

Autoradiographic Studies of the Utilization of Ca^{45} by the Chick Embryo*

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ABSTRACT

Calcium-45 was injected into the dense albumen of fertile hen's eggs, to the extent of 25 μc . per egg. The eggs were incubated under standard conditions and three or more embryos removed daily and fixed in 10 per cent neutral formalin. Stripping-film autoradiograms were prepared from paraffin sections of the tibiofibulae. Exposure varied with the isotope concentration. The tissue sections with their autoradiograms in place were stained with dilute Giemsa, while other sections were stained with hematoxylin-azure-eosin and by von Kossa to demonstrate bone salt.

At about 9 days, Ca^{45} is found in the cartilage template both intra- and extracellularly. Between 9 and 11 days, a primary diaphyseal lamella is deposited which is largely acellular. The lamella is eroded by capillaries from the periosteum and a resorption center is established in the cartilage. New lamellae of bone are deposited centrifugally in an imbricated pattern. Bone matrix formation precedes calcification by about 1 to $\frac{1}{2}$ days, and calcification in a particular lamella is not uniform. Endochondral bone formation is described, as well as calcification of the epiphyseal/diaphyseal cartilage. Calcium-45 occurs intracellularly in the osteocyte during bone formation.

INTRODUCTION

The mineral metabolism of the avian embryo has interested biochemists and embryologists for many years and considerable work has been done to elucidate the problems involved. Needham (1931) should be consulted for a review of the literature. Johnston and Comar (1955), using Ca^{45} as a tracer, determined the time of initial calcification of the skeleton of the chick and the relative contributions of the yolk, albumen, and shell to its development.

Intimately associated with mineral metabolism are, of course, the processes of bone formation and bone remodeling. Fell and Robison (1930, 1934), Robison and Soames (1930), and more recently Sevastikoglou (1957), have studied these phenomena *in vitro*, and others have attacked the problem from various aspects in a variety of forms (See Bourne, 1956). Bloom *et al.* (1941) have

described the changes which occur in the medullary bone during the ovarian cycle in the pigeon.

This report describes an autoradiographic study of the process of bone formation in the chick embryo beginning with the 7th day of incubation and extending through the 21st day or hatching. Since it was not possible to examine all bone-forming areas in detail, the tibiofibula was chosen as an index of osteogenesis, and the descriptions which follow are based on observations made during its development.

Materials and Methods

Embryonated hen's eggs of mixed breeds, mostly White Leghorn, were obtained locally. Each egg was weighed and numbered serially. The eggs varied in weight from 53 to 70 gm. After equilibration with room temperature, each egg was injected with 0.1 ml. of a solution containing 25 μc . of Ca^{45} as chloride.¹ Injection

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¹ Radioactive materials were obtained from the Oak Ridge National Laboratory on allocation from the United States Atomic Energy Commission.

was made into the dense albumen as described earlier (Johnston and Comar, 1955). A reference standard, equivalent to 0.01 per cent of the dose, was prepared from the dosing solution at the time of injection. After a mixing period of several hours, the eggs were incubated under standard conditions of temperature, humidity, and air movement. Beginning with eggs incubated for 5 days, three or more embryos were removed and staged according to Hamburger and Hamilton (1951), and then grouped into the proper morphological day sequence. Fixation was carried out in 10 per cent neutral formalin, and was varied from a few hours to several days, depending upon the age of the embryo. After fixation, both hind limbs were removed and the tibiofibular portions separated from the remainder. Experience showed that better sections were obtained when the muscles and connective tissues were left intact. The tibiofibulae were dehydrated in alcohol-dioxane, cleared in dioxane and xylol, and embedded in paraffin. One tibiofibula was sectioned longitudinally and the other transversely at scale settings of 5 and 7 microns, but mostly at 5 microns. In some instances, entire serials were mounted, whereas in others only those sections showing the greatest degree of marrow cavity formation were used. All sections were mounted on glass slides, using a minimum of albumen fixative and water.

The isotope concentration in the sections was determined from the reference standard, using a thin window (1.19 mg./cm.²) G-M tube. This determination was used to estimate the exposure time for the sections to be autoradiographed. Autoradiograms were prepared according to Doniach and Pelc (1950), as modified by Lotz and Johnston (1953). Exposure time varied from 1 to 21 days. The autoradiograms were developed in Kodak D-19 for 4 minutes and fixed in acid fix for 10 minutes. The preparations obtained were of sufficient density to be interpreted subjectively and did not require grain counting. Because of the special property of the Kodak stripping film which was used, with a developable grain yield of one per incident electron, a relatively accurate judgment of the concentration of isotope in a given section or region could be made on the basis of grain density.

After photographic processing, the tissue sections, with their autoradiograms in place, were stained with dilute Giemsa. Afterward the autoradiograms were mounted in a non-drying immersion oil.

Sections adjacent to those being autoradiographed were stained with azure-eosine (pH 4.5-4.7), and counterstained with hematoxylin. Other sections were prepared according to von Kossa's technique to demonstrate the presence of bone salt.

OBSERVATIONS

The osteogenetic sequence in the tibiofibula of the chick can be divided conveniently into three

phases: (1) the prelamellar; (2) the lamellar; and (3) the postlamellar, or remodeling. The first phase extends from the formation of the cartilage template at about 7 to 8 days, through the formation of the primary diaphyseal lamella at about 11 days. The second phase involves those events occurring between 11 and 17 days, and the third phase consists of those changes which occur subsequent to the 17th day. The observations which follow are described accordingly.

The Prelamellar Phase.—Subsequent to the formation of the cartilage template, a sheath of osteogenic fibers forms beneath the perichondrium. This sheath is particularly well developed in the middle third of the diaphysis and stains bright pink following hematoxylin-azure-eosine staining. Coincidentally, the chondrocytes begin to hypertrophy and the cartilage matrix becomes more basophilic, presumably from an increase in alkaline phosphatase (Fell and Robison, 1934). During this time there is a movement of Ca⁴⁵ into the template, where it occurs both intra- and extracellularly (Fig. 1). The autoradiographic reaction of the cartilage does not suggest a preferential accumulation either in the matrix or in the chondrocytes (Fig. 1). From a comparison of Fig. 1 with Fig. 2, which shows muscle adjacent to cartilage, it may be deduced that reaction above the cartilage was produced by the Ca⁴⁵ in the cartilage. The total calcium present is negligible, and no formation of bone salt was demonstrable using von Kossa's technique. Chemical analyses made earlier (Johnston and Comar, 1955) have shown that the total calcium in the embryo at 9 days was 0.23 mg., and in the tibiofibula was 0.02 mg.

Between the 9th and 11th days the calcium in the tibiofibula increases and is confined very largely to a layer beneath the periosteum (Figs. 3 and 4). Prior to the calcification of this primary lamella, the cartilage template is attacked by chondroclastic buds from the periosteum, resulting in the formation of an uneven peripheral contour (Fig. 4). From Figs. 3 and 4, then, it may be seen that the Ca⁴⁵ is more concentrated in the lamella than elsewhere. The primary lamella is essentially acellular, and inspection of Fig. 4 reveals that no osteocytes are present in the lamella. Osteoblasts apparently differentiate from the innermost cells of the periosteum, and the deposition of the lamella is from one side only. Osteoblasts were not observed between the cartilage template and the

lamella. In Fig. 4 it may also be noted that Ca^{45} is present in the cartilage. It is known from chemical analyses made earlier that at the end of 11 days the calcium of the embryo has increased to 0.76 mg., and that of the tibiofibula to about 0.12 mg. (Johnston and Comar, 1955).

The Lamellar Phase.—After the primary lamella is formed, its medial surface is eroded by blood vessels from the periosteum and a primary cartilage resorption center is established, which later extends toward the epiphyses. The lamellar phase is characteristic of intramembranous ossification. The new lamellae are laid down in an imbricated pattern, which changes in direction of overlap at about the middle of the diaphysis. Because resorption and reconstitution of diaphyseal bone does not occur to any large extent until after hatching, the approximate age of the bone may be determined by counting the successive layers of lamellae.

During this phase several events occur which are significant. The first of these defines the relationship between the matrix of the bone lamellae and its subsequent calcification. Because of the centrifugal character of the growth of the tibiofibula, all facets of lamellae differentiation may be observed in a single cross-section of the bone, if one uses a tibiofibula taken between 13 and 17 days of development. Therefore, a detailed description of such a section appears to be warranted (Fig. 5).

Fig. 5 represents a segment of a cross-section of the tibiofibula of a 13 day embryo. The diaphyseal cartilage may be seen in the upper right portion of the figure, where one of the chondrocytes is clearly visible at *a*. The primary lamella, immediately adjacent to the cartilage, is almost obscured by the autoradiographic reaction above it. Outward and downward from this at *b* there is the first true or 12 day lamella, and it can be seen that the Ca^{45} is not uniformly distributed throughout this lamella. Two areas, *x* and *y*, show a high concentration, whereas the region between them and the region labelled *z* show lesser concentrations. This indicates that calcification does not occur at the same rate everywhere in a particular lamella. The 13 day lamella, *c*, is less well developed than the 12 day one, and it may be noted that these circumferential lamellae begin as isolated nests of osteoblasts which subsequently anastomose. An initial stage of radial lamellar bridging is seen at *y*. That portion of the 13 day lamella

lying to the right of the bridge appears to be only slightly calcified. Most of the Ca^{45} is confined to the center of the lamella, while along its edge osteoblasts and osteocytes are seen in various stages of entrapment. Another portion of the 13 day lamella may be seen at *d*, and here also, between *u* and *v*, there is matrix that is being calcified. Another interlamellar bridge is forming at *e*. Examination of the autoradiogram under higher power showed a considerable fraction of the Ca^{45} to be intracellular in those lamellae in the initial stages of calcification. The same distribution was observed in later stages also. To the left of *c* and below *d* may be seen a nest of osteoblasts, *f*, surrounding a core of matrix. This area represents the beginning of the 14 day lamella, and is descriptive of the manner of formation of the previous lamellae, with the exception of the primary. The slight autoradiographic image above this matrix core is not significant, since it does not exceed background. The periosteal surface may be seen in the lower left hand corner, but is ill defined. Several capillaries, present between the lamellae, are indicated by small arrows.

Another phase in the formation of the lamellae is depicted in Fig. 6. Here the development of an interlamellae bridge may be seen as an extension of one lamella toward the next adjacent lamella. In the region lying between *x* and *y*, it is evident again that matrix formation precedes calcification, for an elongated area may be seen which contains little or no Ca^{45} . This region stains bright pink with Giemsa. Also it should be noted that in certain metachromatic areas (appearing dark grey in the figure) there is unequal deposition of Ca^{45} , with considerable concentrations at some foci. This formation of "hot spots" is also apparent in later stages (Figs. 8 and 10).

A nest of osteoblasts as part of a developing 14 day lamella is depicted in Fig. 7. The periosteal surface lies to the left and above, and the 13 day lamella to the right and below. That portion of the osteocytic nest adjacent to the stroma contains Ca^{45} , indicating that calcification has begun. Within the matrix an osteocyte is visible at *a*, and along the left hand margin of the nest there is a row of osteoblasts.

By the end of the 13th day, the total calcium in the embryo has increased to 6.4 mg., (which represents an 800 per cent gain over that present in the 11 day stage), and about 25 per cent of the

total calcium is contained in the hind limbs (Johnston and Comar, 1955).

Subsequent events, involved in the formation of additional lamellae, repeat those already described, and need not be considered in detail for each day of development. However, some consideration should be given to changes in the staining properties of the lamella during its formation.

When the matrix is first laid down, it is almost colorless to slightly acidophilic. Gradually it becomes more acidophilic, and then the acidophilia gives way to basophilia. It is not until the basophilic stage is reached that calcium begins to move into the matrix. It has been suggested that this increase in basophilia reflects the presence of alkaline phosphatase derived from the osteoblasts (Fell and Robison, 1930, 1934). However others (Bourne, 1956) have contended that the basophilia is due to the formation of mucopolysaccharides and chondroitin sulfate in particular. Regardless of the time source of the basophilia, it disappears as the lamella becomes calcified, so that the fully calcified lamella possesses an almost colorless matrix.

The Postlamellar Phase.—As defined earlier, the postlamellar phase begins about the 18th day and continues subsequent to hatching. It is during this time that resorption of the lamellar bone and endochondral ossification begin. The resorption is characterized by the appearance of osteoclasts and the destruction of the lamellae. In Fig. 9, portions of several lamellae which have been joined together by interlamellar bridges are depicted. Evidence of osteoclasts is provided by the presence of "hollowed out" regions, as well as by the considerable concentration of Ca⁴⁵ in the osteocytes and in the matrix immediately adjacent to them.

During the postlamellar phase, those lamellae that are not destroyed continue to grow by apposition. Since the Ca⁴⁵ has been available to the embryo since 0 time, it can be deduced that a given lamella is composed of bone salt containing calcium of different specific activities. In Fig. 10, for example, which depicts several lamellae that have continued to grow, it may be seen that the central cores of the lamellae contain more Ca⁴⁵ than do the marginal regions. This observation is consistent with that of previous studies in which the day to day change in the specific activity of the blood was determined (Johnston and Comar,

1955). It is apparent that the bone salt, deposited during a particular day or period, reflects precisely the specific activity of the blood calcium for that period.

The formation of endochondral bone also occurs during the postlamellar phase. Its beginning is seen in the "tunneling out" of the epiphyseal cartilage by cords of stromal cells, which results in the formation of finger-like trabeculae of cartilage extending into the marrow cavity. This is followed by the deposition of bone salt in the cartilage matrix (Figs. 11 to 14). Bone is deposited along the borders of the trabeculae with the effect that the unresorbed cartilage becomes entrapped forming a core similar to that in the spongiosa of the long bones of mammals. Fig. 11 represents a portion of one cartilage trabecula that is being overlaid with bone. The newly formed bone appears as the lighter regions of the figure, indicated by arrows, and the dark areas represent the cartilage. From Fig. 11 it may be seen again that the amounts of Ca⁴⁵ vary from one region to the other in the developing bone.

In Fig. 12, of another cartilage trabecula, the plane of focus is slightly above the tissue level, and the cellular details are not apparent. The light circular areas represent the chondrocytes and the light border strips, the forming bone. A considerable concentration of Ca⁴⁵ occurs in the matrix, with a lesser amount being present in the chondrocytes. Little or no activity is present in the bone on the lower side of the cartilage core, whereas a discernible amount is detectable in the bone on the upper side.

Figs. 13 and 14 represent von Kossa preparations of the epiphyseal regions of the tibiofibula in embryos of 19 days, and show the presence of bone salt in the cartilage. Here it may be seen that the deposition of bone salt in the cartilage is very marked and discrete. In Fig. 13 several lamellae of bone are visible to the left of the cartilage. The cartilage is hypertrophic, and in the hematoxylin-azure-eosin-stained preparations the matrix is basophilic to metachromatic. Fig. 14 depicts an area showing one of the buds of stromal tissue which invade the epiphysis. Calcified cartilage is present both to the left and the right of the bud.

DISCUSSION

The distribution of Ca⁴⁵ during bone formation in the chick embryo has been followed by means

of micro autoradiography. The results obtained indicate that the cartilage template of the long bone plays an important role in the initial stages of calcification. The observations made here support the *in vitro* studies of Fell and Robison (1930, 1934). The fact that osteoid formation precedes calcification by as much as $\frac{1}{2}$ day was also demonstrated. The osteoid becomes calcifiable only after it acquires a certain degree of basophilia. The basophilia diminishes during calcification, and the substance responsible for this staining property appears to be consumed or modified in some way. It has been variously suggested that the substance causing the basophilia in the osteoid is perhaps a phosphoric ester (alkaline phosphatase) or chondroitin sulfate (Bourne, 1956). The presence of osteoid has also been demonstrated conclusively by Arnold *et al.* (1956) in the young Haversian system of the rabbit, and, more recently, Sevastikoglou (1957) has described the formation of physiologic osteoid in tissue culture, using explants of the upper tibial and lower femoral epiphyses of 10 to 14 day chicks.

Calcification does not occur uniformly in a given lamella, and the deposition of bone salt in a non-uniform manner results in the formation of "hot spots" of Ca^{45} . It is apparent that the concentration of Ca^{45} in the forming bone is directly proportional to the specific activity of the blood and tissue fluids at the time the bone is deposited. This has been demonstrated in the growth of a particular lamella (Fig. 10). Here, it is possible to determine differences in isotope concentration by autoradiographic density. Also by comparing the autoradiographic reaction above the lamellae with previous values obtained for blood specific activity (Johnston and Comar, 1955), one can assign to a certain autoradiographic density a corresponding specific activity value.

Although Amprino (1955) has reported that the cartilage in the long bones of the chick does not calcify, it is apparent from the observations made here that calcification does occur (Figs.

13 and 14). From Fig. 21 of his account (Amprino, 1955) it is clear that no deposition of silver salts occurs in the cartilage. Possibly, however, if he had stained the same region in later stages (18 to 20 days), he would have been able to demonstrate the presence of bone salt.

The occurrence of calcium in the cartilage template and in the chondrocytes before and during the formation of the primary lamella has not been reported previously, insofar as is known to the present author. Also the demonstration of calcium intracellularly in the osteocyte represents a new finding. The intracellular accumulation of sulfur, another element important in bone formation, also has been demonstrated (Pelc and Glücksmann, 1955, Johnston and Comar, 1957).

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EXPLANATION OF PLATES

All figures are unretouched photomicrographs taken using apochromatic objectives and compensating oculars on Kodak microfilm. Figs. 1 and 2 were taken under phase contrast. Figs. 1 through 12 are autoradiograms; Figs. 13 and 14 are von Kossa's silver preparations. The magnification varies and is indicated.

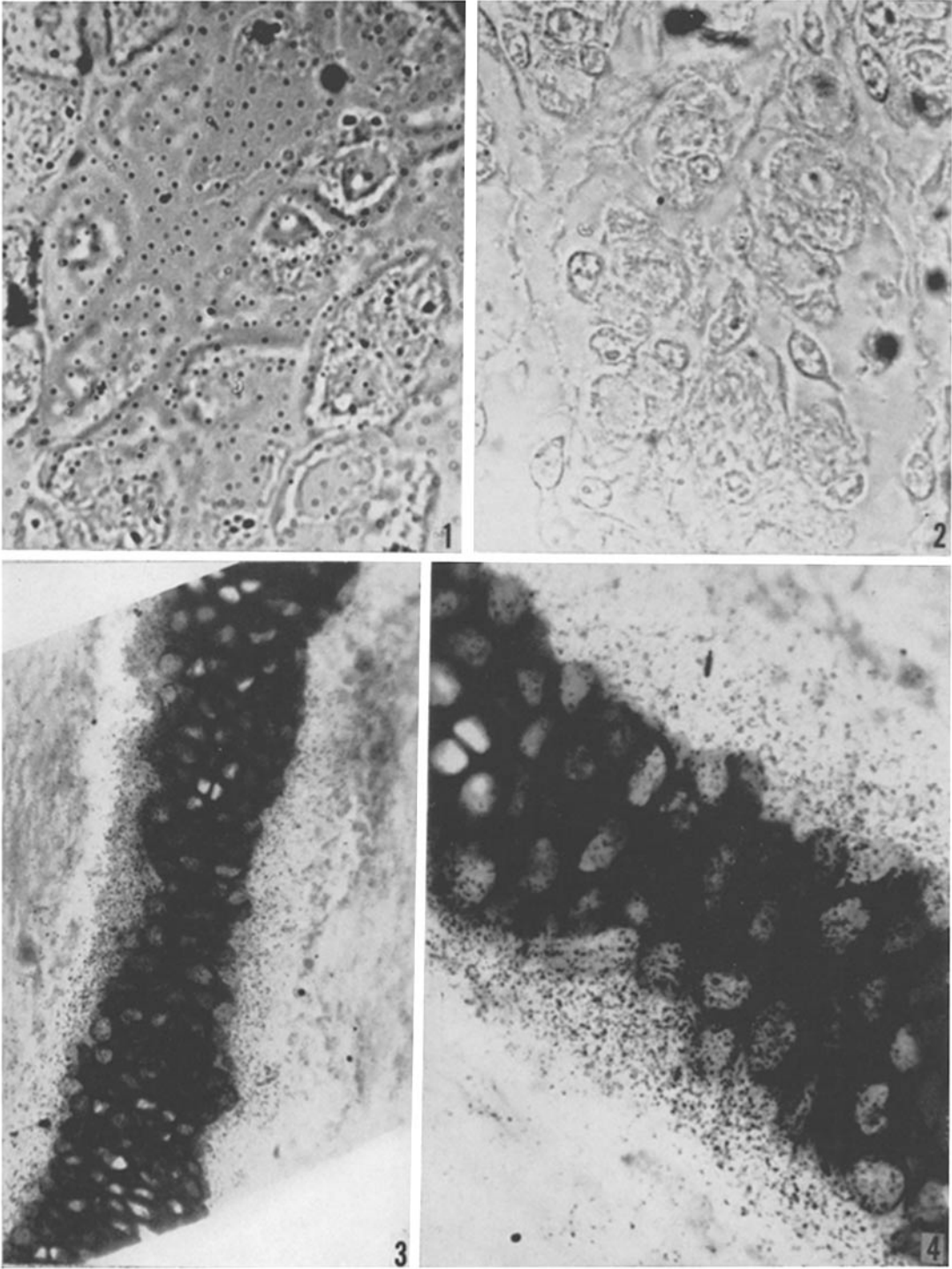
PLATE 85

FIG. 1. A portion of the diaphyseal cartilage of the tibiofibula of an 8 day embryo. The silver grains are clearly visible above the matrix and the chondrocytes. $\times ca. 970$.

FIG. 2. A portion of the muscle adjacent to the cartilage depicted in Fig. 1. It is evident that no photographic grains occur above the muscle. The large dark structures are technical artifacts. $\times ca. 970$.

FIG. 3. A longitudinal section of the diaphysis of the tibiofibula of an 11 day embryo. Note the autoradiographic reaction above the primary lamellae on either side of the cartilage. $\times ca. 200$.

FIG. 4. An enlargement of the middle part of Fig. 3. Note the absence of cellular elements in the primary lamellae, and the concentration of Ca⁴⁵ in the cartilage as revealed by the autoradiographic reaction. $\times ca. 430$



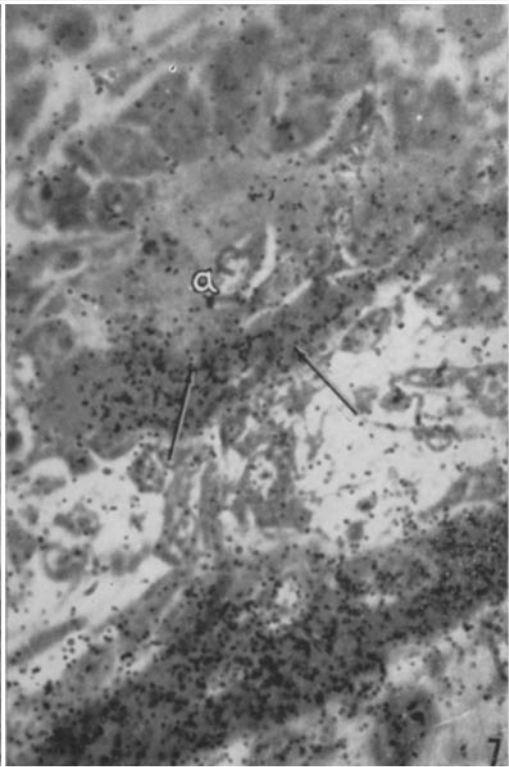
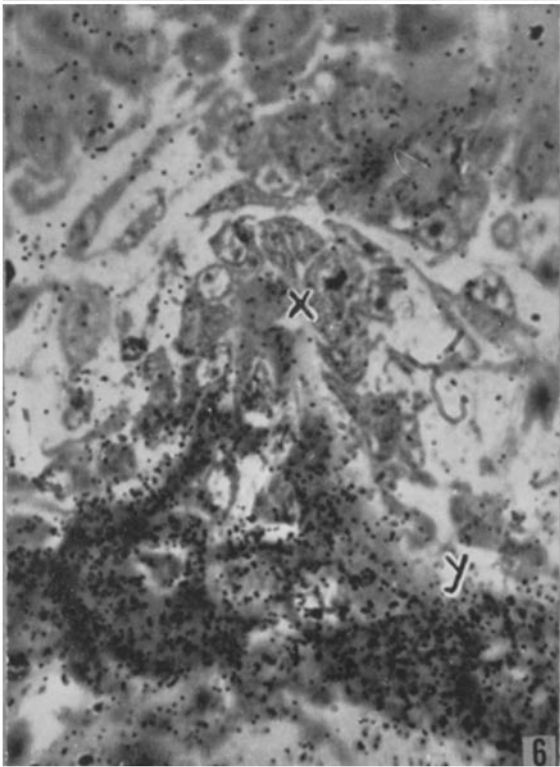
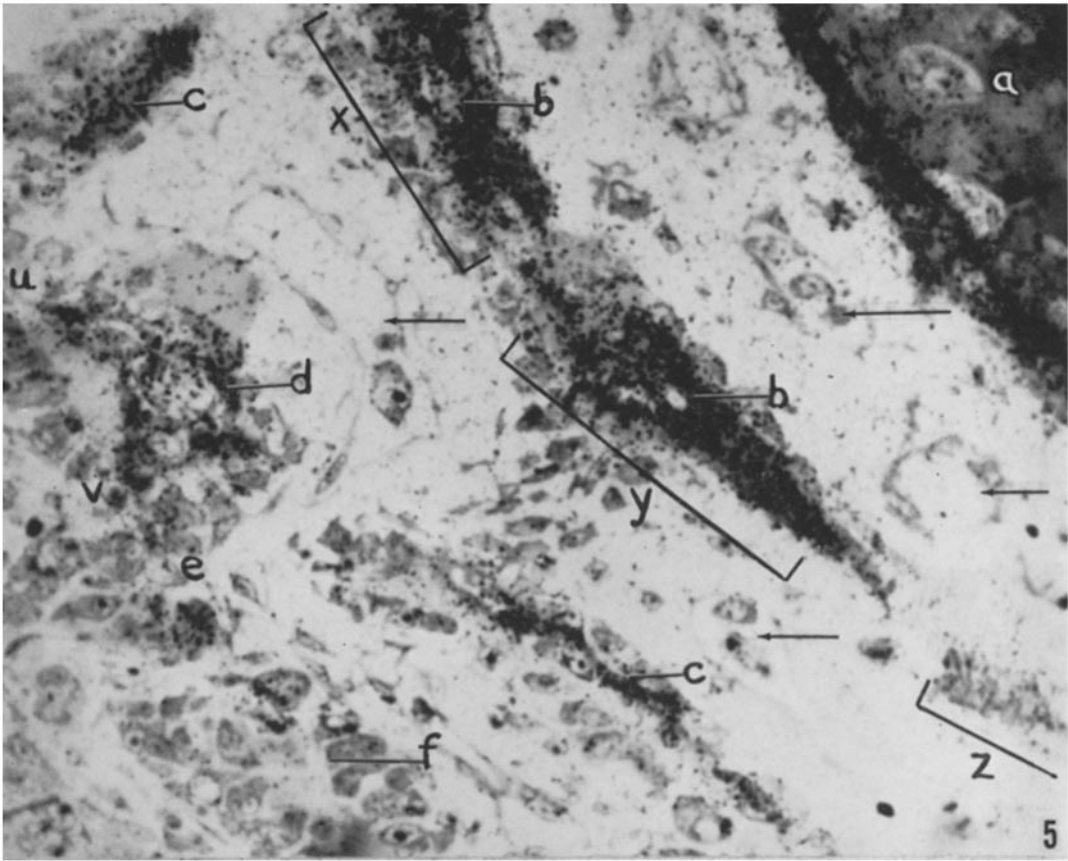
(Johnston: Ca^{45} in chick)

PLATE 86

FIG. 5. A segment of the cross-section of the diaphysis of the tibiofibula of a 13 day embryo. The primary lamella is visible at the upper right, immediately adjacent to the cartilage. The 12, 13, and 14 day lamellae are depicted at *b*, *c*, and *f*, from right to left, in the figure. See text. $\times ca. 200$, enlarged to 1000.

FIG. 6. Another region of the diaphysis of a 13 day embryo. Here may be seen the manner of formation of the radial lamella. See text. $\times ca. 200$, enlarged to 1000.

FIG. 7. Another section of the diaphysis of a 13 day embryo, same as Fig. 6, showing one of the osteocytic nests formed during the development of the circumferential lamellae. An osteocyte is visible at *a*. The 13 day lamella is visible below and to the right. See text. $\times ca. 200$, enlarged to 1000.



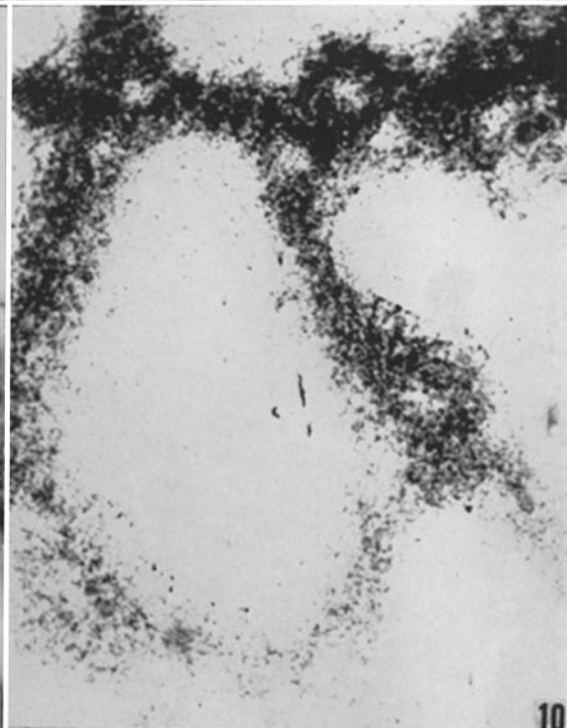
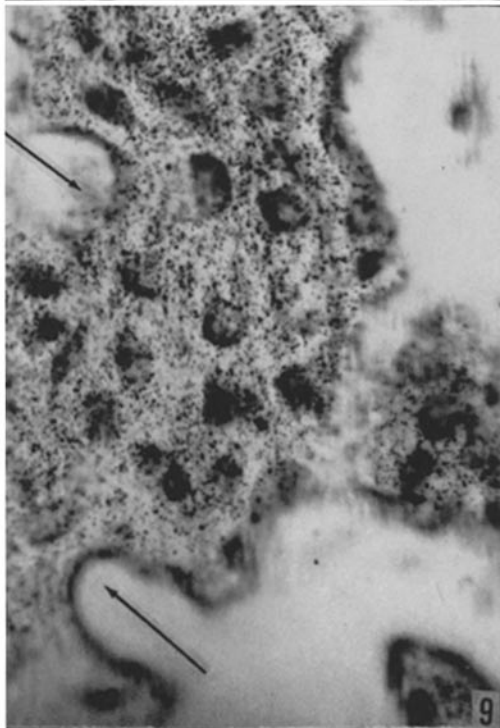
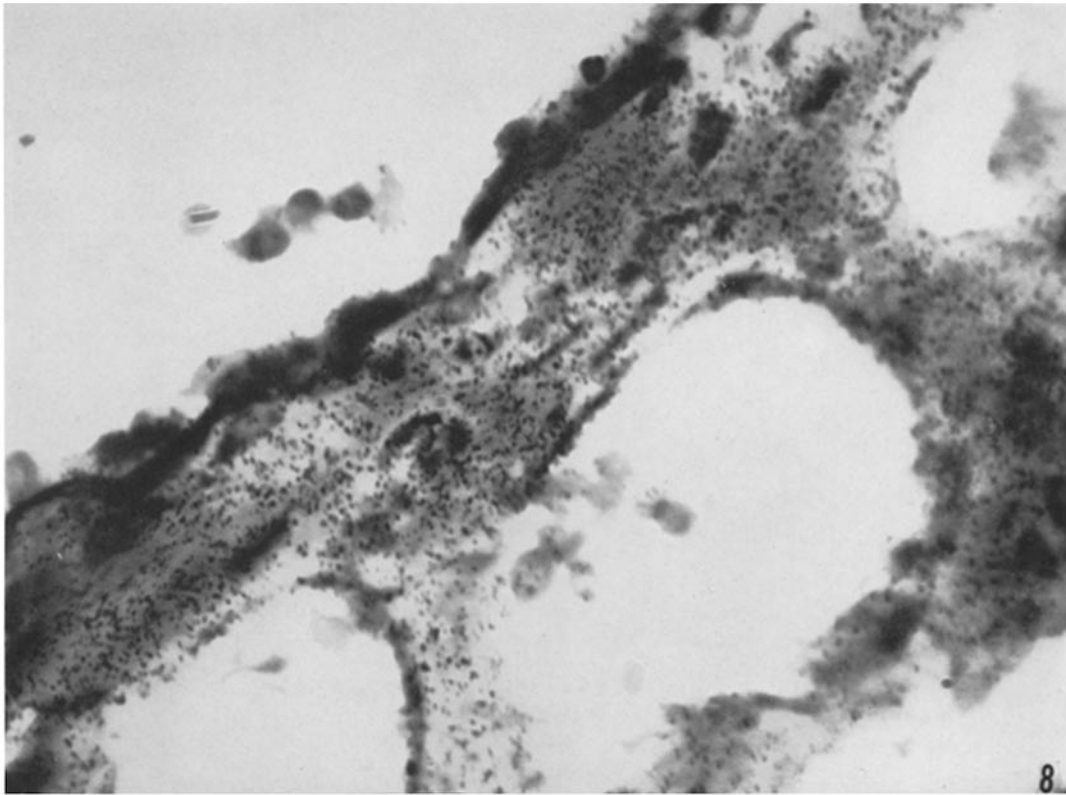
(Johnston: Ca⁴⁵ in chick)

PLATE 87

FIG. 8. A portion of the diaphysis of the tibiofibula of a 16 day embryo. Two circumferential lamellae are visible and are connected by two radial bridges. The focal plane is slightly above the tissue level. Note the uneven distribution of Ca^{45} in the upper lamella in particular. $\times ca. 970$, enlarged to 2000.

FIG. 9. A portion of a cross-section of the diaphysis of the tibiofibula of a 19 day embryo. Resorption of bone has begun as indicated by the "hollowed out" areas, two of which are indicated by arrows. Note the autoradiographic reaction above the osteocytes, and the matrix in their immediate vicinity. $\times ca. 100$.

FIG. 10. A portion of the diaphysis of the tibiofibula of a 16 day embryo. Note the variation in grain density above the lamellae. Most of the Ca^{45} is concentrated in the middle of the lamellae. The osteocytes appear as light areas within the lamellae. The older lamellae lie above, and the younger below. $\times ca. 200$.



(Johnston: Ca⁴⁵ in chick)

PLATE 88

FIG. 11. A portion of one of the cartilage trabeculae in the tibiofibula of an 18 day embryo. The endochondral bone appears as light areas, indicated by arrows, and the cartilage appears dark. Note the difference in concentration of Ca^{45} in the bone as evidenced by the autoradiographic reaction. $\times ca. 100$, enlarged to 1000.

FIG. 12. A part of another cartilage trabecula in the tibiofibula of an 18 day embryo. The focal plane is above the tissue level. Note the Ca^{45} in the cartilage, as evidenced by the autoradiographic reaction. The bone is indicated by arrows. See text. $\times ca. 100$, enlarged to 1000.

FIG. 13. A part of the epiphysis of the tibiofibula of a 19 day embryo stained with von Kossa's method. Note the discrete deposition of bone salt in the cartilage. Note also the absence of spurious silver salts elsewhere in the preparation. Several lamellae of bone are visible. $\times ca. 100$.

FIG. 14. A portion of the epiphysis of the tibiofibula of a 19 day embryo, showing one of the stromal buds from the marrow. Calcified cartilage may be seen both to the right and left of the bud. This is a von Kossa's preparation to illustrate the deposition of bone salt. $\times ca. 100$.

