The Distribution of Alkaline Phosphatase in Relation to Calcification in *Scyliorhinus canicula*

Development of the Endoskeleton

BY

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With one Plate

INTRODUCTION

IN teleosts and all higher vertebrates calcification is a preliminary to ossification. But in elasmobranchs calcified cartilage functionally replaces bone. The histology and biochemistry of elasmobranch cartilage have been reviewed by Weidenreich (1930). In spite of the vast literature on the subject, the relation of the calcium salts to the matrix is not fully understood and there is disagreement regarding the presence and amount of calcium phosphate. However, the following points seem established: calcification always starts at the periphery of a piece of cartilage, at a point remote from the cells. Amorphous granules are first formed which later coalesce into an irregular mosaic of crystalline plates. In *Scyliorhinus* the platelets cover the inner and outer surfaces of the vertebral arches and the skull and the total surfaces of the visceral skeleton. The pattern of calcification in the vertebral column of the higher elasmobranchs is so typical that it has been used as a basis of classification (Hasse, 1893). In view of these facts a study of the distribution of alkaline phosphatase in an elasmobranch seemed of interest. Here normal calcification can be studied without the complications of cartilage hypertrophy and resorption and the simultaneous formation of a new protein matrix which always accompany ossification.

Badansky, Bakwin, and Bakwin (1931) showed that alkaline phosphatase exists in elasmobranchs, but drew no correlation between phosphatase activity and calcification. The question was studied in greater detail by Roche and Bullinger (1939). These authors showed that the phosphatase present in the skeleton of both teleosts and elasmobranchs belongs to the Class IA of Folley and Kay (1936); i.e. it is closely similar to that found in mammalian bones. By means of microchemical tests Roche and Bullinger showed that only the calcifying regions of elasmobranch skeletons contained the enzyme, adjacent soft portions being negative. These results as well as a study of phosphatase in relation to the growth of scales and teeth (Roche, Collet, and Mourgue, 1940) led the authors to conclude that in elasmobranchs, as in higher vertebrates, phosphatase is concerned with rapid calcification.

No histochemical study of the fish skeleton seems to have been undertaken, nor has phosphatase been studied during the embryonic development of lower vertebrates. It therefore seemed desirable to undertake a systematic study of the histological and cytological distribution of alkaline phosphatase in developing teleost (Lorch, 1949) and elasmobranch embryos. Only the endoskeleton is considered here.

**METHODS**

Twenty egg-cases and five hatched specimens of *Scyliorhinus canicula* were obtained living from the Marine Biological Laboratory, Plymouth. After removal of egg-case and yolk-sac the specimens were anaesthetized in 10 per cent. ethyl alcohol, measured, and placed into 80 per cent. ethyl alcohol in distilled water. When sufficiently hardened they were divided into a number of blocks according to the size of the fish and fixation was continued up to 24 hours. It was found that when 80 per cent. alcohol in sea water was used as fixative, considerable destruction of phosphatase occurred. The reason for this is not clear. The heads of three of the hatched specimens were split longitudinally, one half being decalcified by my method (Lorch, 1947b) and the other cut undecalcified. It was found that 4 hours' decalcification (4 changes of buffer at 10° C. and pH 4.7) was adequate, but times up to 4 days did not result in an appreciable loss of enzyme. Reactivation was carried out at room temperature for 24 hours.

All specimens were dehydrated in alcohol, cleared in cedarwood oil or benzene, and embedded in paraffin (m.p. 56° C.). Serial sections (8 μ) were mounted without albumen. For the visualization of alkaline phosphatase Gomori's (1939) method as modified by Danielli (1946) was mainly employed. Incubation times were varied from 1 to 15 hours at 28° C. Some sections were treated by the diazo-method of Menten, Junge, and Green (1944), by means of which phosphatase can be visualized independently from calcium salts. However, only sites of very great enzyme activity can be demonstrated (Lorch, 1947a). The two-colour method involving the use of gallamine blue (Lorch, 1947b) was used to demonstrate calcium salts and phosphatase in the same section. Calcium deposits were also visualized by von Kossa's silver nitrate method, by staining the section for 5–15 mins. in a saturated neutral solution of gallamine blue (Stock, 1949), and by bulk alizarin red S staining. The usual histological stains were employed to elucidate general structure. A combination of methyl green, which stains cartilage metachromatically, and van Gieson's stain was found useful.

**RESULTS**

The description of phosphatase distribution is based on a study of 13 specimens which may conveniently be divided into 6 stages (see opposite). Purely anatomical features will not be described; reference should be made to de Beer (1931, 1937).
in relation to Calcification in Scyliorhinus canicula

<table>
<thead>
<tr>
<th>Stage</th>
<th>Length (mm.)</th>
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<tr>
<td>5</td>
<td>75</td>
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<tr>
<td>6</td>
<td>96–115</td>
<td>5</td>
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1. Cartilage of the Skull

At the earliest stages examined (18–20 mm.) no cartilage is present. The first signs of precartilaginous tissue appear in the region of the future parachordals as condensation of mesenchymal cells. No phosphatase was detected in this tissue, nor in the developing cartilages of the 32–35-mm. specimens. At Stage 3 (47 mm.) the main features of the adult skull are established. The growing regions and the more recently formed cartilages are still negative for phosphatase, but a positive reaction is now given by the chondrocytes of some older cartilages. Centrally placed cells are only faintly positive but the flattened chondrocytes at the periphery stain strongly. Perichondral fibroblasts and fibres display phosphatase activity in most cases but never at the growing zones. In the parachordals, the oldest cartilages of the skull, the cells stain strongly and traces of phosphatase occur also in the matrix. Meckel's cartilage of the lower jaw may be described as typical of this stage: its anterior tip is just distinguishable from the connective tissue. It has no phosphatase activity. Distally, Meckel's cartilage is better defined and has a distinct positively staining perichondrium on the dorsal and ventral surface. Laterally the cartilage merges into connective tissue. Pl. I, fig. 1, shows the distribution of phosphatase in two jaw cartilages of a 47-mm. specimen. In the 58-mm. embryo the anterior parts of Meckel's cartilage are partly surrounded by positively staining perichondrium, leaving the central (growing) surfaces free. But in the mid-orbital region the strongly staining perichondrium forms a complete ring round the cartilage and patches of strongly positive chondrocytes make their appearance near the ventral edge. The matrix is also slightly positive here. Similar patches are seen in the pterygoquadrate and ceratohyal. Apart from these occasional well-localized patches the cartilage matrix is negative at this stage, but nuclear phosphatase occurs in some of the flattened peripheral chondrocytes and in the perichondral fibroblasts.

At Stage 5 a new phenomenon makes its appearance: the jaw cartilages, parachordals, and most of the brain case of the 75-mm. and older specimens display phosphatase activity in a well-defined pattern. The cartilage may be divided into three zones (Text-fig. 1). Immediately below the perichondrium the 2–3 rows of flattened cells continue to display phosphatase activity and the matrix may give a moderately strong reaction (Zone 1). This region shows less affinity for basic dyes than the rest of the cartilage matrix. Passing inwards the cells become more rounded and their phosphatase activity increases
The Distribution of Alkaline Phosphatase

Lorch—The Distribution of Alkaline Phosphatase

sharply. Pericellular 'capsules' of intensely positive matrix are frequent. The matrix between the cells also stains strongly (Zone 2). Centrally to this layer of cells the cartilage abruptly loses its phosphatase activity, although it does not appear in any way different when ordinary staining procedures are used (Zone 3). The zones are best distinguished after relatively short incubation times and it becomes clear that the maximum phosphatase activity is found in Zone 2. When the growing region of a cartilage is examined it is seen that phosphatase appears in the following order: first in the perichondrium as soon as this is clearly defined, secondly in 'Zone 1' (flattened cells), and finally in the cells and matrix of Zone 2 where the reaction is most intense.

FIG. 1
TEXT-FIG. 1. From a decalcified transverse section through the pterygoquadrate of a 110-mm. specimen, showing the distribution of phosphatase at the periphery of the cartilage. Incubation time, 5 hours.

TEXT-FIG. 2. Calcified platelets at the periphery of the parachordal cartilage of a 115-mm. specimen. Stained with gallamine blue and counterstained with safranin. Note position of platelets corresponds to 'Zone' 2 in Text-fig. 1.

As growth proceeds Zone 2 increases somewhat in thickness, but the negative core of cartilage (Zone 3) persists even in adult dogfish. Pl. I, fig. 2, shows the appearance of Meckel's cartilage and the pterygoquadrate at Stage 5.

In the skulls of embryos up to Stage 6, there was no sign of calcification detectable by histochemical methods. But in the hatched specimens (96–115 mm.) calcified platelets are seen at the periphery of some of the cartilages (Text-fig. 2). With regard to the distribution of phosphatase in these specimens there is no qualitative change in the pattern just described. However, Zone 2 is now present in a greater number of cartilages and has increased in thickness in places where it was previously found. The reaction is even more intense and can be detected after very short incubation times (1 hour). It is of interest to note that the calcified platelets are always located within Zone 2. The phosphatase reaction is more extensive than the region of calcification, but the reverse was never the case. The perichondrium, previously positive, now appears negative. Some of the smallest branchial cartilages are still devoid of phosphatase, whereas others show a patchy distribution of Zone 2. Meckel's cartilage, the pterygoquadrate, and the ceratohyal have broad, intense bands of phosphatase activity, and show most extensive peripheral calcification. In the anterior region of the parachordals extracellular phosphatase is present only near the ventral surface, whereas more distally ventral and dorsal positive bands are seen. Calcification is strictly correlated with this phos-
in relation to Calcification in Scyliorhinus canicula

phatase distribution. Decalcified sections treated with haematoxylin and eosin or van Gieson’s stain do not show any change in the matrix in the region of calcification, such as takes place in the calcifying cartilage of mammals, nor is there any hypertrophy of the phosphatase-containing cells similar to that seen during cartilage-bone formation in the trout (Lorch, 1949). In

cartilages of an adult dogfish examined, essentially the same distribution of phosphatase was found as in the calcified cartilages of the recently hatched specimens.

2. The Vertebral Column

Only the trunk region will be described. Up to the 35-mm. stage no phosphatase was detected in the notochord or in the rudiments of the vertebral cartilages. However, at Stage 3 (47 mm.) intense, well-localized areas of phosphatase activity are found. The matrix at the periphery of the neural arch cartilages is strongly positive as well as the chondrocytes both at the periphery and, to a lesser degree, in the centre (Text-fig. 3). Only the cartilage at the junction of neural plate and notochord sheath is entirely free from phosphatase. The intercalary tissue and the perichondrium are negative. Zones of phosphatase activity display an affinity for acid dyes: the periphery

TEXT-FIG. 3. Transverse section through the vertebral column of a 47-mm. specimen. Phosphatase shown black (Gomori method). There is no calcification. Incubation time, 4 hours.

spinal cord

neural plate

cartilage of future centrum

notochord sheath

200 μ
of the neural arches stains pink with eosin or acid fuchsin and red with Heidenhain's Azan.

The spongy tissue of the notochord displays no phosphatase activity at this and subsequent stages, but the fibrous sheath foreshadows the future double-cone pattern of calcification in the vertebral centra by the distribution of phosphatase in the nuclei of spindle-shaped concentrically arranged cells. There is as yet no calcification in the vertebral column.

At Stage 4 (58 mm.) the pattern of phosphatase distribution becomes more sharply defined and the reaction more intense (Pl. I, fig. 3). The strongest reaction (very marked after 20 minutes' incubation) is given by the cells and fibres of the notochord sheath but only in the area of future calcification. The intervening tissue is negative, although as yet no histological differentiation can be seen within the future centrum. In the neural plates the cells and matrix are now positive throughout the cartilage, but short incubation times (1 hour) and application of the diazo-method show that the most intense reaction occurs in the matrix at the periphery (Text-fig. 4). The first signs of the calcification are now seen in the matrix just below the peripheral layer of flattened cells which is very narrow in the vertebral cartilages (Pl. I, fig. 4). No platelets of calcium salts similar to those in the skull have been noted. Sections treated by von Kossa's silver nitrate method show a granular deposit (Text-fig. 5). It is again noted that the phosphatase reaction is more widespread than the zone of calcification, but deposits of calcium salts do not occur in negative regions.

In the 74-mm. embryo calcification of the neural plates has progressed considerably: only the central core of the cartilage is now free from calcified deposits. There is a tendency for the granules to fuse and form a homogeneous calcified zone (Pl. I, fig. 6). In the specimens above 95 mm. granular deposits are no longer seen. The phosphatase activity of the neural plates becomes less as calcification increases: at Stage 5 some positive chondrocyte nuclei are encountered within the most recently calcified zone, but heavily calcified regions are quite negative (Pl. I, fig. 5). At the junction between the calcified outer layer and the uncalcified core of cartilage the matrix continues to display phosphatase activity. Hence it appears that the phosphatase positive zone is progressively pushed inwards by the advance of calcification. There is some overlap when phosphatase and calcium salts occur in the same region.

The matrix of the calcified cartilage becomes increasingly more acidophil, while uncalcified cartilages such as the neural spines show the usual affinity for basic dyes. In the hatched specimens the strongly calcified periphery of the neural plates have irregularly shaped lacunae which contain the chondrocytes. The latter also show various shapes and do not resemble the spherical chondrocytes of the non-calcified cartilage which have basiphil granules in the cytoplasm. In the transition zone (phosphatase positive) some chondrocytes have a strongly acidophil cytoplasm devoid of granules, while others are still granular.
in relation to Calcification in Scyliorhinus canicula

The notochord sheath first shows a zone of calcification in the 75-mm. embryo, i.e. somewhat later than the neural plates. The pattern already established in the 47-mm. specimen by the phosphatase distribution is main-

![Image](image-url)

**Fig. 4**

**Fig. 5**

TEXT-FIGS. 4 and 5. From the neural plate of a 58-mm. specimen. In Fig. 4 phosphatase is shown as Ca α naphthyl phosphate (modified method of Menten, Junge, and Green, 1944). Fig. 5 shows the distribution of calcium salts (von Kossa's nitrate method).

tained. The calcium deposits occur in the middle zone of the fibrous sheath, where a layer 2–3 cells thick stains heavily with silver nitrate (Pl. I, fig. 6). The elongated cells, surrounded by calcium salts, are still well defined and display some phosphatase activity. But in the oldest specimens, where the calcified zone is much thicker, the cells are negative.
Lorch—The Distribution of Alkaline Phosphatase

The inner zone of the fibrous sheath displays strong phosphatase activity in all 74-115-mm. specimens, but the outer zone becomes progressively less positive. In the intervertebral regions there is no calcification and no phosphatase. No secondary calcification of the outer zone of the notochord sheath was noted. This is said to occur (Hasse, 1893).

The relation between phosphatase and calcification in the skeleton of the series of dogfish embryos examined is summarized in Table 1.

**DISCUSSION**

The following points emerge clearly from this study: phosphatase is absent from the cartilages of the young embryos and appears first in the chondrocyte nuclei and in the perichondrium. At a stage just prior to calcification the enzyme is also detected in the cartilage matrix. The maximum extracellular phosphatase activity occurs during the first stages of calcification. The phosphatase positive zone then retreats in front of the wave of calcification. This is most clearly seen in the neural plates where calcification has reached the most advanced state. In the skull the stage of secondary reduction of phosphatase activity has only just been reached in the hatched specimens. It seems, therefore, that in dogfish, as in teleosts and mammals, phosphatase is an essential pre-requisite for calcification. Moreover, it is noted that calcification only occurs in regions where extracellular phosphatase is present, but there is a lag between the appearance of the enzyme in the cartilage matrix (or notochord sheath) and the onset of detectable calcification. It is interesting to note in this connexion that no extracellular phosphatase could be detected in the cartilage and notochord sheath of an adult lamprey (*Petromyzon fluviatilis*), although the peripheral chondrocytes gave a positive reaction as in young dogfish embryos. The skeleton of cyclostomes does not calcify. To save space no description of the distribution of phosphatase in tissues other than the skeleton has been given, but it must be understood that the enzyme is present in tissues other than those here described. Taking the embryo as a whole the conclusions reached with developing trout (Lorch, 1949) are confirmed: nuclear phosphatase shows no relation to calcification, whereas extracellular phosphatase is only found in connexion with calcification or fibre formation.

There is one notable difference between calcification in the dogfish and cartilage bone formation in the trout: in the latter the chondrocytes enlarge, the matrix becomes basiphil and a perichondral layer of pre-osseous substance is formed prior to calcification. However, in *Scyliorhinus* the calcium salts are precipitated within the cartilage and neither the matrix nor the cells show any preliminary change other than the phosphatase distribution. (The change in staining reaction described by Weidenreich [1930] and noted in the vertebrae of the older specimens is secondary to calcification.)

Whereas one cannot exclude the possibility of phosphatase playing a part in the formation of the pre-osseous matrix in teleosts and mammals, such a role is unlikely in elasmobranchs. The simplest hypothesis is therefore that
<table>
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<td>ADULT</td>
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<tr>
<td>Remarks: NO CARTILAGES, PRECARTILAGE NEGATIVE. VERTEBRAE NOT EXAMINED.</td>
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phosphatase is here solely concerned with the precipitation of calcium phosphate.

ACKNOWLEDGEMENTS

I should like to thank Professor J. F. Danielli for reading the manuscript and Professor Samson Wright for providing facilities for this work, which was financed by a grant from the Medical Research Council.

SUMMARY

1. A histochemical study has been made of alkaline phosphatase in the endoskeleton of dogfish embryos of 18–115 mm.
2. The distribution of phosphatase is compared with that of insoluble calcium salts.
3. Phosphatase was first noted in the chondrocytes and perichondrium at 47 mm. and in the cartilage matrix at 58 mm.
4. Calcification occurred first in the neural plates at 58 mm. and in the skull at 74 mm. No calcification was observed in zones devoid of extracellular phosphatase.
5. As the intensity of calcification increased the amount of phosphatase tended to drop.
6. It is concluded that in elasmobranchs, as in higher animals, extracellular phosphatase is an essential precursor of calcification.

REFERENCES

— 1947b. Ibid., 88, 367.
— 1949. Ibid., 90, 183.
—, COLLET, J., and MOURCUE, M., 1940. Enzymologia, 8, 257.

DESCRIPTION OF PLATE I

Fig. 1. Transverse section through the jaws of a 47-mm. specimen. Sites of phosphatase activity black (Gomori method). Incubation time, 3 hours. There was no calcification.
Fig. 2. Transverse section through the jaws of a 75-mm. specimen. Sites of phosphatase activity black (Gomori method). There was no calcification. Note the patchy distribution of phosphatase in the cartilage matrix. Incubation time, 3 hours.
Figs. 3 and 4. Serial transverse sections through the vertebral column of a 58-mm. specimen. Fig. 3 incubated 6 hours for phosphatase visualization (Gomori method). Fig. 4, unincubated control showing zone of calcification.
Figs. 5 and 6. Serial transverse sections through the vertebral column of a 75-mm. specimen. Fig. 5 shows the distribution of phosphatase in a decalcified section (Gomori method, 3 hours' incubation); Fig. 6 shows the extent of calcification (von Kossa's method). Note the regression of phosphatase from heavily calcified zones.