *pre-osteoblasts* represent the immature stages of osteoblastic development. They include (1) small, rounded, closely packed elements with a high mitotic rate, high Feulgen-staining intensity, conspicuous glycogen content but with phosphatase activity confined to the nucleus, as well as (2) a series of cell types approaching the definitive osteoblastic condition with fewer mitoses, reduced glycogen storage, a very high nuclear and cytoplasmic phosphatase activity and increasing cytoplasmic basophilia allied with decreasing intensity of Feulgen staining of the nucleus, and an increasing size of the Golgi element. The pre-osteoblasts as a group contain comparatively few mitochondria. The associated intercellular matrix appears oedematous, with moderate numbers of fine fibres which are argyrophilic, metachromatic, give a positive reaction with the periodic-Schiff test and show conspicuous phosphatase activity.

The medullary osteoblasts vary considerably in size. The larger ones show characteristics almost as pronounced as those of the definitive periosteal osteoblasts. The smaller ones show them in lessened degree.

The interpetation of these facts cannot be finally decided in the present stage of our knowledge, but there are a number of suggestive relationships:

(1) The overall cell enlargement, and increasing eccentricity of the nucleus during osteoblastic differentiation are easily explained as being due to the accumulation of cytoplasmic products: basophilic material (ribonucleoprotein), mitochondria, and the large Golgi element.

(2) There seems little doubt that the juxta-nuclear vacuole is occupied by the Golgi element (in agreement with Fell, 1925).

(3) The decline in mitotic activity is what might be expected in view of the generally observed inverse relationship between cell reproduction and functional activity.

(4) The decline in Feulgen-staining intensity may represent a real or an apparent loss of desoxyribose nucleic acid. In the former case it would agree with the decline in mitotic activity. On the other hand, the increasing nuclear size may be due to invisible chromosome reproduction, as has been found in other nuclei (Salvatore, 1950), associated with the dilution of a constant amount of nucleic acid.

(5) The great increase in cytoplasmic basophilia (ribonucleic acid) and the associated increase in nucleolar size are diagnostic of a cell actively engaged in protein manufacture (Caspersson, 1947). This evidence, taken in conjunction with the simultaneous increase in the intercellular matrix, particularly of its collagenous (protein) component, surely indicates that the osteoblast is actively secreting the protein basis of the matrix (cf. Cappellin, 1948). This view is further supported by the great rise in alkaline phosphatase activity which accompanies the signs of protein manufacture mentioned, for it is probable that the formation of fibrous proteins involves alkaline phosphatase activity (Bradfield, 1949, 1950; Jeener, 1947).

(6) The increase in mitochondria and the hypertrophy of the Golgi element support the thesis that the osteoblast is metabolically very active.

(7) Besides the possible significance of alkaline phosphatase in fibrous-protein manufacture alluded to, alkaline phosphatase on the evidence of this paper may well play a part: (*a*) in the intrinsic metabolism of cell differentiation, for nuclear phosphatase activity has been so interpreted by several workers (cf. e.g. Moog, 1944), (*b*) in the manufacture of cytoplasmic ribonucleic acid (Bradfield, 1950); and (3) in the mechanism of calcification.

Of course phosphatase may be concerned in all the processes mentioned, but at present its role in the mechanism of calcification is best supported by independent evidence, especially when its activity is reviewed in conjunction with the metabolism of glycogen in calcifying tissues. Thus the Gutmans' (1941) discovery of a phosphorylase as well as phosphatase and glycogen in calcifying cartilage prompted the hypothesis that the initial step in calcification is the hydrolysis of glycogen by phosphorylase to produce hexosephosphate esters which are in turn hydrolysed by phosphatase to liberate PO_4 ions. Then according to Roche & Mourgue (1943) and Roche & Deltour (1943) the PO_4 ions are temporarily fixed to the pre-osseous

matrix, only to be liberated again with the formation of the definitive organic matrix, when they combine with calcium ions to form the inorganic matrix. It will be seen that part of the story is derived from the study of calcifying cartilage and part from the investigation of bone formation, but there is no reason to suppose that the mechanisms are very different. Roche's hypothesis explains one formerly puzzling feature of phosphatase histochemistry, namely the observation that enzyme activity is most intense at the pre-osseous stage *before* calcification has begun.

(8) The presence of glycogen in the precalcification stages of bone and cartilage development, and its abrupt disappearance with the actual onset of calcification, seem satisfactorily explained on the above theory, but there are other possible roles for the carbohydrate. Thus both in bone and cartilage, its temporary disappearance from the osteoblast and chondroblast is associated with the appearance of mucopolysaccharides in the intercellular matrices, and it is therefore possible that the breakdown of glycogen is associated with the manufacture of the mucopolysaccharides. However, we know too little about the formation of these substances to pursue this speculation further.

Then again glycogen may be held in the pre-osteoblastic and pre-hypertrophic cartilage cells as a store of potential energy to be used in the intense metabolic processes accompanying cell differentiation and secretion. Or again, as Harris (1933) suggests, glycogen storage may simply be indicative of relatively anaerobic conditions, and it is certainly true that cartilage, which contains much glycogen, is avascular, while the glycogen content of the osteoblastic series of cells seems to fall as the blood supply improves.

The changes undergone by the *cartilage* cell in its maturation from the mesenchyme show several features in common with the osteoblastic series of cells. On the morphological and histochemical evidence it seems justifiable to differentiate the following stages: *pre-chondroblastic* mesenchymal cells, *chondroblasts*, *chondrocytes*, and *hypertrophic chondrocytes*. The chief cytological trends observed during this maturation were: (1) A progressive increase in cell size. (2) A progressive decrease in Feulgen-staining intensity. (3) A very early period of glycogen storage at the pre-chondroblastic, followed by its complete absence at the chondroblastic stage; its reappearance and increasing deposition at the chondrocyte and early hypertrophic stages; followed by its disappearance as the matrix becomes calcified. (4) Phosphatase activity was absent from the early stages and did not appear unless hypertrophic changes supervened. Then it increased rapidly, first in the nucleus, then in the cytoplasm and finally extracellularly as the matrix calcified. (5) Cytoplasmic basophilia steadily increased during the chondroblastic stage of differentiation, but diminished again at the chondrocyte stage. (6) Changes in the Golgi element and mitochondria were not marked.

On the above evidence the participation of glycogen and alkaline phosphatase in the calcification mechanism in hypertrophic cartilage seems likely. The histochemical evidence, however, gives little indication of the way in which the *organic* matrix of cartilage is formed, or of the cellular role therein.

At this stage in our knowledge of bone formation the statement of a working hypothesis about the mechanism of the formation of the organic and inorganic matrices, and the role of the osteoblasts therein, taking into account the cytological,

histochemical and chemical evidence, may be of some value. I would suggest therefore that the osteoblast secretes (1) alkaline phosphatase, (2) a collagen precursor, (3) hexose-phosphoric esters, (4) mucopolysaccharides; that these secretory products constitute the 'pre-osseous substance' of the French School; that the presence of mucopolysaccharides explains the oedematous appearance of the pre-osseous substance owing to their marked water-binding properties (Heringa, 1949); that the collagen is at first in an unaggregated, non-micellar, molecular form; that the hexose phosphates derived from the breakdown of glycogen (Harris, 1932; Gutman & Gutman, 1941) are hydrolysed by extracellular alkaline phosphatase (Robison, 1923) to provide a local excess of PO_4 ions which are at first fixed to the protein matrix (Roche & Deltour, 1943). Possibly also calcium ions are fixed independently to the mucopolysaccharides. Then there is a rather sudden physico-chemical change in the state of aggregation of the collagen and mucopolysaccharide molecules which results in the precipitation of the osteoid matrix, while the calcium and PO_4 ions, liberated from their attachments, combine and precipitate to form the mineral salt (Roche & Deltour, 1943). Later other ions are added to give the mineral its complex structure (Dallemagne, 1943). This hypothesis accounts satisfactorily for most of the evidence we possess about the mechanism of osteogenesis. One difficulty remains, however, for in some situations (Weidenreich, 1928) bone formation apparently occurs with a direct transformation of fibroblast-like cells into osteocytes without the intervention of an osteoblast stage. I feel it would be worth while investigating this atypical method of ossification in order to see whether or not the cells involved show characteristic signs of osteoblastic activity such as intense cytoplasmic basophilia and a high phosphatase content, even if the typical osteoblast morphology is not reproduced in all its features. At any rate, the time is past when one can afford to neglect the cells, as Leriche & Policard (1928) have done, in any consideration of the mechanism of bone formation.

SUMMARY

1. A cytological and histochemical investigation of the processes of ossification and chondrification has been made on foetal rats with the objects of defining the changes which occur during the differentiation of osteoblasts and chondroblasts from their mesenchymal precursors, and of relating these changes to the stages in the deposition of the intercellular matrices.

2. Particular attention was paid to the changes in glycogen storage, alkaline phosphatase activity, cytoplasmic basophilia, the intensity of the Feulgen nuclear reaction, the number of mitochondria and the size of the Golgi element.

3. The cells involved in ossification were classified as periosteal fibroblasts, pre-osteoblasts, definitive periosteal osteoblasts and medullary osteoblasts; and those involved in chondrification as perichondrial fibroblasts, pre-chondroblasts, chondroblasts, non-hypertrophic chondrocytes and hypertrophic chondrocytes.

4. Glycogen storage was negligible in periosteal fibroblasts, intense in pre-osteoblasts, absent or negligible in definitive periosteal osteoblasts and moderate in medullary osteoblasts. It was negligible in perichondrial fibroblasts, intense in pre-chondroblasts, absent in chondroblasts, intense again in non-hypertrophic chondrocytes and greatly diminished in the later hypertrophic chondrocytes.

5. Alkaline phosphatase activity was absent from periosteal fibroblasts, entirely nuclear in early pre-osteoblasts, intense in nucleus, cytoplasm and intercellular fibres at the late preosteoblast stage and of diminished intensity in the nucleus, cytoplasm and fibres at the definitive osteoblast stage. In cartilage enzyme activity was restricted to the hypertrophic stage when it appeared first in the nucleus, then in the cytoplasm and finally in the extracellular matrix at the onset of calcification.

6. Cytoplasmic basophilia, shown to be due to ribonucleic acids, was moderate in periosteal fibroblasts and pre-osteoblasts but intense at the definitive osteoblast stage. It was less intense in medullary osteoblasts. Cytoplasmic basophilia increased in the cartilage series of cells up to the chondrocyte stage when a reduction in intensity was observed.

7. The intensity of the Feulgen nuclear reaction decreased markedly as osteoblasts and chondroblasts were differentiated.

8. Filamentous and rod-like mitochondria were very numerous in periosteal osteoblasts, less numerous in medullary osteoblasts and few in pre-osteoblasts and fibroblasts. They were most numerous at the chondroblast stage in the cartilage cell series.

9. The size of the Golgi element increased markedly as osteoblasts were differentiated from their mesenchymal precursors. The size and position of the element coincided with the size and position of the juxta-nuclear vacuole. The Golgi element showed no conspicuous changes in form and position during the differentiation of the cartilage cells.

10. The intercellular matrix of the fibroblastic layer of the periosteum contained comparatively stout collagen fibres orientated tangentially. That of the pre-osteoblastic layer contained a reticulum of fine fibres which exhibited metachromasia to basic dyes, a positive reaction to the periodic acid-Schiff test and intense alkaline phosphatase activity. That of the osteoblastic layer consisted of sheaves of collagen fibres merging into trabeculae of hyaline osteoid matrix and then into calcified bone. The fibres exhibited phosphatase activity but the osteoid did not. Fibres, osteoid and calcified bone (after decalcification) were all metachromatic and gave a positive periodic acid-Schiff reaction.

11. The evidence warrants the assumption that the osteoblast is an actively secreting cell.

12. These histochemical and cytological findings were discussed in the light of current theories about the biochemical mechanisms involved in bone formation and it was suggested that the osteoblast contributes to the deposition of the matrix by secreting (*a*) alkaline phosphatase, (*b*) a collagen precursor, (*c*) hexose phosphates and (*d*) mucopolysaccharides.

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EXPLANATION OF PLATES

All the illustrations shown were prepared from sections of foetal rat material with the exception of Pl. 1, fig. 3, which was from bony callus after fracture in an adult rat.

PLATE 1 (general morphology and Golgi element)

Fig. 1. Periosteum of neck of mandible (18-day). Note zoning of cells and cytoplasmic basophilia of the deeper-lying osteoblasts. Haematoxylin and eosin. × 425.

Fig. 2. Periosteum of neck of mandible (18-day). Note tangential fibres of the outer layer of the periosteum above and radially orientated pre-osseous fibres below. Wilder. × 425.

Fig. 3. Row of osteoblasts against newly formed bone matrix (12-day callus). Note eccentricity of nucleus, large juxta-nuclear vacuole and cytoplasmic basophilia. Methylene blue and eosin. × 665.

Fig. 4. Periosteum and newly formed bone matrix from mandible (16-day). Note successive fibre zones; tangential above, then reticular, then radial pre-osseous fibres below passing into coarse strands of definitive bone matrix. Wilder. × 540.

Fig. 5. Edge of mandibular ossification centre (16-day). Note central osteoblasts surrounded by pre-osteoblasts. Haematoxylin and eosin. × 425.

Fig. 6. Perichondrium of metacarpal (16-day). Note successive zones of fibroblasts, pre-chondroblasts and chondroblasts from above downwards. Haematoxylin and eosin. × 540.

Fig. 7. Periosteal osteoblasts from the mandible (20-day). Note the conspicuous Golgi elements adjacent to the nucleus. Aoyama. × 540.

Fig. 8. Periosteal osteoblasts from the mandible (20-day). Note the granular character of the Golgi element in this preparation. Aoyama. × 1665.

Fig. 9. Osteoblasts from the parietal bone (18-day). Note the reticular character of the Golgi element in the central cell. Aoyama. × 1665.

Fig. 10. Part of the skull vault near the sagittal suture (18-day). Note the large size of the Golgi elements of the centrally placed osteoblasts compared with those of the more peripherally placed pre-osteoblasts and fibroblasts. Aoyama. × 200.

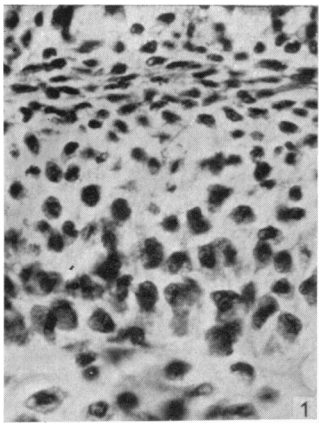
PLATE 2 (glycogen)

Fig. 11. Glycogen of mesenchymal anlagen of mandible and Meckel's cartilage (14-day). Clear oval area marks position of the inferior dental nerve. P.A.S. × 130.

Fig. 12. Glycogen of mandible and Meckel's cartilage (15 day). Note decreased glycogen storage in the central area of differentiating osteoblasts compared with the surrounding pre-osteoblasts. P.A.S. × 130.

Fig. 13. Glycogen of radius and surrounding musculature (16-day). Note glycogen in cartilage cells, pre-osteoblasts and muscles but not in the osteoblasts. Cf. Pl. 3, fig. 29, which shows alkaline phosphatase activity in an adjacent section. Author's silver method. × 100.

Fig. 14. Tangential section of edge of mandible (17-day). Note glycogen in the pre-osteoblasts and muscles but its absence from the fibroblastic and osteoblastic zones of the periosteum. Best's carmine and cobalt sulphide. × 130.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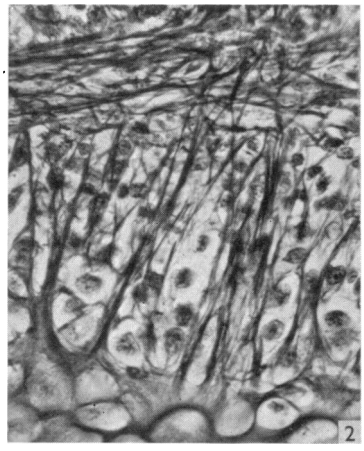
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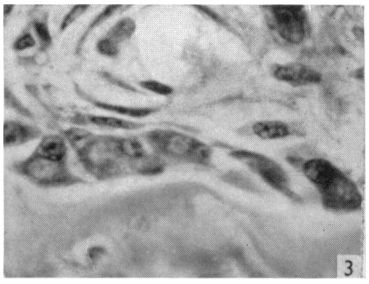
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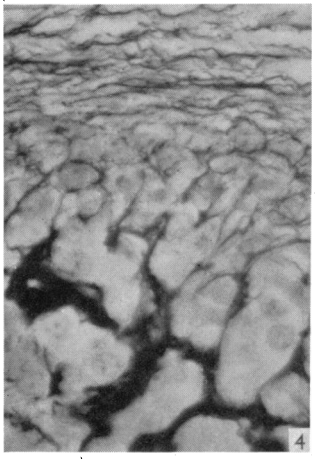
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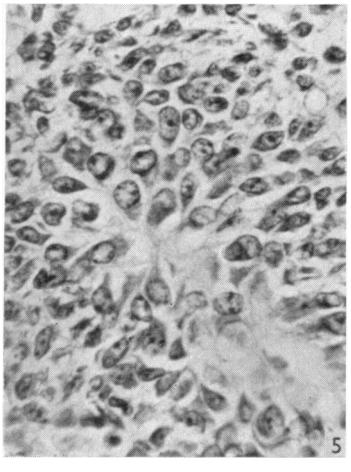
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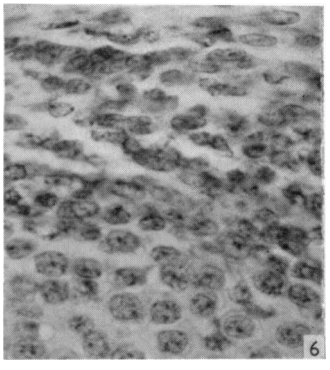
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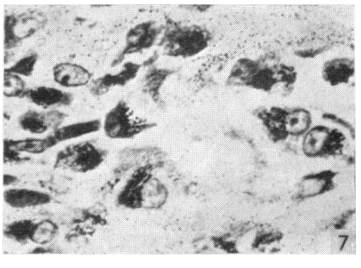
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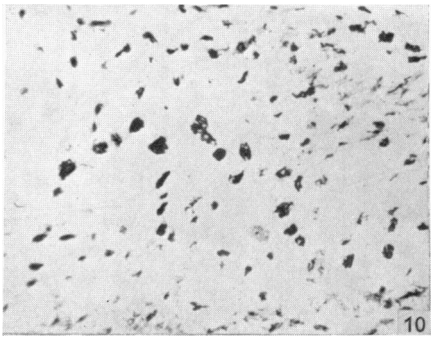
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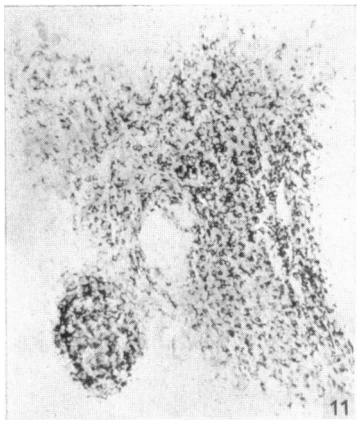
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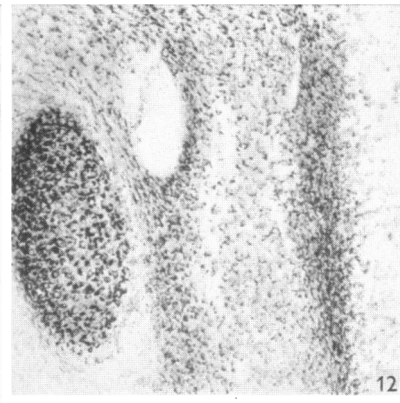
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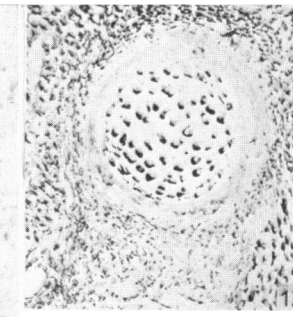
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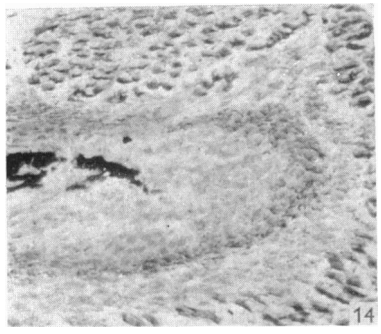
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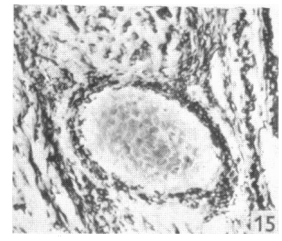
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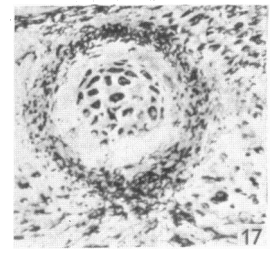
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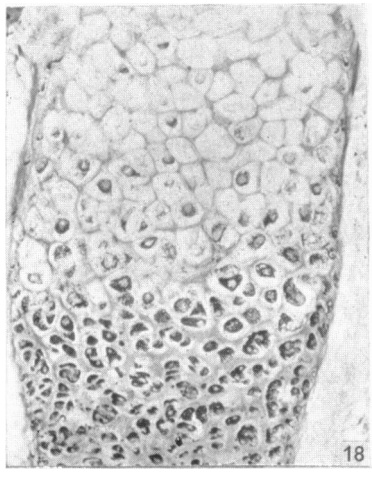
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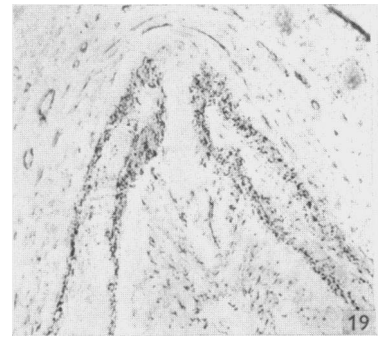
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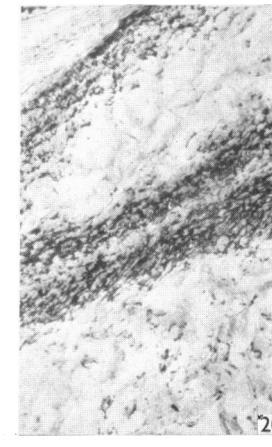
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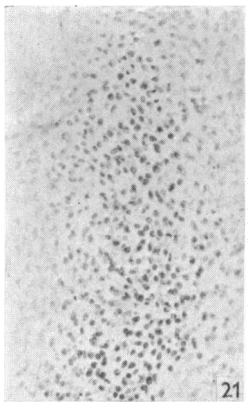
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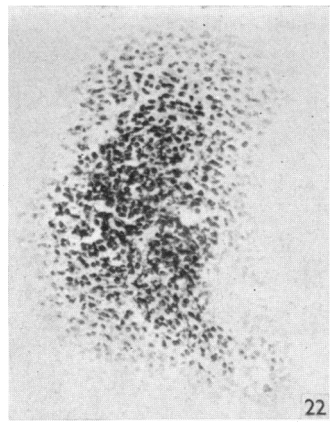
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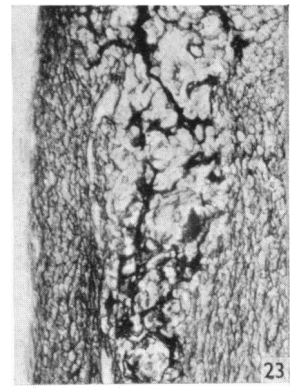
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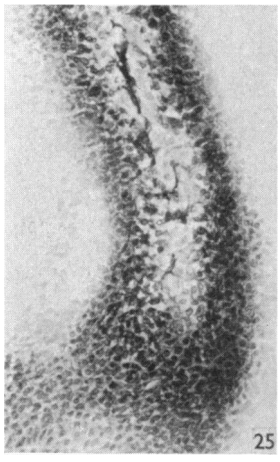
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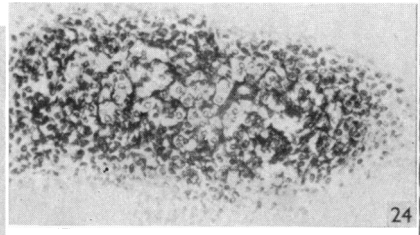
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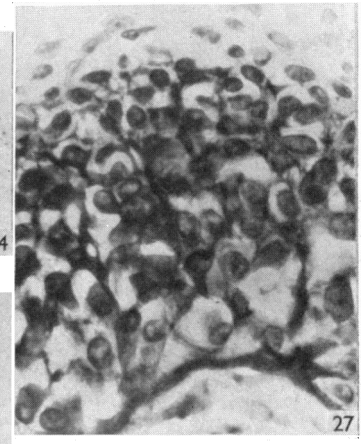
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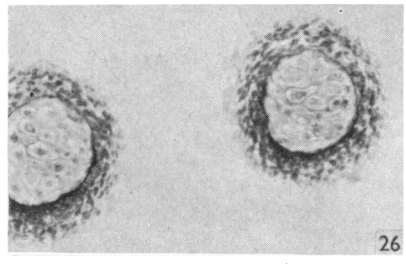
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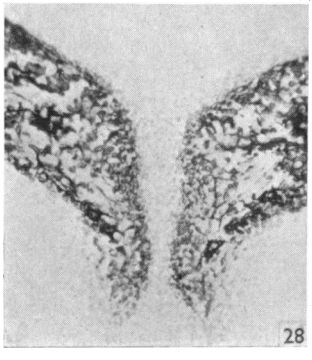
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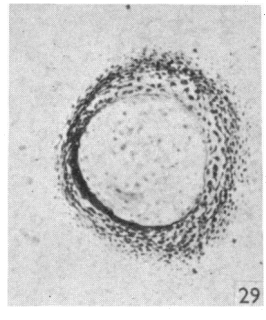
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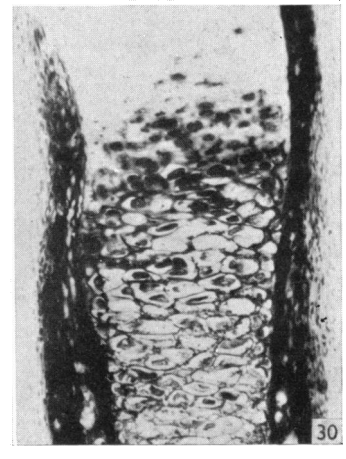
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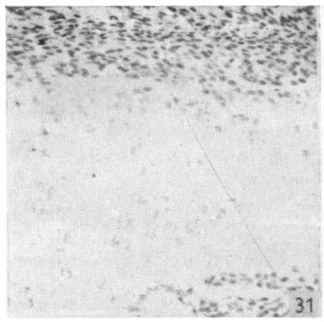

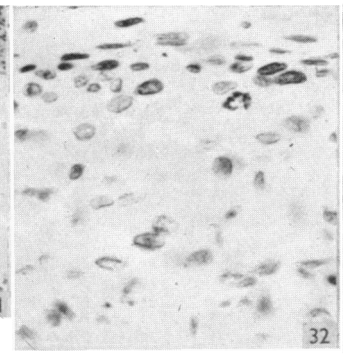

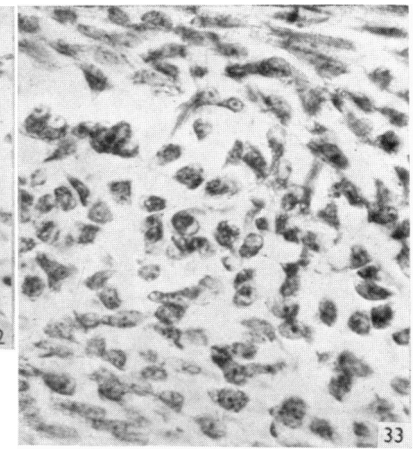

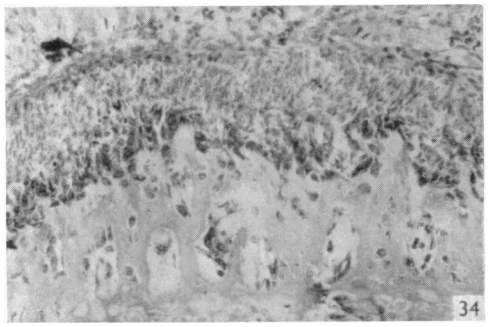

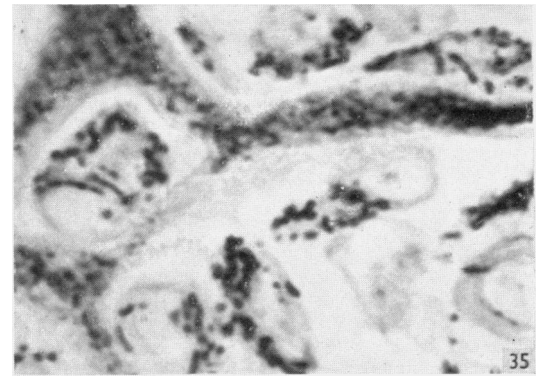

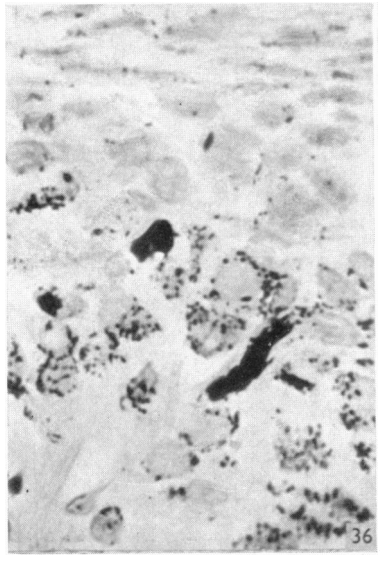

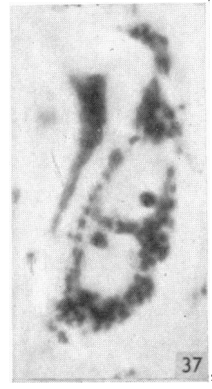

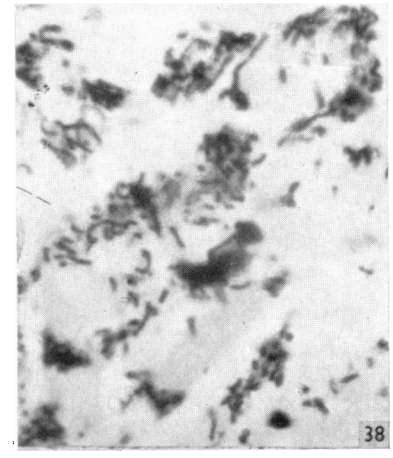

PRITCHARD—Bone and cartilage formation in the rat

Fig. 15. Costal cartilage (16-day). Note presence of glycogen in the pre-chondroblastic zone but its absence from the chondroblasts. Author's silver method. × 130.

Fig. 16. Mandible (17-day). Glycogen present in the pre-osteoblasts above and the medullary osteoblasts below but absent from the column of periosteal osteoblasts. Best's carmine and cobalt sulphide. × 540.

Fig. 17. Rib (16-day). Glycogen in mature chondrocytes and pre-osteoblasts but none in the osteoblasts. Author's silver method. × 130.

Fig. 18. Basisphenoid (19-day). Note decrease in glycogen storage accompanying hypertrophic changes in the chondrocytes preparatory to endochondral ossification. P.A.S. × 130.

Fig. 19. Parietal part of sagittal suture (17-day). Glycogen present in pre-osteoblasts but not in the sutural fibroblasts. Author's silver method. × 130.

Fig. 20. Maxillary-premaxillary suture (19-day). Note heavy glycogen storage in the pre-osteoblasts on either side of the sutural fibroblasts. P.A.S. × 130.

PLATE 3 (alkaline phosphatase)

Fig. 21. Maxillary primordium (14-day). Note nuclear phosphatase of early pre-osteoblasts. × 130.

Fig. 22. Mandibular primordium (14-day). Note increased enzyme activity in the late pre-osteoblasts centrally. × 130.

Fig. 23. Mandible (16-day). Note intense enzyme activity of the fibres of the pre-osteoblastic zone. The central network of bone trabeculae is calcified. × 130.

Fig. 24. Clavicle (14-day). Note decreased phosphatase activity of the definitive osteoblasts centrally × 130.

Fig. 25. Mandible (15-day). Decreased phosphatase activity of the definitive osteoblasts centrally. × 130.

Fig. 26. Ribs (16-day). Note phosphatase activity of the early periosteum and beginning activity of the hypertrophic chondrocytes. × 130.

Fig. 27. Periosteum of humerus (16-day). Note maximal enzyme activity in the pre-osteoblasts with decreased activity in the osteoblasts adjacent to the cartilaginous shaft below. × 540.

Fig. 28. Palatine suture (18-day). × 130.

Fig. 29. Radius (16-day). Cf. Pl. 2, fig. 13. × 100.

Fig. 30. Scapula (19-day). Note heavy enzyme activity in the hypertrophic cartilage and in the periosteum. × 100.

PLATE 4 (nucleoproteins and mitochondria)

Fig. 31. Part of scapula (16-day). Note the marked reduction in Feulgen-staining intensity of the definitive chondrocytes below as compared with that of the perichondrium above. × 130.

Fig. 32. Part of mandible (16-day). Feulgen stain. Staining is relatively heavy in the fibroblast and pre-osteoblast nuclei above but is much less in the osteoblast nuclei below. × 540.

Fig. 33. Mandible (16-day). Note conspicuous cytoplasmic basophilia of the centrally placed osteoblasts as compared with the more peripheral pre-osteoblasts. Pyronin and methyl green × 420.

Fig. 34. Tangential section through periosteum of mandible (18-day). Note conspicuous cytoplasmic basophilia of the osteoblasts adjacent to the newly formed bone matrix in the deepest zone of the periosteum. Methylene blue and eosin. × 130.

Fig. 35. Group of medullary osteoblasts from the mandible (20-day). Note the elongated mitochondria which avoid the vacuolar area of the cytoplasm. Author's silver method. × 1750.

Fig. 36. Periosteum of mandible (20-day). Note the numerous mitochondria of the osteoblasts below and their scarcity in the pre-osteoblasts and fibroblasts above. Author's silver method. × 605.

Fig. 37. An osteoclast from the mandible (20-day). Numerous rodlike and granular mitochondria. Author's silver method. × 1665.

Fig. 38. Periosteal osteoblasts from the mandible (20-day). The filamentous character of the mitochondria is clearly shown. Author's silver method. × 1665.