LXXXVII. THE GROWTH, DEVELOPMENT AND PHOSPHATASE ACTIVITY OF EMBRYONIC AVIAN FEMORA AND LIMB-BUDS CULTIVATED *IN VITRO*.

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Introduction.

It has been shown in previous work [Fell, 1928] that if terminal fragments of 8-day embryonic limb-cartilage with some adherent connective tissue were explanted *in vitro* they differentiated during subsequent cultivation into a diaphysial portion containing hypertrophied cells and an epiphysis containing smaller cells; in some cases bone was deposited around the diaphysial portion. If undifferentiated limb-bud mesenchyme were explanted [Strangeways and Fell, 1926; Fell, 1928] cartilage differentiated during cultivation, but with three exceptions [Fell, 1928] it failed to ossify and was of the small-celled type similar to that composing the normal avian epiphysis [Fell, 1925].

From these results it was clear that some at least of the chemical processes operating *in vivo* in the normal development of cartilage and bone also operated *in vitro* in explants of embryonic skeletal tissue. It seemed possible, therefore, that a study of the growth and differentiation of explants of embryonic cartilage and limb-bud mesenchyme could be correlated with a parallel study in the same material of certain chemical phenomena observed in normal and abnormal bones grown *in vivo*. It was thought that a combined morphological and biochemical study of such cultures might yield interesting information owing to the fact that experimental conditions can be more rigidly controlled *in vitro* than *in vivo* and tissue more easily handled and observed.

Bone and ossifying cartilage have been shown to contain a phosphatase [Robison, 1923], and this enzyme is regarded as an active agent in ossification, effecting the hydrolysis of certain phosphoric esters present in the blood [Kay and Robison, 1924; Martland and Robison, 1926] and thereby raising the concentration of inorganic phosphate in the tissue fluid so that calcium phosphate is deposited. In support of this hypothesis evidence was furnished

that the phosphatase is secreted in the region of the osteoblasts and hypertrophic cartilage cells. When severely rachitic bones were immersed in solutions of calcium hexosemonophosphate or calcium glycerophosphate, deposition of calcium phosphate took place in the periosteum and 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 [Robison and Soames, 1924, 1928]. Similarly, it was shown by examining the cartilages and bones of human embryos and young infants that, while the ossified portions of young normal bones contained phosphatase in high degree, the non-ossifying cartilage was in all cases devoid of the enzyme [Martland and Robison, 1924].

The present series of experiments was undertaken with two objects, (*a*) to study the correlation between the growth, development and phosphatase activity of isolated early embryonic femora and undifferentiated limb-buds cultivated *in vitro* and (*b*) to compare these processes in the explants with the corresponding processes in the normal embryonic limb.

Material and methods.

Material.

The material explanted consisted of the isolated femora of 5½ and 6-day fowl embryos and the entire limb-buds (anterior and posterior) of 3-day embryos.

Culture technique.

The tissue fragments were grown in a relatively large volume of medium contained in a watch-glass. The method[1] 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 30–40 cc. of sterile distilled water was pipetted into the 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-glasses containing fresh medium every 3 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 round 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 in the case of the femora minimised distortion during cultivation. The explants when loosened from the medium were sucked into a wide-bore pipette, washed in extract and with the same pipette placed on the surface of the clot in the new culture-vessel.

[1] This method is a modification of one tried by the late Mr. T. S. P. Strangeways.

The 3-day limb-buds after 3–4 days' growth usually became completely invested by ectoderm which subsequently keratinised. When the cultures were changed on the 6th day after explantation this layer of somewhat impervious keratin was dissected off with fine needles so that only a few small fragments remained attached to the connective tissue.

Chemical technique.

Phosphatase. Experiments were made to determine the most satisfactory method of estimating the phosphatase content of these small tissue fragments, having regard to the rather wide variations which might be anticipated both in their size and enzyme activity. For these control tests corresponding cartilages and bones from the right and left limbs of fowl embryos in varying stages of development were used. Extraction was carried out with chloroform water and with 0·1 % sodium bicarbonate solution for varying lengths of time, both the extracts and the extracted cartilage being separately tested for phosphatase by measuring the hydrolysis of sodium glycerophosphate under standard conditions. The results showed that extraction of the enzyme from these entire cartilages was very slow and incomplete, and that higher and more comparable values were obtained by allowing the cartilage itself to remain in the glycerophosphate solution during the test. The effect of varying the concentration of the substrate was also investigated. As the result of these experiments the following method was adopted for the estimation of phosphatase in the cartilages cultivated *in vitro*.

The tissue fragments were placed in small stoppered tubes containing 2 cc. distilled water saturated with chloroform, in which they were allowed to remain at ordinary temperature for 24 hours being meanwhile transferred from Cambridge to London. 1 cc. of 0·2 *M* sodium glycerophosphate (p_H 8·7) was then added to each tube and 1 cc. of the mixed solution immediately removed for the estimation of inorganic phosphate by the Briggs colorimetric method. The p_H of the remaining 2 cc. containing the tissue fragments was measured by means of the capillator, and, if necessary, was adjusted to 8·5–8·6. The tubes were then placed in a thermostat at 37° for 24 hours, after which the inorganic phosphate was again determined in 1 cc. of the solution, the increase representing the amount of hydrolysis effected by the tissue phosphatase. The 0-hour samples contained only traces of inorganic phosphate (0·001–0·002 mg. P), but some estimate of this was necessary in order to detect small amounts of hydrolysis or confirm its absence after 24 hours.

In calculating the phosphatase activity of the cartilage the assumption was made, based on control experiments, that only 20 % of the enzyme was present in the aqueous extract when the glycerophosphate was added, one-third of this 20 % being, therefore, removed in the 0-hour sample. Any error involved in this assumption will probably lie within the general limitations of the method. The increase of inorganic phosphate (mg. P) in 1 cc. × 2·14

gives, therefore, a measure of the hydrolysis effected by the tissue phosphatase in 24 hours at 37° and p_H 8·5. This value is denoted by A.

For the investigation of phosphatase production during growth of the embryonic bones *in vivo* a slight modification of the above method was considered advisable in view of the much larger size and phosphatase activity attained by the bones. Parallel estimations were carried out on femora from several embryos at each stage of development, the quantity of glycerophosphate being suitably increased for the larger bones. The 0-hour estimations were omitted, the necessary small corrections being found from control experiments with femora of similar age.

Weight. It was desired to correlate the phosphatase activity with the weight of the cartilage, but the determination of the moist weight was found to be impracticable owing to the very rapid evaporation from the surface of these minute fragments. It was also thought undesirable to dry the tissues before carrying out the phosphatase estimations, but a practicable compromise was found in the determination of the air-dry weight of the cartilage after the completion of the enzyme test. The tissues were removed from the glycerophosphate solution, rinsed with distilled water and the surface moisture removed with hardened filter paper. They were then placed on a coverslip, which had been previously weighed on a micro-balance, and were left exposed in the balance case until the weight became constant. Control tests carried out on the right and left limb-buds and cartilages of the embryos showed that considerable loss in weight occurred during the extraction, the loss being relatively greatest in the case of the smallest (and youngest) tissues. Thus, the air-dry weights of corresponding femora of a 6-day fowl embryo, the first untreated, the second after extraction and phosphatase test, were 0·061 mg. and 0·016 mg. respectively, while for the femora of a 21-day embryo the weights were 68·5 mg. and 60·0 mg. Some portion of this loss in weight is due to removal of the soluble constituents of the tissue fluids but autolysis of the tissue proteins may also account for some of the loss. No correction has been made for this loss in weight but the probable effect of such correction is indicated in discussing the results.

Histological technique.

Explants were removed from the watch-glasses at different periods of cultivation and were fixed in Zenker's fluid. The explanted femora required for a study of gross anatomy, after being fixed in Zenker's fluid and washed in water and in alcoholic iodine in the usual way, were stained in dilute haematoxylin, and were then dehydrated, cleared in xylene or clove oil and mounted whole in glass cells containing Canada balsam. Explants for histological investigation were 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.

PART I. THE GROWTH AND DEVELOPMENT *IN VITRO* OF ISOLATED FEMORA OF 5½-DAY AND 6-DAY EMBRYOS.

Growth.

The rate of growth of the femora during cultivation *in vitro* was studied by determining the increase in length at intervals of 3 days, the measurements being made by the aid of a micrometer eye-piece 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 Fig. 1 are plotted from the average lengths of the femora, those in which serious curvature developed

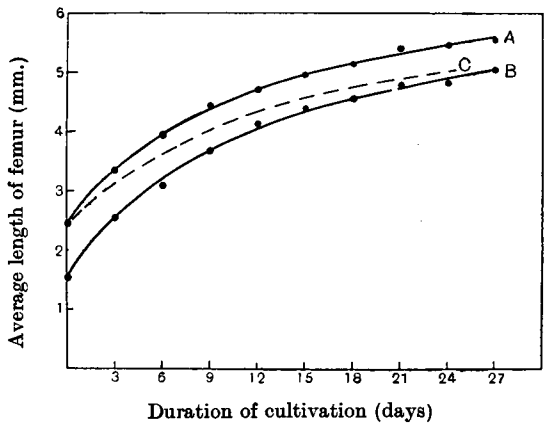

Fig. 1.

Curve A. Average increase in length of 6-day embryonic femora during 27 days' cultivation *in vitro*. Equation of curve: $y - 2{\cdot}47 = \frac{4{\cdot}5}{x+12}x$.

Curve B. Average increase in length of 5½-day embryonic femora during 27 days' cultivation *in vitro*. Equation of curve: $y - 1{\cdot}54 = \frac{5{\cdot}1}{x+12{\cdot}3}x$.

Curve C. Increase in length of 5½-day femora reckoned from the point at which their length was equal to that of the 6-day femora at 0-day. This length (2·47 mm.) was reached after 2·7 days' cultivation. Curve *C* is obtained from curve *B* by shifting each point 2·7 days to the left.

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 femora. Relatively to their initial size, however, the 5½-day femora grew much more rapidly, increasing in length by 67 % during the first 3 days against 36 % for the 6-day femora, while in 27 days' cultivation the increase amounted to 226 % and 124 % respectively. The average weights (air-dry, after extraction) of the 5½-day and 6-day femora after 27 days' cultivation *in vitro* were 0·244 mg. and 0·482 mg. 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 3 days *in vivo* exceeding that during 27 days *in vitro*. At 21 days, the chicks having hatched, the average length of the femora was 23·3 mm. while the average dry weight was 60·0 mg.

Morphological development.

(*a*) *Anatomical.* 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 maintained their characteristic shape to a remarkable extent (cf. Plate III, figs. 6 and 7).

When first explanted (0-day) the 5½-day femur of the normal embryo (Plate III, 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, projecting 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 III, 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 III, 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 III, figs. 5 and 6).

During cultivation *in vitro* the explanted femora did not increase in width to the same extent in all regions (Plate III, figs. 2–6). After the 3rd 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 had 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 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 only 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 IV, fig. 12).

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, this second factor could be largely eliminated by removing as much of the zone of outgrowth as possible when transferring the explants to fresh medium, and thus preventing the formation of an increasingly large mass of organised connective tissue around the femur.

The anatomical development of 6-day femora was also studied in the same way and gave very similar results to those obtained in the case of the $5\frac{1}{2}$-day specimens.

(*b*) *Histological.* 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 the $5\frac{1}{2}$-day specimens described in the preceding section.

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 for the investigation of phosphatase activity and the third being fixed and sectioned. In this way it was possible to study the correlation between histological differentiation and enzyme activity; the latter aspect of the experiment is dealt with in the next section.

Sections of one of these 6-day embryonic femora (0-day) showed a very early type of cartilage (Plate IV, fig. 9). 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 very 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 IV, fig. 10) showed a marked advance in development as compared with the control. The outline of the cartilage was much 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 [Fell, 1925] 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. Two 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 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 (possibly uncalcified) 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 IV, fig. 11) 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 epiphyses 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 IV, fig. 12) was found to be

much less well developed than that fixed after 12 days *in vitro*; 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 epiphyses. A loose, highly fibrous mass of connective tissue surrounded the shaft and was continued over the articular surfaces of the epiphyses as a thin, compact perichondrium.

The femur from the 21-day culture (Plate IV, fig. 13) 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 showed 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 V, fig. 15) the tissue was still relatively healthy. This degeneration was still more extensive in the oldest (27-day) femur (Plate IV, fig. 14) 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 Kossar'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 III, fig. 8).

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*. This phenomenon will be investigated further.

For purposes of comparison a series of normal embryonic femora were histologically examined. It was found that differentiation proceeded more rapidly *in vivo* 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 2 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 *in vivo* 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 begun 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 almost the same stage of differentiation. The zone of hypertrophic cells occupied rather less relative space in the explant than in the normal bone, 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 the 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.

Phosphatase.

The formation of phosphatase during growth *in vitro* of 6-day embryonic femora was investigated in parallel with the study of the histological development recorded above. Two femora from each culture were used for the phosphatase estimations but the values of A are given as the amounts of hydrolysis, in mg. P, effected by one femur in 24 hours under the specified conditions (p. 770). These values, plotted against the period of cultivation, are shown in Fig. 2, curve A.

The result of the 0-day test was completely negative, that is, the tissues before cultivation contained no phosphatase.

After 3 days' cultivation a definite though very small amount of phosphatase was found, $A = 0{\cdot}0026$; after 6 days', A had risen to 0·006; after 9 days' to 0·015 and after 27 days' to 0·135. An exception to the regular increase in phosphatase was shown by the 15-day culture, for which A was only 0·014 against 0·030 for the 12-day culture. It was noted in the previous section that the histological differentiation of the femur was much less advanced in the 15-day than in the 12-day culture.

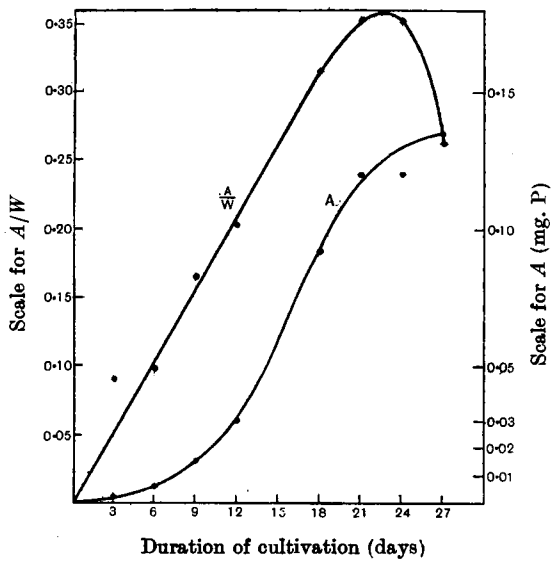

Fig. 2. Production of phosphatase during the development *in vitro* of femora from 6-day fowl embryos.

A = Phosphatase per femur; given as the amount of hydrolysis (mg. P) of sodium glycerophosphate in 24 hours at 37° and p_H 8·5.

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

(The values of A and A/W for the 15-day culture, which was exceptionally backward in development, fall far below the curves and have been omitted for the sake of clearness.)

Weight. During 27 days' cultivation the dry weight of one femur (after extraction) increased from 0·014 mg. to 0·52 mg. Judging from the data of the control experiments, the corresponding dry weights of the unextracted femora would have been about 0·05 mg. and 0·9 mg. respectively. The femora of the 15-day culture were slightly heavier than those of the 12-day culture and in respect of weight, therefore, were not so backward as in histological development and phosphatase synthesis.

A/W. The ratio A/W expresses the amount of hydrolysis, as mg. P, effected in 24 hours by 1 mg. of tissue (dry weight). If phosphatase production is proportional to tissue growth, this ratio should be constant, but, if the enzyme is formed by hypertrophic cartilage and osteoblasts, and not by cartilage of the

early, small-celled type, the ratio should vary according to the degree of histological differentiation attained. The values of this ratio, plotted against the period of cultivation, are shown in Fig. 2, curve A/W. The value rises from zero at 0-day to a maximum of 0·35 after 21 days, remains constant for the next 3-day period and then falls to 0·26 at 27 days. Correction for the loss in weight of the femora during extraction would reduce all values of A/W, this reduction being relatively greatest for the youngest explants. The shape of the curve, however, would not be materially altered. (Compare the two curves A/W and A/W_1 for normal femora in Fig. 3.)

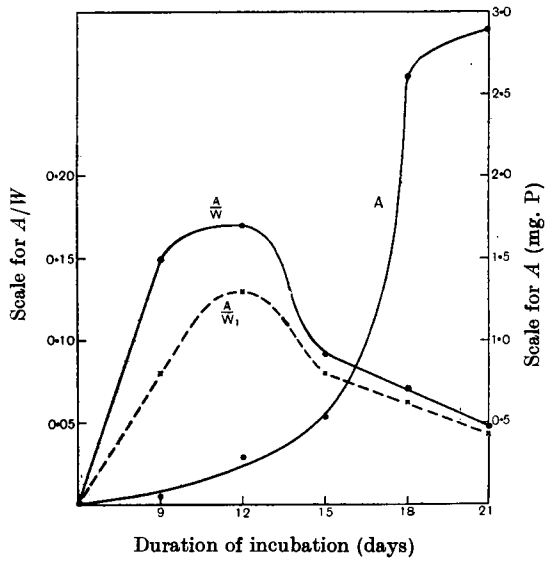

Fig. 3. Production of phosphatase in the femur of the embryonic fowl during normal development *in vivo*.

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).

In order to compare these results with the rate of growth and phosphatase production *in vivo*, femora of normal fowl embryos were examined after the eggs had been incubated for different periods (3-day intervals) from 6 to 21 days. Phosphatase estimations were carried out in triplicate on single femora, one femur from each embryo being thus tested while the femur of the opposite limb was dried and weighed without extraction.

The increase in length (1·5 mm. to 23·3 mm.) of these normal femora between the sixth and twenty-first day *in vivo* has already been discussed (p. 772).

Weight. The average weight of one femur, after extraction, increased from 0·016 mg. at 6 days to 60 mg. at 21 days; the weights of the corresponding unextracted femora were 0·06 mg. and 68·5 mg. respectively.

Phosphatase. The values of A are plotted in Fig. 3, curve A. The 6-day femora were again devoid of phosphatase but by the ninth day *in vivo* the value of A had reached 0·045, increasing to 0·285 at the twelfth day and 2·90 at the twenty-first day (after hatching). By the twelfth day, therefore, the length, weight and phosphatase content of these normal embryonic femora considerably exceeded the maximum values attained by the explanted 6-day femora after 27 days' cultivation *in vitro*.

A/W. The ratio of phosphatase to the dry weight (A/W) rose from zero at the sixth day to a maximum of 0·17 at the twelfth day, thereafter falling to 0·05 at the twenty-first day. The values of this ratio are plotted in Fig. 3, curve A/W. The curve is of similar type to that showing the variation of this ratio during growth *in vitro* (Fig. 2), but differs from the latter in two respects, (*a*) the maximum value of A/W is reached in 12 days *in vivo* as against 6 days *in vivo* + 21 days *in vitro*, (*b*) this value is only 0·17 against 0·35 *in vitro*. The first point of difference is sufficiently explained by the more rapid rate of growth *in vivo*, the second can probably be accounted for by the presence in the normal embryonic femora of solid calcium salts and of marrow, which increase the weight without corresponding increase of phosphatase.

The curve A/W_1 (Fig. 3) also shows the ratios of phosphatase to dry weight, but here the weight, W_1, is that of the corresponding unextracted femur from the opposite limb of the same embryo. Assuming that the two femora were equal in weight this curve shows the effect of correcting the values of A/W, as usually obtained in these experiments, for the loss in weight during extraction and test. It is seen that the general shape of the curve is not greatly altered.

PART II. THE GROWTH AND DEVELOPMENT *IN VITRO* OF ISOLATED LIMB-BUDS OF 3-DAY EMBRYOS.

Growth and histological development.

The 3-day limb-buds (0-day) used in this experiment varied slightly in size in different embryos, the largest consisting of small rounded projections of the body wall whilst the smaller rudiments were somewhat flatter and more ridge-like. The leg-buds of each embryo were larger than the wing-buds. Histological sections showed no trace of cartilage or muscle and the buds were composed of undifferentiated mesenchyme.

After 21 days' cultivation *in vitro* the buds had enlarged considerably, giving rise to irregular nodules of cartilage adhering to the fibrin clot by an extensive outgrowth of perichondrial connective tissue. One explant from each of the five watch-glasses was fixed and sectioned whilst the remainder were tested for phosphatase activity.

The histological structure of the explants was almost precisely similar to that previously described by Strangeways and Fell [1926] in the case of the 3-day limb-buds cultivated in tubes. No bone or hypertrophic chondroblasts

occurred and the explants consisted of one or more nodules of small-celled cartilage, surrounded by a layer of fibrous connective tissue in which islets of keratinised ectoderm were sometimes seen (Plate V, fig. 16).

Phosphatase.

Eleven explants were tested for phosphatase after 21 days' cultivation and gave a completely negative result. The total dry weight of the cartilage after extraction was 0·36 mg. so that the amount of material was ample for the detection of the enzyme even if the relative activity of the tissue were only one-tenth of that of the 6-day femora cultivated for a similar period. Negative results were similarly obtained from four other cultures from 3-day limb-buds, the period of cultivation ranging from 9 to 41 days.

These experiments, therefore, confirm the view that phosphatase is not formed by cartilage of the small-celled type.

Discussion.

The self-differentiating capacity of the avian limb has already been demonstrated from the anatomical standpoint by Murray [1926] and Selby and Murray [1928]. These workers grafted limb-buds and fragments of limb-buds from embryos of 3–5 days on the chorio-allantoic membranes and found that the gross structure of the skeletal tissue which developed in the grafts was remarkably normal. Their results led them to conclude that the limb-rudiment of 3–5 day fowl embryos constitutes a self-differentiating, mosaic system. The experiments recorded in the present communication have shown that not only a fragment of the limb-bud but also a single cartilage segment, such as the femur, has a remarkable capacity for self-differentiation. When explanted *in vitro* by the technique described above, the early embryonic femur is completely deprived of a vascular system, nervous connections, adjacent skeletal structures and, if we except the few isolated muscle fibres scattered in the surrounding connective tissue, of association with the limb musculature. Nevertheless, as the results of the present investigation have shown, the explanted femur during cultivation *in vitro* is able to continue its anatomical development on the same general lines as in the normal limb, and at the same time undergoes a histological differentiation which is correlated with at least one of the chemical activities of the normal ossifying cartilage. The femora cultivated *in vitro* differed from normal embryonic femora of the same age mainly in their much smaller size, in having relatively larger epiphyses, in the absence of a marrow cavity and in being encased by a considerably thinner and more compact sheath of periosteal bone. All these differences may probably be attributed, in part at least, to the absence of certain mechanical and nutritional factors normally supplied by the blood vessels.

Throughout the whole period of growth *in vitro* the raw material required by the cells for the building up of new tissue must be obtained from the

culture medium by diffusion through the surface of the tissue fragment. But as the femur increases in size so the ratio of the surface area to the volume of tissue diminishes. To what extent the effect of this diminishing ratio on the food supply would account for the falling-off in the growth rate cannot be judged from our data. The curves shown in Fig. 1 give the increase in length only and these femora do not increase in the same proportion in all directions. Certain points, however, may be noted from these curves.

During cultivation the smaller 5½-day femora increased in length more rapidly than the larger 6-day femora, both rates being measured from 0-day. If the increments are calculated in percentages of the 0-day lengths the difference is still more strikingly in favour of the 5½-day femora. When, however, after 2·7 days *in vitro* the 5½-day femora had reached the same length, 2·47 mm., as the 6-day femora at 0-day, their growth rate, calculated from this point (2·7 days), was actually slower than that of the 6-day femora calculated from 0-day. This is seen by comparing curves *A* and *C*. Curve *C* shows the growth rate of the 5½-day femora reckoned from 2·7 days, that is, each point on curve *B* has been shifted 2·7 days to the left.

The regularity of these growth curves is interesting. Curves *A* and *B*, which fit the observed points very closely, are actually drawn from the equations of the hyperbolic curves:

$$A.\ y - 2{\cdot}47 = \frac{4{\cdot}5}{x+12}x. \qquad B.\ y - 1{\cdot}54 = \frac{5{\cdot}1}{x+12{\cdot}3}x.$$

For $x = \infty$ the values of y are 6·97 for curve *A* and 6·64 for curve *B*. These values of y correspond with the lengths of the femora at which growth ceases. For the working out of these equations we are indebted to Professor J. C. G. Ledingham, F.R.S.

The contrast between the histological development *in vitro* of the 6-day femora and the 3-day limb-buds is interesting. As in previous experiments [Strangeways and Fell, 1926; Fell, 1928] no differentiation into structures corresponding to the epiphysis and diaphysis took place in the cartilage formed *in vitro* from originally undifferentiated 3-day mesenchyme. On the other hand, the explanted 6-day femora which, at the time of explantation, were composed of very simple, early cartilage, developed epiphyses and diaphysis during the same period of cultivation. Thus, under the conditions of the experiments, the self-differentiating capacity of the 6-day femur was considerably greater than that of the 3-day limb-bud. The reasons for this fact are being investigated.

It has been shown that these femora, although entirely devoid of phosphatase when removed from the 6-day embryo, synthesise this enzyme during cultivation *in vitro* and that the production of the phosphatase follows very closely the progress of histological differentiation in the cartilage. Thus, the first appearance of phosphatase in the explanted 6-day embryonic femora, after 3 days' cultivation, coincided with the first appearance in the cartilage

of a zone of enlarged cells. The extension of this zone and increased enlargement of the cells in the later cultures were accompanied by a corresponding increase in the amount of phosphatase. The parallelism between enzyme synthesis and histological differentiation is, however, most clearly shown by considering the ratio of phosphatase to the dry weight of the femur (A/W). With the increasing proportion of hypertrophic cells and the gradual development of periosteum and periosteal bone observed up to the twenty-first day of cultivation there was found an increase in the value of this ratio, A/W, from zero to a maximum of 0·35. The degeneration which was revealed in the 24-day and still more extensively in the 27-day cultures was accompanied by a halt in the increase of this ratio followed by a fall to the value 0·26 at 27 days. Further evidence of this parallelism was provided by the chance inclusion in the series of one culture (15-day) in which, for some reason, the histological differentiation was very backward and for which the value A/W proved to be correspondingly low. On the other hand, it was shown that the cartilage formed during cultivation *in vitro* of 3-day embryonic limb-buds is entirely of the small-celled (undifferentiated) type and that even after 21 days' cultivation this cartilage synthesises no phosphatase.

The results of these experiments on the development *in vitro* of skeletal tissue confirm the view that phosphatase is synthesised by cartilage, only if hypertrophic cells are present.

The values for weight and phosphatase measured in this investigation and quoted above are, in some cases, of such small dimensions that it would be unjustifiable to claim for them any high degree of accuracy. For the older explanted femora and normal embryonic bones the amounts of hydrolysis were large enough for accurate estimation, but some caution is still needed in making the assumption that these amounts are proportional to the phosphatase present in the tissue. It is believed, however, that the possible errors, even on a generous scale, would not affect the significance of the results or invalidate the conclusions drawn from them.

The very considerable phosphatase activity attained by the explanted 6-day femora may be better appreciated if expressed in the following terms. The maximum value of A/W was 0·35, found after 21 days' cultivation *in vitro*. Under the conditions of the test, therefore, the amount of inorganic phosphate set free from the phosphoric ester by this femur in 24 hours was equivalent, in terms of calcium phosphate, $Ca_3(PO_4)_2$, to 1·75 times the dry weight of the femur. The corresponding values for the normal embryonic femora are 0·85 at 12 days and 0·24 at the time of hatching, or 0·65 and 0·22 if calculated on the dry weights of the unextracted femora.

SUMMARY.

1. The isolated femora of 5½-day and 6-day fowl embryos were found to have a remarkable capacity for self-differentiation.

2. During cultivation in a watch-glass the 5½-day femora increased to more than three times their original length.

3. During cultivation the gross anatomy of the femora remained comparatively normal.

4. The explanted femora underwent a remarkably normal histological differentiation. The three characteristic cell zones—small-celled epiphysis, intermediate belt of flattened cells and region of hypertrophic cells—appeared in the cartilage during cultivation. Periosteal bone and a periosteal membrane were also formed.

5. During cultivation *in vitro* the isolated femora synthesised a phosphatase; no phosphatase was present in the tissue at the time of explantation and the gross amount formed increased with the age of the culture.

6. The ratio of phosphatase to the dry weight of the femur increased during cultivation from zero to a maximum value and then declined. The increase corresponded with the progress of histological differentiation and the decline with degeneration. The maximum value of this ratio was higher than that found for normal embryonic femora.

7. Undifferentiated 3-day embryonic limb-buds cultivated *in vitro* gave rise to small-celled cartilage only.

8. Such explants after cultivation were completely devoid of phosphatase, thus confirming the view that the enzyme is formed by cartilage, only if hypertrophic cells are present.

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DESCRIPTION OF PLATES.

The outline of all the figures was drawn with the aid of either a camera lucida or a Leitz projection prism. All the figures on Plate III except Fig. 7 are drawn to the same scale and all the figures on Plate IV are to the same scale.

b., bone; *c.*, condyle; *ca.*, calcification; *c.t.*, connective tissue; *ep.*, epiphysis; *f.l.*, fibrous layer; *h.*, head; *h.c.*, hypertrophied chondroblast; *k.*, keratin; *my.*, myoblasts; *o.l.*, osteoblastic layer; *p.*, periosteum; *s.c.c.*, small-celled cartilage; *t.*, trochanter; *z.f.c.*, zone of flattened cells; *z.h.c.*, zone of hypertrophied cells.

PLATE III.

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

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

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

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

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

Fig. 6. Femur from 5½-day embryo after 27 days' cultivation *in vitro* (whole mount). 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.

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

Fig. 8. Femur from 5½-day embryo after 27 days' cultivation *in vitro* (whole mount). This specimen has been stained by von Kossar's silver nitrate method. Note the sheath of calcified bone enclosing the middle region of the shaft.

PLATE IV.

Fig. 9. Section of normal femur from 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. (Safranine and picro-indigo-carmine.)

Fig. 10. 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. (Safranine and picro-indigo-carmine.)

Fig. 11. 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's stain.)

Fig. 12. Section of a 6-day embryonic femur after 15 days' cultivation *in vitro*. This 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.)

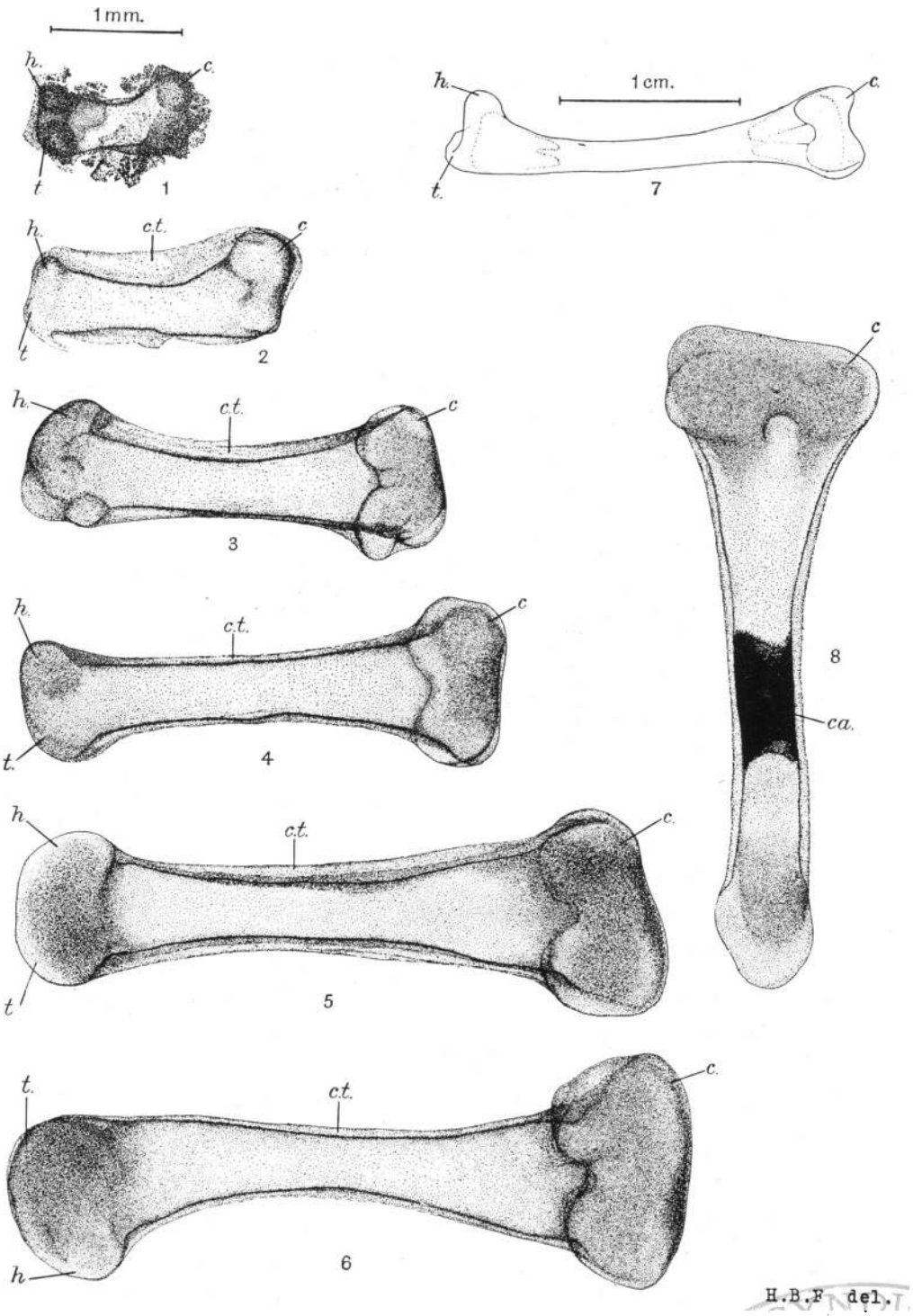
Fig. 13. Section of a 6-day embryonic femur after 21 days' cultivation *in vitro*. The epiphysis is sharply marked off from the diaphysis by the zone of flattened cells; a sheath of bone invests the extensive zone of hypertrophied chondroblasts. (Safranine and picro-indigo-carmine.)

Fig. 14. Section of a 6-day embryonic femur after 27 days' cultivation *in vitro*. (Safranine and picro-indigo-carmine.)

PLATE V.

Fig. 15. Section of a 6-day embryonic femur after 24 days' cultivation *in vitro*. Hypertrophied chondroblasts, bone and the two-layered periosteum are seen. (Safranine and picro-indigo-carmine.)

Fig. 16. Section of a 3-day embryonic limb-bud after 21 days' cultivation *in vitro*. An irregular mass of small-celled cartilage has been formed during cultivation; no bone is present. (Mallory's triple stain.)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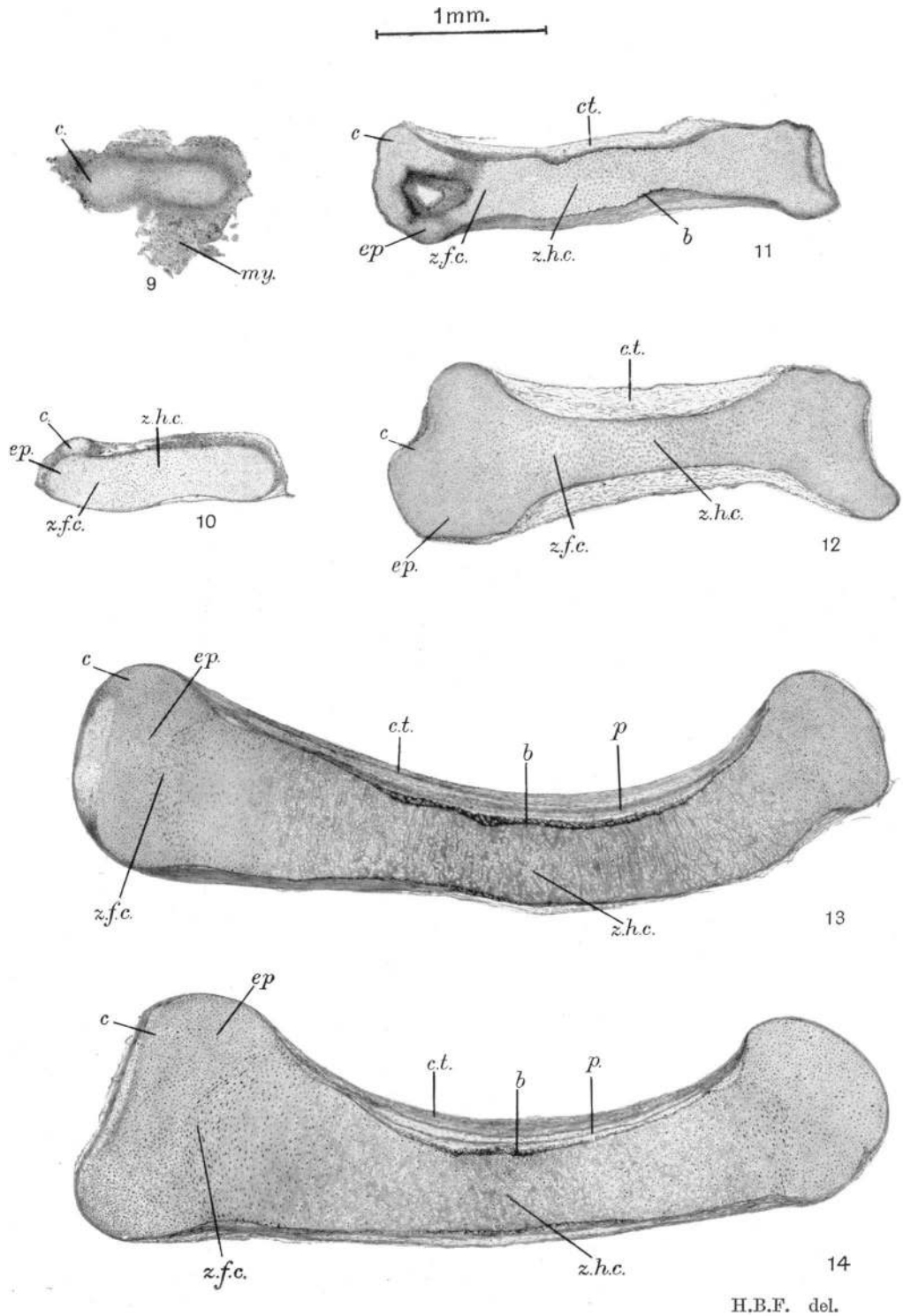
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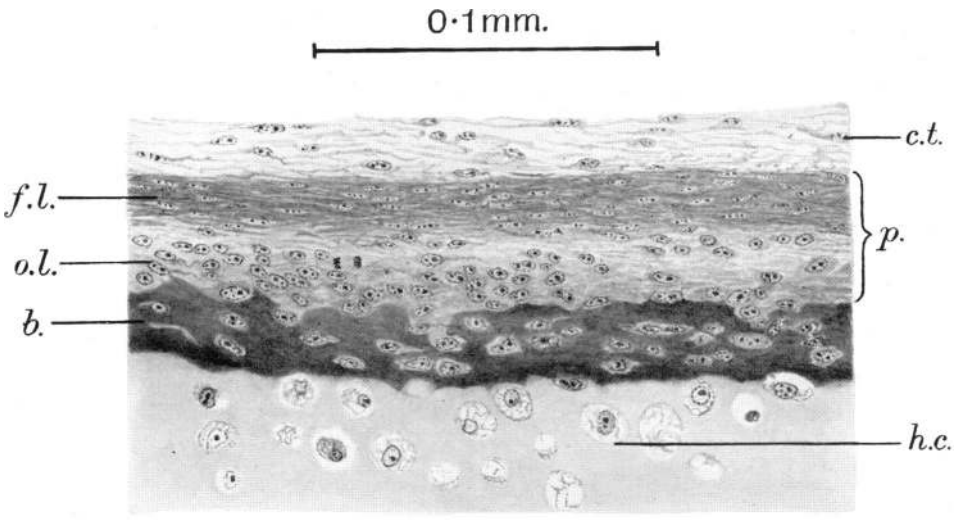

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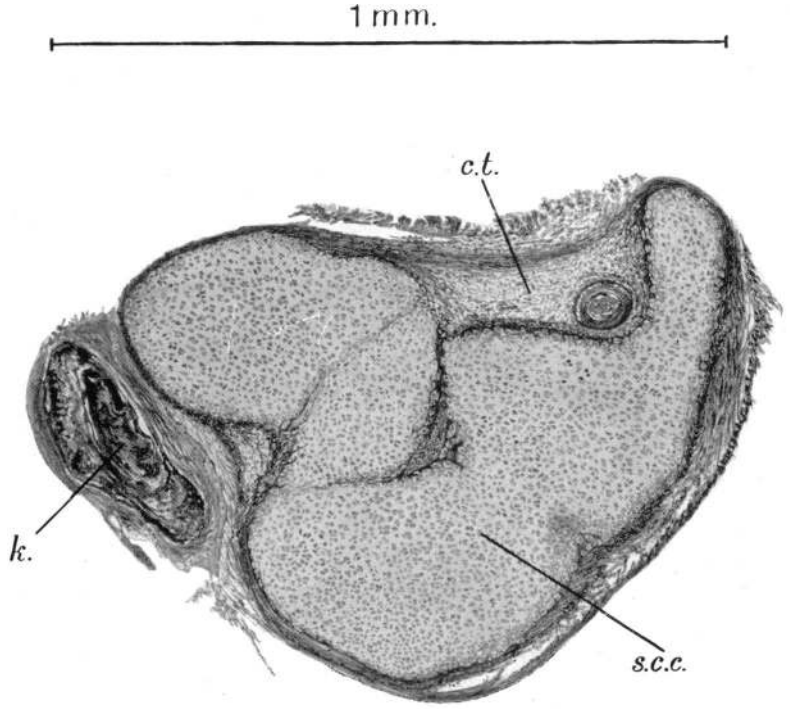

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