THE ROLE OF MECHANICAL STRESSES IN BONE FORMATION IN VITRO

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INTRODUCTION

In animal experiments on the effect of mechanical conditions on skeletal development, it is almost impossible to define the minute stresses set up in skeletal tissue, as the physical conditions are so greatly complicated by the presence of muscles, nerves and blood supply. Moreover, there is evidence that mechanical stresses may have an indirect effect on the tissue by altering its blood supply and thereby its nutrition (Löschke & Weinnold, 1922; Leriche & Policard, 1928; Greig, 1931).

Many of these complicating factors can be eliminated by using the tissue culture method. Bone and cartilage can be grown in vitro in the absence of blood vessels, nerves and muscles, and the direct effect of various known stresses on the histological architecture of skeletal tissue can therefore be studied with much greater precision than is possible in vivo.

In earlier work (Glucksmann, 1938) it was found that a regular pattern could be imposed on irregularly formed osseous tissue in vitro by mechanical agents, and also that perichondrium and periosteum reacted to pressure stresses by forming cartilage both at the site of pressure and the site of displacement (Glucksmann, 1939). In the present investigation the effect of pressure and tension on cartilage and of tension on periosteum has been studied.

MATERIAL AND METHODS

Skeletal tissue from chick embryos was grown by either the hanging-drop or watch-glass technique in a mixture of 1 drop of fowl plasma and 2 drops of extract of a 10-day chick embryo. The explants were transferred to fresh medium every 2–3 days. Each culture was either drawn or photographed at the beginning and end of the culture period.

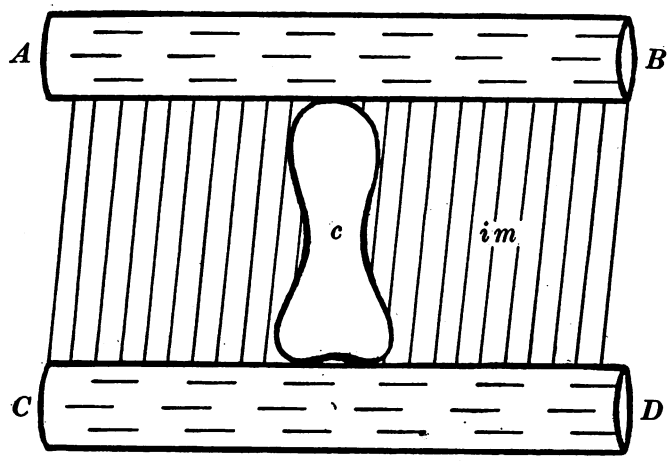
The cultures were fixed in Zenker's fluid or in Susa and when ossified were decalcified in formol-nitric acid. Serial sections were cut and stained with Azan, haematoxylin-eosin or by Wilder's method.

RESULTS

The effect of pressure and tension on cartilage

Experimental conditions. Two methods of applying pressure and tension to cartilage were used; they have been described in detail elsewhere (Glucksmann, 1939).

(*a*) *Direct application.* The rudiment was implanted between a pair of 17-day embryonic ribs in culture. The ribs, which were connected by intercostal muscles, gradually drew together during cultivation, thus exerting pressure on the rudiment implanted between them on the surface of the muscle (Text-fig. 1). Plates of scleral cartilage from 11-day embryos, 4-day embryonic femora and tibiae, and 7–12-day embryonic metatarsals and phalanges were implanted in this way; thirty-two experiments were made.



Text-fig. 1. Diagram showing the experimental arrangement for the direct application of pressure to a cartilaginous rudiment. The rudiment (*c*) is placed on the intercostal muscle (*im*) between the two ribs (*AB* and *CD*). During the period of cultivation the ribs approach each other, thus exerting a pressure on the cartilaginous rudiment.

(*b*) *Indirect application.* Barriers were placed in the direction of expansion of the rudiment which thus had to grow against a resistance (Text-fig. 2). Four-day embryonic femora and tibiae and the metatarsals and phalanges of 7–12-day embryos were used both as experimental cartilages and as barriers. Fifty-four experiments were made.

The effect of both these methods is to bend or even break the growing cartilage. The convex side is thus subjected to tension and the concave side to pressure, so that the action of both types of stress can be considered together.

Results. The degree of distortion produced depends on the type of cartilage and the duration of pressure. Scleral cartilage bends easily and usually breaks on the convex side by about the 6th day of cultivation, while 4-day embryonic femora and tibiae, though readily bent, seldom break. The metatarsals and

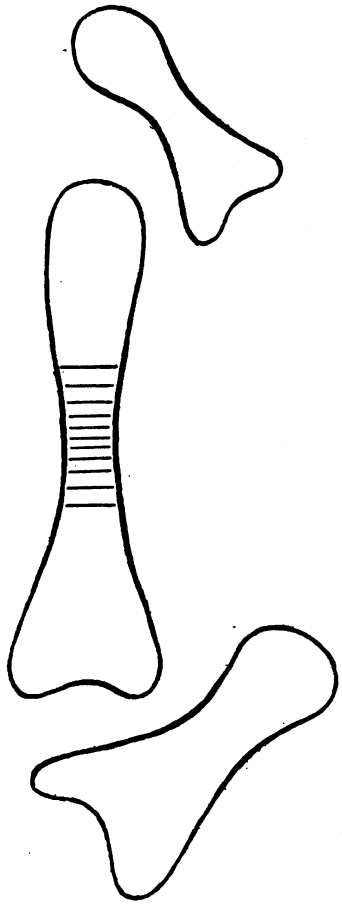
phalanges of older (7–12-day) embryos, which are usually covered by a thin osseous sheath, bend only slightly. As might be expected, curvature is greatest in the oldest cultures.

Evidence of pressure stresses on the concave and tension stresses on the convex side of the rudiment is seen as soon as bending begins and is expressed in an alteration of the arrangement of the cells, in the structure of the ground substance, and in the relationship between cells and ground substance. These changes increase with the curvature.

(*a*) *The arrangement of the cells.* On the concave side of the rudiment the long axes of the cells shift through 90° from a direction parallel with the surface. The cells also become pressed closely together (Pl. 1, fig. 1), and in very bent cartilage some degenerate (Pl. 1, fig. 4), while others are squeezed out into the surrounding tissue and may assume the appearance of fibroblasts. On the convex side the cells maintain their normal orientation but become flattened and more widely separated. If the bending is very great the cells either degenerate or are freed from their capsules.

When the curvature is slight, the cells in the interior of the cartilage are not affected, but as it increases their arrangement alters according to whether they lie nearer the concave or the convex surface of the rudiment. If the cartilage breaks under pressure the cells in the break degenerate and the neighbouring cells lose their orientation.

These changes in cellular arrangement are only seen in those parts of the cartilage which are subjected to pressure and tension stresses; elsewhere in the rudiment the cells maintain their normal orientation (Pl. 1, figs. 1, 2, 3, 5).



Text-fig. 2. Diagram showing the experimental arrangement for the indirect application of pressure to a growing rudiment. In this combination the two terminal 'barrier' rudiments prevent free longitudinal expansion of the middle rudiment and thus force it to bend.

(*b*) *Structure of the ground substance.* In response to pressure and tension, fibrils appear and the chondromucoid matrix disappears in the ground substance. In slightly bent cartilage, fibrils occur on or near the surface and lie parallel to the elongated cells; they are restricted to the intercapsular substance. When the curvature is great, the whole ground substance, including the capsules, and even intracapsular spaces left empty by the degeneration of

the enclosed cells, becomes fibrillar. This fibrillar system originates at the vertex of the concave surface (Pl. 1, figs. 2, 3), whence it runs into the interior and spreads fan-wise near the convex side so that the superficial fibres run parallel to the surface. The convex surface is pitted with shallow erosions (Pl. 1, fig. 5) lined with elongated cells, which are produced by dehiscence of the ground substance. The perichondrium is disrupted in this area.

When chondroblasts are squeezed out at the concave surface, the accompanying fibres are also extruded and may disappear in the surrounding mesenchyme, make contact with perichondrial fibres or, in ossifying regions, become continuous with bone or periosteal fibres (Pl. 1, fig. 4). This third condition is well seen when cartilage rods covered by a thin osseous coat are subjected to pressure. The bony sheath is folded in at the concave vertex (Pl. 1, fig. 2) and in very bent rudiments tends to break inside the cartilage (Pl. 1, fig. 3). The broken edges of the bone become the central points of attachment of the fibrillar system of the cartilage, and the bone and cartilage fibrils seem to be continuous.

As fibre formation progresses, the interfibrillar matrix of the ground substance disappears. Fibrillation does not appear in neighbouring parts of the rudiment not subjected to abnormal mechanical stresses, and its amount depends on the degree of stress applied, showing clearly that the fibrils are formed in direct response to the mechanical factors.

Conclusion. Pressure and tension exerted on cartilage in vitro cause the reorientation of the cells, the disintegration of the hyalin ground substance, and its replacement by a fibrillar system.

The effect of tension stresses on bone-forming tissue

Three series of experiments were made and will be considered separately.

Series 1. The rudiments were bent by blocking their longitudinal expansion by terminal barriers (see p. 2 and Text-fig. 2). The tibiae and femora of 4-day embryos and the metatarsals and phalanges of 7–12-day embryos were used; fifty-four experiments were made.

Both the effect and the degree of bending depend on the degree of differentiation at the beginning of the experiment. Unossified rudiments from 4-day embryos bend much and form little or no bone on the convex side but an abnormally large amount on the concave surface (Pl. 1, fig. 5). On the other hand, the partly ossified rudiments from older embryos bend very slightly so that the hour-glass-shaped cartilage is rarely curved sufficiently to produce more than a flattening of one side instead of forming a strictly convex contour, and ossification is always greater on the 'convex' than on the concave side (Pl. 1, fig. 6).

This difference in the distribution of new bone between the younger and older rudiments is due to the difference in behaviour between perichondrium and periosteum. Perichondrium is readily detached from the cartilage, and,

as the rudiment bends, the membrane is drawn right away from the shaft on the concave side by the natural elasticity of the tissue. In the space thus formed between the perichondrium and the cartilage, tension lines are set up which radiate outwards from the vertex of the concave surface of the cartilage (Murray, 1936; Studitsky, 1934). Bone deposition follows these radiating tension lines, so that midway along the shaft the bone is orientated at right angles to the cartilage (Pl. 1, fig. 5). On the convex surface the perichondrium becomes very attenuated or even disrupted and shallow erosion cavities often appear in the cartilage.

The periosteum investing the older rudiments, however, is firmly attached to the osseous sheath encasing the shaft and therefore does not become drawn away from the concave side of the cartilage. The increased bone-formation on the convex side follows tension stresses running parallel with the surface of the cartilage (Pl. 1, fig. 6).

Series 2. In each experiment two or more rudiments were explanted parallel to each other and a short distance apart. They soon became enclosed in a common fibrous and later osseous capsule which gradually contracted during cultivation, thus drawing the rudiments towards each other and altering the direction of the tension stresses in the capsule (Text-fig. 3). The greatest contraction and consequent change in stress occurred during subcultivation when the centrifugal stresses of the plasma clot were released and no longer counteracted the forces pulling the rudiments together (Glucksmann, 1938, 1939). The object of the experiments was to study the effect of these changes of stress on the architecture of the newly formed bone.

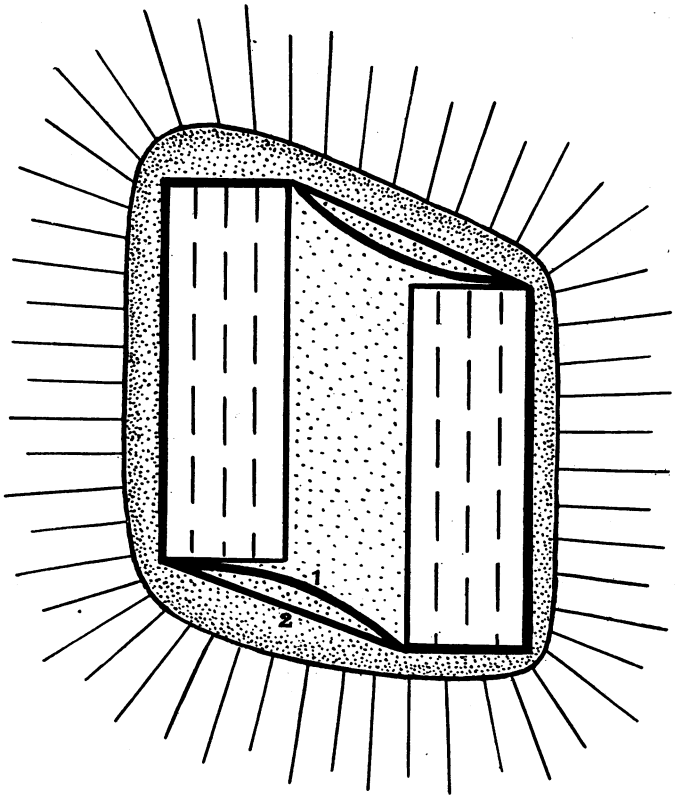
Metatarsals of 7–12-day chick embryos and parts of tibiae from 2-day hatched chicks were used; seventy-seven experiments were made.

Sections showed that the successive layers of bone formed in the common capsule correspond in number with the changes of culture medium, i.e. the periods of greatest tension. For example, if the explants have been subcultivated twice, i.e. have been in three different media, three distinct layers of bone, often separated by non-osseous tissue, are formed (Pl. 2, fig. 7). The three layers are deposited at an angle to each other according to the degree of contraction of the distance between the explanted rudiments.

Series 3. In this series of experiments the degree or direction of the normal tension stresses in the ossifying explant was altered by removing all or part of one epiphysis. The epiphyses expand in two directions, parallel to the long axis of the shaft and at right angles to it. Since the periosteal fibres are attached distally to the epiphyses, this expansion exerts tension stresses on the periosteum in two directions. Thus, by cutting off one epiphysis the lateral component of these forces is removed and the tension greatly reduced. When only half the epiphysis is removed the tension stresses are, of course, affected on one side only.

If tension promotes bone formation, then reducing the tension by excision of all or part of one epiphysis might be expected to reduce ossification. The

results of fourteen such experiments, in which phalanges and metatarsals of 10–12 day chick embryos were used, showed that this is true. Removal of one complete epiphysis greatly reduces the amount of bone deposited round the diaphysis (Pl. 2, fig. 8). When half the epiphysis is cut off, no bone develops on the operated side of the rudiment, but on the intact side ossification is normal (Pl. 2, fig. 9). In cases where less than half the epiphysis was removed, bone formation proceeded normally on the intact side. On the operated side



Text-fig. 3. Diagram showing two distinct layers (1 and 2) in an osseous capsule enclosing two bone rudiments. The inner layer became bent when the distance between the original explants contracted during subcultivation.

bone was not deposited on the surface of the diaphysial cartilage in the usual way but developed in the superficial periosteal tissue in a region which was subjected to tension stresses in the capsule formed around a prominent part of the diaphysis (Pl. 2, fig. 10).

Conclusion. (1) Tension stresses promote bone formation in osteogenic tissue in vitro.

(2) The histological structure of developing bone in vitro is orientated along the lines of tension in the osteogenic tissue.

DISCUSSION

It has been shown in earlier work that pressure applied to perichondrium or periosteum in vitro causes the disappearance of collagen fibres and the formation of cartilage. In the present experiments it was found that both pressure and tension applied to differentiated cartilage produce the reverse effect, viz. the disintegration of the hyaline matrix and its replacement by fibrillar tissue. It can hardly be assumed that the mechanical stresses merely mask or unmask pre-existing fibres by causing the deposition or resorption of interfibrillar matrix. In the first place the fibres of perichondrium or periosteum subjected to pressure actually disintegrate during cartilage formation; in the second place, in cartilage subjected to pressure fibres appear even in empty *intracapsular* spaces where they were obviously not pre-existent.

Weiss (1933) suggested that the micellae of intercellular substance tend to become transformed into a more stable fibrillar structure if the mechanical conditions remain fairly constant. It is possible that, when perichondrium or periosteum is transformed into cartilage in response to mechanical factors, the fibres are reduced to a micellar structure and the micellae become impregnated with chondromucoid matrix. Conversely, pressure and tension stresses may lead to the orientation of the micellae in the differentiated cartilage, to the formation of fibres along the pressure and tension lines, and to their separation from and the resorption of the chondromucoid matrix.

The results described above indicate that tension stresses affect the development of the osseous architecture by determining where bone shall be laid down in the osteogenic tissue. As shown elsewhere, young differentiated bone also responds to mechanical conditions in vitro so that an irregular structure may be forced into a regular pattern by pressure stresses.

Thus mechanical factors influence the structure of bone in vitro both during its formation and after it has differentiated. They also influence the amount of bone which is formed in any given region since, as described above, increasing the normal tension of the periosteum also increases bone formation, while reducing the tension correspondingly diminishes ossification.

The effects of mechanical stresses on the development of skeletal tissue which have been observed in vitro are not peculiar to tissue culture conditions, and similar results have been obtained in vivo by other workers. For example, when early bone rudiments are grafted on the chorio-allantoic membranes of fowl eggs (Murray, 1936; Studitsky, 1934) some of the grafts become very bent. In these bent rudiments bone formation is increased on the concave side and follows the radiating lines of tension set up between the cartilaginous shaft and the detached perichondrium just as in the bent explants in vitro. If half-bone rudiments are grafted, only a thin veil of bone is formed, whereas grafts of entire rudiments ossify almost normally (Murray, 1936); this result agrees with that obtained in vitro when an entire epiphysis is removed at the time of explantation. In experiments on fractures in dogs, Krompecher (1937)

showed that in callus, as in cultures of osteogenic tissue, tension stresses promote direct bone formation while pressure initiates cartilage formation, but if the pressure persists the cartilage disintegrates and is replaced by bone.

It is clear, therefore, that the effect of mechanical stresses on skeletal tissue developing under the extremely simplified conditions of culture in vitro are essentially the same as those produced by similar mechanical factors on skeletal tissue developing in vivo where the situation is complicated by the presence of muscles, blood vessels and nerves. This affords evidence that many of the structural effects resulting from mechanical stresses in vivo are due to the direct action of the stresses on the skeletal tissue itself.

SUMMARY

1. Pressure and tension stresses exerted on cartilage in vitro cause the reorientation of the cartilage cells and lead to the disintegration of the hyaline ground substance and its replacement by a fibrillar system.

2. Tension stresses promote bone formation in osteogenic tissue in vitro and determine the pattern of osseous architecture.

3. Skeletal tissue cultivated in vitro responds directly to mechanical stresses.

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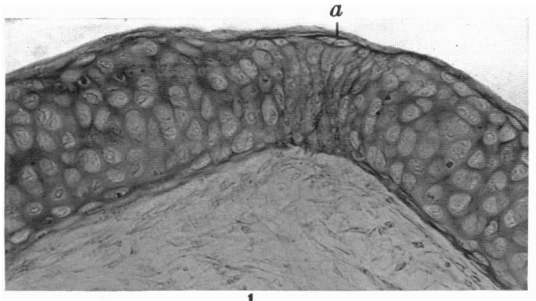
EXPLANATION OF PLATES 1 AND 2

PLATE 1

Fig. 1. Section through a culture of scleral cartilage from an 11-day chick embryo, implanted between the ribs from a 17-day chick embryo and cultivated by the hanging-drop-method for 6 days. ×270. Azan. The cartilage cells are compressed at the curvature. Cells at the convex surface are flattened (*a*), while on the concave side the long axis of the cells is shifted through 90° from a direction parallel with the surface.

Fig. 2. Section through the metatarsal of an 11-day chick embryo cultivated with two barrier rudiments for 8 days by the watch-glass technique. ×155. Azan. Note the cellular arrangement, the folding in of the osseous sheath (*o.s.*) and the appearance of a fibrillar system originating on the concave surface.



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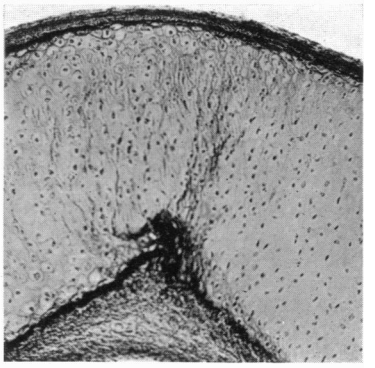

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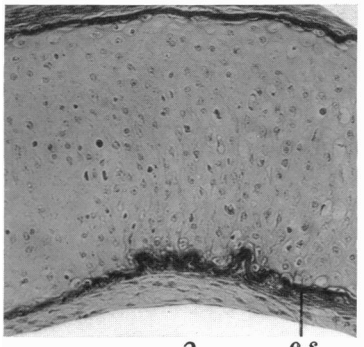

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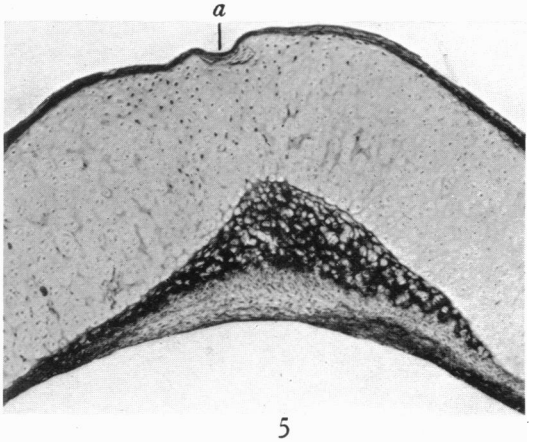

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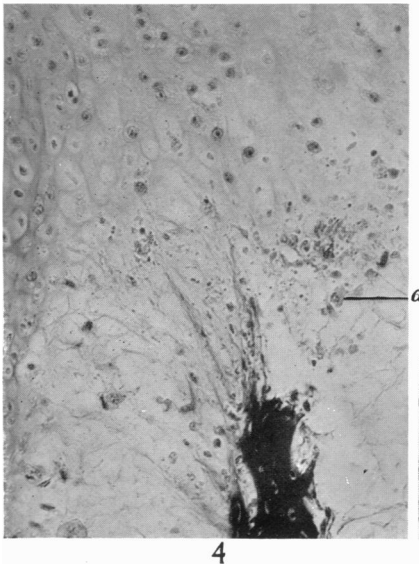

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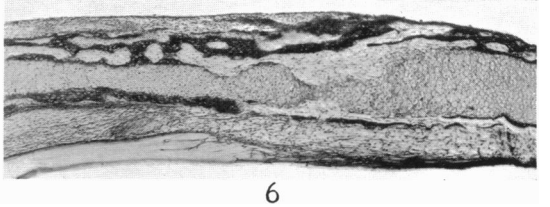

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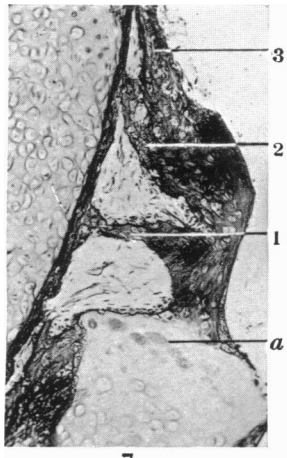

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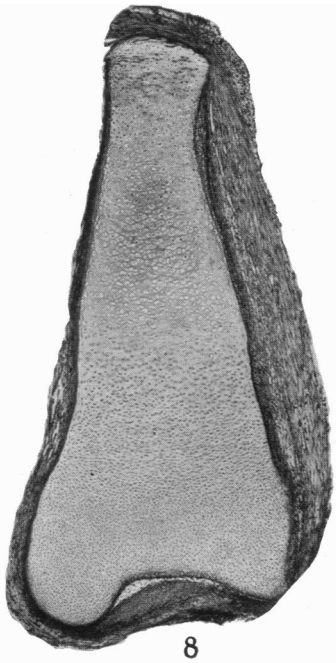

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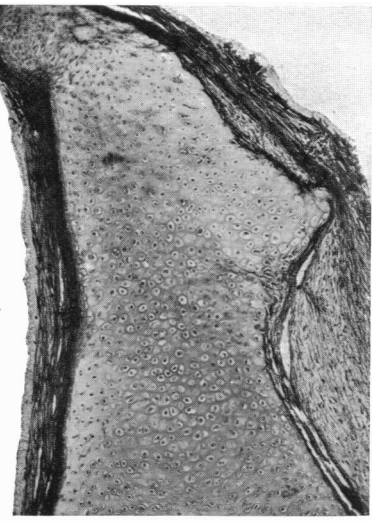

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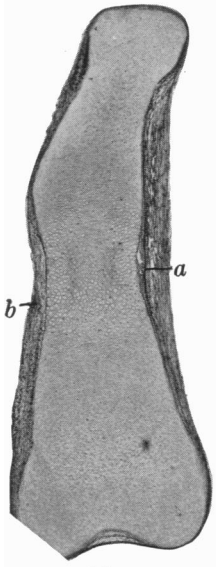

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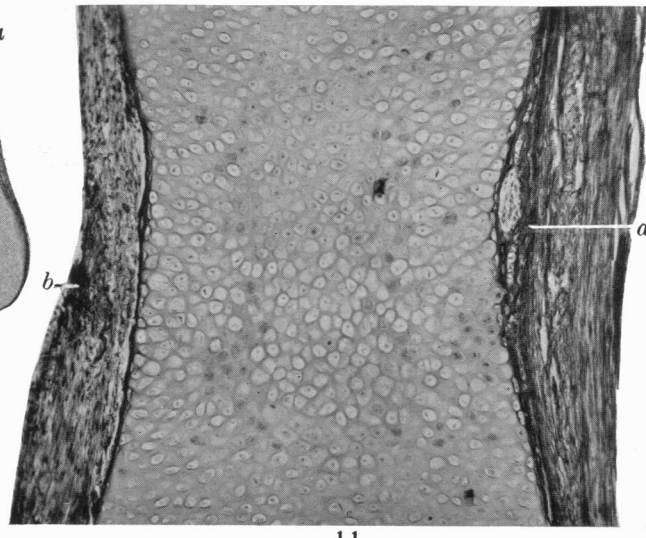

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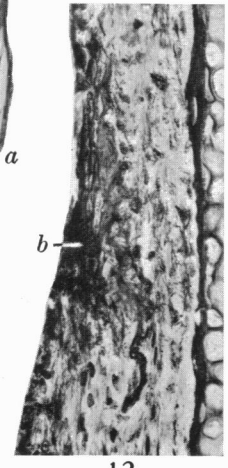

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Fig. 3. Section through a metatarsal of a 10-day chick embryo cultivated with two barrier rudiments for 10 days by the watch-glass technique. ×95. Azan. The folded in osseous sheath is broken and the broken edges form the central points of attachment of the fibrillar system in the cartilage.

Fig. 4. Section through a femur of a 4-day chick embryo implanted between the ribs of a 17-day chick embryo, cultivated by the watch-glass technique for 11 days. ×270. Azan. The cartilage fibres are continuous with bone fibrils and periosteal fibres. At (*a*) degenerate cartilage cells are seen.

Fig. 5. Section through a metatarsal cultivated in combination with phalanges of the same 7-day chick embryo by the watch-glass technique for 12 days. ×90. Azan. The convex surface of the cartilage is pitted with shallow erosions (*a*). On the concave side bone formation is increased. The bone trabeculae are radiating outwards from the vertex of the curvature.

Fig. 6. Section through a metatarsal cultivated in combination with phalanges from the same 11-day chick embryo by the watch-glass technique for 8 days. ×40. Azan. Bone formation is increased on the convex surface. The newly-formed bone runs parallel with the surface of the cartilage.

PLATE 2

Fig. 7. Section through a combination of parts of an 11-day metatarsal cultivated by the hanging-drop method for 9 days. ×150. Wilder-carmalum-light green. Three successive layers (1, 2, 3) are seen in the osseous capsule corresponding to two subcultivations, i.e. cultivation on three culture media. Note intracapsular fibres formed in spaces vacated by degenerating cartilage cells (*a*).

Fig. 8. Section through a phalanx from an 11-day chick embryo cultivated by the watch-glass technique for 10 days. ×50. Wilder-carmalum-light green. The upper epiphysis has been removed. The amount of bone formation on both sides of the diaphysis is reduced.

Fig. 9. Section through the phalanx from an 11-day chick embryo cultivated by the watch-glass method for 10 days. ×90. Wilder-carmalum-light green. The right half of the upper epiphysis has been removed in an oblique line. There is no bone formation on the right side of the diaphysis while on the left side ossification has proceeded.

Figs. 10, 11, 12. Section through the phalanx from an 11-day chick embryo cultivated by the watch-glass technique for 8 days. Wilder-Azan. ×30 (Fig. 10), ×130 (Fig. 11), ×265 (Fig. 12). Only the left half of the upper epiphysis has been removed. Normal bone formation (*a*) has occurred on the right side of the diaphysis. On the left side bone (*b*) has been formed on the margin of the culture but not immediately adjacent to the diaphysis. Note the difference in density and arrangement of the periosteal tissue on the left and right side.