MORPHOLOGICAL AND EXPERIMENTAL STUDIES ON THE SKELETOGENESIS OF THE FOWL.

by

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C O N T E N T S

Chapter 2.

GENERAL INTRODUCTION

The skeleton of the embryonic fowl is particularly favourable material both for the study of the normal histogenesis of cartilage and bone and for experiments with the tissue culture technique. Fowl embryos are easy to obtain at any stage of development required, the bones are less hard than those of most mammalian embryos so that except in the later stages they can be sectioned without previous decalcification and fowl embryonic tissue is more suitable for cultivation than any other owing partly to the ease with which large quantities of suitable culture medium can be prepared and partly to the readiness with which the tissue grows under the conditions of life *in vitro*.

The first part of this thesis consists of an account of the normal histogenesis of cartilage-bone, of non-ossifying cartilage and of membrane bone; the long-bones of the limbs, the distal part of Meckel's cartilage and the mandibular membrane bones have been selected as examples of these three types of tissue. This work was undertaken in order to provide a basis for a series of tissue culture experiments of which the morphological results are described in Parts II and III. Some of these latter studies were correlated with biochemical investigations by Dr. R. Robison of the Lister Institute and the relation between Dr. Robison's results and my own morphological observations is considered in a section of the discussion.

Tissue culture, which provides a valuable method for approaching an immense number of biological problems, was initiated by Ross Harrison in 1907 when he published his classical experiments on nerve outgrowth *in vitro*. Harrison's original technique has been developed

by Burrows and Carrel and others so that it is now possible to cultivate many types of tissue from many species of animal and in some cases to maintain the cultures indefinitely.

The essential principles of the tissue culture technique are simple. A fragment of tissue - the explant - is removed from the body, and is transferred to a small quantity of nutritive medium contained in a suitable glass vessel. The culture medium which permits the most prolific and consistent growth is a semi-solid clot formed by a mixture of blood plasma and saline extract of embryonic tissues. For growth to take place freely the explanted tissue must be kept at the normal body temperature of the animal from which it has been taken. If the cultures are to be maintained for a long time the medium is renewed every few days and the tissue fragment on reaching a certain size is cut out of the clot and explanted in fresh vessels sometimes after being subdivided into two or more pieces. Strict asepsis has to be maintained in all manipulations.

Tissues <u>in vitro</u> show two types of growth which are sometimes termed "uncontrolled" and "somatic" respectively. Uncontrolled growth is perhaps more characteristic of tissues cultivated <u>in vitro</u> and has been more extensively studied. It involves a progressive simplification of the original histological structure of the explant correlated with a diffuse outgrowth of the cells into the culture medium and increase in volume is by cell multiplication only. The experiments recorded in this thesis refer almost entirely to the second type of growth viz. somatic growth, also known as "controlled" (Thomson '14) or "organotypic" (Maximow '25). A tissue fragment undergoing somatic growth behaves to a great extent as if it were still part of the body. It may increase in size by any or all of the three methods observed <u>in vivo</u> viz. by the multiplication of

cells, by the enlargement of cells and by the formation of intercellular material, and if it is undifferentiated when explanted, its histological development may proceed almost normally. Both uncontrolled and somatic growth often occur simultaneously in the same explant, uncontrolled growth taking place at the periphery and somatic growth in the centre.

Tissue culture as a morphological technique has two important advantages: (a) it enables the observer to study the structural changes in the living tissue with a precision that is impossible by any other means and (b) it provides him with a method of investigating the developmental potentialities of undifferentiated or partly differentiated tissue when removed from the general influence of the body, i.e. when deprived of a vascular system, nerve supply and association with normally adjacent parts.

Comparatively little was known concerning the behaviour in culture of the various types of skeletogenous tissue and it seemed likely that a study of these tissues under the conditions of life in vitro might afford particularly interesting information in view of the fact that the histological and gross anatomical development of the skeleton are often regarded as being controlled largely, if not entirely by local environmental factors. The experiments described below were accordingly undertaken and fall into two groups, one (recorded in Part II) to investigate the capacity of different forms of embryonic skeletal tissue for histological differentiation in vitro and the other (recorded in Part III) to investigate the capacity of isolated skeletal rudiments for gross anatomical development in vitro.

PART I.

NORMAL HISTOGENESIS

Object of investigation. To study the normal histogenesis of typical examples of cartilage bone, of non-ossifying cartilage and of membrane bone.

Chapter I.

The cytology and histological development of cartilage and bone in the normal embryonic long-bones.

Introduction. Work on the development of avian cartilage and bone is limited and references to this subject are rare in recent literature. The general histology of cartilage resorption and osteogenesis in the embryo of the bird was first described by Strelzoff in 1873. Van der Stricht ('90), working on the articular cartilage of birds, refers briefly to cartilage resorption and the fate of the cartilage cells. In 1893 Brachet published a more detailed account of the resorption of cartilage and the development of the long-bones in birds, his study extending from the tenth day of incubation until the tenth day after hatching. Parsons ('05), in a paper on pressure epiphyses, gives a short description of the process of ossification in the limb bones of the pigeon, but supplies no histological detail. I have been unable to find any publications previous to my own (Fell '25), which deal with the cytology and finer histology of cartilage and bone in birds, and such records are comparatively rare in the literature of mammalian osteogenesis.

Material and methods. Thirty embryos of ages ranging from fifty-two hours, and one day-old chick were used for this investigation, the bones of the leg being usually selected for study.

With the exception of the tibiotarsus and knee-joint of the day-old chick and the wing and leg bones of a twenty-day-old embryo, which were fixed in Bouin's solution and decalcified in picro-nitric acid, the material was cut without previous decalcification. In order to insure good penetration in the case of older embryos, the

bones were rapidly stripped of muscle before fixing, but the limbs of very young embryos were not detached from the body.

For the study of nuclear structure and general histology, material was fixed in Allen's modification of Bouin's fluid at 38°C. dehydrated by the drop method, and embedded in paraffin wax. Most of the sections were stained either with Mallory's triple stain or with iron haematoxylin alone or followed by van Gieson's stain, but other methods were also employed. In order to demonstrate mitochondria, tissue was fixed in Flemming's solution less acetic acid, post-chromed for twenty-four hours, and the sections were stained with iron haematoxylin (long method). Da Fano's silver impregnation method was employed successfully for the demonstration of the Golgi apparatus, and preparations fixed by this method and stained with methylene blue were found to afford excellent material for the study of the cartilage matrix.

Sections were usually cut at thicknesses ranging from 4 μ, in the case of very young specimens, to 7 μ, in the case of older embryos.

The structure and cytology of the primitive mesenchyme. The limb-bud of an embryo of fifty-two hours consists of a mass of undifferentiated mesenchyme cells slightly condensed in the proximal region and covered externally by the columnar epithelium of the epidermis. When fixed with Allen's solution, the mesenchyme cells appear to form a syncytium, the cell bodies being connected by delicate threads of protoplasm, but in tissue fixed in Flemming's solution without acetic acid and stained for mitochondria with iron haematoxylin this structure is found to be artifact and the cells are seen to consist of a central oval or pyriform mass and one or more much elongated filaments which, while usually intertwined, are not fused with those of neighbouring

cells; the complete structural independence of each cell is readily demonstrated (Plate I, Fig. 1).

The nucleus forms the main bulk of the mesenchyme cell. It is oval in shape and contains two large, irregular nucleoli and several small chromatin granules. Numerous long, filamentous mitochondria are present in the cytoplasm and a Golgi apparatus which appears to have a reticular structure lies to one side of the nucleus.

As described by Johnson ('83), the first trace of chondrification appears in the proximal portion of the limb and the development of the skeleton is always most advanced in this region. As a preliminary to cartilage formation the mesenchyme condenses to form a compact mass of cells which marks the site of the future cartilage and merges at the periphery with the surrounding undifferentiated tissue.

In some areas, both of the prechondral mass and of the surrounding tissue, numerous groups of cells are to be seen which are undergoing degeneration. The nucleoli and chromatin granules enlarge greatly, while the nuclear membrane shrinks and in some cases appears to dissolve. The cytoplasm rounds off and the cell appears as a small spherical structure occupied by several large, deeply staining, spherical or elliptical bodies; in some instances the cytoplasm breaks down completely, leaving the chromatin granules lying free among the unaffected cells. With Da Fano's technique these degenerate cells are conspicuous objects, owing to their heavy impregnation.

The development and degeneration of the chondroblasts. As in mammals, chondrogenesis and osteogenesis begin at the centre of the shaft. When the prechondral mass of mesenchyme is first formed, the cells are still undifferentiated and are arranged according to no definite plan. The cells at the centre, however, soon become elongated in

a direction at right angles to the long axis of the cell mass (Plate I, Fig. 2.), while at the same time narrow intercellular spaces appear denoting that the formation of cartilage matrix has commenced. The structure and development of this material will be considered in the following section. Toward its extremities the bone-rudiment is still composed of undifferentiated mesenchyme which grades imperceptibly into the surrounding tissue, but the boundary of the cartilage is now fairly sharp in the central region where the perichondrial membrane is beginning to appear. The transverse elongation of cells soon spreads to the ends of the diaphysial portion of the shaft, and simultaneously a slightly different arrangement of the cells is seen. Their long axes no longer lie along straight, transverse lines, but on ellipses convex to the future epiphyses; at the centre of the diaphysis is a narrow lozenge-shaped area, occupied by more or less polyhedral cells, where the two series of ellipses meet. This elliptical configuration is most marked toward the end of the diaphysis where the curvature of the lines is greatest, and serves to indicate the future dividing line between the diaphysis and the epiphysis. The latter region is composed of stellate cells scattered throughout the matrix, which merge imperceptibly into the surrounding undifferentiated embryonic connective tissue. At this stage the cytological features of the young cartilage cells are still almost identical with those of the original mesenchymatous elements. Each possesses a large nucleus with two nucleoli, surrounded by a thin film of cytoplasm which is prolonged into delicate processes. The Golgi apparatus usually appears to consist of a coarse network at one side of the nucleus, but in certain cells the apparatus has the appearance of a loose aggregation of rods rather than of a reticulum. Mitotic fig-

ures are everywhere abundant.

The next stage in development (Plate I, Fig. 3) is characterised by the formation of three zones of cells and the elliptical arrangement is lost except at the extreme ends of the diaphysis. The cells in and near the central lozenge-shaped area, referred to above, enlarge and cease to divide. They are at first polyhedral in outline, but soon become rounded and they show no linear or columnar arrangement, being evenly distributed throughout the matrix (Plate I, Fig. 4). Some exhibit degenerative changes: chromatolysis and partial disintegration of the cytoplasm. This constitutes the first zone. The second zone lies on either side of the first and consists of longitudinally flattened, plate-like cells (Plate I, Fig. 5) many of which are in mitosis; at the extreme periphery the cells are polyhedral. The third zone is the epiphysial region which is composed of round or polyhedral cells (Plate I, Fig. 6) in very active proliferation. The Golgi apparatus of these elements is shown in Plate I, Fig. 7. Toward the extremity the cells of this zone are closely approximated and at the surface become slightly flattened, thus delimiting the cartilage of the epiphysis from the still undifferentiated tissue by which it is surrounded. There is no sharp line of division between the three zones which are all connected by transitional elements. The first signs of ossification appear at this stage, being represented by a thin lamina of osteoid tissue beneath the perichondrium investing zone 1 and part of zone 2.

As growth proceeds the hypertrophied cells in the centre of zone 1 increase to nearly three times their original diameter (cf. Figs 2 and 4, Plate I); the nucleus is clear and contains somewhat enlarged nucleoli; a few long mitochondria are present in the highly

vacuolated cytoplasm; the centrosome appears as a more or less crenated sac in which lie two large centrioles; a few small fat globules are sometimes seen in between the vacuoles. These large cells eventually degenerate (Plate II, Fig. 1); the nucleoli are often extruded from the nucleus, which then appears as an inconspicuous shrivelled vesicle, the centrosome becomes considerably enlarged, and the Golgi apparatus, which appears granular and condensed, shows a corresponding increase in size (Plate II, Fig. 2). It is difficult to ascertain how far this granulation and condensation of the Golgi apparatus is an artifact, since the extreme density of the matrix at this stage no doubt renders cartilage very unfavourable material for the study of the Golgi apparatus. The mitochondria break down into small particles and finally disappear. As previously recorded by Brachet ('93), mitotic figures are only occasionally found in this zone; in some instances the chromosomes are broken down into granules and are hardly recognisable, while in other cases they appear comparatively normal. Binucleate cells are not uncommon. Camera-lucida drawings show that the cells of zones 2 and 3 are also appreciably larger than those of very early cartilage (cf. Figs. 2, 5 and 6, Plate I).

The cellular hypertrophy characterising zone 1 gradually extends into zone 2, the flattened cells of which lose their compressed appearance and become vacuolated and distended. The zone of enlarged cells thus increases in size until it comes to occupy about one-half of the total length of the shaft. The epiphysial portion (zone 3) is now quite sharply defined. The flattened cells at the articular surface become still more compressed and form a distinct layer which is inwardly continuous with the ordinary cartilage.

Erosion of the diaphysial cartilage begins at this stage, and this process and the further development of the epiphysis will be described later.

The structure and formation of the cartilage matrix. In preparations fixed and stained by ordinary histological methods, the matrix of the cartilage either remains colourless or stains so faintly that the structure is indistinguishable. In later cartilage the stain is more readily taken and the matrix then appears to be composed of a close network of fine fibres. If material is fixed by Da Fano's silver impregnation method, however, the cartilage becomes highly chromaphil and presents a remarkable appearance. The best differentiation, in the writer's experience, is obtained by staining sections with methylene blue. By this method the cells appear a vivid emerald green and the matrix a deep bluish violet. By employing this technique the matrix can be demonstrated in its earliest stages of formation.

Cartilage matrix in such specimens first appears as a delicate film over the surface of the closely aggregated chondroblasts and their protoplasmic processes. The capsules of adjacent cells are in continuity and, as the cells separate, the capsules appear to be drawn out into strands and irregular laminae and tubes perforated by circular and oval holes (Plate II, Fig. 3). As the amount of matrix increases, a close, intercellular sponge-work is seen which becomes progressively denser as development advances.

In the older cartilage the matrix composing the intercellular partitions in zone 1 appears yellowish brown in unstained, untoned preparations fixed by Da Fano's technique, thereby contrasting with the colourless matrix in the other regions. At this late stage it

stains very deeply with methylene blue; the sponge-work is extremely dense (Plate II, Fig. 2), the small interstices being almost obliterated; the outlines of the partitions have become very sharp and the cells are usually invested by lightly staining, loose, spongy matrix connected with the intercellular wall by numerous radiating strands. In zone 2 a similar appearance is often seen, the flattened cells being separated on either side from the dense matrix with which they are connected by numerous branching strands of intercellular material. These strands can also be distinguished in deeply stained preparations fixed by ordinary methods. The matrix, though at first looser than in zone 1, ultimately becomes equally dense. In the epiphysial region (zone 3) the rounded cells lie in a close, regular network which in a young embryo becomes fainter and more diffuse towards the periphery, finally being indistinguishable as the cartilage merges with the undifferentiated tissue with which it is surrounded. As the epiphysis assume a definite contour, the matrix becomes of equal density throughout. In the articular cartilage the matrix is present in very small quantities only, and toward the free surface appears to vanish completely.

The structure of the matrix of hyaline cartilage has for long been a matter of dispute. The earliest observers regarded cartilage matrix as a simple, homogeneous substance, but later histologists recorded the presence of fibrils permeating the ground-substance and fibres proceeding from the chondroblasts into the matrix were also noted. The nature of these latter structures was the subject of considerable diversity of opinion and by some they were regarded as solid fibres, by others as hollow lymph channels interconnecting the capsules.

Retterer ('17) describes the matrix of mammalian hyaline cartilage as a spongy network, the meshes of which contain amorphous material, and his account of the structure and development of this network corresponds fairly closely with that described above in the case of the fowl. Retterer, however, considers the intercellular substance as an extension of the cytoplasm of the cartilage cells. My own results fail to endorse this view and suggest that the matrix is merely the product, or secretion of the chondroblasts. Although in ordinary preparations the reticulum of cartilaginous material extending from the chondroblasts to the wall of the capsule appears to be continuous with the cytoplasm of the cell, in sections fixed by Da Fano's method and stained with methylene blue the contrast in colour between the bright green protoplasm and the purple matrix is so sharp that it is comparatively easy to tell where the cytoplasm ends and the reticulum of matrix begins. It is found that an irregular film, like a thin coagulum, covers the surface of the cell, being closely adherent to the cytoplasm and that this film extends into branching strands continuous with the wall of the capsule. Although special attention was paid to this point, I was unable to observe any sign of the strands from the surface film of the matrix being in direct continuity with the cytoplasm, and no material intermediate in staining reaction was to be found uniting the two substances.

Whether the reticular structure of the matrix seen in sections of fixed material really represents the structure of the matrix in life or is merely a coagulation artifact due to fixation, is at present impossible to decide. As will be described later no such reticulum is distinguishable in the intercellular substance of living cartilage growing <u>in vitro</u>. This, however, is no evidence that

such a structure does not exist.

An interesting characteristic of the matrix of ossifying cartilage in avian long-bones noted by Niven (unpublished) is the complete absence of calcification except in the distal parts of the shaft during the later stages of development. In this respect avian material differs from ossifying cartilage in the mammal.

The development of the perichondrium. As previously stated, the cartilage when first formed has no definite perichondrial membrane, but passes gradually into undifferentiated mesenchyme. The first trace of this structure appears around the middle region of the diaphysial part of the cartilage, where the mesenchyme gives rise to an indistinct layer of oblong cells with their long axes parallel with that of the cartilage. This layer is outwardly continuous with the surrounding undifferentiated tissue and inwardly merges with the young cartilage through a gradation of intermediate elements and a short distance on either side of the middle region its identity is lost in the general mass of embryonic connective tissue investing the cartilage.

This rudimentary perichondrium enveloping the middle region of the cartilage (zone 1 and part of zone 2) soon begins to exhibit a two-layered structure (Plate I, Fig. 3 and Plate II. Fig. 4). The outer, oblong cells become drawn out into a fusiform shape and are gradually transformed into fibroblasts, while certain of the intermediate elements show an increased amount of cytoplasm, assume an oval or rounded form, and finally give rise to osteoblasts. The two layers are fairly sharply defined, both from one another and from the carti-

lage. Irregular, cleft-like spaces lined by endothelium appear which represent developing blood vessels, and are fairly numerous in the outer layer of the perichondrium. The fibroblastic layer, although inwardly compact, is very diffuse toward the periphery and fades into the general connective tissue of the limb. Although the perichondrium is now distinguishable as far as the future epiphysis, where it passes into the undifferentiated tissue surrounding that region (Plate I, Fig. 3), its double structure is lost around the distal half of zone 2, where it is composed entirely of undifferentiated, elongated cells.

As soon as the two-layered condition of the perichondrium is attained, the first signs of ossification appear, and at the same time delicate white fibres begin to form in the fibroblastic region. The development of the osteoblasts and fibroblasts surrounding the middle portion of the cartilage is now complete and no further change takes place in these cells except the transformation of certain of the former elements into bone cells. This will be dealt with later. The cytology of the perichondrium, which can now be termed the periosteum, may therefore be conveniently described at this point.

The osteoblasts are usually oval in shape and slightly compressed in a direction at right angles to the surface of the cartilage (Plate II, Fig. 5), and are seen best in tangential sections. The spherical nucleus usually lies toward one end of the cell and contains one or more nucleoli and several smaller chromatin granules. On one side of the nucleus is the spherical centrosome, in which lie two minute centrioles and round which are aggregated large numbers of filamentous mitochondria. Preparations made by Da Fano's method show that the centrosphere is associated with a Golgi apparatus simi-

lar in structure to that of the chondroblasts. The fibroblasts are very similar to the osteoblasts in structure, though not in shape. They consist of an elongated nucleus with nucleoli and chromatin granules, enclosed in a film of cytoplasm prolonged at either end into filamentous processes. A small reticular Golgi apparatus lies near the nucleus, and mitochondria are present both in the processes and in the perinuclear film of cytoplasm.

As osteogenesis proceeds, the fibroblastic layer becomes more condensed and more sharply defined at the periphery, where, however, it is continuous with the intramuscular connective tissue. It also becomes very tough, owing to the formation of increasing quantities of intercellular fibres. The vascular clefts mentioned above become more regular and develop into small blood vessels of the usual type. As the epiphysis begins to assume its characteristic contour and to differentiate from the embryonic connective tissue around it, the perichondrium by which it is covered becomes much more distinct and is seen to be continuous with the cap of flattened cells investing the articular surface of the cartilage. The perichondrium investing the ends of the diaphysis is still composed of a single coat consisting of undifferentiated, elongated cells, and remains in this condition until cartilage erosion is far advanced. Ultimately, however, the double-layered structure appears throughout the diaphysial region but the osteoblastic layer stops rather abruptly at the boundary of the epiphysis and diaphysis. The outer layers of the fibroblastic coat are in continuity with the fibrous tissue of the epiphysial perichondrium, while the inner layers pass into the cartilage of the epiphysis where the fibres merge with the matrix.

Periosteal ossification. The progress of bone formation is readily

followed in preparations fixed in Allen's or Flemming's solution and stained with Mallory's triple stain. In such material the osseous matter stains a deep blue.

As stated above, the first signs of ossification appear in the osteoblastic layer investing the middle region of the cartilage, where the osteoid tissue is formed as delicate, undulating laminae composed of fine interwoven fibres cemented together by an amorphous material which becomes more conspicuous as development advances (Plate II, Fig. 4). These laminae are intercellular structures running parallel with the long axis of the cartilage, and are first developed near the line of junction of the cartilage and perichondrium. The chondroblasts at the extreme circumference of the cartilage are flattened transversely, and it is sometimes difficult, therefore, to distinguish between a central osteoblast and a peripheral chondroblast with the ordinary techniques. In preparations fixed by Da Fano's method and stained with methylene blue, however, the line of division is fairly sharp, since the cartilage stains a deep purplish blue, while the osteoid tissue is either not coloured or stains a light green.

The central osteoid laminae become gradually thicker, other lamellae - as yet very delicate - appear among the peripheral osteoblasts and the ossification begins to spread toward the ends of the diaphysis, where the process is always much less advanced than in the middle region. In transverse or longitudinal sections the laminae are almost indistinguishable from young white fibres, and their lamellar structure is only demonstrable in tangential sections.

The central laminae soon come in contact with each other both terminally and laterally, and fuse to form a thin, compact osteoid

cylinder between the perichondrium and the cartilage (Plate I, Fig.3). Many osteoblasts are included in the substance of this osseous layer as it increases in thickness and give rise to bone cells.

Osteoblasts imprisoned in this way become compressed in a direction at right angles to the bone, and are therefore seen best in tangential sections (Plate II, Fig. 6). The nucleus remains structurally unchanged, but assumes a still more eccentric position, so that it comes to lie at one end of the cytoplasm. The centrosome appears as a small circular area rather more lightly staining than the rest of the protoplasm and contains two minute centrioles. Large numbers of filamentous mitochondria radiate from the neighbourhood of the centrosome and show a fairly regular fan-like arrangement. At intervals the surface of the cytoplasm is produced into small points; these are gradually prolonged into delicate branching processes extending into the intercellular substance.

The central bony cylinder increases in thickness partly by the deposition of fresh material on its outer surface by the contiguous osteoblasts and partly by fusion with the younger peripheral lamellae, until a fairly stout layer of bone is formed around the middle region of the cartilage. At this stage the osteoblastic layer becomes somewhat diffuse. This is probably due to the fact that cell division ceases early in the middle region of the cartilage, with the result that the diameter of the future diaphysis is now nearly half as large again at the extremities as at the middle. The membranous fibroblastic layer becomes drawn away from the cartilage in the middle region by the enlargement of the ends of the diaphysis and in longitudinal sections appears as two almost straight lines on either side of the somewhat hour-glass shaped cartilage. The osteoblasts cease to form a compact layer around this middle part of the cartilage and

become scattered in the enlarged space between the fibroblasts and the bone, with the result that osseous deposition upon the central cylinder becomes very irregular. At the same time the osteoblastic layer begins to be richly vascularised. The osteoblasts become broken up into intervascular groups which give rise to short iregular trabeculae of bone fused at one end with the central cylinder and having a general tendency to radiate from the original centre of ossification. As the vessels increase in length and diameter, the space intervening betweem the fibroblastic layer and the inner tube of bone widens, the transverse trabeculae become correspondingly longer, and at the same time a second series of laminae is formed concentric with the circumference of the cartilage. This second series is formed in the layer of osteoblasts lying between the fibrous layer of the periosteum and the developing vascular plexus, and does not give rise to a continuous tube. At this stage of development the first trace of the erosion of the cartilage appears (see next section).

From the foregoing account it will be seen that ossification always follows the plane of least resistance. In the earliest stages of osteogenesis this plane must obviously lie between the compact perichondrium and the cartilage, since the direction of pressure will be at right angles to the surface of the latter; as we have seen, bone first appears in this region and gives rise to the continuous tube described above. When the intervascular groups of osteoblasts are formed, the plane of least resistance will be tangential to the walls of the growing vessels. As the blood vessels develop, the intervascular osteoblasts become arranged in two ways: (a) in irregular transverse columns and, (b) in an irregular layer between the

vessels and the fibrous coat of the periosteum. In (a) the line of least resistance will be more or less at right angles to the surface of the cartilage, while in (b) it will lie between the fibrous tissue and the vascular plexus. Ossification begins in the inner ends of the columns of intervascular osteoblasts, thus forming the short transverse trabeculae, and extends to the layer of osteoblasts wedged between the fibroblastic coat and the plexus of the blood vessels where the second series of lamellae concentric with the original bony cylinder appear. It has been stated that the transverse trabeculae show a tendency to radiate from the original centre of ossification. This tendency increases as development proceeds and, as suggested by previous authors, is probably the result of tension due to the terminal growth of the cartilage.

Ultimately the cartilage enclosed by the osseous tube is completely removed except at the extremities, while early periosteal ossification can now be seen as far as the epiphyses. In the middle region the radiating transverse trabeculae become so much inclined that many are actually parallel with the long axis of the bone. At the same time the various protuberances projecting from the surface of the trabeculae become enlarged until they fuse with neighbouring trabeculae. A series of incomplete tubes is thus gradually built up around the vessels, which are continuous on the one hand with the original cylinder and on the other hand with the peripheral laminae beneath the fibrous layer (Plate III, Fig. 1). The blood vessels occupying the spaces (Haversian spaces) between the trabeculae are composed of a single layer of much flattened endothelial cells surrounded by delicate strands of connective tissue. Toward the periphery the bony network is thickly covered by osteoblasts, but in the

more central parts these cells are comparatively sparsely scattered. The original tube of bone retains its continuity except in those places where an irrupting blood vessel has forced a passage into the central cavity which is occupied by the developing marrow. As ossification continues, the bony trabeculae become thicker and their interconnections increase in size and number until at length the blood vessels become completely enclosed in continuous radiating tubes - the rudimentary Haversian canals. The periosteal bone increases in thickness partly by the formation at the periphery of additional osseous trabeculae and partly by the enlargement of those already formed.

The formation of the marrow cavity. A striking feature of bone formation in the fowl is the absence of endochondral ossification in all but the extremities of the diaphysis. As soon as the formation of the periosteal bone is fairly well advanced, the enclosed cartilage begins to be eroded and is not replaced by bony trabeculae as in the mammal. Erosion begins in the middle region of the cartilage. The blood vessels of the periosteal bone at intervals burst through the central osseous cylinder carrying with them osteoblasts and strands of connective tissue, and make their way into the substance of the cartilage, the matrix of which appears to dissolve before their passage. As the vessels penetrate farther inwards, they branch repeatedly and come to occupy large irregular cavities. Marrow tissue now begins to increase in quantity and non-granular lymphoid elements in active mitosis are to be seen both in the vessels and in the intervascular tissue which they appear to reach by penetrating the walls of the vessels. Erythroblasts and granular cells in all stages of formation are also seen both in the central marrow cavity and in the spaces of the periosteal bone. As the excavations in the cartilage

enlarge, they gradually come in contact and fuse to form a single large, irregular cavity occupying the middle region of the diaphysis within the central osseous cylinder (Plate III, Fig. 1). The space contains a complex system of anastomosing thin-walled vessels communicating with several larger longitudinal veins and arteries. The intervascular tissue becomes more abundant, large numbers of granular cells are formed, and young adipose cells of the usual type also begin to appear. Numerous osteoblasts, many of which are in degeneration, are scattered among the marrow cells or lie between the marrow tissue and the eroded cartilage. Multinucleated giant cells are also present, both lying freely in the marrow and applied to the surfaces of the periosteal bone and the degenerating cartilage. The structure and origin of these elements will be considered later. At the surface of the erosion there is a belt of rather degenerate tissue, the nature and composition of which can only be determined by the study of very well-fixed material; less favourable preparations render reliable observation impossible. The results of the present investigation indicate that this tissue is composed partly of the invading cells of the marrow and partly of liberated chondroblasts. The liberated cartilage cells form a narrow diffuse layer between the marrow and the surface of the matrix (Plate III, Fig. 2). Many of the chondroblasts when first liberated are already in a degenerate condition and soon disintegrate altogether. The cytoplasm becomes very granular and diffuse and the nucleus so shrunken and distorted as to be almost indistinguishable; the nucleus finally breaks down and the cells are represented by floccular masses of protoplasmic debris. Owing to the rapidity with which the cartilage cells degenerate and disappear after being liberated from their capsule, they occupy a very

restricted region and the progress of their degeneration is easily overlooked and can only be followed satisfactorily by the careful study of material in which shrinkage due to fixation is negligible.

The excavation of the cartilage soon spreads from the middle region towards the ends of the diaphysis. This extension is the result of erosion by the vessels already present in the central cavity and the lateral irruption of new vessels from the periosteal bone is comparatively rare. Finger-like, vascular processes grow outward toward the extremities and in transverse section are seen to occur at fairly regular intervals around the circumference of the cartilage immediately within the inner tube of the periosteal bone, while one or sometimes two pass up the centre; in this way are formed the 'cartilaginous cones' described by previous workers. At the same time the marrow cells multiply steadily and at length give rise to a compact mass of tissue composed mainly of granular and adipose cells. The fate of the cartilage cells is much less readily studied in the distal extensions of the marrow cavity, as the degeneration of the chondroblasts before liberation is not so far advanced toward the ends of the diaphysis as in the middle region. The condition of these cells when first set free varies. Some are comparatively normal but in others the nucleus is reduced to an empty vesicle or has become pycnotic, and in certain cases the whole cell has shrunk into a crenated, deeply staining mass showing no cytological structure. It is often impossible to distinguish the less abnormal chondroblasts from the other elements composing the mass of somewhat degenerate tissue covering the surface of the cartilage.

According to some authorities (Van der Stricht '90, Brachet '93) the liberated chondroblasts develop into osteoblasts or cells of the

marrow reticulum. I have been unable to find any evidence of this in my material.

In many regions there is no zone of degeneration such as that described above. This is particularly the case in the later stages of cartilage excavation where the marrow tissue is dense. Connective tissue cells of the marrow, osteoblasts, and in some cases small blood vessels crowd into the breaking-down capsules and the enclosed cartilage cells can be seen disintegrating in situ, while the invading tissue shows little degeneration.

The destruction of the cartilage matrix is very well seen in preparations fixed by Da Fano's method and stained with methylene blue. As described above, the matrix at this late period appears as an extremely dense sponge-work which stains a deep violet with methylene blue. For a short distance beneath the eroded surface, however, the matrix is almost colourless, and in some areas masses of granular substance, stained a pale violet, are seen among the neighbouring cells, possibly representing the debris of the matrix. It would seem that several types of marrow cells exert a chondrolytic action. It is possible that the multinucleated giant cells may have a destructive effect upon the cartilage, but in the earlier stages of the formation of the marrow cavity these structures are not sufficiently numerous to constitute the only, or indeed the chief agents of erosion, and the excavations at this period usually contain only blood vessels, connective tissue, and osteoblasts. In some areas the tissue forming the walls of the blood vessels appears to be chiefly involved in the resorption of the cartilage; it is pressed closely against the matrix and fills the cavities of the capsules opening on the eroded surface. Elsewhere the blood

vessels are separated from the cartilage by a fairly wide space which, as described above, is occupied by connective tissue, hoemopoietic cells, and osteoblasts some of which have forced their way into the disintegrating matrix.

The later development of the epiphysis. While the marrow cavity is being formed in the diaphysis, medullary spaces are also being excavated in the epiphyses. Blood vessels make their way into the cartilage where in longitudinal section they are seen as a row of branching cavities in the inner part of the epiphysial region. These vessels are not derived from those of the disphysial marrow cavity, but for the most part pass into the epiphysis from its posterior aspect, while others enter at various points on the surface. For some time, therefore, as Brachet points out, there is no communication between the marrow spaces of the diaphysis and those of the epiphysis. Besides blood vessels, the epiphysial marrow spaces contain only connective tissue, liberated cartilage cells and non-granular wandering cells; osteoblasts were not observed. These cavities soon ramify throughout the epiphysis, forming a system of more or less cylindrical canals (Plate IV. Fig. 1).

The finger-like vascular processes which extend toward the extremities of the diaphysis eventually penetrate into the epiphysis, but have not advanced far in material from the day-old chick.

As the diaphysial excavations enlarge and become more numerous, the cells of the terminal regions of the diaphysis lose their former compressed appearance and become swollen and vacuolated. At length the zone of flattened cells becomes reduced to a belt of cartilage which grades imperceptibly into the region of hypertrophied cells on the one side and into the epiphysis on the other.

The zone of flattened elements gradually becomes more restricted and more sharply demarcated both from the epiphysis and from the rest of the diaphysis. At the same time the cells become more closely approximated and begin to show a linear arrangement, the columns being parallel with the long axis of the bone. In the day-old chick the boundaries of this intermediate cartilage are quite definite and the linear arrangement of the discoidal cells very marked (Plate IV, Fig. 1). Some of the cells show degenerative changes, but mitotic figures are occasionally to be found.

The epiphysis at this stage consists of a somewhat narrow, flattened cap of cartilage varying in shape in different bones. The cells, which are evenly distributed through the matrix, are rounded, contain a large spherical nucleus, and, although mitosis is rare, show no sign of degeneration; the matrix is extremely dense and stains more darkly with aniline or methylene blue, the acid fuchsin of van Gieson's stain, etc., than does that of the diaphysial cartilage.

The layer of flattened cells covering the articular surface seen in younger embryos ultimately develops into the articular cartilage of the adult. Fibres begin to appear among the cells and are continuous with the matrix of the underlying hyaline cartilage in which their identity is gradually lost. As development proceeds the number of these fibres increases considerably and in the day-old chick they form fairly stout bundles between which lie columns of oblong or, toward the sides of the epiphysis, spindle-shaped cells. On its inner side this fibrous articular cartilage passes imperceptibly into the hyaline cartilage through a series of intermediate elements.

It is usually stated in anatomical publications dealing with the subject that the long bones of birds are devoid of epiphyses. Le Damany ('03) writes: "Les femurs des oiseaux n'ont pas de points épiphysaires". Parsons ('05) states that "as far as I know there is only one instance of a true epiphysis in their long bones and that is in the upper end of the tibio-tarsus of the Gallinaceae and Ratitae". Other writers state that there are independently ossifying structures associated with the lower end of the tibiotarsis and the upper end of the tarso-metatarsus which, however, are to be regarded as fused tarsal elements, and not as true epiphyses.

Brachet, on the other hand, refers to the epiphyses of birds without discussing the question of whether or not such structures exist in this group of vertebrates. This disparity between the records of Brachet and those of anatomical workers is probably due to the fact that in most of the avian long bones the epiphysis is small, remains cartilaginous, and can only be distinguished in histological preparations. A histogenetical study of the long bones, however, affords almost convincing evidence of the formation of a structure homologous with the epiphysis of mammals. Up to a certain point the embryonic development of the epiphysis in the fowl and in the mammal is identical in all essential points. In both, the epiphysis at first consists of a region of cartilage with rounded cells, which is directly continuous with the diaphysis through a zone of elements intermediate in type. In the later embryo, in both fowl and mammal, this epiphysial region gives rise to a more or less independent structure sharply separated from the diaphysis by a well-defined belt of cartilage known in mammalian histology as the intermediate cartilage (Schafer '29) or proliferative zone. In the case

of the mammal, however, the intermediate cartilage usually persists for a considerable period after birth, while the epiphysial cartilage undergoes independent ossification. In the fowl, on the other hand, the intermediate cartilage is a relatively transitory structure which is soon penetrated by the numerous longitudinal outgrowths of the diaphysial marrow. Moreover, with the exceptions referred to above, the epiphysis does not ossify independently of the diaphysis. Parsons, in his account of ossification in the pigeon, writes: "As life goes on, the ossification extends towards the bases of the cones (of cartilage - H.F.) but for a short time it is checked at some little distance from the articular end: this of course is the equivalent of the epiphysial line of other vertebrates, but it is comparatively transitory and ossification creeps on until the articular end is reached, except for a narrow strip of articular cartilage".

Parsons employs the term "articular epiphysis" to mean "an ossification in the cartilage at the articular end of a long bone", and in this sense the limb bones of the fowl certainly cannot be said to possess 'true' epiphyses. On the other hand, if by 'true' epiphysis we mean a structure homologous with the epiphysis of mammals, then the fact that in the young chick the distal cartilage of all the long bones is differentiated into a distinct cap precisely similar in structure and development to the unossified mammalian epiphysis, seems to justify the conclusion that in early life true, if poorly developed, epiphyses occur in all the long bones of the fowl.

Endochondral ossification. The endochondral ossification at either end of the diaphysis occupies about one-sixth of the total length of the diaphysis and is not very far advanced in the bones of the day-old chick. It begins in the interhal part of this region and is not

complete at the end for a considerable time after hatching.

As previously stated, finger-shaped outgrowths from the marrow penetrate into the cartilaginous extremities of the diaphysis, giving rise to longitudinal marrow spaces. At first these outgrowths occur at fairly regular intervals round the periphery, not more than one or two being present in the centre of the cartilage. They usually contain one or more small arteries, several large, thin walled vessels, hoemopoietic cells, giant cells, and osteoblasts most of which are arranged in a layer applied to the surface of the cartilage. A thin lâmina of bone is deposited on the eroded cartilage matrix (Plate IV, Fig. 1). In the internal portion of the region of endochondral ossification large simple cavities are formed by rapid lateral erosion, and the osseous wall by which they are lined is continually broken through and removed as soon as it is formed. In the terminal parts of the longitudinal marrow spaces, however, a fairly stout layer of bone is deposited in which osteoblasts become included and form bone corpuscles. This layer is very incomplete, as it is ruptured at frequent intervals by outgrowths of the marrow.

The finger-shaped marrow cavities soon become connected by irregular transverse spaces and at the same time the central mass of cartilage is penetrated by numerous small blood vessels accompanied by marrow cells, connective tissue, and osteoblasts. The small canals thus formed in the central cartilage enlarge, while the larger, elongated cavities extend into longitudinal and transverse branches until the distal end of the diaphysis becomes honeycombed by a system of longitudinal, intercommunicating marrow spaces which increase in number and complexity as they approach the extremity (Plate IV, Fig.1).

In the meantime bone formation is proceeding rapidly in the internal parts of the region of endochondral ossification. As the

lateral excavation of the cartilage advances, the bony lining of the longitudinal marrow spaces, as described above, is in some areas broken through and destroyed. Part, however, remains and resorption of the cartilage proceeds on the other side. This ruptured osseous barrier is often reinforced by further deposition by the osteoblasts on its outer side, and finally develops into a fairly stout, irregular trabeculum. In this way the cartilage is gradually replaced by cancellated bone derived from the incomplete osseous walls of the marrow spaces. This replacement is not very extensive in the day-old chick. The osseous lining of the marrow spaces becomes very thin as the excavations approach the extremity of the diaphysis, while only in the internal part of the area of endochondral ossification are complete trabeculae occasionally found. In this region, where large simple marrow cavities are formed by rapid lateral erosion of the cartilage, very little endochondral bone is deposited and the trabeculae are few, becoming progressively more numerous and more complex in structure as ossification spreads toward the end of the diaphysis where the marrow cavities are smaller and more numerous.

The cartilage matrix surrounding the marrow cavities is almost always heavily infiltrated for a short distance inward with calcium salts. In some areas osteoblasts enter the capsules opening to the eroded surface, and the capsule and the surrounding chondroid matrix become infiltrated with osseous material. This, however, does not take place to any great extent and usually only the superficial capsules are involved.

The vacuolated and swollen chondroblasts are for the most part destroyed <u>in situ</u> in the region of endochondral ossification, and the capsules at the eroded surface of the cartilage may often be seen to contain a disintegrating cartilage cell along with one or more osteo-

blasts or the pseudopodium of a giant cell. The chondroblasts are finally broken down into a diffuse mass of granular detritus. Degenerating chondroblasts are also seen between the layer of osteoblasts and the laminae of bone laid down on the cartilaginous matrix and, more rarely, lying free in the marrow spaces.

The structure and formation of the multinucleated giant cells. In the earlier stages of osteogenesis giant cells are comparatively rare and do not become numerous until the resorption of the cartilage is well advanced. The giant cells in the embryonic bones of the fowl differ little in structure from those of the mammal (Plate IV, Fig. 2). They usually consist of large irregular masses of protoplasm extending into long branched processes and containing from ten to twenty nuclei; the central cytoplasm is crowded with degenerate mitochondria which have a swollen appearance and are often reduced to a globular condition similar to that of the granular mitochondria which Dubreuil ('10) describes in the osteoclasts of the (?) mammal. Fat globules are almost always present in the peripheral cytoplasm, sometimes in very large quantities. Many of the nuclei are grouped together in the centre of the cytoplasm and in structure are for the most part identical with those of the osteoblasts. The multinucleated cells are usually found closely applied to the resorption surface of the cartilage or to a trabeculum of bone. They appear to be strongly phagocytic and often contain erythrocytes.

Many views as to the origin of osteoclasts have been put forward, the most important of which are summarised in a publication by L.B.Arey ('20). Most modern observers regard the multinucleated giant cells of developing bone as the products of cell fusion. Arey described these elements as being formed by the fusion of a large mass of agglutinated osteoblasts. Lacoste ('23), however, working on

the osteoclasts of the skull of the foetal sheep, states that "les ostéoclastes dérivent des cellules rondes mobiles du tissue conjonctif" and describes as the stages in their formation bi-, tri-, and small plurinucleated cells, which last "peuvent s'accroître en incorporant dans leur masse d'autres cellules jeunes uni ou plurinuclées". According to Lacoste, the osteoclasts develop in 'régions formatrices' of the connective tissue of the marrow.

The results of the present study accord with the observations of Arey. In the fowl the first stage in the formation of the multinucelated giant cells is represented by groups of osteoblasts in close association, but still showing the cell boundaries; the mitochondria in such cells are aggregated around the nucleus instead of around the centrosome. The next stage is seen in a mass of osteoblasts in which the outlines of the component cells, although indistinguishable in places, have largely disappeared, while the mitochondria show the swollen and distorted appearance mentioned above. Finally, the cell boundaries vanish completely, and the mass of agglutinated cells thus gives rise to a multinucleated giant cell. Although in the later stages of osteogenesis the osteoclasts are the chief, they are not the only elements involved in the formation of giant cells; connective tissue cells of the marrow are also to be found in association with the fusing masses. In the earlier stages of osteogenesis the few giant cells present at that period appear to be composed mainly of connective tissue cells, as Arey and other workers have already pointed out in the case of the mammal.

The bi- and trinucleated cells described by Lacoste in the sheep are also to be found in the long bones of the fowl. In the present study the possibility that these elements might represent the earliest stages in the formation of the multinucleated giant

cells was investigated but no satisfactory intermediate stages could be found linking the bi- and trinucleated cells with the giant cells, and the examination of serial sections almost invariably showed that what had appeared to be a small multinucleated cell was, in reality, the process of a large giant cell.

Brachet regards the multinucleated cells in the embryonic bones of the fowl as 'bourgeonnements vasculaires' and describes them as being in continuity with the blood vessels of the marrow. It is true that these cells are not uncommonly found adhering to the walls of vessels, as they adhere to any solid object, but I have been unable to find any evidence whatever of their origin from such a source.

The swelling and distortion of the mitochondria and the somewhat extensive elaboration of fat indicate that the giant cell is a degenerative structure. In these cells the nuclei degenerate later than the mitochondria. The first sign of nuclear degeneration is the enlargement of the nucleolus which continues to increase in size, while the nucleus shrinks. The nuclear membrane at length disappears and the nucleolus is seen as a large granule lying in the general mass of protoplasm. These observations lend support to Arey's view of the formation of osteoclasts in the mammal, viz. that "the entire course from the time of osteoblastic coalescence is one of progressive decline". Jordan ('21), on the other hand, states that "the younger osteoclasts exhibit no signs of degenerative changes, either cytoplasmic or nuclear". In material fixed by ordinary histological methods such as those employed by Jordan degeneration cannot be detected in the younger giant cells, but, as shown above, if technique suitable for the demonstration of mitochondria is employed, pathological changes are to be seen in the agglutinated cells even before fusion has taken place.

Summary of stages in the development of cartilage and bone.

Stage.	Synchronous events.
1.	Condensation of prechondral mesenchyme.
2.	First appearance of cartilage matrix.
3.	Transverse elongation of cells in middle of prechondral mass.
	First signs of perichondrium appear around middle region of mass.
4.	Formation of three zones of chondroblasts: epiphysial, flattened and hypertrophied.
	Differentiation of middle region of perichondrium into fibroblastic and osteoblastic coats.
	Appearance of osteogenic fibres among osteoblasts.
5.	Union of fibrous, osseous lamellae to form bony cylinder enclosing cartilage.
	Demarcation of epiphysis from surrounding mesenchyme, and formation of rudimentary, fibrous articular cartilage.
	Perichondrium distinguishable as far as epiphysial region.
6.	Cartilage resorption begins at middle of shaft.
	Formation of short, radiating, intervascular, bony trabeculae and thickening of central cylinder.
	Increased restriction of zone of flattened cells.
	Future boundary between diaphysis and epiphysis becoming distinguishable.
7.	Cartilage enclosed by periosteal bone completely excavated in middle two-thirds of shaft and replaced by marrow.
	First signs of endochondral ossification in proximal part of cartilaginous extremities.
	Epiphysis sharply marked off from diaphysis.
	Zone of flattened cells reduced to well-defined belt of cartilage between epiphysis and diaphysis.
	Osteoblastic and fibroblastic layers of periosteum recognisable as far as limit of diaphysis.
	In middle region of shaft intervascular trabeculae of bone thickened and extended to form Haversian canals.

Chapter 2.

The histological development of the normal embryonic mandible.

Introduction. Although there are several accounts of the general anatomical development of the avian mandible, I am unaware of any literature dealing with its histogenesis.

Material and methods. The lower jaws of a series of thirty embryos ranging in age from the fifth day of incubation up to the time of hatching were used in this study.

The material was fixed in Bouin's or Zenker's solutions and sections were stained with Mallory's triple stain or with safranin and picro-indigo-carmine. A number of specimens were fixed in neutral formalin, treated by von Kossa's method for the demonstration of calcification, and mounted whole after being cleared in cedar wood oil.

General structure of the embryonic mandible. In the late embryo the lower jaw consists of a rod of cartilage, known as Meckel's cartilage, which is surrounded by a sheath composed of four membrane bone - the angulare, supra angulare, operculare and dentale (Plate V, Fig. 1). Meckel's cartilage itself is not ossified except at the proximal end where it gives rise to a typical cartilage bone - the articulare (Lillie, '19). In the mandible, therefore, it is possible to study not only the development of cartilage bone similar to that described above in the case of the limb, but also the histogenesis of non-ossifying cartilage and of membrane bone.

Histogenesis of Meckel's cartilage. At the fifth day of development Meckel's rod is distinguishable as a short, stout bar of very early cartilage in which comparatively little matrix is present and which is

not sharply defined from the surrounding mesoderm.

In a well-developed six-day embryo Meckel's cartilage is very distinct (Plate XIII, Fig. 1). At this stage it consists of small, rather irregular cells scattered in fairly abundant matrix and is covered by a cellular perichondrium; at a short distance from each end of the cartilage rod a zone of rather flattened cells is seen. The rod increases in size partly by apposition of new cells from the perichondrium and partly by the formation of additional intercellular material.

After the 8th day of development the perichondrium surrounding the non-ossifying distal part of Meckel's cartilage becomes less conspicuous, the outer region growing rather diffuse and indistinguishable from the surrounding connective tissue whilst the inner cells give rise to an extremely thin fibrous membrane. The development of this part of the perichondrium is therefore different from that of the perichondrium investing an ossifying cartilage such as the femur or quadrate, which differentiates into a periosteum composed of an outer fibrous and inner osteoblastic layer.

The ossification centre of the articulare does not appear until about the 14th day of incubation but is usually fairly distinct by the 15th day. At this stage of development the ossifying cartilage shows a sharp demarcation into a middle region of large rounded, vacuolated cells, on either side of which is a broad belt of flattened cells passing into a region of small, round chondroblasts. The area of hypertrophic cells is covered by a two-layered periosteum which has formed a sheet of osteoid tissue on the surface of the cartilage. As the histological development of the ossifying articular cartilage closely resembles that of the distal part of an ossifying long-bone

rudiment from a limb it need not be described in detail. The cells in the non-ossifying distal rod show no sign of hypertrophy (Plate V, Fig. 1).

In a 2-day post-embryonic mandible the histological structure of Meckel's cartilage is essentially the same as in a 15-day specimen, except that the ossification of the articulare (Plate V, Fig. 2) is much more advanced. The elongated distal part of the cartilage (Plate V, Figs. 3 & 4) shows no sign of periosteal ossification and contains somewhat flattened but irregularly shaped chondroblasts whihh are larger than the cells of the unossified parts of the proximal end, but are smaller than and differ markedly in appearance from the swollen, vacuolated cells of the ossifying portion (cf. Figs. 4 & 5, Plate V.)

Histogenesis of membrane bone. The formation of membrane bone begins near the articulation of the jaw. In a well-developed 6-day embryo osteoblasts and a few fine osteogenic fibres can usually be distinguished in this region, but elsewhere the sites of the future membrane bones are indicated by rather diffuse condensations in the connective tissue. From the 6th to the 9th day ossification advances very rapidly and by the end of this period osteoid tissue is present as far as the apex of the jaw. Calcification begins near the articulation at about the 8th day and calcified bone is well developed throughout the mandible by the 15th day of incubation.

The histogenesis of the bone is briefly as follows.

The cells composing a condensed area of connective tissue, representing one of the four rudimentary membrane bones, are at first similar to those of the surrounding tissue. Very shortly, however, the cells in the centre of the mass enlarge somewhat and their cyto-

plasm becomes more deeply staining. These cells are the osteoblasts and a delicate intercellular network of osteogenic fibres rapidly forms amongst them.

The osteogenic fibres increase in number and give rise to rather irregular lamellae (Plate VI, Fig. 1). Blood vessels then grow into the ossification centre and the osteoid tissue assumes an irregular trabecular structure. The intercellular material increases in density, its fibrous structure being less obvious than in the earlier stage, and at the same time many osteoblasts become enclosed in the matrix as embryonic bone cells (Plate VI, Fig. 2).

The process of ossification, which is always more advanced in the original centre of ossification, extends outwards until the whole rudiment of the membrane bone is involved.

During the subsequent stages of development the intercellular material becomes more and more plentiful whilst at the same time the bone undergoes a continual resorption and redeposition resulting, at about the 15th day of incubation, in the formation of a large central marrow cavity running parallel with the length of the jaw, from which radiate fairly stout trabeculae between the medullary spaces (Plate VI, Fig. 3).

In the 2-day embryonic chick these trabeculae are very thick and regular and the bone cells are scattered rather sparcely in the matrix. The marrow spaces are also larger and better defined.

From this account of the ossification in the jaw it will be seen that there is no essential difference between the histogenesis of the mandibular membrane bone and that of the so-called 'cartilage bone' of which the long bones of the limb are composed.

Summary of Results (Part I).

1. Ossifying cartilage, non-ossifying (Meckel's) cartilage and membrane bone all develop from localised condensations of the primitive mesoderm.
2. Ossifying cartilage differentiates into three zones: small cells (epiphysial), flat cells (intermediate) and hypertrophic cells.
3. Bone is deposited on the surface of the zone of hypertrophic cells and the cartilage is then excavated and replaced by marrow.
4. Endochondral ossification is very slight in the fowl.
5. The cartilaginous epiphyses of the long bones do not ossify independently in the fowl.
6. The non-ossifying part of Meckel's cartilage does not differentiate into three cell zones but contains stellate chondroblasts only.
7. A sheath of membrane bone is formed around the non-ossifying part of Meckel's cartilage but is not laid down directly on the surface of the cartilage.
8. The non-ossifying part of Meckel's cartilage persists throughout embryonic life and is not replaced by marrow.
9. The structure and development of embryonic mandibular membrane bone and the periosteal bone of 'ossifying' cartilage are essentially the same.

PART II.

HISTOGENESIS IN VITRO

Object of experiments: To investigate the capacity for histological differentiation of various types of skeletogenous tissue when deprived of a circulatory system, nerve supply and association with normally adjacent structures.

Chapter 1.

Chondrogenesis in vitro.

Introduction. The behaviour in vitro of cartilage has been investigated by several workers. Carrel and Burrows ('10) cultivated conjugal cartilage and described the outgrowth from the original fragment of "a piece of new cartilage 2 mm. long". Fischer ('22) worked on cultures of the sclerotic cartilage of the embryonic fowl and Chlopin ('22) explanted the limbs of mammalian embryos; both these workers found that the cartilage matrix disintegrated during growth in vitro whilst a proportion of the chondroblasts wandered into the medium and became free amoeboid cells. Demuth ('28) described calcification in cartilage cultures; he also studied the effect of various chemical reagents upon the growth in vitro of isolated cartilaginous segments from the limb-skeleton.

The behaviour in vitro of undifferentiated chondrogenic mesenchyme was first studied by Strangeways and Fell ('26) who cultivated entire limb-bud rudiments in tubes. The technique employed in this work, the results of which will be described in a later chapter, precluded any microscopic study of the living tissue and I therefore made further experiments ('28) using a different culture technique.* These later investigations together with some unpublished results are described in the present chapter.

Material and methods. The tissue was obtained from the undifferentiated limb-buds of 3-day fowl embryos.

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* These experiments were undertaken at the suggestion of Dr. W. H. Lewis of Baltimore, who pointed out that it would be interesting to know whether cartilage would develop in mesenchyme undergoing active outwandering on a coverslip.

Cultures were made by Carrel's hanging drop method using No. 1 $\frac{7}{8}$ in. square coverslips and 3 x 1 inch hollow ground slides. The explants which were grown in a mixture of equal parts of plasma and embryo extract, were transferred to fresh medium every 48 hours and were cultivated for periods up to 3 months. Most of the mesenchyme cultures were divided into two after either 48 hours' or 8 days' growth but otherwise the original explant was kept intact throughout the period of cultivation so that in transplantation only the zone of outwandering was cut.

The cultures were fixed at various stages of growth in either Bouin's fluid or Zenker's solution less acetic acid. Some of the specimens were then mordanted in $2\frac{1}{2}\%$ iron alum, stained in dilute thionin and mounted whole. The remainder were serially sectioned; in the case of the older cultures fixation was followed by immersion for 3 hours in picro-nitric acid. In order to embed the cultures with the minimum damage the explants were not removed from the cover-glass on which they had been growing until they had been cleared and infiltrated with paraffin wax. The coverslips were then removed from the paraffin and the adherent wax was allowed to set, but not completely to harden. The explant, infiltrated and covered with wax, was readily peeled off the glass with a sharp knife after which it was dropped back into hot molten wax and embedded and cooled in the usual way. Tissues removed from the coverslip in this manner showed comparatively little distortion, tearing or folding.

Sections were stained with iron haematoxylin, haematoxylin and van Gieson's stain, safranin and picro-indigo-carmine or Mallory's triple stain.

In order to investigate the chondrogenic capacity <u>in vitro</u> of very small fragments of mesoderm, a series of experiments (unpublished)

were made in which each limb-bud was teased into numerous pieces which were then explanted in a hanging drop culture in a fluid medium. Each culture thus contained a large number of small explants. The culture medium was prepared as follows. Two parts of plasma were mixed in a sterile tube with three parts of embryo extract and the mixture was allowed to clot. The clot was then broken up with a sterile rod or knife and incubated at 38° C. for about 10 minutes, at the end of which time a considerable amount of fluid had exuded. This fluid was removed with a capillary pipette for use. The cultures were incubated with coverslips downwards for the first 48 hours of growth in order to allow the tissue to become adherent to the glass. Every 48 hours the explants were washed on the coverslip with a large drop of embryo extract which was then pipetted off and replaced with a drop of fresh culture medium. The tissue was maintained undisturbed in this way for 14 days at the end of which time the cultures were fixed, stained with dilute thionin and mounted whole.

Observations on living cultures. Cartilage was seen to develop in over sixty of the large explants cultivated in plasma and extract.

When first explanted the tissue consisted of a small, compact mass of mesenchyme. After 24 hours' growth most of the explants whilst showing a certain amount of outgrowth, were still somewhat rounded but by the end of 48 hours' *in vitro* the entire fragment of tissue had spread out into a thick plate. Sometimes the explants spread out more rapidly and during the first day's incubation, gave rise to thin plates of tissue which after 48 hours' growth had expanded into delicate sheets of mesenchyme containing slight local thickenings of precartilage, and bordered by a ragged margin of migrating cells. The blood vessels could still be distinguished as flattened, branching

cavities containing a few blood cells. In some cultures the ectoderm spread out over the coverslip in an 'epithelial veil' but in most cases, although partially covering the membrane, it tended to contract into flat pockets and islets in the substance of the explant.

After 4 days' cultivation the explants were still spread out into thin sheets but in many of the cultures small areas of 'epithelioid' cartilage had appeared in the centre of the masses of pre-cartilage mentioned above. This cartilage, which consisted of polyhedral cells separated from each other by thin partitions of refractile, colourless and apparently homogeneous matrix, passed imperceptibly into the surrounding mesenchyme.

The masses of cartilage were much larger after 6 days <u>in vitro</u> and towards the centre of the nodules the cells were separated by broader partitions of matrix. In places the cartilage still merged with an aggregation of undifferentiated cells, but elsewhere the surrounding tissue had given rise to a perichondrial membrane of elongated cells, which was continuous with, but clearly differentiated from the cartilage. The rest of the explant was still expanded into a sheet; there was usually an extensive zone of migrating cells which were of two types - fibroblasts and small, round, wandering cells.

A small proportion of the cultures became completely invested by partly keratinised ectoderm after about 8 days' growth. In such cases the ectoderm was dissected off and the explant continued to form zones of outwandering.

The cartilaginous nodules enlarged fairly rapidly until about the tenth day, after which growth proceeded more slowly. The amount of cartilage matrix had greatly increased by the 10th day and most of the nodules were completely surrounded by a definite perichondrium in

which fibres could be distinguished. The bread zone of growth consisted of elongated, radiating fibroblasts and wandering cells; the wandering cells were larger and more branched than those of the younger cultures and had the appearance of typical macrophages. Chlopin ('22) also notes this change in the size and appearance of the wandering cells in his cultures of early embryonic mammalian limb-tissue.

In cultures of 28 days' growth the cartilage was very hard to the touch and was usually enclosed by a capsule of extremely tough fibrous tissue. The zone of growth was rather variable in extent and tended to become organised into fibrous tissue unless the clot was very firm; mitosis was active and numerous branched wandering cells were seen.

During the last few weeks of the culture period very little increase in size took place; the maximum length attained by nodules of cartilage was 2.5 - 3 mm.

In some cases the cartilage matrix remained extremely hard but in a small proportion of the cultures it underwent a curious softening so that the nodules felt gelatinous when touched with a sterile knife.

The cartilage was usually surrounded by a very tough fibrous capsule. Patches of what appeared to be fat cells occurred in the connective tissue and seemed to be derived from wandering cells. The zone of outgrowth was usually smaller than in the younger cultures, but consisted, as before, of fibroblasts and wandering cells.

In the case of the teased limb-buds grown in a fluid medium, some of the fragments adhered to the coverslip and spread out as small sheets whilst others floated in the medium and were lost when the culture medium was changed. Cartilage was seen to develop in about

thirty of the attached explants, the course of development being the same as described above. Some of the chondrifying fragments were extremely small and at the time of fixation usually consisted of a tiny central nodule of cartilage from which radiated strands of amoeboid cells. Many of the explants, however, showed no sign of chondrogenesis and it was interesting to note that the occurrence of chondrification was not in any way correlated with the size of the explants.

Observations on fixed material. a. Whole mounts. A study of twenty-eight cultures stained with dilute thionin and mounted whole confirmed the observations made on living material.

Forty-eight hour specimens were expanded into thin sheets of tissue (Plate VII, Fig. 1) incompletely covered by partly contracted ectoderm. The masses of early precartilage and the remains of the blood vessels were well seen. There was no trace of the red, metachromatic staining characteristic of cartilage matrix.

In 4-day cultures (Plate VII, Fig. 2) the areas of precartilage were larger and even more dense and in some cases matrix which stained a purplish red had appeared in the centre of the thickenings. The contraction of the ectoderm was usually more pronounced.

After 6 day's growth (Plate VII, Fig. 3) almost all the precartilage had become transformed into cartilage as shown by the metachromatic staining. Where the cartilage was covered by a definite perichondrium the purple colouration terminated abruptly at the surface of the nodule but where the nodule was still continuous with precartilage the metachromatic staining faded away gradually. The rest of the explant retained its sheet-like form.

By the 10th day the much enlarged nodules of cartilage were

usually completed invested by perichondrium and their limits were therefore clearly defined by the metachromatic staining of the matrix.

In 24 days old cultures the cartilage nodules were stained almost black and contrasted very sharply with the green fibrous perichondrium, and the broad zone of outgrowth.

In specimens fixed after 10-12 weeks' growth (Plate VII, Fig.4) the metachromatic staining was sometimes confined to the central regions of the nodules of cartilage, the peripheral matrix being colourless. The chondroblasts were well seen in these unstained areas and appeared normal; in one specimen they were arranged in groups as in adult cartilage. The cartilage in one culture consisted of a small mass which was known to have been soft and gelatinous in life; the intercellular substance was colourless throughout this nodule and was seen to contain large numbers of closely distributed, unshrunken cells. In most of the older explants, however, the metachromatic staining was very pronounced. Each cartilaginous nodule was surrounded by a thick capsule in which yellowish fibres could be seen.

The fixed preparations of the cultures of teased mesoderm showed pronounced metachromatic staining of the matrix in the smallest nodules of cartilage. Some of the nodules were so minute that the number of chondroblasts present could be counted. The smallest explant studied consisted of a tiny central mass of cartilage containing only thirty chondroblasts, from which radiated strands of amoeboid cells attached to the surface of the glass.

b. Histological sections. Serial sections were made of fifty-one of the large explants grown in clotted medium.

A section through a typical 4-day culture showed a thin plate of mesenchyme passing gradually into a delicate zone of outgrowth. The blood vessels were seen as irregular cavities lined by a single layer of endothelium and containing rather degenerate blood cells. Numerous degenerating cells were scattered in the inner part of the explant. A mass of late precartilage was present and consisted of compactly arranged cells which were rather larger than those of the surrounding mesenchyme.

In a culture of 6 days' growth early cartilage was present but at the periphery faded into the surrounding mesenchyme.

After 10 days' cultivation considerably more matrix was present in the cartilage which was surrounded by a perichondrium containing numerous intercellular fibres; the surrounding connective tissue was looser and showed a reticulum of fine but well marked fibres. The cavities of the blood vessels were still visible. The ectoderm showed early keratinisation.

The cartilage in 18 day cultures contained rather more intercellular substance and in some cases the chondroblasts were rather larger; the perichondrium was denser and more fibrous.

After 28 days' growth the large, rounded or oblong nodules of cartilage were still in a healthy condition. The peripheral chondroblasts were often arranged in groups of two or four but the more central cells were still diffusely scattered. In a few cultures the cartilage contained numerous small, spherical cavities which were lined by flattened cartilage cells and had no communication with each other or with the surface. Some of the larger cavities contained a fairly dense coagulum. The histological appearance in such cases suggested that the chondroblasts themselves were dis-

solving the matrix.

Three of the fifteen cultures fixed and sectioned after 10-12 weeks *in vitro* were partially ossified and will be described in the next chapter. Of the remaining twelve, two contained relatively healthy cartilage (Plate VIII, Figs. 1 & 2) whilst in the other ten many of the chondroblasts were greatly shrunken. Sometimes the degenerate cells occurred in definite tracts, but in other explants they were scattered throughout the cartilage nodule. Numerous amoeboid chondroblasts often occurred in the degenerate tracts having apparently migrated there from normal regions. In one specimen there was a curious thin, superficial layer of healthy cartilage containing round cells, which overlaid but was sharply marked off from an area of degeneration; the origin and significance of this layer was obscure. The cartilage in all the non-ossified cultures was of the small-celled type found in the normal epiphysis and there was no sign of localised cellular hypertrophy. The chondroblasts were either evenly distributed throughout the matrix or formed small groups of two to four cells (Plate VIII, Fig. 1); the grouping was usually most conspicuous near the surface of the nodule. The connective tissue enclosing the cartilaginous nodules sometimes formed a loose network and sometimes a dense fibrous capsule.

Chapter 2.

Ossification of cartilage in vitro.

Introduction. As stated above Demuth ('28) observed calcification in his cultures of isolated embryonic limb-bone rudiments but he makes no mention of bone formation. This worker, who prepared the cultures primarily for biochemical investigations, records very few histological data and gives no account of the histological development of the explants during cultivation.

The development of true bone in association with limb cartilage was first described by myself (Fell '28) and I later (Fell & Robison '30) studied the histological development and ossification *in vitro* of the isolated femur rudiment. The results of these investigations are described in this chapter.

My results obtained in the cultivation of the femur rudiment, have since been confirmed by M.Levi ('30) who has cultivated various parts of the cartilaginous skeleton of early fowl embryos and by Niven ('31) in her work on the repair *in vitro* of embryonic cartilage and bone. In the case mammalian cartilage, Freidheim ('30) has recently found that the perichondrium of explanted fragments of limb cartilage from the embryonic rat forms bone when cultivated in association with leprosy bacillus.

Section A. Ossification of small masses of cartilage in hanging-drop cultures.

Material and methods. The cartilage used in the following experiments consisted mainly of the unossified extremities of the long bones of 8-8½ day fowl embryos; the epiphysial region and often a very small part of the diaphysis was removed from either end of each of the larger bones. As stated in the previous chapter ossification of

cartilage was also observed in three cultures (referred to as Nos. 1, 2 and 3) originally derived from the chondrogenic mesenchyme of the undifferentiated 3-day limb-bud.

The cultural and histological techniques were the same as those described in the previous chapter except that all the cultures were sectioned and none were mounted whole. The cultures were maintained for 10-12 weeks.

Observations on the living cultures. Although the greater part of the surrounding tissue had been dissected away from the 8-8½ day cartilage at the time of explantation a certain amount of perichondrium remained adherent and after 48 hours *in vitro* gave rise to a broad zone of outgrowth consisting of fibroblasts and numerous highly amoeboid wandering cells. This active outgrowth continued, with intervals of depression, throughout the maximum period of cultivation. In order to maintain this outwandering of cells it was necessary to cultivate the tissue in a relatively stiff clot; when explanted in a softer medium the zone of growth tended to invest the nodule of cartilage and become organised into a capsule of fibrous tissue instead of migrating outwards.

During cultivation *in vitro* the cartilage nodules usually increased to three or four times their original size; growth was most rapid during the first ten days *in vitro* after which it proceeded at a progressively slower rate and towards the end of the culture period altogether ceased.

Six of the explants underwent ossification during cultivation *in vitro*. In five of these cultures the process to some extent could be followed in life. For convenience of description the ossifying cultures are numbered 1-6.

The first indication of ossification *in vitro* was noted in explant No. 1; after about three weeks' cultivation a patch of whitish, opaque material which appeared black when observed by transmitted light, was seen on the surface of the diaphysial portion of the cartilage. This deposit, at first not very dense, gradually became thicker and spread over the surface of the nodule until it formed a sheath investing the fragment of diaphysis but stopping short at the border of the diaphysis and epiphysis. In Nos. 2-5, which belonged to another series of cultures, a similar deposition was observed after about a month's cultivation. This deposition took place over the entire surface of the cartilage in the case of No. 2, but in Nos. 3, 4 and 5 it was confined to about one half of the nodule - the diaphysial fragment; the bone was not very regularly laid down, in some places forming comparatively thick mounds, elsewhere a thin sheet, whilst in some places it was altogether absent. The bony deposit was found to be very hard when touched with a sterile knife.

In three of the ossifying explants Nos. 1, 2 and 3, the cartilage enclosed by the bone was largely or completely excavated by the surrounding tissue after 6-9 weeks *in vitro*. This excavation in the case of Nos. 1 and 3 was greatly accelerated, if not actually begun by the zone of outgrowth which, after the culture had been changed on one occasion, suddenly migrated into the cartilage within the sheath of periosteal bone, instead of wandering out into the medium in the usual way; it is probable that the diaphysial cartilage enclosed by the bone had become softened before this invasion took place. This phenomenon was perhaps most strikingly seen in No. 1, a section of which is reproduced in Plate IX, Fig. 2; in this

specimen in the course of 48 hours the bony tube surrounding the invaded cartilage was pulled downwards by the inwandering tissue until it made an angle of about 60° with the epiphysis and the coverglass; eventually the diaphysial bone was completely reflected over the surface of the epiphysial cartilage thus becoming once more parallel with the coverglass after a rotation of 180°. The removal of the cartilage was rather more gradual in the case of No. 2 in which the cartilage was completely covered with bone, and the progressive softening of the contents of the irregular, bony capsule could be followed by touching the explant with a sterile knife when changing the culture; the sensation resembled that of touching an egg in which the membranes are intact but the shell broken (a section of this culture is shown in Plate IX, Fig. 3).

The excavation of the cartilage caused a contraction in the diameter of the sheath of bone but the thickness and the consequent opacity of the bony wall continued to increase.

During the removal of the cartilage the zone of outgrowth, as might be expected, was greatly reduced and in the case of Nos. 1 and 3 was practically absent for a considerable time; this was doubtless due to the large number of cells that were migrating into the cartilage instead of into the medium.

Osteogenesis was seen in the living tissue in only two of the three ossifying explants (Nos. 1 and 3) originally derived from undifferentiated limb-bud mesenchyme. Ossification was first observed in No. 1 (Fig. 4, Plate IX. was drawn from a section of this explant) after about 5 weeks' cultivation. The cartilage in this specimen consisted of a single pear-shaped nodule the apex of which was twisted to one side. A saddle-shaped deposit of bone was seen to form on the surface of the nodule in the angle of this apical bend

and gradually increased in thickness and extent. In the case of No. 2 (a section of which is shown in Fig. 1, Plate X) the cartilage was composed of several nodules fused together. On the smallest of these nodules a white sheet of bone appeared in the usual way but as this part of the explant was rather obscured by connective tissue it was not possible to observe the progress of ossification in detail.

Ossification of the cartilage formed in cultures of 3-day mesoderm was greatly deferred as compared with normal ossification in vivo. In normal development the first signs of bone-formation are to be observed around the middle portion of the larger cartilage shafts at about the 6th day of incubation in well developed embryos; in the explanted limb-bud fragments, on the other hand, ossification was not seen until the third week of cultivation. This delay was probably due to the less favourable conditions of growth in vitro as compared with the conditions in the embryo. As will be seen from the next section the delay was greatly reduced when cartilage was grown in a large volume of medium.

Histological structure of fixed specimens. Sections of the five cultures of 8-day embryonic cartilage in which suspected ossification had been observed in every case showed the presence of well developed, typical bone; very early ossification was also found in another specimen (No. 6). Two of the cultures (Nos. 1 and 6) were fixed after 84 days' cultivation, the remainder after 70 days'.

No. 6 showed the earliest stages of ossification. This explant consisted of an epiphysis fitting cap-like over a small fragment of diaphysis. The diaphysial cartilage containing hypertrophied cells was shrply marked off from the small-celled epiphysis and there was no intermediate region of flattened cells. Towards the apex of the

nodule the cartilage had begun to break down; apparently under the influence of the chondroblasts themselves; the matrix showed large branched cavities having no connection with the surface and containing amoeboid cells some of which could be seen just migrating from the capsules. The centre of these cavities was sometimes occupied by a large, round mass of coagulum. A thin, irregular deposit of bone overlaid by a somewhat diffuse layer of osteoblasts covered one side of the diaphysial cartilage. The entire nodule was enclosed by a dense, rather thick capsule of fibrous tissue.

Two other specimens, Nos. 4 and 5, showed ossification without extensive erosion of the cartilage. Both explants were comparatively elongated and were differentiated into an epiphysial region, which was rather degenerate, an intermediate zone of flattened cells and a diaphysial region of hypertrophied cells; in No. 4 the three regions were sharply defined but in No. 5 their boundaries were indefinite. The periphery of the diaphysial fragment in both cases consisted of a broad tract of smaller-celled, probably calcified cartilage. In No. 4 the tip of the diaphysial fragment remote from the true epiphysis was covered by a small cap or typical and very healthy small-celled cartilage. The bony deposit varied in thickness; in No. 4 it formed patches on the surface of the diaphysis but in No. 5 (Plate IX, Fig. 1) it was laid down in a continuous sheath; a definite osteoblastic layer was not seen in either culture. No. 4 showed the beginning of cartilage erosion. In this explant various irregular cavities occurred near the surface of the cartilage which were filled with amoeboid cells consisting partly of invading fibroblasts from the perichondrium and partly of liberated chondroblasts. The ossification was wholly of the periosteal type in the case of No. 5, but in No. 4 it was partially endochondral since the deposition of bone sometimes took place on the

walls of the small excavations described above.

In three specimens, as previously stated, the cartilage enclosed by the bone had largely (Nos. 1 and 3) or completely (No. 2) disappeared. No. 3 was fixed three days after the invasion of the cartilage by the entire zone of outgrowth. Sections of this culture showed that the cartilage was highly degenerate and that the boundaries of the three zones were imperfectly defined. The diaphysial region was enclosed by a fairly stout, incomplete tube of bone. One side of the diaphysial cartilage had completely disappeared, leaving the bony wall intact; the space previously occupied by the cartilage contained a loose coagulum amongst which lay a large, dense mass of cells representing the former zone of outgrowth. At one point the mass of invading cells was continous with the superficial connective tissue. In No. 1 the epiphysial cartilage though partly necrotic was present, but the cartilage of the diaphysis had completely disappeared and was represented by bone only. (Plate IX, Fig. 2). The bone in this culture was remarkably healthy and well developed and consisted of an incomplete, deeply staining outer tube enclosing an irregular, trabecular mass of more lightly staining bone; a loose reticulum of connective tissue filled the cavities. No. 2 (Plate IX, Fig. 3) contained no vestige of cartilage and was composed of an irregular block of very healthy bone showing a two-layered structure, the outer layer being thick and deeply staining, the inner layer irregular and more lightly coloured. In one part (shown in the figure) the inner layer was absent and its place was occupied by loose connective tissue. Multinucleate osteoblasts (Plate IX, Figs. 2 and 3) were fairly numerous in both Nos. 1 and 2.

The three ossifying explants derived from undifferentiated limb-bud mesenchyme were fixed after 84 days' growth <u>in vitro</u>.

In one specimen (No. 3) only the earliest stages of ossification were seen. This explant consisted of a large, complex, rather degenerate nodule of cartilage surrounded by fibrous tissue. In one arm of the nodule two ossification centres were present. The cartilage of one of these centres contained typical hypertrophied chondroblasts; at the periphery it showed a few small cavities filled with invading connective tissue cells and on the walls of these excavations a thin layer of bone had been laid down. In the other ossification centre only the peripheral chondroblasts showed a limited enlargement, the inner cells being shrunken and degenerate; endochondral bone-formation, similar to that observed in the centre described above, occurred here also.

A section of the deposit of bone formed on the single pear-shaped cartilage nodule of explant No. 1 is shown in Plate IX, Fig. 4. The broad end of the nodule displayed a median tract of healthy, rounded cells sandwiched between two extensive areas of degeneration. The chondroblasts in the apical portion were hypertrophied and enclosed in deeply staining capsules; there was no sharp boundary between the regions of small and large cells. As already described, the bone formed a thick, compact layer on one side of the apical part of the cartilage. Rather dense fibrous tissue enveloped the entire nodule and the connective tissue cells had already begun to invade the cartilage of the ossification centre forming pocket-like excavations in the matrix.

Explant No. 2 contained a relatively large, complex block of cartilage formed by the fusion of several smaller masses. Comparatively advanced ossification was seen at one end of this nodule (Plate X, Figs. 1 and 2) where a stout but incomplete layer of bone had been laid down on the surface of the cartilage. As in the

previous culture the cartilage of this ossification centre consisted of a large, degenerate chondroblasts encapsuled by deeply staining matrix. Much of the cartilage in this area had been excavated, however, its place being represented partly by an oval space containing coagulum and partly (Plate X, Fig. 1) by numerous invading connective tissue cells. The rest of the cartilage nodule, which was in a fairly healthy state, showed no ossification and the cells were not enlarged. The nodule was surrounded by rather loose connective tissue.

The histological structure of the bone formed *in vitro* differed very little from that of normal embryonic bone. It was deposited in a less regular manner but in histological structure it was almost indistinguishable from the bone of the late fowl embryo (Fell '25). The bone cells (Plate X, Fig. 2) possessed the same cytoplasmic branches running into the intercellular material; the intercellular substance itself had the same closely fibrous texture and exhibited precisely the same staining reactions as in normal bone.

The principal difference between ossification in the cultures and ossification *in vivo* was the absence in all the explants of the definite two-layered periosteum seen in the normal embryonic long-bones.

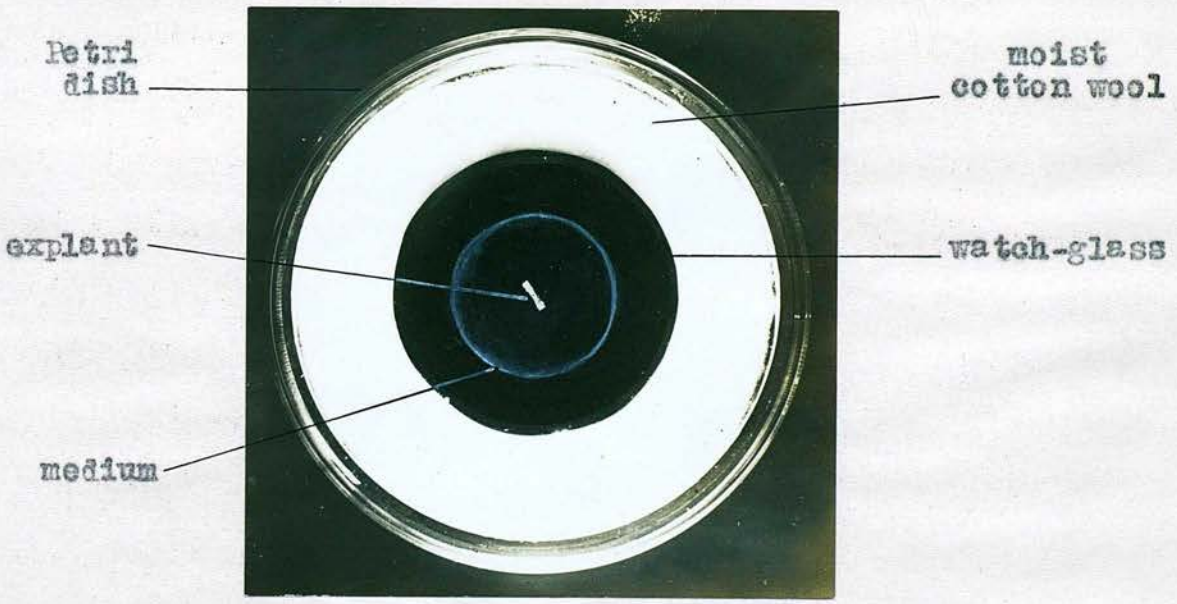
It was interesting to note that in all the cultures showing ossification the bone was invariably laid down in association with an area of hypertrophied chondroblasts.

Section B. Histological development and ossification of isolated femur rudiments in watch-glass cultures.

Material and methods. The material explanted consisted of the isolated femora of $5\frac{1}{2}$- and 6-day fowl embryos.

The tissue fragments were grown in a relatively large volume

of medium contained in a watch-glass. The culture method which is a modification of one tried by the late Mr. T.S.P.Strangeways, was as follows. A watch-glass, with its convex surface painted black to facilitate macroscopic observation, was placed on a layer of cotton wool at the bottom of a Petri dish 8 cm. wide and 1.5 cm. deep. After sterilisation of the culture vessel 30-40 c.c. of sterile distilled water was pipetted into the Petri dish where it was absorbed by the cotton wool; in this way a highly effective moist chamber was produced. The culture medium,consisting of 10 drops of plasma and 10 drops of embryo extract,was then introduced into the watch-glass with capillary pipettes and allowed to clot. Either three or four explants were placed on the surface of the clot in each watch-glass which was then incubated. The tissues were transferred to watch-



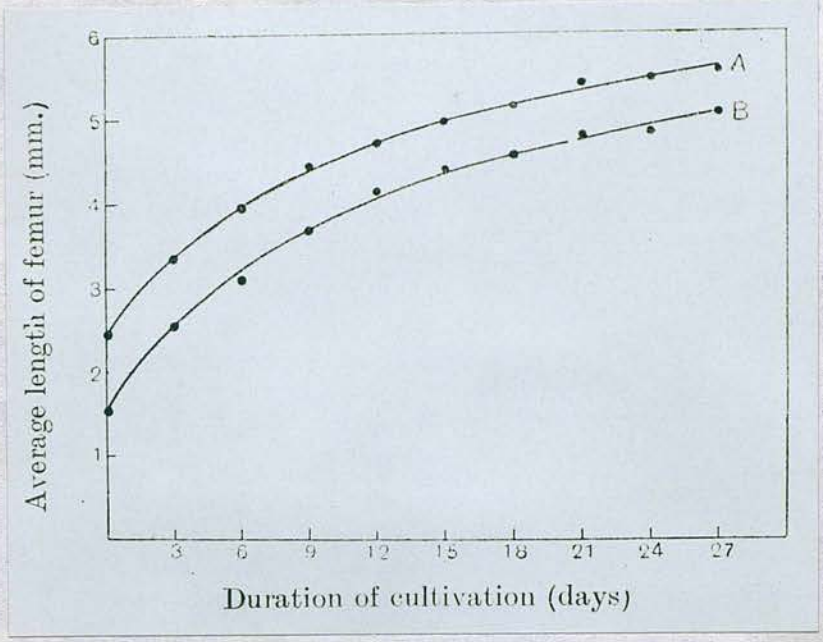
Text-fig. 1.

Photograph of a watch-glass preparation to show the arrangement of the culture vessel, culture medium and explant. (for photographic reasons this was not taken from an actual living culture).

glasses containing fresh medium every three days. This was effected in the following way. Each explant was first loosened from the clot either by lifting with a pair of fine forceps or by running the point of a cataract knife around the periphery of the cartilage so as to separate it from the zone of outgrowth; this latter method prevented the formation around the cartilage of a large mass of connective tissue and minimised distortion during cultivation. The explant when loosened from the medium was sucked into a wide-bore pipette, washed in extract and with the same pipette placed on the surface of the clot in a new culture vessel.

Explants were removed from the watch-glasses at different periods of cultivation and were fixed in Zenker's fluid. They were then embedded in paraffin wax and cut into serial sections; the slides were stained with haematoxylin and van Gieson's stain, safranine and picro-indigo-carmine or with Mallory's triple stain.

Observations on living cultures. The rate of growth of the femora during cultivation *in vitro* was studied by determining the increase in length at intervals of three days, the measurements being made by the aid of a micrometer eyepiece without removing the cartilages from the culture medium. For this experiment 16 femora of 5½-day embryos and 18 femora of 6-day embryos were used, cultivation being continued during 27 days. The growth curves shown in Text-fig. 2 are plotted from the average lengths of the femora, those in which serious curvature developed being omitted from the calculations. The 5½-day femora increased in length from 1.54 mm. to 5.03 mm. during 27 days' while the 6-day femora increased from 2.47 mm. to 5.54 mm. during the same period. The absolute increase in length was therefore slightly greater for the 5½-day than for the 6-day



Text-fig. 2.

Curve A. Average increase in length of 6-day embryonic femora during 27 days' cultivation in vitro.

Curve B. Average increase in length of 5½-day embryonic femora during 27 days' cultivation in vitro.

femora. Relatively to their initial size, however, the 5½-day femora grew much more rapidly, increasing in length by 67% during the first three days against 36% for the 6-day femora, while in 27 days' cultivation the increase amounted to 226% and 124% respectively.

The rate of growth in vivo was also determined by measuring the length of a number of femora of fowl embryos after periods of incubation from 6 to 21 days. In this series the length of the 6-day femora was 1.5 mm. and of the 9-day femora 6.0 mm., the increase during three days in ovo exceeding that during 27 days

in vitro. At 21 days, the chicks having hatched, the average length of the femora was 23.3 mm.

Although the watch-glass technique makes it possible to cultivate a much larger volume of tissue than can be grown by the hanging drop method, it is not suitable for a detailed microscopic study of the living tissue. Thus the only evidence of ossification distinguishable in the living material was the appearance after a few days' cultivation of a whitish band across the middle of the shaft. This band increased in width and opacity during subsequent cultivation.

On the other hand, the general anatomical development of the femora which is described in Part III, could be readily followed in the living cultures.

Observations on fixed material. The 6-day embryonic femora used for this part of the work were all slightly subnormal in development, having reached about the same stage as an ordinary 5½-day specimen.

In this series of cultures three femora were explanted in each watch-glass. Every three days one of the cultures was sacrificed, two of the explanted femora (the largest and smallest specimens) being used by Dr. Robison for biochemical investigations (see discussion) and the third being fixed and sectioned.

Sections of one of these 6-day embryonic femora (0-day) showed a very early type of cartilage (Plate XI, Fig. 1). The shaft was better developed than the two ends and contained slightly larger cells separated by rather broader partitions of matrix. Most of the cells in the shaft appeared more or less oval with their long axes at right angles to the long axis of the femur but towards the end of the cartilage the chondroblasts were rounded and irregularly disposed. There was no sign of demarcation into epiphysis and diaphysis so that the

young cartilage of the future epiphysis merged gradually into the better developed cartilage of the future diaphysis. The periphery of the shaft was not sharply defined but passed into a fairly broad, compact region of indifferent cells intervening between the cartilage on one side and the rudimentary muscle on the other. The outlines of the diaphysial regions were still less distinct and faded imperceptibly into a dense mass of undifferentiated mesenchyme. There was no trace of bone and no definite perichondrial membrane.

The femur fixed after 3 days' cultivation (Plate XI, Fig. 2) showed a marked advance in development as compared with the control. The outline of the cartilage was more distinct, the matrix was relatively abundant and the first indication of the three regions of cartilage cells - rounded, flattened and hypertrophic - characteristic of normal development had appeared. The region of hypertrophied cells occupied about one-fifth of the total length of the cartilage; the cells were very irregular in form and the enlargement was not very great. The zones of flattened cells compressed in the direction of the long axis of the shaft, lay one on either side of the region of hypertrophy, each extending through about one-fifth of the length of the femur. The two ends of the cartilage contained smaller, rounded cells. There was no sharp border line between the three zones which passed into each other very gradually. A perichondrium of somewhat elongated cells covered the surface of the femur and was continuous inwardly with the cartilage and outwardly with a thin layer of loose connective tissue.

After 6 days' cultivation the zone of hypertrophic chondroblasts had come to comprise about one-third of the length of the femur whilst the cells were much larger, rounded in form and showed

the characteristic vacuolation of the cytoplasm. The flattened elements were much more plate-like than in the 3-day explant and occupied a more sharply defined area which ended at the border of the epiphysial region containing small, rounded cells; there was thus a fairly distinct division into epiphysis and diaphysis. A very thin, conspicuous, rather sinuous sheet of early bone (or osteoid tissue) covered the surface of the cartilage in the region of the zone of hypertrophy. This sheet was overlaid by a layer of irregularly shaped cells resembling osteoblasts and these again were covered by a dense layer of spindle-shaped fibroblasts extending over the entire surface of the femur.

The specimen from the 9-day culture (Plate XI, Fig. 3) showed no striking histological change.

In the 12-day explant the cartilage matrix was everywhere more abundant than in the younger specimens and the demarcation between epiphysis and diaphysis was more pronounced. The cartilage was enveloped by a layer of connective tissue which was thin and compact over the surface of the epiphysis and loosely reticular over the shaft. The zone of enlarged cells was overlaid by a thin layer of bone covered by a fairly well-defined periosteum.

The femur from the 15-day culture (Plate XI, Fig. 4) was found to be much less developed than that fixed after 12 days <u>in vitro</u>; the reason for this imperfect development was not clear but it was probably due to some technical flaw in that particular culture. The region of hypertrophic cells was very restricted, there was no sharp division between epiphysis and diaphysis and an osseous layer could hardly be distinguished. ~~As will be seen later,~~ This subnormal differentiation was interesting in view of the fact that the two corresponding femora from the same culture showed a lower phosphatase

activity than those from the 12-day culture.

In the femur fixed after 18 days *in vitro*, a comparatively stout layer of bone had been laid down on the surface of the cartilage around the zone of hypertrophy; this zone composed about one-half of the total length of the femur. The bone was thickest around the middle of the shaft where it contained typical branching bone-cells and was covered by a periosteum consisting of an outer fibrous and an inner osteoblastic layer. The two zones of flattened chondroblasts were relatively narrow and terminated abruptly at the borders of the small-celled epiphysis. A loose highly fibrous mass of connective tissue surrounded the shaft and was continued over the articular surfaces of the epiphysis as a thin compact perichondrium.

The femur from the 21-day culture (Plate XI, Fig. 5) showed a much thicker layer of bone than was seen in the 18-day specimen. In one place the ossification had spread into the surrounding fibrous tissue and had formed a sheet of less densely staining (probably uncalcified) bony material running parallel with the sheath of periosteal bone covering the cartilage; a similar phenomenon was observed in an older (27-day) culture from the same series and in another 27-day culture from a different series.

The cartilage itself presented little change.

Up to the 21st day *in vitro* the explants showed no more degeneration than is seen in the normal embryonic limb-skeleton; indeed it was surprising to find that such a relatively large volume of tissue could remain so remarkably healthy under the conditions of life *in vitro*. In the 24-day explant, however, a limited number of shrunken cells were seen in places in both bone and cartilage, although elsewhere (Plate XII, Fig. 1) the tissue was still relatively healthy. This degeneration was still more extensive in the oldest

(27-day) femur (Plate XI, Fig. 6) which otherwise resembled the 21-day explant in histological structure; similar degenerative changes were seen in four 27-day cultures from another series. It seemed probable that this necrosis was due to an increasing density of the intercellular material of the cartilage, bone and connective tissue, which no doubt interfered with the proper diffusion of food material and excretory products and with the gaseous exchange.

In order to determine whether the osseous material laid down *in vitro* was calcified, four femora (from 5½-day embryos) from another series of cultures were fixed after 27 days' growth *in vitro* and were then stained whole by von Kossa's silver nitrate method. Of these one showed no blackening, another showed a single patch, whilst the remaining two displayed a broad belt of calcification around the middle section of the shaft (Plate XII, Fig. 2).

In most of the older explants a few scattered muscle fibres of considerable length and showing beautiful cross striation were seen in the connective tissue surrounding the shaft. At the time of explantation only very simple early myoblasts were present so that the muscle fibres of the explants, which were often indistinguishable from isolated normal fibres, had differentiated during cultivation *in vitro*.

For purposes of comparison a series of normal embryonic femora were histologically examined. It was found that differentiation proceeded more rapidly *in ovo* than *in vitro*, especially with regard to bone-formation.

In an 8-day normal femur, which would be equivalent in age to an explanted 6-day femur cultivated for two days, the region of hypertrophic cells was better developed than in the 3-day culture (equivalent in age to a 9-day normal embryonic femur) described above

and was already covered by a two-layered periosteum and delicate sheath of bone similar to that seen in the 6-day culture. By the twelfth day of growth the periosteal bone had formed a fairly thick and highly vascular, trabecular layer enveloping the zone of hypertrophy whilst at the same time ingrowing blood vessels, connective tissue and marrow had bgeun to excavate the cartilage enclosed by the bone. In the equivalent 6-day culture, as previously mentioned, the bone formed merely a delicate membranous sheath around the middle segment of the shaft.

On the other hand, the cartilage in the normal 12-day femur and in the equivalent 6-day culture was at the same stage of differentiation. The zone of hypertrophic cells occupied rather less relative space in the explant than in the normal bone rudiment, but the epiphyses were quite as clearly defined and the zone of flattened cells was on the whole rather more pronounced in the explant. By the eighteenth day of incubation two-thirds of the diaphysial cartilage of the normal femur had been excavated and replaced by marrow whilst trabeculae of periosteal bone between the blood vessels were relatively long and stout. A marrow cavity was not formed in any of the femora explanted in vitro and ossification was never so advanced as in vivo; even in the oldest cultures the periosteal bone was represented by no more than the single compact layer described above in the case of the 21-27-day cultures.

Chapter 3.

The development in vitro of the distal (non-ossifying) part of Meckel's cartilage.

Introduction. I have been unable to find any previous work on the cultivation in vitro of Meckel's cartilage.

The results recorded in this chapter were obtained by myself and form part of a morphological and biochemical study published by Fell & Robison ('30). As described in Part I, Chapter 3, the histological structure of the non-ossifying part of Meckel's cartilage in the normal jaw differs from that of ossifying cartilage in the absence of the zone of large, vacuolated chondroblasts which is so conspicuous in a developing cartilage bone. It seemed possible that this difference in histological structure might be due, not to any inherent difference in the two types of cartilage, but to some unknown factor in their respective environmental conditions. For this reason it was decided to investigate the development of Meckel's rod when removed from its normal environment and cultivated in vitro.

Material and methods. The explants were taken from 5½- and 6-day embryos and in every case the articular end of the cartilage, which normally ossifies, was cut off and rejected. Each batch of cultures of Meckel's rods was controlled by a similar batch of cultures of either the palato-quadrates or femora from the same embryos.

The tissue was cultivated by the watch-glass technique described above. The culture vessels (1½ inch watch-glasses in 3 inch Petri dishes) were somewhat smaller than those used before, and the culture medium consisted of only four drops of embryo extract and four drops of plasma. Four explants were grown in each watch-glass.

In some experiments the specimens were transferred to fresh medium every three days but better results were obtained when the medium was changed every 48 hours.

Explants were removed from the culture medium at intervals and fixed in either Bouin's or Zenker's solution. Sections were stained with Mallory's triple stain or with safranine and picro-indigo-carmine. In all, sections were made of 36 Meckel's rods controlled by 36 palato-quadrates and of 10 Meckel's rods controlled by 9 femora.

Observations on living cultures. Meckel's cartilages isolated from 6-day embryos increased considerably in length during cultivation in vitro, although this increase was very much less than in the case of the explanted femora.

In one experiment the entire cartilage rods from both sides of the jaw were removed from a number of 6-day embryos, explanted in a series of watch-glasses and measured at 3-day intervals during 21 days' cultivation. Twenty-four rods were explanted, 8 of which became curved and distorted during subsequent growth and had to be rejected. The average length of the rods at the time of explantation was 3.4 mm. and after 21 days' cultivation was 5 mm., showing an increase of 47%. The greatest increase in length of any individual specimen was 80%. The original average length of the explanted 6-day femora (Fell & Robison, '29) was 2.47 mm. and during 21 days' cultivation this length increased by 118%.

The experiments with palato-quadrate controls were perhaps the more interesting owing to the fact that the quadrate and Meckel's rod are adjacent structures.

In a 6-day embryo the palato-quadrate already shows its typical tri-radiate form which was usually preserved during the first 8 days'

growth *in vitro* during which time the quadrate increased considerably in size. After this period, however, the explants usually became rather deformed owing to twisting and bending of the arms. The explants of Meckel's cartilage also tended to become slightly distorted after this stage (Plate XIII, Fig. 2) usually by a hook-like bending of one or both ends. This distortion was probably due to the increasing toughness of the fibrous capsule by which the growing cartilage was surrounded.

After each subculture the explants became attached to the clotted culture medium by an outgrowth of cells from the perichondrial tissue.

Observations on fixed material. No histological difference can be distinguished between the quadrate and the Meckel's rod in the 6-day embryo (Plate XIII, Fig. 1). Both consist entirely of small-celled cartilage showing no trace of periosteal ossification.

After 2 days' *in vitro* the structure was still essentially the same in both types of explants. Both showed an increased amount of matrix and contained rather irregularly shaped chondroblasts, some of which showed mitotic figures. All the explants were covered by a layer of connective tissue.

After 4 days *in vitro* the Meckel's rods and some of the smaller quadrates were unchanged in microscopical appearance but in the larger quadrate controls a slight swelling of the cells occupying the middle region of one of the three arms could be observed, associated with a flattening of the cells bordering this area.

In 8-day cultures a striking difference in the histological structure of the two sets of explants was seen. Sections of the six palato-quadrates fixed at this stage all showed, as in the normal

development of this cartilage in vivo, a well marked differentiation into three cell zones, viz. a middle region of round vacuolated, hypertrophic cells bounded on each side by a belt of flattened cells which in turn passed into an area of small, round chondroblasts. In some cases a delicate layer of osteoid tissue had been deposited on the surface of the hypertrophic region. The Meckel's cartilages, on the other hand, still consisted of irregularly shaped cells scattered in fairly plentiful matrix.

In the older (16-20 day) explants the same contrast between the Meckel's rods and the palato-quadrates was observed. All the quadrates (Plate XIII, Fig. 3) showed the three definite regions of small, flattened and hypertrophic chondroblasts characteristic of normal "cartilage bone" rudiments (Plate XIII, Fig. 4), whilst all the Meckel's cartilages (Plate XIII, Fig. 2) contained only irregularly shaped cells similar to those seen in Meckel's cartilage of a normal late embryo (Plate V, Figs. 1 and 4). Most of the quadrates showed a certain amount of periosteal ossification. The only ossification ever observed in the explants of Meckel's cartilage were small areas of membrane bone in the surrounding tissue, which were quite unassociated with the cartilage; such areas only occurred in a few isolated cases and disappeared during the later stages of cultivation.

In those experiments where the femora instead of the quadrates were used as controls, similar results were obtained, i.e. well developed zones of small, flattened and hypertrophic cells were seen in all the femoral cultures but did not appear in a single explant of the jaw cartilage.

From these results it was clear that the striking contrast in histological structure between the non-ossifying part of Meckel's

rod and ossifying cartilage such as the embryonic quadrate or femur cannot be due to the influence of environmental factors since each type develops its characteristic histology when isolated from the body and growing in a watch-glass.

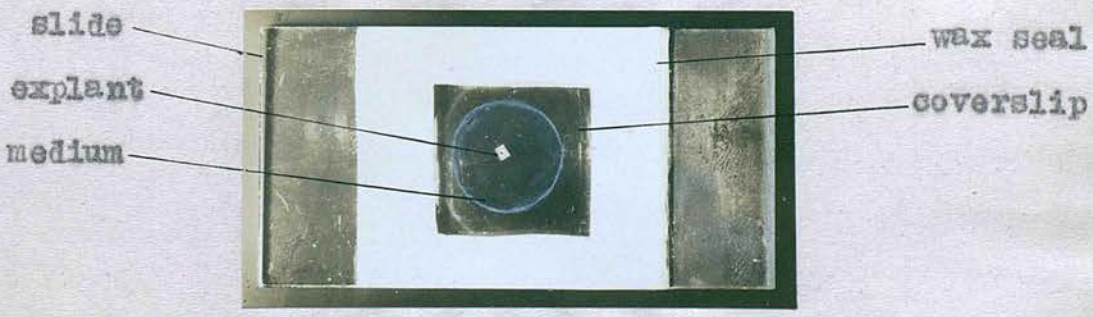
Chapter 4.

The osteogenic capacity in vitro of isolated embryonic periosteum.

Introduction. The behaviour in vitro of periosteum isolated from the bone has been very little studied. Policard and Boucharlat ('25) grew periosteum which in some experiments was denuded of bone, but state that osteogenesis did not occur. Several investigators have cultivated fragments of bone to which periosteum was attached but ossification was never observed.

The primary object of the present study (Fell '31, in press) was to investigate the osteogenic capacity in vitro of embryonic periosteum of the limb-bones of the fowl at different stages of development.

Material and methods. The periosteum was removed with fine needles and cataract knives from the middle third of various long bones the ends of which were cut off and rejected. The hanging-drop method was employed throughout the investigation. Large 1¼ inch square coverslips were used and 3 x 1½ inch hollow ground slides. The



Text-fig. 3.

Photograph of a hanging-drop preparation to show the arrangement of the culture vessel, culture medium and explant. (this was not taken from an actual living culture).

culture medium consisted of a mixture of equal parts of fowl plasma and embryo extract and the explants were usually transferred to fresh medium every 48 hours. Throughout the culture period the central part of the culture was kept intact and at subcultivation only the peripheral growth was cut away. The object of this procedure was to leave the middle region of the culture undisturbed and thus provide favourable conditions for organisation and differentiation.

Both cultures and controls were fixed in acetic Zenker's solution. Heavily ossified material was hardened in alcohol after a short fixation in acetic Zenker and was then decalcified with formol-nitric acid. This technique gave very satisfactory results.

A few of the cultures were stained with dilute phosphotungstic acid haematoxylin and mounted whole. Others were fixed in neutral formalin and were mounted whole after treatment with either sodium alizarin sulphonate or von Kossa's silver nitrate method, to demonstrate calcification. The silver nitrate specimens were photographed immediately after mounting.

The cultures to be sectioned were not removed from the coverslips until after infiltration with paraffin wax (Fell and Robison '30). They were cut either parallel with or at right angles to the plane of the coverslip. Most of the slides were stained with safranin and picro-indigo-carmine, but Mallory's phosphotungstic acid haematoxylin was also used.

A. Behaviour in vitro of 6-day embryonic periosteum.

Controls. The 6-day embryos from which the material was obtained were very well developed for their age. Tissue for explantation was taken from all the limb-bone rudiments except the tarsal and

carpal elements and digits, but the best results were obtained from the femur, tibia and humerus.

In order to determine the exact state of development of each rudiment from which periosteal tissue was removed, one cartilage was usually taken for dissection whilst the corresponding cartilage from the opposite limb of the same embryo was fixed and sectioned. Similarly in order to ascertain how much of the periosteum had been explanted, the fragment of cartilage from which the tissue had been stripped was, in many cases, also fixed and sectioned. The periosteal tissue removed from each rudiment was explanted separately so that each culture had thus a double control (Plate XIV, Fig.s 1 and 2).

Serial sections of the first set of controls, i.e. the cartilage rudiment fixed entire, usually showed the following histological structure (Plate XVI, Fig. 1). There was a middle portion occupying one-third of the total length of the rudiment, in which the cells had begun to hypertrophy and to assume the vacuolated appearance characteristic of the chondroblasts of "ossifying" cartilage; this region of enlarged cells was continuous on each side with two regions of flattened cells, which in turn passed gradually into the small-celled areas of the future epiphyses the articular parts of which were still composed of undifferentiated mesoderm. The first sign of osteogenesis had appeared around the middle third of the rudiment where the perichondrium had become differentiated into two layers - and inner stratum of polyhedral cells, the early osteoblasts, and a thick outer layer of elongated cells representing the rudimentary fibrous coat of the periosteum. A very delicate sheet of osteogenic fibres was present on the surface of the cartilage immediately beneath the osteoblastic layer; in some cases osteogenic fibres also ran outwards among the osteoblasts. The two-layered structure of the

perichondrium did not persist throughout the length of the shaft but become much less distinct over the area of flattened cells and disappeared on approaching the future epiphyses. In some of the controls development was less advanced; the enlargement and vacuolation of the cells occupying the middle third of such rudiments was only very slightly marked and the layer of osteogenic fibres was hardly distinguishable; the perichondrium covering the middle region had, however, assumed its two-layered structure. Examination of the second set of controls, i.e. the fragments of cartilage from which the periosteum had been removed for explantation (Plate XIV, Fig. 2) showed that the region from which the periosteum had been taken usually consisted of the area of hypertrophic cells and a small part of the two zones of flattened cells. The cartilage was rarely denuded completely of osteogenic fibres and osteoblasts and in most cases all or most of the layer of osteogenic fibres and sometimes a large proportion of the osteoblastic layer were left behind. Thus the actual explant usually consisted of ~~a~~ myoblasts and connective tissue, the rudimentary fibrous coat of the periosteum and part of the osteoblastic layer.

Observations on living cultures. Ossification centres developed in many of the cultures; in some cases cartilage appeared as well and in other specimens cartilage alone differentiated. In the most successful experiment out of a total of thirty-six cultures, eighteen developed ossification centres and no cartilage, three formed both cartilage and bone, five gave rise to cartilage only and ten formed no skeletogenous tissue, although growth was as vigourous as in the ossifying or chondrifying specimens.

The tissue when first explanted consisted of one or more

rather diffuse, irregular fragments in which the original site of attachment to the cartilage was sometimes distinguishable as a smooth semi-circle.

During the first 24 hours' growth the explants usually spread out into flat plates with a very narrow, marginal fringe of amoeboid cells. Many specimens contained a compact mass of rather rounded cells which later proved to be osteogenic or chondrogenic, from which radiated strands of myoblasts interspersed with capillary vessels and loose connective tissue; in other cultures it was impossible to distinguish different histological types.

After 48 hours' *in vitro* the explants had expanded into very delicate, more or less circular sheets 1-2 mm. in diameter. In some cases the cells composing the compact areas mentioned above (Plate XV. Fig. 1) had enlarged somewhat and had assumed the appearance of osteoblasts; they contained a large spherical nucleus with one or more rounded nucleoli, showed a finely granular cytoplasm and were separated from one another by delicate, slightly refractile partitions in which a fibrous structure was not apparent. These osteogenic regions were not sharply demarcated, but merged into the surrounding tissue; they varied in thickness, in some cases being as thin as the rest of the explant and in other cases forming definite lumps.

Occasionally one of the condensed areas seen after 24 hours' cultivation, by the end of the second day had differentiated into very early cartilage. These cartilage nodules which were rounded or oval in shape, differed from the ossification centres in the more regular, rounded shape of the cells and in the higher refractive index of the developing matrix which gave the nodule a characteristic glistening appearance.

Almost all the two-day cultures showed rather extensive cell degeneration which was probably due to the injury inflicted on the tissue by the original dissection.

After four days' cultivation most of the degenerate cells had vanished and the tissue seemed to have recovered completely. The ossification centres previously observed were considerably more advanced (Plate XV, Fig. 2). Delicate osteogenic fibres had appeared among the cells, forming rather irregular, very refractile, branching bundles. When the tissue was transplanted to fresh medium it was found that this early intercellular material had a certain amount of rigidity even in an extremely thin ossification centre. After being cut out of the plasma clot the delicate sheet-like explant contracted greatly except in the region of the ossification centre which, though showing a few narrow creases, remained as a fairly flat plate around which the soft, contracted mesoderm was crumpled in a ring like the parchment cover of a jam pot.

Occasionally osteogenesis began as late as the 7th day *in vitro*, but most of the ossification centres were distinct by the 6th day of cultivation, although their degree of development varied considerably. Bone -formation in cultures of 6-day embryonic periosteum was almost invariably diffuse, i.e. the osteoblasts spread out into the surrounding tissue so that osteogenic fibres radiated into the explant in all directions (Plate XV. Fig. 4). As a result almost every stage in osteogenesis could often be seen simultaneously in the same ossification centre. Thus in a six-day culture the middle region might consist of compact osteoid tissue in which the fibrous structure of the matrix was obscured by the deposition of a refractile, interfibrillar cement (cf. the development *in vitro* of membrane bone, Fell & Robison '30) whilst towards the periphery only a fine network

of osteogenic fibres could be distinguished among the osteoblasts.

It was interesting to note how the deposition of bone was modified by the mechanical conditions of cultivation. As stated above, narrow folds sometimes formed across the early, fibrous, semi-rigid ossification centre owing to the contraction of the explant when removed from the plasma clot for subcultivation. The rapid expansion of the explant which always followed transplantation did not cause these folds to flatten out again and they gave rise to hard ridges which usually persisted throughout the culture period. In the larger cultures the contraction also sometimes formed irregular more or less concentric folds separated from one another by comparatively flat areas of osteoid tissue (Plate XV. Figs. 1-4). These concentric folds, one of which appeared after each subcultivation, were probably due to the fact that the middle region of an ossification centre, as described above, was further developed and therefore more rigid than the fibrous outer zone, so that the latter under the influence of the general contraction of the explant, tended to form a circular wrinkle around the former. Before the re-expansion of the explant could pull this fold flat again, advancing osteogenesis transformed it into a hard, permanent ridge.

The osteoid tissue frequently appeared healthy after eighteen days' cultivation. Small ossification centres in the periosteal explants often disappeared before the eighteenth day but the larger ones continued to increase in size partly by the deposition of more intercellular material and partly, as described above, by the formation of new bone at the periphery, until in some cases they reached a diameter of over 1 mm.

Calcification could sometimes be detected by the 8th or 9th day and in good specimens was quite conspicuous by the 12th day.

Calcification was seen as a deposit of brilliantly refractile granules in the matrix of the oldest, more highly developed parts of the ossification centre. Calcified areas appeared a chalky white by reflected light and almost black by transmitted light (Plate XV. Fig. 5).

In a few cultures an ossification centre differentiated very close to the margin of the explant, some days after osteogenesis was well advanced in the other specimens; occasionally an ossification centre would form in the interior of the explant at the usual time and a peripheral one would develop several days later. It is probable that at the time of explantation osteoblasts were sometimes present at the edge of the tissue fragment and wandered into the medium in the zone of outgrowth. When the tissue was subcultivated the inner cells of the original zone of outwandering became incorporated with the main explant, whilst the outer cells again crawled into the culture medium. If osteoblasts were present in the original zone of outgrowth they would be unlikely to form bone until they had become included in the organised tissue of the culture, since cells undergoing uncontrolled growth in vitro seldom differentiate. This might possibly account for the delayed appearance of these peripheral ossification centres.

As described above, cartilage nodules developed in a minority of the cultures. Such nodules were not introduced into the cultures along with the periosteal tissue at the time of explantation, but differentiated in vitro, all stages of chondrogenesis being seen in the living material. Sometimes cartilage and osteoid tissue occurred in the same explant, in which case the two tissues were not associated but formed separate nodules; in other specimens cartilage only was present. The cartilage generally became invested by a fibrous perichondrium but occasionally merged directly with the surrounding tissue.

Some of the nodules, usually those without a perichondrium, disappeared after a few days' growth but the majority continued to grow and remained healthy up to the end of the culture period.

As development advanced the explants increased in thickness and the general stroma became denser and more fibrous. The original capillaries persisted as numerous branching clefts lined by endothelium and the myoblasts were also distinguishable after eighteen days' growth. Large numbers of wandering cells crawled out after each subcultivation and divided actively in the medium.

Observations on histological preparations. Histological preparations were made of most of the specimens containing ossification centres or cartilage, and many of the cultures in which skeletogenous tissue did not develop. Thirty-two explants containing ossification centres only were serially sectioned and three mounted whole, ten cultures containing both cartilage and bone and eight containing cartilage only, were sectioned. Most of these cultures were controlled by sections of the corresponding limb-bone rudiment from the opposite side of the same embryo and sixteen had the 'double controls' described above.

The histological findings confirmed the observations made on osteogenesis in the living material.

The youngest specimen fixed and sectioned was a 2-day culture in which an ossification centre had just appeared. The osteogenic tissue consisted of an elongated mass of cells which at one end of the mass were similar to those of the surrounding stroma and at the other end were differentiated into typical osteoblasts among which were seen narrow, irregular partitions of matrix stained bright blue with picro-indigo-carmine. The rest of the explant consisted of spindle-

shaped connective tissue cells, scattered myoblasts, and blood vessels which formed irregular spaces lined by endothelium and containing a few blood cells; no connective tissue fibres were seen. Mitosis was active throughout the culture.

Slightly older (three- and four-day) specimens presented a similar appearance but the ossification centres contained more intercellular material.

In the six- and seven-day explants the osteoid tissue was very well developed and contained typical stellate bone cells scattered in abundant matrix; it was covered externally by a layer of osteoblasts and usually had a somewhat trabecular structure owing to the presence of large, irregular cavities lined by osteoblasts. The ossification centre was not covered by a fibrous membrane. In some cases a thin sheet of rather diffuse osteoid tissue ran out from the main mass into the surrounding tissue. The general stroma of the explant was denser at this stage and contained intercellular fibres and sometimes compact masses of myoblasts.

Ten-day cultures (Plate XV. Fig. 6) showed little change, except that the stroma was still more dense and fibrous and the cavities lined by endothelium were more complicated.

Sections of the older (eighteen- and nineteen-day) specimens showed that the ossification centres were usually still healthy (Plate XVI. Fig. 2) and two specimens which were stained with alizarin and mounted whole exhibited a fairly extensive central region of calcification surrounded by a peripheral uncalcified zone. The general stroma was loosely reticular in some regions but elsewhere, especially in the neighbourhood of an ossification centre, it showed dense fibrous areas; the osteoid tissue was not, however, surrounded by a continuous fibrous membrane. In one specimen the ossification

centre had a rather interesting histological structure. A large plate of bone had been formed, which at an earlier stage had been bent over at one end. The internal angle of this bend was occupied by a small mass of tissue having the staining reaction and histological appearance of cartilage. The bone was not deposited on the surface of the cartilaginous tissue as in the normal development of the limb-skeleton, but gradually merged with it. The cartilaginous material seemed to have been formed from the stroma, with which it was internally continuous.

The isolated nodules of cartilage which, as previously described, sometimes appeared in cultures of 6-day periosteum, were found on section to be of the hyaline type and were enclosed by a fibrous perichondrium. Even in eighteen-day cultures the cartilage showed neither cell hypertrophy nor periosteal ossification. When a nodule of bone was present in the same culture (Plate XVI. Fig. 3) the cartilage and bone were quite separate.

B. Behaviour in vitro of 10-day embryonic periosteum.

Controls. Material was obtained from all the long bones from one side of a 10-day embryo with the exception of the digits, and from the femur and tibia from one leg of another embryo. Several periosteal explants were made from each of the larger bones. The corresponding bones from the opposite side of each embryo and in most cases the pieces of shaft from which the periosteum had been removed were fixed and sectioned as controls.

The embryo from which the tibiae and femora were removed was rather better developed than the embryo from which all the long-bones were taken. In both embryos the periosteum (Plate XVII. Fig. 1) consisted of a fairly thick and tough fibrous layer overlying an

osteoblastic layer which was rather irregular in thickness and somewhat diffuse along its inner surface. The periosteal bone was markedly trabecular in all the larger bone-rudiments but in the carpals and tarsals of the less advanced embryo the trabecular structure had only just begun to appear and the osteoblastic layer of the periosteum was consequently more compact and regular than in the larger rudiments. In the case of the more advanced embryo the cartilage was completely excavated from the middle region of the femur and tibia but in the less developed chick excavation in this region was only partial in the larger bones and had not begun in the tarsal and carpal elements.

Sections of the fragments of shaft from which the periosteum had been dissected for explantation showed that the fibrous coat had been removed and the osteoblastic layer partially so.

Observations on living cultures. In the best experiment bone-formation took place in seven cultures out of twelve and all stages in the process of ossification could be studied in the living material.

The original explants consisted of rather tough, irregular fibrous fragments to which minute pieces of bone sometimes adhered. After 24 hours' cultivation the original fragments which were still rather dense, had become surrounded by a zone of outwandering cells. During the next 24 hours' growth the culture began to spread out over the surface of the coverslip, whilst at the same time the fibrous tissue showed signs of disintegration, the intercellular material assuming a characteristic granular appearance indicating that resorption of the fibres was in progress. Early ossification centres were sometimes visible in 2-day cultures as rather large, compact masses of cells in which, 24 hours later, osteogenic fibres appeared.

In 4-day cultures the remains of the original fibrous tissue were still visible but the explants were usually much more homogeneous, cellular and expanded, although a few contained a thick opaque central region in which very little histological detail could be distinguished. As a rule, the explants of 10-day periosteum did not spread out into such thin sheets as the explants of 6-day material, and in many cases it was therefore difficult to follow the stages of osteogenesis in detail. Sometimes, however, the whole course of bone-formation could be observed very readily.

Most of the ossification centres were already present by the 6th day of cultivation, but in one specimen an ossification centre appeared near the periphery of the growth on the 14th day. Some of the centres were compact, i.e. they consisted of a mass of osteoid tissue covered by a layer of osteoblasts enveloped by a fibrous membrane; others were compact at one end and diffuse at the other, whilst others again were entirely diffuse. The ossification centres seemed to arise quite independently of the small spicules of bone which were sometimes accidently included in the explant and which showed no further growth and eventually disappeared.

The ossification centres formed <u>in vitro</u> by 10-day embryonic periosteum were rather thick and by continued deposition in some regions and resorption in others usually developed a trabecular structure. The details of osteogenesis were essentially the same as in the cultures of 6-day periosteum and need not be described.

By the 12th day <u>in vitro</u> all the ossification centres distinguishable were seen to be partially calcified. Outwandering from the margin of the explant still continued actively, with the formation of a broad zone of outgrowth, but the middle region had usually become so thick and opaque by this stage of cultivation that in several

cases an ossification centre observed at an earlier period had become completely obscured.

Observations on histological preparations. Nine of the cultures containing ossification centres were fixed and sectioned and one was stained with alizarin to show calcification, and mounted whole.

The youngest specimen fixed, which had been growing in vitro for 6 days (Plate XVII, Fig. 2), contained a large oblong mass of osteoid tissue in which were several large irregular cavities filled with osteoblasts. The surface of the developing bone was covered by a compact layer of osteoblasts outside which was a continuous fibrous membrane. The rest of the culture consisted of a rather thin, dense sheet of cells and intercellular fibres with a few small clefts lined by endothelium.

Most of the explants were fixed after fourteen days' cultivation, although one was carried on until the sixteenth day. In these cultures the bone and osteoid tissue showed a marked trabecular structure. In the centre of the ossification centre there was usually a region which stained very deeply with picro-indigo-carmine and probably represented true bone; this was surrounded by a broad, more lightly stained zone of probably uncalcified osteoid tissue. Large, fairly dense masses of fibrous tissue were sometimes present in the inner part of the explant, which were readily distinguishable from the ossification centres.

One fourteen-day specimen in which no ossification centre was present, contained a curious nodule which in life strongly resembled hyaline cartilage. In sections stained with safranin and picro-indigo-carmine this nodule was very similar to hyaline cartilage in its histological structure and consisted of rather rounded cells embedded in an apparently amorphous matrix. On the other hand,

unlike truehyaline cartilage stained by the same technique, the matrix was green in colour instead of bright pink. This was the only case in which any structure resembling cartilage was found in a culture of 10-day embryonic periosteum.

C. Behaviour in vitro of late embryonic and early post-embryonic periosteum.

Controls. The periosteum was obtained from the femur and tibia of a 17-day embryo, the femur of a 21-day embryo and the tibia of a day-old chick. In each experiment the femur or tibia from one side of the body was fixed and sectioned whilst the periosteum of the corresponding bone from the opposite side was removed and cut up into fragments for explantation. The shaft from which the periosteum had been taken was also fixed and sectioned.

Sections of the entire bones showed that the periosteum (Plate XVII. Fig. 3) consisted of a fairly thick coat of dense fibrous tissue covering and merging with a rather thinner layer of very flat cells. Between the flattened cells and the bone was a thin stratum of osteoblasts. The underlying bone contained numerous large, rather irregular Haversian spaces tending to radiate from the centre of the shaft and bounded by fairly thick walls of well developed bone. The spaces were lined by a layer of osteoblasts and enclosed thin-walled blood vessels. Only the middle third of the shaft was completely filled with marrow, the future medullary cavity in the distal thirds being occupied by partially eroded cartilage.

In sections of the shaft from which the periosteum had been stripped for cultivation, it was seen that the fibrous coat and its associated layers of flattened cells had been completely removed. In some places a thin layer of osteoblasts remained attached to the

bone which in other areas was covered by the remains of torn and degenerate cells; elsewhere the outer surface of the bone was completely denuded.

Observations on living cultures. The periosteum in the old embryos and newly-hatched chick, which was more firmly attached to the bone than in the case of the 10-day embryos, was removed and cut into small square explants to which the ends of muscle fibres usually adhered.

A few cells emerged from the fibrous explant after about 24 hours in vitro, and by the third day the original fragment had become surrounded by a halo of rather scattered growth. After 6 days' cultivation the scattered outgrowth had increased to form a broad sheet composed of spindle-shaped fibroblasts and a small proportion of wandering cells; the outline of the original explant appeared less sharply defined near the periphery and the intercellular fibres were undergoing a granular disintegration.

A broad zone of actively dividing cells continued to wander into the medium after each subcultivation whilst the cells in the interior of the culture became heaped up around the now greatly contracted original fragment, to form a central lump of organised tissue.

In most cases the culture was cut in half after 6 days in vitro, the two halves being placed side by side at a little distance from each other. The halves rapidly fused and the final result of this procedure was to produce a relatively large mass of organised tissue derived from new growth, in which the original half explants were seen as small contracted islets which became smaller and more opaque as the age of the culture advanced.

Over fifty cultures were observed for periods up to one month

but although the majority grew profusely and appeared healthy, osteogenesis was seen in two cases only. In one culture it was noted after nine days in vitro as a patch of enlarged cells embedded in refractile matrix and lying in the new growth near one half of the original explant; the specimen was fixed immediately. The other case of bone formation was not observed until the 20th day of growth when a small fibrous mass was noticed in the outer part of the organised region of new growth. Typical osteogenic fibres began to spread from this nodule into the surrounding tissue and by the 27th day a large kidney-shaped ossification centre similar to those seen in cultures of 10-day periosteum, had been formed. The ossification centre was not associated with the halves of the original explant which were conspicuous as two very opaque masses in the centre of the growth. The culture was fixed at this stage.

Observations on histological preparations. Fourteen cultures of the late embryonic (17- and 21-day) periosteum were fixed after periods of cultivation ranging from 12 to 28 days and were serially sectioned.

In the younger (12-14 day) specimens the original explant was seen as a dense fibrous mass in the centre of the culture. It was surrounded by much less fibrous, very cellular connective tissue derived from new growth which was sometimes compact and sometimes loosely reticular. Sections made at right angles to the plane of the coverslip showed this central region as a hump tapering on either side into the narrow zone of unorganised outgrowth. In some places, especially in the case of the cultures which had been cut in half at an earlier stage, the original explant or half explant, whilst often preserving its outline intact, had begun to lose its fibrous nature

and to give rise to a compact mass of cells. Mitosis was usually active throughout the culture. In some specimens numerous degenerative cells were present in the interior but others showed little or no degeneration.

In the two oldest (28-day) cultures the fibrous character of the original fragment had completely disappeared and only a flattened oval nodule composed of lightly staining cells was seen in the middle of the organised new growth.

Osteoid tissue occurred only in the two cultures in which it had been observed during life. The younger specimen, fixed after 12 days' cultivation, consisted of a relatively large central mass of organised new growth on either side of which lay two rounded fibrous lumps - the two halves of the original explant, which had already begun to disintegrate. The ossification centre lay in the newly formed organised tissue between the halves of the original explant and consisted of a rounded group of typical osteoblasts among which early osteoid matrix had been laid down. The surrounding tissue contained no fibres and consisted merely of a mass of healthy cells. In the older (26-day) specimen the original half explants had become reduced to small, compact areas in which no fibrous structure was seen, surrounded by a large mass of connective tissue with rather lightly staining, gelatinous intercellular material, formed by the new growth. The ossification centre which lay to one side of this fibrous tissue, was much larger and better developed than that in the younger culture and the matrix stained more deeply with picro-indigo-carmine. The nodule appeared healthy and showed very few degenerate osteoblasts.

Criticism of results. From the foregoing results it appeared that the osteogenic capacity *in vitro* of isolated embryonic periosteum

diminished with age. This required explanation. The diminished osteogenic power might be attributed to various possible causes :-

(a) To complete absence of osteoblasts in the original explant.

This was unlikely, since histological examination of the controls showed that the layer of osteoblasts which is present under the fibrous membrane even in the early post-embryonic periosteum, was at least partially removed when the periosteum was dissected away for explantation.

(b) To a less healthy condition of the cells in cultures of older periosteum.

The majority of cultures of late embryonic and early post-embryonic periosteum, however, grew actively and appeared as healthy as the bone-forming cultures derived from 6- and 10-day embryos.

(c) To the osteoblasts in late embryonic life being less able to form bone under the abnormal conditions obtaining in vitro, than the osteoblasts from early embryos.

This possibility was supported by the fact that on the whole, early embryonic tissue is more readily adaptable to the conditions of life in vitro than similar tissue from late embryos.

(d) To severe mechanical damage being inflicted in the osteoblastic layer when removing the periosteum for explantation, and thus preventing the multiplication and development of the osteoblasts during cultivation in vitro.

In late embryos and young chicks the periosteum is tough and rather firmly attached to the bone, so that mechanical damage might easily result from stripping off the membrane. Surviving groups of osteoblasts might have little chance of recovery and differentiation owing to the profuse over-growth of fibroblasts from the fibrous coat.

The only way to decide which of the last two alternatives was

correct, was to obtain undamaged osteoblasts from the late embryonic and early post-embryonic limb-skeleton and investigate their osteogenic capacity under the same experimental conditions. The experiments recorded in the next chapter were undertaken with this object.

Chapter 5.

The osteogenic capacity in vitro of late embryonic and early post-embryonic endosteum.

Introduction. The endosteum of the Haversian spaces seemed ideal material for obtaining undamaged osteoblasts from late embryos and young chicks. From the work of previous investigators (quoted in chapter 4) it was clear that the soft tissue contained in the cavities of bone migrated into the culture medium and there grew actively. As mentioned in chapter 4, the Haversian spaces of a normal late embryonic long-bone are lined by a layer of osteoblasts (the endosteum). It seemed probable, therefore, that a large proportion of the cells forming the zone of outgrowth from a bone fragment would consist of osteoblasts which, being protected by the bony walls of the Haversian spaces, would have sustained little or no damage when the bone was cut into small pieces suitable for cultivation.

Method. The tibia was dissected from the leg of a late embryo or newly hatched chick and the two ends of the bone was cut off and rejected, leaving only the middle third. The periosteum was then stripped off and in three of the seven experiments performed was cut up into fragments which were explanted *in vitro* as a control series of cultures.

The piece of shaft from which the periosteum had been removed was split open longitudinally and the marrow was carefully scraped away. The bone thus cleaned was cut into small oblong fragments, the long axes of which were at right angles to the long axis of the tibia, as this method of cutting involved the greatest number of Haversian spaces and was found to give the best outgrowth on subsequant cultivation. The fragments were then explanted into a large

drop of plasma and extract in a large hanging drop culture and incubated for three days, by which time a fairly broad zone of outgrowth had appeared. The cultures were cut out of the clot at this stage, transferred to fresh medium and grown for another two or three days.

After a total of either five or six days' cultivation the original bone fragment was removed from the zone of outgrowth by means of a needle and cataract knife and was rejected. The ring of growth left behind was cut out of the clot and transferred to fresh medium. The cultures thus produced were maintained for various periods up to twenty-eight days. The tissue which was transferred to fresh medium every 48 hours, was never subdivided and only the outer part of the growth was cut away in subcultivation. Owing to the very profuse growth of these cultures and their tendency to liquefy the clotted medium it was found necessary to grow them in as large a volume of culture medium as the coverslip would hold and to make the clot rather stiff by mixing a drop of unusually concentrated embryo extract with a rather large drop of plasma.

In the three experiments in which a control series of periosteal explants were cultivated simultaneously with the explants of bone, the periosteal cultures were treated in a precisely similar fashion to the bone cultures. There was usually more variation in the size of outgrowth in the periosteal cultures. The original explants of the most actively proliferating specimens were removed and rejected at the same time as the original explants of the bone growth, but cultures showing less active proliferation were allowed to grow for a few days longer before removal of the original tissue.

Observations on living cultures. The bone when first explanted was seen as a small, flat, rectangular mass in which the Haversian

spaces were distinguishable as lighter bands running parallel with the shorter sides of the rectangle. Two days after explantation a rather sparse outgrowth composed of cells resembling fibroblasts and of a number of leucocytes and other wandering cells had appeared from the two longer sides of the explant, i.e. the two sides which cut the Haversian spaces at right angles; very little growth took place from the other (shorter) sides. The periosteal controls usually showed considerably less outgrowth at this stage than the bone cultures. By the third day the bone had become surrounded by a fairly broad zone of cells, but the outgrowth from the periosteal controls was still sparse. The cultures were then transferred to fresh medium.

Growth became much more profuse after the first subculture and by the fifth or sixth day in vitro the bone and many of the periosteal explants had become surrounded by quite a wide halo of cells. It was interesting to note the difference in habit of growth between the bone and periosteal cultures. The bone (Plate XVIII. Fig. 1) was always surrounded by a broad sheet of irregularly shaped, compactly arranged cells forming a ring of tissue, from the periphery of which strands of elongated cells radiated into the clot. The outgrowth in the periosteal cultures, on the other hand, only rarely showed traces of a definite sheet of tissue and consisted entirely of long radiating cords of spindle-shaped cells running out from the explant in all directions.

As stated above, the original bone was removed either on the fifth or sixth day of cultivation. In many cases no trace of bone was left, but in other specimens a few minute spicules were present which underwent no further development during subsequent cultivation, remaining in the culture as foreign bodies. In the later experiments

owing to improvement in the manipulative technique, it was usually possible to re-explant the zone of growth after it had been cut out of the old clot, as a complete ring; in the earlier experiments the ring was often twisted and distorted when transferred to the fresh coverslip. The same was true of the control periosteal cultures.

It was interesting to find that these cultures ossified in 100 per cent of cases, and bone or osteoid tissue has now been observed in over fifty specimens. None of the periosteal controls, however, showed any trace of bone formation.

For the sake of convenience the cultures derived from bone have been termed "endosteal", but this does not imply that osteoblasts only were present in the growths which also included cells derived from the Haversian vessels.

All stages of osteogenesis could be observed in the living tissue (Plate XVIII, Figs. 1-4).

When the cultures were examined about 20 hours after the removal of the bone (Plate XVIII, Fig. 2) the peripheral cells were seen to be wandering outwards in the usual way, whilst the hole left after dissecting away the explant, was completely or partially filled by a centripetal ingrowth of cells from the inner margin of the ring. In exceptional cases the medium liquefied in the central hole, in which case the ingrowth of cells did not take place until the culture was changed. The periosteal controls showed the same outward and inward migrations of cells.

Forty-eight hours after dissection the hole was in most cases completely filled by cells, although its site was usually still distinguishable as a thin patch in the centre of the culture. At this stage of growth, and sometimes even earlier, the first signs of osteogenesis became visible, and when the tissue was examined under a high

power a network of delicate osteogenic fibres was seen to permeate the culture. These fibres were sometimes very clearly and beautifully seen in the thin central patch. In the thick ring of tissue surrounding this area, they often formed circular bands, whilst in the outer parts of the culture, where they were much more sparse, they tended to radiate towards the margin. No such fibrillary network was distinguishable in control periosteal cultures of the same age and from the same tibia.

On the fourth day following the removal of the original bone, osteoid tissue was easily recognisable in all the endosteal cultures. The matrix at this stage appeared in the middle region of the culture as an irregular mass of refractile lumps and ridges in which the original fibrous structure was almost indistinguishable. The site of the hole left by the removal of the explant seldom vanished completely, as the osteoid matrix usually remained sparse and diffuse in this area. A network of osteogenic fibres radiated outwards from the main osteoid mass into the broad sheet or organised tissue by which it was surrounded. The osteoid tissue in the middle of the culture sometimes showed thick corrugations radiating from the centre. This was a mechanical effect similar in origin to the concentric ridges described above in the case of cultures of 6-day embryonic periosteum, and was produced as follows. When the ring of new growth was removed from the original bone, the tissue, which like most forms of tissue culture had been growing under tension, contracted considerably, and if the circular sheet was broad and thin it became puckered into radiating folds when released from the clot. Before these folds could be straightened out again by the re-expansion of the tissue, they became partially ossified and persisted as rigid bars.

In the earlier experiments the endosteal cultures gave rise to

osteoid tissue only - calcification was never observed. This was found to be due to an inadequate supply of culture medium and consequently in the later experiments, as described in the section on methods, the size and stiffness of the clotted drop was considerably increased with much better results. The cultures appeared healthier, grew more actively, and in almost every case formed true calcified bone (Plate XVIII, Fig. 4).

In these later experiments calcification was frequently well advanced by the fifth day after removal of the original bone fragment (Plate XVIII. Fig. 3). It often progressed very rapidly, and it was not uncommon to see only a slight opacity in the matrix one day and quite a dense calcification the next. A typical periosteal control culture at this stage appeared as a flat mass of elongated cells somewhat resembling a heap of straw and presenting a marked contrast to the regular sheet of compactly arranged cells which surrounded the new bone in the endosteal cultures (Plate XVIII. Fig.3).

As the age of the culture advanced the mass of bone in the centre enlarged, partly by peripheral deposition of new bone and partly by increase in the quantity of intercellular material. Uncalcified osteoid tissue was often resorbed some ten or fourteen days after the removal of the original fragment, but calcified bone could persist intact for at least a month after the dissection (the longest period for which the cultures were maintained).

The control periosteal cultures grew very vigorously during the first fortnight after removal of the explant, often forming a large mound of cells in the middle, and completely obliterating all traces of the hole from which the original explant was taken. A degenerate spot sometimes appeared in the centre of this lump of tissue, and some of the innermost cells were seen to be fatty or

necrotic. Sometimes this rather opaque, necrotic area would spread to a comparatively large size and then at a later stage of growth would apparently be largely resorbed and eventually reduced to a small, rather hyaline nodule in which nuclei were embedded. A nodule of this type was readily distinguishable from osteoid tissue.

Observations on histological preparations. The observations made on the living material were checked by histological examination of cultures fixed at various stages of growth. Most of the endosteal cultures were sectioned, but others were mounted whole after either staining with dilute phosphotungstic acid haematoxylin or treatment by von Kossa's silver nitrate method for demonstrating calcification. In all, thirteen cultures were fixed and sectioned before removal of the explant and three were stained with phosphotungstic acid haematoxylin and mounted whole, fifty-three were sectioned at various stages after removal of the original bone, and whole mounts were made of three specimens stained with Mallory's haematoxylin and of eleven treated by von Kossa's method. The periosteal controls were also histologically studied, three cultures being fixed and sectioned before removal of the explant and thirty-nine being sectioned after removal.

As previously described, bone from the middle region of a late embryonic or early post-embryonic tibia contains numerous more or less cylindrical Haversian spaces tending to radiate from the centre of the shaft; each Haversian space is lined by a layer of osteoblasts (the endosteum) and encloses delicate, endothelial vessels.

Sections of bone cultures fixed after five or six days' growth, that is, at the stage immediately before removal of the explant (Plate XIX. Figs. 1. and 2), showed that both the osteoblasts and

endothelial cells of the Haversian spaces had migrated actively into the surrounding medium. A large proportion of the actual bone cells appeared degenerate. It is possible that some of those near the cut edges of the bone or close to the Haversian spaces might have emerged from the intercellular material and entered the culture medium, but by far the greater part of the zone of outgrowth was obviously derived from the tissue in the Haversian spaces. In one experiment in which growth had been particularly active, all three cultures fixed on the fifth day showed osteogenic fibres already formed by the new tissue around the original bone, and in two cases the fibres were quite well marked in certain areas of the culture. Osteogenesis was not observed in the living specimens before fixation, owing probably to the thickness of the zone of outgrowth in the neighbourhood of the explant. In another experiment where growth had been rather less profuse, no sign of newly formed osteogenic fibres was seen in any of the eight cultures fixed after six days in vitro.

Osteogenic fibres were present in seven out of the eight cultures fixed 14-24 hours after removal of the bone fragment (Plate XIX. Fig. 3). They were seldom distinguishable in the living cultures at this stage, owing probably to the contracted condition of the tissue during the first 24 hours following removal of the bone. The hole from which the bone had been dissected was filled either by a rather loose network of cells or by a plug of culture medium, and the osteogenic fibres formed a diffuse mat, stained blue with picro-indigo-carmine, in the tissue surrounding this area. Specimens cut at right angles to the coverslip were seen as a rather flat plate of tissue in which the osteogenic fibres formed either thin flat lamellae running parallel to the coverslip and sandwiched between layers of osteoblasts or fairly thick, irregular masses in which lay scattered osteoblasts.

Both the specimens fixed three days after removal of the bone were thicker than the 24-hour cultures, and the osteoid tissue was more abundant. In some areas the osteoid matrix was comparatively dense and deeply staining, but elsewhere it consisted merely of early osteogenic fibres.

By the fifth day after dissection (Plate XIX. Fig. 4) the osteoid tissue in the interior of the culture had in places assumed the character of young bone, and by the seventh day (Plate XVIII. Fig. 5) most of the osteoid tissue in the centre had become transformed into true bone which, as described above, was seen to be heavily calcified in life.

Eleven days after the removal of the bone fragment (Plate XX. Fig. 1) quite broad and dense partitions of bone matrix had been laid down which stained a very deep blue with picro-indigo-carmine. Osteoblasts in mitosis (Plate XX. Fig. 1) were still seen even in the best developed parts of the bone. In some cases the deposition of fresh bone was taking place fairly extensively at the periphery of the main mass, but usually the cultures showed signs of a diminished osteogenic activity at this stage, and the zone of newly formed osteoid tissue surrounding the central mass of bone was more restricted than in younger cultures.

One culture was fixed 21 days after removal of the original bone (Plate XX. Fig. 2). The nodule of bone which had formed during this period was very well developed and healthy, but osteogenesis appeared to have stopped as the outline of the bone was sharply marked off from the rest of the culture. The bone was surrounded by a broad, lightly staining, fibrous region which resembled ordinary connective tissue rather than osteogenic tissue.

Four cultures were fixed on the twenty-eight day after dissection. In two of them the bone was fairly healthy, but in the other two it showed signs of degeneration.

The eleven cultures treated by von Kossa's method were fixed at periods ranging from eight to eleven days after removal of the original bone. All the specimens showed dense blackening in the areas where calcification was suspected in the living cultures (Plate XX. Figs. 3 and 4).

The histology of the periosteal controls was very different from that of the endosteal cultures.

Three periosteal cultures were fixed after 6 days in vitro, at the stage immediately before removal of the explant. They showed a dense, deeply staining, coarsely fibrous explant, on one side of which, in one case, were tags of muscle, and from the edges of which extended the broad zone of outgrowth.

Four specimens fixed 14-24 hours after removal of the original tissue consisted of a sheet of rather loosely arranged cells among which no intercellular fibres were seen. By the fifth day the cells had become much more closely packed and the cultures had increased in thickness forming, as previously described, a kind of hump in the centre which was very conspicuous in sections made at right angles to the plane of the coverslip. A few fine intercellular fibres, stained light blue with picro-indigo-carmine, could just be distinguished in the denser parts of the tissue.

The central lump had become relatively large ten or eleven days after removal of the explant, and the lightly staining intercellular fibres running among the densely packed cells were more plentiful. In two cultures an area of degeneration occurred in the interior of the central lump. In one of these cases, where degeneration was

less advanced, the cells in the necrotic area were highly vacuolated and in life probably contained fat. The intercellular material, which stained more deeply than in the surrounding healthy tissue, appeared as rather stout, sinuous bands running among the vacuolated cells. The other culture showed a later stage in this degenerative process. In this specimen the necrotic region was smaller and more compact; the cells were highly vacuolated and the intercellular material, which stained a dark green with picro-indigo-carmine, was less plentiful than in the healthy parts of the culture and appeared to have contracted. In two of the cultures fixed on the twelfth day this type of degeneration was even more clearly shown. In the middle of these specimens was an oval nodule composed of the granular remains of cells in which somewhat shrunken nuclei were still seen, and among which ran numerous narrow, intercellular partitions of amorphous, deeply staining material representing the contracted remains of the lightly staining intercellular fibres seen in healthy cultures. Workers unused to the histology of this material might at first mistake such necrotic areas for some differentiation of a chondroid or osteoid nature.

Vacuolated, degenerate cells occurred in most cultures fixed after the eleventh or twelfth day following removal of the explant, although in some instances only very few degenerate cells were present and the centre of the culture was comparatively healthy (Plate XX. Fig. 5).

No sign of ossification was seen even in the oldest cultures which had been maintained for twenty-five days after removal of the explant. Except in the necrotic areas described above the intercellular substance remained as a lightly staining, finely fibrous material totally different from that of the bone or osteoid tissue

present in endosteal cultures of the same age.

Criticism of results. From the results recorded in this chapter it is clear that undamaged endosteal osteoblasts from the limb skeleton of late embryos and young chicks form bone *in vitro* as readily as periosteal osteoblasts from early embryos. It might be argued that in the writer's experiments the endosteal osteoblasts formed bone *in vitro* and the periosteal osteoblasts failed to do so because the former were cultivated for some days in asssociation with differentiated bone which in some way induced an osteogenic capacity in the surrounding cells. On the other hand, the two positive results obtained with cultures of isolated late embryonic periosteum, together with the fact that ossification took place readily in cultures of periosteum derived from 6-day embryonic limb-bone rudiments in which true bone had not yet been formed, showed clearly that contact with differentiated bone is not an essential condition for osteoblasts to manifest their osteogenic function *in vitro*.

The results described in this chapter also fail to exclude the possibility that the capacity for bone formation, although possessed by the endosteal osteoblasts, might be partially or completely lost by the periosteal osteoblasts in the later stages of development. In this case the two positive results obtained with the late embryonic periosteum might be explained by assuming that endosteal osteoblasts had been dragged out of the Haversian spaces when the periosteum was stripped off. Histological examination of normal late embryonic and early post-embryonic limb bones shows, however, that osteogenesis is very active immediately beneath the fibrous periosteum which makes it difficult to believe that the normal osteogenic capacity of the periosteal osteoblasts is seriously diminished at this stage.

It seems more probable that in the writer's experiments the failure of late embryonic and early post-embryonic periosteum to form bone *in vitro* except in rare cases, was merely due to mechanical damage inflicted on the delicate osteoblastic layer when stripping off the tough fibrous covering membrane.

Chapter 6.

The development in vitro of mandibular membrane bone.

Introduction. Very little work has been done on the cultivation *in vitro* of membrane bone. Dolschansky ('29) studied cultures of embryonic fowl membrane bone, but although the soft tissue in the bone grew readily forming broad zones of outgrowth, the formation of new bone was not observed. Fischer and Parker ('29) cultivated for prolonged periods tissue derived from the os frontalis of the embryonic fowl. They found that such cultures completely lost their original histological structure and when grown in the usual culture medium consisting of plasma and embryo extract showed active proliferation and migration of cells but no signs of differentiation. On the other hand, if such "dedifferentiated" cultures were transferred to a medium consisting of plasma only which restricted all proliferation, hard nodules, regarded by the authors as chondroid or osteoid in nature, developed in the centre of the explants. The results of Fischer and Parker are, however, open to criticism as some, at least, of the appearances which they figure and describe suggest degeneration rather than differentiation.

The development *in vitro* of membrane bone from undifferentiated osteogenic mesoderm was studied by myself (Fell and Robison '30) and the results obtained are described below.

Material and methods. The material was obtained from 6-day fowl embryos. The mandible was removed from the head and its articular ends were cut off and rejected. As much as possible of the ectoderm was stripped away from the underlying mesoderm which was then carefully dissected from Meckel's cartilage and cut into fragments for explantation.

The explants were grown in large hanging drop preparations ($1\frac{1}{4}$ inch square coverslips, 3 x $1\frac{1}{2}$ inch hollow ground slides) in a medium composed of equal parts of plasma and embryo extract. In the earlier experiments the tissue was subcultivated every three days but better results were obtained by transplanting to fresh medium every 48 hours. During subcultivation the central region of the culture was carefully kept intact and not subdivided. Two series of cultures were prepared in which neutral red was present in the medium in the proportion of 1 in 50,000 and 1 in 100,000 respectively.

Some of the cultures of osteogenic mesoderm were fixed in neutral formalin, treated by von Kossa's silver nitrate method for the demonstration of calcification and examined whole after being mounted in Farrant's medium. Most of the specimens were fixed in either Flemming's, Zenker's or Bouin's solution, they were then embedded in paraffin wax by the method described in Chapter 1 and were serially sectioned in a plane at right angles to that of the coverslip. Sections were stained with Mallory's triple stain or with safranin and picro-indigo-carmine.

Controls. In most of the experiments two explants were made from each half jaw and two entire jaws were fixed and sectioned to serve as controls. In some experiments cultures were made from half the jaw whilst the other half was fixed and sectioned so that each pair of cultures had a control. Twenty-three entire or half jaws were used as controls in this way.

Examination of the jaws and half jaws fixed as controls showed that in some cases there was no trace of ossification at the time of explantation, but in the better developed specimens a small area of osteoblasts with a few osteogenic fibres could be distinguished near the articulation of the jaw though elsewhere the future membrane

bones, as described above, were indicated only by diffuse condensations in the connective tissue surrounding Meckel's cartilage.

Observations on living cultures. About 65% of the explants developed ossification centres. Ossification was observed in over 180 cultures and the various stages in the process which closely resembled that seen in the periosteal and endosteal cultures, could be readily studied in the living tissue.

When first explanted the tissue, consisting mainly of connective tissue and myoblasts, was usually rather diffuse, but after 24 hours' growth *in vitro* it had contracted into a more or less compact mass of cells of which the outermost had begun to wander into the medium forming a narrow fringe of outgrowth around the central fragment.

During the next 24 hours' cultivation the compact mass gradually spread over the coverslip in a sheet of tissue which usually showed in places one or more fairly large aggregations of cells merging imperceptibly into the surrounding tissue (Plate XXI. Fig. 1). Subsequent observation showed that in many instances such a group of cells represented the first appearance of an ossification centre. In these cases the group rapidly became more distinct and more restricted and after 3 days *in vitro* was often seen as a well defined, usually oval area of somewhat closely packed cells which, as in the case of the periosteal and endosteal cultures, seemed to be separated from each other by a slightly refractile and apparently amorphous intercellular substance. The cells of the ossification centre took up neutral red to about the same extent as the surrounding connective tissue cells.

The next stage in development, which usually took place within

the following 24 hours, was the differentiation of the cell mass into two regions - an inner part composed of polyhedral cells and an outer capsule of elongated cells continuous with the surrounding tissue. In cultures in which neutral red was present in the medium the inner cells were conspicuous owing to the readiness with which they stained with the dye.

A few hours later (Plate XXI. Fig. 1.b) very delicate intercellular fibres - the osteogenic fibres - appeared in the region of the ossification centre. These increased in number until they formed a fairly fine network between the closely packed cells. Usually the inner region now consisted of a single peripheral layer of osteoblasts surrounding a central mass of early bone cells embedded in the osteogenic fibres, whilst the outer region had given rise to a typical fibrous capsule of connective tissue. The osteoblasts and the young bone cells in the neutral red cultures stained even more deeply and examination under high power showed that the cytoplasm was filled with rather fine, spherical red granules.

This type of structure was attained by about the 4th or 5th day *in vitro* (Plate XXI. Fig. 1.c).

The next stage observed (Plate XXI. Fig. 1.d) was the formation of an apparently amorphous cement between the osteogenic fibres. In one culture where a very thin ossification centre was present the various stages in this process could be followed particularly well in the living material. It was found that the criss-cross network of delicate fibres originally seen became aggregated into refractile bundles in which the individual fibres were difficult to distinguish. Focussing on the surface of the capsule with the high power of the microscope the matrix was seen to have a matted structure very different in appearance from the delicate network observed some 24 hours

previously. The density and refraction of the matrix progressively increased whilst the fibres became less and less conspicuous, being probably obscured by the cement in which they were embedded.

As the age of the ossification centre advanced it was found that in some areas the original intercellular partitions were resorbed and replaced by small cavities filled with osteoblasts, in other places they remained apparently unaltered, whilst elsewhere they became thickened to form fairly stout, irregular walls and columns. In this way the developing bone gradually assumed an irregular trabecular structure which was usually well seen about the 9th or 10th day in vitro. Whilst these changes were taking place in the general structure of the matrix, a certain proportion of the osteoid nodules underwent partial and occasionally (Plate XXI. Fig. 3) extensive calcification. Refractile granules appeared in the intercellular material and increased in number until the bone appeared almost black when viewed under the low power of the microscope by transmitted light. When examined under high power the bony trabeculae were seen to have a nodular appearance owing to the brilliant granules embedded in the tissue.

Besides the compact ossification centres described above, diffuse ossification centres also occurred, though less frequently. In such cases there was no definite periosteum and at the margin of the developing bone the osteogenic fibres passed into the surrounding connective tissue in all directions. A compact and a diffuse centre were sometimes present in the same culture, and in other cases the same ossification centre might be compact at one end and diffuse at the other.

The rate of development of the bone varied considerably in different cultures so that one explant might take 9 days to reach a

stage of differentiation attained by another after 6 days in vitro.

The tissue surrounding the ossification centres was complex and consisted of connective tissue, myoblasts, wandering cells and, when embryos of coloured breeds were used, branched pigment cells.

In some cases the bone remained in a fairly healthy condition up to the 14th and more rarely up to the 24th day of cultivation. Usually, however, the ossification centres were more prone to resorption than those found in the periosteal and endosteal cultures and began to disappear after about the 12th day in vitro.

Observations on fixed material. Histological preparations of cultures fixed at different stages of development confirmed the observations made on the living explants (Plate XXI. Fig. 1).

An early ossification centre such as that sometimes present in a 2-day culture (Plate XXII. Fig. 1) appeared in sections as an oblong mass of cells of somewhat irregular shape and with rather deeply staining cytoplasm, which were slightly flattened in a direction at right angles to the coverslip. A few osteogenic fibres which stained dark blue with Mallory's stain or with picro-indigo-carmine could be distinguished among the cells.

In sections of a 4-5 day culture (Plate XXII. Fig. 2) in which ossification was more advanced the fibrous capsule and adjacent layer of osteoblasts described in the case of the living cultures were well seen. The inner mass of developing bone was about 4-8 cells thick; the oblong or polyhedral bone cells were set closely and fairly regularly in the fibrous intercellular material which with Mallory's stain and with safranin and picro-indigo-carmine gave exactly the same staining reactions as in the normal jaw.

In cultures fixed at about the 6th day of growth (Plate XXI.

Fig. 2 and Plate XXII. Fig. 3) the bone was seen to contain irregular spaces filled with osteoblasts and connective tissue cells. The bone cells were normal in appearance and scattered throughout the dense matrix which was hard and difficult to cut.

The structure of the bone in older (12-14 day) cultures was similar but more intercellular material was present (Plate XXII. Fig. 4).

Some of the specimens which in life appeared to be calcified, when treated by von Kossa's silver nitrate method showed a dense granular deposit of silver in the regions where calcification had been suspected (Plate XXI. Fig. 3).

Summary of Results (Part II)

1. Undifferentiated mesoderm from the 3-day embryonic limb-bud, even when teased into minute fragments, is able to chondrify during cultivation *in vitro*.
2. Cartilage formed *in vitro* from such undifferentiated mesoderm, with rare exceptions, is of the small-celled type and does not ossify.
3. Unossified cartilage from the distal parts of 8-day embryonic limb-bone rudiments, may ossify during cultivation *in vitro* and the original cartilage may then be completely removed from the shell of periosteal bone by invasion of the surrounding connective tissue.
4. The early (5-6 day) femur rudiment undergoes extensive histological development including ossification when cultivated *in vitro*.
5. Undifferentiated mesoderm from the 6-day embryonic mandible ossifies during growth *in vitro*.
6. Periosteum removed from 6-10 day embryonic limb-bone rudiments is able to ossify *in vitro*.

7. Periosteum from late embryos and young chicks may grow profusely *in vitro* but only ossifies in rare cases, probably owing to severe damage being inflicted mechanically on the osteoblastic layer when stripping away the tough, firmly attached fibrous membrane.
8. Undamaged (endosteal) osteoblasts derived from the Haversian spaces of late embryonic and early post-embryonic tibiae readily form bone *in vitro*.

PART III.

ORGANOGENESIS IN VITRO

Object of experiments: To investigate the importance of extrinsic factors in the development of gross anatomical form.

Chapter 1.

The anatomical development in vitro of the undifferentiated limb-bud.

Introduction. The self-differentiating capacity of the isolated limb-bud of the embryonic fowl has been investigated mainly by means of grafts. Spurling ('23) describes a case of accidental but successful autotransplantation of the posterior limb-bud in a fowl embryo. Murray and his collaborators (Murray and Huxley '25, Murray '26, Selby and Murray '28), using the method of chorioallantoic grafting, have made important contributions to our knowledge of the developmental mechanics of the early avian limb-bud and as a result of their investigations they have shown that the undifferentiated limb-rudiment is a self-differentiating system.

The development *in vitro* of the undifferentiated limb-bud was recorded by Strangeways and Fell ('26)* and the results obtained in this study are described below.

Material and methods. The limb-buds for explantation were obtained from embryos of 72-hours incubation.

The culture medium consisted of a mixture of embryo extract and plasma diluted, in the earlier experiments, with Pannett and Compton's isotonic salt solution; the ingredients were mixed in three different proportions approximately as below :-

Medium.	Plasma.	Emb. Ex.	Saline.
1.	7 drops	2 drops	50 drops per tube
2.	8 drops	8 drops	8 " " "
3.	10 drops	10 drops	0 " " "

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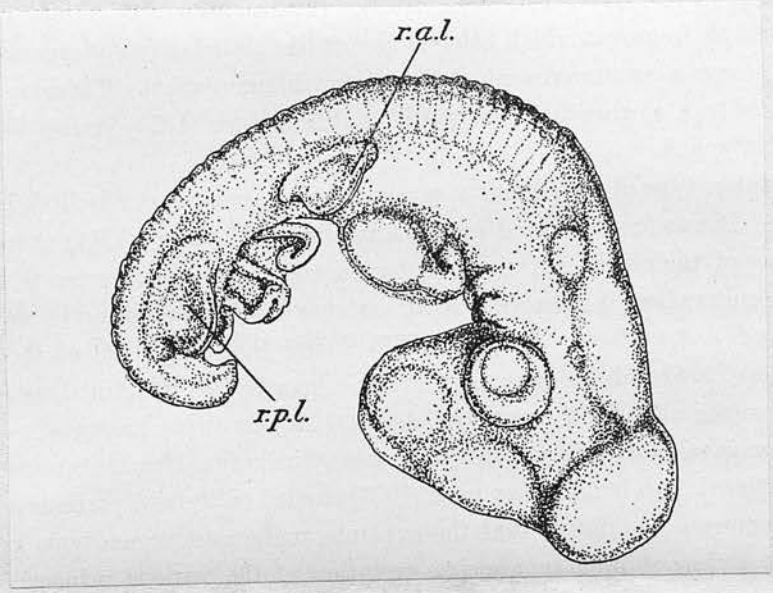
* My personal share in this work was the histology and part of the tissue culture.

Small centrifuge tubes were employed as culture tubes, and the medium was placed in these and allowed to clot. Medium 1. gave rise to fibrin clots floating in a large quantity of serous fluid; medium 2. formed a soft jelly covered by a layer of supernatant fluid, and medium 3 a tough clot with a mere film of supernatant fluid. An embryo was removed and both the anterior and posterior limb-buds were dissected off from one side of the body - usually the right. The buds on the opposite side were fixed in Allen's solution as controls. The buds to be cultivated were each placed in a separate tube on the surface of the medium and the tube was then tightly corked. In all, 67 cultures were prepared, of which 50 gave satisfactory growths. Explants cultivated in medium 1. sometimes adhered to the clot but more frequently sank to the bottom of the tube, whilst those grown in media 2. and 3. almost always remained on the surface.

The cultures were periodically changed into fresh medium; in the earlier experiments this was done at intervals of two to three days, but in the later half of the work regularly every 48 hours. The explants were removed from the old plasma by means of a wide-mouthed pipette, washed in a drop of saline in a hollow-ground slide, and transferred to one of a new series of culture tubes.

At different periods of growth the explants were removed from the culture tube with a wide-mouthed pipette and fixed immediately in Allen's modification of Bouin's fluid warmed to 38° C. Complete serial sections both of the explants and of the control limb-buds were cut, and stained with iron haematoxylin either alone or followed by van Gieson's stain, haematoxylin and eosin or Mallory's stain.

The structure of the limb-bud at the time of explantation. The size of the limb-buds of embryos of 72-80 hours' incubation is variable and may be greater in an individual of 72 hours than in one of 80 hours. The largest limb-buds used formed well marked, elongated projections from the body wall, whilst the smallest were almost indistinguishable with the naked eye: most of these very minute



Text-fig. 4.

Drawing of 80 hrs. embryo showing the limb-buds (r.a.l. right anterior bud, r.p.l. right posterior bud).

buds grew and differentiated in vitro almost as readily as the larger rudiments. In most cases the limb-buds were intermediate in size between these two extremes. The controls (Plate XXIII. Fig. 1) were found to consist histologically of completely undifferentiated,

rather loose mesenchyme, in which were numerous irregular vascular spaces; even in the largest buds the mesenchyme showed no sign of becoming dense to form precartilage. The ectoderm was two-layered and along the outer margin was thickened to form a faint ridge. The anterior bud was very similar in shape and structure to the posterior rudiment, but was slightly smaller.

Observations on the living explants. During the first few days of life in vitro most of the explants showed outwandering of cells from the cut surface, but later they usually, though by no means invariably, became rounded off and covered by a continuous membrane of connective tissue or epithelium. Liquefaction of the fibrin in the immediate neighbourhood of the explants almost always occurred. In most cases growth proceeded very rapidly until the fifth day, continued rather less actively until the ninth or tenth day, and then diminished until it became inappreciable and in some cases probably stopped altogether. When cultivated in medium 1. the limb-buds, at first solid, after a short time usually became cystic. Sometimes a thin-walled vesicle appeared as a bulge on the surface of the explant, whilst in other cases the entire growth gradually became hollow, giving rise to a uni- or multi-locular cyst with delicate translucent walls. This cystic structure usually developed very rapidly, and many of the explants were completely cystic by the third day of growth in vitro. Such vesicular cultures sometimes attained a considerable size and the largest, which was fixed after 20 days' cultivation in vitro, had reached a diameter of about 4 mm. Those buds growing in medium 1. which remained solid did not enlarge to any great extent. With media 2. and 3. this cyst formation was not nearly so marked; only two completely cystic cultures were formed, and the majority of the explants

either remained solid or showed only slight cyst-formation. Unlike the solid explants grown in a thin medium, those cultivated in the less dilute plasma grew rapidly and sometimes increased to several times their original size. As differentiation proceeded, cartilage began to form and the cartilaginous nodules could usually be faintly distinguished in the living tissue.

The histological development of the explants. As stated in the description of technique, two types of dense medium were employed, one more solid than the other, but as both gave essentially the same results the growths will be described in a single series.

In the younger explants, i.e. in those which had been cultivated for not more than four days in vitro, it was usually possible to distinguish roughly the original contour of the limb-rudiment and to determine which was the proximal end and which was the distal region of the bud owing, in the first place, to the persistance for the first few days of cultivation in vitro of that ridge of thickened ectoderm which runs along the outer margin of the bud, and in the second place, to the fact that the cut surface of the bud usually became attached to the clot of fibrin by the outwandering of the mesenchyme cells, and was therefore not covered with ectoderm. The first signs of tissue differentiation appeared after two days' growth in vitro. In two of the three specimens fixed at this age the mesoderm composing the proximal* part of the limb-bud had condensed into an oblong mass of cells representing precartilage (Plate XXIII. Fig.2). This elongated mass was slightly broader at one end than at the other, the larger end lying near the cut surface of the limb-bud. It was

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* The words "proximal" and "distal" are used with reference to the original relationship of the limb-bud to the embryo.

interesting to note that as regards both shape and position in the limb-bud this block of precartilage resembled the earliest rudiment of the posterior appendicular skeleton in the normal embryo. In one specimen another small, more or less spherical nodule was situated near the larger end of the main mass of precartilage. The general stroma of the explants was composed of a network of stellate cells, as in the normal early limb. There was comparatively little cell degeneration, and mitotic figures were very numerous in both the mesoderm and ectoderm. The vascular sinuses were seen as irregular branching cavities and were considerably distended.

After three days' cultivation in vitro the cells occupying the central region of the precartilaginous mass became less densely packed together, enlarged somewhat and at the same time became slightly flattened in a direction at right angles to the long axis of the mass; at the periphery the precartilage faded into undifferentiated mesenchyme.

By the fourth day of cultivation the greater part of the precartilage had assumed the character of early cartilage. In the single specimen (Plate XXIII. Fig. 3) fixed at this stage of development the skeletal mass was broken up into a series of oblong segments. One of the nodules (c.1) was situated at the extreme end of the explant, in what appeared to have been originally the proximal portion of the limb-bud, and consisted of fairly well developed cartilage. The adjacent cartilaginous segment (c.2) was also comparatively well differentiated towards the proximal end, but about halfway along its length it was still at the precartilaginous stage. Two other small skeletal masses were present in this culture which are not shown in the figure. The histological structure of the larger cartilaginous nodules in this specimen was almost identical with that

of the posterior appendicular skeleton in a normal embryo after four days' incubation. The principal difference lies in the fact that in the normal embryo the rudiments of a perichondrium are seen, whilst in the culture there was no sign of such a structure. Chondrogenesis in the explant also resembled the process in the normal embryonic limb in that chondrification was most advanced in what was originally the basal region of the limb-bud.

In cultures which had been growing in vitro for five or six days the cartilage was fairly well developed. The ectoderm had also undergone a certain amount of differentiation, and the superficial cells showed commencing keratinisation. At this stage, also, various areas of the ectoderm began to show a peculiar cystic structure, which occurred to some extent in almost all the limb-bud cultures, sometimes on a very extensive scale.

By the seventh day after explantation the histological structure of the cartilage showed a comparatively advanced stage of differentiation, and the cells were separated by broad partitions of matrix; the chondroblasts themselves, however, showed little structural change. The cartilage near the periphery of the nodules was of the epithelioid type but had a fairly distinct outline, and did not merge into a mass of undifferentiated mesenchyme as in the younger cultures.

After the seventh day of growth in vitro, the limb-buds underwent little further development, although they might continue to exist in a comparatively healthy condition for another fourteen days. Mitosis usually occurred to some extent, even in the oldest culture (Plate XXIII. Fig. 4), but as a rule diminished in amount after the tenth or eleventh day of growth. In the oldest cultures there was usually a greater quantity of matrix present, but otherwise the

histological structure of the cartilage showed little change and the large vacuolated cells characteristic of normal ossifying cartilage were not present. In the older specimens many of the nodules were partially or completely covered by a perichondrium (Plate XXIV. Fig. 1) composed entirely of fibrous tissue, which on the inside faded into the cartilage. Ossification was never observed. As the period of cultivation lengthened the amount of white fibrous tissue in the stroma increased and a close network of white fibres were formed (Plate XXIV. Fig. 2). Differentiated muscle was absent in all the explants. The keratinisation of the epidermis continued and the explants became invested by a stratified coat of horny material which became progressively thicker as the age of the culture advanced.

The shape and arrangement of the precartilaginous and cartilaginous nodules in different limb-bud cultures was by no means constant. In most cases, however, it was found to conform to one of three general types. The first and most common of these, which might be termed the "axial" type, was very well illustrated by the four days' old culture (Plate XXIII. Fig. 3) described above. In such cases the cartilage consisted of (1) a single rod, or (2) of two or three elongated cartilages set end to end, or (3) of an elongated cartilage at one end of which lay one or more irregular nodules.(Plate XXIV. Fig. 1. and Plate XXV. Fig. 1). In the second type of arrangement two rounded nodules of equal or unequal size lay side by side, and in the third type a single large nodule was present (Plate XXV. Fig.2). In a few of the explants the distribution of the nodules was quite irregular. In some of these specimens (e.g. Fig. 1, Plate XXIV) in which the axial configuration of the nodules was most marked it was found that the degree of differentiation of the cartilage was con-

siderably more advanced at one end of the explant than at the other.

The ectoderm in many of the cultures gave rise to cords and solid tubules running into the substance of the growth. Some of the cords became transformed into epithelial cell nests. These bodies were solid spheres of tissue composed of concentric layers of flattened epithelial cells. Several of the cultures were completely or partially devoid of an epidermal covering and contained instead a large ectodermal sac entirely enclosed by the connective tissue of the explant (Plate XXIII. Fig. 3).

The cultures grown in a thin medium (Plate XXVI. Figs. 1 and 2) differed from those grown in a firm clot (1), in showing a greater tendency to cyst-formation; (2) in the comparatively poor development of cartilage; (3) in the relatively slight keratinisation of the epithelium investing the older cultures, and (4) in that all the tissues were more liable to degeneration. Of the sixteen successful cultures, three only were solid, four were partly cystic and nine completely cystic.

The anatomical structure of the skeletal tissue formed in the explants as compared with that of the normal embryonic limb-skeleton. In the case of the cystic explants cultivated in medium 1, there was no resemblance in shape or mutual arrangement between the cartilage nodules of the explants and the developing cartilage bones of the normal embryonic limb. In six of the younger and nine of the older explants cultivated in media 2 and 3, however, the precartilage or cartilage - whichever happened to be present - showed an axial arrangement; in such cases it would seem that a morphological relationship did undoubtedly exist between the skeletal tissue of the explants and of the normal limb. As in the normal limb-rudiment

(Johnson, 1883) an elongated condensation of mesenchyme appeared in what was originally the proximal region of the explanted bud, and gradually extended towards the distal part; this mass usually became subdivided into two and sometimes three segments, segmentation beginning in the proximal part of the bud, as in the embryo. In the explants, however, the gross morphological development proceeded no further although the cartilage continued to differentiate histologically for some time longer. This arrest of the anatomical development of the skeleton in the explanted limb-bud may be explained as follows. In the cultures, growth and differentiation both proceeded at considerably less than the normal rate. During the first three days of life in vitro, growth and differentiation lagged behind the normal to about the same extent. Soon, however, it was found that as compared with normal development, the process of tissue differentiation rapidly outstripped that of growth; for instance, the degree of histological differentiation of the cartilage in an explant of eleven days' growth might approximately correspond with that of the cartilage in the limb of an embryo of five days' incubation, although the explant itself was only one-third the size of the five days' old limb. In the culture, therefore, the precartilaginous rudiment of the limb-skeleton, owing to its extremely slow growth, became completely chondrified long before it had had time to elongate and complete its segmentation in the normal way.

Those cases in which the skeletogenous tissue of the explanted limb-bud failed to develop along normal lines may probably be attributed to one or more of the following causes: (1) to the distortion of the original arrangement of the mesenchyme my internal cyst formation or by ingrowth of an epithelial sac; (2) to the explantation of an incomplete or damaged bud; (3) to placing the bud upside down

on the surface of the clot; (4) to the bud being closely embedded in the clot during some stage of development.

Chapter 2.

The anatomical development in vitro of the embryonic femur.

Introduction. Demuth found that cartilaginous segments of the embryonic limb-skeleton when cultivated *in vitro* increased in length and for a time maintained more or less their normal shape. Demuth gives no information as to whether progressive anatomical development took place in his cultures and his illustrations suggest that the general form of the rudiment was already differentiated at the time of explantation.

By the 7th day of incubation the gross anatomical structure of the long-bones is to a great extent already developed, so that in my experiments on the embryonic femur (Fell & Robison '29) the rudiments were taken for explantation at a very early stage (5½ days' incubation) when their characteristic form was not fully differentiated. This was done in order to determine whether the explants would merely enlarge during cultivation or whether they were also able to continue their general anatomical development. The results obtained in this investigation have since been repeated and with the aid of Dr. R.G.Canti, recorded by means of cinematography. They have also been confirmed and considerably extended by the interesting researches of Murray and Selby ('30) who used the method of chorio-allantoic grafting.

Material and methods. The material and culture technique employed have already been described in Part I, Chapter 2. As mentioned above, the anatomical development of the femur *in vitro* was studied in a series of explants obtained from 5½-day embryos. Many of the explants after being fixed in Zenker's fluid and washed in water and in alcoholic iodine in the usual way, were stained in dilute haema-

toxylin and were then dehydrated, cleared in xylene or clove oil and mounted whole in glass cells containing Canada balsam.

The culture which I prepared for cinematography consisted of a 4½-day embryonic femur grown by the watch-glass technique. In order to permit of illumination from below the watch-glass was not painted black and was placed over a circular hole cut in the centre of the carpet of cotton wool covering the floor of the Petri dish. The explanted femur was transferred to fresh medium every 48 hours, care being taken at each transference to replace the femur in the original position, i.e. with the posterior surface uppermost. During photography the culture was kept in a thermo-regulated box at 38° C. and photographs were taken automatically at intervals over a period of 14 days. Growth as shown in the film is enormously accelerated and 14 days' development is seen in less than five minutes. The photography was done entirely by Dr. R. G. Canti on apparatus of his own design and construction and I am indebted to him for the photographs shown in Plate XXVIII, which are taken from his cinema film.

Results. The gross anatomy of the explants was studied partly by observation of the living cultures and partly by means of the series of whole mounts.

During cultivation in vitro the majority of the isolated femora which, as stated above, showed an average increase in length of 226%. developed their characteristic shape to a remarkable extent. (Plate XXVII, cf. Figs 1 & 6 and Plate XXVIII, cf. Figs. 1 & 8).

When first explanted (0-day) the 5½-day femur of the normal embryo (Plate XXVII. Fig. 1) was a short thick rod showing at the distal end two rounded processes representing the developing condyles and at the proximal end two rather smaller knobs, one of which, pro-

jecting somewhat at right angles to the shaft, was the head and the other, more terminally placed, was the early trochanter.

After 3 days' cultivation (Plate XXVII. Fig. 2) the condyles had assumed a more definite shape whilst the head and trochanter were also more pronounced. The diameter at the middle of the shaft was only slightly larger than at the time of explantation but had considerably increased in the two epiphysial regions.

By about the 15th day of growth in vitro (Plate XXVII. Fig.4) the characteristic pulley-like form of the condyles was very distinct. The head was also fairly well developed but the trochanter was usually less marked. The condyle, head and trochanter continued to enlarge during subsequent cultivation but showed no further changes in shape (Plate XXVII. Figs. 5 & 6).

During cultivation in vitro the explanted femora did not increase in width to the same extent in all regions (Plate XXVII. Figs. 2 & 6). After the third day in vitro the diameter of the femur at the centre of the shaft increased very little and in 27-day explants was only about 25% larger than at the time of explantation. On the other hand, the diameter of the proximal epiphysis in the same explants usually enlarged by at least 130% and the distal (condylar) epiphysis by at least 180% of their original size. This difference between the relative growth rates of the epiphysial and diaphysial diameters was greater than in normal development, although in vivo also the percentage increase in diameter is much higher in the epiphysis than in the middle of the shaft. The relatively greater size of the epiphyses in the explanted femora was probably due partly to the limited periosteal ossification and partly to the absence of a marrow cavity. Ossification in vivo produces a mass of trabecular bone in the interstices of the network of periosteal

blood vessels, whilst the cartilage in the shaft is removed and replaced by the expanding marrow cavity. In vitro, on the other hand, ossification gave rise merely to a single compact layer of periosteal bone and the lateral expansion of the shaft could only have taken place by the growth of the highly differentiated, large-celled cartilage of which it was composed. It was also probable that the small-celled cartilage of the epiphysis actually grew more readily in vitro than the large-celled cartilage of the shaft; this was indicated by the fact that one of the commonest forms of abnormality encountered among the explanted femora was an attenuation of the shaft correlated with the presence of relatively large, mushroom-like epiphyses (Plate XI. Fig. 4).

A minority of the explanted femora showed marked abnormalities, of which the commonest form has been described above. Twisting and distortion of the shaft sometimes occurred which was due to two main causes. The first of these was the outgrowth of the epiphysial perichondrium into the fibrin clot; in some cases this outgrowth was so prolific as to anchor each epiphysis firmly to the medium so that increase in the length of the shaft could only take place by bending. The second cause of distortion was the formation around the shaft of extremely tough bands of fibrous tissue which, extending between and attached to the epiphyses, prevented the femur from elongating along its normal axis. As previously stated, the second factor could be largely eliminated by removing as much of the zone of outgrowth as possible when transferring the explant to fresh medium, and thus preventing the formation of an increasingly large mass of organised connective tissue around the femur.

In the cinema film (Plate XXVIII) the anatomical development of the femur rudiment which was rather less differentiated than the

majority of those in the series described above (Plate XXVIII, Fig.1), is very beautifully demonstrated. As the femur rapidly elongates the small, knob-like rudiments bulge up on either side of the distal extremity forming between them a well marked intercondylar notch (Plate XXVIII. Fig. 6) - a structure which is indistinguishable at the time of explantation, whilst at the proximal end the head and trochanter are seen gradually pushing outwards. The relative proportions of the different parts of the femur alter during cultivation as described in the case of the previous series. The general form of the explant does not change very markedly after about the 4th day of cultivation although enlargement continues until the end of the culture period. Growth is seen to be most active during the earlier part of the culture period, and to be greatly diminished by the 14th day. At about the 7th day (Plate XXVIII. Fig. 4) an opacity appears on the surface of the middle region of the diaphysis and probably represents the onset of ossification. The film also shows very strikingly the partial liquefaction of the clotted medium by the explant and the profuse outward migration of the connective tissue cells surrounding the cartilage (Plate XXVIII. Fig. 8).

Summary of Results (Part III)

1. Undifferentiated limb-buds from embryos of 72 hours' incubation when cultivated *in vitro* frequently show the early stages in the development of a normal limb-skeleton.
2. Femoral rudiments isolated from 4½-5½ day embryos usually develop a remarkably normal form during cultivation *in vitro*.

DISCUSSION

The factor controlling chondrogenesis. The problem of the relative importance of intrinsic and extrinsic factors in the primary development of the limb-skeleton has been admirably discussed by Murray and Selby ('30). These authors state that the question to be considered "is whether the limb-bud at a stage prior to the origin of chondrogenesis contains cells which are specifically determined for chondrification, or whether those cells which later take part in the formation of the cartilaginous skeleton are indifferent, so that the nature of their differentiation depends wholly upon conditions extrinsic to them".

The fact that undifferentiated limb-bud mesenchyme chondrifies readily in vitro enables us to exclude several possible factors which might otherwise be regarded as partly or wholly responsible for cartilage-formation in the embryo. It is obvious from the writer's tissue culture experiments that chondrogenesis is not due to the influence of a circulation or nervous system nor, since only a few scattered myoblasts developed in the limb-bud cultures, is it due to mechanical conditions resulting from simultaneous myogenesis in the surrounding mesoderm.

It remains to consider whether there are any other extrinsic factors originating within the normal limb-bud which might also operate under the conditions of growth in vitro. In this connection Murray and Selby point out that "the cells which give origin to the membranous and cartilaginous skeleton occupy a central position around the axis of the bud. Whatever the nature of the extrinsic factors [if any] may be, it is evident that they are associated with this position, for otherwise chondrification would occur in the

peripheral as well as the central cells". If we are to assume that the environmental conditions (mechanical or otherwise) of the cells occupying the axial region of the normal limb-bud, induce chondrification, we must also be able to conceive of the existence of corresponding conditions in the chondrifying explants. In the case of the tube cultures of entire limb-buds undergoing 'organotypic' growth this presents no difficulty but at first sight it is not easy to see how internal environmental conditions equivalent to those present in the interior of the normal limb-rudiment could possibly occur in the coverslip cultures of limb-buds which, as described above, chondrified whilst in course of expansion into thin sheets. When first explanted, however, the limb-buds each consisted of a fairly compact mass which did not spread out very much during the first 48 hours' cultivation and by the end of this time the precartilaginous condensations had usually appeared. The chondrogenic tissue might thus have been determined during the first two days <u>in vitro</u> when the environmental conditions of the cells in certain areas might be much the same as in the axial region of the normal limb-bud. This, however, can hardly apply in the case of the teased limb-buds grown in a fluid medium; since in the same culture a relatively large and apparently healthy explant might show no trace of cartilage formation whilst a minute explant sometimes chondrified readily. On the assumption that chondrogenesis in normal development is induced by extrinsic factors associated with an axial position of the cells, we should have expected that all the large, but none of the minute explants would form cartilage. It therefore seems clear that the chondrogenic cells must be already determined, although histologically indistinguishable, in the three-day limb-bud and are self-differentiating from this stage. If this conclusion is true, the failure of some

of the explants to form cartilage would be due to the fact that they did not contain chondrogenic cells whilst those minute explants which chondrified, did so because they happened to be derived from a chondrogenic area of the original bud.

It is very possible that at an earlier stage of development these chondrogenic areas of the mesoderm may be determined by extrinsic factors. In adult life, cartilage can apparently differentiate as a result of environmental influence, as indicated by the common occurrence of cartilage in bone-fractures where there is movement between the fragments, but how this differentiation is induced is not yet understood.

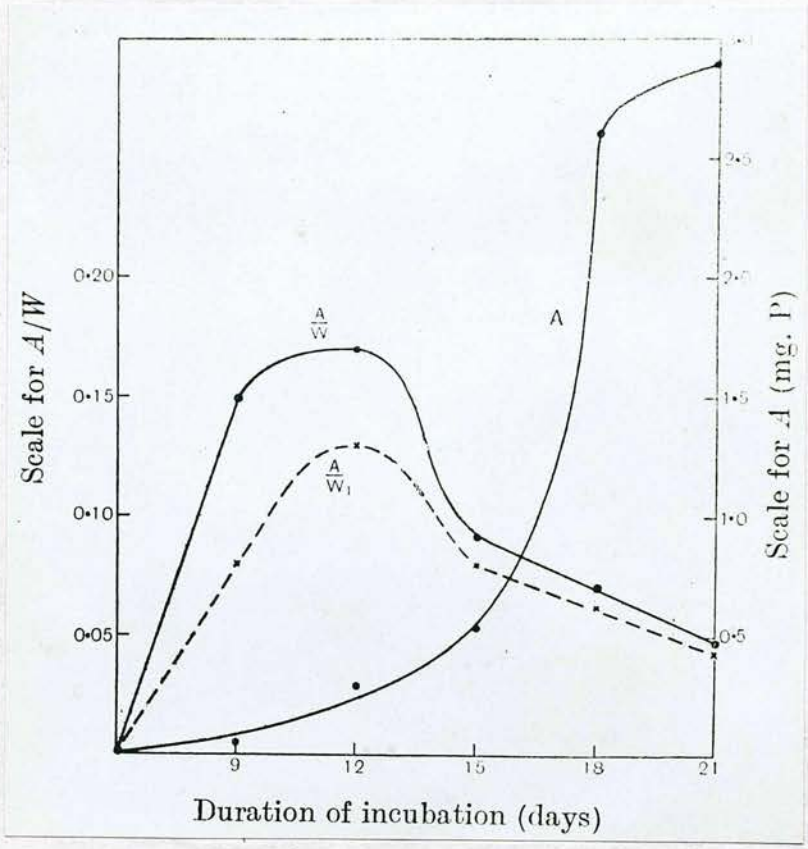
The significance of the hypertrophic chondroblasts. The most striking histological characteristic of ossifying cartilage is the remarkable hypertrophy and vacuolation of the chondroblasts. The question naturally arises as to whether this histological change has any general physiological significance and also whether it is to be regarded as a degenerative process or as a form of cellular differentiation.

The work of Robison sheds light on the first part of this problem. Robison ('23) demonstrated the presence of a phosphatase in mammalian bone and ossifying cartilage. He and his co-workers (Kay & Robison '24, Martland & Robison '26) adduced evidence to show that this enzyme is an active agent in ossification, effecting the hydrolysis of certain phosphoric esters supplied by the blood stream and thereby raising the concentration of inorganic phosphate in the tissue fluid so that solid calcium phosphate is deposited. It was found (Robison & Soames '24, Martland & Robison '24, Robison & Soames '28) that when severely rachitic bones were immersed in solutions of calcium hexosemonophosphate or calcium glycerophosphate

deposition of calcium phosphate took place in the periosteum and also in the matrix of the proliferating and hypertrophic cartilage; on the other hand, no deposit was observed in the small-celled cartilage of the epiphysis. From these results the authors concluded that the phosphatase is probably secreted both by the osteoblasts and by the hypertrophic chondroblasts but not by the small cartilage cells.

An investigation of the phosphatase activity of various forms of avian skeletal tissue both from the normal embryo and from cultures in vitro seemed likely to prove interesting and I therefore provided normal and explanted material which Dr. Robison studied from this point of view.

Dr. Robison's first experiments were made with the femur grown in vivo and in vitro (Fell & Robison '30). The phosphatase activity was measured by the amount of glycerophosphoric ester hydrolysed in 24 hours at 37°C. and pH 8.4, the results being expressed in mg. phosphorus. The total hydrolytic activity (A) of the normal femur at different stages of development was plotted against the age of the embryos from which the specimens were taken and an S-shaped curve was obtained rising from zero in the case of the 6-day femur to a value of 2.90 in the 21-day bone. The ratio of phosphatase activity per unit dry weight of the extracted femora (A/W) rose from zero at the 6th day to a maximum of 0.17 at the twelfth day, after which the value declined.



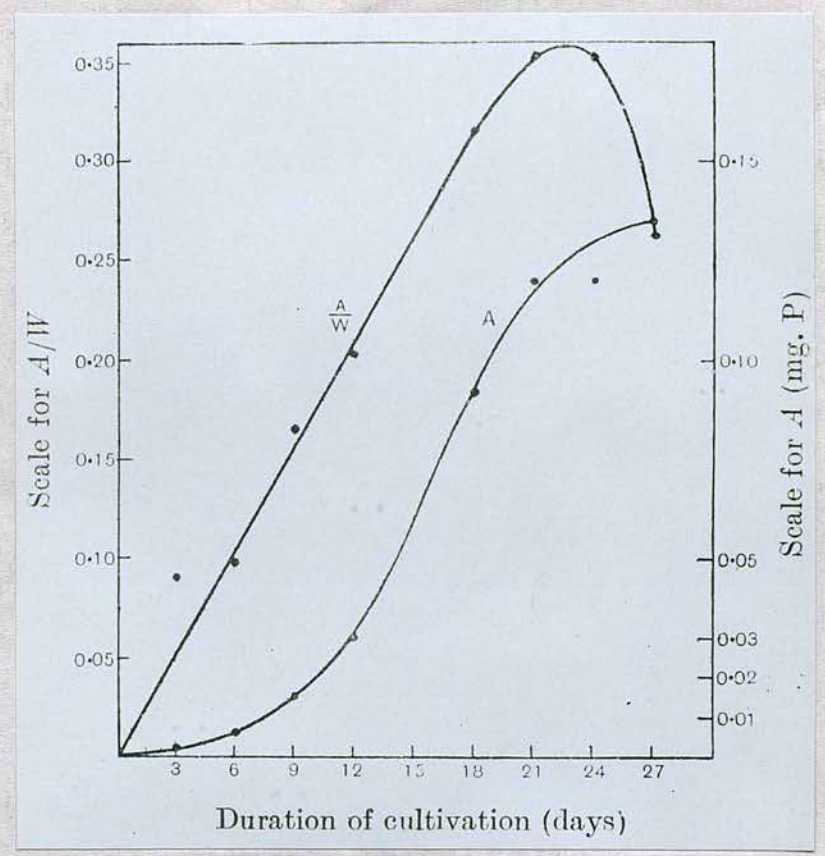
Text-fig. 5.

Production of phosphatase in the femur of the embryonic fowl during normal development in vivo.

A - Phosphatase per femur.
A/W - Phosphatase per mg. dry weight of femur (weighed after extraction).
A/W_1 - Phosphatase per mg. dry weight of femur (calculated on dry weight of corresponding unextracted femur).

The phosphatase activity of the explanted femora at different stages of growth gave similar results. The total phosphatase activity plotted against the age of the cultures produced an S-shaped curve rising from zero in the case of the normal 6-day femora tested before explantation, to a value of 0.135 at the 27th day of cultivation. The curve of phosphatase activity per unit dry weight of the extracted explants showed a fairly steady increase from zero at the beginning

of cultivation up to a maximum of 0.35 at the 21st day *in vitro*. The value then decreased and this diminution corresponded with the appearance of necrotic areas in the cartilage.



Text-fig. 6.

Production of phosphatase during the development *in vitro* of femora from 6-day fowl embryos.

A - Phosphatase per femur; given as the amount of hydrolysis (mg.P) of sodium glycerophosphate in 24 hours at 37°C. and pH 8.5.

A/W - Phosphatase per mg. dry weight of femur (weighed after extraction).

Dr. Robison also investigated (Fell & Robison '30) the phosphatase activity *in vivo* and *in vitro* of the non-ossifying part of

Meckel's cartilage and, as controls, of the adjacent palato-quadrate which, as previously mentioned, is an ordinary ossifying cartilage containing hypertrophic chondroblasts. It was found that in normal development the non-ossifying Meckel's cartilage which, as described in Part I, contains no areas of hypertrophic cells, gave a negative result at every stage of growth from the 6th day of incubation up to the 13th day after hatching, whilst all the palato-quadrates tested after the 7th day of incubation gave a positive value which in the later stages of embryonic life was comparatively high. Similar results were obtained with the explants. At the time of explantation i.e. at the 6th day of embryonic development, both Meckel's cartilages and palato-quadrates gave a negative result. After a period of 20 days' cultivation in vitro, however, Dr. Robison found that whereas the unossified Meckel's cartilages still showed no trace of phosphatase activity, the palato-quadrates in which, as recorded above, an area of hypertrophic cells had appeared during growth in vitro, gave a value for phosphatase activity per unit dry weight which was even higher than the maximum attained in normal development. The higher value in the explants was probably due to the presence in the normal palato-quadrate of solid calcium salts and of marrow which increased the dry weight without a corresponding increase in phosphatase.

Dr. Robison also tested for phosphatase (Fell & Robison '29) cultures derived from undifferentiated mesoderm, which as already described, with very rare exceptions, gave rise to cartilage of the small-celled, non-ossifying type. No phosphatase was formed by such material.

Dr. Robison's results have made it clear that the presence of hypertrophic chondroblasts both in normal and explanted cartilage

is always associated with the secretion of phosphatase and that, conversely, phosphatase is never produced by small-celled, non-ossifying cartilage. How much of the phosphatase present in ossifying cartilage is formed by the hypertrophic chondroblasts and how much by the osteoblasts it is impossible to say, but the very high concentration of phosphatase in the explanted palato-quadrates which, as described in Part I, showed only very slight traces of ossification, make it probable that the hypertrophic cells secrete phosphatase actively.

It is sometimes thought that chondroblastic hypertrophy and the formation of phosphatase are degenerative phenomena and I myself in earlier work (Fell '25) supported this view. The hypertrophic cartilage cells in the normal long bones, at least in the middle region of the shaft, eventually assume a shrunken and distorted appearance which is undoubtedly degenerative, and it was therefore thought that the preceding swelling and vacuolation were merely earlier stages in the same regressive process.

My experimental results, however, have compelled me to discard this interpretation of chondroblastic hypertrophy. It seems impossible to explain the phenomenon as merely a form of degeneration, in view of the fact that Meckel's rod and the palato-quadrate, although showing no histological difference when first explanted, invariably developed in the one case, small-celled cartilage only, and in the other a large area of hypertrophic cells precisely as in normal development, although both sets of rudiments were isolated from the body and growing <u>in vitro</u> under identically the same environmental conditions. If the hypertrophy were a degenerative change, we should expect it to occur in unhealthy cultures of Meckel's cartilage. This

was never observed, and in those explants where cell degeneration occurred, it always took the form of shrinkage.

It would seem, therefore, that the appearance of hypertrophic cartilage represents a true tissue differentiation and that, as in most forms of differentiation, the histological destiny of the tissue is determined before its characteristic histological structure becomes distinguishable.

Although the future histological structure of the non-ossifying part of Meckel's rod and of a future cartilage bone such as the palato-quadrate or femur is apparently firmly determined by the 6th day of embryonic life, yet at an earlier stage of development the ultimate structure of the chondrogenic tissue of the limb-rudiment is determined less definitely. As described in Parts II and III, the undifferentiated 3-day limb-bud when cultivated in vitro, with rare exceptions, gave rise to nodules of small-celled, non-ossifying cartilage. On the other hand, Murray and Selby ('30) state that the early (2-day) embryonic limb-rudiment, when removed from the body and grafted on the chorio-allantoic membranes, may form 'ossifying' cartilage with typical hypertrophic cells, showing clearly that the isolated rudiment, even at this early stage, has the potentialities for its characteristic histological differentiation when growing under suitable environmental conditions. The histological similarity between the non-ossifying part of Meckel's rod and the non-ossifying cartilage formed by the explanted 3-day limb-bud does not necessarily mean that the two types of cartilage are identical, and it is probable that the same histological result is differently produced in the two cases. The factors responsible for determining whether chondrogenic tissue shall develop into ossifying cartilage with the characteristic hypertrophic cells are at present quite obscure.

If the development of the hypertrophic cartilage cells represents a specific differentiation, the next question to be considered is what part these cells play in ossification. I thought at first that they might be concerned in some way with the earliest stages of bone-formation, but since the isolated 6-day embryonic periosteum readily forms bone in vitro when deprived of all association with the cartilage, this is obviously not the case. It is conceivable that as the osteoblastic layer of the periosteal rudiment develops pari passu with the hypertrophic cartilage, the latter may induce the development of the former. This, however, is pure speculation and at present there is no evidence as to the function of the hypertrophic chondroblasts in osteogenesis.

The nature and function of the osteoblast. Many views have been expressed as to the nature and function of the osteoblast. According to the classical theory osteoblasts are healthy cells constituting a specific histological type whose primary function is the formation of bone. Some workers, on the other hand, regard bone-formation as the result of a degeneration of the osteoblasts, whilst Leriche and Policard ('28) maintain that the osteoblasts are merely altered fibroblasts and that their formation is only the secondary result of ossification. These authors state that "the creation of an ossifiable medium and preosseous condensation seems to be the consequence of circulatory changes" and they consider that "the appearance of osteoblastic deposits expresses, in short, reactionary attempts of the cells against the preosseous transformation which congeals their medium". Leriche and Policard even affirm "that the cell is injurious to bone since, on resuming activity, this cell may cause a retrogression of the bone". Greig ('31) largely endorses the conclusions of Leriche and Policard, and believes that the osteoblasts are identical with

fibroblasts and are not essential to bone-formation, although their disintegration products may in some way assist ossification.

The results recorded in this thesis strongly support the orthodox view namely, that the osteoblasts in normal osteogenesis are healthy cells whose specific function is bone formation.

In the case of adult bone, the extreme hardness of the intercellular material renders it impossible to obtain an accurate cytological picture of the osteoblasts and bone cells and it is therefore very difficult to determine whether these elements are healthy or not. The embryonic skeleton of the fowl, on the other hand, can be studied by adequate cytological methods and in this material there is no evidence that the osteoblasts are in any way degenerate. They may undergo mitosis even when enclosed by early osteoid matrix, the nuclei show no regressive changes and in well-fixed material the filamentous mitochondria of the osteoblasts and young bone cells display no trace of those granular changes which are usually one of the first signs of cell degeneration. Moreover, as described in Part II, unhealthy cultures of endosteum formed abnormal osteoid tissue only, whilst active, healthy growths gave rise to true calcified bone; this would be difficult to explain on the assumption that cell degeneration promotes ossification.

That the osteoblasts are specific bone-forming elements and are not physiologically identical with fibroblasts, is indicated by the fact that bone only differentiated <u>in vitro</u> when osteoblasts or presumptive osteogenic cells were present in the original explants. Ordinary connective tissue or, as described above, the fibroblastic layer of the periosteum does not form bone during cultivation, so that any possibility of the ossification of the explanted osteoblastic tissue being caused by the environmental or mechanical conditions of

growth *in vitro* is thus eliminated.

The fact that very active osteoblastic proliferation tends to be associated with relatively slight ossification or even with bone resorption, is no evidence that the osteoblasts are antagonistic to ossification as claimed by Leriche and Policard. It is well known that *in vitro* very active proliferation in any tissue is usually correlated with unorganised cell migration and absence of histological structure - a general principle which is illustrated by cultures of osteogenic tissue. As previously stated, in cultures of 6-day embryonic periosteum, peripheral ossification centres always appeared later than those in the interior of the explant; this was probably due to the fact that the cells composing the original margin of the culture, being in a state of active proliferation and unorganised growth, were unable to differentiate until, after successive subcultivations, they had ceased to form part of the zone of outgrowth and become incorporated in the inner, organised region. Similarly in the endosteal cultures, ossification always began and was most advanced in the middle of the culture, where mitosis and cell migration were minimal.

If bone is formed by osteoblasts and the osteoblasts represent a specific histologiwal type, we are faced with the difficult problem of determining what factors are responsible for the differentiation of these cells. The innumerable examples of the occurrence of heteroplastic bone-formation in adult life, show clearly that cells in ordinary connective tissue, under certain conditions, can be transformed into osteoblasts. We have yet to discover the nature of these conditions. It is found that many cases at least, of heteroplastic ossification are associated with a previous deposition of calcium salts in the tissue and Greig adduces considerable evidence in support

of his view that a local calcific excess is a fundamental factor in inducing osteogenesis. This, however, can hardly apply in the case of the ossifying cultures of mandibular mesoderm or of 6-day embryonic periosteum which had never been in contact with calcified material either before or during cultivation (the limb-cartilage of 6-day fowl embryos is not calcified - Niven, unpublished observation). Until further information has been obtained it is impossible to formulate any satisfactory theory concerning the causes of osteoblastic differentiation, as at present the ascertained facts cannot be correlated.

The exact nature of the osteoblastic influence in bone-development is also very obscure. Robison has shown (Robison & Fell '30) that the osteoblasts of avian membrane bone, whether growing in vivo or in vitro, actively secrete a phosphatase but this cannot be their only function in osteogenesis since the osteoid matrix (collagenous fibres and interfibrillar cement) is formed before calcium phosphate is deposited. The mechanism by which the osteoblasts produce this osteoid matrix is not known.

The anatomical development in vitro of the embryonic limb-skeleton. The results recorded in Part III show that the embryonic limb-skeleton of the fowl possesses a remarkable capacity for anatomical self-differentiation. As described above, the undifferentiated three-day limb-bud may exhibit the earlier stages in the formation of a normal skeleton, when growing apart from the body and cut off from a blood and nerve-supply, but the development in vitro of anatomical form is still more strikingly demonstrated by the explanted femur. Although deprived not only of a vascular system and nervous connections, but also of normally adjacent skeletal rudiments and, with the exception of a few isolated muscle fibres, of association with the limb musculature, the

femur rudiment is able to continue its anatomical development *in vitro* along the same general lines as in the normal limb. Much of the superficial sculpturing of the normal femur is undoubtedly due to mechanical factors such as muscular contraction and pressure, and is not seen in the explants, but the differentiation of the general shape of the cartilaginous rudiment is clearly shown to be independent of extrinsic mechanical influences, a conclusion which has also been expressed by Murray and Selby ('30).

Although the form of the cartilaginous femur is largely self-differentiating, the architecture of the bone by which it is normally replaced is probably controlled entirely by extrinsic factors. Both *in vivo* and *in vitro* the bone is deposited, as in a mould, between the fibrous layer of the periosteum and the cartilaginous rudiment but whereas in the normal limb, the bone soon becomes trabecular owing to the ingrowth of blood vessels into the osteoblastic layer of the periosteum, in the cultures it persists as a single compact layer. As pointed out in Part I, ossification appears always to follow the plane of least resistance.

I should like to acknowledge my deep indebtedness to the late Mr. T.S.P.Strangeways who taught me the tissue culture technique and whose inspiration initiated the investigations described in these pages.

I also wish to express my thanks to Dr. R. Robison, F.R.S., Miss J. S. F. Niven and Dr. F.G.Spear for valuable constructive criticism during the writing of this thesis.

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P L A T E S

All the drawings were made with the aid of a camera lucida or a projection apparatus. The photomicrographs are by Mr. V. C. Norfield, Strangeways Research Laboratory and Mr. H. P. Hudson, Department of Pathology, Cambridge University.

Abbreviations

am. amnion.
am.c. amoeboid cells.
art.c. articular cartilage.

b. bone.
ba. base of limb-bud.
b.c. bone cell.
b.m. bone matrix.
b.v. blood vessel.

c. cartilage.
co. condyle.
ca. calcification.
ca.a. calcified area.
cart. cartilage.
c.b. cell boundary.
cent. centrosome.
ch. chondroblast.
ck. cork.
c.m. culture medium.
c.o.cyl. central osseous cylinder.
c.t. connective tissue.
cy. cyst.
cy.pr. cytoplasmic process.

d.c. degenerate cartilage.

eb. endochondral bone.
ec. ectoderm.
ect. ectoderm.
ep. epiphysis.
ep.t. epithelial tube.

f.c. flattened chondroblasts.
fi. fibroblasts.
fib. fibrous layer.
f.l. fibrous layer.
f.t. fibrous tissue.

g. Golgi apparatus.

h. head.
Ha.s. Haversian spaces.
h.c. hypertrophied chondroblasts.
hyp.c. hypertrophied cells.

i.c.t. invading connective tissue cells.

k. keratin.

m. marrow.
ma. matrix.
m.b. membrane bone.
m.c. Meckel's cartilage.
m.c.d. marrow cavities of the diaphysis.
m.c.e. marrow cavities of the epiphysis.
mes. mesenchyme.
mi. mitosis.
mr. cells of the marrow reticulum.
my. myoblasts.
mch. mitochondria.
n. nucleus.
n.b. new bone.

oc. osteoclast.
o.c. ossification centre.
o.c.t. outwandering connective tissue cells.
o.f. osteogenic fibres.
o.l. osteoblastic layer.
o.m. osteoid matrix.
or.b. original bone.
ost. osteoblasts.
os.t. osteoid tissue.
o.t. osteoid tissue.

p. periosteum.
p.b. periosteal bone.
pc. precartilage.
pch. perichondrium.
p.q. palato-quadrate.

r. terminal ridge of ectoderm.
rt. ring-like thickenings.

st.mat. strands of matrix.
s.c. small chondroblasts.

t. trochanter.

z.f.c. zone of flattened cells.
z.h.c. zone of hypertrophied cells.
z.o. zone of outgrowth.

Plate I.

Fig. 1. Mesenchyme cells from the posterior limb-bud of an embryo of 52 hours. Note the complete structural independance of each cell. (iron haematoxylin).

Fig. 2. Early cartilage cells from the middle region of the skeletal rudiment in the posterior limb of a 4½-day embryo. The cells are elongated at right angles to the axis of the limb (indicated by arrow). (iron haematoxylin).

Fig. 3. Section of tibia from a 5½-day embryo (this specimen is abnormally well developed for its age). The first indication of the three zones of chondroblasts can be distinguished and a layer of early osteoid tissue has been deposited over a large part of the diaphysial region. The perichondrium covering the future diaphysis is differentiated into an inner osteoblastic and an outer fibroblastic coat. The epiphysial region is still continuous with the surrounding undifferentiated mesoderm. (iron haematoxylin).

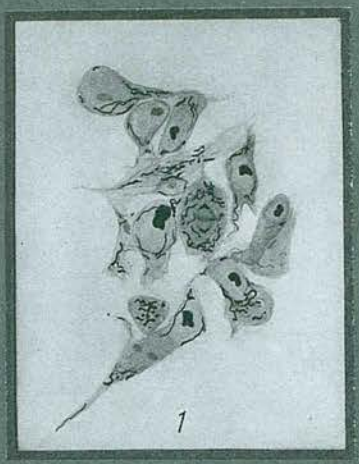
Fig. 4. Hypertrophied cartilage cells from the middle region of the radius of a 6½-day embryo (cf. Fig. 2, same scale). Note the vacuolated cytoplasm, large clear nucleus, vesicular centrosome and long, stout mitochondria. (iron haematoxylin).

Fig. 5. Chondroblasts from the zone of flattened cells in the same radius, drawn to the same scale as Fig. 2. (iron haematoxylin).

Fig. 6. Rounded cells from the epiphysial region of the same radius drawn to the same scale as Fig. 2. (iron haematoxylin).

Fig. 7. Epiphysial chondroblasts from one of the phalanges of an 11-day embryo showing Golgi apparatus (fixed by Da Fano's method, toned and faintly stained with methylene blue.

PLATE I.



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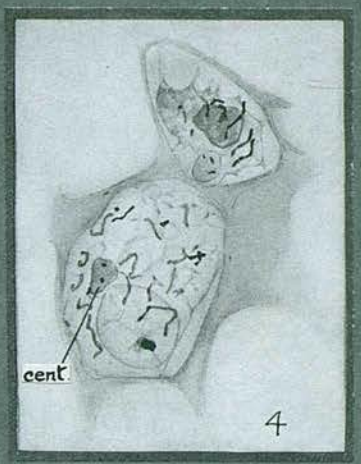

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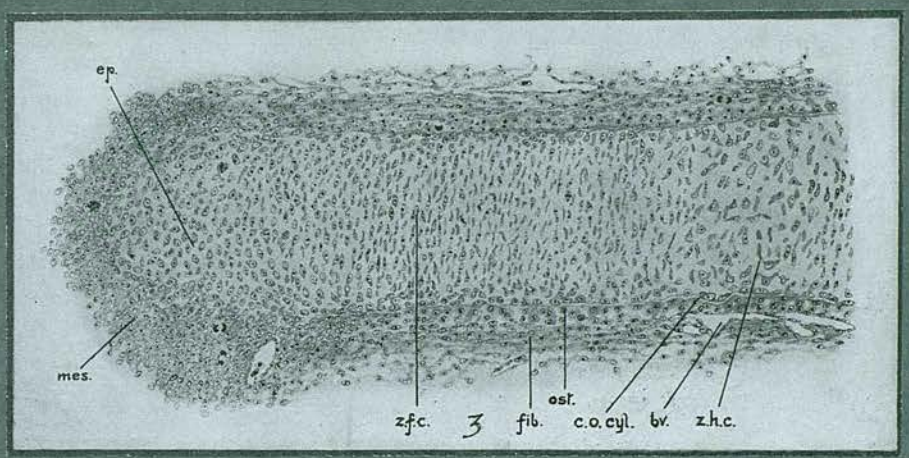

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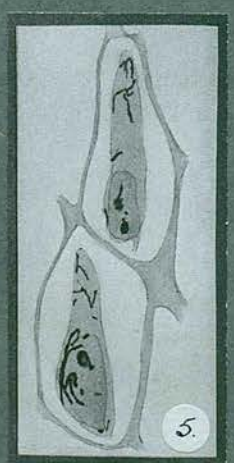

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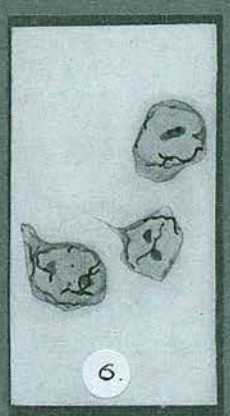

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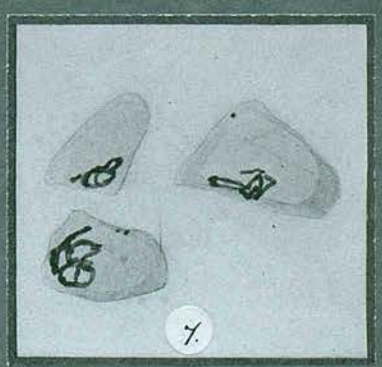

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Plate II.

Fig. 1. Hypertrophied cartilage cells from the middle region of the femur of an 8½-day embryo showing degenerative changes (shrinkage of cytoplasm and nucleus, enlargement of centrosome, and reduction in number of mitochondria). (iron haematoxylin).

Fig. 2. Hypertrophied cells in the tibia of a 14-day embryo showing the dense Golgi apparatus. In this specimen (fixed by Da Fano's method and stained with methylene blue) the matrix appears as a close spongework which is well seen in the capsule cut tangentially. Note the strand of matrix passing from the surface of the chondroblasts to the intercellular partitions.

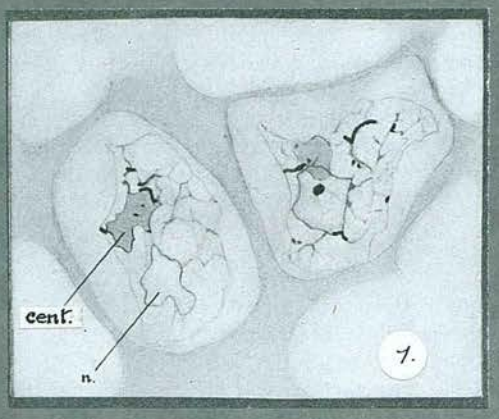
Fig. 3. Young cartilage from a specimen fixed by Da Fano's method and stained with methylene blue. Note the Golgi apparatus in the cells and the loose spongework of early intercellular material.

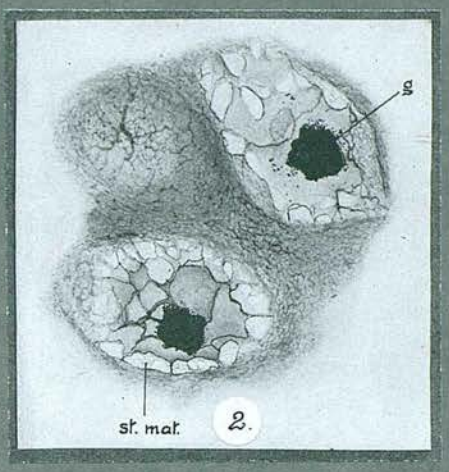
Fig. 4. Early bone-formation in the middle region of a posterior phalanx from a 9½-day embryo. Delicate lamellae of osteoid fibres are seen among the osteoblasts. (Mallory's triple stain).

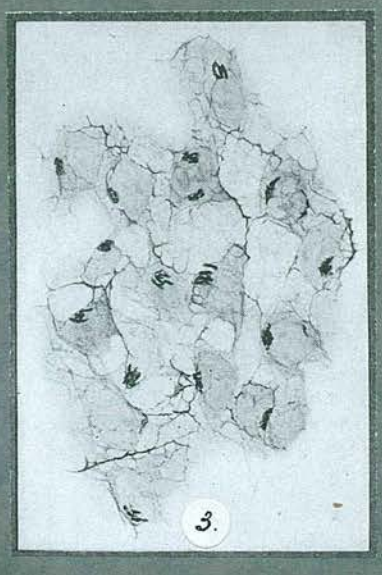
Fig. 5. Osteoblasts applied to a trabeculum of bone. (iron haematoxylin).

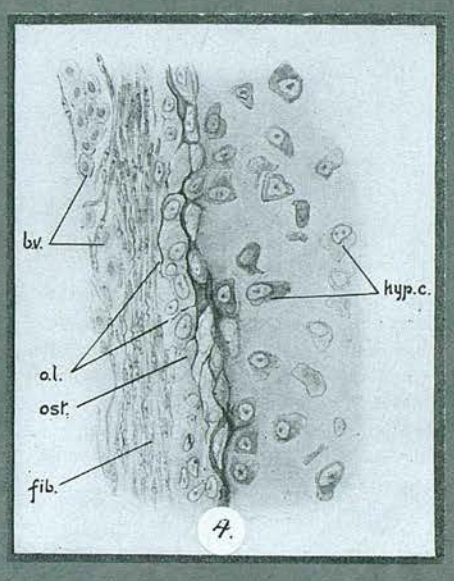
Fig. 6. Young bone cells from the fibula of an 8½-day embryo, showing centrosome, mitochondria and cytoplasmic processes penetrating the ground substance of the bone. (iron haematoxylin).

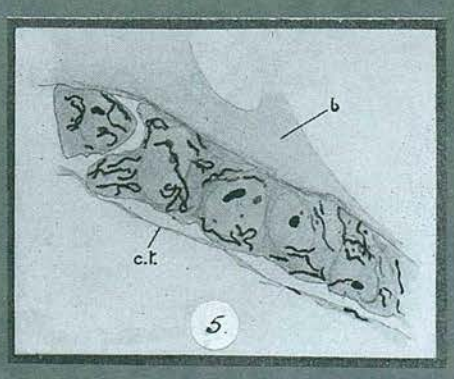
PLATE II.











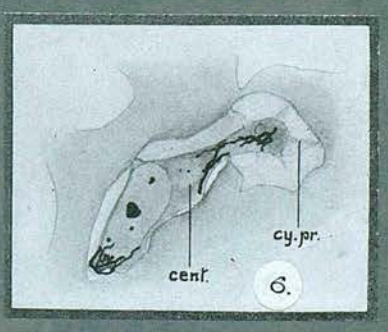


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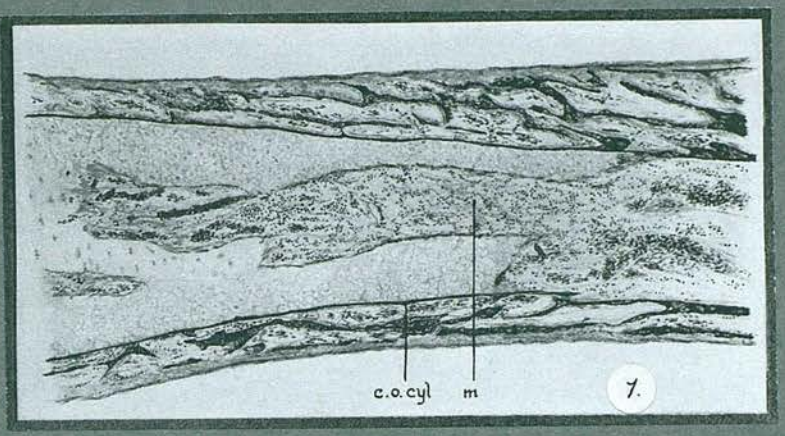
Plate III.

Fig. 1. Cartilage resorption in the tibia of a 14-day embryo. The matrix enclosed by the periosteal bone is being excavated without simultaneous endochondral ossification as in mammals. (Mallory's triple stain).

Fig. 2. Zone of degeneration at the erosion surface of cartilage in the course of resorption, from the femur of a 14-day embryo. To the right are seen disintegrating chondroblasts and degenerate cells of the marrow reticulum and to the left the normal cells of the invading marrow. (iron haematoxylin).

PLATE III.



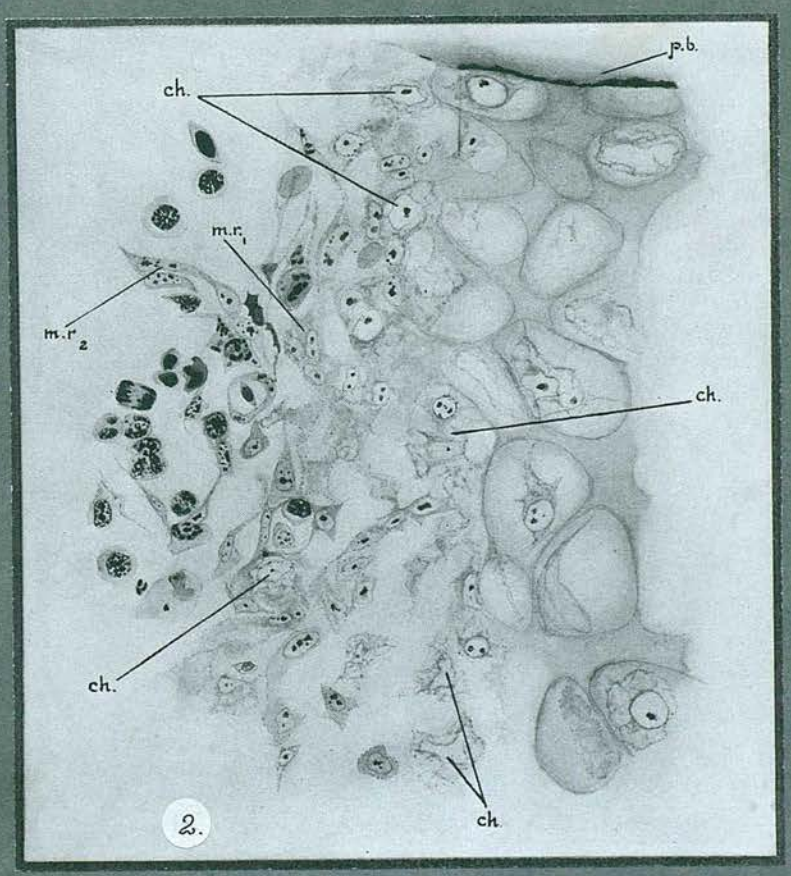


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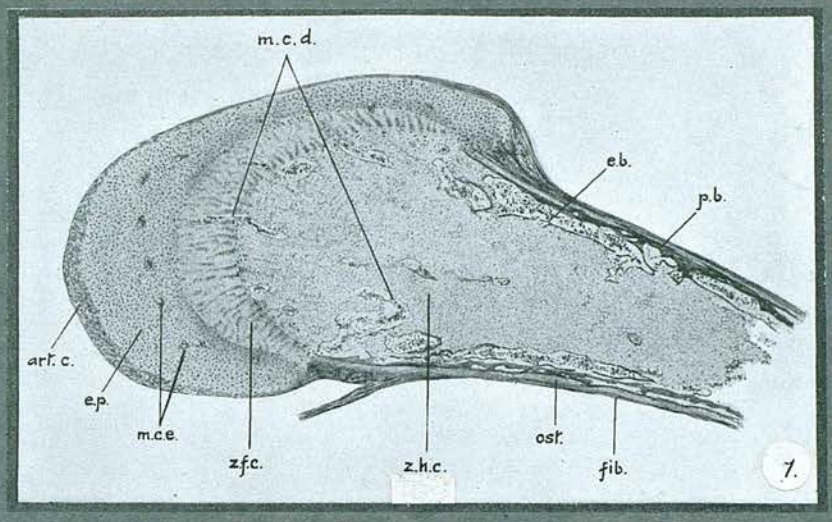
Plate IV.

Fig. 1. Section through the condyle and distal end of the femur of a day-old chick, showing the articular cartilage, epiphysis, intermediate cartilage containing flattened cells and diaphysial cartilage containing hypertrophic cells. Note also the diaphysial and epiphysial marrow cavities and the early endochondral ossification. (Mallory's triple stain).

Fig. 2. Osteoclast from the tarso-metatarsus of a 10½-day embryo showing the swollen and distorted mitochondria. The cell boundaries are still distinguishable in places. (iron haematoxylin).

PLATE IV.



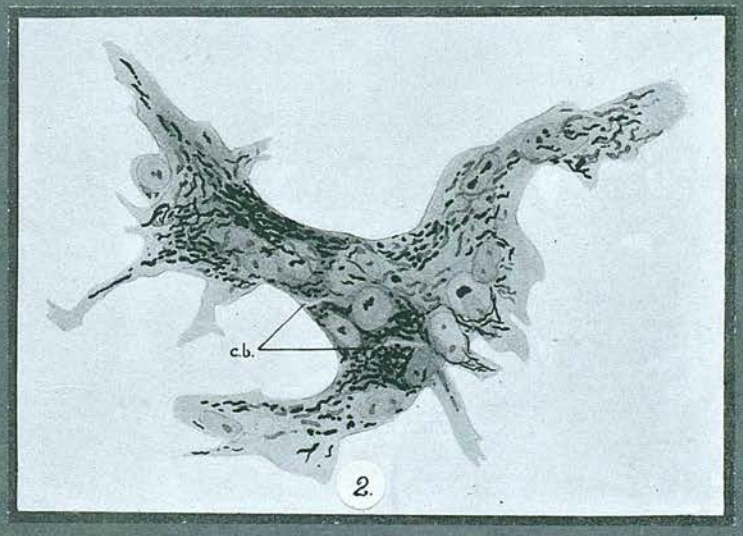


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Plate V.

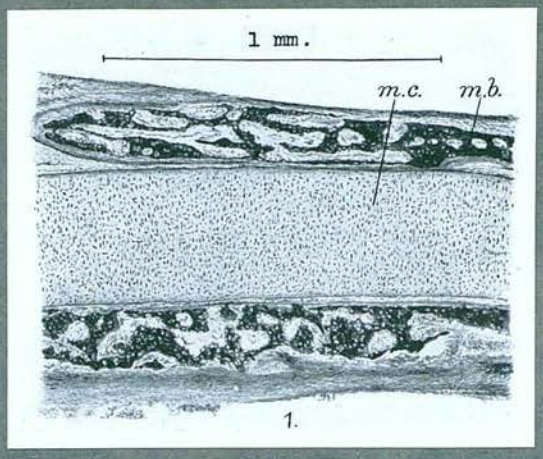
Fig. 1. Longitudinal section through the mandibular skeleton of a 15-day embryo. Note the rod of unossified cartilage enclosed by a sheath of membrane bone. (Mallory's triple stain).

Fig. 2. The articulare from a two-day chick showing ossifying cartilage. (photomicrograph). (Mallory's triple stain).

Fig. 3. The distal part of Meckel's cartilage from the same chick, showing the absence of ossification in the cartilage (photomicrograph). (Mallory's triple stain).

Fig. 4. The distal part of Meckel's cartilage from the same chick. Note the small oval chondroblasts which present a marked contrast to the large vacuolated cells of ossifying cartilage, cf. Fig. 5. (photomicrograph). (Mallory's triple stain).

Fig. 5. Hypertrophic chondroblasts from the articulare of the same chick. (photomicrograph). (Mallory's triple stain).



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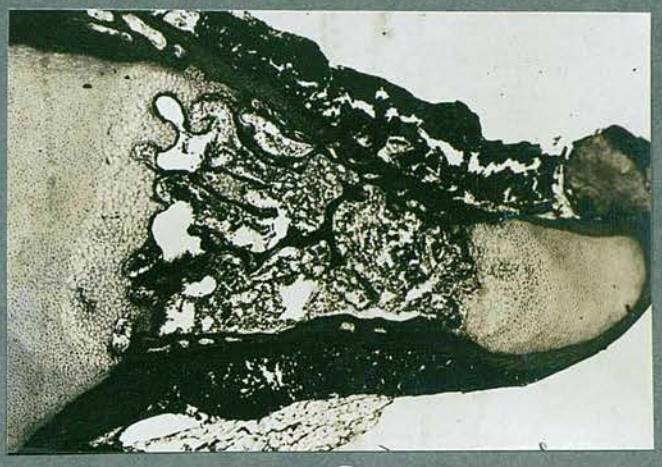

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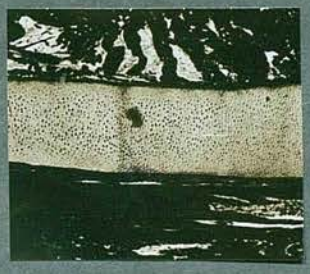

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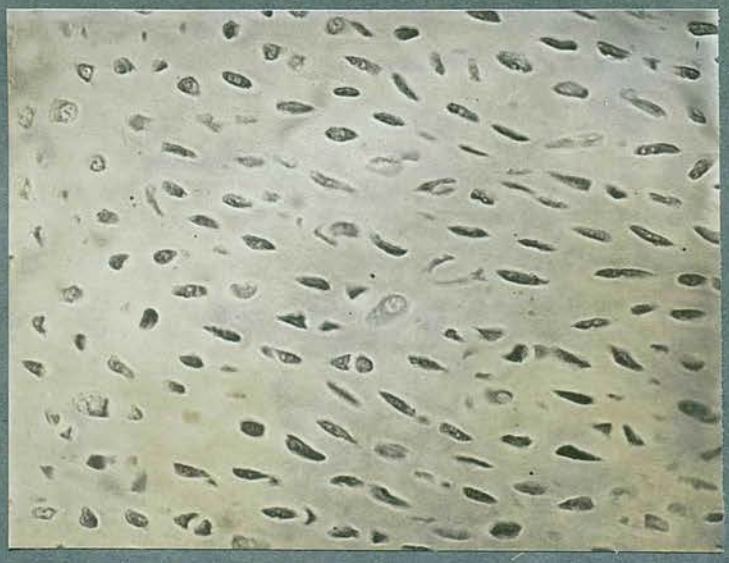

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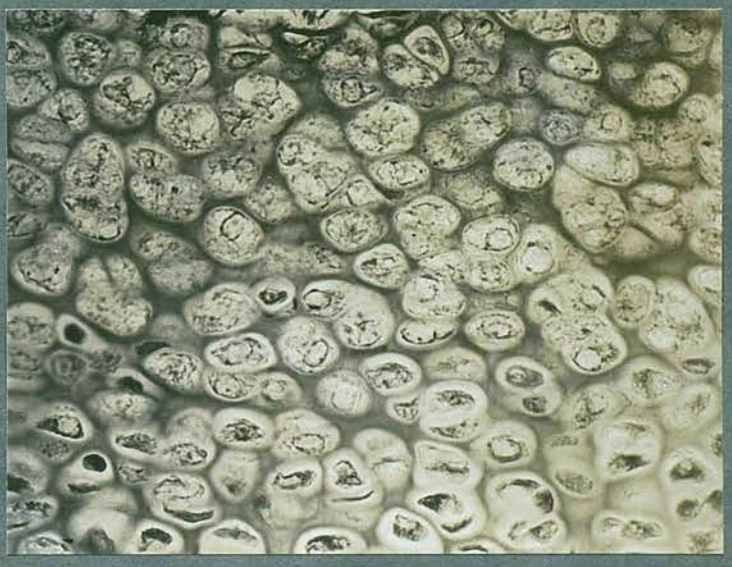

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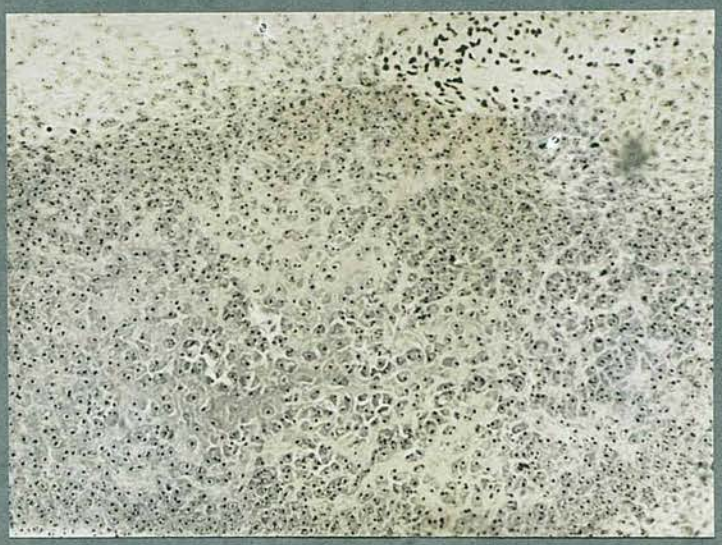
H.B.F. del.

Plate VI.

Fig. 1. Early osteoid tissue from the mandible of a 7-day embryo (photomicrograph). (safranin and picro-indigo-carmine).

Fig. 2. Membrane bone from the mandible of an 11-day embryo (photomicrograph). (safranin and picro-indigo-carmine).

Fig. 3. Membrane bone from the mandible of a 14-day embryo (photomicrograph). (safranin and picro-indigo-carmine).



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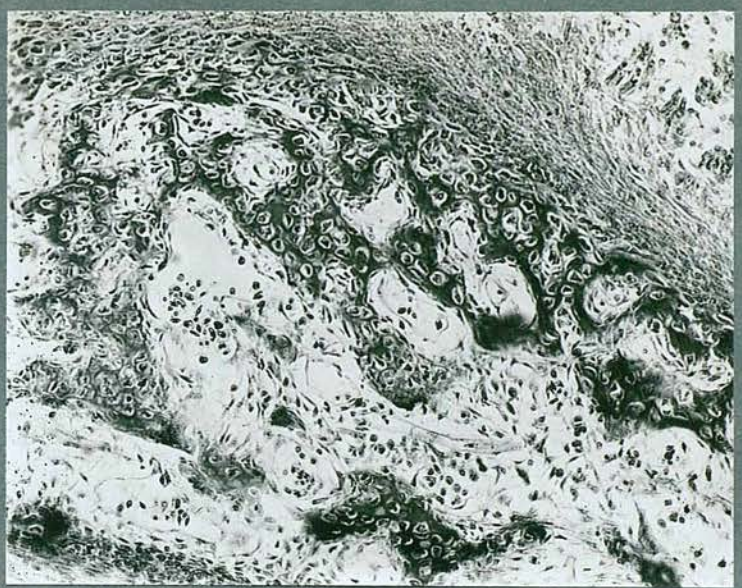

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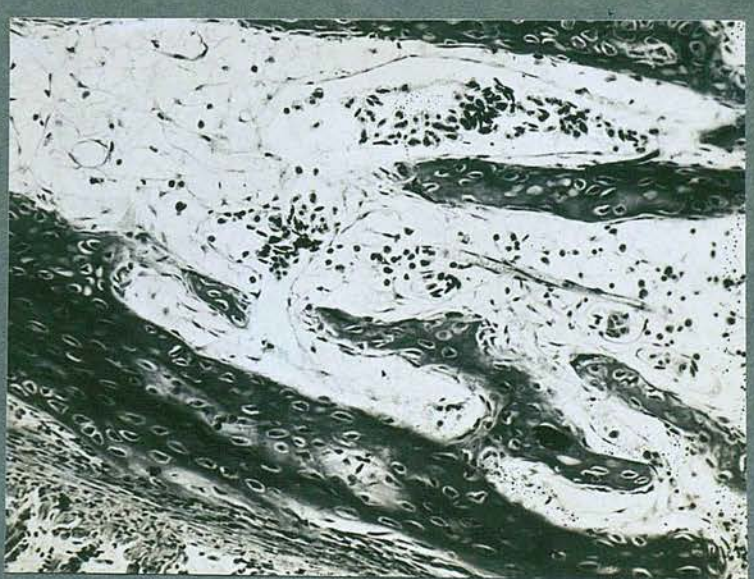

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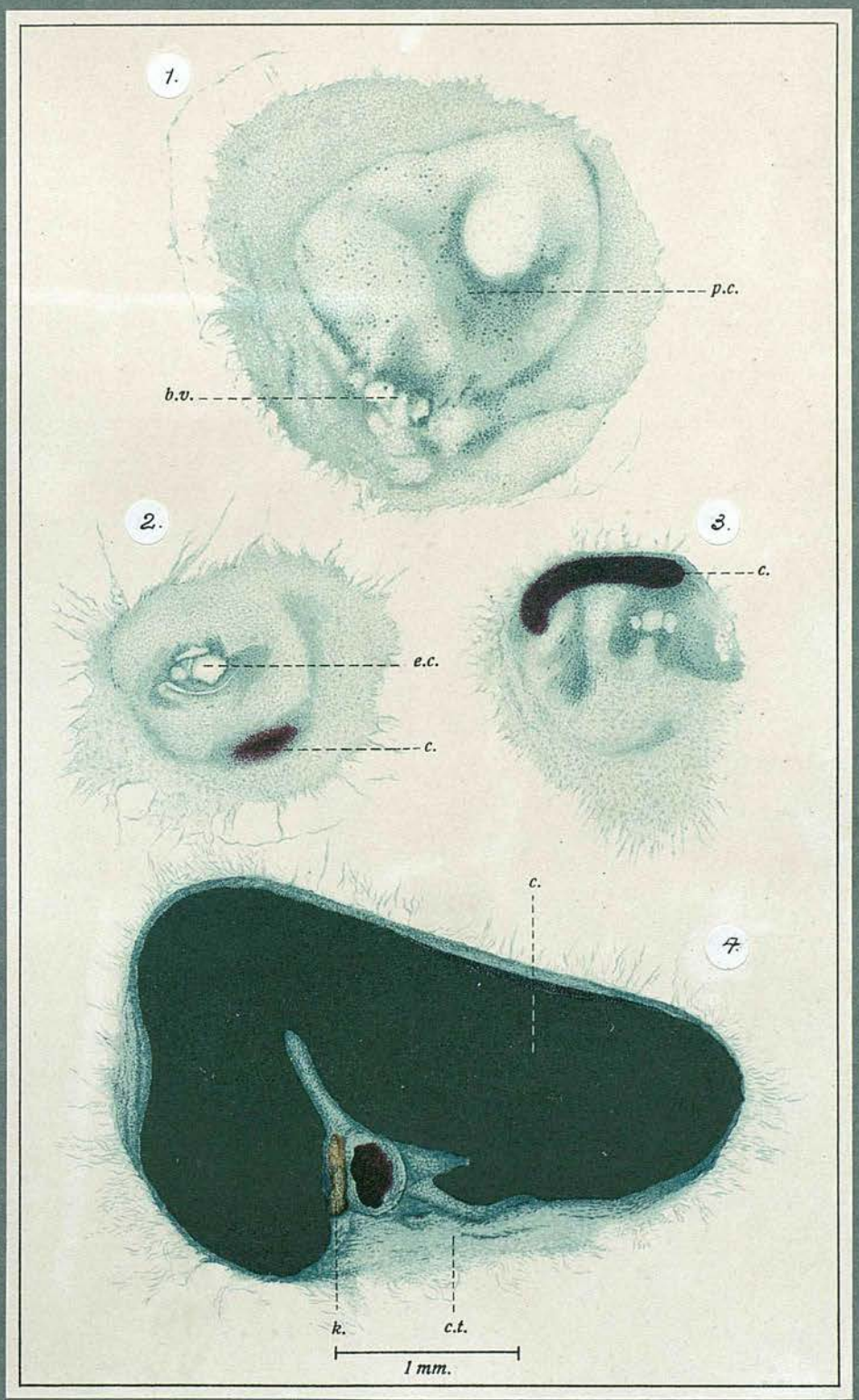
Plate VII.

Fig. 1. Whole-mount of a 48 hour culture of undifferentiated limb-bud mesenchyme. Early precartilage is present but cartilage has not yet developed (dilute thionin).

Fig. 2. Whole-mount of a 4-day culture of originally undifferentiated limb-bud mesenchyme. Note the small nodule of cartilage, the matrix of which shows the characteristic metachromatic colouration (dilute thionin).

Fig. 3. Whole-mount of a 6-day culture of originally undifferentiated limb-bud mesenchyme. A rod-like nodule of cartilage has been formed one end of which is sharply demarcated from the surrounding tissue. (dilute thionin).

Fig. 4. Whole-mount of an 84-day culture of originally undifferentiated limb-bud mesenchyme. Note the large, densely stained nodule of cartilage which has developed *in vitro*. (dilute thionin).

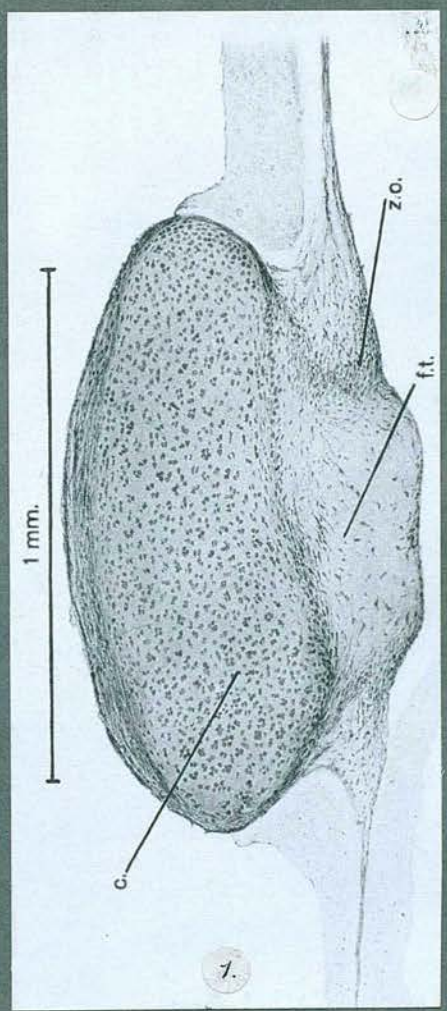


H.B.F. del.

Plate VIII.

Fig. 1. Section through a 70-day culture of originally undifferentiated limb-bud mesenchyme. Note the large nodule of healthy cartilage, dense mass of fibrous tissue and abundant zone of outgrowth. (safranin and picro-indigo-carmine).

Fig. 2. Section through an 84-day culture of originally undifferentiated limb-bud mesenchyme. A large nodule of relatively healthy cartilage has been formed during cultivation. (safranin and picro-indigo-carmine).



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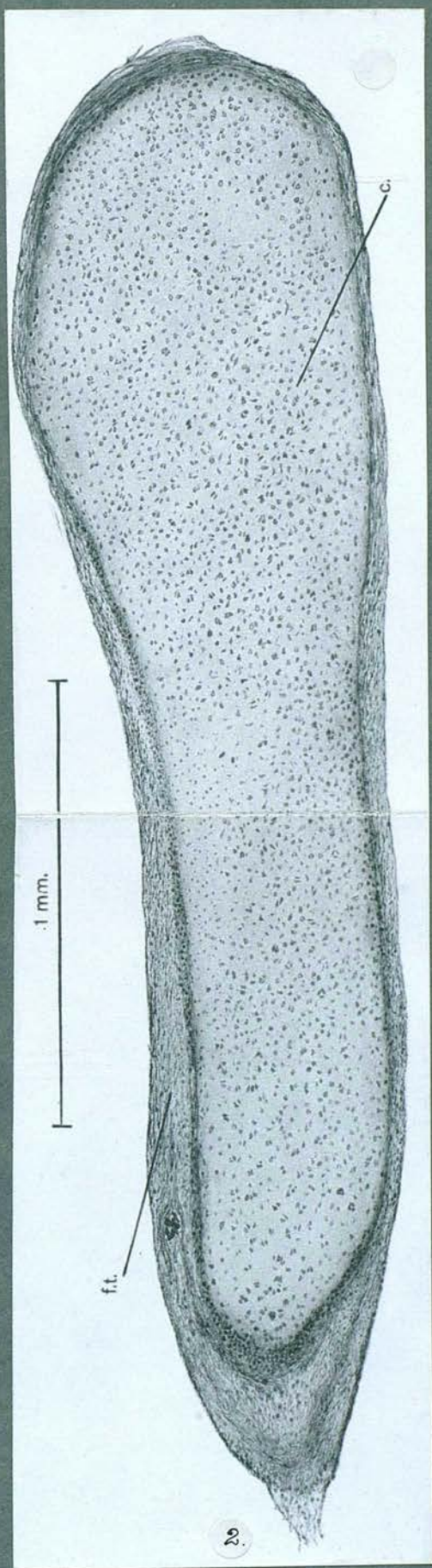

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H.B.F. del.

Plate IX.

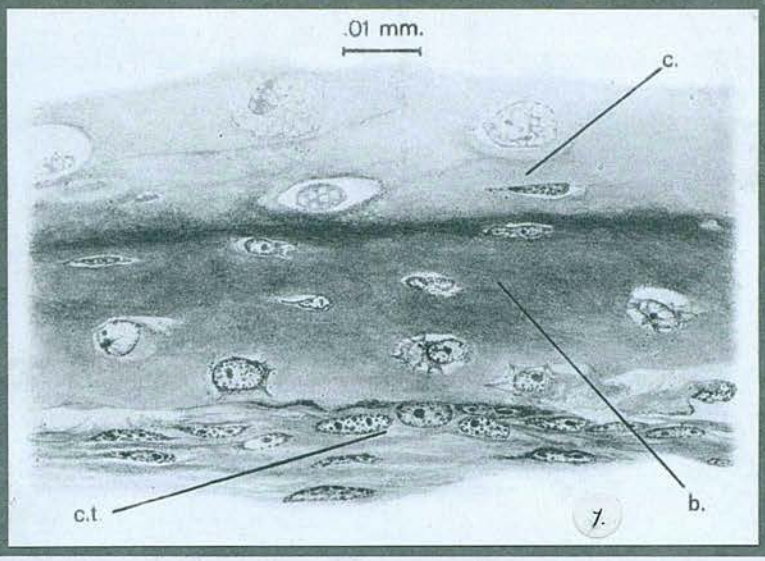
Fig. 1. Section showing ossification in a 70-day culture of embryonic cartilage (No. 5). Note the comparatively thick layer of bone. (safranin and picro-indigo-carmine).

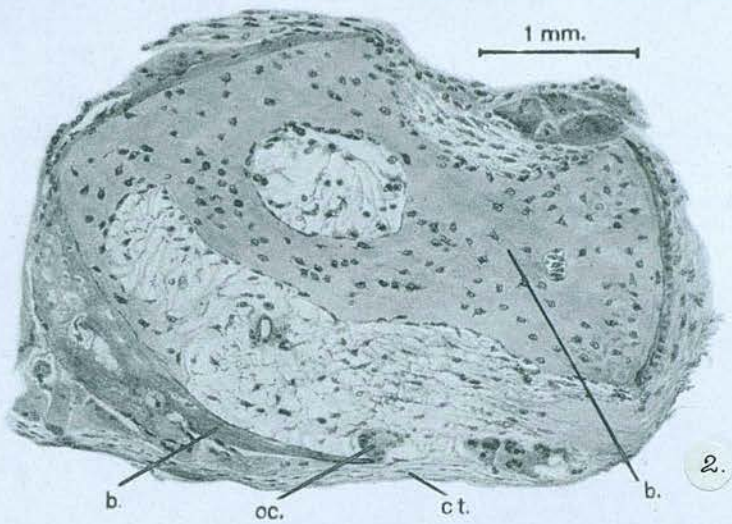
Fig. 2. Section of an 84-day culture of embryonic cartilage (No. 1) showing healthy bone which has been formed *in vitro*. The cartilage has completely disappeared from this part of the explant and the cavities in the bone contain loose connective tissue and occasional osteoclasts. (haematoxylin and van Gieson).

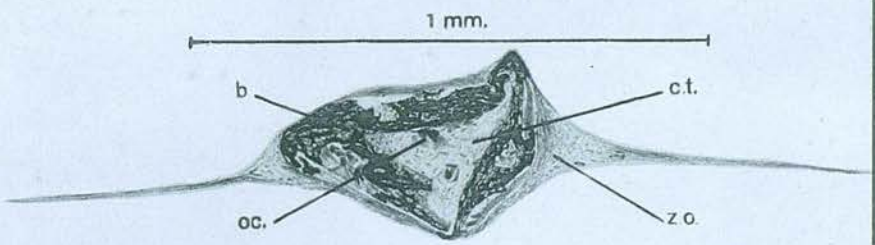
Fig. 3. Section of a 70-day culture (No. 2) originally of embryonic cartilage. An irregular mass of well developed bone has been deposited *in vitro* and the cartilage has been entirely removed. Note the abundant zone of outgrowth. (Mallory's triple stain).

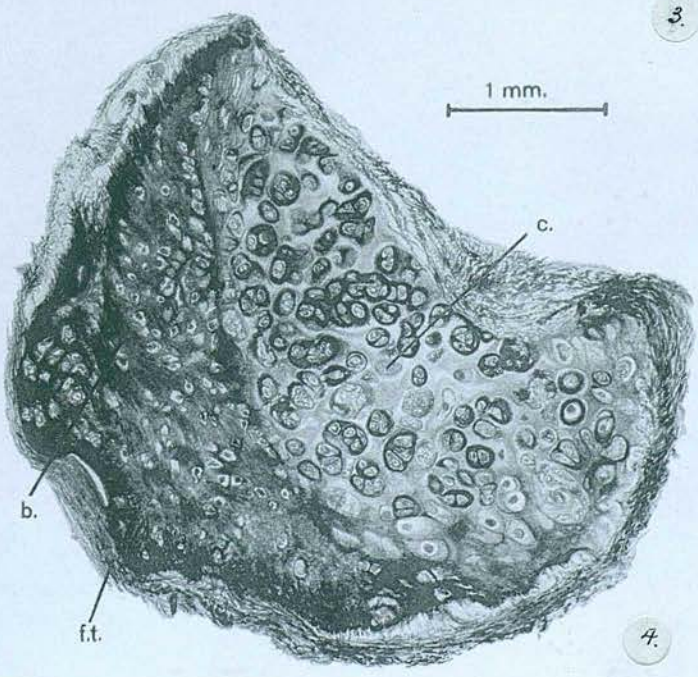
Fig. 4. Section of an 84-day culture (No. 1) of originally undifferentiated limb-bud mesenchyme. The section has been made through the apex of a nodule of cartilage on the surface of which a thick deposit of bone has been formed. (Mallory's triple stain).

PLATE IX.







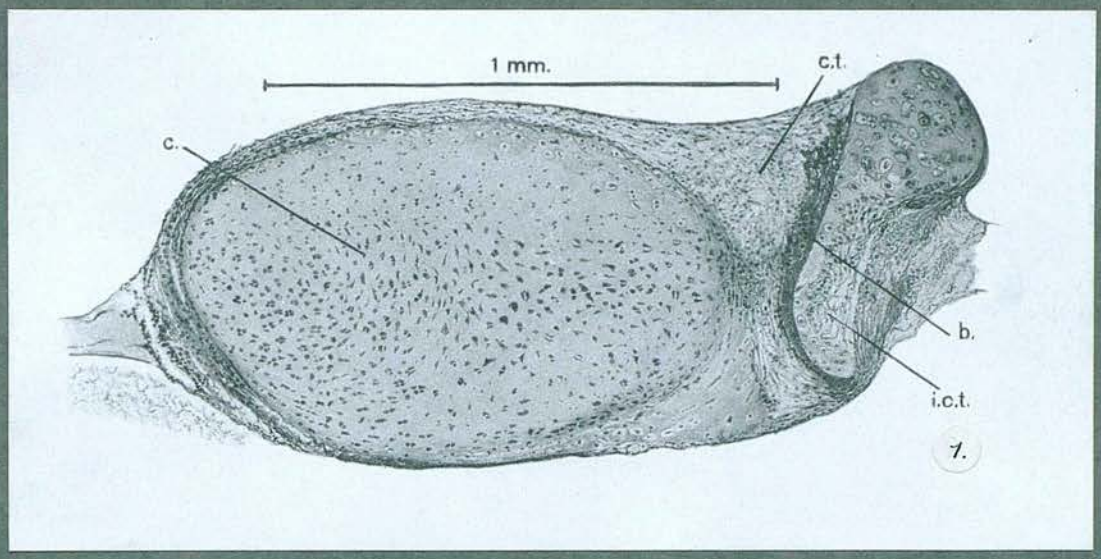


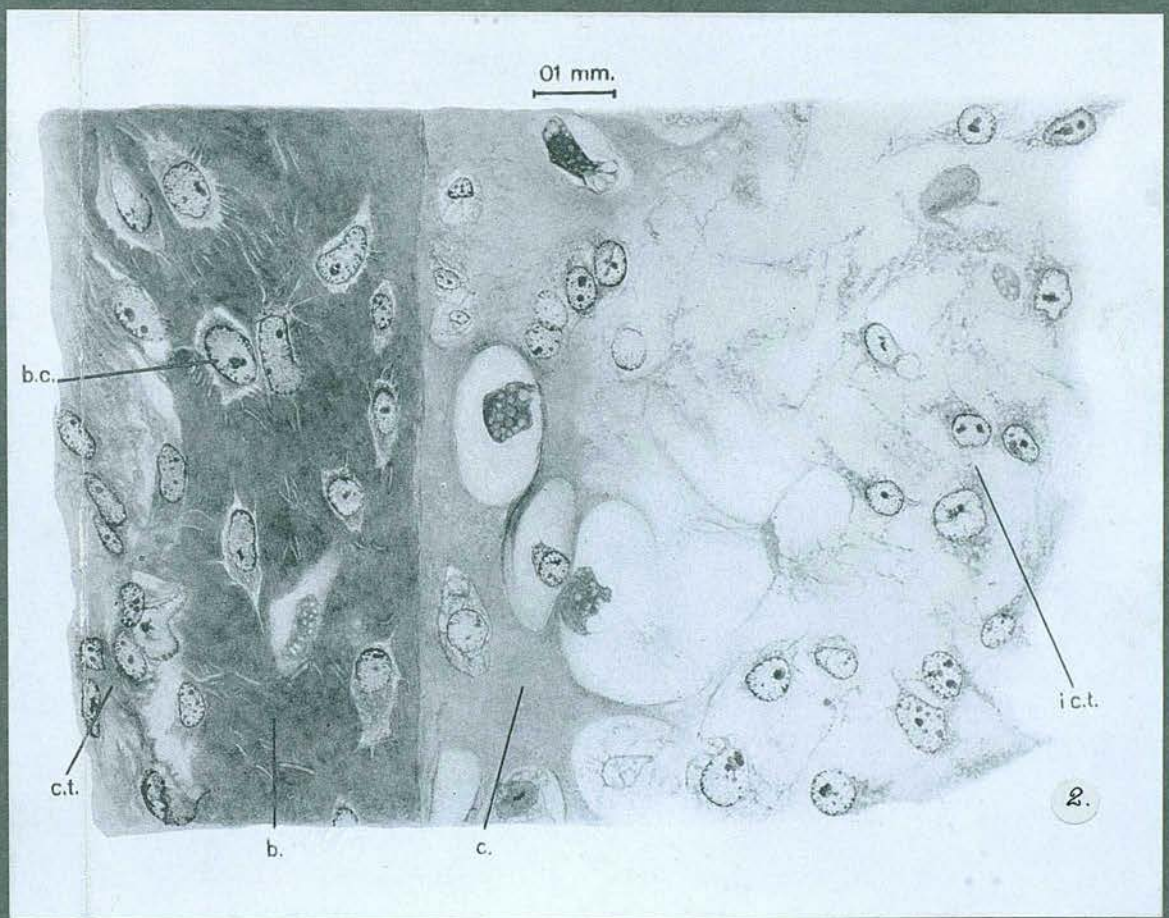
H.B.F. del.

Plate X.

Fig. 1. Section of an 84-day culture of originally undifferentiated limb-bud mesenchyme (No. 2). A layer of bone has been deposited over the surface of part of the cartilage; the cartilage covered by the bone has been partially excavated by invading connective tissue. (haematoxylin and van Gieson).

Fig. 2. Section through the same culture as that shown in Fig. 1. Note the well developed layer of bone containing typical bone cells; the cartilage is being invaded and destroyed by connective tissue. (safranin and picro-indigo-carmine).





H.B.F. del.

Plate XI.

Note. The embryos from which these femora were taken were markedly subnormal in development.

Fig. 1. Section of a normal femur from a 6-day embryo. The femur is composed of a very simple type of cartilage which shows no hypertrophied cells and no differentiation into epiphysis and diaphysis. (safranin and picro-indigo-carmine).

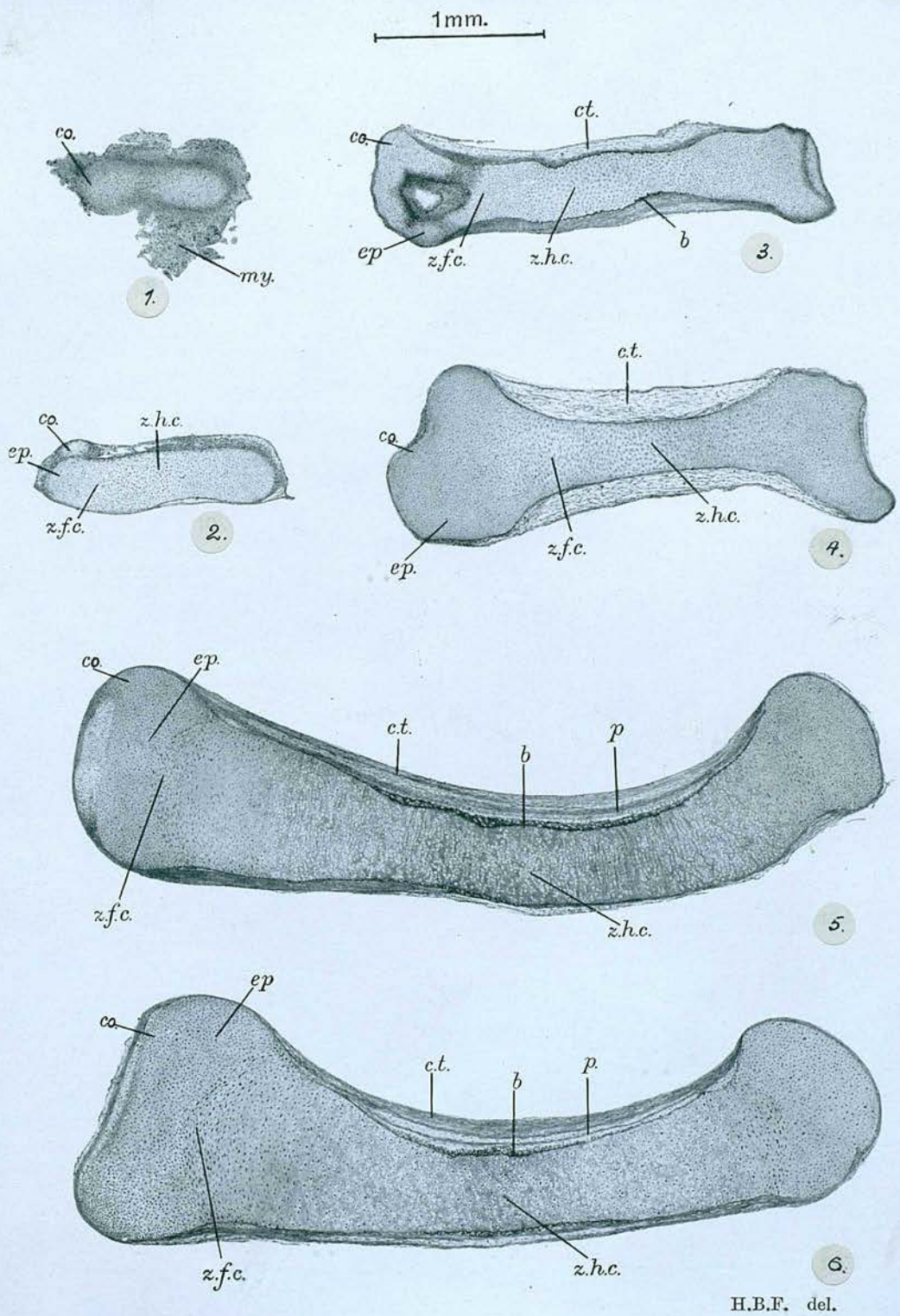
Fig. 2. Section of a 6-day embryonic femur after 3 days' cultivation in vitro. More matrix is present than in the previous specimen and indications of a differentiation into epiphysis, zone of flattened cells and zone of hypertrophied cells are seen.

Fig. 3. Section of a 6-day embryonic femur after 9 days' cultivation in vitro. The region of hypertrophied cells is now fairly distinct and is overlaid by a delicate layer of very early bony material. (haematoxylin and van Gieson).

Fig. 4. Section of a 6-day embryonic femur after 15 days' cultivation in vitro. The explant is subnormal in development as compared with other explants; the zone of hypertrophied cells is not quite so extensive as in the 9-day culture and there is no definite boundary between epiphysis and diaphysis. Note the large size of the epiphysial regions relative to the shaft. (Mallory's triple stain).

Fig. 5. Section of a 6-day embryonic femur after 21 days' cultivation in vitro. The epiphysis is sharply marked off from the diaphysis by the zone of flattened cells; a sheath of bone invests the extensive zone of hypertrophied chondroblasts. (safranin and picro-indigo-carmine).

Fig. 6. Section of a 6-day embryonic femur after 27 days' cultivation in vitro. (safranin and picro-indigo-carmine).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H.B.F. del.

Plate XIII.

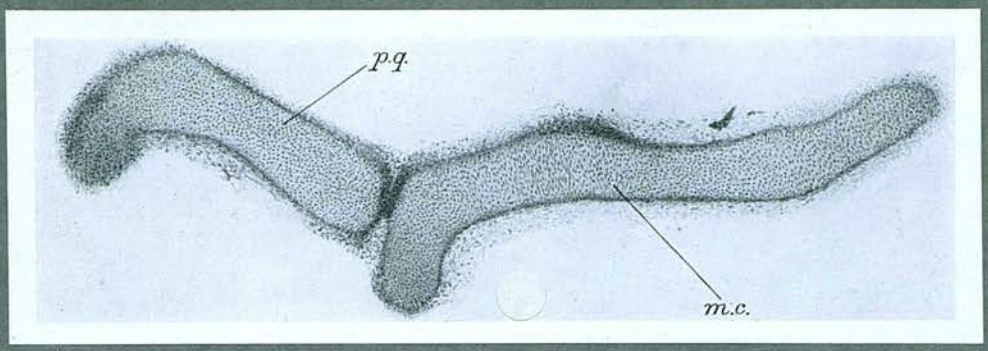
Fig. 1. Longitudinal section through the normal palato-quadrate and Meckel's cartilage of a 6-day fowl embryo (control specimen). The three regions of small, flattened and hypertrophic cells have not yet appeared in the palato-quadrate, the histological structure of which is almost indistinguishable from that of the adjacent Meckel's cartilage. (Mallory's triple stain).

Fig. 2. Longitudinal section of an explant of the distal part of Meckel's cartilage from a 6-day embryonic jaw after 20 days' cultivation *in vitro*. This explant was obtained from the same set of embryos as the control shown in Fig. 1. There is no differentiation into the three zones of small, flattened and vacuolated hypertrophic cells seen in normal ossifying cartilage and the histological structure of the explanted Meckel's rod resembles that of the normal specimen shown in Plate V. Fig. 1. (safranin and picro-indigo-carmine).

Fig. 3. Section through an explant of the palato-quadrate from a 6-day embryo after 20 days' cultivation in a watch-glass, cf. Fig. 4. The explant was taken from the same set of embryos as the two preceding specimens. Note the three zones of cartilage cells (small, flattened and hypertrophic) which have developed during cultivation although (cf. control) these regions were not differentiated at the time of explantation. This specimen also shows slight periosteal ossification over the area of hypertrophic cells. (safranin and picro-indigo-carmine).

Fig. 4. Section of a normal palato-quadrate from a 15-day embryo. Note the zones of small, flattened and hypertrophic cells and the periosteal ossification (photomicrograph). (safranin and picro-indigo-carmine).

PLATE XIII.



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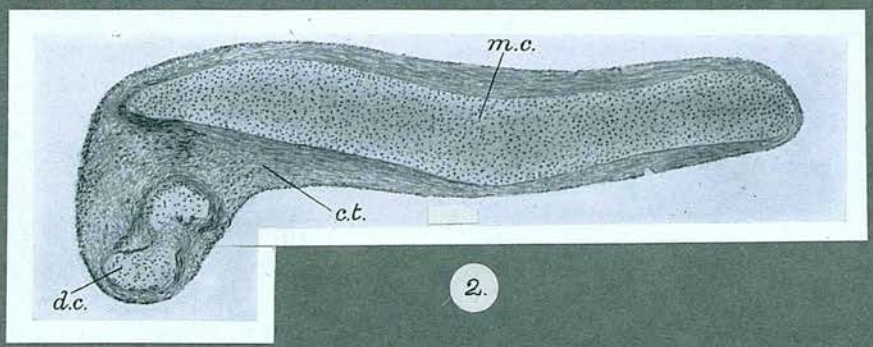

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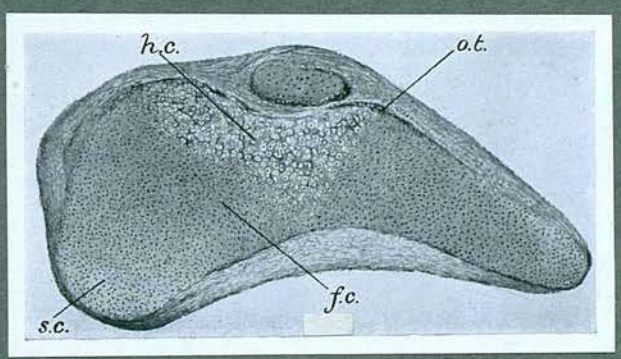

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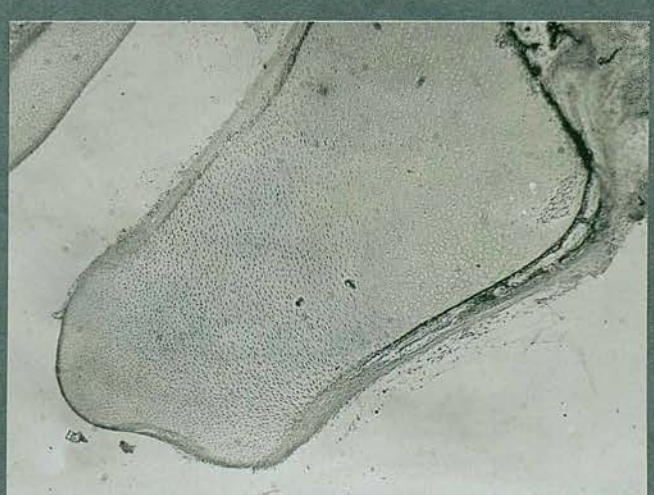

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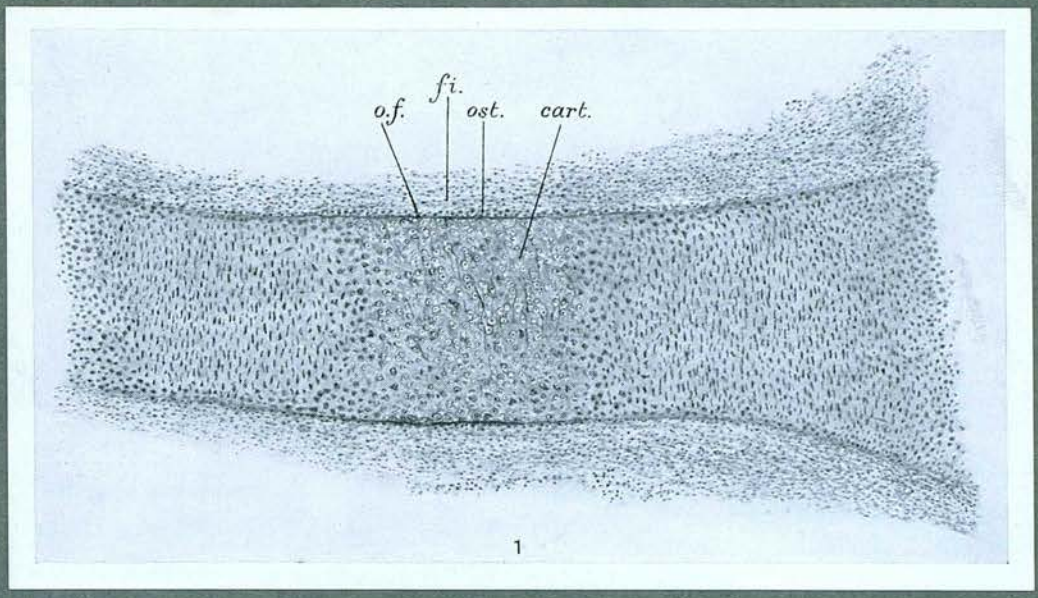
H.B.F. del.

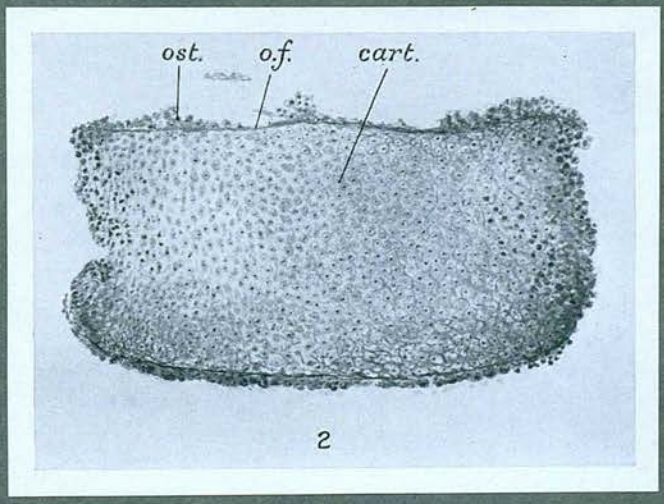
Plate XIV.

Fig. 1. Longitudinal section of a normal (control) tibia from a 6-day embryo showing the general histological structure of the shaft. (safranin and picro-indigo-carmine).

Fig. 2. Longitudinal section of the middle region of the opposite tibia from the same embryo, after removal of the periosteum for explantation. The osteogenic fibres and part of the osteoblastic layer remain adherent to the cartilage. (safranin and picro-indigo-carmine).

PLATE XIV.



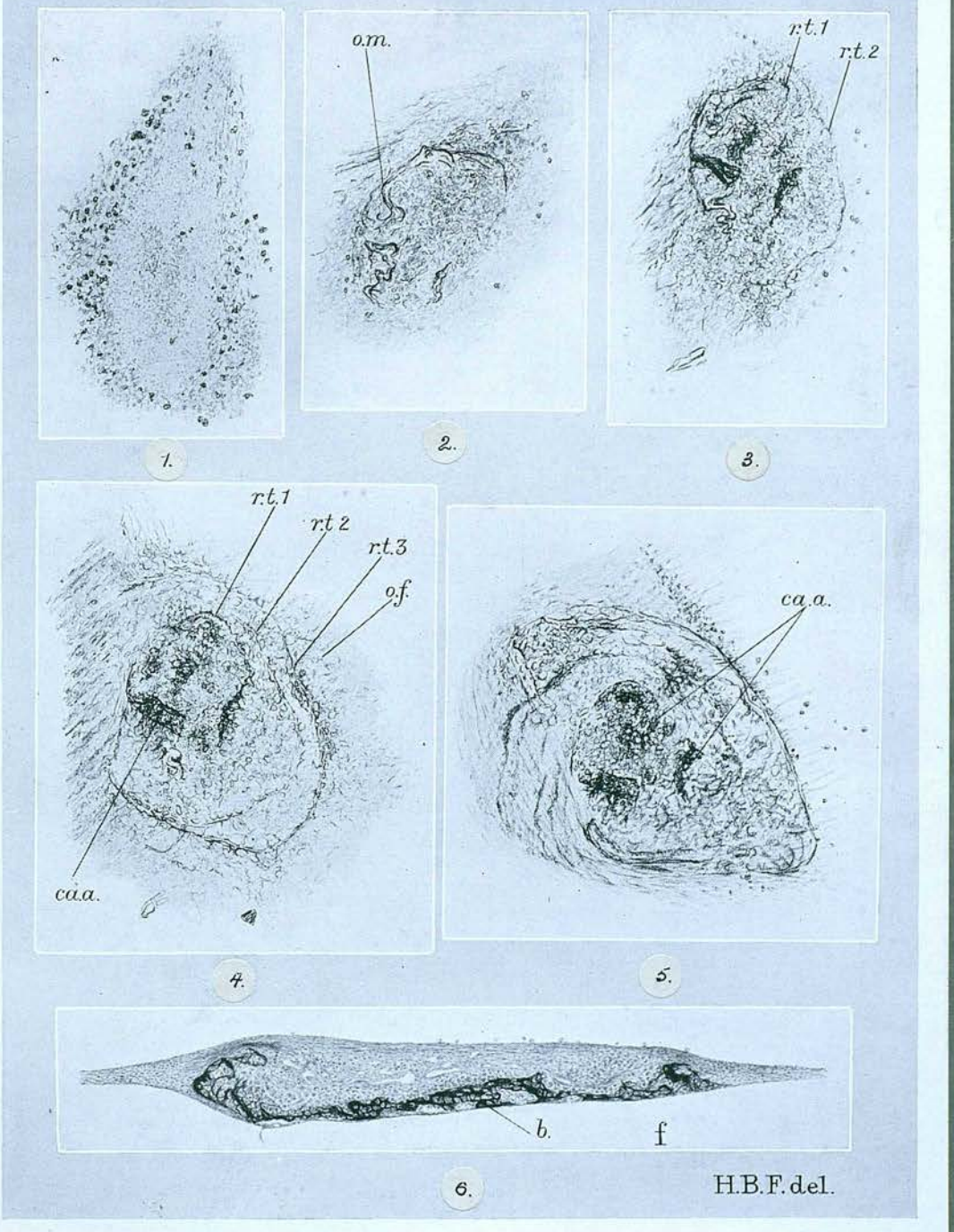


H.B.F. del.

Plate XV.

Figs. 1 - 5. Camera lucida sketches showing the development of an ossification centre which appeared in the culture made from the rudimentary periosteum taken from the tibial fragment shown in Plate XIV. Fig. 2. The plate of bone which differentiated during cultivation shows three concentric, ring-like thickenings, each ring corresponding with a subculture.

Fig. 6. Section of the same culture fixed after 10 days' growth in vitro. (plane of section at right angles to the plane of the coverslip). (safranin and picro-indigo-carmine).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ca.a.
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H.B.F. del.

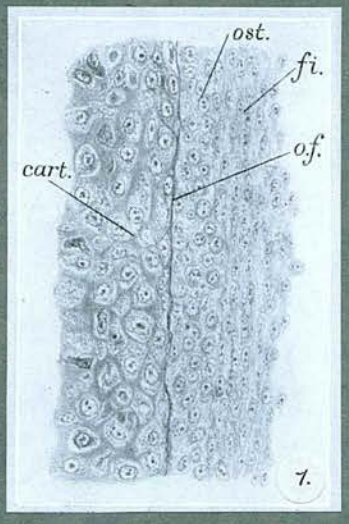
Plate XVI.

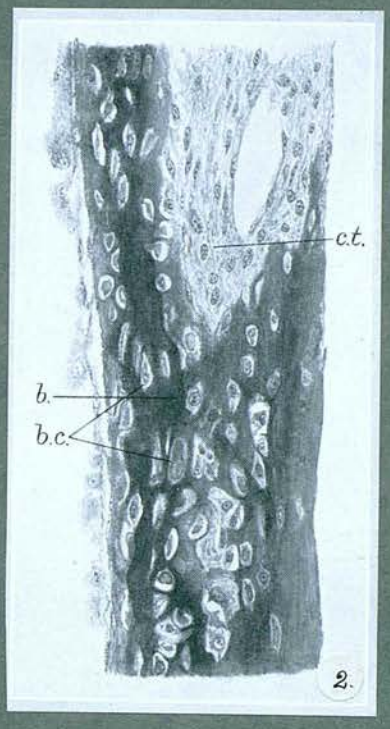
Fig. 1. Longitudinal section through a normal (control) tibia from a 6-day fowl embryo. Note the rudimentary periosteum composed of an outer fibroblastic and inner osteoblastic coat, and the layer of osteogenic fibres. The periosteum from the opposite tibia of the same embryo was explanted *in vitro* and is represented in Fig. 2. (safranin-and picro-indigo-carmine).

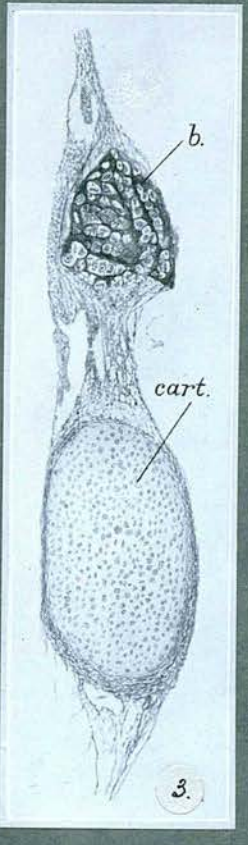
Fig. 2. Section of a periosteal culture made from the corresponding tibia to that shown im Fig. 1. The specimen was fixed after 18 days' growth *in vitro*. Note the bone which has developed during cultivation. (plane of section at right angles to the plane of the coverslip) (safranin and picro-indigo-carmine).

Fig. 3. Section of a 10-day culture of periosteum from a 6-day embryonic femur. In this specimen both cartilage and bone have developed as independent nodules. (plane of section at right angles to the plane of the coverslip). (safranin and picro-indigo-carmine).

PLATE XVI.







H.B.F. del.

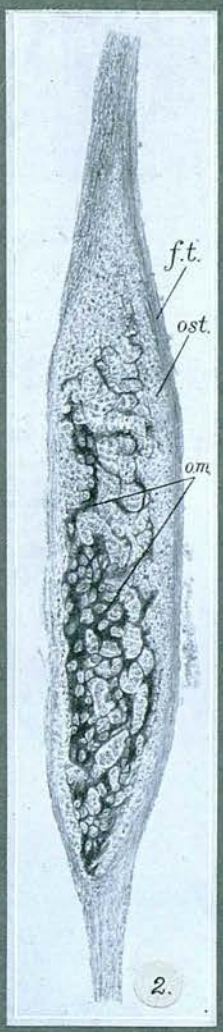
Plate XVII.

Fig. 1. Longitudinal section of a normal (control) tibia from a 10-day embryo. Note the fibrous and osteoblastic layers of the periosteum overlying early bone trabeculae. The periosteum from the opposite tibia of the same embryo was explanted *in vitro* and a culture is shown in Fig. 2. (safranin and picro-indigo-carmine).

Fig. 2. Section of a 6-day periosteal culture obtained from the corresponding tibia to that shown in Fig. 1. Note the compact ossification centre which appeared during cultivation. (plane of section at right angles to the plane of the coverslip). (safranin and picro-indigo-carmine).

Fig. 3. Longitudinal section of a (control) tibia from a day-old chick. Note the outer coat of fibrous tissue merging with an inner layer of flattened cells beneath which lie the osteoblasts. (safranin and picro-indigo-carmine).

PLATE XVII.



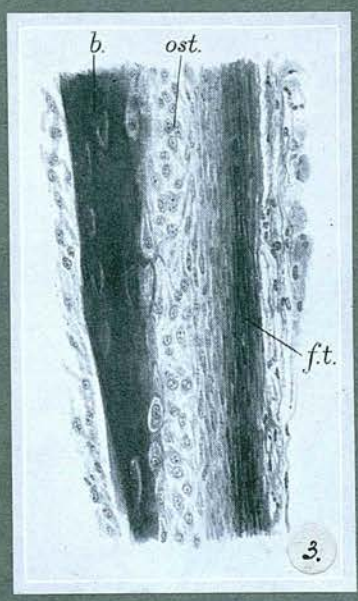


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Plate XVIII.

Figs. 1 - 4. Photomicrographs of a living culture of tibial bone from a 21-day embryo at different stages, showing the formation of bone by endosteum. The culture was obtained from the same tibia as those shown in Plate XIX. Fig. 1, 2 & 4 and Plate XX. Figs. 1, 2, 3 & 4.

1. 5 days in vitro, immediately before removal of the original bone fragment.
2. Same culture, 20 hours after removal of the original bone. The hole left by the removal of the bone is still seen.
3. Same culture, 5 days after removal of the original bone. Note the mass of new bone which has developed in the middle of the tissue.
4. Same culture, 7 days after removal of the original bone. At this stage the culture was fixed and sectioned. A section is shown in Fig. 5.

Fig. 5. Section through central mass of new bone. (section parallel to the plane of the coverslip). (safranin and picro-indigo-carmine).

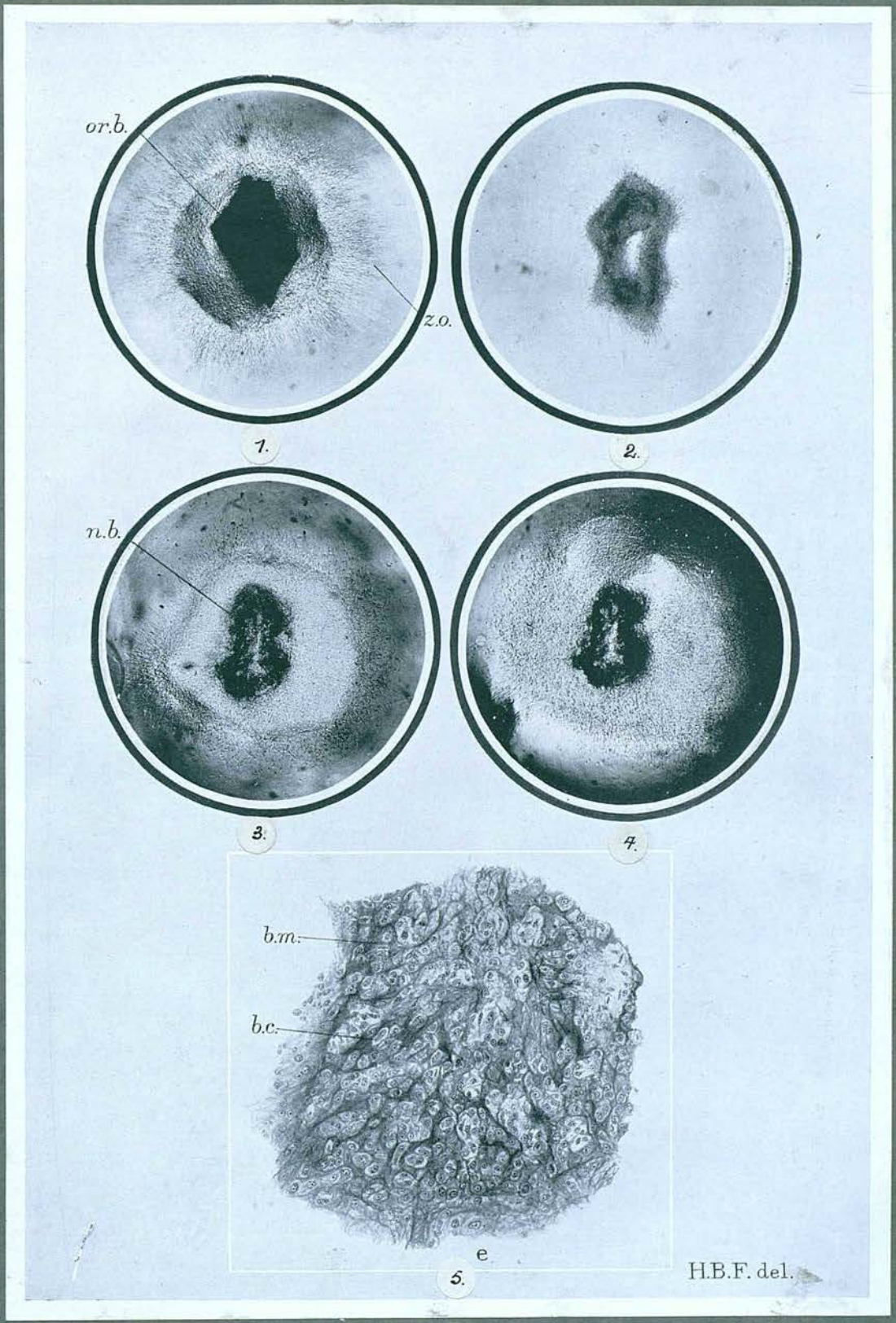
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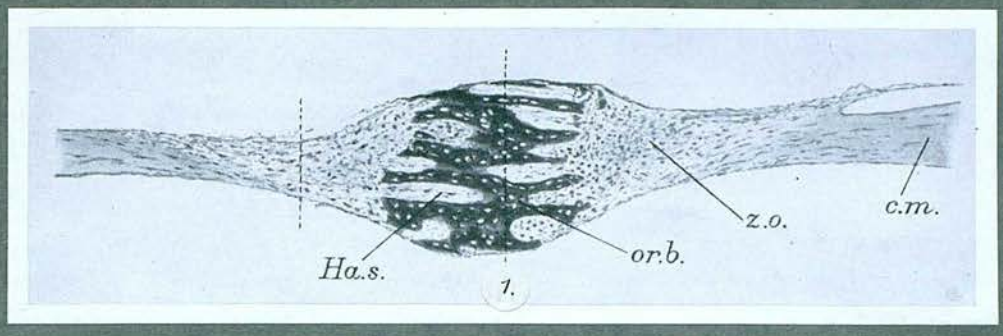
Plate XIX.

Fig. 1. Section of a 5-day culture of bone from the tibia of a 21-day embryo (plane of section at right angles to the plane of the coverslip). (safranin and picro-indigo-carmine).

Fig. 2. Same under higher magnification. The cells composing the zone of outgrowth are mainly if not entirely derived from the Haversian spaces. Most of the actual bone cells are degenerate.

Fig. 3. Section of a similar culture 20 hours after removal of the original fragment of bone. Note the areas of osteogenic fibres which have appeared during cultivation. (plane of section parallel to the plane of the coverslip). (safranin and picro-indigo-carmine).

Fig. 4. Section of a similar culture 5 days after removal of the original fragment of bone. Two concentric rings of osteoid tissue have been formed since the removal of the original bone (plane of section parallel to the plane of the coverslip). (safranin and picro-indigo-carmine).



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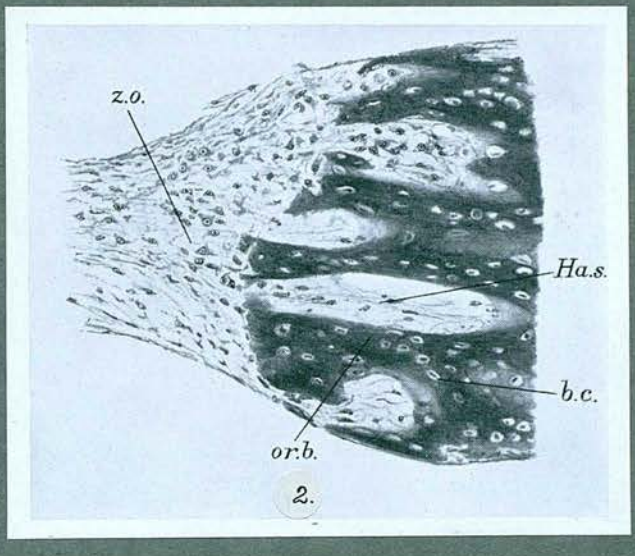

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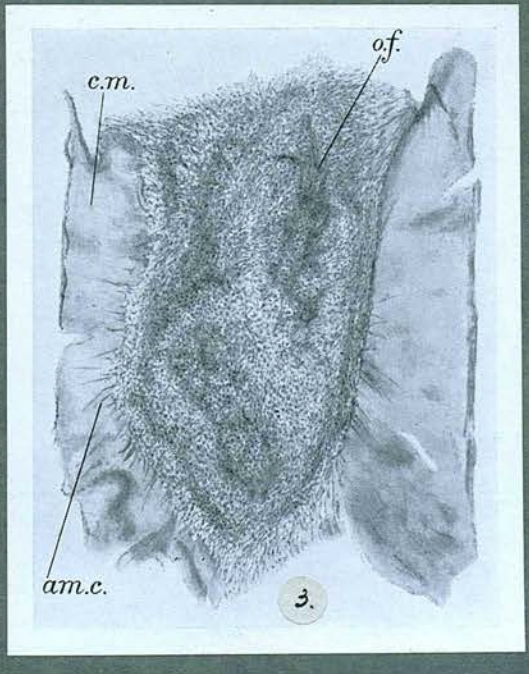

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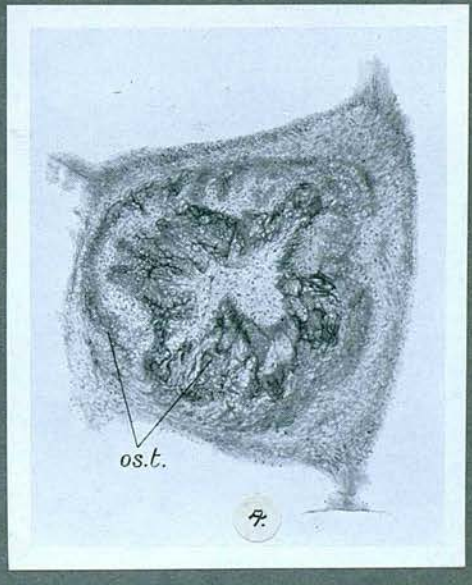


4.

Plate XX.

Fig. 1. Section of a similar culture 11 days after removal of the original bone. Note the well developed bone which has differentiated during cultivation. A mitotic figure is seen in one of the bone cells (plane of section parallel to the plane of the coverslip). (safranin and picro-indigo-carmine).

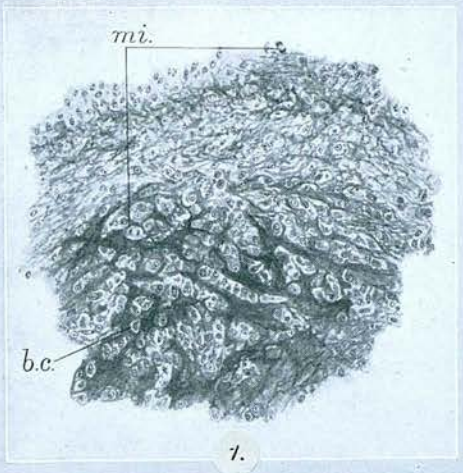
Fig. 2. Section of a similar culture 21 days after removal of the original bone. A well developed nodule of bone has formed during cultivation. Osteogenesis has ceased as indicated by the sharp outline of the bone. (plane of section parallel to the plane of the coverslip). (Safranin and picro-indigo-carmine).

Fig. 3. Photomicrograph of culture treated with von Kossa's silver nitrate method, 8 days after the removal of the original bone (whole mount).

Fig. 4. Photomicrograph of culture treated with von Kossa's silver nitrate method, 10 days after the removal of the original bone (whole mount).

Fig. 5. Section of a culture of periosteum from the tibia of a day-old chick. This specimen was cultivated for 6 days after which the original explant was removed and the zone of growth was cultivated for a further period of 14 days. No sign of osteogenesis is seen and only fibrous tissue has been formed (plane of section parallel to the plane of the coverslip). (safranin and picro-indigo-carmine).

PLATE XX.



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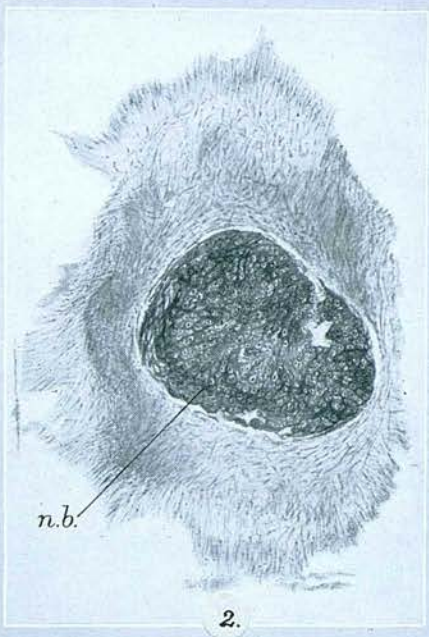

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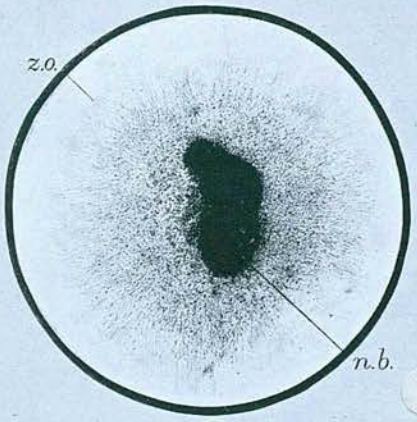

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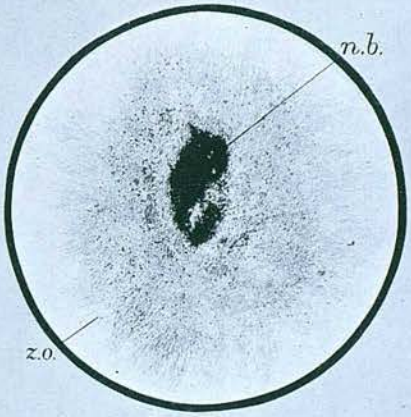

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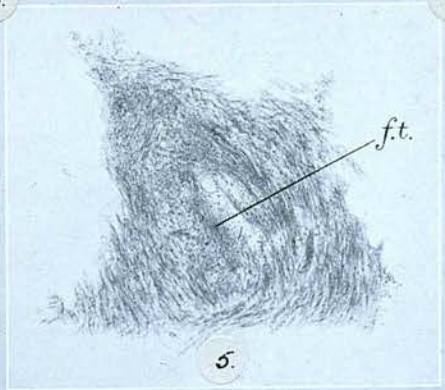

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H.B.F. del.

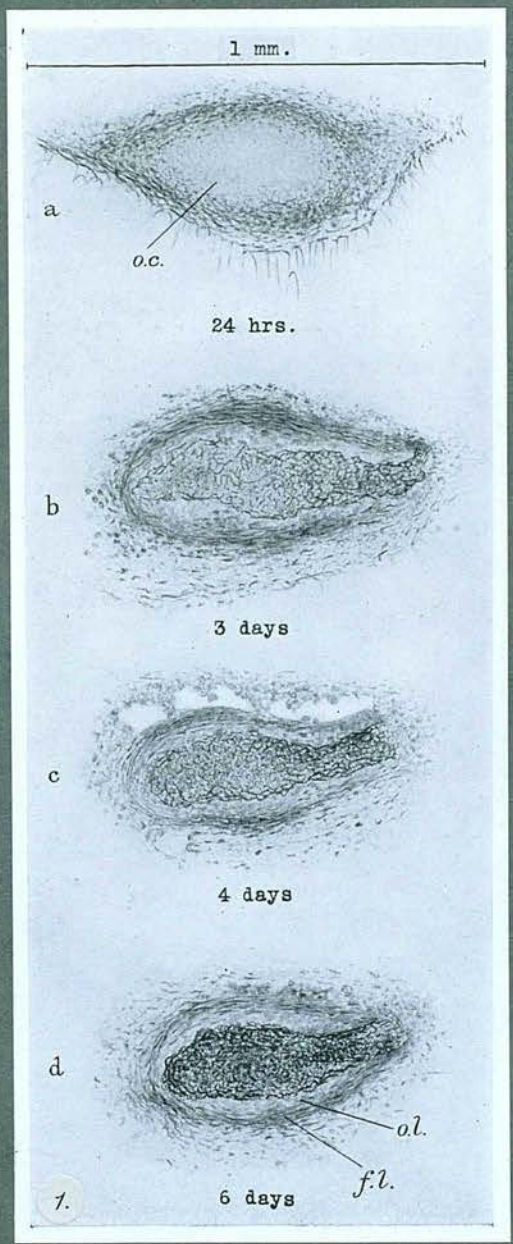
Plate XXI.

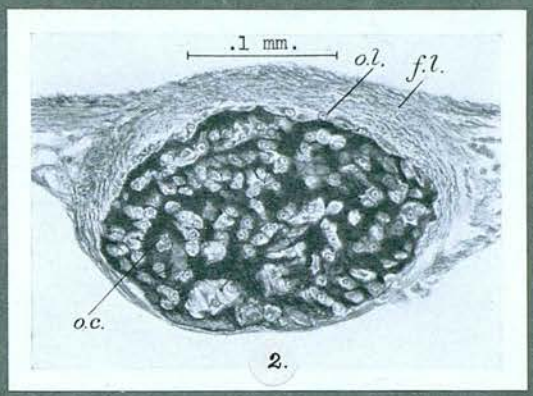
Fig. 1. Osteogenesis in a hanging-drop culture of mesoderm from a 6-day embryonic jaw. The figures are drawn from camera lucida sketches of a living culture and were made at different stages of growth to show the development of an ossification centre. After 6 days' cultivation this specimen was fixed and sectioned. A section is shown in Fig. 2.

Fig. 2. Histological section of culture shown in Fig. 1 after 6 days' growth *in vitro*. (Mallory's stain).

Fig. 3. Photomicrograph of an 8-day coverslip culture of the soft tissue from a 6-day embryonic jaw. This specimen was stained with silver nitrate and mounted whole. An ossification centre, which developed during cultivation, stained black with the silver nitrate indicating that true calcified bone had been formed.

PLATE XXI.





H.B.F. del

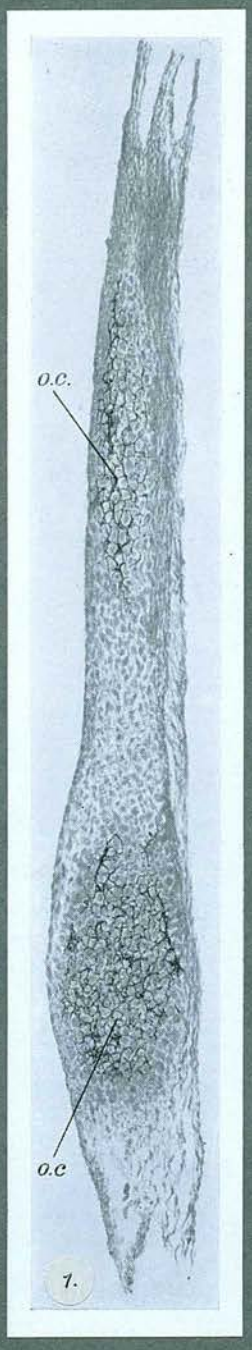
Plate XXII.

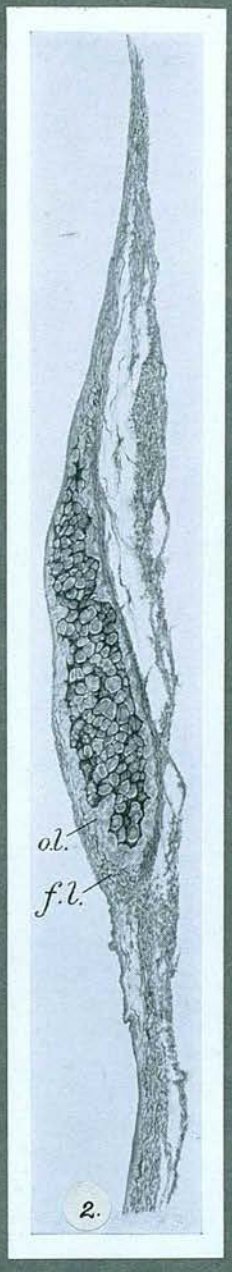
Fig. 1. Section of a culture of mandibular mesoderm from a 6-day embryo after 2 days' growth *in vitro*. Note the two very early ossification centres which appeared during cultivation. Only a few osteogenic fibres are present at this stage. (Mallory's triple stain).

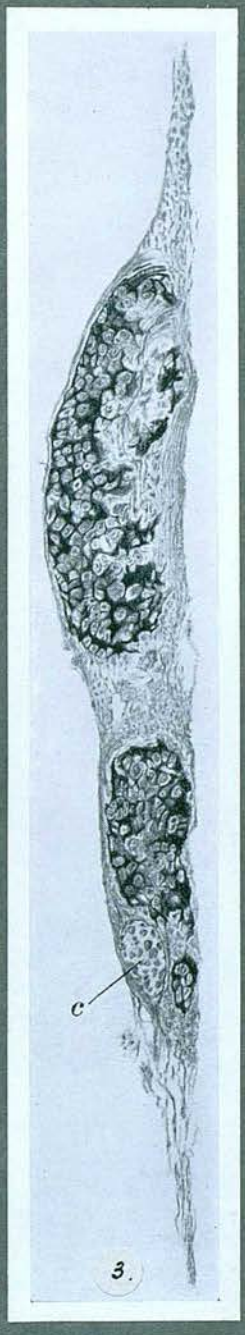
Fig. 2. Section of a similar culture after 6 days' growth showing an ossification centre which developed *in vitro*. The osteoid tissue has reached a higher stage of differentiation than that represented in Fig. 1. (Mallory's triple stain).

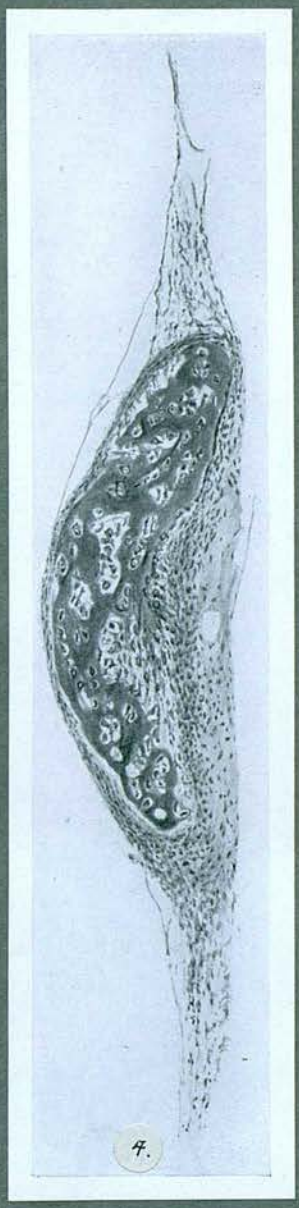
Fig. 3. Section of a similar culture after 6 days' cultivation. Two ossification centres and a small nodule of cartilage are shown (the presence of cartilage is exceptional). (Mallory's triple stain).

Fig. 4. Section of a similar culture after 14 days' growth *in vitro*. Note the mass of trabecular bone which has differentiated during cultivation.









H.B.F. del.

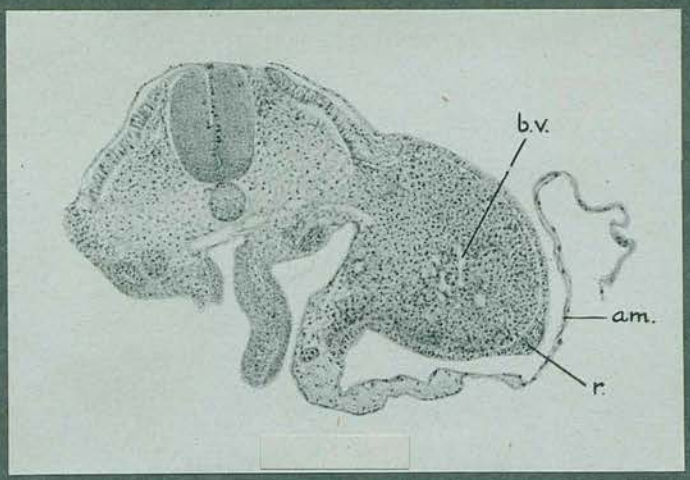
Plate XXIII.

Fig. 1. Transverse section through posterior trunk region of embryo of 72 hours. On the right-hand side of the figure is seen a section of the left posterior limb-bud; the right posterior limb-bud has been amputated for explantation *in vitro* and is shown in Plate XXV. Fig. 1 after 14 days' cultivation. (haematoxylin and eosin).

Fig. 2. Anterior limb-bud after two days' growth *in vitro*. Shows elongated mass of precartilage, dilated blood vessels and terminal ridge of ectoderm. (haematoxylin and eosin).

Fig. 3. Posterior limb-bud after four days' cultivation *in vitro*. The specimen here figured is from the same embryo as the explant represented in Fig. 2. Note the two nodules of early cartilage, showing the "axial" type of arrangement, the ectodermal sac and mass of fibrous tissue. (Mallory's stain).

Fig. 4. Cartilage cells from explant of 21 days' growth. The nucleus of one of the cells is in anaphase. (iron haematoxylin).



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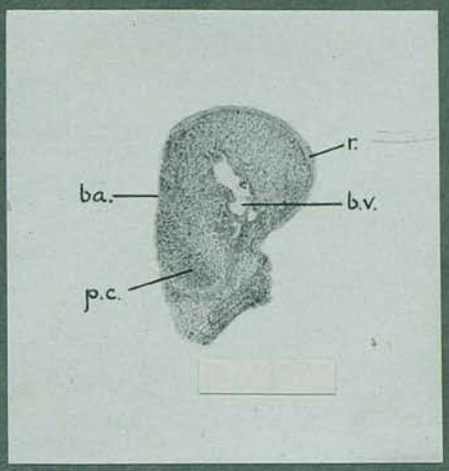

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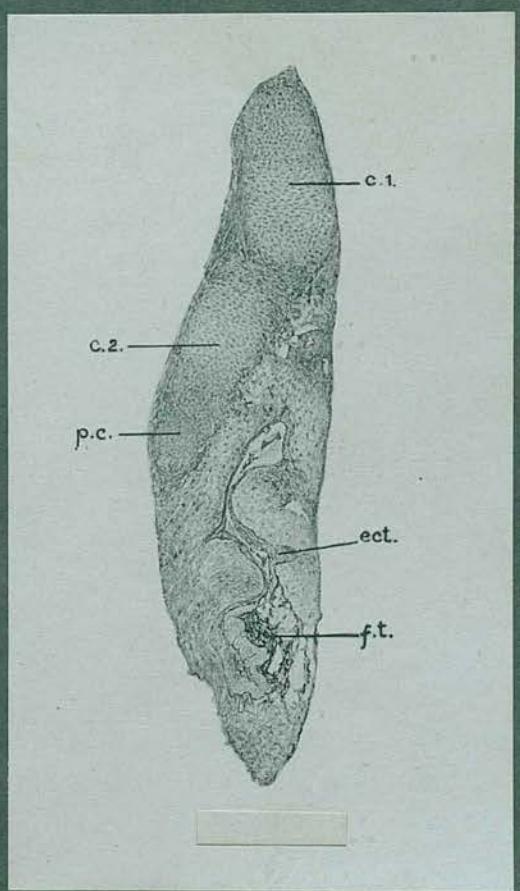

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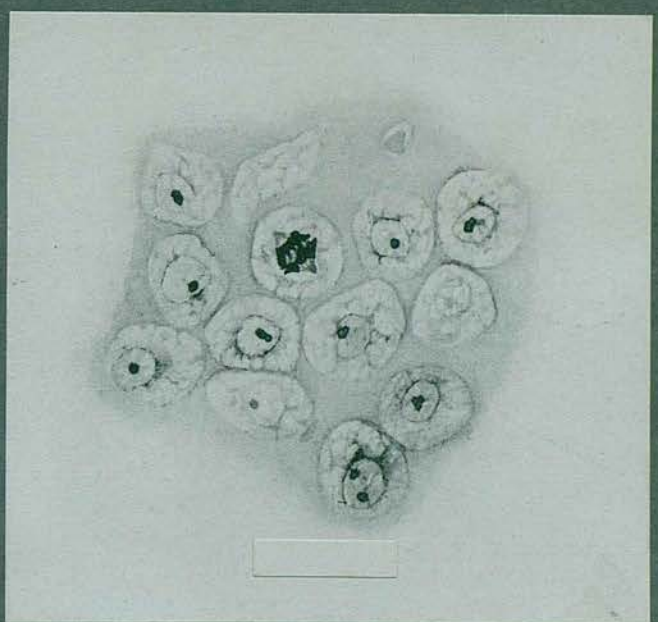

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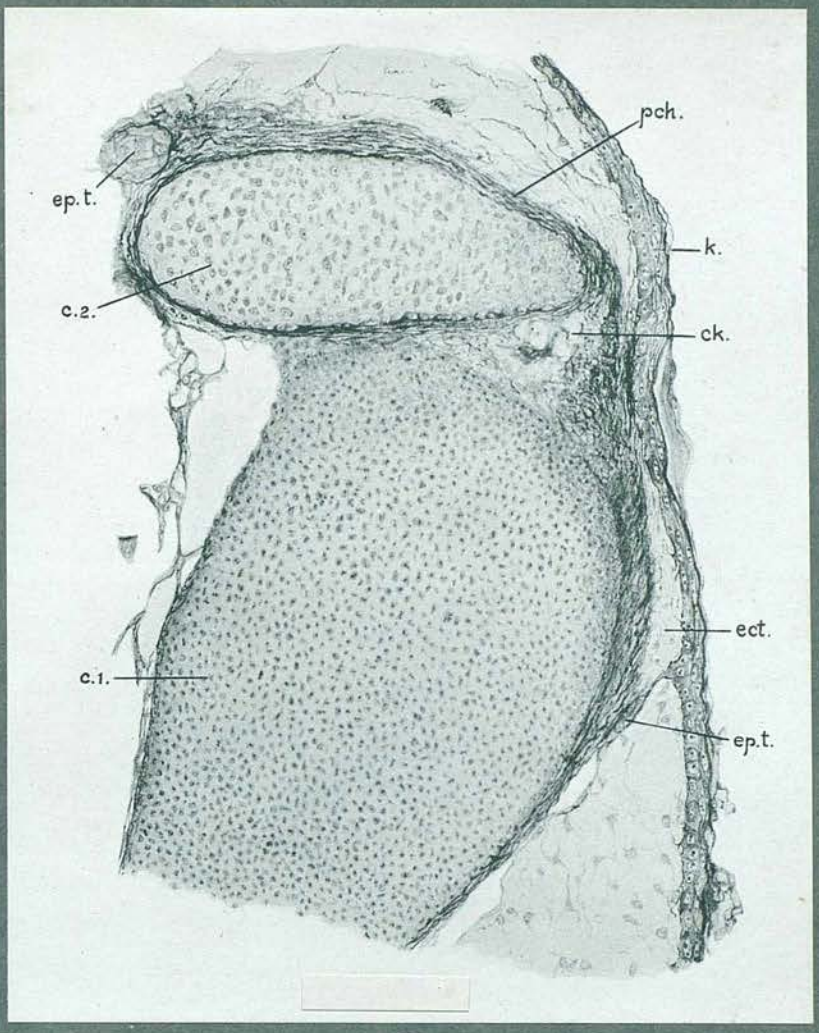
Plate XXIV.

Fig. 1. Portion of 11 days' culture of a 3-day limb-bud. The nodules of cartilage show the "axial" arrangement. Note the thick, fibrous perichondrium and epithelial tubules. (Mallory's stain).

Fig. 2. Stroma from explant of 22 days' cultivation showing dense network of fine white fibres.

PLATE XXIV.



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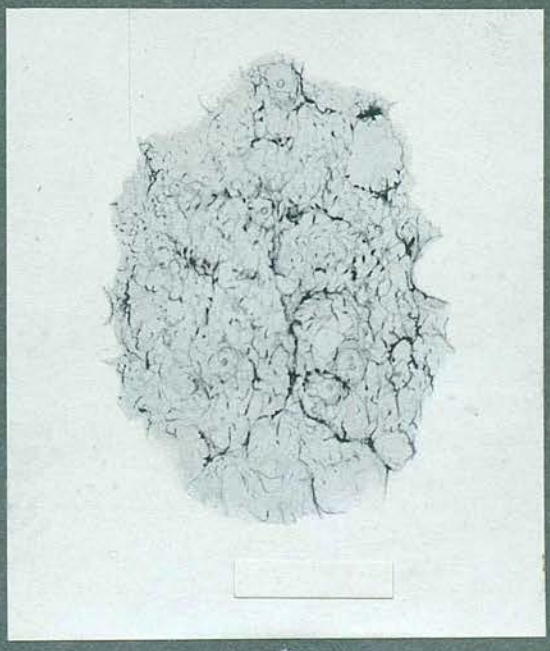

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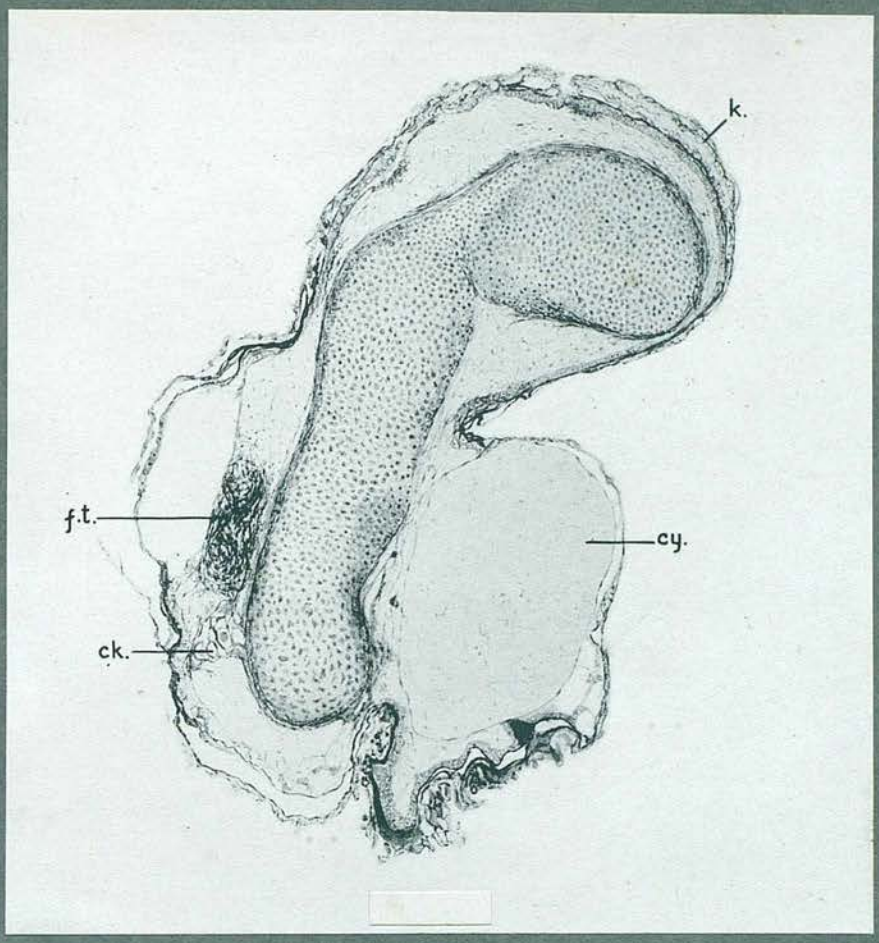
H.B.F. del.

Plate XXV.

Fig. 1. Right posterior limb-bud after 14 days' cultivation. Cf. Plate XXIII. Fig. 1 which shows the left posterior (control) limb-bud from the same embryo, and note the extensive differentiation which has taken place *in vitro*. The cartilage nodules show the "axial" form of arrangement. Note the cystic cavity, dense mass of fibrous tissue and keratin. (iron haematoxylin).

Fig. 2. Explant after 10 days' cultivation. This culture is firmly embedded in the plasma owing to the outwandering (uncontrolled growth) of the connective tissue; a single nodule of cartilage is present; there is no epithelial covering. (iron haematoxylin and van Gieson).



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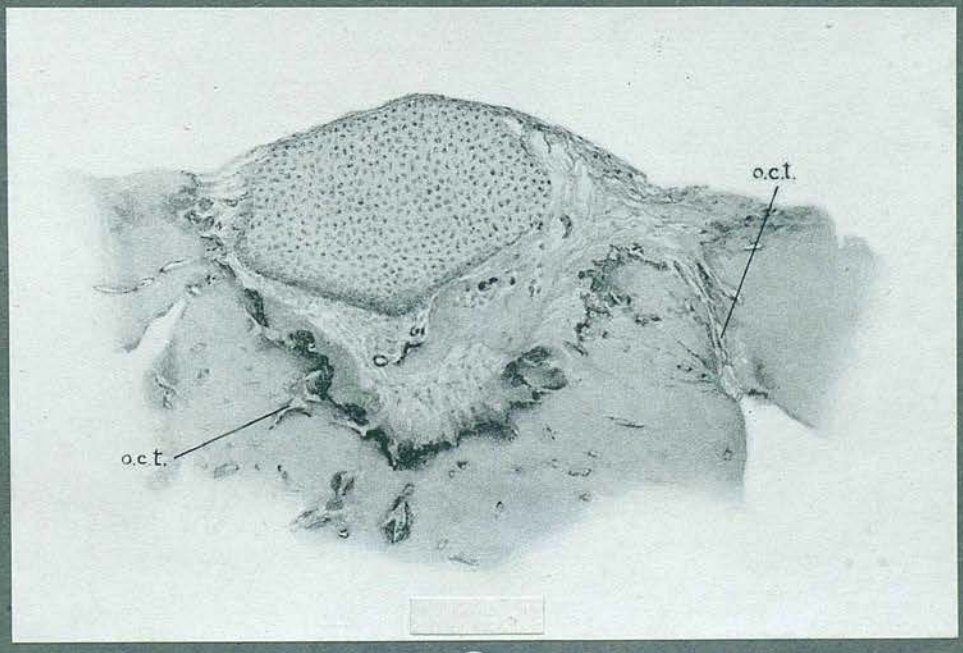

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H.B.F. del.

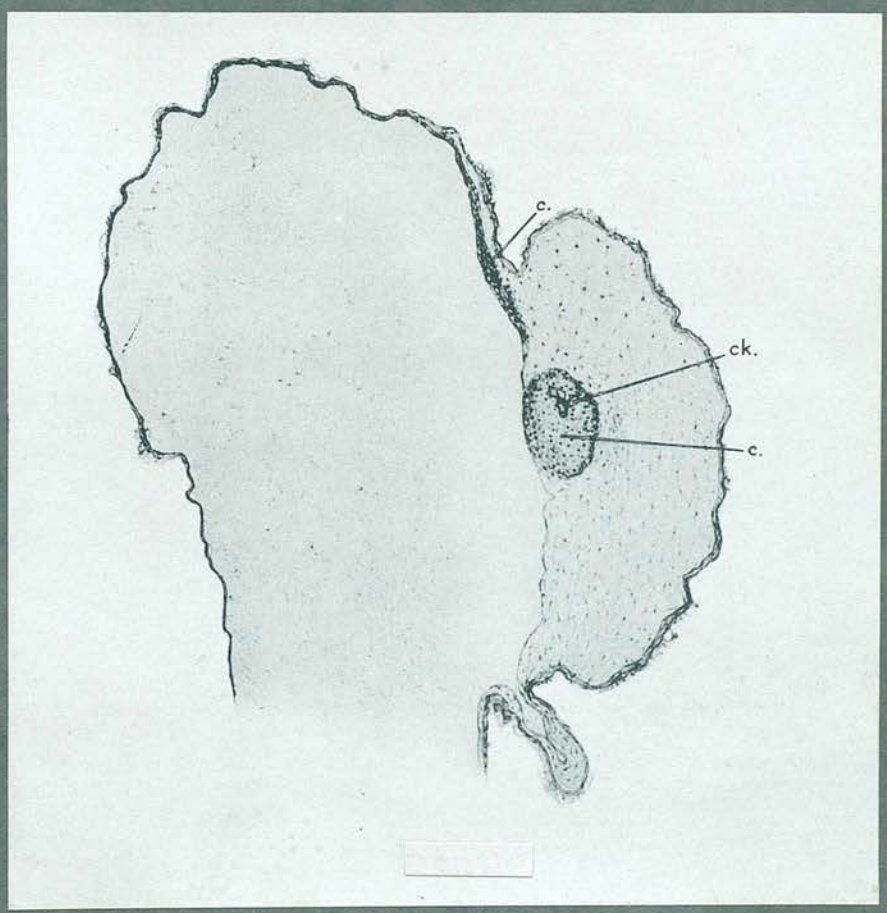
Plate XXVI.

Fig. 1. Early cyst-formation, owing (probably) to extreme dilation of blood vessels, in a limb-bud culture of 3 days' growth in medium 1. Note the terminal ridge of the thickened ectoderm. (iron haematoxylin)

Fig. 2. Portion of largest cyst obtained; about half the section is shown in this figure. (cf. Plate XXIII, Fig. 1 drawn to the same scale). Two nodules of cartilage are present in one of which is embedded a small piece of cork. This explant was cultivated for 20 days in medium I. (Mallory's stain).

PLATE XXVI.

1.



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H.B.F. del.

Plate XXVII.

Fig. 1. Normal 5½-day embryonic femur as dissected for explantation. Small processes representing the condyles, head and trochanter are present. (whole mount; dilute haematoxylin).

Fig. 2. Femur from 5½-day embryo after 3 days' cultivation in vitro. The condyles, head and trochanter are more distinct. (whole mount; dilute haematoxylin).

Fig. 3. Femur from 5½-day embryo after 9 days' cultivation in vitro. (whole mount; dilute haematoxylin).

Fig. 4. Femur from 5½-day embryo after 15 days' cultivation in vitro. (whole mount; dilute haematoxylin).

Fig. 5. Femur from 5½-day embryo after 21 days' cultivation in vitro. (whole mount; dilute haematoxylin).

Fig. 6. Femur from 5½-day embryo after 27 days' cultivation in vitro. Note the relatively normal appearance of this femur as compared with the normal specimen (Fig. 7). It will be seen that the increase in width is much greater in the region of the epiphyses than in the middle of the shaft; this is more marked than in the normal femur. (whole mount; dilute haematoxylin).

Fig. 7. Normal femur from 21-day embryo. (whole mount; dilute haematoxylin).


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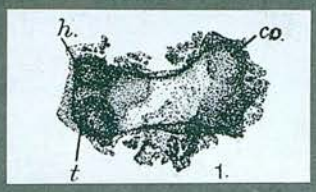
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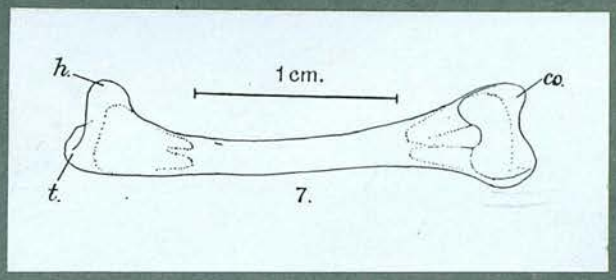
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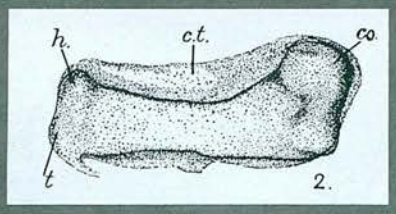
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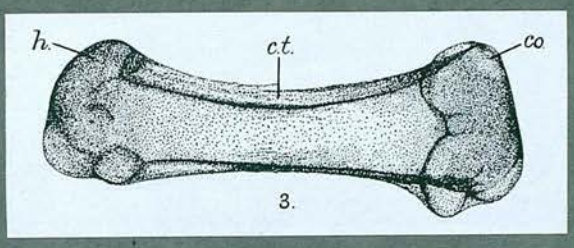
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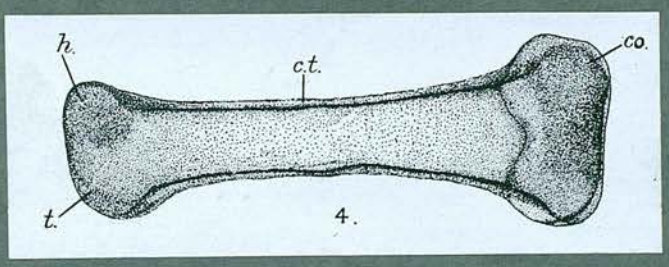
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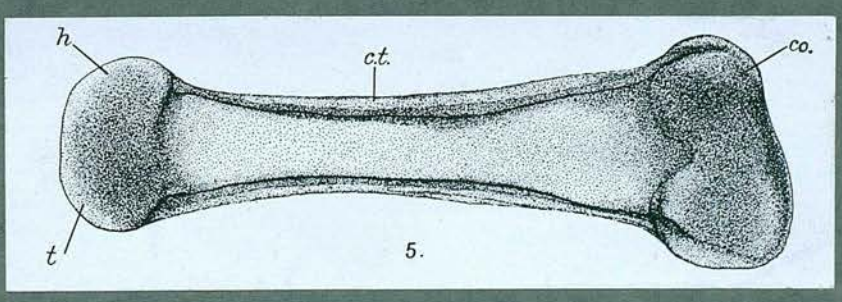
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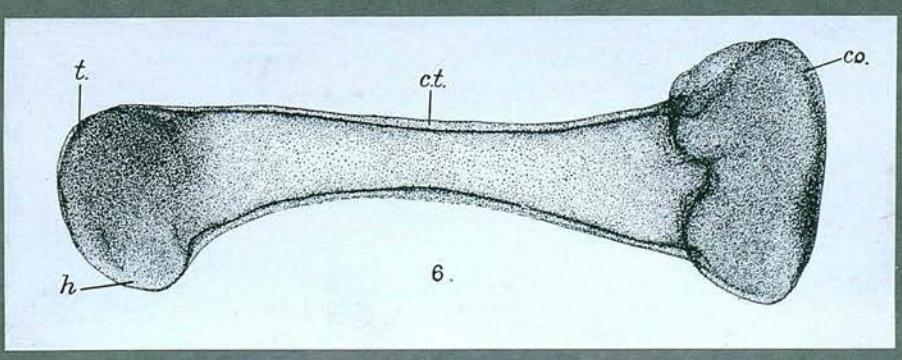
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Plate XXVIII.

Photographs of a living femur rudiment from a 4½-day embryo showing its enlargement and progressive anatomical development during cultivation for 14 days in a watch-glass. The photographs are taken from a cinema film made by Dr. R. G. Canti.

Fig. 1. Femur when first explanted. Faint indications of the condyles, head and trochanter are just distinguishable.

Fig. 2. Same after 48 hours' growth, immediately after the first transference to fresh medium.

Fig. 3. Same after 4 days' growth, immediately after the second transference. Note the slight opacity in the middle of the shaft, which probably represents early ossification.

Fig. 4. Same after 6 days' growth, immediately after the third transference.

Fig. 5. Same after 8 days' growth, immediately after the fourth transference.

Fig. 6. Same after 10 days' growth, immediately after the fifth transference. The intercondylar notch is now very distinct. Growth has become much less rapid.

Fig. 7. Same after 12 days' growth, immediately after the sixth transference.

Fig. 8. Same after 14 days' growth. Note the zone of outgrowth from the perichondrial tissue (as the preceding photographs were taken immediately after transferring the explant to fresh medium no zone of outgrowth is present).

PLATE XXVIII.

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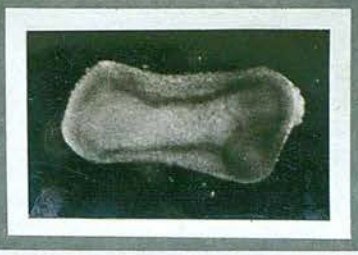

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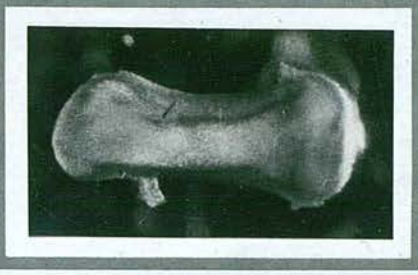

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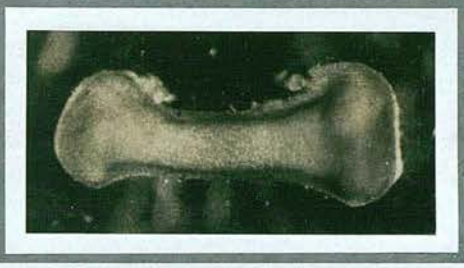

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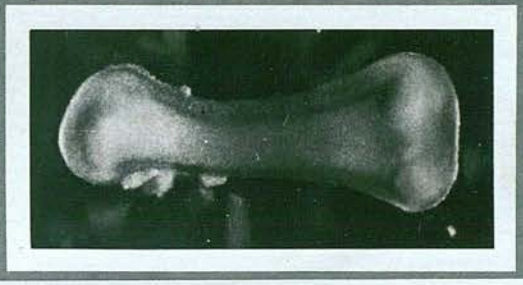

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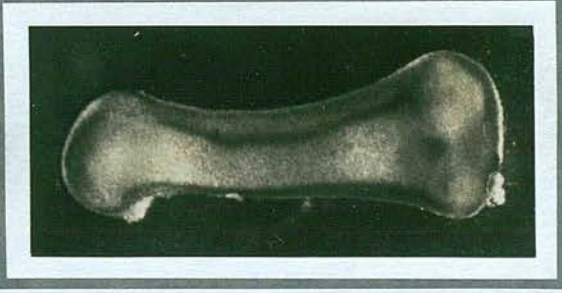

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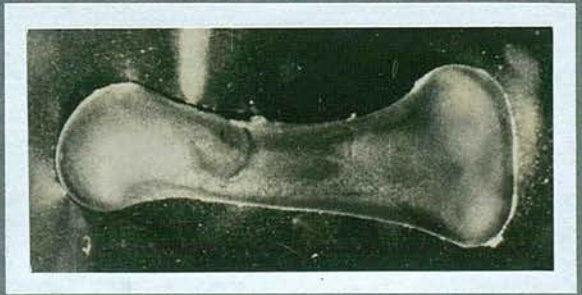

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