The Distribution of Alkaline Phosphatase in the Skull of the Developing Trout

BY

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(With two Plates)

INTRODUCTION

THIS work is part of a comparative study of the histological and cytological distribution of alkaline phosphatase in developing teleost and elasmobranch fishes, made with a view to throwing some light on the mechanisms involved in calcification and ossification. Whereas the adult mammalian skeleton is almost completely ossified, in the lower vertebrates cartilage persists side by side with bone. In elasmobranchs bone does not occur but calcium deposits are formed in the cartilage. Here calcification can be studied independently from ossification.

No histochemical study of phosphatase in lower vertebrates has so far been published. There is, however, evidence that phosphatase exists in teleosts as well as elasmobranchs, and is closely similar to the alkaline phosphatase found in ossifying parts of mammals (Bodansky, Bakwin, and Bakwin, 1931; Roche and Bullinger, 1939). The latter authors found a correlation between the degree of ossification and the phosphatase content in different species of fish and at different stages of development in the same species. A study of phosphatase in relation to the growth of scales in elasmobranchs and teleosts leads Roche, Collet, and Mourgue (1940) to conclude that here, as in the higher vertebrates, phosphatase is concerned with rapid osteogenesis.

These results suggest that the same biological mechanism is operative in the calcification of the skeleton in fish as in mammals. In view of this a systematic study of the distribution of phosphatase and calcium salts in developing teleost and elasmobranch embryos seemed of interest. This incidentally provides material for the study of phosphatase in the embryo generally. Studies so far undertaken in this direction were confined to young fowl embryos (Moog, 1944) and the heads of rat embryos (Horowitz, 1942).

This paper deals only with ossification in a typical teleost. The results obtained with elasmobranch embryos will be published separately. The trout (Salmo spp.) was chosen because it can conveniently be reared in the laboratory. Moreover the development of Salmo has been intensively studied from

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[Quarterly Journal Microscopical Science, Vol. 90, part 2, June 1949]
the morphological point of view (Schleip, 1903; Gaupp, 1906; Böker, 1913; Saunderson, 1935; de Beer, 1927 and 1937). Emphasis is here placed on the phosphatase content and degree of calcification rather than on the anatomical aspects of bone formation.

In the trout as in all teleosts, bones arise in a variety of ways. They may on the whole be classified into membrane bones, cartilage bones, and mixed ossifications.

I am here mainly concerned with the early stages of bone formation, i.e. the formation of the pre-osseous matrix—either within the mesenchyme as in membrane bone formation, or below the perichondrium as in cartilage bone formation—and the subsequent calcification of the matrix with its accompanying variations in phosphatase content and distribution. Examples of the different types of ossification will be described in detail. There was complete agreement of the phosphatase distribution and general appearance among fish at the same stage of development (not necessarily of the same age, but usually of the same length). The structures described were therefore chosen from whichever specimens showed the particular features best. The nomenclature used is that of de Beer (1937).

The following cartilages are suitable for the study of phosphatase in relation to their development and ossification:

Neurocranium. The anterior wall of the auditory capsule and the lateral commissure give rise to the pro-otic bone which later involves the basal plate and the anterior end of the parachordals. The basi-occipital appears in the form of perichondral lamellae of the hind portion of the basal plate on each side of the notochord.

Splanchnocranium. Meckel's cartilage and the autodentary; the pterygoid-quadrate giving rise to the autopalatine, the metapterygoid, and the quadrate; the hyosymplectic cartilage giving rise to the hyomandibula and the symplectic; other visceral cartilages.

The following structures illustrate membrane bone development: (i) the parasphenoid, a typical flat membrane bone which develops in the mesenchyme between the trabecula and the mucous membrane of the mouth. (ii) The dermodentary, i.e. the membrane bone portion of the dentary which is a mixed ossification. Since it develops in close relationship to Meckel's cartilage the structures composing the lower jaw will be described together after Stage 1. (iii) The maxilla, a membrane bone of the upper jaw entering into relationship with teeth. The premaxilla develops on similar lines. (iv) The pre-opercular which lodges part of the mandibular lateral line canal and lies postero-lateral to the symplectic cartilage.

Details of the above structures are described in my thesis (Lorch, 1948). Here only examples of the different types and stages of ossification are given. The terms 'positive', 'strongly staining', 'black', or 'grey' refer to the presence of phosphatase. No attempt has been made to give a strictly quantitative estimate of the phosphatase content of the tissues. The degree of staining after various incubation times is the sole criterion for stating that a tissue is
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'strongly positive' or 'contains little phosphatase'. A 'negative' Gomori reaction does not necessarily imply complete absence of the enzyme since there is a considerable loss during the preparation of the tissues.

Material

Specimens of brown and rainbow trout were reared from 'eyed' ova in the laboratory. It was decided to use length rather than time from hatching as an indicator of development. About 40 specimens ranging from 10 to 40 mm. were examined.

It was found convenient to divide the developing trout into groups of approximately the same length as follows:

<table>
<thead>
<tr>
<th>Stage (for reference)</th>
<th>Length (mm.)</th>
<th>No. of specimens examined</th>
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<tr>
<td>1</td>
<td>10–12</td>
<td>6</td>
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<td>2</td>
<td>15.5–17</td>
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<td>3</td>
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<td>6</td>
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<td>4</td>
<td>21–23</td>
<td>11</td>
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<td>5</td>
<td>29–38</td>
<td>10</td>
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Methods

Samples of trout were removed at intervals after hatching. Any yolk present was dissected off and the length of the specimen measured to the nearest millimetre. Younger alevins were anaesthetized in 10 per cent. alcohol before fixation to prevent curling and to facilitate measurements. Specimens intended for visualization of phosphatase were fixed in 80 per cent. ethyl alcohol. Some specimens of each stage were fixed in Bouin's fluid for general histology and some in 5 per cent. neutral formalin for the preparation of whole mounts of the skeleton stained with alizarin red S by the method of Hollister (1934). The latter helped in identifying the calcified structures and gave an indication of the areas in which calcification first occurred.

Most of the specimens intended for the phosphatase technique were cut undecalcified. Some of the older specimens were decalcified using citrate buffer for 2 to 5 hours according to my method (Lorch, 1947). There was a slight reduction in the phosphatase content. Groups of serial sections (8–10μ) were mounted on alternate slides, one being incubated and the other used as control. The slides were taken through celloidin to distilled water. Phosphatase was visualized by the method of Gomori (1939) and Takamatsu (1939). Minor changes in the composition of the substrate were made as follows:

2 per cent. calcium nitrate 10 ml., 2 per cent. magnesium chloride 10 ml.,
4 per cent. sodium β-glycerophosphate 10 ml., 1 per cent. sodium barbitone 70 ml.
Slides were incubated 1–18 hours at 28° C. and pH 9.4. Subsequent treatment was as described by Danielli (1946). Some sections were counterstained with dilute eosin.

Where the amount of preformed phosphate was considerable (specimens over 20 mm.) it became desirable to show the calcium salts and phosphatase in different colours, hence the gallamine blue technique was applied. The reasons for choosing this method as well as its limitations are discussed in my paper on mammalian bones (Lorch, 1947).

The following histological stains were used: Heidenhain’s ‘Azan’, van Gieson’s connective tissue stain, Ehrlich’s haematoxylin and eosin, and von Kossa’s silver nitrate method for bone salts.

RESULTS

Stage 1 (10–12 mm.)

A. General Distribution of Phosphatase

In all tissues which contain phosphatase the reaction is most marked in the nuclear membranes and nucleoli. The cytoplasm on the whole is negative, but it is not always easily recognizable since the degree of shrinkage and distortion due to alcohol fixation is considerable in the younger embryos. The mesenchyme stains irregularly—nearly all areas show some degree of activity—and the most marked concentration of positive nuclei occurs at the angles of the mouth and in the vicinity of the jaw cartilages. The central nervous system displays positive nuclei and fibres; the intensity of the reaction is variable. The fibrous membranes of the brain are strongly positive. The cells of the retina display slightly positive nuclei at some levels. Nuclei of striated muscle, and of the endothelial cells lining blood-vessels, are positive. The epithelium is negative.

B. Skeletal Tissues

All the cartilages of the chondrocranium display nuclear phosphatase, more or less marked, and in some places the matrix is also slightly positive, more so in the 10-mm. than in the 12-mm. specimens.

Neurocranium. Anterior to the region of the articulation of the lower jaw only the chondrocyte nuclei in the trabeculae are positive, but posteriorly, i.e. near the parachordals, the matrix too displays slight phosphatase activity. The cartilage cells are large and the area occupied by matrix relatively very small. The nuclei have a granular appearance. The cytoplasm is very faintly positive, and can just be observed in slides incubated 15 hours. The perichondral fibroblasts are strongly positive and fine black fibrils are seen in the surrounding mesenchyme which stains most strongly dorsally to the trabecula (Pl. 1, fig. 1). The parachordals contain strongly positive nuclei. The matrix stains positive in well-defined zones where the cartilage is cut near its surface. In the auditory capsules only the nuclei are positive. The perichondrium is still strongly positive on the dorsal (brain) side. More distally the para-
chordals lose their extracellular phosphatase except in the zone adjacent to the notochord: the nuclei remain positive throughout.

The notochord sheath consists of 3 layers: an inner layer of cubical cells with strongly staining nuclei, a middle layer, seemingly structureless and free from phosphatase, and an outer layer of elongated fibroblasts closely packed and strongly positive. The substance of the notochord itself is free from phosphatase.

_Splanchnocranium._ Meckel's cartilage is completely negative in its anterior tip where the pair of cartilages join. The middle and posterior regions contain nuclear phosphatase, the enzyme being most concentrated in the nucleoli and nuclear membranes. There is no extracellular phosphatase in Meckel's cartilage. The distribution of phosphatase in the mesenchymal nuclei of the lower jaw is interesting: they are strongly positive lateral to, moderately so ventral to, and negative dorsal to Meckel's cartilage. This is shown in Text-fig. 1, and will be further discussed under membrane bone development.

Pl. 1, fig. 2, shows the quadrate process and the hyosymphlectic cartilage of a 12-mm. specimen. The mesenchymal nuclei are strongly positive. The hyosymphlectic also shows nuclear phosphatase and some extracellular phosphatase at the periphery. The basihyal displays positive nuclei only after long incubation times, while the other branchial cartilages show strongly staining chondrocytes and perichondral fibroblasts after 6 hours' incubation. There is phosphatase in the striated muscle nuclei.

The first stages in the formation of _membrane bone_ are illustrated by the dermodentary. This is seen as a minute fragment of uncalcified osteoid at some levels only. It forms a thin lamella central and lateral to Meckel's cartilage, i.e. in the region where the mesenchymal nuclei are most strongly positive. The osteoid contains phosphatase (Text-fig. 1).

The pre-opercular resembles the dermodentary. The maxilla is present as a very small incompletely calcified rod angular in cross-section. There is as yet no sign of the parasphenoid.

**Summary of Stage 1**

Nuclear phosphatase is widespread in skeletal as well as non-skeletal tissues. There are occasional traces of enzyme in the cartilage matrix. Osteoid is present in some phosphatase-rich areas of mesenchyme. There is no calcification.

**Stage 2 (15.5–17 mm.)**

_A. General Distribution of Phosphatase_

This does not differ greatly from the distribution at the previous stage. But a new zone of strongly positive mesenchyme is seen just below the epithelium of the lower jaw anterior to the basihyal, and between the anterior end of the basihyal and the mouth epithelium. It is interesting to note that this is the site of the future dermentoglossum bone and tooth buds. Another intensely active zone of mesenchyme is that above and below the trabecula communis. In the latter zone the parasphenoid has its origin.
TEXT-FIG. 1. From the lower jaw of a 12-mm. trout. Incubation time 6 hours. The highest concentration of phosphatase in the mesenchymal nuclei is in the vicinity of the dentary. No tooth papillae are formed yet. The epithelium is negative. The dentary is not calcified. Cf. Text-fig. 2.

All text-figs. except diagrams are camera-lucida drawings of undecalcified sections treated by the Gomori (1939) method, unless otherwise stated. Areas of phosphatase activity are shown black.

TEXT-FIG. 2. From the lower jaw of a 16-mm. trout. Incubation time 15 hours. The pair of Meckel's cartilages are just separated. The dermodentary is very close to the cartilage but not in apposition. Note the positive reaction of the mesenchymal nuclei and of the epithelium in the region of the tooth papilla. The cartilage matrix is slightly positive. The dentary is calcified.
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B. Skeletal Tissues

In embryos of about 17 mm. the chondrocranium has almost reached its full development. The trabeculae, and the anterior part of the parachordals, display little or no phosphatase activity except in the dorsal perichondrium, and this applies to all subsequent stages. These parts do not ossify.

There is a strongly positive reaction of the cartilage matrix in apposition to the notochord, in the region of the future basioccipital. The auditory capsules display little or no phosphatase apart from the perichondrium.

Splanchnocranium. The extreme tip of Meckel's cartilage is still not calcified. It shows phosphatase in the nuclei and also some in the matrix, especially at the periphery, where a thin layer of osteoid, the first sign of the autotodentary, can be seen. The dermodentary is first visible at a level where the two cartilages just separate (Text-fig. 2). This stage shows the earliest calcification in the dentary. It is interesting to observe that the ground substance of the bone here contains phosphatase (visualized in decalcified sections), whereas in the older specimens this is rarely the case. Only the central portion of the bone is calcified as shown by treatment with silver nitrate. A rim of osteoid remains at the periphery of all growing bones. In Text-fig. 4 Meckel's cartilage and the dentary are cut longitudinally. Calcification decreases in intensity towards the distal end of the bone, which consists of a shred of uncalcified osteoid. The mesenchymal cells and fibres surrounding the dentary are strongly positive in this region. Meckel's cartilage has positive nuclei throughout but extracellular phosphatase only at the anterior end, i.e. where the dentary is in apposition to it. There are no new developments in the other branchial cartilages.

Membrane Bone. The dermodentary has already been described and its appearance is typical for all membrane bones at an early stage in their formation. The appearance of the maxilla is as at Stage 1. The parasphenoid is now seen ventral to the trabecula communis. With regard to the presence of calcium salts and phosphatase it resembles the early dermodentary.

Summary of Stage 2

The general distribution of phosphatase does not differ greatly from that at Stage 1. The perichondrium of some cartilages is strongly positive. Extracellular phosphatase occurs in the cartilage matrix where perichondral osteoid is present or about to be formed, e.g. at the anterior end of Meckel's cartilage. The first stages of calcification are observed in membrane bones.

Stage 3 (20 mm.)

A. General Distribution of Phosphatase

The appearance of the central nervous system is unchanged. The retina displays a well-localized zone of high phosphatase activity in the nuclei of the light-receptive cells. The reaction is somewhat weaker at the periphery of the retina than in the central region. The lens epithelium is positive. The
sensory epithelium of the nasal grooves has positive nuclei, and the fine cilia protruding from the olfactory cells stain heavily. The epithelium of the mouth and skin is negative.

Phosphatase in the mesenchyme is localized in the zones described for the previous stage. Tooth buds have now appeared at the angles of the mouth and in the strongly positive zone above the basihyal. The epithelium is thrown into folds and papillae of the strongly staining mesenchyme project into it.

As in previous stages the mesenchyme surrounding the growing membrane bones is strongly positive.

B. Skeletal Tissues

The general distribution of phosphatase within the chondrocranium tends towards more localization of the enzyme in certain areas and reduction in others. Whereas in Stage 1 all cartilages have positive nuclei this is no longer the case; some are completely negative, others have very high concentrations...
of phosphatase in both nuclei and matrix. As will be seen below this accumulation of enzyme can always be correlated with the development of a bony shell round the cartilage. Pl. 1, fig. 3, shows that the hyomandibula is positive in patches and the ceratohyal stains intensely. Portions of the chondrocranium which serve as attachment for muscles tend to give a positive reaction. There is a strongly staining region in the walls of the foramen of the facial nerve where the pro-otic bone can be seen in slightly older specimens (Stage 4).

**Splanchnocranium.** The extreme anterior end of Meckel's cartilage is now completely surrounded by a shell of bone, the mentomeckelian ossification, which merges into the autodentary posteriorly. The chondrocytes in the anterior portion show strongly positive nuclei and there is some phosphatase in the matrix, especially at the point where the pair of cartilages is just separating. The intensity of the phosphatase reaction decreases from the strong nuclear and extracellular reaction in the anterior tip to a weak nuclear reaction at a level where the cartilages are widely separated and the dentary is merging from its cartilage bone portion into the membranous portion. Distal to this the cartilage is completely negative apart from a few isolated nuclei chiefly at the periphery.

The appearance of the cartilage varies in the regions of different phosphatase activity: in the anterior portion the chondrocytes are large and spherical, their nuclei appear either completely black or granular, the cytoplasm gives a positive reaction, and the cells are surrounded by a rim of strongly staining ground substance, the rest of the matrix being moderately positive. In the eye region where the cartilage is negative the chondrocytes are arranged in longitudinal rows and are bilaterally compressed, in contrast to the spherical cells of the anterior zone. In the region of the optic chiasma the thick outer lamella of the dermodentary is fully calcified, while the inner thin lamella consists of uncalcified or feebly calcified osteoid. Both parts are embedded in strongly positive mesenchyme.

**The Pterygoquadrate.** The chondrocyte nuclei of the pterygoid process are moderately positive on the dorsal and external surface, i.e. where the metapterygoid bone is due to develop. In some of the specimens the endopterygoid (membrane bone) is seen as a thin layer of osteoid central to the inner perichondrum of the pterygoid process. It is not yet calcified and gives a positive phosphatase reaction.

In the region of its articulation with Meckel's cartilage and with the hyosymplectic cartilage the appearance of the quadrat process changes to that described for the anterior portion of Meckel's cartilage, and this is typical for all ossifying zones. The extracellular phosphatase activity is most marked near the lateral edge of the cartilage where a broad zone of feebly calcified osteoid, the first sign of the quadrat bone, can now be seen (Pl. 1, fig. 4). Phosphatase is never found in the matrix of cartilages which have no perichondral layer of osteoid or bone. Thus the characteristic features of ossifying cartilage in the trout are: (a) Greatly enlarged spherical cells with round nuclei, which later appear to degenerate and stain only faintly with haematoxylin.
These cells have very marked phosphatase activity. (b) The presence of phosphatase in the cartilage matrix, especially in the newly secreted ground substance round the chondrocytes which therefore appear to have black 'haloes'. The matrix is strongly basiphilic and in staining reaction resembles mammalian hypertrophic cartilage. However, no calcification of the matrix itself has been observed.

Since this type of cartilage is constantly met with, and always in conjunction with perichondral bone formation, it will be briefly referred to as 'positive cartilage', the above characteristics (including bone or osteoid) being implied in that expression. The term will not be used to describe cartilage containing phosphatase in the cells and perichondrium only. In diagrams of sections 'positive cartilage' is represented by areas with black rings, whereas 'negative cartilage' is stippled. Bone or osteoid is shaded. Thus the appearance of Meckel's cartilage described on p. 191 may be represented by a series of diagrams shown in Text-fig. 3.

In specimens of 15 mm. or over all cartilages fall into one of the following categories: (a) cartilages displaying no phosphatase activity whatever, (b) cartilages displaying some phosphatase in nuclei and perichondrium, and (c) cartilages with considerable phosphatase activity in cells, perichondrium, and matrix ('positive type'). Only the latter are ossifying.

The Hyoid Arch. The anterior zone of the symplectic process has the same appearance as the quadrate in this region, i.e. the perichondrium and chondrocyte nuclei are positive. Slightly distal to this there is an abrupt transition to the 'positive type' of cartilage. This is the first sign of the symplectic bone which ossifies in the distal portion of the hyosymplectic cartilage. Pl. 1, fig. 5, shows the posterior end of the quadrate and immediately below it the hyosymplectic cartilage with its layer of bone. The contrast between the adjacent cartilages is very marked.

The hyomandibular portion of the cartilage is not yet calcified but shows a thin perichondral lamella of pre-osseous tissue. The perichondrium is strongly positive especially where the cartilage is pierced by the hyomandibular branch of the facial nerve. The chondrocyte nuclei are positive throughout. A few enlarged cells of the type found in ossifying cartilages are seen and are surrounded by positively staining 'haloes' of ground substance. The mesenchyme enclosing the pre-opercular bone is intensely positive.

Other Branchial Cartilages. The ceratohyal is partly surrounded by a thin shell of bone (the epihyal) and presents the usual picture of an ossifying cartilage. No other cartilages have extracellular phosphatase, but some display positive nuclei and perichondrium.

Membrane Bone. The dermodentary has been described in conjunction with Meckel's cartilage. The maxilla is now heavily calcified especially in its anterior portion and is best studied in decalcified sections where its relationship to the surrounding mesenchyme is more clearly seen. It may be described in some detail as typical of membrane bone at this stage of development. In sections incubated for 14-16 hours the very heavy deposit of calcium salts
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observes the individual cells since the mesenchyme at the angles of the mouth is strongly positive. But in sections incubated for 4–6 hours only, the cells close to the maxilla (or other membrane bone studied) are positive, hence these cells have the highest concentration of phosphatase. The bone itself is negative after this incubation time but may appear grey after 18 hours. Three types of cells can be distinguished in the vicinity of the bone: long fibroblasts with narrow nuclei, large oval cells with prominent nuclei, and polymorphous cells of an intermediate size. The latter make up the bulk of the mesenchymal cells. The large cells are found close to the bone. They may be osteoblasts. The phosphatase content of these cell types is very variable. The cytoplasm only displays phosphatase after very long incubation times. The fibroblast nuclei stain deep black, most other nuclei appear granular. Nucleoli and nuclear membranes are prominent in all cell types. Text-fig. 5 shows a typical field of membrane bone and surrounding cells. In the bone itself two regions of different refractive index can sometimes be distinguished: a central and peripheral zone. In stained preparations, too, the heterogeneous nature of the ground substance becomes evident, the older ground substance having a different appearance from that newly secreted.

Summary of Stage 3

Ossifying cartilages can be distinguished from non-ossifying ones by the presence of high concentrations of phosphatase in the matrix as well as in the enlarged chondrocytes. In non-ossifying cartilages phosphatase, if present, is confined to the nuclei. Membrane bones display phosphatase only in the newly formed portions.

Stage 4 (21–3 mm.)

A. General Distribution of Phosphatase

The decline of nuclear phosphatase in tissues such as muscle, peripheral nerves, mesenchyme, and non-calcifying cartilage, already noted at the previous stage, continues. The skin and mouth epithelium, except at the tip of the lower jaw, remain negative. The anterior region of the brain, the retina, lens epithelium, and the nasal mucosae are strongly positive. The reaction becomes weaker in the mid-brain, the rest of the central nervous system being only feeble positive.

B. Skeletal Tissues

At the exit of the facial nerve, where the pro-otic is now seen in the form of two perichondral lamellae, the anterior wall of the auditory capsule is positive in cells and matrix. The bony lamellae, like all perichondral ossifications, are homogeneous and sharply separated from the cartilage as well as from the connective tissue. (Appearance similar to parachords in Text-fig. 6.) A few small spindly shaped cells form the periosteum. Ossification of the lateral commissure and the walls of the foramen for the trigeminal nerve has also begun.
TEXT-FIG. 5. Membrane bone and associated cell types of a 20-mm. trout. Decalcified section. Incubation time 15 hours. Note that only the growing-tip contains phosphatase.

TEXT-FIG. 6. Transverse sections through the notochord, parachordals, and basi-occipital of a 23-mm. trout. Incubation time 15 hours. Section (b) is slightly distal to (a). The perichondral lamellae of the basi-occipital are seen in (b) and the cartilage matrix displays phosphatase activity in that region.
The parachordals in the region of the myodome between the pro-otic and the basi-occipital are negative. The anterior tip of the notochord is strongly positive and a little farther back the central part of the parachordals also displays phosphatase activity, first in the nuclei only, then throughout the matrix. The space between the parachordals contains strongly positive connective tissue (Text-fig. 6). This, according to Schleip, ossifies later. The basi-occipital is now seen in the form of two perichondral lamellae round the central ends of the parachordals (Text-fig. 6). Still farther back the parachordals are adjacent to the notochord and the bony lamellae continuous with the notochord sheath.

The splanchnocranium of this stage shows very clearly the correlation between perichondral bone and extracellular phosphatase and the absence of any correlation between (a) perichondral bone and nuclear phosphatase, and (b) membrane bone and phosphatase within adjacent cartilages. The reaction in the matrix is never as intense as the reaction given by the enlarged chondrocytes in the ossifying zone.

The appearance of Meckel’s cartilage is as described for the previous stage. Teeth at various degrees of development are present. The mesenchyme round the tooth buds and beneath the mouth epithelium is strongly positive (Text-fig. 7). As the autodentary decreases in thickness posteriorly, the concentration of phosphatase in Meckel’s cartilage gets less as already
described for the younger specimens. In Text-fig. 8 there is a thin layer of mesenchyme between cartilage and bone, and the extracellular phosphatase in the former has completely disappeared. The nuclei are still positive but the intensity of this reaction diminishes and finally the cartilage is completely negative in its distal part, although the dentary approaches it again. But there is always some mesenchyme between the two structures. Thus it is seen again that mere proximity of bone is not correlated with a positive phosphatase reaction in the cartilage, whereas actual apposition of bone is associated with a marked staining of chondrocytes as well as matrix.

TEXT-FIG. 8. From the lower jaw of a 22-mm. trout. Decalcified section. Incubation time 15 hours. Shows a more distal region of Meckel’s cartilage than Text-fig. 7. The dentary is here separated from the cartilage by connective tissue. The cartilage matrix is negative. In Text-figs. 7 and 8 the bone itself shows no phosphatase activity.

This is also illustrated by the pterygoquadrate and the hyomandibula. Ossification in the other branchial cartilages has made marked progress since the previous stage examined. The distribution of extracellular phosphatase and perichondral bone is shown in Text-fig. 9. The two perichondral ossifications of the ceratohyal cartilage have increased in thickness and are heavily calcified. The ceratobranchials display phosphatase activity and perichondral bone in their central portions only. Their anterior and posterior tips remain negative. Hence the appearance in transverse sections varies according to the level. The hypobranchials are negative throughout and have no osteoid or bone. Nuclear phosphatase is present in most of the visceral cartilages and the perichondrium is positive in some regions especially on the dorsal surface of the copula where the dermentoglossum (membrane bone) is now developing ventrally to a set of teeth to which it becomes attached. The mesenchyme in this zone is strongly positive as has already been pointed out at earlier stages. A detailed description of the chondrocranium is given in my thesis (Lorch, 1948).

Membrane Bone. A number of new membrane bones have now taken shape and some of the bones previously described are beginning to assume the
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appearance of a meshwork of ground substance interspersed with cells which are penetrating from the surrounding mesenchyme. The newly formed bones, whether they arise in connexion with the lateral line canals such as the nasals and frontals, or as flat plates to be fused later with teeth such as the vomer and pre-maxilla, do not differ from the description given for developing membrane bones of earlier stages.

Summary of Stage 4
There is little or no phosphatase in non-skeletal tissues with the exception of the central nervous system and some sensory organs which remain strongly positive. All ossifying cartilages display nuclear as well as extracellular phosphatase, while the matrix of non-ossifying cartilages is always negative. The perichondrium of most cartilages displays enzyme activity. The phosphatase content of a cartilage is in no way correlated with the proximity of a membrane bone. The latter are positive at the growing-points.

Stage 5 (29–38 mm.)
From the point of view of phosphatase distribution the change from the 23-mm. embryo to the relatively mature state reached at 38 mm. is so gradual that it is best to describe the appearance of the oldest specimens so as to bring out major developments.

The following account is based on serial sections through a 38-mm. trout (about 3 months after hatching), which was decalcified for 5 hours. Sections were incubated for 6 or 16 hours, and counterstained with eosin. A 36-mm. specimen, cut undecalciﬁed, served for comparison, especially of the calcified structures, but was found histologically inferior owing to the diﬁculty of cutting thin serial sections through such brittle material. With regard to the somewhat smaller specimens, fairly good sections were obtained and the
two-colour technique, using gallamine blue for phosphatase, was found most useful, in that the sites both of calcification and of phosphatase activity could be visualized in the same section.

A. General Distribution of Phosphatase

The general distribution of phosphatase in non-skeletal tissues is mainly as described for Stage 4. The mesenchyme has changed somewhat in appearance in the 38-mm. specimens: fibres have developed between the cells and the latter may now be called connective tissue cells rather than undifferentiated mesenchyme. It has previously been noted that phosphatase in the mesenchyme was at first widespread but then tended to become localized in the regions of membrane bone or tooth development. There seems to be a slight reversal of this tendency: areas of connective tissue not in immediate contact with calcifying structures are found to be strongly positive in the cells—many of which have processes—and in the fibres. Since there is an extensive formation of fibres at this stage the reappearance of phosphatase in the connective tissue may be related to collagen formation.

B. Skeletal Tissues

The classification of cartilage described for Stage 4 applies here equally.

The trabecula communis and the paired trabeculae are negative. The anterior parts of the parachordals remain strongly positive in the region of the pro-otic. This bone has developed extensively and comprises perichondral lamellae of the anterior end of the parachordals, the anterior wall of the auditory capsules, the roof of the myodome, and the lateral commissure. The inner lamella of the pro-otic lies below the cerebrum and the outer represents ossification of the major part of the base of the skull.

The development of the pro-otic is a typical example of perichondral and endochondral ossification with participation of ossifying connective tissue and will therefore be described in some detail.

With regard to the distribution of phosphatase in the region of the pro-otic, the same principles hold as for cartilage bone formation elsewhere in so far as the pro-otic is represented by perichondral lamellae. Where it is formed by ossification of the membranes of the brain, the latter display strong phosphatase activity. The bone itself—whether perichondral or membranous—only displays very slight phosphatase activity at the edges. Examples of transverse sections through the region of the pro-otic will show its complicated structure and its relationship to cartilage, membranes, and to the parasphenoid bone: Text-fig. 11 shows the left half of the anterior region of the myodome and the exit of the trigeminal nerve. The pro-otic is seen as a perichondral lamella of the anterior auditory capsule and extending inwards towards the parasphenoid. Above this extension, the pro-otic has formed a network of interlacing trabeculae connecting dorsally with the ossified membranous base of the brain and forming the lateral wall of the myodome. The nuclei of the cells filling the spaces between the bony
in the Skull of the Developing Trout

Trabeculae are positive, as are also the nuclei of all other tissues in the region shown: ganglion cells of the trigeminal nerve nucleus, Schwann cells, nuclei of striated muscle, connective tissue cells, and erythrocytes. The only marked concentration of extracellular phosphatase is seen in the ossifying cartilage.

Slightly distal to the level shown in Text-fig. 11, the lateral commissure becomes visible. It is strongly positive in its middle portion where it is covered dorsally and ventrally by perichondral lamellae of the pro-otic. But its central tip is negative. The parasphenoid extends below this part of the lateral commissure but is separated from it by the perichondrium.

Text-fig. 11 and 12. Diagrammatic cross-sections through the posterior myodome region of a 38-mm. trout to show the distribution of phosphatase in the cartilage and its relation to associated bones. Key to shading as in Text-fig. 3. Description in text.

In Text-fig. 12 the cartilaginous roof of the myodome is seen. Its cells are arranged in transverse rows. At the point shown only the perichondrium and the chondrocyte nuclei have phosphatase activity. But distal to this region, where the pro-otic grows over the cartilage, the latter is strongly positive. The entry of the abducens nerve into the myodome is seen. The lateral walls of the myodome are formed by the anterior ends of the parachordals. Ventrally the parasphenoid is seen to curve inwards and partially surrounds the parachordals. The latter are positive only where the pro-otic has formed a perichondral lamella.

The extreme tip of the notochord is strongly positive and surrounded by an ossified sheath which is continuous with the basi-occipital. More distally only the notochord sheath contains phosphatase, the central tissue being negative. The basi-occipital forms perichondral lamellae dorsally and ventrally to the parachordals which are now positive. The ventral wall of the posterior semicircular canal is negative but becomes positive laterally where the exoccipital bone is developing. A bony lamella lying in the strongly positive membranes covering the ventrolateral aspect of the brain connects the basi-occipital with the roof of the brain. The exoccipital is well developed round the jugular foramen and the cartilage in that region is positive.
**Splanchnocranium.** Calcification of the autodentary although intense in places is not complete. The growing-surfaces are free from calcium salts. The bone and osteoid are relatively poor in phosphatase, but the connective tissue surrounding the dentary is strongly positive especially at the growing-tips. The phosphatase seems to be mainly nuclear, the cytoplasm of the osteoblasts and connective tissue cells is negative. But in areas of very great phosphatase activity some positive fibrils are seen.

The middle portion of Meckel's cartilage contains no extracellular phosphatase in these older specimens but the chondrocyte nuclei and the perichondral fibroblasts are positive in some areas. In the oldest fish examined (36–8 mm.) Meckel's cartilage shows two regions of phosphatase activity in its posterior portion: just before articulation with the quadrate there is a patch of positive cartilage which at first sight seems to have no relation to any perichondral bone since it is mainly in the centre of the cartilage. But closer inspection of consecutive sections shows that in some places the angular does come into direct contact with the cartilage laterally. The angular, according to Haines (1937), has taken the place of the articular in most teleosts. Like the dentary it has a cartilage and a membrane bone portion, the former being regarded by previous authors as the articular (autoarticular of Böker).

**The Pterygoquadrate.** At all previous stages the palatine process of the pterygoquadrate was completely negative or only showed slight phosphatase activity in nuclei and perichondrium. The palatine bone was then separated from the cartilage by a thin layer of connective tissue. In the 38-mm. specimen a different picture is obtained: anteriorly the palatine is composed of a very thin perichondral lamella and a membrane bone portion to which the teeth are fused. The two parts of the bone are partially separated by a very thin layer of connective tissue but are fused laterally. The cartilage is positive at its ventral edge, i.e. where the autopalatine is in apposition (Text-fig. 13). The dermopalatine extends farther backwards than the autopalatine. Hence
more distal sections show no perichondral lamella and also no phosphatase in the cartilage (Text-fig. 14). The dermopalatine is separated from the palatine process by a thin layer of connective tissue, the nuclei of which are positive. In the pterygoid region the cartilage is negative and the perichondrium positive, being in close relation to the two membrane bones (ecto- and endopterygoid) of that zone.

The distribution of phosphatase in the quadrate is similar to that at the previous stage. The tendinous tissue connecting the quadrate with the symplectic is strongly positive. It is said to ossify.

The symplectic bone is becoming thicker at the expense of the central cartilage which is very strongly positive and shows much-enlarged cells with degenerating nuclei. There is resorption of cartilage and formation of marrow spaces near the foramen for the hyomandibular branch of the facial nerve. The cartilage in that region is strongly positive. The nuclei of the perichondral and periosteal cells show phosphatase activity; Schwann cell nuclei as well as those of bone-marrow cells are also positive. In all the branchial cartilages where perichondral bone is formed there are well-defined zones of extracellular phosphatase. Areas free from bone have no phosphatase. The degree of phosphatase activity does not seem to depend on the thickness of the perichondral bone. Text-fig. 10 is a diagram of part of the branchial skeleton showing the position of 'positive cartilage' in a 38-mm. trout. This is based on serial sections, an example of which is given in Pl. 1, fig. 6. With regard to the two membrane bones dorsal to the copula the following point is again illustrated: there is no correlation between a positive reaction of the cartilage and the proximity of a membrane bone.

Membrane Bone. In the oldest specimens examined, the histological structure of the membrane bones is now clearly that of an 'adult' trout. There is no sharp transition to adult condition such as is found, for instance, in mammalian long bones where the epiphyseal cartilage is replaced by bone. Since fish continue growing throughout their life if conditions are favourable, the term 'adult' must be applied with reservations. The chief difference between teleost bone and mammalian bone lies in the relative scarcity of osteocytes. According to Kölliker (1859) these are entirely missing in some teleosts. He called the acellular tissue 'osteoid', a term which I have used here to describe uncalcified bone irrespective of the presence of osteocytes. Schmid-Monnard (1883) admits that the primary bony lamella is a structureless acellular mass, but in adult bones osteocytes are occasionally seen. Stéphan (1900) points out that both acellular and cellular bone is found in teleosts and that the former invariably consists of thin lamellae through which nutrients could diffuse, thus eliminating the necessity for a vascular system such as the Haversian systems of mammalian compact bone. The last observation is confirmed by the present series: thin, bony lamellae—whether perichondral or membrane bone—are devoid of cells. Many membrane bones, e.g. the nasals and frontals, remain thin plates and only very few osteocytes could be found in such bones. But bones which rapidly increase in bulk such
as the pre-maxillae and maxillae and the perichondral ossification of the symplectic show a fair number of cells within the ground substance (Pl. 2, fig. 1). Marrow spaces are seen in some bones. The ground substance shows lines separating the older from the more recently secreted matrix. Near the outer limit of the bone there is often a black line indicating phosphatase activity. Osteoblasts, if present, are ranged outside the osteoid layer and are usually strongly positive, as for instance at the distal end of the maxilla (Pl. 2, fig. 4). The same relationship as has just been described for bone, osteoid, and osteoblasts exists between calcified dentine, uncalcified dentine, and odontoblasts.

The relationship between membrane bone and cartilage at this stage is illustrated by the vomer in the region where it forms a continuous lamella roughly following the shape of the cartilage from which it is separated by a layer of very cellular, intensely positive connective tissue (Pl. 2, figs. 2 and 3). Below this bony lamella the connective tissue is less cellular, but the nuclei also display strong phosphatase activity.

The frontal may be mentioned here as a typical 'canal bone'. It consists of a bony tube surrounding a lateral line canal and a flat lamella extending inwards towards the mid-dorsal line. Another shorter process extends outwards from the canal. The bony plates are separated from the cartilage of the tectum cranii by a very thin layer of tissue which is both perichondrium and periosteum. It is never thicker than three layers of fibroblasts. As has constantly been noted for connective tissue between cartilage and bone, it has marked phosphatase activity. The underlying cartilage is negative. The frontal bone itself is negative in its thicker (peripheral) portions. It tapers to a thin end centrally and here, i.e. at the growing-point, it displays the 'positive lines'. The walls of the tube surrounding the lateral line canal are somewhat thicker than the flat part of the frontal and occasional osteocytes are seen. Again the growing (dorsal) tips of the bone have some phosphatase peripherally and accumulations of osteoblasts are seen. These are strongly positive, but so are most other connective tissue cells and fibres. Groups of the large round cells, noted also in connexion with the pre-opercular, are seen at the junction of the canal bone portions and the flat part of the frontal. These cells are devoid of phosphatase except for the nucleoli which stain faintly. Minute fragments of bone are sometimes found between them, and these fragments, unlike the newly formed osteoid, are free from phosphatase. The large cells suggest a possible osteolytic function. Although the connective tissue between bone and skin epithelium is strongly positive, the epithelial cells themselves display on the whole no phosphatase activity.

**Summary of Stage 5**

Further examples of the correlation between perichondral ossification and extracellular phosphatase are given. The structure of some membrane bones is described.
DISCUSSION

The following points emerge from the study of the distribution of phosphatase and 'bone salts' in growing trout.

In the early stages of development the enzyme is widely distributed and is on the whole confined to the nuclei. As differentiation proceeds phosphatase becomes more concentrated at sites of bone or fibre formation, while the nuclei of the undifferentiated mesenchyme and non-calcifying cartilage display less phosphatase activity. With the appearance of perichondral osteoid the chondrocytes undergo a marked change in appearance and, simultaneously, phosphatase activity spreads from the cells to the matrix. The change is reminiscent of that observed in mammalian hypertrophic cartilage. It is significant that bone is never formed in the absence of extracellular phosphatase. This observation is in agreement with that previously made on mammalian bones (Lorch, 1947). It must be noted that the maximum concentration of phosphatase in the cartilage occurs before there is any sign of calcification and at sites which do not themselves calcify. The pre-osseous substance seems to contain very little phosphatase. However, the perichondrium (which becomes the periosteum) is always strongly positive. A speculation regarding the source of phosphatase in cartilage bone formation is of interest. In mammalian endochondral ossification the enzyme is said to be derived from osteoblasts as well as hypertrophic cartilage cells. In the trout, osteoblasts are not prominent and are especially rare in connexion with perichondral bone formation. Therefore the most likely sources of phosphatase are the enlarged cartilage cells which may secrete the enzyme into the matrix with which the bone is in contact. The strongly positive reaction of these cells and the adjacent matrix favours this view. The fact that the cartilage itself does not calcify at the stages examined is surprising, but may be a necessary condition for the diffusion of the enzyme from the cells to the periphery.

In membrane bone formation increased phosphatase activity was also noted in the mesenchyme well before the onset of calcification. The enzyme occurred in fibres as well as cells, but the change from purely intracellular to extracellular phosphatase was not as marked here as in cartilage bone formation.

However close a membrane bone is to a cartilage, the latter never displays phosphatase in the matrix, unless it is itself ossifying. Hence the two types of bone can easily be distinguished by means of the Gomori method.

The absence of phosphatase from the calcifying osteoid and the fact that calcification starts at the centre, whereas the highest concentration of enzyme is found at the periphery, may seem surprising. However, if it be considered that osteoid is a dense avascular tissue surrounded on all sides by a zone of high phosphatase activity, it seems likely that the inorganic phosphate liberated at the periphery tends to accumulate within the osteoid and so a high level of phosphate ions may be reached without the presence of phosphatase at the actual site of calcification.
As calcification proceeds, new layers of osteoid are formed at the periphery. Once the bone is well defined, its phosphatase content is usually very low, except at the growing-tips and edges. Very few cells are enclosed in the bony matrix and they contain no phosphatase. The phosphatase content of the mesenchymal cells and fibres stays high as ossification proceeds although the bone itself may be quite negative.

The biochemical studies of phosphatase in fish, mentioned in the introduction, must necessarily deal with organs or parts of organs and no information regarding the distribution of phosphatase within a tissue or its intracellular distribution can be gained from them. The facts previously established may be briefly reviewed in the light of the present work: Roche and Bullinger (1939) found that 'phosphatase was present in all teleost bones examined and high concentrations of enzyme were present in scales and teeth'.

Roche and Collet (1940), working on the sardine, found that there was a seasonal increase in phosphatase activity in the whole skeleton during spring and early summer, i.e. when optimum conditions for growth prevail. They emphasize that this constitutes evidence for the physiological regulation of phosphatase activity in the skeleton as a whole, possibly by an endocrine mechanism. Without wishing to contradict this hypothesis for which there is independent evidence (Roche and Filippi, 1938), I think it should be pointed out that the increase of phosphatase activity in different parts of the skeleton may be 'simultaneous' when reckoned in terms of months, but histochemical studies of the ossification of the chondrocranium of the trout show that the increase of phosphatase activity of each cartilage—or portion of cartilage—is exactly correlated with the appearance of perichondral osteoid round the particular cartilage and in no way influenced by ossification in an adjacent cartilage or within the mesenchyme.

With regard to the distribution of phosphatase in the embryo generally a comparison with results obtained by Moog (1944) on chick embryos and by Horowitz (1942) on the heads of foetal rats is interesting.

According to Moog 'phosphatase persists as long as a tissue remains undifferentiated. As differentiation proceeds, phosphatase in some cases disappears and in others accumulates in higher concentrations than in the primitive phase.' Although the trout embryos examined did not include the very early stages of development comparable to Moog's chick embryos, her statement is on the whole confirmed and similar observations were made on the marine teleost Cottus bubalis, early stages of which were examined as a preliminary study (unpublished) to the present work.

Horowitz (1942) commences his study of phosphatase and glycogen with rat foetuses at the gill arch stage (13 days) and finds them 'devoid of phosphatase'. However, incubation was only carried out for 2 hours.

In 15-day foetuses Horowitz notes that prospective regions of calcification show a marked phosphatase activity, i.e. they become chemically differentiated before the occurrence of any morphological differentiation. This is in accord with the present results. Also the irregular distribution of phosphatase in the
central nervous system, and its high concentration in the linings of blood-vessels, in taste-buds, and in the lens epithelium are paralleled in the trout embryos.

With regard to ossifying cartilage Horowitz's results are in agreement with previous descriptions for other species: hypertrophic cartilage containing high concentrations of phosphatase in cells, matrix, and perichondrium is invariably associated with ossification.

It is seen that apart from minor differences, there is a striking parallelism between phosphatase distribution in developing embryos belonging to species as widely different as chicks, rats, and trout. It is therefore indeed likely, as suggested by Moog, that phosphatase plays a fundamental role in histogenesis, apart from its function in the development of calcified structures.

I should like to thank all those who have helped me by their advice and criticism, particularly Dr. J. F. Danielli for his encouragement throughout this work.

I am grateful to Professor Samson Wright for providing facilities, to Dr. P. D. F. Murray and Dr. A. Stock, of St. Bartholomew's Medical College, for their kindness and hospitality, to Professor G. R. de Beer for helpful suggestions, to Messrs. F. W. Steven and R. A. Isaacs of the Gloucestershire Fisheries for their co-operation in obtaining specimens, to Dr. P. Pincus and to Mr. D. Stevenson-Clark of Ilford, Ltd., for their advice on photomicrography, and to Mr. H. S. Edwards for preparing the drawings for publication.

The work was financed by a grant from the Medical Research Council to Professor Samson Wright.

The work described in this paper formed part of a thesis approved for the degree of Ph.D. in the University of London.

**SUMMARY**

1. The histological and cytological distribution of alkaline phosphatase in developing trout has been studied with special reference to membrane and cartilage bone formation in the skull.

2. Nuclear phosphatase is widely distributed in the youngest stages examined, but decreases as differentiation proceeds.

3. Extracellular phosphatase is always associated with ossification or fibre formation.

4. No deposition of calcium salts in the absence of phosphatase was observed.

5. Alkaline phosphatase is probably connected with histogenesis in general apart from its special function in calcification.
REFERENCES

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DESCRIPTION OF PLATES 1 AND 2

All figures are unretouched photomicrographs of undecalcified sections treated by the Gomori (1939) method, unless otherwise stated. Areas of phosphatase activity are shown black. No counterstain was used.

PLATE 1

Fig. 1. Trabecula communis of a 12-mm. trout. Incubation time 6 hours. The chondrocyte nuclei are positive. Note the strong reaction of the mesenchyme dorsal to the trabacula.

Fig. 2. Quadrature and hydraulic cartilage of a 12-mm. trout. Incubation time 6 hours. Note the strongly staining mesenchyme lateral to the cartilages. This is the site of the future pre-opercular bone. Chondrocyte nuclei and perichondrium are positive.

Fig. 3. Transverse section through the anterior auditory region of a 20-mm. trout. Incubation time 2 hours. Note the intense reaction of the ceratohyal and the absence of phosphatase from the parachordals and auditory capsule. The hyomandibula shows patches of phosphatase activity.

Fig. 4. Quadrature process of a 20-mm. trout showing perichondral bone. Incubation time 2 hours. Note the strongly positive reaction of the matrix near the zone of ossification.

Fig. 5. Distal end of quadrature cartilage at its articulation with the symplectic process. 20-mm. trout. Incubation time 6 hours. Note the difference in appearance and phosphatase content between non-ossifying and ossifying cartilage: only the symplectic is surrounded by a layer of osteoid.

Fig. 6. Copula and hypohyals of a 38-mm. trout. Level in Text-fig. 10. Decalcified transverse section, incubation time 6 hours. Counterstained with eosin. Note the positive reaction of the copula which has a shell of perichondral bone. The hypohyals are only positive ventrally where ossification is beginning.
PLATE 2

Fig. 1. Undecalcified premaxilla of a 30-mm. trout. Bone salts visualized as cobalt sulphide. 
Not incubated for phosphatase visualization. Note cell spaces in the bone.

Fig. 2. Trabecula communis and vomer of a 32-mm. trout. Incubation time 15 hours. 
Note the strongly positive connective tissue in the area of tooth formation.

Fig. 3. Detail from centre of previous figure. Note the clear zone of osteoid (free from both 
calcium salts and phosphatase) on both sides of the vomer. The perichondrium on the ventral 
aspect of the trabecula is strongly positive.

Fig. 4. Distal end of maxilla of a 29-mm. trout. Incubation time 4 hours. The maxilla 
is not calcified in this zone. It displays faint phosphatase activity. Osteoblasts are seen on 
both sides of the osteoid. The surrounding connective tissue is strongly positive.