CCXV. THE DEVELOPMENT AND PHOSPHATASE ACTIVITY *IN VIVO* AND *IN VITRO* OF THE MANDIBULAR SKELETAL TISSUE OF THE EMBRYONIC FOWL.

BY HONOR BRIDGET FELL
(*Senior Beit Memorial Research Fellow*)

AND ROBERT ROBISON.

*From the Strangeways Research Laboratory, Cambridge, and the
Biochemical Department, Lister Institute, London.*

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INTRODUCTION.

IN a previous communication [Fell and Robison, 1929] observations were recorded on the development and phosphatase activity of embryonic femora and limb-buds cultivated *in vitro*. It was found that the isolated femur of the 5-6 day embryonic fowl continued both its anatomical and histological development when explanted *in vitro*, underwent periosteal ossification and also synthesised a phosphatase which normally occurs in considerable quantities *in vivo* in bone and ossifying cartilage [Robison, 1923]. Explants of the 3-day mesenchymatous limb-bud, on the other hand, gave rise to cartilage of a different type which neither ossified nor synthesised phosphatase.

As a continuation of these earlier experiments it was decided to investigate the histology, development and phosphatase activity *in vivo* and *in vitro* of two other types of skeletal tissue: (a) cartilage which normally does not ossify, and (b)¹ membrane bone, *i.e.* bone which develops in a membrane of connective tissue and is not deposited on the surface of the cartilage as in the case of a so-called "cartilage-bone" such as the femur.

The lower jaw of the embryonic fowl provided admirable material for such a study. In a late fowl embryo the mandible consists of a rod of cartilage, known as Meckel's cartilage, which is surrounded by a sheath of membrane bone. Meckel's cartilage only ossifies in the articular region and the greater part of it persists, until the end of development, as an unossified cartilaginous rod.

The chief objects of the present study were as follows.

(1) To find whether any histological difference exists *in vivo* between non-ossifying cartilage such as the distal part of Meckel's rod and ossifying cartilage such as the palato-quadrate.

¹ These experiments were undertaken at the suggestion of Prof. J. T. Wilson.

(2) To determine whether the non-ossifying Meckel's cartilage synthesises phosphatase.

(3) To investigate the histological development and phosphatase activity of Meckel's cartilage when isolated from its normal environment and explanted *in vitro*.

(4) To compare the histological behaviour and phosphatase activity of normal and explanted mandibular osteogenic mesoderm.

MATERIAL AND METHODS.

Material.

Fowl embryos and chicks were used throughout the investigation. The normal material examined was obtained from embryos and chicks of various ages up to the 13th day after hatching. The explants were taken from 5½ and 6-day embryos.

Culture technique.

The cultures of Meckel's rods, palato-quadrates and femora were made by the watch-glass method described in our previous communication [Fell and Robison, 1929]. 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 3 days but better results were obtained when the medium was changed every 48 hours.

The watch-glass method was found to be unsuitable for the cultivation of the mandibular mesoderm owing to the very diffuse habit of growth of this tissue and its close adherence to the fibrin clot which made transference to fresh medium difficult. Large hanging-drop preparations (1¼ inch square coverslips, 3" × 1½" hollow-ground slides) were therefore used. The explants were grown in equal parts of plasma and embryo extract. In the earlier experiments the tissue was subcultivated every three days but better results were obtained in the later experiments by transplanting to fresh medium every 48 hours. During subcultivation the central region of the explant 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.

Histological technique.

Some specimens of the normal jaw and 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 cleared in cedar wood oil or (in the case of the cultures) mounted in Farrant's medium. Many normal specimens and explants were serially sectioned. Some of the osteogenic mesoderm cultures were fixed in Flemming's solution without

acetic acid, but the rest of the material, both normal and explanted, was fixed in Bouin's or Zenker's solution. Sections were stained with Mallory's triple stain or with safranin and picro-indigocarmine.

All the explants of osteogenic mesoderm which were sectioned were cut in a plane at right angles to that of the coverglass. The explants were not removed from the coverglass 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.

Chemical technique.

In the previous investigation of the development of phosphatase activity in relation to the histological differentiation of embryonic limb-buds and femora grown *in vitro* and *in vivo* it was realised that the ratio of phosphatase activity to the dry weight of the tissue (A/W) was of even greater significance than the activity (A) itself. The technique employed was as follows: the phosphatase activity was first determined by measuring the amount of glycerophosphoric ester hydrolysed in 24 hours at 37° and p_H 8.6, after which the tissues were removed from the solution of glycerophosphate, rinsed in distilled water, allowed to dry in air and weighed on a micro-balance. Determination of the dry weight after measurement of the phosphatase involved a certain error, since the tissues during immersion in the glycerophosphate solution underwent some autolysis with consequent loss of weight, which was relatively greater in the early embryonic tissues than in those of later development. It has been shown [Martland and Robison, 1929] that an aqueous extract of bone phosphatase can be evaporated to dryness in an evacuated desiccator and the dry residue stored for many weeks without any loss of activity. It therefore seemed possible that the small embryonic tissue fragments and explants might be dried and weighed before estimating the phosphatase without affecting either the phosphatase content or the accuracy of its estimation. Comparative tests were carried out by both methods on corresponding bone rudiments from the right and left limbs of avian embryos at different stages of development. The values of A found by the two methods were in satisfactory agreement, and the second method was accordingly adopted for the present study. The dry weights of the tissues so determined were always greater, and the values of A/W consequently smaller, than those found by the previous method.

Details of the technique employed are as follows. The embryonic tissue or explant was placed for a moment on a piece of hardened filter paper to remove surface fluid, then transferred to a coverslip and dried for a few hours in an evacuated desiccator. The coverslips with the adherent tissues were packed in

glass tubes and transferred from Cambridge to London. They were weighed on a micro-balance after being left in the balance case until the weight remained constant. The tissues were then removed from the coverslip with a sharp knife and transferred to a small stoppered tube containing 2 cc. of water, together with a very small drop of chloroform. By reweighing the coverslips the air-dry weight (W) of the tissue was found. The tissues were kept in the chloroform water at room temperature for 3 days; 1 cc. of a 0.2 M solution of sodium glycerophosphate (p_H 8.7) was then added, together with another small drop of chloroform. The further procedure for the estimation of phosphatase and the method of calculating the value of A were the same as described in the previous paper [Fell and Robison, 1929, p. 769].

It should be noted that, although the value of A is quoted in the tables to 3 places of decimals, a value of 0.001 corresponding with an estimated increase in inorganic phosphate of only 0.0005 mg.P. is almost beyond the limit of experimental certainty. For this reason the more important value A/W is given only to 2 places of decimals, and when shown as less than 0.01 may in some cases have been zero.

PART I. HISTOGENESIS AND PHOSPHATASE ACTIVITY OF THE NORMAL MANDIBULAR SKELETON.

Histogenesis.

As previously stated, the mandibular skeleton of a normal late embryo (Plate VII, fig. 1) consists of Meckel's cartilage, surrounded by a sheath composed of four membrane bones—the angulare, supra-angulare, operculare and dentale [Lillie, 1919, p. 433]. Meckel's cartilage itself is not ossified except at the proximal end where it gives rise to a typical cartilage bone—the articulare.

The histogenesis of the cartilage and bone of the lower jaw was investigated in a series of 28 normal embryos ranging in age from the 6th day of incubation up to the time of hatching.

In a well developed 6-day embryo, Meckel's cartilage which is already very distinct (Plate VII, fig. 2) 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 (Plate VII, fig. 2). The rod increases in size partly by apposition 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, and the general histological structure of the ossifying articular cartilage closely resembles that of an ossifying long-bone rudiment from one of the limbs [Fell, 1924]. The long rod composing the distal part of Meckel's cartilage (Plate VII, fig. 1) contains somewhat flattened but irregularly shaped chondroblasts which 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.

In a 2-day post-embryonic mandible the histological structure of Meckel's cartilage is essentially the same as in a 15-day specimen. The ossification of the articulare is much more advanced, but the elongated distal part of the cartilage shows no sign of cell hypertrophy or periosteal ossification.

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 (Plate VII, fig. 1).

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 cytoplasm 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. 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.

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.

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

Phosphatase.

The phosphatase activity of Meckel's cartilage and of membrane bone from normal embryonic and post-embryonic mandibles at different stages of development is shown in Table I. The specimens of membrane bone did not in

Table I. *Phosphatase activity of Meckel's cartilage and membrane bone from mandibles of fowl embryos and of young chicks.*

Age of embryo (days)	No. of specimens ($\frac{1}{2}$ -mandibles)	Meckel's cartilage			Membrane bone		
		W mg.	A mg. P	A/W	W mg.	A mg. P	A/W
6	6	—	—	—	1.20	0.032	0.03
6	6	—	—	—	1.06	0.024	0.02
6	8	0.12	0.001	<0.01	—	—	—
7	6	0.27	0	0	1.38	0.042	0.03
8	2	0.09	0	0	0.52	0.007	0.01
9	2	0.15	0	0	0.57	0.035	0.06
9	2	0.20	0	0	0.75	0.055	0.07
10	2	0.20	0.001	<0.01	1.10	0.146	0.13
11	2	0.25	0.001	<0.01	1.54	0.274	0.18
11	1	0.18	0	0	1.03	0.198	0.19
13	1	0.25	0	0	2.08	0.474	0.23
15	1	0.35	0	0	2.87	0.546	0.29
Age of chick (days)							
13	1	0.62	0	0	9.04	0.68	0.08
13	1	0.60	0	0	12.77	0.92	0.07
13	1	0.94	0.011	0.01	14.20	0.74	0.05

all cases represent the entire bone of the half jaw, and the articular end of Meckel's cartilage was always rejected. It has been shown in the preceding section that during its embryonic and early post-embryonic development the distal part of Meckel's cartilage shows no sign of cell hypertrophy and does not ossify. It is seen from the table that this cartilage does not synthesise phosphatase. Specimens were examined from embryos of ages ranging from 6 to 15 days and also from 13-day chicks. In the majority of these no trace of phosphatase activity was detected and in no case did the value of A/W exceed 0.01.

Phosphatase tests on the mandibular mesoderm or membrane bone from the same embryos gave very different results, confirming the conclusions drawn from tests on human embryonic parietals [Martland and Robison, 1924] that

membrane bone as well as cartilage bone synthesises phosphatase. The 6-day and 7-day embryonic mandibles showed small but definite phosphatase activity corresponding with the presence of osteoblasts even at this early stage (*vide* p. 1909). Exceptionally, the activity of the 8-day specimen was somewhat lower, but at 9 days there was a marked increase in the value of A/W which corresponded with the rapid development of osteoid tissue noted in the preceding section. From this stage A/W rose rapidly to the high value of 0.29 in the 15-day embryos. By the 13th day of post-embryonic life the value of A/W had fallen very considerably, which can readily be accounted for by the increased proportion of inactive material (calcium salts and marrow) then present.

The maximum value of A/W found for normal embryonic femora [Fell and Robison, 1929, p. 778] was reached at the 12th day and was only 0.13 (based on the weight before extraction). The femur and another cartilage bone, the palato-quadrates, which articulates with the lower jaw, provided convenient controls in the study of the development *in vitro* of Meckel's cartilage (see next section). For this reason the phosphatase activity of normal embryonic palato-quadrates was investigated and the results are shown in Table II. The value of A/W was only 0.01 at 7 days but had risen to 0.18 in one of the 13-day specimens.

Table II. *Phosphatase development in normal embryonic palato-quadrates.*

Age of embryo (days)	No. of specimens	Palato-quadrates		
		W mg.	A mg. P	A/W
7	3	0.40	0.004	0.01
12	1	0.47	0.031	0.07
13	1	0.92	0.128	0.14
13	1	0.94	0.169	0.18
17	1	2.70	0.334	0.12

PART II. THE GROWTH, DEVELOPMENT AND PHOSPHATASE ACTIVITY OF MANDIBULAR SKELETAL TISSUE DURING CULTIVATION *IN VITRO*.

A. MECKEL'S CARTILAGE.

It seemed possible that the difference in histological structure and phosphatase activity between the non-ossifying portion of Meckel's cartilage and the cartilaginous rudiment of so-called cartilage bones such as the palato-quadrates and femur 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*.

Growth and histological development.

Meckel's cartilages isolated from 6-day embryos increased considerably in both length and weight during cultivation *in vitro*, although this increase was very much less than in the case of the explanted femur.

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. Four rods were dried and weighed at the beginning of the experiment.

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 and Robison, 1929] was 2.47 mm. and during 21 days' cultivation this length increased by 118 %.

The average dry weight of the control Meckel's cartilages was 0.048 mg. and that of the explants after 21 days' cultivation was 0.091 mg. The increase during growth *in vitro* thus amounted to 90 % of the original weight.

In order to study the histological development and phosphatase activity of the non-ossifying part of the mandibular cartilage when cultivated *in vitro*, a number of 6-day embryonic Meckel's rods from which the articular end had been removed were explanted in watch-glasses. An equal number of cultures of either the palato-quadrates or the femora from the same embryos were made as controls. In most of the experiments some of the explants were fixed and sectioned after 18-20 days' cultivation and the remainder were tested for phosphatase. In one experiment explants of Meckel's cartilage and of the palato-quadrates were fixed after 2, 4, 8 and 16 days' growth *in vitro* in order to observe the histological structure at different stages of development. 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.

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

In a 6-day embryo the palato-quadrates 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 VII, fig. 3) usually by a hook-like bending of one or both ends. This distortion of the explants was probably due to the increasing toughness of the fibrous capsule by which the growing cartilage was surrounded.

There is no conspicuous histological difference between the quadrate and the Meckel's rod in the 6-day embryo (Plate VII, fig. 2). 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 explant. 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 quadrates 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 VII, fig. 4) showed the three definite regions of small, flattened and hypertrophic chondroblasts characteristic of normal "cartilage-bone" rudiments, whilst all the Meckel's cartilages (Plate VII, fig. 3) contained only irregularly shaped cells similar to those seen in Meckel's cartilage of a normal late embryo (Plate VII, fig. 1). 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.

Phosphatase.

The results of phosphatase tests on explants of the distal part of Meckel's cartilage cultivated 17–19 days *in vitro* are shown in Table III, and are there compared with the corresponding values for explants of palato-quadrates or femora from the same embryos cultivated *in vitro* for the same length of time. The results are in entire agreement with the histological differences described in the preceding section.

Table III. *Phosphatase development in cultures of Meckel's cartilages compared with that in palato-quadrates and femora of 6-day fowl embryos.*

Exp.	Duration of cultivation (days)	No. of explants	Meckel's cartilages			Palato-quadrates		
			W mg.	A mg. P	A/W	W mg.	A mg. P	A/W
1	0	6	0.18	0.001	<0.01	0.24	0.002	<0.01
1*	17	18	0.74	0.003	<0.01	1.00	0.099	0.10
2	0	8	0.12	0.001	<0.01	0.16	0	0
2†	19	18	1.16	0.003	<0.01	1.48	0.48	0.33
Femora								
3	19	3	0.17	0.001	<0.01	1.22	0.154	0.13
3	19	3	0.20	0.001	<0.01	1.03	0.081	0.08
3	19	3	0.19	0.001	<0.01	1.12	0.084	0.08
3	19	3	0.25	0.001	<0.01	—	—	—
4	19	18	0.91	0	0	—	—	—
4	19	3	—	—	—	0.81	0.035	0.04
4	19	3	—	—	—	1.07	0.162	0.15
4	19	3	—	—	—	0.93	0.093	0.10
4	19	3	—	—	—	1.06	0.087	0.08
4	19	3	—	—	—	0.80	0.045	0.06
4	19	3	—	—	—	0.85	0.046	0.05

* Transplanted every 3 days.

† Transplanted every 2 days.

In the first three experiments the very slight traces of phosphatase found in the explants were no greater than those found in the controls, and represented values of A/W of less than 0.01. In order to obtain a more critical test the whole of the 18 explants in Exp. 4 were tested together, giving a total air-dry weight of 0.91 mg., but no phosphatase at all was detected, in agreement with the complete absence of hypertrophic cells and ossification. On the other hand, the explants of palato-quadrates and femora in which cell hypertrophy and ossification occurred were found to have synthesised phosphatase during cultivation. It was interesting also to find that the value of A/W determined for these explants corresponded very closely with the histological condition observed in explants from the same cultures, which had been fixed and sectioned. Thus, in Exp. 1, in which the culture medium was changed every 3 days, some degeneration of the palato-quadrates explants was seen, and a relatively low value of A/W (0.10) was found. In Exp. 2 the culture medium was changed every 48 hours and very little degeneration of the palato-quadrates was observed; the value of A/W found for 3 explants was 0.33, the highest yet recorded. Similarly in the cultures of femora in Exp. 4 the rather variable values of A/W corresponded with the general superficial appearance of the explants, those showing the least abnormality giving the highest phosphatase values.

B. MEMBRANE BONE.

Histological development.

It was found that when the soft tissue surrounding Meckel's cartilage in the 6-day embryonic jaw was dissected away and explanted *in vitro*, about 65 % of the explants developed ossification centres. Ossification was observed in over 180 cultures and the various stages in the process could be seen in the living tissue.

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 this control material showed that in some cases there was no trace of ossification 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.

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 VIII, fig. 5 a). 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 seemed to be separated from each other by 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.

Histological sections of a culture containing such a centre showed the latter to consist of an oblong mass of cells of somewhat irregular shape and rather deeply staining cytoplasm which were slightly flattened in a direction at right angles to the coverslip; the intercellular spaces contained a loose coagulum.

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 VIII, fig. 5 *b* and Plate IX, fig. 8) very delicate intercellular fibres—the osteogenic fibres—appeared in the inner 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 VIII, fig. 5 *c*). Sections of such a specimen (Plate IX, fig. 9) showed the fibrous capsule and adjacent layer of osteoblasts described in the case of the living cultures. 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-indigocarmine gave exactly the same staining reactions as in the normal jaw.

The next stage observed (Plate VIII, figs. 5 *d* and 6) in developing ossification centres 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 capsules 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 VIII, fig. 7) 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. Such a culture fixed in neutral formalin and stained with silver nitrate showed a dense, granular deposit of silver in the bone (Plate VIII, fig. 7).

Sections of a culture at this stage of development (Plate IX, fig. 10) confirmed the observations made on the living material. A nodule of bone was seen surrounded by a capsule of very cellular connective tissue within which was a layer of osteoblasts. The bone itself contained irregular spaces filled with osteoblasts and connective tissue cells. The bone corpuscles were normal in appearance and scattered throughout the dense matrix which was hard and difficult to cut.

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 (Plate IX, fig. 11) 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 began to disappear after about the 12th day *in vitro* and eventually vanished. In this respect the membrane bone cultures differed from the cultures of periosteal bone described in a previous communication [Fell, 1928], as in some instances the latter contained healthy bone and osteoid tissue after 84 days' cultivation. This point is being investigated further.

Phosphatase.

The results of phosphatase tests on the explants of mandibular mesoderm grown *in vitro* for periods of 6 to 8 days are shown in Table IV. Ossification centres had developed in the majority of these explants during cultivation and an increase in the phosphatase activity of the tissues was therefore expected. In all these experiments a considerable increase in the value of A/W was actually observed for the explants as compared with that for the controls. The maximum increase was found in Exp. 2, in which the value of A/W for explants after 6 days' growth *in vitro* was 0.13. It will be seen that in Exp. 1 a higher value was obtained for those explants which contained ossification centres than for those in which no such centres had developed.

Table IV. *Phosphatase development in membrane bone grown in vitro from the mandibular mesoderm of 6-day embryos.*

(Four explants made from one mandible.)

Exp.	Duration of cultivation (days)	No. of specimens	W mg.	A mg. P	A/W	Remarks
1	0	1 mandible	0.31	0.003	0.01	
	6	14 explants	0.23	0.016	0.07	{All contained ossification centres
	6	7 explants	0.22	0.005	0.02	{Contained no ossification centres
2	0	3 mandibles	1.06	0.024	0.02	
	6	12 explants	0.27	0.036	0.13	
3	0	3 mandibles	1.20	0.032	0.03	
	8	15 explants	0.30	0.033	0.11	

The proof that phosphatase was synthesised during cultivation of the explants *in vitro* is not, however, so clear as in the experiments with femora and palato-quadrates, since in spite of the marked increase in the ratio A/W the total phosphatase activity (A) of the explants after cultivation was, in two of the experiments (Exps. 1 and 3), actually less than that of the controls (bearing in mind that four explants were made from one mandible). This was due to the fact that the weight of the explants was much smaller than that of the controls. The difference in the weight resulted from two causes; in the first place it was necessary to dissect off the ectoderm from the tissue which was to be explanted and this somewhat difficult operation involved the removal of a considerable quantity of the underlying mesoderm; in the second place, during each subcultivation, only the central part of the tissue was transplanted, a considerable portion of the outgrowth being rejected. If the rejected tissue were entirely devoid of phosphatase the increase in the ratio A/W could be accounted for without assuming any synthesis of the enzyme. In Exp. 2, however, although the weight of the 12 explants was only one quarter of the weight of the 3 mandibles used as controls, the total phosphatase activity (A) was increased by 50 %. It is therefore very probable that synthesis of phosphatase occurred during development of this membrane bone *in vitro* as it certainly does during the normal growth of the bone *in vivo*.

DISCUSSION.

The results recorded in this communication indicate that by the 6th day of embryonic development the future histological structure of the non-ossifying part of Meckel's rod and of a future cartilage bone such as the palato-quadrates or femur is already firmly determined, although at this stage no histological differences between the two types of cartilage have yet appeared. It has been shown [Strangeways and Fell, 1926; Fell, 1928] that at an earlier stage of development the histological destiny of the chondrogenic tissue of the limb-bud when cultivated *in vitro* usually gives rise to nodules of small-celled, non-

ossifying cartilage. On the other hand, Murray and Selby [1930] state that the early (2-day) embryonic limb-rudiment, when removed from the body and grafted on to 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 in 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 non-ossifying cartilage or into ossifying cartilage with the characteristic hypertrophic cells are at present quite obscure.

The close correlation which exists between the histological structure and the occurrence of phosphatase in cartilage has again emerged from this study of the palato-quadrates and of Meckel's rod. In the normal embryonic palato-quadrates, as in the femur, the progressive development of hypertrophic cartilage cells and of osteoid tissue was accompanied by rapidly increasing phosphatase activity, while the non-ossifying cartilage from Meckel's rod remained inactive even in early post-embryonic life.

The specific biochemical character associated with these different types of cartilage was equally manifested when the 6-day embryonic rudiments were removed from the body and allowed to develop *in vitro*. At the time of explantation both the palato-quadrates and Meckel's cartilage were almost devoid of phosphatase, but during cultivation, marked synthesis of the enzyme occurred in the palato-quadrates along with chondroblastic hypertrophy and ossification, whilst in the Meckel's cartilage neither hypertrophic chondroblasts, periosteal ossification, nor phosphatase were found.

The fact that synthesis of phosphatase also accompanies ossification in membrane bone found similar confirmation from the cultivation of such bone *in vitro*. Although the evidence for the synthesis of phosphatase *in vitro* was less clear than that obtained in the cultivation of femora and palato-quadrates, the appearance of ossification centres in explants of mandibular mesoderm was accompanied by a large increase in the ratio of phosphatase to the dry weight of tissue.

SUMMARY.

1. The histological development of the normal embryonic mandibular skeleton, consisting of Meckel's cartilage surrounded by a sheath of membrane bone, was investigated.
2. The non-ossifying distal part of Meckel's rod in a late embryo differs from the cartilage of the palato-quadrates or femur in the absence of the region of vacuolated, hypertrophic cells characteristic of ossifying cartilage.
3. The correlation between the histological structure of cartilage and

phosphatase synthesis is made evident in these three cartilage rudiments. The palato-quadrates and femur develop high phosphatase activity while the non-ossifying part of Meckel's rod remains devoid of phosphatase up to an early stage of post-embryonic life.

4. The membrane bone surrounding Meckel's cartilage possesses a high phosphatase activity.

5. The distal part of Meckel's rod was removed from a number of 6-day embryos and explanted *in vitro* by the watch-glass method. Similar cultures were made of either the palato-quadrates or femora from the same embryos. As in the normal embryo, the distal part of Meckel's cartilage when cultivated *in vitro* neither ossified nor formed a region of hypertrophic chondroblasts, whilst in the explanted palato-quadrates and femora an area of hypertrophic cells appeared which was usually associated with a layer of osteoid tissue.

6. During cultivation *in vitro* the explants of Meckel's cartilage synthesised no phosphatase while the explanted palato-quadrates and femora developed high phosphatase activity.

7. The mandibular mesoderm surrounding Meckel's cartilage was removed from a large number of 6-day embryos and explanted *in vitro* by the hanging-drop method. Ossification centres developed in about 65 % of the cultures. It was possible to observe the different stages of osteogenesis in the living material.

8. There was evidence that explants of mandibular mesoderm in which ossification took place also synthesised phosphatase.

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DESCRIPTION OF PLATES.

c., cartilage; *c.t.*, connective tissue; *d.c.*, degenerate cartilage; *f.c.*, flattened chondroblasts; *f.l.*, fibrous layer; *h.c.*, hypertrophic chondroblasts; *m.b.*, membrane bone; *m.c.*, Meckel's cartilage; *p.q.*, palato-quadrate; *o.c.*, ossification centre; *o.l.*, osteoblastic layer; *o.t.*, osteoid tissue; *s.c.*, small chondroblasts.

PLATE VII.

- Fig. 1. Longitudinal section through the normal mandibular skeleton of a 15-day fowl embryo. Note the rod of unossified cartilage and the early membrane bone.
- Fig. 2. 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.
- Fig. 3. 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. 2. 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 Fig. 1.
- Fig. 4. Section through an explant of the palato-quadrate from a 6-day embryo after 20 days' cultivation in a watch-glass. 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.

PLATE VIII.

- Fig. 5. 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. 6.
- Fig. 6. Histological section of culture shown in Fig. 5 after 6 days' growth *in vitro*.
- Fig. 7. Photograph 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 IX.

- Fig. 8. Section of a similar culture 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.
- Fig. 9. 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. 8.
- Fig. 10. 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).
- Fig. 11. Section of a similar culture after 14 days' growth *in vitro*. Note the mass of trabecular bone which has differentiated during cultivation.