THE CELLULAR BASIS OF CARTILAGE MORPHOGENESIS IN EMBRYONIC CHICK LIMBS

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BY

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Abstract

Cartilaginous long bone rudiments, of the chick embryo, were used as a model for the mammalian epiphyseal growth plate. Both contain 3 zones of chondrocytes which, therefore allows the cartilage rudiment to be considered as an expanded growth plate. The involvement of each zone in the growth of the rudiment was determined, at the cellular level, by counting cell numbers in histological sections of the ulna. Studies on the cell kinetics of the rudiments, in vitro, demonstrated that cell division was confined, mainly to the zone of rounded cells, with little or no division observed in the zones of cell flattening and hypertrophy.

It is proposed that the morphogenesis of the early cartilage long bone rudiment is influenced by the structure of it's surrounding perichondrium, rather than by a property intrinsic to the constituent chondrocytes. The perichondrium is thought to exert it's influence through a process termed "directed dilation", whereby circumferential expansion is resisted and longitudinal growth is favoured. Ultrastructural examinations show that the perichondrium of a long bone rudiment has a variable structure: distinct at the diaphysis and loose at the epiphysis. By contrast, the perichondrium surrounding Meckel's cartilage, which has only one type of chondrocyte, appears to have a uniform structure.

Evidence for the lack of an intrinsic property determining morphogenesis comes from the observation that chondrocytes, from various cartilage elements, behave identically, in vitro, under appropriate conditions. In addition, contrary to reports that the expression of a cartilage phenotype is dependant on high cell densities and histogenic interactions, it is proposed that the maintenance of a rounded cell configuration is sufficient to elicit phenotypic expression.

Experimental evidence suggests that the 3 zones of cells are set up by a combination of interactions with the perichondrium and by a signal specific to the cartilage matrix.

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GENERAL INTRODUCTION

Cartilage is an ubiquitous tissue which occurs in a wide range of forms - rods, nodules, sheets or hollow capsules. The morphogenesis of this tissue is of fundamental importance in that the whole of the vertebrate endoskeleton has a cartilaginous origin though much of it is later replaced by bone. Only the dermal skeleton does not have a cartilaginous phase. Even once a cartilage element, such as a long bone, has ossified, subsequent growth in length is based on cartilage growth within the epiphyseal growth plate.

Growth and morphogenesis of cartilage are intimately related and can be viewed in three phases: pattern formation, morphogenesis - the generation of form, and growth via the growth plate (Wolpert, I982).

Pattern Formation

Before cartilage is observed in the limb, the limb itself has to undergo considerable growth and development and it is during limb outgrowth that pattern formation occurs. Limb outgrowth, therefore, has to be considered first.

I) Limb outgrowth

The chick embryo wing bud first appears as a slight swelling on the flank, opposite somites I5-20, at stage I7 (stages are taken from Hamburger and Hamilton, I95I), which is equivalent to 52 hours of development as reported by Fell (I925). The early development of the wing bud is characterised by rapid outgrowth and expansion which transforms the initial, simple beehive shape into an elongated structure slightly flattened dorso-ventrally (Zwilling, I96I). At stage I8, both the wing and the leg bud consist of a mass of undifferentiated mesenchyme surrounded by an ectodermal covering (Fell, I925); the ectoderm is thickened along the distal rim and is

known as the Apical Ectodermal Ridge (AER). The AER is necessary for development since removal results in truncated limbs (Saunders, 1948; Janners and Searls, 1971; Summerbell, 1974; 1977).

Elongation of the limb bud is due to proliferation of the mesenchyme cells; at stage 18 the mitotic index throughout is high, about 10% but this decreases steadily to 2% by stage 30 (Hornbruch and Wolpert, 1970). Mitotic cells are uniformly distributed until stage 24 when there is a significant decrease in mitotic index of proximal cells when compared with distal cells. The ectoderm has a more or less constant mitotic index of approximately 4% throughout stages 18-30. Ede and Law (1969) have suggested that growth occurs in a proximo-distal direction and this is consistent with the proximo-distal gradient of mitotic activity observed. Several authors have proposed that mesenchyme proliferates within boundaries imposed by the ectoderm and that limb elongation occurs via distal mesenchyme cells dividing and moving into free space provided by ectodermal growth (Amprino, 1965; Hornbruch and Wolpert, 1970; Summerbell and Wolpert, 1972; Summerbell, 1977).

During development, the skeletal and muscular elements of the limb differentiate within the mesenchyme in a proximo-distal sequence. How the spatial organisation of these elements is specified is the process of pattern formation.

2) Specification of pattern

Pattern formation governs the process of spatial differentiation in which individual cells within a population are specified to undergo a particular molecular differentiation (Wolpert, 1969). The spatial organisation of tissues, such as muscle and cartilage, can account for the structural differences observed between the chick embryo wing and leg bud. Molecular differentiation of cartilage is similar in

these limbs and it is their spatial organisation which is different.

Pattern may be already present in the fertilised egg (see pre-pattern model of Turing, I952), which implies that, in a limb field, each cell is different, or it may come about at a later stage when differences between identical cells may be specified resulting in the pattern observed (Wolpert, I969). Both of these models could specify some kind of skeletal pattern but only the latter is able to explain how reduplicated limbs can come about (see later), and therefore only this type of model will be considered in detail.

Wolpert (I969) introduced the concept of Positional Information by which individual cells within a population each received a Positional Value, the way in which each cell interpreted this positional value resulted in appropriate cyto-differentiation. Two classes of models based on positional information have been put forward; short-range specification where local neighbour-neighbour interactions take place (French, Bryant and Bryant, I976; Maden, I977), and long-range specification where at least one special signalling region is present in the system studied (Wolpert, I97I; Gierer and Meinhardt, I972).

The Polar-coordinate model of French et al (I976) was proposed from the results of studies on regeneration in insect and amphibian legs and insect imaginal discs (Bryant, Bryant and French, I977), and required that cells had already undergone some process to specify positional values. The model is based on intercalation, i.e. when cells with different positional values come into contact, under experimental circumstances, generation of new structures with the missing values ensues. The model looks at the limb circumferentially and the results are interpreted as the limb maintaining a "complete circle" of positional values. Recently this model has been proposed to apply to the chick limb (Iten and Murphy, I980; Iten, I982), however, this proposal is controversial, and has been criticised by

the experimental results of Wolpert and Hornbruch (I98I) and Honig (I98I). Since it cannot account for the initial specification of positional values, it is a model more suited to regeneration than to pattern formation.

Positional values could be set up by long-range signalling systems. Two models of such long-range specification have been put forward; Tickle, Summerbell and Wolpert (I975) suggest that the specifying signal may be a diffusible morphogen whilst Gierer and Meinhardt (I972) have suggested that the signal arises from a reaction-diffusion mechanism. The major differences between these models are when and how a signalling region can appear, Wolpert (I97I) proposes that the signalling region is specified early in development and subsequently affects later cells making the population of cells in the limb field heterogenous, on the other hand Gierer and Meinhardt (I972) suggest that the limb field is almost homogenous and that the specifying region forms by autocatalysis. Both of these models could explain the spatial organisation observed in limbs but only the Wolpert model has dealt extensively with the chick wing and only this will be considered in detail below.

The way in which positional values are established along two of the axes of the chick wing have been investigated and it turns out that each axis is specified by a different mechanism. In addition to a long-range signal, a clock mechanism has also been proposed.

i) <u>The proximo-distal axis</u>

The AER has been found to specify and maintain a region about 300um thick at the distal tip of the limb, known as the Progress Zone (Summerbell, Lewis and Wolpert, I973), - if the AER is removed the progress zone is no longer maintained, resulting in truncated limbs as mentioned above. The cells in the progress zone are constantly leaving due to cell proliferation, and it is proposed that specification

occurs just as the cells leave the zone. Cyto-differentiation can only occur once cells have left the progress zone. The final position of a cell in the limb depends on the time spent in the progress zone; the longer it stays, the more distal the structure it will participate in forming (Summerbell et al, I973) (Fig. I).

ii) The Antero-posterior axis

Positional value along this axis is thought to be specified by a graded signal (Wolpert, I969), originating from a small group of mesenchymal cells found at the posterior edge of the progress zone, known as the Zone of Polarising Activity, or Polarising Region (Saunders and Gasseling, I968; Balcuns, Gasseling and Saunders, I970).

Evidence that a signal from the polarising region specifies digits comes from experiments where polarising regions were grafted to a more anterior level of a host limb, in contact with the AER (Saunders, Gasseling and Gfeller, I958; Tickle, Summerbell and Wolpert, I975; Summerbell and Tickle, I977). These grafts resulted in reduplication of cartilage elements in the antero-posterior axis (Fig. 2). Only cells in the progress zone are susceptible to the influence of the polarising region (Summerbell, I974) and, considering the results obtained from successive grafts to different positions along the antero-posterior axis, it seems likely that elements are specified by their distance from the polarising region when they leave the progress zone (Tickle et al, I975). The signal from the polarising region has been found to be universal since grafts of this region from mice (Tickle et al, I976), hamsters (MacCabe and Parker, I976), and snapping turtles (Fallon and Crosby, I977) were all able to induce chick limb reduplication.

The specifying agent is thought to be a diffusible morphogen (as proposed by Tickle et al, I975) originating at the polarising region and decreasing in concentration postero-anteriorally, a high

concentration would specify digit 4 whilst a low concentration would specify digit 2 (Tickle et al, 1975; reviewed by Summerbell and Honig, 1982). The chemical nature of the signal is unknown but recent work (Tickle, Alberts, Wolpert and Lee, 1982) has shown that the vitamin A derivative, retinoic acid, mimics the action of the polarising region. Vitamin A appears to be a morphogenetically active chemical since it can also alter the pattern of structures regenerated in amphibian limbs (Maden, 1982).

iii) The Dorso-ventral axis

The mechanism of pattern formation along the dorso-ventral axis is thought to be under the control of the ectodermal cell sheath, but is little understood (MacCabe, Errick and Saunders, 1974).

Although several models exist to explain pattern formation the positional information model proposed by Wolpert (1969) provides the most useful way of considering limb development. Interpretation of positional values will result in molecular differentiation which, for cartilage, is identified as secretion of cartilage matrix.

3) Molecular differentiation of cartilage

Cartilage matrix is composed of water, collagen, proteoglycans and other proteins. In the type of cartilage found in long bone rudiments (hyaline cartilage) the only collagen present is Type II which is made up of three identical α II chains ($(\alpha\ II)_3$) and is specific to cartilage (Miller and Matukas, 1974; Prockop et al, 1979; von der Mark and Conrad, 1979). Proteoglycans consist of a small amount of protein convalently linked to glycosaminoglycans (GAGs), the GAGs found in cartilage are chondroitin sulphate, keratin sulphate and hyaluronic acid. Each of the GAGs bind together via the link protein to form one proteoglycan molecule (see Stockwell, 1979 for review). Chondroitin sulphate is the most abundant GAG in cartilage and is scarce

elswhere in the embryo, as opposed to hyaluronic acid which is less abundant in cartilage and is found in many other tissues. Chondroitin sulphate is characterised by the position of the sulphate group on it's repeating disaccharide units. The disaccharide unit consists of Glucuronic acid bound to N-acetylgalactosamine (Stockwell, I974); chondroitin sulphate A has a sulphate at the C4 position and chondroitin sulphate C has a sulphate at the C6 position on the galactosamine moiety (Mathews, I958). Any incorporation of sulphate into cartilage is thought to reflect increased synthesis of chondroitin sulphate; incorporation of radioactively labelled $^{35}SO_4$ has been recorded as early as stage 22 but metachromatic matrix is not visible until stage 25 (Searls, I965). Differentiation of cartilage can be identified by the incorporation of $^{35}SO_4$ into chondroitin sulphate (Searls, I965; Abbot and Holtzer, I966), by the presence of large amounts of chondroitin sulphate proteoglycan (Crawford, I980), or by the presence of Type II collagen (von der Mark et al, I98I).

After pattern formation has specified that a particular group of cells will become cartilage these cells interact with each other in some way to produce a cartilage element. How this element takes shape is the process of morphogenesis and this shall be considered below.

Morphogenesis

Cartilage occurs in a wide range of shapes, and cartilage morphogenesis has to account for all of these. One thing every cartilage element has in common is that each element arises from a mesenchymal condensation, and it would appear that the shape of the initial condensation may reflect the shape of the final element formed, e.g. the condensation of a long bone is an elongated structure whereas that of a wrist element is more rounded. Since the shape of a condensation may be an important first step in the morphogenesis of that tissue, the

formation of the condensation, in the chick limb, shall be considered.

I) Pre-cartilage mesenchymal condensations

The first study of chondrogenesis in the chick limb was by Fell in I925, who stated that a mesenchymal condensation was observed in the proximal part of the limb at 4 days, with the first signs of a cartilaginous matrix appearing in the centre of the future diaphysis of the femur at 5 days. Within 30 hours of chondrogenic initiation, three zones of cells were observed in the developing rudiment (Fell and Canti, I934), these were a zone of small, rounded, actively dividing cells, a zone of cells flattened at right axis to the long axis of the limb and a zone of large hypertrophic cells. (Fig. 3).

This initial condensation process is thought to be very important for normal chondrogenesis to proceed, but as yet the actual mechanism involved has not been fully elucidated. Two views of the mechanism involved in the condensing process together with two views of the ultrastructure of the condensation are hotly debated. A third possibility, that the condensation is due to a localised increase in mitosis, as has been proposed by Wessells (I965) to account for dermal papillae condensations in feather development, has been shown to be unlikely in the limb by Janners and Searls, (I970) and by Hornbruch and Wolpert (I970).

It is generally agreed that a condensation results in a area of high cell density appearing in the region of presumptive cartilage (Ede and Agerbak, I968; Gould, Day and Wolpert, I972; Searls, Hilfer and Mirow, I972; Thorogood and Hinchliffe, I975). It is the way in which this increase in cell density comes about that is controversial. The view of Ede and Agerbak(I968) is that increase in cell density comes about by active cell migration of pre-cartilage cells into the region of presumptive cartilage with a concomitant increase in intimate cell-cell

contacts. On the other hand, Gould et al (1972) suggest that the increase in cell density is due to lack of movement away of pre-cartilage cells after cell-division, with no increase in intimate cell-cell contacts.

a) Centripetal movement mechanism

The increase in cell density in the central core is thought to come about through centripetal movement of peripheral mesenchyme cells and Ede and Agerbak (1968) liken this process to the aggregation phase of amoebae of the slime mould Dictyosteliun discoideum. No migration of mesenchyme cells can be observed in vivo, therefore the behaviour of dissociated limb mesenchyme cells has been studied in the hope that this may provide an analogous mechanism.

Ede, Wilby and Colquhoun (1977) have demonstrated that re-aggregating limb mesenchyme cells growing in culture form chondrogenic foci with surrounding cells moving towards them. These foci are thought to be similar to the whorl-like arrangements observed in transverse sections of whole limbs (Ede and Flint, 1972). The migration of mesenchyme cells in culture is once again likened to aggregation of slime mould amoebae. In normal limb development the centripetal movement proposed is considered to involve a change in adhesiveness of some mesenchyme cells. This would result in pre-cartilaginous mesenchyme cells migrating past non-cartilaginous cells until they came into contact with cells of a similar adhesiveness, stick together and form a condensation.

Evidence for increased adhesiveness playing a major role in the condensation process comes from ultrastructural studies of normal chondrogenesis and from in vitro studies of the chick mutant talpid.

Increased adhesion between cells in a condensation in vivo has been reported by Thorogood and Hinchliffe (1975) who studied the process of condensation in the chick hind-limb. These authors, recorded an increase in cell density of 62% at the region of presumptive cartilage, -40 cells/unit area at stage 20 to 65 cells/unit area at stage 24. (These

results have been calculated as an increase from I2-I9 cells/I000 μm^2 by Hall (I978)). Although cell density has been increased at stage 24 no increase in cell-cell contact was observed, but if an increase in adhesiveness, as proposed by Ede and his colleagues, was a transient step then looking at condensations from various stages may miss this step. To overcome this, Thorogood and Hinchliffe (I975) looked at different regions of a stage 26 tibia where the central region, which had already undergone matrix secretion, was considered to represent an advanced stage of chondrogenesis, and regions distal to this were considered to be less advanced. Cells at the distal end of a stage 26 tibia exhibited the highest cell density, 70 cells/unit area, were very close together and became intimately associated. They state that this region is similar to the classical condensation observed under the light microscope and propose that close apposition occurs by active migration of cells into the presumptive cartilage region. However, it is not clear if the stage 26 distal cells are exhibiting extensive cell-cell contacts.

Some observations on the mutant talpid^3 may also be related to the condensation process. Talpid^3 is a mutant caused by an autosomal recessive gene which is lethal, between 7-I4 days of development, in homozygous embryos (Ede and Kelly, I964a;b), and is characterised by short, very wide limb buds (Cole, I942) with abnormal cartilage condensations. Many of the condensations are found to remain fused when they should normally have separated (Ede, I97I). When normal and talpid^3 limbs were dissociated and allowed to re-aggregate separately, normal mesenchyme cells formed a few large aggregates, whilst talpid^3 cells formed numerous small clusters. This was interpreted to mean that talpid^3 cells were more adhesive and therefore less motile, than normal cells so that when they came into contact with each other they stuck together and remained in that position. In the

limb the extra adhesiveness of talpid^3 mesenchyme would hinder migration and therefore inhibit formation of condensations; any condensations which did form would contain mutually adhesive cells which would tend to remain together resulting in the fused elements observed.

b) Non-movement mechanism

The mechanism of centripetal migration can be contrasted with the model proposed by Gould et al (I972) in which it is suggested that the increase in cell density observed in a condensation arises, not through movement into the condensation, but through lack of movement away after cell division. This lack of movement could be related to the phenomenon of contact inhibition of locomotion as exhibited by cells in culture (Abercrombie and Heaysman, I954). The major point of this report is that, although there is an increase in cell density of central core cells from IIcells/I000 μm^2, at stage 2I, to I5cells/ I000 μm^2, at stage 24 (an increase of 36%), there is no concomitant increase in intimate cell-cell contacts. This result has been supported by ultrastructural studies of Searls et al (I972) who observed a decrease in extensive cell-cell contacts between stages I8-24. Gould et al (I972) and Searls et al (I972) both record an increase in the total number of cell contacts during this period, but these are via filopodia and rarely extend more than 0.5 μm. They suggest that the increase in cell density observed under the light microscope is due mainly to the many filopodia and not solely to the close packing of cells. Gould et al (I972) state "that to talk of condensation of the pre-cartilage mesenchyme as if it involved a close packing of cells resulting in a considerable increase in cell contact is misleading"., "true" condensations only appear in pre-myogenic mesenchyme.

Very little cell movement has been demonstrated in the chick limb in vivo (Searls, I967), and when embryonic cells were implanted into

the chick wing they exhibited a lack of invasiveness (Tickle, Goodman and Wolpert, 1978). There is also a precedent for lack of active migration causing condensation from studies of a different system, the amphibian neural crest (Epperlein and Lehmann, 1975). After the initial contact between cultured neural folds and pharyngeal ectoderm, neural crest cells proliferate and form a cluster solely due to lack of movement away after cell division.

During the condensation process, pre-cartilaginous mesenchyme cells undoubtedly get closer and may come into contact therefore adhesiveness may be an important factor but a difference in adhesion, between cartilaginous and non-cartilaginous mesenchyme cells, as proposed by Ede and Agerbak (1968) is unlikely. Whatever role adhesiveness plays in the condensation process it is not sufficient to cause segregation of cells *in vitro* (Searls, 1972; 1973). Mixtures of stage 24, 25 or 26 central core (or cartilage) cells with central core cells from stages 20-22 did not exhibit the "sorting-out" phenomenon described by Steinberg (1964).

It is not yet clear which mechanism is involved in the actual condensation process, but experimental evidence suggests that the lack of cell movement after cell division (Gould *et al*, 1972) is the most likely.

A major controversy, however, is whether there is increased cell-cell contact in the condensations. The apparently contradictory ultrastructural observations can be explained if examined closely. One possibility is that the differences may simply be due to the fact that Gould *et al* (1972) studied the wing bud whilst Thorogood and Hinchliffe (1975) studied the leg bud; wing development lags behind leg development by about 12 hours, and it could be that the close proximity observed in the leg had not yet occurred. A second possibility, that

the differences observed are due to different methods of fixation causing shrinkage of chondrogenic cells, therefore causing them to appear further apart, (as suggested by Thorogood and Hinchliffe (1975) to be the case in the electron micrographs of Gould et al (1972)), is unlikely since myogenic cells exhibit no shrinkage and show large areas of cell fusion.

A third possibility, and the most likely, is that the distal region of a stage 26 tibia does not represent an accurate model of an early condensation. The secretion of matrix has already began in the centre of a stage 26 tibia and therefore, cells distal to the centre will be under excess pressure due to the matrix secreted. It is probably this extra pressure which causes the very close apposition observed. Indeed, when Thorogood and Hinchliffe (1975) look at a stage 24 condensation they observe that "there is an increase in cell number but without the close apposition of cell surfaces" which is identical to the result of Gould et al (1972). It would seem likely, therefore, that at stage 24, when the condensation is most pronounced, pre-cartilage cells do not come into close contact with each other, and the mechanism proposed by Gould et al (1972) must be considered as being the most probable.

The signal which triggers off the condensation process is not known but some evidence has been produced to suggest that a change in glycosaminoglycan proportions is involved. Hyaluronic acid (HA) is at a maximum just prior to matrix secretion (stage 24) and it is at this point that synthesis of hyaluronidase dramatically increases (Toole, 1972). Breakdown of HA into oligosaccharides has been found to stimulate chondroitin sulphate synthesis (Wiebkin and Muir, 1973; 1975), therefore, removal of HA may be a controlling step in cartilage differentiation.

Hyaluronic acid may be involved in the condensation process itself

but the evidence for it's involvement can be interpreted to support both mechanisms of condensation and is therefore unclear. Toole and Trelstad (I97I) proposed that HA provided a substratum over which mesenchyme cells migrated during corneal development and Toole (I972) suggested that limb mesenchyme cells moved into the centre of the limb, at stage 22 utilising a similar mechanism. On the other hand, HA has been found to mask the protein fibronectin in the mouse (Silver, Foidart and Pratt, I98I) and it is possible that mesenchyme cells in a condensation are being held close together by fibronectin (which appears as HA) after cell division. This interpretation has been supported by observations in the chick where levels of fibronectin seem to increase as HA is broken down (Newman and Frisch, I979; Dessau et al, I980).

Hyaluronic acid has been known to be involved in cell proliferation and mobility since I952 when Maurer and Haduck demonstrated it's presence in considerable quantities in the early stages of callus formation during repair of fractured long bones. Recently Solursh et al (I979) demonstrated the importance of HA in the morphogenesis of the sclerotome but suggested that HA acted by expanding and pushing mesenchyme cells "en masse" with no individual cell migration. In solution, HA expands greatly to occupy a volume I0,000 times that of the molecular chain (Preston, Davies and Ogston, I965; Laurent, I970), and this could account for the pushing phenomenon. Until further studies are carried out on the condensation process, the role of HA in it must remain unclear.

Since it is difficult to determine the conditions required for cartilage differentiation in the living embryo many studies have been performed in vitro.

2) In vitro chondrogenesis

Cell cultures of mouse or chick embryo pre-cartilage limb mesenchyme have been found to produce histologically identifiable cartilage only when grown at densities greater than confluence (Umansky, I966; Caplan, I970). A very useful technique for ensuring greater than confluent density has been developed by Ahrens, Solursh and Reiter (I977) and this involves plating out cells in a volume of I0/ul containing $2xI0^5$ cells, (i.e. $2xI0^7$ cells/ml.) - a micro-mass culture. At this density limb mesenchyme cells from stage 24 embryos forms discrete aggregates during the first day of culture, by 72 hours these aggregates stain positively with alcian blue (pH I) indicating the presence of cartilage matrix. Positively staining aggregates are called "cartilage nodules" and nodules will not form without the preceding aggregation step.

The ability to form cartilage nodules is stage dependent: mesenchyme cells from limbs of stages 2I-24 will form a relatively constant number of nodules, irrespective of the stage, but cells from stage I7-I9 limbs will only form aggregates without subsequent nodules. Stage 20 cells may or may not produce nodules suggesting that this is a transitional stage during which the cells acquire the ability to form nodules. Thus it has been suggested that the ability to form aggregates and the ability to form nodules seem to be two distinct steps in vitro.

The ability to form nodules seems to be related to levels of cyclic AMP since the addition of dibutryl cyclic AMP (an analogue) or theophylline (an inhibitor of cyclic AMP phosphodiesterase, the enzyme responsible for cyclic AMP breakdown), both of which elevate levels of cyclic AMP, results in nodule formation in stage I9 cultures. The action of cyclic AMP in nodule formation is unclear, it would seem, from the above results, that elevation of cyclic AMP enhances differentiation but Kosher (I976) has found the opposite result when somitic

mesoderm is exposed to cyclic AMP - cartilage differentiation is suppressed. Whitfield et al (I979) have shown that many differentiating tissues, other than cartilage, also have increased levels of cyclic AMP, but the role of cyclic AMP here, as in nodule development, is unclear.

In addition to the stage dependency, the ability to form cartilage is also region dependent. Central core mesenchyme from stage 24 embryos produced virtually a carpet of cartilage whereas similarly staged peripheral cells formed only a few nodules in addition to fairly extensive areas of myogenic tissue. This implies that by stage 24 the chick limb consists of a heterogenous population of mesenchyme cells (Ahrens et al, I979).

Solursh, Ahrens and Reiter, (I978) have extrapolated their in vitro results to correspond with in vivo events and have produced a model outlining the steps involved during in vivo chondrogenesis. Their model states that cells develop the capacity to form cartilage before the condensation phase, but that aggregation must occur before cartilage matrix is produced and this has led Newman (I977), and Solursh and Reiter (I980) to conclude that histogenic, or cell-cell interactions are a pre-requisite for cartilage cell differentiation. Individual cells, able to secrete cartilage matrix without histogenic interactions, only appear after overt differentiation has begun (Solursh and Reiter, I975). We have recently suggested (Archer, Rooney and Wolpert, I982) that cell-cell interactions are not required for cartilage differentiation and that isolated pre-cartilage mesenchyme cells are capable of producing a metachromatic matrix if they remain rounded during culture. Cells were maintained in a rounded configuration by culturing on a semi-adhesive substratum (poly (HEMA)) and these cells synthesised more sulphur-containing extracellular matrix than cells allowed to flatten on normal tissue culture plastic. It is proposed (Archer et al, I982) that the high density involved in micro-mass cultures favours a rounded

cell configuration, and it is this, not histogenic interactions, which is the pre-requisite for cartilage matrix production.

Irrespective of the mechanism involved in the condensation process, pre-cartilage cells start to secrete matrix and the final cartilage element begins to take shape. The factors involved in transforming a long bone condensation into a long bone rudiment will be considered below.

3) Morphogenesis of the chick embryo long bone rudiment

Fell (1925) observed that soon after the first sign of matrix secretion chondroblasts, in the centre of the presumptive rudiment, became orientated perpendicularly to the long axis of the limb. From this stage onwards growth of the rudiment is due mainly to cell division, cell hypertrophy and matrix secretion. Cell division, cell hypertrophy and matrix secretion would only be important in morphogenesis if differential rates of expression were observed for each factor in each zone of the rudiment.

a) Cell division

A cartilaginous, chick long bone rudiment, between stages 30-34, consists of three zones of cells, similar to those of the epiphyseal growth plate, and can be considered as being an elongated growth plate. Differential rates of cell division have been observed in the cartilaginous epiphyseal growth plate of rats by Kember (1972; 1973; 1978), mitoses were abundant in the zone of flattened cells, few in the zone of resting cells and none in the zone of hypertrophy.

Cell division does not appear to be the most important factor in cartilage rudiment growth since a rudiment continues to grow to 80% of controls after exposure to 4000 rads (40 Greys) of X-irradiation (Biggers and Gwatkin, 1964; Archer, personal communication). (40 Greys is considered to be sufficient to knock out cell division, of mammalian cells in culture, without killing the cells). Cell division,

however, does seem to play some role in morphogenesis since the shape of the epiphysis was found to be abnormal, and 20% of the growth was affected.

b) Cell hypertrophy

If cell hypertrophy is prevented by storage in glycerol saline at -79°C for I½ hours (Biggers, I957), the increase in length observed, once thawed, is dramatically reduced compared with controls. Freezing kills hypertrophic cells preferentially and stops flattened cells producing further hypertrophic cells. Blockage of hypertrophy was found to affect the diaphysis only since cell division and morphogenesis in the epiphysis returned to normal.

The ability of chondrocytes to hypertrophy may provide an explanation as to why some rudiments, such as the ulna, elongate extensively whilst others, such as the wrist elements, which do not hypertrophy, hardly elongate at all (Summerbell, I976; Wolpert, I98I). The difference in the increase in length observed between the ulna and the wrist is a clear example of non-equivalence as proposed by Lewis and Wolpert (I976). This theory states that, for cartilage, each element is different and each will grow according to the individual growth programme of that element. Each element is laid down at the same initial size, about 300 µm, (Lewis, I975) and it is the growth programme which determines the final length.

c) Matrix secretion

Every cartilage cell is capable of secreting matrix and this may be the most important factor in cartilage growth and morphogenesis. Thorogood (I983 in press) has outlined three ways in which matrix secretion could affect cartilage morphogenesis;

i) Differential rates of secretion

ii) Differential rates of matrix accululation

iii) Polarised secretion of matrix.

Very little evidence is available for the first two points but some information is available for the third.

Orientated matrix deposition is thought to occur in cartilage rudiments (Thorogood, I983 in press) but there is some controversy about how it comes about. Gould, Selwood, Day and Wolpert (I974) believe that matrix secretion causes cellular orientation whereas Holmes and Trelstad (I980) believe that cellular orientation is present before matrix secretion begins.

Transverse sections of early cartilaginous rudiments show a whorl-like arrangement of cells (Ede and Flint, I972; Gould et al, I974), cells at the centre are rounded and separated by metachromatic matrix, whilst peripheral cells appear as elongated cresent shapes with very little extracellular matrix and many extensive cell contacts. The elongated peripheral cells eventually form the perichondrium and secrete type I collagen, whilst rounded cells secrete cartilage type II collagen (von der Mark and von der Mark, I977; von der Mark et al, I980). In longitudinal sections the central cells appear flattened, as observed by Fell (I925), suggesting that they are in fact disc-shaped. Gould et al (I974) suggested that the pressure exerted by the matrix, associated with growth in the rudiment, caused chondroblasts to become disc-shaped and peripheral cells to form the perichondrium and proposed a model to explain the orientation observed. (Fig. 4). Further growth in length is now enhanced by the constraining effect of the perichondrium as suggested by Carey (I922) and Fell and Canti (I934).

If cell orientation precedes matrix secretion, as proposed by Holmes and Trelstad (I977; I980), then some factor demonstrating cell polarity might be expected to be present. This factor has been identified by Holmes and Trelstad (I977; I980) to be the nucleus - golgi body axis. In the 9 day old mouse embryo hind-limb bud non-cartilaginous mesenchyme cells were orientated with their golgi body

towards the basement membrane, but as development proceeded this orientation was reversed so that, by the onset of matrix secretion (I3 days), the golgi body was orientated away from the basement membrane (Holmes and Trelstad, I977). Pre-cartilage mesenchyme cells at I2 days were orientated with their golgi body towards the longitudinal axis of the condensation i.e. away from the basement membrane (Holmes and Trelstad, I980). However, this orientation was only clear in pre-cartilage condensations of the hind limb and even here was only significant in proximal (upper leg) or distal (foot) regions of the limb, cells in the central (tibia/fibula) region condensations exhibited virtually random orientation prior to matrix secretion.
The orientation decreased in the proximal and distal regions at I2.5 days which is not consistent with orientation being present before matrix secretion. However, orientation returned after matrix secretion which is consistent with the proposal of Gould et al (I974).
Interestingly, Trelstad (I977) states that somitic mesoderm is orientated in very early (stage I2) chick embryos and after matrix secretion has begun (stage 27) but orientation cannot be found in an intermediate stage (stage I6). This could suggest that waves of orientation exist, with no orientation just prior to matrix secretion.

If cellular orientation is present before matrix secretion, the pressure effect proposed by Gould et al (I974) would still take place as described. Which factor is the cause and which factor is the effect is unknown, but both models result in cellular orientation and this would allow polarised secretion of matrix to play a major role in cartilage morphogenesis.

The importance of matrix secretion has been demonstrated in the mandible of embryonic rats by interrupting the processes of matrix secretion and matrix organisation. The synthesis of glycosaminoglycans was inhibited by the addition of Diazo-nor-leucine (DON) (Diewert and

Pratt, I979), the utilisation of sulphate was prevented by 6-Aminonicotinamide (6-AN) (Diewert, I979) and collagen cross-linkage was inhibited by B-Aminopropnonitrile (BAPN) (Diewert, I980 a; b), all treatments resulted in the production of a cleft palate due to lack of increase in length of Meckel's cartilage. Meckel's cartilage from each side of the jaw has to come close together for normal palate development. DON and 6-AN both exerted their effects on the growth of the cartilage whilst BAPN allowed growth to occur, but caused bending of the cartilage, therefore they could not come close together. This implies that collagen secreted by chondroblasts may play a greater role in morphogenesis than it does in growth, possibly by altering the organisation and therefore the rigidity of the matrix.

d) Other factors

Some attention has been paid to other factors which may play a role in morphogenesis, these factors have been considered by Thorogood (I982 in press) and include appositional growth, recruitment of cells from the surrounding mesenchyme and physical constraints.

Appositional growth is thought of as differentiation of the inner layer of the perichondrium into chondroblasts which merge into the cartilage, but such an incorporation of perichondral cells has yet to be clearly demonstrated. Increase in transverse diameter of epiphyseal growth plate cartilage occurs by interstitial growth of proliferative chondroblasts (Rigel, I962; Hert, I972). Meikle (I975) demonstrated that the third metacarpel of a 7 day rat, when transplanted into an intracerebral site, exhibited a marked lack of increase in transverse diameter when compared with controls. He stated that "perichondrial chondrogenesis" was unaffected, but does not make clear how this phenomenon was assessed. The lack of increase in transverse diameter was due to inhibition of cell division of proliferative chondroblasts. He concluded that "perichondrial chondrogenesis" played a very minor

role in the growth of cartilage.

Cell recruitment is a similar phenomenon to that of appositional growth except that the cells are thought to come from the surrounding mesenchyme and the recruitment occurs before the cartilage element has formed. Two examples have been put forward.

Ede and Agerbak's (1968) centripetal movement mechanism for the process of condensation is essentially a recruitment of cells. As discussed above, there is no direct evidence to verify any recruitment. The other example has been put forward by Wolff (1958) and Hampé (1959; 1960) to explain why the tibia grows much larger than the fibula. Wolff (1958) proposed a "principle of competition" which stated that the larger the anlagen the greater its "field of influence" i.e. its ability to recruit cells. According to Hampé (1959, 1960), the tibia is more able to recruit mesenchyme cells, leaving a smaller pool for the fibula to draw cells from, therefore the tibia gets longer whilst the fibula does not. Recent work, however, suggests that the fibula gets smaller relative to the tibia because the distal epiphysis of the fibula fuses to the distal epiphysis of the tibia and breaks away (Archer, Hornbruch and Wolpert, personal communication). The rate of increase in length of the fibula decreases, because it lacks an epiphyseal end, and it gets left behind as the tibia continues to grow.

Very few studies have been carried out on the effect of physical constraints on cartilage morphogenesis. An increase in pressure exerted on whole tibiae (Rodan, Mensi and Harvey, 1975), on slices of tibiae (Rodan, Bourret, Harvey and Mensi, 1975) or on dissociated cells from each zone of the tibia (Bourret and Rodan, 1976), all resulted in a change in the intracellular levels of cyclic AMP and affected the uptake of radioactively labelled ^{3}H-thymidine. How this pressure could be exerted *in vivo* is unknown but it is probable that the perichondrium plays a vital role.

Little is known about the role of the perichondrium in cartilage growth and morphogenesis which is surprising since every cartilage element is surrounded by a perichondrium. Carey (1922) and Fell and Canti (1934) suggested that the perichondrium might act as a constraining sheath preventing circumferential expansion and thereby favouring longitudinal growth. The idea of the perichondrium acting as a constraining sheath has been modified and has been termed Directed Dilation (Wolpert, 1982; Archer Rooney and Wolpert, 1982), on the basis of experimental evidence which will be considered in detail in later chapters. Relevant to this, some work has been performed on the constraining effect of the periosteum. The periosteum arises from the perichondrium and consists of an inner layer of osteogenic cells and an outer layer of fibroblastic cells. Crilly (1972) demonstrated that if a circumferential incision was made in the periosteum of the radius of an immature chicken, the radius overgrew in length compared with controls. Similar results have been presented for the immature rat (Houghton and Dekel, 1979). Both groups conclude that a growing long bone is held under considerable pressure by the periosteum, and that release of this pressure, by circumferential incision of the periosteum, allows excess growth in length to occur.

It appears likely that physical constraints exerted by the perichondrium play an important role in transforming the main factors of cartilage growth and morphogenesis, cell division, cell hypertrophy and matrix secretion, into the appropriate morphogenesis observed during development. The role of the perichondrium in cartilage morphogenesis may turn out to be of fundamental importance.

This thesis will only deal with chondrogenic tissue after it has been committed and when it's morphogenesis appears to be autonomous. The autonomy of cartilage morphogenesis will be considered below.

4) Autonomy of cartilage morphogenesis

Once chondrogenic tissue has become committed it will continue to differentiate and exhibit an almost normal morphogenesis even if it is transplanted to non-chondrogenic regions or cultured in vitro. This autonomy is expressed whether the tissue is complex, as in a whole limb, or simple, as in an isolated long bone rudiment (Murray and Huxley, 1925; Murray, 1926; Strangeways and Fell, 1926; Fell and Canti, 1934; Biggers, 1964; Holder, 1977a; b; c). Autonomy of morphogenesis is not restricted to limb cartilage and has been shown for scleral cartilage (Weiss and Amprino, 1940), Meckel's cartilage (Jacobson and Fell, 1941), the sternum (Chen, 1952; 1953) and ribs (Kieny, Mauger and Sengel, 1972). Although cultured elements retain their gross morphology, long bone rudiments do not form a marrow cavity, hypertrophic cells do not get re-absorbed and joints may fuse together (Fell and Robinson, 1929; Holder, 1978), which implies that some balance must exist between intrinsic and extrinsic factors in the normal development of detailed features of the mature element.

Weiss and Moscona (1958), however, suggest that the autonomy of cartilage morphogenesis is due to properties intrinsic to the cartilage cells themselves. Their experiment showed that committed periocular mesenchyme, when dissociated, always formed a flat sheet of chondroblasts but, in contrast, dissociated $3\frac{1}{2}$ day committed limb core mesenchyme always formed rods of cartilage similar to that produced in the limb. Wolpert (1982) would suggest that, although every cell is different by virtue of its positional information, the morphogenesis of cartilage depends, not on the nature of the positional values of the constituent cartilage cells, but on the instructions recieved from the perichondrium surrounding each element. Possible differences in perichondrial structure would account for the various cartilage shapes produced, and, their autonomy could therefore be controlled simply by the

perichondrium. In normal development the perichondrium would interact with local extrinsic properties to produce the final element observed.

Once a long bone has ossified, continued growth is still due to growth of cartilage; in mammals each long bone forms a cartilaginous epiphyseal growth plate and growth of the bone is due mainly to growth within this growth plate.

Growth and the Growth Plate

The growth plate consists of three zones of cells with proliferation occurring only in the zone of flattened cells (Kember, I972, I973, I978). The rate of proliferation is similar for each growth plate of different long bones and it is the size of the proliferating population which determines the rate of increase in length of the bone (Kember, I978). The mechanism by which the three zones of cells are set up is therefore very important in determining the rate of growth.

The growth plate is very easily damaged (e.g. any interference with the vascular supply causes severe damage), and for this reason it has not proved possible to study the growth plate *in vitro*. However, as mentioned above, the chick embryo cartilaginous long bone rudiment is essentially an enlarged growth plate, and since these rudiments are accessible to direct manipulation both *in vivo* and *in vitro* they produce an excellent model for the study of the cellular basis of cartilage growth within the growth plate.

The aim of this thesis will be to discuss the factors involved in cartilage morphogenesis with reference to the role played by physical constraints. Particular attention shall be paid to the role of the perichondrium in eliciting these constraints. An attempt will be made to describe how three zones of cells are set up in the long bone rudiment, thus providing a model to describe the setting up and functioning of the epiphyseal growth plate.

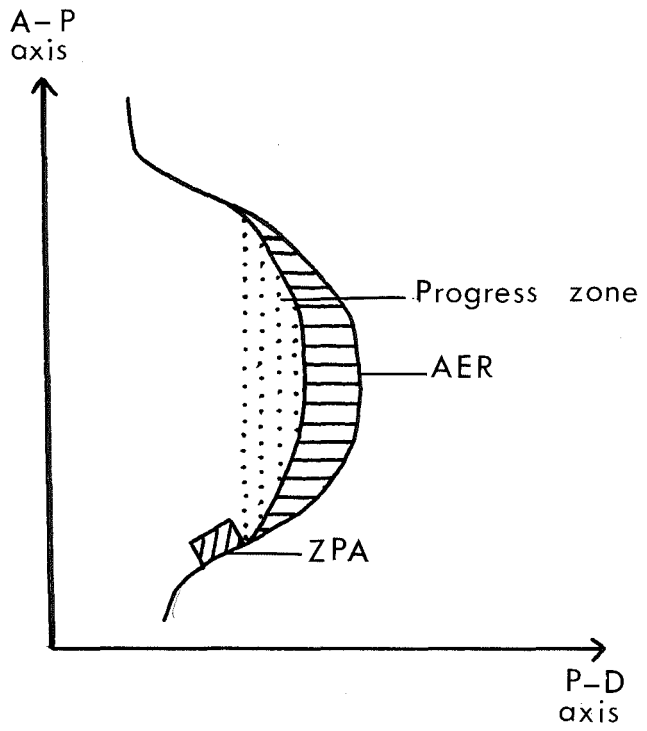
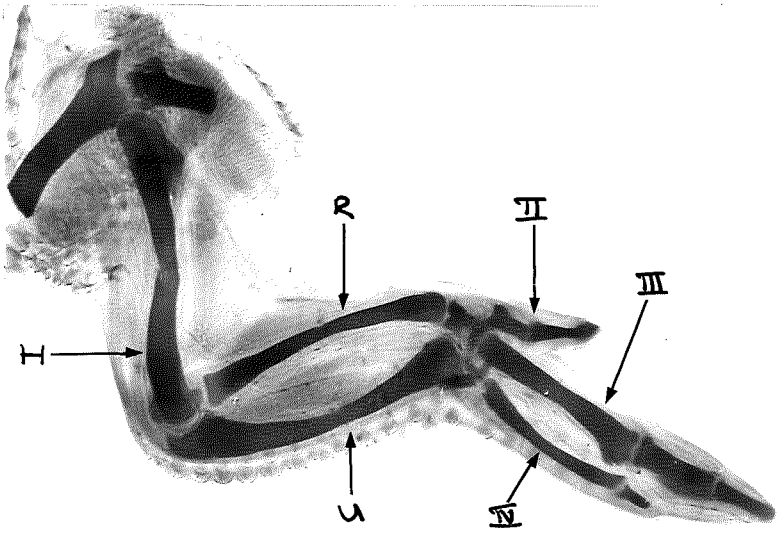


Fig. I. The Apical Ectodermal Ridge (AER) maintains the cells in the Progress Zone in a labile state. As the cells leave this zone they have positional values assigned to them. Position in the proximo-distal axis is determined by the amount of time spent in the progress zone. In the case of the antero-posterior axis, position is determined by the distance of the cell from the Zone of Polarising Activity (ZPA) when it leaves the progress zone.

Fig. 2a. Whole mount of a 10 day wing stained with alcian green to show the cartilage elements. H - Humerus, R - Radius, U - Ulna, II - Digit II, III - Digit III, IV - Digit IV.

Fig. 2b. Grafting an excess polarising region to an anterior position on a wing bud results in the formation of supernumerary digits. The digits formed are mirror-image symmetrical to the natural digits of the host.

Fig. 3. Three zones of cells can be clearly recognised in a developing long bone rudiment - in this case half of the central phalange of digit 3 at stage 32. Note the zones of rounded (R), flattened (F), and hypertrophic (H) cells. Each cellular zone is contained within a multilayered perichondrium (P). Toluidine blue stain. Mag. x 200.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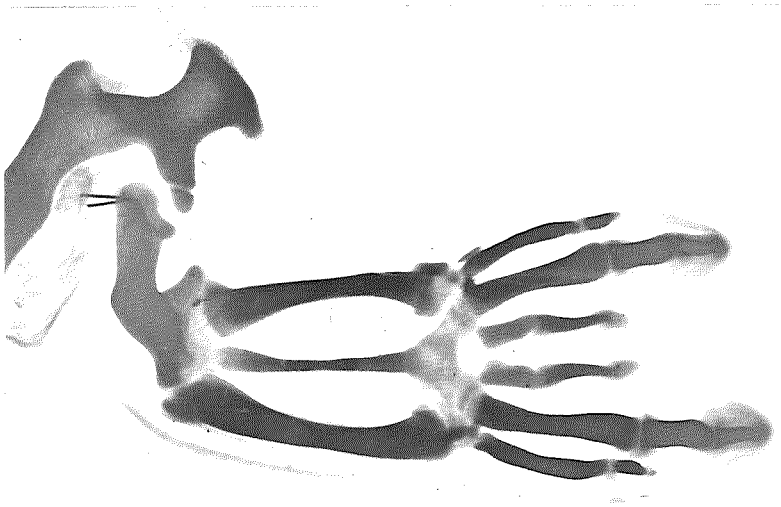

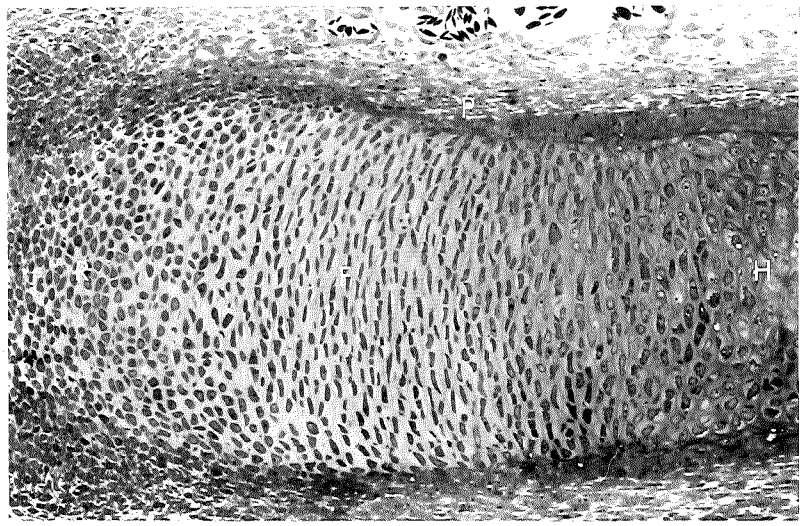
P
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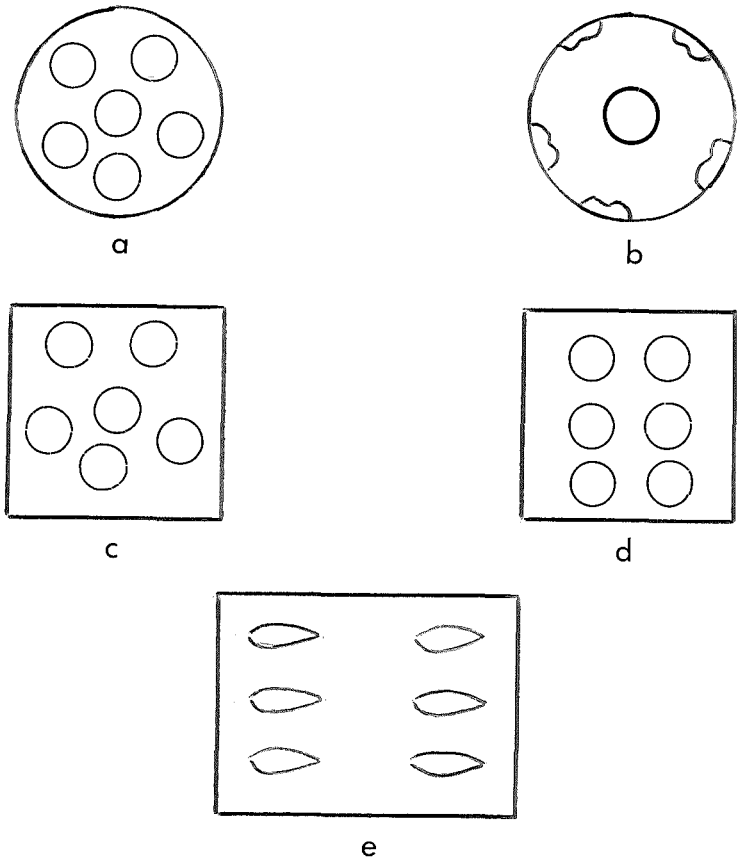


Fig. 4. Diagramatic Representation of Cellular Orientation in Long Bones.

(a) A pre-cartilage condensation, in cross-section, is considered to be a cylinder with randomly spaced cells.

(b) If only central cells begin to secrete cartilage matrix then peripheral cells with be forced to flatten. The flattened cells will eventually form the perichondrium.

(c) A longitudinal section view of the central cells at a stage similar to that above.

(d) If every central cell secretes matrix and the cylinder remains the same diameter then the cells will become evenly spaced.

(e) If the walls of the cylinder expand, in a radial direction, at the same time as central cells secrete matrix then flattening of the cartilage cells would occur.

In a developing rudiment the width is narrowest at the diaphysis but increases towards the epiphyseal region. this increase in width begins at the position of flattened cells and could account for the flattening observed.(see fig. 3).

Redrawn from Scott et al (1954).

GENERAL MATERIALS AND METHODS

General Materials and Methods

I) Eggs

Fertilised White Leghorn chicken eggs were obtained from a local source (Needle Farm, Enfield), and stored in a cooled incubator, at 12°C, for up to 7 days. To restart embryonic development, eggs were transferred to a humidified incubator at 37-39°C. On the third day of incubation the eggs were removed from the incubator and the blunt end was pierced to puncture the air sac. The eggs were then windowed by cutting a square into the shell using a diamond-edged cutting disc on a dentists drill, the shell was wiped with 70% alcohol and the square was removed. The shell membrane was carefully torn, causing the yolk to collapse into the air sac, and the embryo was exposed. The embryos were staged according to Hamburger and Hamilton (1951), the window was sealed with sellotape and eggs containing normal embryos were returned to the incubator until the desired stage was reached. Approximately 90% of the eggs were found to be fertile and normal.

2) Media

i) BGJb - Fitton Jackson modified medium

BGJb - Fitton Jackson modified medium is a chemically defined medium used specifically for growth of cartilage explants (Biggers, Gwatkins and Heyner, 1961).

100ml of BGJb - Fitton Jackson modified medium (Gibco) was supplemented with 10ml of Foetal Calf Serum (Gibco), 1.1ml of 200mM L-Glutamine (Gibco), to a final concentration of 292 µg/ml, 1.1ml of a 100 x Antibiotic-Antimycotic solution (Gibco), to a final concentration of 100 units Penicillin, 100 µg Streptomycin and 0.25 µg Fungizone/ml, and 30mg of Ascorbic acid (AnalaR).

ii) Nutrient mixture F-I2 (Ham).

Hams F-I2 is a chemically defined medium used specifically for cell culture, particularly clonal cell culture (Ham, I965).

Hams F-I2 (Gibco) was supplemented with the same constituents as BGJb except, in some cases, Foetal Calf Serum was replaced by Chicken Serum (Gibco).

All media were sterilised by filtration through a sterile Millipore filter with a pore size of 0.22 /um.

3) Enzymes

i) Trypsin

30mg of trypsin (I:250 Difco, U.S.A.) was dissolved in I0ml of phosphate buffered saline (PBS) to make a 0.3% solution (W/V). The pH was adjusted to 7.4, the solution was centrifuged at I250 r.p.m. for 5 minutes and the supernatant was filtered through a sterile Millipore filter. The solution was made into aliquots which were kept frozen until required.

ii) Collagenase

20mg of Type IA collagenase (Sigma) was dissolved in I0ml of PBS to make a 0.2% solution (W/V). The solution was filtered through a Millipore filter and kept frozen until required.

4) Organ culture

Wings were removed from suitably staged embryos and placed in sterile PBS. The ectoderm and surrounding muscles were removed from skeletal elements with watchmakers forceps, isolated long bone rudiments were cleared of any remaining connective tissue either by needles made from 500 /um diameter tungsten wire (Goodfellow Metals) or by carefully rolling the rudiment on a piece of dry Millipore filter. Dissections were carried out under a Zeiss Stereo IV microscope with illumination from a Schott Mainz KL I50B fibreoptic lamp. Dissections for both

organ and cell culture were carried out under sterile conditions in a Microflow hood (Flow Laboratories).

The organ culture technique used was a modification of that described by Jensen, Gwatkin and Biggers (1964). The rudiment was cultured on a piece of sterile Millipore filter which was placed on top of a stainless steel gauze grid. The grid was placed into a pool of 1.5ml of BGJb medium in a sterilin 35mm tissue culture petri dish and incubated in a humidified incubator with a 5%CO2 : 95% air mixture at 38°C (± 0.5°C) (Forma Scientific) Medium was changed every second day when the rudiment was revolved 180° to prevent tissue adhering to the filter.

5) Cell culture

i) Limb bud mesenchyme

Wing buds were removed from embryos between stages 22-24 and placed in a sterile PBS. The ectoderm and peripheral mesenchyme was cut away so that only an oblong, central core of presumptive cartilage and local connective tissue remained. This central core was treated with 0.1% trypsin (10min.) at 37°C (diluted from 0.3% trypsin with PBS). The tissue was not agitated and was therefore loose but still whole, it was then centrifuged at 1000 r.p.m. (10min.) and the supernatant which contained very few cells, was discarded. The pellet was resuspended in 0.2% collagenase (10min.) at 37°, vigourously agitated on a whirlymixer every 2-3 minutes, centrifuged at 3000 r.p.m. (10min.), resuspended, and washed twice in Hams F-12 medium. This procedure resulted in the production of a population of single cells. Cells were counted in a haemoacytometer and plated out on glass coverslips, or on Sterilin 35mm tissue culture dishes, at various concentrations ranging from $1x10^6$ cells/ml - $2x10^7$ cells/ml. Cells, at the initial concentration of $1x10^6$ cells/ml, were diluted with Hams F-12 such that the final

concentration ranged from $IxI0^4$ - $IxI0^6$ cells/dish. Micro-mass cultures (Ahrens et al, I979) were set up by plating out a I0 ul drop containing $2xI0^5$ cells (i.e. $2xI0^7$ cells/ml), into the centre of a tissue culture dish. The cells were allowed to settle for 2 hours after which the dish was gently flooded with Iml of Hams F-I2 medium.

All dishes were then incubated in a 5% CO_2 incubator at 37°C, and the medium was replaced every day.

ii) Long bone cartilage

Wing rudiments were removed from 7,8 and I6 day old embryos and rolled on Millipore filters as described for organ culture. The cartilage areas of each rudiment were cut into fragments approximating the size and position of each of the 3 zones of cells. (The size and position of each zone can be estimated by studying histological sections - see results section of Chapter I). Fragments from each region were pooled and, for rounded and flattened cell regions, peripheral areas were cut away leaving a rectangular shaped piece of cartilage with very little connective tissue.

a) 7 and 8 day rudiments

Trimmed fragments from the rounded and flattened cell regions were immediately treated with 0.3% trypsin (I0min.) at 37°C, vigourously agitated every 2-3 minutes and centrifuged at 3000 r.p.m. (I0min.) to settle clumps of tissue. This treatment loosened any connective tissue from the fragments but had virtually no effect on the structure of the cartilage. The supernatant, containing the connective tissue, was discarded, the pellet was resuspended in 0.2% collagenase (30min.) at 37°C, and vigourously agitated every 5 minutes. After 30 minutes the clumps had almost totally dissociated into single cells and these were centrifuged, washed and plated out as above.

Cells were obtained from the hypertrophic cell fragments by utilising one of two methods. The first method was to trim the

fragments and dissociate them in the same way as described above. The second method was to gently squash the hypertrophic cell fragments under a glass coverslip prior to any enzymatic treatment. This resulted in a piece of hypertrophic cartilage, free from any connective tissue, being expelled from each end of the fragment (see Chapter 3). The expelled pieces of cartilage were pooled and dissociated in the same way as the rounded and flattened cell fragments.

Both methods gave equal numbers of hypertrophic cells which appeared identical in culture, but, since squashing the cartilage gave a completely homogenous population of chondrocytes, this method was most often used for the isolation of hypertrophic cells. This method was not used for rounded or flattened cell fragments since the connective tissue seemed to stretch and the cartilage was never found to be free of connective tissue.

b) 16 day rudiments

The cartilaginous areas were cut away from the bony centre with a scalpel blade before they were cut into fragments. Every fragment was trimmed into rectangular shaped pieces because hypertrophic cartilage at this stage could not be squashed under a coverslip. The pieces of cartilage were dissociated in the same manner as for younger rudiments but the treatment times differed; treatment with 0.3% trypsin was for 15 minutes, and treatment with 0.2% collagenase varied from 1-2 hours depending on the region being treated (Hypertrophic cells took longer to dissociate then rounded cells). This dissociation procedure always left some clumps of cartilage which were removed by centrifugation at 100 r.p.m. for 3 minutes. The supernatant contained a population of more or less single cells which were then centrifuged and plated out as above.

6) Fixation and Histology

i) Long bone rudiments

a) Wax sections

Rudiments were fixed immediately, or after various days in culture, for 2 hours in Bouins general fixative, dehydrated through a graded series of alcohols (2 changes in 50%, 70% and 90% - 15 minutes each, 2 changes in 100% - 30 minutes each), cleared in xylene (2 changes of 30 minutes each) and embedded in paraffin wax (2 changes of 30 minutes each, followed by final embedding) (Solmedia Ltd.). Serial sections, 7 µm thick, were cut on a rotary microtome (Reichart Instruments, Austria), hydrated and stained either with Harris's hematoxylin and eosin or 0.2% toluidine blue (Humason, 1979).

b) Araldite sections

I) Light microscope

Rudiments were fixed for 2 hours in ice cold, half strength Karnovsky, cacodylate buffered, formalin/gluteraldehyde mixture (Karnovsky, 1965), rinsed in 0.1M cacodylate buffer and dehydrated through the alcohols as above (Rudiments could be stained, en bloc, with 0.1% alcian green 2GX, (made in 1% acid alcohol), for 1 hour between changes in 70% alcohol, to faciliate orientation during sectioning). After dehydration, rudiments were cleared in propylene oxide (2 changes of 15 minutes each), placed in a 1:1 mixture of propylene oxide : Araldite resin (30min.) and embedded in Araldite (Agar aids). Serial sections, 1 µm thick, were cut on a Cambridge Huxley MKII ultramicrotome and stained with 0.1% toluidine blue.

2) Electron microscope

Rudiments were fixed and washed as for light microscope sections, they were then post-fixed in cacodylate buffered 1% osmium tetroxide for 1 hour at 4°C. The dehydration and embedding procedure was identical to that described above except that the rudiments did not require staining with alcian green. 1 µm thick sections were stained with 0.1%

toluidine blue for the light microscope whilst ultrathin sections, 0.1 µm thick, were picked up on rhodium - coated copper grids (Graticules Ltd. - 200 mesh), stained with 4% aquous uranyl acetate (5min.) (AnalaR) and/or lead citrate (3min.) (Reynolds, 1963), and examined using a Phillips EM300 electron microscope.

ii) Cell cultures

Micro-mass cultures were rinsed in serum-free Hams F-12, gently loosened and freed from the tissue culture dish and processed as above for sectioning in araldite. To visualise non-micro-mass cultures, in situ, the cell cultures were rinsed in serum-free Hams F-12, and fixed by adding 1ml of an ice cold 3:1 alcohol : glacial acetic acid mixture to the culture dishes for 10 minutes. The dishes, or coverslips, were rinsed twice in ice cold trichloro-acetic acid, rinsed twice in ice cold double distilled water and allowed to dry in air. Cells were stained with alcian blue (pH 2.8) and nuclear fast red or with 0.2% toluidine blue (Humason, 1979).

7) Autoradiography

i) ^{3}H-thymidine

a) Labelling

Excised 7 day old (stage 32) cartilage rudiments were grown in organ culture for at least 1 hour before the medium was removed and replaced by 1.5ml of BGJb medium containing 15 µ Ci/ml of 6-^{3}H-thymidine (Amersham International, specific activity 23 Ci/m.mol). ^{3}H-thymidine is incorporated into DNA during the S phase of the cell cycle and is therefore a marker for those cells which passed through the S phase during the period of incubation with the label. The incubation period varied from 1-24 hours after which the rudiments were fixed and sectioned (wax and araldite) as described above. Sections from the centre of each rudiment were floated on subbed slides (Rogers, 1967),

allowed to dry and dipped in nuclear emulsion gel. (I5 µ Ci of ^{3}H-thymidine was sufficient to label chondrocytes *in vivo* (Lewis, I977) and this was chosen arbitrarily to ensure a high degree of incorporation over a short period of time).

Cell cultures, growing on glass coverslips, were labelled by replacing the medium with Hams F-I2 containing 2 µ Ci/ml of ^{3}H-thymidine. The period of labelling varied from I-I6 hours, after which the cells were fixed and dried as described above. After drying, the coverslips were mounted onto subbed slides and dipped. (2 µ Ci/ml of ^{3}H-thymidine has been shown by many workers to be sufficient to label cells *in vitro*, including chondrocytes from the vertebrae of I0 day old chick embryos (Murison, I972).

b) Dipping

All procedures were carried out in the darkroom under safe light F-904 (Ilford). Nuclear emulsion gel, type L4 (Ilford), was melted at 43°C in a water bath and diluted I:I with a solution of 2% glycerol (AnalaR) in distilled water. The mixture was gently stirred, allowed to settle for 2 minutes and excess bubbles were removed by dipping test slides into the mixture. When no bubbles were present, the slides were dipped vertically into the mixture, removed and drained of excess emulsion. The back of the slide was wiped clean and the slides were placed, sections up, onto a cooled metal tray to dry and solidify. When dry, the slides were placed in light-tight boxes, sealed in black plastic bags and stored at 4°C.

The path distance of ^{3}H-thymidine (Beta particles) is approximately 3 um (Rogers, I967), therefore, wax sections (7 µm thick) and labelled cells (at least 5 µm thick) contained, effectively at least twice as much radioactivity as Araldite sections (I µm thick). Therefore, the exposure time varied depending on the type of section dipped; wax sections and cell cultures only required 2 weeks exposure whilst

Araldite sections required 4 weeks.

c) Developing

Slides were allowed to reach room temperature, still sealed in light-tight boxes, before being developed for 7 minutes in Kodak DI9 developer, at 20°C, in the darkroom. Developed slides were washed in running water (IOmin.) and fixed in a I:4 dilution of Amfix (IOmin.) (May and Baker Ltd.). Slides were then re-washed in running water (IOmin.), rinsed twice in double distilled water (IOmin. each), stained as above and examined under the light microscope.

ii) ^{3}H-thymidine - scintillation counter

After 2 days of culture, ^{3}H-thymidine (2 µCi/ml) was added to stage 23-24 limb bud mesenchyme for I6 hours. The labelled medium was then removed and the cells were washed 3 times in P.B.S. before being fixed in absolute methanol (5 minutes at 0°C). After fixation the attached cells were washed in TCA and dissolved in 0.5ml of 0.3M NaOH which in turn was dissolved in 5ml of "Aquasol 2" liquid scintillation cocktail (NEN) and the activity measured in a Packard Tri-Carb liquid scintillation counter.

iii) $Na_2\,^{35}SO_4$

The synthesis of sulphated glycosaminoglycans by stage 23 limb bud mesenchyme cells, and stage 32 chondrocytes, in vitro was determined by incubating the cells in the presence of $Na_2\,^{35}SO_4$ (Amersham International). Cells were grown at a concentration of $2xI0^5$ cells/35mm sterilin petri dish for 2 days, before the medium was replaced with medium containing $Na_2\,^{35}SO_4$ at a concentration of 2 µCi/ml and incubated for a further I6 hours.

Sulphated glycosaminoglycans may be deposited into the extracellular matrix or may be released into the medium in soluble form, thus the synthesis must be measured in different ways.

a) Matrix deposition

After the 16 hour labelling period, the cultures were washed 4 times in P.B.S. and digested, at 37°C, for 2 hours in 1ml of hyaluronidase (0.5mg/ml) (Sigma) made up in Sorensens buffer (pH 5.6). The digests were dissolved in 5ml of "Aquasol 2" liquid scintillation cocktail (NEN) and the activity measured in a Packard Tri-Carb liquid scintillation counter. This is essentially the method of Meier and Solursh (1972).

b) Soluble glycosaminoglycans

Medium, pooled from 3 identical cultures, was centrifuged at 3000 r.p.m. (5min.) to remove any floating cells. 1ml of the supernatant was removed and into this was added 0.2ml of 2M Tris buffer, 0.7ml of double distilled water, 0.1ml of chondroitin sulphate (10mg/ml) and 1ml of 1% cetylpyridinium chloride consecutively. The mixture was allowed to stand for 5 minutes and was then centrifuged at 12000g for 15 minutes, on an ultra-centrifuge, at room temperature. The precipitate was resuspended in ice-cold distilled water, centrifuged at 12000g for 15 minutes at 0°C (to prevent the precipitate dissolving), resuspended and centrifuged again. The final precipitate was dissolved in 1ml of absolute methanol and counted in 5ml of "Aquasol 2" as described above. This method is similar to that of De la Haba and Holtzer (1965).

CHAPTER ONE

CARTILAGE GROWTH IN VIVO AND IN VITRO

Introduction

Growth of cartilage long bone rudiments occurs by a combination of cell division, cell hypertrophy and secretion of extracellular matrix. Hall (1978) defined growth as "a permanent increase in the size of any perameter that is measurable," in the case of cartilage, growth is manifested as an overall increase in length and/or an increase in rudiment diameter. The earliest growth of a cartilage rudiment can be represented as the proliferation of precursor cells and the accumulation of progenitor cells into pre-cartilage condensations. The mitotic index of cells during and after the formation of condensations in the chick wing bud has been investigated extensively (Janners and Searls, 1970; Hornbruch and Wolpert, 1970 and Lewis, 1975), and the results indicate that a proximo-distal increase in cell division occurs. Cell kinetic studies have also been performed on the epiphyseal growth plate of juvenile rats (Kember, 1972; 1973; 1978) and these have demonstrated that, within one animal, rates of proliferation in every growth plate are similar although the labelling indices are different. Kember found that incorporation of tritiated thymidine was confined almost entirely to the proliferation zone, and suggested that the size of the proliferating cell population may be an important factor controlling long bone growth. Very little work, however, has been performed on the intermediate stage of the cartilaginous long bone rudiment.

Each long bone rudiment has a different rate of increase in length and can be ranked, in ascending rates of growth, as follows: radius, ulna, humerus, tibia and femur (Fell and Mellanby, 1955; Summerbell, 1976). The growth of cartilage rudiments *in vitro* has also been comprehensively studied (Fell and Robinson, 1929; Fell and Mellanby, 1955 and Holder, 1978) and once again, ascending rates of

growth are found. However, in both cases the cellular changes which bring about the increase in length have been little studied. Stocum et al (1979) examined the distal half of the chick tibiotarsis by measuring the length of each histogenic zone of cells, but paid no attention to any changes in cell number or distribution within the zones as the tibia developed.

This chapter aims to study the growth of long bone rudiments of the chick wing, particulary the ulna, in terms of increases in length, changes in number and distribution of cells within the rounded, flattened and hypertrophic zones and the pattern of cell labelling in these zones after incubation with tritiated thymidine both in vivo and in vitro. The ulna has been chosen because it appears at the same time as the humerus but does not have a kink in the centre of the diaphysis (Summerbell, 1976), also, the radius has already been examined for cell number changes (Holder, 1978).

Materials and Methods

Rudiments were removed asceptically from the wings of embryos between stage 30 - stage 36 (6-10 days), measured immediately using a calibrated eyepiece graticule, and then cultured for up to 4 days, as described previously. Cultured rudiments were measured daily and samples were fixed, in $\frac{1}{2}$ strength Kamovsky fixative, for histological examination.

Cell counts were only carried out on longitudinal sections of ulnae; sections were selected from the centre of each rudiment and cells were counted using a x40 objective and an eyepiece grid divided into 100 squares. Total numbers of cells in each histological zone were counted together with the number of cells along the longitudinal axis, the number of cells across the transverse axis and the density of cells present in each zone. The total length of each zone was also measured,

however, these measurement are slightly arbitary since the boundaries between zones are not clear. Zonal boundaries were defined as the regions where the cell type of the adjacent zone began to predominate.

Labelling indices were calculated from stage 30 and stage 33 ulnae which had been incubated with I5 μCi/ml of ^{3}H-thymidine *in vitro* for times ranging from 2-6 hours. Again, only labelled cells in central sections were examined. Grain counts were not necessary since the cell was either heavily labelled or not labelled at all. Cell counts for labelling indices could only be carried out on cultured rudiments because of the impracticability of labelling *in vivo* - see Discussion.

Results

I) Increase in length

i) In vivo

The mean lengths and the rates of increase for each long bone rudiment of the wing are shown in Table I. Each rudiment increased in length by approximately 275% from stage 30 - stage 36. The mean lengths and mean growth rates appear similar for each rudiment (Table I) but this is due to slight differences in times of development between specimens; when an individual wing was measured, the humerus was always longer than the ulna which in turn was always longer than the radius.

Each rudiment exhibited a similarly shaped growth curve (Fig. I), with the growth rate being uniform until stage 32 when a large increase was observed. This higher rate was maintained until stage 34 when another increase in rate was observed (Fig. I).

ii) In Vitro

Table II shows an increase in length of approximately I30% for stage 30 rudiments over a 4 day period in culture. (Stage 30 rudiments

would be expected to increase by approximately I90% over the same period *in vivo*). The pattern of growth seems autonomous since the radius retained the lowest growth rate and the humerus retained the highest. *In vitro* growth curves (Fig. 2) are very different from those for *in vivo* rudiments (Fig. I). Initially, the *in vitro* growth rate approaches the *in vivo* growth rate for the first day of culture but there is a constant decrease in growth rate thereafter, if rudiments are cultured for I0 days or more linear increments cease and a plateau is observed (not shown). The general shape of an *in vitro* growth curve, for any given rudiment, was similar irrespective of the age of the rudiment prior to culture.

2) Histology

i) In vivo

At stage 28 the ulna appears to have only two distinct types of cartilage cell, a central zone of flattened cells with a much smaller zone of rounded cells at each end (Fig. 3). Some flattened cells in the centre of the rudiment are beginning to enlarge but no recognisable hypertrophic cells are yet present. At this stage a loose perichondrium can be observed around the central region, but at the "epiphyseal" ends the chondrogenic cells merge imperceptibly into the surrounding mesenchyme. By stage 30, however, several distinct hypertrophic cells are present and the ulna resembles a typical cartilage long bone rudiment with 3 histological cell types - rounded, flattened and hypertrophic (see Fig. 4). The perichondrium around the hypertrophic cell region is more distinct than at stage 28, the perichondrium around the flattened cell regions appears loose and at the epiphyseal ends the cartilage continues to merge into the mesenchyme (Fig. 4).

The periosteum first appears around the centre of the region of hypertrophic cells at stage 32 and is closely followed by sub-periosteal

osteoid formation (Fig. 5). The perichondrium is fairly distinct around the flattened cell regions and by now a loose perichondrium is observed covering the epiphyseal ends. At the proximal end of the ulna, the beginnings of a protrusion, the olecranon, is observed. In vivo the olecranon forms a groove for the ulna to fit into at the elbow (Fig. 5). At stage 34 (Fig. 6), 3 zones of cells are still present and the bilayered periosteum surrounds the whole of the hypertrophic cell region. At the junctions of the hypertrophic cell zone with the flattened cell zones the periosteum is replaced with a perichondrium (Fig. 6). The olecranon is more pronounced and appears as an asymmetric extension of the epiphysis.

The invasion of blood vessels into the cartilage and the formation of a marrow cavity begins, in the ulna, at stage 35 (Fig. 7) when central hypertrophic cells are resorbed. (The region where the blood vessels will enter can be recognised at stage 34 (Fig. 6).) From this stage onwards the gross histology changes little except that the bony area becomes progressively larger at the expense of cartilaginous regions.

Although the gross internal configuration of the chondrocytes changes little between stage 30 - stage 34, a transitory change in the orientation of cells within zones occurs in some rudiments between stage 30 - stage 31. At this stage, flattened cells in the centre of the rudiments (which are aligned with their long axis perpendicular to the long axis of the rudiment) appear to re-orientate whilst they are in the process of hypertrophying (they now have their long axis parallel to the long axis of the rudiment). This re-orientation is particularly evident in the humerus (Fig. 8) and is not apparent by stage 32. A similar orientation has been observed in the tibia (Archer, unpublished observation).

If intact, non-sectioned rudiments are looked at, between stage 32 - stage 34 it is possible to identify the position of each cellular zone by examination using incident illumunation (Fig. 9). The junctions of the zone of hypertrophic cells with the zones of flattened cells occurs at the point where the diameter of the diaphysis begins to increase, also, because of the periosteum there is a difference in contrast between the two zones. The periosteum surrounding the hypertrophic cell zone appears opaque whereas the perichondrium surrounding the flattened cell zones appears clearer and lighter in colour. The boundary between the zones appears almost as a straight line (Fig. 9). The junction between the zone of flattened cells and the zone of rounded cells is not so readily identified but it can still be seen and can be accurately estimated. This means of identifying each zone can be used to set up cell cultures of individual chondrocyte cell types. The efficiency of this gross method of zone separation is shown in Figure IO.

ii) In vitro

When a stage 30 ulna is grown in vitro for four days it increases in length such that it approaches the length of a stage 35 ulna in vivo (Tables I and II) (stage 30 - stage 35 takes $2\frac{1}{2}$ - 3 days in vivo). The "dumb-bell" shape of the rudiment has been retained and the cultured rudiment develops a gross morphology similar to that of the non-cultured stage 35 ulna (Fig. IIa). The histological structure, however, is quite different (Fig. IIb, compare with Fig. 7).

Sub-periosteal bone formation and the spread of hypertrophy have begun as in vivo but no invasion of blood vessels or resorption of cartilage is observed (Fig. IIb). Also, the olecranon, visible in non-cultured rudiments (Figs. 6 and 7), fails to appear so that both epiphyseal ends now appear rounded (Fig. IIa).

The increase in length observed in culture (up to 70% of the in vivo increase), together with the continuing spread of hypertrophy suggests that a large proportion of the increase in length in vivo is due to properties of the cartilage rudiment itself. It would appear, however, that enviromental factors are necessary for the process of osteogenesis to proceed further than the first collar of sub-periosteal bone.

3) Cellular changes during cartilage growth

Along the proximo-distal axis of a cartilage rudiment, 5 zones of cells can be observed - rounded, flattened, hypertrophic, flattened and rounded (see Fig. 6). Tables III - VII present data for the ulna, between stage 30 - stage 34, detailing the lengths of each individual zone, the number of cells, end to end, along the length of each zone, the number of cells across the width of each zone the total number of cells in each zone, and the density of cells in each zone. Each table also includes the same data for a stage 30 ulna which has been cultured for 3 days. Ulnae were cultured for 3 days so that they would be approximately equal in length to a stage 34 non-cultured ulna (compare Table I with Table II). For ease of understanding non-cultured and cultured data will be considered separately.

i) In vivo

Although each table presents differing data there are several similarities in the general trends observed. These are outlined below.

a) Proximal zones are generally larger than their corresponding distal zones: they are longer (table III), contain more cells end to end (Table IV), contain more cells across their width (Table V) and have a greater cell number (Table VI) for every stage studied. However, there is no difference in cell density between proximal and

distal zones (Table VII).

b) Each zone increases in length during the period studied (Table III) but only the hypertrophic zone increased in length proportional to the whole rudiment. By definition if one zone increases in proportion, others must decrease, in the case of cartilage long bone rudiments the hypertrophic zone increases in proportional length at the expense of flattened zones (particularly the proximal zone) and to a lesser extent rounded zones (Table III). The hypertrophic zone is also the only zone to show a proportional increase in cell number end to end (Table IV), once again at the expense of flattened and rounded zones.

c) Each zone shows an increase in total cell number (Table VI). Both the hypertrophic cell zone and the proximal rounded cell zone show a proportional increase in total cell number at the expense of both flattened cell zones and the distal rounded cell zone.

d) Each zone shows an increase in the number of cells across the width of the rudiment and this increase is always greater in rounded zones than in flattened zones (table V). The hypertrophic zone only increases in cell number across it's width until stage 32 when it remains fairly constant until the cells are resorbed (Table V).

e) The hypertrophic zone starts out as one of the smallest zones but from stage 32 - stage 34 it is clearly the longest individual zone (Table III). This spread of hypertrophy can be best observed as the increase in the number of cells end to end along the longitudinal axis of the zone. For the ulna, this spread proceeds at a rate of approximately 50% of the existing number of cells per developmental stage (Table IV).

f) Rounded cell zones have a higher cell density than flattened cell zones which in turn have a higher cell density than hypertrophic

zones (Table VII). Cell densities tend to decrease from stage 30 - stage 34 in every zone except for an increase in cell density of rounded cells between stage 32 - stage 33 (Table VII).

ii) In vitro

Tables III - VII show that when a stage 30 ulna is cultured for 3 days the overall picture is similar to that of a stage 34 non-cultured ulna. However there are several small differences which can be grouped together as follows:

a) The cultured rudiment is very similar in length to the stage 34 non-cultured ulna but it has longer zone lengths (Table III), has more cells end to end (Table IV) and has an increased total cell number (Table VI). Proximal zones are now similar in length to distal zones making the rudiment more symmetrical (Table III, Fig. I2).

b) When individual zones are looked at, it is observed that every zone, except the proximal rounded zone, increases in length (Table III), contains a higher number of cells end to end (Table IV) and has a higher total cell number (Table VI) when compared with non-cultured ulnae. The proximal rounded zone, however, is smaller (Table III), has fewer cells end to end (Table IV) and has a lower total cell number (Table VI) when compared with a stage 34 non-cultured ulna. The lack of rounded cells is compensated for by an increase in the total number of proximal flattened cells present (Table VI).

c) Much of the increased cell number in distal zones (Table VI) can be accounted for by an increase in the number of cells across the width of these zones (Table V). Proximal zones do not increase in width when compared with stage 34 non-cultured rudiments (Table V).

d) The hypertrophic zone does not increase in the number of cells across it's width (Table V) but it does increase dramatically in the number of cells end to end (Table IV). This results in the hypertrophic

zone becoming the longest zone (Table III) with the greatest number of cells (Table VI).

e) The cell density in each zone is lower than that found in corresponding zones of stage 34 non-cultured ulnae (Table VII).

4) Labelling Indices

Calculations of labelling indices were carried out on the radius, ulna and humerus at stage 30 and stage 33 (6 and 8 days) after incubating the rudiments in tritiated thymidine for 2, 4 or 6 hours. Table VIII shows the overall labelling index for each whole rudiment. The percentage of cells labelled at any given time was remarkably similar for each rudiment studied. The labelling index increased when labelling time was increased from 2 to 4 hours but remained static between 4 and 6 hours for both ages studied. There would appear to be a decrease in the overall labelling index between stage 30 and stage 33 for each rudiment at each time interval. Since 4 hours of labelling produces the highest percentage of labelled cells, Figures I3 - I8 show the pattern of labelling in each rudiment at this time.

When the labelling index of each individual zone is looked at (Tables IX - XIV), it is observed that there is always a higher percentage of rounded cells labelled than flattened, and always more flattened labelled than hypertrophic. Hypertrophic cells hardly label at all in the radius and ulna (Figs. I3, I4, I6 and I7). Hypertrophic cells which do label are near the border with the flattened zones and perhaps should be considered as hypertrophying flattened cells. The only rudiment to show any significant labelling of hypertrophic cells is the stage 30 humerus (Table XI, Fig. I5); and it is important to note that this is the time when re-orientation of flattened cells is taking place as mentioned in section 2 above.

For any labelling time in both the stage 30 and stage 33 radius

and ulna, the labelling index of cells in the distal rounded cell zone is almost always higher than in the proximal rounded cell zone (Tables IX, X, XII and XIII). The humerus, on the other hand has a slightly higher index in the proximal rounded cell zone (Tables XI and XIV). However, if the rudiment is considered as two separate halves and the overall labelling index of the rounded plus flattened cell zone in each half is calculated, very little differences are observed between proximal and distal halves (Tables IX - XIV).

Discussion

The results presented in this chapter provide a detailed account of the growth of cartilaginous long bone rudiments of the chick wing. Growth is dealt with in terms of increases in length, increases in cell number and the labelling index of cells at any given time.

The hierarchy of growth rates shown in Table I is in general agreement with that observed by Fell and Mellanby (1955) and Summerbell (1976), however, the values presented here are from living, unfixed material and therefore require no consideration of shrinkage factors. The increase in length observed per stage is not uniform (Fig. I) with spurts in growth occurring between stage 32 - stage 33 and between stage 34 - stage 35. This observation may be corroborated by a careful study of Figure 4 in Summerbell's 1976 paper which shows growth curves for various chick wing rudiments. If tangents are drawn on these curves at the points where the slope of the curve changes, then at least 2 points are found - one between 7-8 days and one between 8-9 days of development, which correspond to the stages mentioned above. It also seems likely that a spurt in growth occurs between stage 29 - stage 30, but because of the difficulty in obtaining accurate linear measurements of stage 27 and stage 28 rudiments this additional

acceleration in growth cannot be verified precisely.

The growth spurts shown in Figure I may be correlated with histological observations. The perichondrium is replaced by a periosteum around the middle of the diaphysis at stage 32 coinciding with the first appearance of osteoid and osteogenic tissue. From this stage onwards, the central cartilage core of the diaphysis ceases to expand in width (Table V) and this would cause any further increase in volume to be represented as an increase in length. Therefore, a sudden increase in length, as shown in Fig. I, might be expected.

Osteogenesis, associated with the onset of vascular invasion, occurs between stage 34 - stage 35 in the ulna, (Fig. 7), and it is possible that factors, present in the blood, accelerate growth at this time. This may be similar to the action of growth hormone on the growth plate of hypophysectomised rats (Isaksson, Jansson and Gause, 1982). The possible spurt in growth which may occur between stage 29 - stage 30, could be explained simply by the onset of cell hypertrophy (Figs. 3 and 4) causing a rapid increase in cell volume which is again manifested largely as an increase in length.

When grown in organ culture, cartilage rudiments exhibit a high degree of autonomous growth in that the gross morphology is very similar to an equivalently sized in vivo sample (Fig. IIa). The overall growth rate observed was higher than that observed by Fell and Mellanby (1955) which implies that the culture system used here is more efficient (Table II). No growth spurts were observed in cultured rudiments - after the first day of culture the rate of elongation constantly decreases so that by 10 - 14 days in culture a plateau is reached and the rudiment ceases to increase in length (data not shown). A cultured stage 30 ulna forms a periosteum during the first day and this may play some role in keeping the rate of increase similar to that found in vivo.

Histological studies show that at an early stage (stage 28) the *in vivo* ulna consists of only two cell types, rounded and flattened (Fig. 3), with the third cell type, the hypertrophic cell, appearing at stage 30 (Fig. 4). It is generally accepted that at any given stage the ulna is more advanced than the radius but less advanced than the tibia. However, Holder (1978) states that 3 types of cell are present in the radius by stage 28 and Stocum *et al* (1979) do not mention the existence of the 3 cell types in the tibia until day 8 (stage 34).

The histological appearance of embryonic chick limb rudiments has been well documented (e.g. see Fell, 1925 and Holder, 1978) and therefore will not be considered in detail here. For the purpose of this chapter the main points are that between stage 30 - stage 34 an embryonic rudiment consists typically of 3 types of chondrocytes, the hypertrophic zone begins as the smallest zone, in the centre of the diaphysis, at stage 30 and spreads along the diaphysis towards the epiphysis so that by stage 32 it is the largest individual zone. (Figs. 4 and 5, Table III).

The problem as to why a rudiment increases in length much more than in width may be explained in part by an understanding of the process of hypertrophy. Hypertrophic cells arise from flattened cells and examination of any rudiment shows, that the long axes of flattened cells are arranged perpendicularly to the adjacent perichondrium and therefore roughly perpendicular to the long axis of the rudiment (Fig. 6). The process of hypertrophy involves a massive increase in volume of a flattened cell which can be most simply envisaged as an increase in the size of the small axis of the cell, i.e. the width, (Fig. 19). Skalaslek (personal communication) has pointed out that such an increase in volume would be transferred, almost totally, into

longitudinal growth. Also, once a perichondrium/periosteum has formed, this may form a constraining barrier resisting transverse expansion and, therefore, favour growth in a longitudinal direction (Lutfi, 1974). The role of the perichondrium/periosteum as a constraining barrier will be considered in a later chapter.

It is obvious from histological examination that during the process of growth and development changes take place in the configuration of the cellular zones. These changes have been studied *in vivo* and *in vitro*. Analysis of the lengths of each zone of a non-cultured ulna is in general agreement with the results of Stocum *et al* (1979) on the tibia in that the length of each zone increases as the embryo develops (Table III). However, in the case of the ulna only the hypertrophic zone increases by such a degree that it increases in length in proportion to the original size of the rudiment (Table III). This suggests that the spread of hypertrophy throughout the rudiment may be one of the most important factors in the overall increase in length observed.

The spread of hypertrophy can be observed clearly when the number of cells end to end along the longitudinal axis are counted for each developmental stage. This spread occurs at a uniform rate such that, at any given stage, the number of hypertrophic cells end to end will be 50% greater than the number of cells end to end at the previous stage (Table IV). The importance of cell hypertrophy has been shown by Biggers and Gwatkin (1964), who prevented cell division by X-irradiation and observed that a rudiment continued to increase in length by up to 80% of the controls. However, this reduction of 20% in length and the lack of a uniform growth curve (Fig. I) suggests that the other zones must also play a role in the growth process.

Much of the growth effect of the rounded cell zones is to produce a large increase in the number of cells across the width of the zones

(Table V). In the case of the distal rounded cell zone it would appear that almost all of it's increase in cell number (Table VI) is used to widen the zone since this zone shows the smallest increase in length (Table III) or number of cells along the longitudinal axis (Table IV). This is consistant with the findings of Hert (1972) who stated that interstitial growth within an epiphysis contributed to a rudiment's increase in width. The proximal rounded zone undoubtedly plays a role in the increase in length by producing the olecranon beginning at stage 32 (Fig. 5). This coincides with a large increase in rounded cell number between stage 32 - stage 33 (Table VI) and a large increase in the number of cells end to end (Table IV).

Both of the flattened zones produce a similar pattern in their amount of growth per stage except that the proximal zone is always larger (Tables III and IV) and contains more cells (Table VI) then the distal zone. In these flattened zones the cells appear to be arranged in overlapping rows across the width of the zone (Fig. 5) and since the width of these zones does not increase by as much as the rounded zones (Table V), polarised secretion of matrix (Trelstad, 1977; Holmes and Trelstad, 1980), if it exists, would allow these zones to play a greater role in an increase in length rather than in width.

As every zone increases in size during development, the cell density in each zone decreases (Table VII). The decrease in cell density in rounded and flattened cell zones between stage 30 - stage 34 suggests that the increase in total cell number which occurs (Table VI) is insufficient to keep up with the general increase in size of the zones (Tables III, IV and V). The area of rounded or flattened cells change little during this period (see next chapter), therefore the lower cell density must be due to an increased accumulation of extracellular matrix around each cell. Although the hypertrophic cell zone increases in size

between stage 30 - stage 34, so too does the hypertrophic cell. This increase in cell area can account for the decrease in cell density.

The transient increase in cell density observed in the rounded cell zones between stage 32 - stage 33 (Table VII) coincides with a large increase in the number of cells which are present at that time (particularly in the proximal rounded cell zone) (Table VI). This increase in cell number and cell density may account, in part, for the growth spurt observed at this time (Fig. I).

When a stage 30 ulna which has been cultured for 3 days is studied the first impression is that it is only slightly larger than a stage 34 non-cultured rudiment. However, the lengths of most zones are increased (Table III) and there is a greater number of cells overall (Table VI). It must be noted that the proximal rounded cell zone is shorter (Table III), has fewer cells end to end (Table IV) and has a lower cell number (Table VI) than the corresponding zone in a stage 34 rudiment. This discrepancy can be accounted for by the fact that the cultured rudiment does not develop an olecranon (Fig. I2) and the zone appears more uniformly round. The width of this zone does not decrease (Table V) which suggests that the zone is growing efficiently in the culture system and that the olecranon may form *in vivo* due to some factor extrinsic to the ulna. The olecranon fits around the elbow joint *in vivo* and it may be that mechanical pressure from the humerus is required for the development of this protrusion. The lack of a protrusion in cultured ulnae is similar to the lack of a groove across the femoral head, housing the acetabular ligament found when femora were cultured (Murray and Selby, I930).

If a stage 30 ulna were to continue developing for 3 days *in vivo* it would become a stage 35/36 ulna which, would have many more cells than a stage 34 ulna. When a stage 30 ulna is grown *in vitro* for 3 days it does have more cells than a stage 34 non-cultured ulna (Table VI)

which suggests that the cultured rudiment may be continuing to grow at a cellular rate similar to that *in vivo*.

If each zone, from a cultured rudiment, is considered individually it is found that both flattened zones, the hypertrophic zone and the distal rounded zone all increase in size and cell number (Tables IV and VI). The large increase in proximal flattened cells observed when compared with proximal rounded cells (Table VI) may simply be due to the lack of the olecranon extending from the rounded zone, however, several different rudiments exhibit a large increase in flattened cells when cultured (Archer, personal communication) and this may be a property of the culture system. The hypertrophic zone continues to spread at a rate which is similar to that observed *in vivo* (Table IV).

The density of cells in each zone of a stage 30 cultured rudiment is lower than that found in the corresponding zones of a stage 34 non-cultured rudiment (Table VII). Non- cultured rudiments show a decrease in cell density with increasing developmental stage (Table VII) and it is possible that the lower cell densities observed in cultured rudiments are just a continuation of this process.

These results indicate that the culture system used is efficient and allows the cartilaginous zones to develop at a rate similar to that of *in vivo* rudiments. It proved technically difficult to incorporate ^{3}H-thymidine into cartilage rudiments *in vivo* (injection of labelled medium into the blood system resulted in labelled mesenchyme, sometimes labelled perichondrium but never labelled cartilage - probably because of the avascular nature of early cartilage) but if the culture system allows development to continue as *in vivo* then incorporation of ^{3}H-thymidine *in vitro* for short periods of time should provide useful information as to labelling indices *in vivo*. Tables IX - XIV present values for the labelling indices of the radius, ulna and humerus at stage

30 and stage 33. The general conclusions from these tables are:

I) Rounded cell zones have a higher labelling index than flattened cell zones which in turn have a higher labelling index than hypertrophic cell zones.

2) Distal halves of the radius and ulna tend to have a higher labelling index than proximal halves.

3) In the case of the humerus, proximal halves tend to have a higher labelling index than the distal halves.

4) Stage 30 rudiments have a higher labelling index for each individual zone (and for the whole rudiment) than a stage 33 rudiment. (To achieve the overall labelling index for each rudiment the total number of cells were counted, it was found that the total cell number of a stage 33 radius cultured for 2 hours - 3836 was remarkably similar to that found by Holder (1977) in a stage 33 non-cultured radius - 3627. The excess cells found here are probably due to growth in vitro).

Kember's work on the kinetics of growth plates in bones of juvenile rats (1972; 1973; 1978) also included data on the labelling indices of the individual growth plates. It is difficult to directly compare Kember's results with those listed above since cartilage long bone rudiments do not have epiphyseal growth plates. However, if a long bone rudiment is considered as two separate halves and each half (rounded plus flattened cell zones) is thought of as being equivalent to an individual growth plate, general comparisons can then be made. When this is done, important similarities are found:

i) the labelling index for any given half, or individual zone (or growth plate) of a particular rudiment shows little variation from embryo to embryo i.e. the labelling index observed is typical of that rudiment, not just of that embryo

ii) at a given age, and time of incubation with ^{3}H-thymidine, the

labelling index in any half rudiment (rounded plus flattened zone values) is similar no matter which rudiment is looked at (the actual labelling indices found are very similar to that found by Kember e.g. stage 30 radius, proximal half, 4 hours incubation, labelling index = 17%; 6 week old rat, proximal tibia labelling index = 16%). This is consistant with the findings that the labelling indices of growth plates in the rat pelvis are similar to those of long bones (Kember 1972, 1973). The results from the chick embryo go further in that the overall labelling index for a whole rudiment is also similar for any rudiment at any particular age and time of incubation with ^{3}H-thymidine. These results suggest that cartilaginous rudiments of the chick wing provide an excellent model for the mammalian epiphyseal growth plate.

The conclusions from Kember's work were that the cell cycle time and the percentage of cells entering S phase prior to entering the cell cycle varied little from growth plate to growth plate (Kember and Walker, 1971; Kember, 1978). Therefore variations in the growth rate between individual bones are due to variations in the size of the proliferating cell population. Similar conclusions can be made from chick embryo results (see later chapter for cell cycle times). The decrease in the overall labelling index in older (stage 33) rudiments, when compared with younger (stage 30) rudiments, can be explained by the fact that a large proportion of cells have become hypertrophic and have withdrawn from the cell cycle, therefore they do not incorporate ^{3}H-thymidine.

It is important to know what determines the size of the proliferating population. It is unlikely that the change from rounded cell to flattened cell is due to some diffusible signal originating from the epiphyseal region for two reasons:

I) If a concentration gradient existed, no labelled flattened or hypertrophic cell should be found.

2) If a diffusible signal did exist, one would expect the size of the rounded cell zone, i.e. the number of cells end to end, to remain constant but this does not happen (Table IV). (Factors which may control the change from rounded to flattened cell will be dealt with in a later chapter).

It would appear that there are differences between the labelling indices of proximal and distal halves of each rudiment. Distal halves of the radius and ulna (particularly rounded cell zones) have higher labelling indices than their larger proximal halves which implies that the distal halves of these rudiments may grow at a faster rate than the proximal halves. The humerus, on the other hand, has a higher labelling index in proximal halves. These results are consistant with the findings of several workers on the relative growth rates of each growth plate in several different species (see Stockwell, 1980 for review).

It is not known why the overall percentage of labelled cells does not increase when exposure time to ^{3}H-thymidine is increased from 4-6 hours (Table VIII) but examination of Tables IX-XIV show that there is a tendency for the labelling index of rounded cell zones to decrease with a concomitant increase in the labelling index of flattened cell zones. It may be that many rounded cells exhibit synchronous spurts of S phase and that between 4 and 6 hours these cells mature into flattened cells. It may also be that different cell types commence waves of DNA synthesis at different times. (Cell cycle times for each cell type both in the rudiment and when cultured free from the extracellular matrix will be presented in a later chapter). The observation that flattened cells closest to the zone of hypertrophy incorporate ^{3}H-thymidine just prior to hypertrophy suggests that DNA synthesis may be a prerequisite to cell hypertrophy.

In conclusion, the results presented here suggest that the chick

embryo ulna does not increase in length at a uniform rate but comes about via a series of spurts in growth. These spurts in growth do not necessarily involve cell division since the process of hypertrophy combined with periosteal bone formation at stage 32 would produce a rapid increase in length. Cell labelling data indicates that the overall labelling index is similar in each rudiment of the wing and that it is variations in the size of the proliferating population which produce differences in the rate of growth. This implies that the size of the initial condensation, or initial rounded zone, may be of paramount importance in determining the final size of the rudiment.

During the time period studied in this chapter the rudiment maintains its characteristic "dumb-bell" shape. How this shape is formed in the first place and how it is subsequently maintained throughout development will be the subject of the following chapter.

Fig. I. In vivo growth curves of the long bone rudiments of the chick embryo wing.

x---x Radius

o---o Ulna

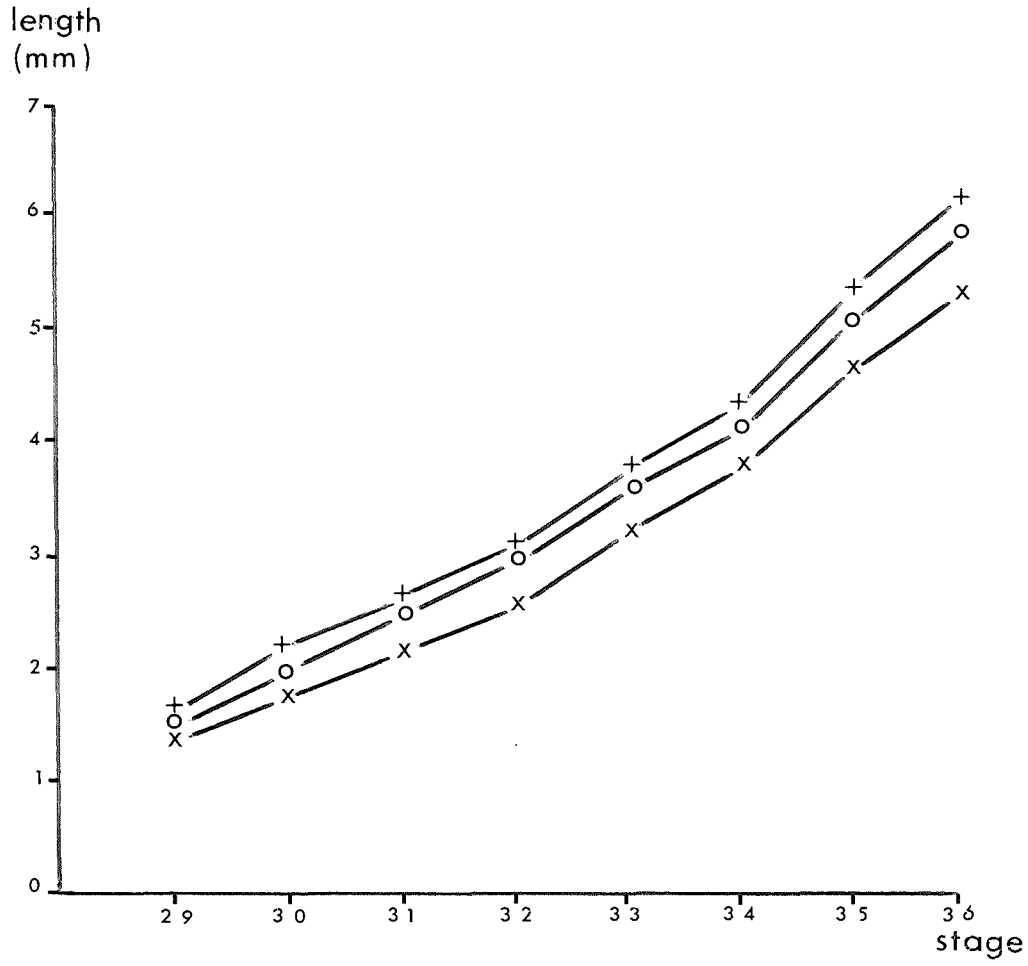
+---+ Humerus

Fig. 2. In vitro growth curves of the long bone rudiments from a stage 30 wing.

x---x Radius

o---o Ulna

+---+ Humerus


length
(mm)
7
6
5
4
3
2
1
0
29
30
31
32
33
34
35
36
stage

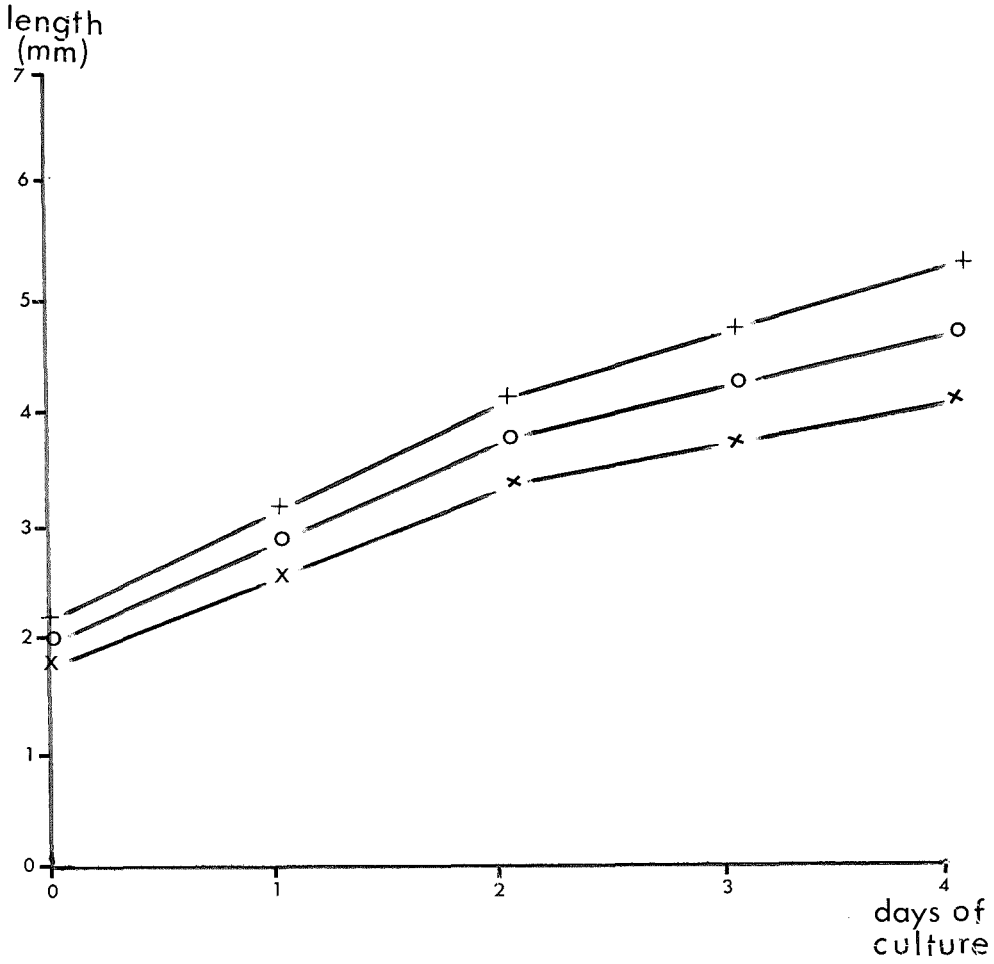
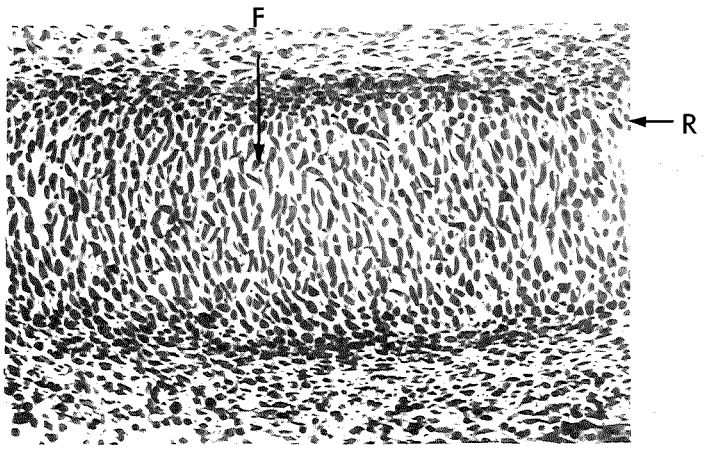
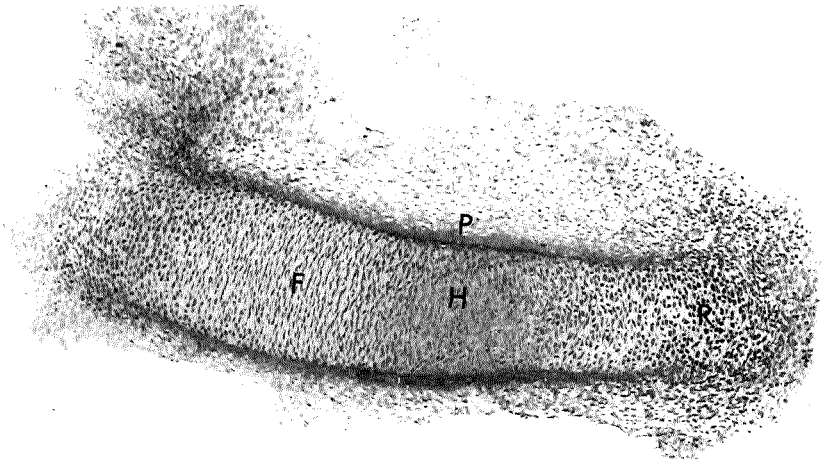

length
(mm)
7
6
5
4
3
2
1
0
0
1
2
3
4
days of
culture

Fig. 3. Stage 28 ulna. Only 2 cell types are present, rounded and flattened. The flattened cell zone is surrounded by a very weak perichondrium whilst the rounded cell zones merge into the mesenchyme.
Tol. blue stain. Mag. x 125.
R = rounded, F = flattened.

Fig. 4. Stage 30 ulna. 3 cell types are now present, rounded, flattened and hypertrophic. Note that the perichondrium appears more distinct around the hypertrophic cell zone.
Tol. blue stain. Mag. x 50.
R = rounded, F = flattened, H = hypertrophic, P = perichondrium.

Fig. 5. Stage 32 ulna, proximal half. Cells in the centre of the rudiment are now surrounded by an early periosteum. Flattened and rounded cell zones continue to be surrounded by a perichondrium. The proximal rounded cell zone shows the beginings of a protrusion - the olecranon.
Tol. blue stain. Mag. x 40.
R = rounded, F = flattened, H = hypertrophic, P = perichondrium.


F
R


P
F
H
R

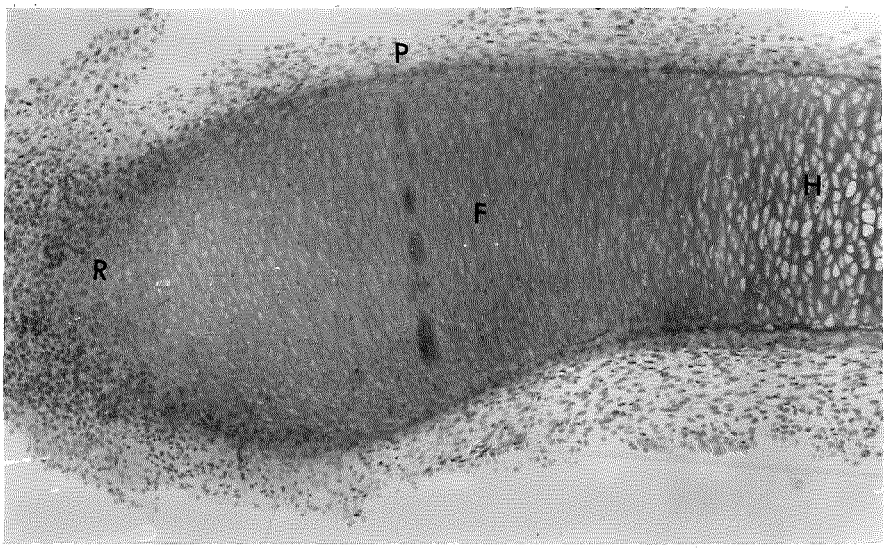
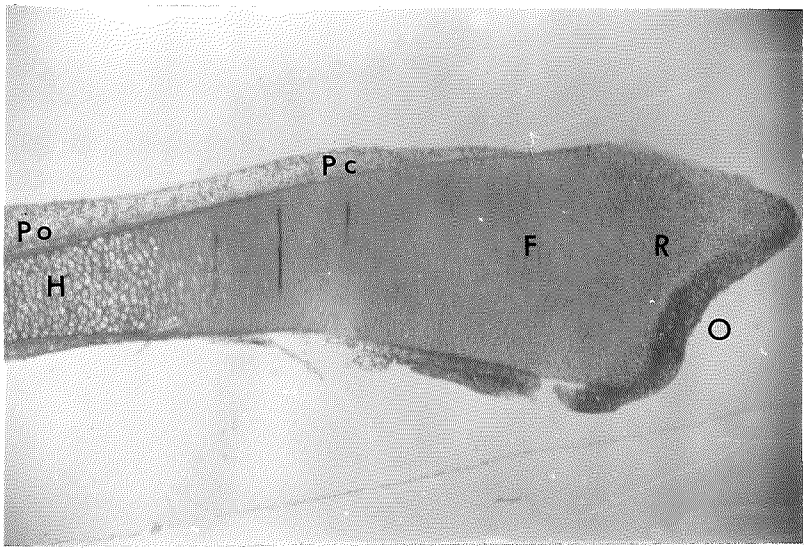

P
F
H
R

Fig. 6. Stage 34 ulna, proximal half. The periosteum now extends as far as the zone of hypertrophy. The lighter stained area near the centre of the hypertrophic cell zone (x) marks the position where the invasion of blood vessels begins. The olecranon process is more distinct in the proximal rounded cell zone.
Tol. blue stain. Mag. x 32.
R = rounded, F = flattened, H = hypertrophic, Pc = perichondrium, Po = periosteum, O = olecranon.

Fig. 7. Stage 35 ulna. Central hypertrophic cells are resorbed by blood vessels which gradually spread throughout the hypertrophic region.
Tol. blue stain. Wax section of whole wing. Mag. x 32.

Fig. 8. Stage 30/31 humerus. Reorientation of central flattened cells just prior to cell hypertrophy. This orientation is not apparent by stage 32.
Tol. blue stain. Mag. x 125.


Pc
Po
H
F
R
O

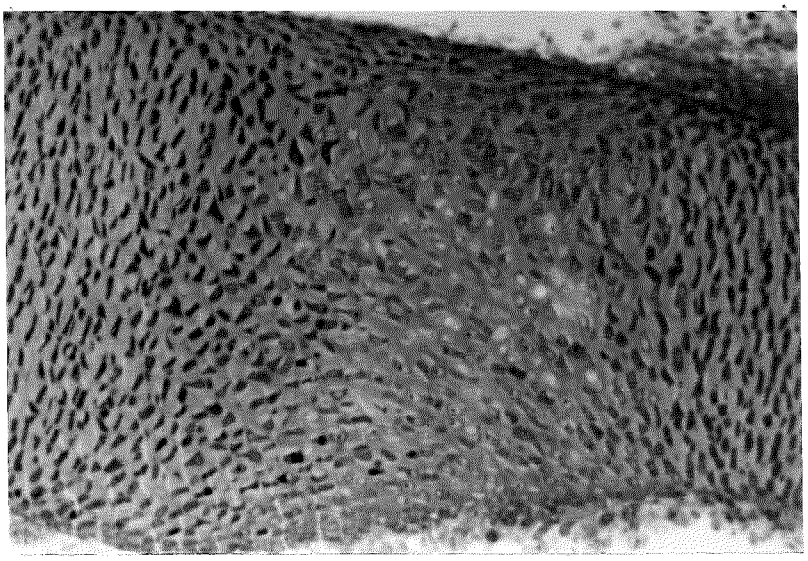
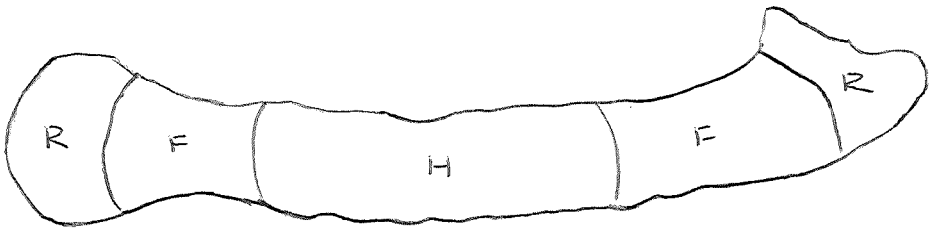
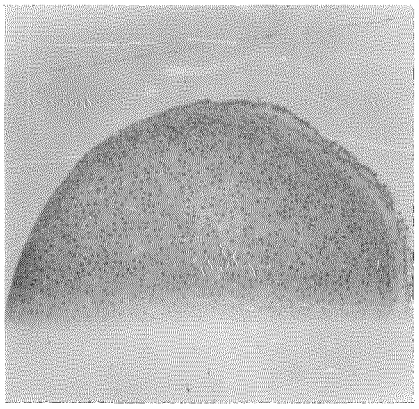


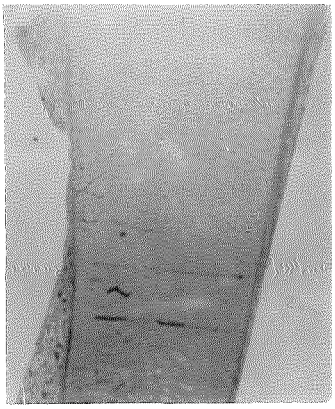
Fig. 9. Stage 3+ ulna. Camera lucida drawings of an intact ulna to show the demarcations of the individual cellular zones. The boundaries between the hypertrophic and flattened cell zones appear almost as straight lines whilst that between flattened and rounded zones is more difficult to distinguish.
R = rounded, F = flattened, H = hypertrophic.

Fig. 10. Individual zones of a stage 32 ulna isolated by cutting into pieces at the 2 junctions shown in the previous Figure.
Tol. blue stain. Mag. x 50.
r = round cell zone, f = flattened cell zone, c = hypertrophic cell zone.

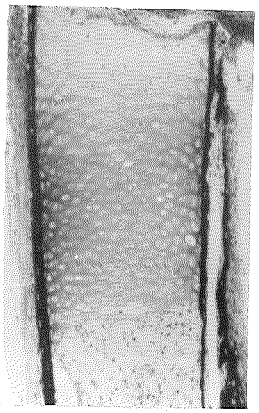




a



b

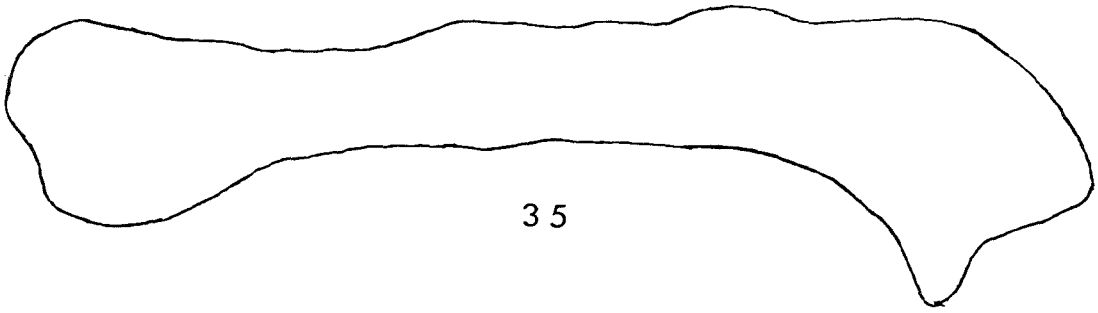


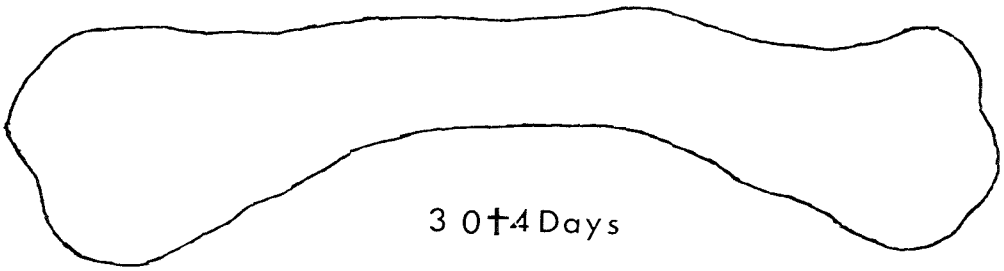
c

Fig. IIa. Camera lucida drawings to show the similarity between shape and size of a stage 35 ulna and a stage 30 ulna grown in vitro for 4 days.

Fig. IIb. Stage 30 ulna plus 4 days in vitro growth. Sub-periosteal bone has continued to cover the entire zone of hypertrophy but no invasion of blood vessels has occurred. Note that the olecranon has not formed and that the proximal epiphysis is almost spherical in shape.
Tol. blue stain. Mag. x 40.
R = rounded, F = flattened, H = hypertrophic.

Fig. I2. Stage 30 ulna plus 3 days in vitro growth. Note that the olecranon is much reduced but that there are more proximal flattened cells than normal. No invasion of blood vessels has occurred.
Tol. blue stain. Mag. x 40.
R = rounded, F = flattened, H = hypertrophic.


35


30+4 Days


H
F
R

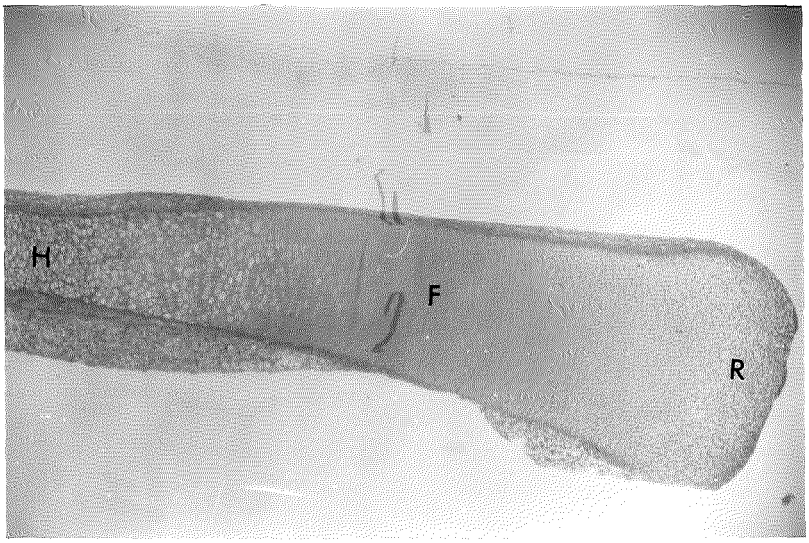
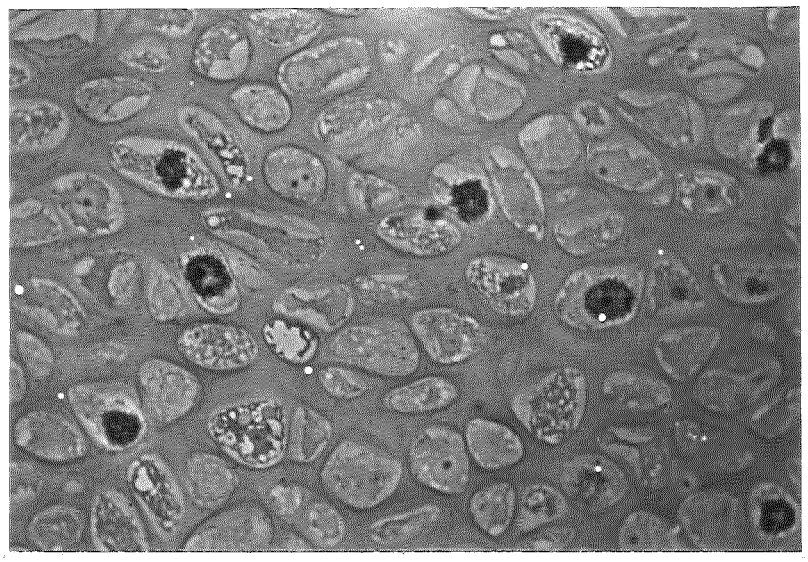
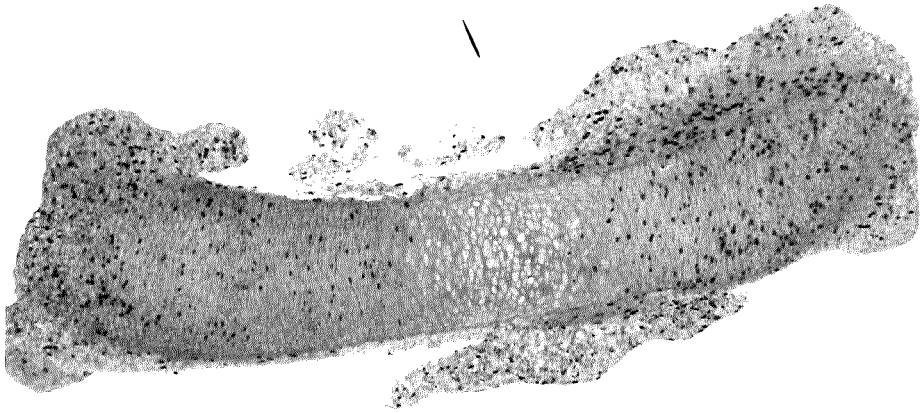

H
F
R

Fig. I3. Stage 30 radius labelled for 4 hours. The distribution of labelled nuclei can be seen to be maintained almost exclusively to the rounded cell zones, very few flattened cells are labelled and no hypertrophic cells have incorporated label.
Tol. blue stain. Mag. x 50.

Fig. I4. Stage 30 ulna labelled for 4 hours. The pattern of labelling is very similar to that for a stage 30 radius.
Tol. blue stain. Mag. x 50.

Fig. I5. Stage 30 humerus labelled for 4 hours. The pattern of labelling is similar to that for the radius and ulna but in this case a few hypertrophic cells have incorporated label.
Tol. blue stain. Mag. x 320.





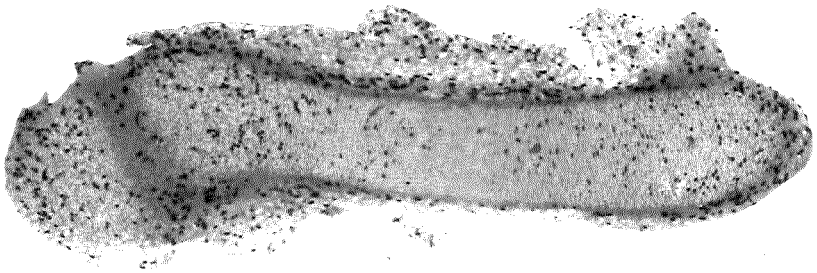
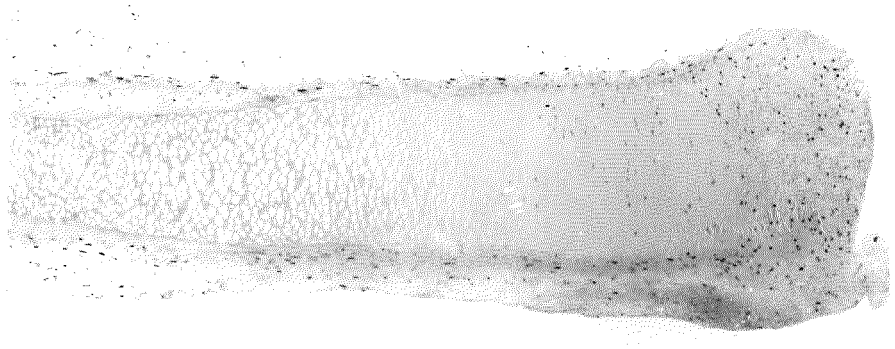
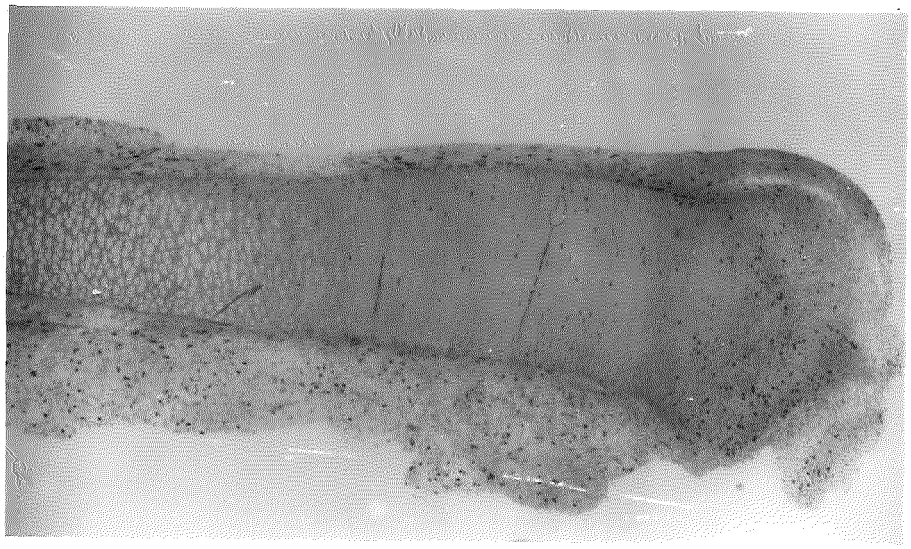


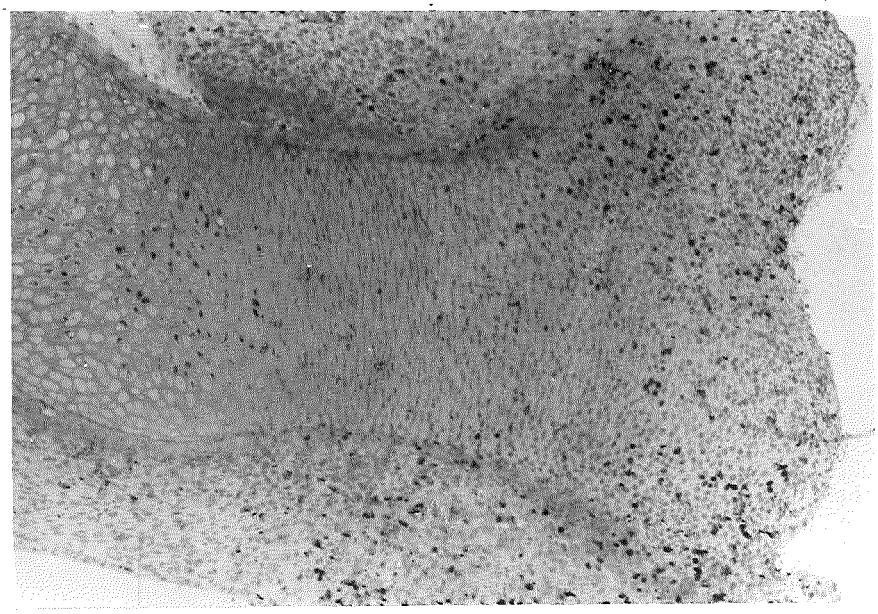
Fig. I6. Stage 33 radius labelled for 4 hours. The overall pattern is similar to that for a stage 30 rudiment. Note that the distinct perichondrium is heavily labelled compared to the cartilage cell zones.
Tol. blue stain. Mag. x 40.

Fig. I7. Stage 33 ulna labelled for 4 hours. Similar to the stage 33 radius. Note that small clusters of labelled cells are present in the proximal rounded cell zone - these may aid the production of the olecranon.
Tol. blue stain. Mag. x 40.

Fig. I8. Stage 33 humerus labelled for 4 hours. No hypertrophic cells have labelled.
Tol. blue stain. Mag. x 40.







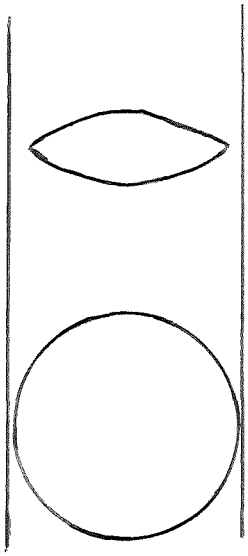


Fig. 19. Diagramatic representation of the process of cell hypertrophy.

A flattened cell, when it hypertrophies, hardly increases in length at all along it's long axis but increases dramatically along it's short axis. Since the flattened cell zone does not increase much in width this expansion would almost entirely be transformed into an increase in length.

If such an expansion as shown in the example occurred, the net result would be approximately a 4 fold increase in volume which could play a considerable role in the longitudinal elongation of the long bone rudiment. (If a flattened cell is considered as a disc with a depth equal to the long axis then the volume can be calculated as $4/3\pi abc$ (where $a = \frac{1}{2}$ x long axis, $b = \frac{1}{2}$ x short axis and $c = \frac{1}{2}$ x depth, J. Lewis, personal communication). If the hypertrophic cell is considered as a sphere then the volume can be calculated as $4/3\ \pi\ r^3$).

Table I Mean lengths of cartilage long bone rudiments of the developing chick wing.

	Length of rudiment (mm)		
Stage	Radius	Ulna	Humerus
29	I.44 ± 0.07*	I.50 ± 0.08	I.65 ± 0.04
30	I.80 ± 0.I0	2.0I ± 0.I3	2.I9 ± 0.I8
3I	2.I8 ± 0.I4	2.53 ± 0.I3	2.6I ± 0.I8
32	2.60 ± 0.I3	2.97 ± 0.I4	3.08 ± 0.I6
33	3.24 ± 0.I8	3.59 ± 0.II	3.77 ± 0.I4
34	3.82 ± 0.I5	4.I3 ± 0.I9	4.26 ± 0.26
35	4.63 ± 0.2I	5.04 ± 0.20	5.28 ± 0.I9
36	5.26 ± 0.I8	5.80 ± 0.2I	6.I5 ± 0.I9
Growth rate (mm/stage)	0.546	0.6I5	0.643
(mm/day)+	0.955	I.053	I.I25
% increase in length	265	287	273

Unfixed rudiments were excised and measured immediately using a calibrated eyepiece graticule. Values are presented for measurements made on at least 30 specimens for each rudiment at each developmental stage.

* Standard deviation of the mean.

+ Stage 29 - stage 36 = 6-I0 days (Hamburger and Hamilton, I95I).

Table II Mean lengths of stage 30 cartilage long bone rudiments cultured *in vitro*.

	Length of rudiment (mm)		
Day of culture	Radius	Ulna	Humerus
0	1.80 ± 0.10*	2.01 ± 0.13	2.19 ± 0.18
1	2.58 ± 0.08	2.93 ± 0.16	3.15 ± 0.16
2	3.32 ± 0.18	3.74 ± 0.08	4.05 ± 0.05
3	3.69 ± 0.18	4.24 ± 0.06	4.71 ± 0.09
4	4.02 ± 0.20	4.64 ± 0.12	5.28 ± 0.13
Growth rate (mm/day)	0.555	0.657	0.773
% increase in length	123	131	141

Unfixed rudiments were measured daily, using a calibrated eyepiece graticule, whilst being cultured on top of Millipore filter rafts. Values are presented for measurements made on at least 30 specimens for each rudiment.

*Standard deviation of the mean.

Table III Mean lengths of histological zones of the ulna.

	Length of zone (mm)					
	Stage					
Zone	30	31	32	33	34	30+3 days*
Rounded (Prox.)	0.13 (13.8)+	0.16 (16.0)	0.17 (10.5)	0.20 (10.3)	0.37 (12.7)	0.25 (6.5)
Flattened	0.33 (35.1)	0.31 (31.0)	0.47 (29.0)	0.51 (26.1)	0.64 (21.9)	0.78 (20.3)
Hypertrophic	0.17 (18.1)	0.20 (20.0)	0.52 (32.1)	0.69 (33.4)	1.22 (41.8)	1.85 (48.2)
Flattened	0.22 (23.4)	0.20 (20.0)	0.31 (19.1)	0.39 (20.0)	0.51 (17.5)	0.74 (19.3)
Rounded (Dist.)	0.09 (9.6)	0.13 (13.0)	0.15 (9.2)	0.16 (8.2)	0.18 (6.1)	0.22 (5.7)

Zone lengths were measured from 1 µm thick araldite sections using a calibrated eyepiece graticule. Values were calculated from 3 central sections, 10 µm apart, from 6 different rudiments for each stage. No attempt is made to give total lengths for each stage since younger rudiments were found to shrink much more than older rudiments.

*A stage 30 ulna cultured for 3 days is comparable in size to a stage 34 non-cultured ulna. Values are presented so that histological progression can be compared *in vivo* and *in vitro*.

+Figures in parentheses give the percentage of the total fixed length occupied by each zone.

Standard deviations are left out for clarity, they ranged from 1-7% of the length.

Table IV Cell numbers along the longitudinal axis of each zone of the ulna.

	Number of cells end to end*					
	Stage					
Zone	30	31	32	33	34	30+3 days
Rounded (Prox.)	31 (18.4)+	37 (17.4)	42 (15.8)	53 (15.7)	72 (16.9)	55 (11.4)
Flattened	50 (29.8)	63 (29.7)	78 (29.3)	93 (27.6)	105 (24.6)	115 (23.8)
Hypertrophic	26 (15.5)	38 (17.9)	56 (21.0)	86 (25.6)	127 (29.8)	173 (35.7)
Flattened	36 (21.4)	43 (20.3)	55 (20.7)	69 (20.5)	86 (20.2)	101 (20.9)
Rounded (Dist.)	25 (14.9)	31 (14.6)	35 (13.2)	35 (10.4)	36 (8.5)	40 (8.3)
Total	168	212	266	336	426	484

*Values were calculated from the same specimens as in Table III. Cells in the proximal rounded zone were counted from the tip of the olecranon to the first flattened cell.

+Figures in parentheses give the number of cells end to end along each zone as a percentage of the total number of cells along the rudiment.

Standard deviations ranged from 2-9%.

Table V Cell numbers across the transverse axis of each zone of the ulna.

	Number of cells across each zone*					
	Stage					
Zone	30	31	32	33	34	30+3 days
Rounded (Prox.)	29	38	49	66	83	83
Flattened	28	31	38	46	58	59
Hypertrophic	18	20	22	23	23	22
Flattened	23	28	32	35	39	44
Rounded (Dist.)	27	35	36	49	54	73

*Values were calculated from the same specimens as in Table III. Rounded and flattened cells were counted at the widest part of the zone whilst hypertrophic cells were counted in the centre of the zone.

Standard deviations ranged from 4-9%.

Table VI Total cell numbers of histological zones of the ulna.

	Number of cells/zone*					
	Stage					
Zone	30	3I	32	33	34	30+3 days
Rounded (Prox.)	426 (22.5)+	609 (24.6)	759 (20.3)	I256 (24.2)	I767 (25.7)	I5I0 (I6.3)
Flattened	498 (26.3)	558 (22.6)	950 (25.4)	I223 (23.5)	I297 (I8.8)	2055 (22.2)
Hypertrophic	3I5 (I6.6)	429 (I7.3)	756 (20.3)	I00I (I9.3)	I726 (25.I)	2505 (27.I)
Flattened	336 (I7.7)	396 (I6.0)	649 (I7.4)	885 (I7.0)	I068 (I5.5)	I88I (20.4)
Rounded (Dist.)	32I (I6.9)	480 (I9.4)	6I9 (I6.6)	837 (I6.I)	I026 (I4.9)	I287 (I3.9)
Total	I896	2472	3733	5I96	6884	9238

*Values are calculated from the same specimens as in Table III.

+Figures in parentheses give the number of cells in each zone as a percentage of the total cell number.

Standard deviations ranged from 2-II%.

Table VII Cell densities in histological zones of the ulna.

	Number of cells/unit area*					
	Stage					
Zone	30+	3I	32	33	34	30+3 days
Rounded	I99	I82	I40	I55	I45	I23
Flattened	I7I	I49	II0	II4	87	7I
Hypertrophic	I54	I27	84	79	79	6I

*Values are calculated from the same specimens as in Table III. The area looked at was I7956μm^2 (I34 μm x I34 μm) and two readings were taken from each zone. No difference was found in cell density between proximal and distal zones, therefore, the values are grouped together.

+The zones in stage 30 rudiments were too small for two readings of I7956 μm^2, therefore an area of 7I82 μm^2 (53.6 μm x I34 μm) was looked at and the values were extrapolated to fit the larger area.

Standard deviations ranged from 2-7%.

Table VIII Overall labelling indices of cartilaginous long bone rudiments of the chick wing cultured *in vitro*.

	Labelling index		
	Hours labelled		
Stage 30	2	4	6
Radius	II.55 ± 0.30*	I5.28 ± 0.70	I5.33 ± 0.65
Ulna	II.5I ± 0.83	I5.96 ± 0.75	I5.74 ± 0.85
Humerus	I2.24 ± 0.69	I5.43 ± 0.82	I5.00 ± 0.58
Stage 33	2	4	6
Radius	9.07 ± 0.85	I2.54 ± 0.78	I2.54 ± 0.63
Ulna	9.00 ± 0.48	I2.59 ± 0.93	I3.22 ± 0.68
Humerus	9.96 ± 0.62	I3.09 ± 0.58	I3.78 ± 0.83

Values represent the number of cells which incorporate ^{3}H-thymidine (I5 uCi/ml) as a percentage of the total cell number. Values were calculated from 3 central sections (I /um thick) I0 /um apart, from 4 different rudiments for each labelling period.

*Standard deviation of the mean.

Table IX Labelling indices of histological zones of a stage 30 radius cultured in vitro.

	Labelling index*						Number of cells end to end along each zone**		
	Hours labelled								
Zone	2		4		6		2	4	6
Rounded (Prox.)	I2.80 (336)		20.74 (376)		I9.86 (428)		24	23	25
		II.74		I7.03		I6.88			
Flat.	I0.68 (394)		I3.32 (428)		I3.90 (489)		54	52	53
Hyp.	2.I9 (I37)		I.78 (I69)		0.08 (236)		I0	I5	I8
Flat.	II.73 (324)		I0.82 (342)		I3.04 (368)		33	35	38
		I3.58		I7.09		I8.I4			
Rounded (Dist.)	I5.43 (298)		23.36 (32I)		23.24 (370)		I5	I6	I6
Total	II.55 (I489)		I5.28 (I636)		I5.33 (I89I)		I37	I42	I50

*Values were calculated from the same sections as in Table VIII. Numbers in parentheses are the total cell number in each zone.

Standard deviations are not shown for clarity, they ranged from 2-II%.

**These values allow comparison with those for the ulna (Table IV).

Table X Labelling indices of histological zones of a stage 30 ulna cultured in vitro.

	Labelling index*						Number of cells end to end along each zone**		
	Hours labelled								
Zone	2		4		6		2	4	6
Rounded (Prox.)	I3.54 (443)		22.49 (498)		20.52 (536)		30	33	33
		II.88		I9.45		I6.94			
Flat.	I0.23 (508)		I6.4I (5I8)		I3.36 (554)		5I	50	53
Hyp.	2.42 (330)		I.6I (373)		I.28 (39I)		27	29	26
Flat.	I3.I2 (343)		I3.36 (358)		I9.24 (395)		36	34	38
		I5.4I		I7.22		20.70			
Rounded (Dist.)	I7.8I (348)		2I.07 (408)		22.I7 (469)		26	25	29
Total	II.5I (I972)		I5.96 (2I55)		I5.74 (2345)		I7I	I7I	I79

*Values were calculated from the same sections as in Table VIII.

Standard deviations ranged from 4-8%.

**When compared with Table IV, these values appear very similar which suggest that the addition of ^{3}H-thymidine for short periods of time does not affect cell numbers.

Numbers in parentheses are the total cell number in each zone.

Table XI Labelling indices of histological zones of a stage 30 humerus cultured in vitro.

	Labelling index*						Number of cells end to end along each zone		
	Hours labelled								
Zone	2		4		6		2	4	6
Rounded (Prox.)	14.75 (1023)		22.90 (1144)		20.90 (1245)		52	51	49
		11.57		16.14		15.83			
Flat.	8.39 (527)		9.37 (587)		10.76 (604)		53	51	53
Hyp.	7.76 (528)		6.34 (567)		2.36 (592)		31	31	32
Flat.	12.48 (705)		11.20 (732)		12.57 (756)		42	40	42
		13.60		15.48		16.30			
Rounded (Dist.)	14.73 (740)		19.76 (754)		20.04 (923)		31	30	33
Total	12.24 (3568)		15.43 (3784)		15.00 (4120)		206	203	209

*Values were calculated from the same sections as in Table VIII.

Standard deviations ranged from 3-9%.

Numbers in parentheses are the total cell number in each zone.

Table XII Labelling indices of histological zones of a stage 33 radius cultured *in vitro*.

	Labelling index*						Number of cells end to end along each zone		
	Hours labelled								
Zone	2		4		6		2	4	6
Rounded (Prox.)	14.16 (918)		20.02 (1084)		19.07 (1173)		34	34	38
		10.98		14.98		14.66			
Flat.	7.80 (1013)		9.93 (1086)		10.31 (1125)		72	74	75
Hyp.	0 (698)		0 (757)		0 (923)		59	61	68
Flat.	7.28 (563)		11.09 (698)		12.05 (672)		51	53	53
		11.17		15.57		16.30			
Rounded (Dist.)	15.06 (644)		20.06 (698)		20.55 (764)		24	23	24
Total	9.07 (3836)		12.54 (4178)		12.54 (4637)		240	249	258

*Values were calculated from the same sections as in Table VIII.

Standard deviations ranged from 2-10%.

Numbers in parentheses are the total cell number in each zone.

Table XIII Labelling indices of histological zones of a stage 33 ulna cultured in vitro.

	Labelling index*						Number of cells end to end along each zone		
	Hours labelled								
Zone	2		4		6		2	4	6
Rounded (Prox.)	I5.II (III2)		2I.96 (II93)		I9.98 (I35I)		5I	50	54
		II.85		I7.II		I6.4I			
Flat.	8.59 (896)		I2.26 (938)		I2.84 (989)		88	90	93
Hyp.	0 (1223)		0 (I27I)		0 (I334)		86	91	95
Flat.	6.79 (898)		I0.II (910)		I3.20 (932)		66	66	70
		II.84		I5.49		I7.59			
Rounded (Dist.)	I6.89 (8I7)		20.86 (858)		2I.98 (992)		35	35	36
Total	9.00 (4946)		I2.59 (5I70)		I3.22 (5598)		326	332	348

*Values were calculated from the same sections as in Table VIII.

Standard deviations ranged from 2-I0%.

Numbers in parentheses are the total cell number in each zone.

Table XIV Labelling indices of histological zones of a stage 33 humerus cultured in vitro.

	Labelling index*						Number of cells end to end along each zone		
	Hours labelled								
Zone	2		4		6		2	4	6
Rounded (Prox.)	I4.2I (I372)		24.97 (I482)		24.02 (I653)		57	58	60
		II.53		I7.II		I7.54			
Flat.	8.86 (I083)		9.26 (II45)		II.06 (I248)		80	82	85
Hyp.	0 (I098)		0 (II56)		0 (I293)		99	I02	I09
Flat.	9.29 (I0I2)		9.06 (I060)		II.23 (I2II)		66	67	70
		I2.54		I3.37		I5.I2			
Rounded (Dist.)	I5.78 (II98)		I7.67 (I290)		I9.0I (I387)		4I	42	43
Total	9.96 (5763)		I3.09 (6I33)		I3.78 (6794)		343	349	367

*Values were calculated from the same sections as in Table VIII.

Standard deviations ranged from 3-9%.

Numbers in parentheses are the total cell number in each zone.

C H A P T E R T W O

THE ROLE OF THE EXTRACELLULAR MATRIX IN CARTILAGE MORPHOGENESIS

Introduction

Cartilage morphogenesis, unlike morphogenesis in early embryos, is always associated with growth. In the case of cartilage long bone rudiments, growth involves a massive increase in volume with little increase in width except at the ends of the rudiment, therefore, morphogenesis results in the formation of a long narrow diaphysis with epiphyseal swellings - the typical "dog-bone" shape.

As mentioned in the General Introduction, cartilage morphogenesis could be controlled by various factors; cell division, cell hypertrophy, matrix secretion and organisation, cell configuration, appositional growth and physical constraints (Thorogood, 1983). Several of these factors have already been studied in detail, e.g. cell division (Biggers and Gwatkin, 1964), cell hypertrophy (Biggers, 1957), cell orientation and configuration (Holmes and Trelstad, 1980), whilst other factors, such as appositional growth, have been difficult to determine and probably play a minor role (Miekle, 1975). Surprisingly, little attention has been paid to the roles played by matrix secretion, matrix organisation and physical constraints in the early morphogenesis of long bone rudiments.

The importance of matrix secretion and organisation in maintaining the shape of a cartilage element has been shown by Diewert, in the developing rat mandible, when she added the teratogens 6-Amino-nicotinamide (6-AN) (Diewert, 1979), Diazo-oxo-norleucine (DON) (Diewert and Pratt, 1979), and B-aminoproprionitrile fumerate (BAPN) (Diewert, 1980a) to pregnant mothers. The general conclusion was that all three teratogens produced a cleft palate, but that 6-AN and DON both affected the growth rate, and therefore the size, of Meckel's cartilage, whereas BAPN affected the shape of Meckel's cartilage causing it to bend (Diewert, 1980b).

The organisation of cartilage matrix has also been shown to play an important role in maintaining shape in long bone rudiments. Fitton-Jackson (1970) treated 12 day old chick embryo tibiae with collagenase for 2 days, *in vitro*, and then returned the rudiments to enzyme-free medium. By 5 days of culture, the cartilaginous regions had completely lost their characteristic anatomical features although the size was similar to that of controls. This result was interpreted as indicating that the structural organisation of the collagenous componant of the extracellular matrix is a vital factor in maintaining the definite shape of a rudiment.

The object of this chapter is to study the early formation of a cartilaginous long bone rudiment (the chick embryo ulna) and to evaluate the relative roles played by cell division, cell hypertrophy and matrix secretion in relation to the overall increase in volume observed during growth. Particular attention will be paid to the role of the collagenous component of the extracellular matrix (ECM) in the formation and maintainance of the characteristic histological distribution of cells and the anatomical shape of the ulna.

Materials and Methods

The early formation of the ulna (between stages 24-27) was studied histologically using 1 µm sections (see General Methods). Particular attention was paid to the shape, orientation and distribution of the prechondrogenic and chondrogenic cells. In addition, the roles of cell division, cell hypertrophy and ECM secretion were evaluated *in vivo* from slightly older rudiments (stage 31-34) when 3 distinct cell types - rounded, flattened and hypertrophic chondrocytes - were present. The relative role of each parameter was evaluated by calculating the cell volume, the number of cells/unit volume the volume of each cellular

zone and the volume of ECM/cell at each stage. These calculations were performed as follows:-

a) cell volume - rounded and hypertrophic cells were considered as spheres and therefore had a volume of $4/3\ \pi r^3$. Flattened cells were considered as discs which appeared spherical in transverse section, similar to a coin, and therefore the volume was calculated as $4/3\ \pi$ a b c, where a = $\frac{1}{2}$ x longitudinal axis, b = $\frac{1}{2}$ x transverse axis and c = $\frac{1}{2}$ x depth - since the cell is spherical in transverse section, b = c. (J. Lewis, personal communication).

b) number of cells/unit volume - calculated from the equation $n = x/2r \div s$, where x = number of cells/unit area, r = radius of nuclei (or cells in the case of hypertrophic cells) and s = the thickness of the section studied (I/um) (J. Lewis, personal communication).

c) volume of cellular zones - calculated directly from camera lucida drawings of fixed and sectioned ulnae. If the rudiment is considered as a cylinder with a partial sphere at either end then the volume of the hypertrophic cell zone can be calculated as $\pi r^2 h$, where h = the length of the zone and r = $\frac{1}{2}$ x the mean width of the zone, obtained by taking at least 4 measurements of the width at different regions of the zone for each rudiment. The volume of the flattened cell zone was also calculated as $\pi r^2 h$ but in this case the mean width was determined by taking at least 8 measurements at different regions since the width tapered in towards the hypertrophic zone. The volume of the rounded cell zone was calculated by estimating what proportion of a sphere the zone occupied and then performing the equation vol. = a x $4/3\ \pi r^3$, where a = the proportion of the sphere and r = $\frac{1}{2}$ x maximum diameter of the zone. a was estimated by measuring the distance from the mid-point of the maximum diameter to at least 12 points on the periphery of the zone and expressing the mean distance as a proportion of the maximum diameter.

d) volume of ECM/cell - calculated from the equation

$$\text{ECM/cell} = \frac{\text{total volume of zone - total volume of cells}}{\text{total number of cells}}$$

where the total volume of the zone = volume of proximal zone plus volume of distal zone, total volume of cells = mean volume of the cell x total number of cells in the two zones, total number of cells = volume of the zones (prox. + distal) x number of cells/unit volume. In the case of the hypertrophic zone only one set of values was required.

In each case mean values for the parameters were obtained from 3 central 1 µm sections, 10 µm apart, from each of 4 different rudiments for each stage.

The role of the collagenous component of the ECM was evaluated in two ways:

i) by culturing stage 32 ulnae in the presence of teratogenic drugs (all of which affect collagen synthesis and assembly) for 3 days continuously or for 1 day followed by removal to complete control medium.

ii) by treating stage 30 and stage 32 ulnae with the enzyme collagenase (0.2% w/v. Sigma) for time intervals ranging from 15-60 minutes followed by culture in control medium for 2 days.

The teratogenic drugs used were chosen because they were known to induce cartilage malformations when added to chick embryos *in ovo* (G. Shellswell, personal communication). They were L - azetidine - 2 - carboxylic acid (LACA) - an analogue of L - proline which results in the synthesis of abnormal collagen which cannot be secreted from the cell, B-aminoproprionitrile fumerate (BAPN) - an inhibitor of the enzyme lysyl oxidase which prevents cross-linkage between collagen fibres after secretion, and $\alpha\,\alpha'$ dipyridyl ($\alpha\,\alpha'$) - an iron chelating agent which inhibits prolyl and lysyl hydroxylase (all from Sigma).

Each drug was used in concentrations of I, 5 and I0 mg/ml, and each drug was dissolved in complete BGJb medium.

Results from collagen disruption experiments suggested that collagen might be present in an organised manner within non-treated rudiments, therefore, this was looked for using the Electron Microscope. 0.I/um araldite sections were cut and stained as described in the General Methods.

Results

I) Formation of the early cartilaginous ulna

Figures I-4 show histological sections, cut longitudinally, of the position of the ulna in embryos aged between stages 24-27 respectively. Figure I shows an increased cell density in the central region of the fore-limb, the so-called condensation, with no apparent cellular orientation. At stage 25 these cells appear more densly packed but still no overt orientation is observed (Fig. 2). Metachromasia cannot be identified in the ulna at this stage but it can be observed in the humerus of the same wing (Fig. 2a). However, by stage 26, distinct metachromatic matrix can be observed between the now oriented cells of the ulna. (For the remainder of this thesis, a condensation will be considered to have become a rudiment upon secretion of metachromatic matrix.) In longitudinal section this orientation appears to be at right angles to the long axis of the rudiment, hence the cells appear flattened (Fig. 3). This orientation is more pronounced in a stage 27 ulna (Fig. 4) and the rudiment at this stage consists virtually of one zone of flattened cells with a few rounded cells merging into the mesenchyme at what will be the "epiphyseal" ends (Fig. 4). It is also at stage 27 that the first clear presence of a barrier is observed between flattened cells in the "diaphysis"

and the surrounding mesenchyme. This early perichondrium is very loose and appears to be composed of round or polygonal cells in longitudinal section (Fig. 4). However, in transverse section the perichondrial cells appear cresent shaped and are arranged around the circumference of the rudiment (Fig. 5). It should be noted that flattened cells in transverse section appear polygonal and therefore are really disc-shaped (Fig. 5).

The very early rudiment at stage 26 is a cigar-shaped tissue comprising of approximately 25±2 cells across it's diameter and 90±4 cells along it's length (Fig. 3). It should be noted that the number of cells across the width at this stage is greater than the number of flattened chondrocytes across the width of a stage 27 rudiment - 17±1 cells (Fig. 4). (It is also greater than the number of hypertrophic cells across the width of rudiments aged between stage 30-34 - see Table V in chapter I). Interestingly, the number of cells making up the early perichondrium is approximately 4 deep on each side of the diaphysis (Fig. 4). Thus it appears that the outer cells of the condensation may form the perichondrium.

Once the perichondrium has formed the rudiment increases in length with little increase in width until flattened cells in the centre begin to hypertrophy at stage 30 with the result that perichondrial cells become stretched in a longitudinal direction (Fig. 6).

2) Further growth of the ulna

By the time cell hypertrophy begins the ulna has already assumed it's "dog-bone" shape and any future growth is predominately in a longitudinal direction (Fig. 7). This growth produces a large increase in volume which must be due to a combination of the 3 main parameters involved in cartilage growth - cell division, cell

hypertrophy and matrix secretion. Table I presents values for the cell density, the cell volume and the volume of ECM associated with each cell type in fixed ulnae from stage 3I - stage 34 (a 2 day growth period when the rudiment consists solely of rounded, flattened and hypertrophic chondrocytes). Also shown are values for the total volume of cellular zones plus an estimate of the number of cells present in each zone.

The findings of this table can be summarised as follows:

i) There is a general tendency for each cell type to exhibit a decrease in the number of cells/unit volume with increasing age. Concomitantly, each cell type undergoes an increase in cell volume and an increase in the volume of ECM associated with each cell. (No difference was found in any parameter between proximal and distal zones).

ii) Assuming that every hypertrophic cell originates from a rounded cell, then the process of cell hypertrophy from stage 3I - stage 34 involves a 27 - fold increase in cell volume.

iii) If the volume occupied by a rounded cell plus its associated ECM, at stage 3I, is compared with the volume occupied by a hypertrophic cell plus its associated ECM, at stage 34, then there is approximately a 9 - fold dilation during growth.

iv) During development the ratio of ECM volume : cell volume changes little for rounded and flattened cells but the roles for hypertrophic cells reverses. This implies that although the hypertrophic ECM increases in volume, the hypertrophic cell volume increases by a greater amount so that, unlike rounded and flattened cells, hypertrophic cell volume at stage 34 is greater than the volume of ECM associated with it. However, it should be noted that the ECM volume of hypertrophic cells almost doubles during this period whilst the increase in

ECM volume/cell for rounded and flattened cells is not so marked.

v) The total volume of the rudiment increases by approximately 6-fold ($67 \times 10^{-3}mm^3$ - $393 \times 10^{-3}mm^3$) but the total cell number only increases 3-fold (107058-319030).

3) Growth of the ulna in vitro

Stage 32 ulnae increase in length by an average of 105% when grown in vitro for 3 days (Table II). Histologically it can be seen that cell hypertrophy and sub-periosteal bone formation has continued to spread along the diaphysis but no invasion of blood vessels or resorption of cartilage is observed (In this way the rudiment is similar to that of a stage 30 rudiment cultured for the same time period - see chapter I). The stage 32 cultured ulna has one major difference from the stage 30 cultured ulna in that the olecranon at the proximal epiphysis has continued to develop (Fig. 8).

When cellular parameters are compared with those of a stage 32 non-cultured ulna (Table I) it is found that for each cell type there is a decrease in cell density concomitant with an increase in cell volume, and an increase in the volume of ECM associated with each cell (Table II). The largest increase in cell volume is found in hypertrophic cells which double in size to a volume which is greater than that found in stage 34 hypertrophic cells in vivo. The ratio of ECM volume : cell volume of rounded and flattened cells is virtually unchanged from stage 32 non-cultured ulnae but the ratio for hypertrophic cells has been reversed, that is the ratio has changed from 52:48 to 47:53, (Tables I and II). If the cellular distributions of cultured ulnae are studied (Table V) it is found that the cultured rudiment has more cells end to end, more cells across the width of the rounded zones and a greater total cell number than non-cultured rudiments (Table I this chapter and Tables IV-VI in chapter I).

4) The effect of teratogenic drugs on stage 32 ulnae cultured in vitro

Tables II-IV present data for the cell density, the cell volume, the volume of ECM associated with each cell and the overall increase in length observed when stage 32 ulnae were cultured in the presence of the teratogenic drugs LACA, BAPN or $\alpha\,\alpha$ either continuously or for I day followed by removal to control medium. The results of these tables can be summarised as follows

i) LACA - (a) Img/ml - Ulnae grown in LACA for 3 days increased in length by a mean of 47% (i.e. by approximately 50% of control ulnae) (Table II). The rudiments appear normal in histological section and clear distinctions can be observed between cellular zones. Several mitotic figures can be seen in the rounded cell zones. Closer examination under higher power shows that many flattened cells appear to be enclosed in large rounded lacunae rather than lacunae outlining the shape of the cell (Fig. 9).

When cellular parameters are compared with those of cultured controls it is found that each cell type exhibits a decrease in the number of cells/unit volume (Table II). To compensate for this, rounded cells show an increase in cell volume (this increase in volume is statistically significant, $P<0.05$) but no change in the volume of ECM association with each cell; flattened cells retain the same cell volume but the volume of ECM/cell increases ($P<0.05$); and hypertrophic cells show an increase in both cell volume and volume of ECM/cell ($P<0.05$). The ratio of ECM volume : cell volume decreased for rounded and hypertrophic cells but remained fairly constant for flattened cells (Table II). (For the remainder of the results, if increases or decreases in parameters are mentioned, statistical significance is implied and can be checked by referring to Tables II-VII).

(b) 5 mg/ml - Ulnae grown in LACA at 5mg/ml for 3 days increase in length by a mean of 25% (Table II). Histologically the rudiment appears far from normal and differs from cultured controls in several ways, the most obvious being the lack of flattened cells. Flattened lacunae are observed but the cells within these are rounded and are indistinguishable from cells in the rounded cell zone (Fig. I0).

When cellular parameters are compared with those of cultured controls it is found that the number of cells/unit volume decreases for each cell type whilst the volume of ECM associated with each cell increases. However, rounded and hypertrophic cells also increase in cell volume but flattened cell volume decreases. The ratio of ECM volume : cell volume decreases for rounded cells (i.e. cells are becoming proportionally larger) but increases for both flattened and hypertrophic cells (Table II).

(c) IOmg/ml - Ulnae cultured in LACA at IOmg/ml for 3 days increase in length by a mean of 9% (Data not shown). Histologically the rudiment appears dead with many cells containing pycnotic nuclei and many lacunae containing no cells at all (Fig. II). Cellular parameters were not measured in these rudiments.

(d) Img/ml plus 2 days control - Ulnae treated with LACA at Img/ml for I day and then cultured in control medium for 2 days increased in length by a mean of 96% (Table III). Histologically the rudiment is very similar to cultured controls (Fig. I2).

When cellular parameters are studied it is found that rounded and hypertrophic cells both show a decrease in the number of cells/unit volume concomitant with an increase in cell volume and an increase in the volume of ECM associated with each cell. Flattened cells, on the other hand, show an increase in the number of cells/unit volume

with a decrease in cell volume, however, they do show an increase in the volume of ECM/cell. The ratio of ECM volume : cell volume decreases for rounded cells but increases for flattened and hypertrophic cells (Table III).

ii) BAPN - (a) Img/ml - Ulnae grown in BAPN at Img/ml for 3 days increase in length by a mean of 46% (Table II). Histologically, rudiments appear quite different from cultured controls in that several cells appear dead, empty lacunae are present, the perichondrium is thickened and contains vacuolated cells and the diaphysis is very narrow with hypertrophic cells aligned longitudinally (Fig. I3).

When cellular parameters are compared with those of stage 32 cultured controls it is found that there is a general tendency for cell volume and the volume of ECM associated with each cell to increase whilst the cell density in each zone decreases. The ratio of ECM volume : cell volume decreases for rounded cells, increases for hypertrophic cells and remains constant for flattened cells (Table II).

(b) 5mg/ml - Ulnae grown in BAPN at 5mg/ml for 3 days increase in length by a mean of I0% (Data not shown). Histologically these rudiments appeared dead with almost every cell in the rounded and flattened zones containing a pycnotic nuclei (Fig. I4).

(c) I0mg/ml - Ulnae grown in BAPN at I0mg/ml for 3 days increase in length by a mean of 4% (Data not shown). Histologically the rudiments appear dead and are very similar to that of the 5mg/ml treated rudiment except that the matrix stains very faintly with toluidine blue (see Fig. I4).

(d) Img/ml plus 2 days control - Ulnae grown in BAPN at Img/ml for I day and then cultured in control medium for 2 days increase in length by a mean of 97% (Table III). Histologically, rudiments appear very different from cultured controls in that they become a z shape,

they have very few flattened cells (which are always preceeded and followed by round shaped cells) and they have extremely narrow diaphyses full of longitudinally aligned hypertrophic cells (Fig. I5).

When cellular parameters are compared with cultured controls, it is found that rounded cells have values very similar to controls. Flattened cells also have the same cell density as controls but the cell volume has decreased and the volume of ECM/cell has increased. Hypertrophic cells show a decrease in cell density but show a large increase in both cell volume and volume of ECM : cell. The ratio of ECM volume : cell volume has remained constant for rounded cells has increased for flattened cells but has decreased for hypertrophic cells (Table III).

(iii) ∝∝' - (a) Img/ml - Ulnae grown in ∝∝' at Img/ml for 3 days increase in length by a mean of 29% (Table II). Histologically the rudiments look different from controls in that no mitotic figures are observed, cells stain very darkly (with toluidine blue), some cells in the flattened zones are rounded, and there is virtually no perichondrium (Fig. I6).

When cellular parameters are compared with cultured controls it is found that rounded cells show a decrease in cell density, retain a similar cell volume but show an increase in the volume of ECM/cell. Flattened and hypertrophic cells both show an increase in cell density, and a decrease in cell volume of ECM/cell to that of controls. The ratio of ECM volume : cell volume changes little for rounded cells, but increases for both flattened and hypertrophic cells (Table II).

(b) and (c) - 5 and I0mg/ml - Ulnae grown in ∝∝' at 5 or I0mg/ml for 3 days did not increase in length. Histologically the rudiments appear dead, every cell has a pycnotic nucleus, hypertrophic cells are very small, and no distinct perichondrium is observed (Fig. I7).

(d) Img/ml plus 2 days control - Ulnae grown in ∝∝' at Img/ml for

I day and then cultured in control medium for 2 days increase in length by a mean of 31% (Table III). Histologically the rudiments appear very similar to those cultured continuously in ∝∝' at Img/ml (Figs. I6 and I8).

If cellular parameters are studied it is found that all 3 cell types increase in cell density and all 3 decrease in cell volume, whilst the volume of ECM/cell remains similar for rounded cells but increases for flattened and hypertrophic cells. The ratio of ECM volume : cell volume also increases for each cell type (Table III).

The above results show that ulnae cultured in the presence of teratogenic drugs exhibit changes in the cellular parameters of each type of chondrocyte plus a reduction in the overall increase in length observed during culture. The effects of these drugs on the distribution of cells within each rudiment are shown in Tables IV and V.

Stage 32 ulnae cultured in control medium for 3 days increased in total volume, total cell number, the number of cells end to end along the longitudinal axis and the number of cells across the transverse axis of each zone when compared with stage 32 non-cultured ulnae (Table IV compare with Table I this chapter and Table IV and V in chapter I). Ulnae cultured continuously in the presence of teratogenic drugs also increased in total volume and total cell number when compared with stage 32 non-cultured controls, but only those ulnae which were cultured in LACA at Img/ml increased in volume and cell number to a value greater than that for stage 32 cultured controls (Table IV).

Every treatment produced a decrease in the number of cells end to end along the longitudinal axis, when compared with cultured controls (Table IV) and, since the cell density almost always decreased with a

concomitant increase in cell volume (Table II), this implies that the increase in total volume and total cell number observed when ulnae were cultured in LACA at Img/ml must come about due to an increase in width. Ulnae treated with LACA at Img/ml increased in width at their epiphyseal ends by at least 50% more than controls (data not shown) and this is represented by an increase in the number of cells across the width of rounded and flattened zones (Table IV).

Treatment of ulnae with BAPN at Img/ml most closely approached the effects of LACA at Img/ml in that the total volume of rounded and flattened zones and the number of cells across the width of these zones increased with respect to cultured controls (Table IV). However, the volumes of the cells within these zones also increased considerably during the culture period, as did the volume of ECM associated with each cell, (Table II), and this resulted in the actual number of cells present within each zone being reduced when compared with cultured controls (Table IV). The hypertrophic cell zone also contained fewer cells than controls but in this case much of the decrease is due to a reduction in the overall volume of the zone (Table IV).

Treatment of ulnae with LACA at 5mg/ml and $\alpha\alpha'$ at Img/ml severly restricted growth. The number of cells end to end along the longitudinal axis and the number of cells across the width of each zone hardly increased with respect to stage 32 non-cultured controls and therefore, contained far fewer cells than cultured controls (Table IV).

A similar series of results was found when ulnae were cultured in the presence of teratogenic drugs for I day only and then allowed to recover for 2 days in control medium. In these cases, however, the increase in volume observed in the rounded zones of ulnae treated

with LACA at Img/ml was insufficient to match the increase in cell volume (Table II), therefore these zones contain fewer cells than controls (Table V). In addition the volumes of rounded and flattened cells in ulnae treated with BAPN at Img/ml did not increase with respect to cultured controls and, since the total volumes of these zones did increase, these zones were found to contain more cells than controls (Table V).

5) The effects of collagenase on ulnae grown in vitro

Table VI presents data for the increase in length and increase in epiphyseal width observed when stage 30 and stage 32 ulnae were treated with 0.2% (w/v) collagenase, at 37°C, for time periods ranging from 15-60 minutes and then allowed to recover in control medium for I or 2 days.

The general trend observed was that treated ulnae increased less in length and more in epiphyseal width than controls. Diaphyseal width did not increase significantly for either stage studied - data not shown. Also, there would appear to be a time and age related response in that the longer the time of exposure to collagenase, or the younger the rudiment, the greater the increase in epiphyseal width (Table VI).

Table VI shows that the maximum effect of collagenase was observed in stage 30 ulnae treated for 45 minutes and in stage 32 ulnae treated for 60 minutes - only these rudiments were studied in detail. In both cases the rudiment appeared to be recovering after 2 days in control medium, therefore cellular parameters were only measured after I day of recovery.

(i) Stage 30 - 45 minutes treatment. By I day of recovery, the proximal epiphysis has increased in width by 72% compared with 26% in controls (Table VI). Histologically the proximal rounded cell zone consists of a layer of closely packed cells in the centre which

is sandwiched between two regions of rounded cells at a slightly lower density (Fig. I9). A similar but smaller region is observed in the distal rounded cell zone. Cells in the flattened regions appear less elongated and are no longer arranged in discrete rows across the rudiment - several of the cells have their long axis parallel to the long axis of the rudiment. Hypertrophic cells appear small and very closely packed and no perichondrium can be seen around this zone whereas the perichondrium appears fairly normal around the flattened and rounded zones. All 3 zones stain faintly with toluidine blue (Fig. I9).

Examination of the cellular distribution in treated rudiments shows that the number of cells end to end along the longitudinal axis is similar to controls. However, the number of cells across the width of both rounded cell zones increased significantly ($P<0.05$). Similar increases were observed in flattened and hypertrophic cell zones but the increases were not so great (Table VII).

When cellular parameters were compared with cultured controls it was found that the cell density and cell volume of rounded cells both increased whilst the volume of ECM associated with each cell decreased. By contrast, the cell density and cell volume of flattened cells decreased whilst the volume of ECM/cell increased.

The cell density of hypertrophic cells also decreased but the cell volume increased resulting in the volume of ECM/cell remaining similar to controls (Table VII).

(ii) Stage 32 - 60 minutes treatment. By I day of recovery the proximal epiphysis has increased by 64% compared with 20% in controls (Table VI). Histologically these ulnae also have a layer of closely packed cells in both rounded cell zones whilst flattened cells tend to be thicker and more round (Fig. 20). Flattened cells are no

longer arranged in discrete rows across the rudiment and, in the distal half, several lacunae have broken open and some flattened cells appear almost to be in contact with each other. The junctions between the rounded and flattened cell zones is indistinct, very irregular and can no longer be considered as being a smooth arc. Both flattened and rounded cell zones are surrounded by a thick perichondrium in which the cells do not appear to be very elongated. By contrast, no perichondrium (or periosteum) can be seen surrounding the hypertrophic cell zone, but the thin layer of sub-periosteal bone, which was present prior to treatment, is still intact (Fig. 20).

Studies on the cellular distribution of treated rudiments show that the number of cells end to end along the longitudinal axis have decreased when compared with controls. The number of cells across the transverse axis of the proximal rounded zone has increased but all other zones either have values similar to or less then controls (Table VII).

When cellular parameters were compared with cultured controls it was found that treatment of stage 32 ulnae with collagenase gave results very similar to those shown by stage 30 treated rudiments. Rounded cells behaved identically in that cell density and cell volume both increased whilst the volume of ECM/cell decreased. Flattened cells decreased in cell density (as in stage 30) but the cell volume increased such that the volume of ECM/cell remained similar to controls. Hypertrophic cells decreased in cell density and increased in cell volume (as in stage 30) but in this case the volume of ECM/cell also increased (Table VII).

6) Collagen fibre alignment within the ulna

An Electron Micrograph (EM) of cells in the centre of the rounded cell region shows the chondrocytes to be small, rounded cells

containing a relatively large, round nucleus (Fig. 21). The cartilage matrix between rounded cells appears to be more granular than fibrillar (the granules are presumably glycosaminoglycans attached to their appropriate proteins) and higher magnification shows that collagen fibres are sparse and any which are present seem to be randomly arranged (Fig. 21a).

An S.E.M. of cells in the flattened cell region shows the cells to be large and elongated with an elongated nucleus (Fig. 22). The cartilage matrix between flattened cells appears fibrillar in nature, and at a higher magnification these fibres can be identified as collagen which are aligned in the same direction as the long axis of the cells when viewed in longitudinal section (Fig. 23). These fibres are never seen orientated in the direction of the short axis of the cell. In transverse section the collagen fibres are interwoven to form a meshwork with no apparant orientation (Fig. 23a).

When hypertrophic cells are looked at under the S.E.M. the cells appear large, usually rounded, and the cell nucleus often appears discontinuous (Fig. 24). The cells are widely separated by a very fibrillar cartilage matrix in which collagen fibres can be seen to be arranged around the circumference of the cell (Fig. 24).

Discussion

Every cartilage long bone rudiment arises from a condensation of mesenchyme cells which can be seen in histological section at 4 days of incubation (Fell, 1925) or stage 23/24 (Hamburger and Hamilton, 1951). Cells in the condensation are rounded and seem randomly orientated (Figs. 1 and 2). However, Holmes and Trelstad (1980) state that, in the mouse, condensation cells are in fact orientated by means of their Golgi [illegible] and similar observations have been

reported in the chick (see Ede, 1983 for review) but the significance of this to long bone formation has not yet been fully ascertained. The first overt orientation of condensation cells in the chick wing is observed in the humerus at stage 25 (Fig. 2a) when central cells in the future diaphysis become aligned at right angles to the long axis of the rudiment. Cell orientation is first observed in the ulna at stage 26 (Fig. 3). In both the humerus and the ulna, extracellular spaces are found which stain metachromatically with toluidine blue suggesting the presence of cartilage matrix. This implies that cartilage cell orientation is associated with matrix secretion (as suggested by Gould et al, 1974). Also, the short time period between stage 25 and stage 26 (about 6 hours) suggests that there is no stage when the cartilage rudiment is composed solely of rounded cells secreting cartilage matrix, nor is there a stage when pre-chondroblasts are already orientated. The nucleus-golgi body alignment observed by Trelstad (1977) and Holmes and Trelstad (1980) before matrix secretion is therefore unlikely to play a role in the initial orientation of cartilage cells.

A long-bone condensation consists of a "cigar-shaped" mass of cells which exist at a higher density than the surrounding mesenchyme but which do not come into extensive contact with each other (Gould et al, 1972; Thorogood and Hinchliffe, 1975). The ulnal condensation at stage 25/26 consists of approximately 25 cells across the width of the transverse axis (Figs. 2 and 3) but the number of flattened cells across the diaphysis of a stage 27 ulna is only 17 (Fig. 4). The number of cells which constitute the perichondrium at stage 27 is 8 (4 on either side), thus the total number of cells across the width of a stage 27 ulna is also 25. It would seem likely, therefore, that the cells at the periphery of the stage 25 condensation may give

rise to the early perichondrium. This observation reinforces the suggestion of Gould et al (1974) that the perichondrium forms as a result of peripheral cells being passively forced out and flattened circumferentially due to the accumulation of matrix secreted by central cells.

The early perichondrium is a very weak structure which allows expansion in both the longitudinal and transverse axes (as proposed by Carey, 1922) and it is not until stage 30/31, when cell hypertrophy begins, that the perichondrium becomes consolidated and increases in rudiment length exceed increases in width (Figs. 6 and 7). From this time onwards the rudiment consists of 3 types of chondrocytes and growth occurs by a combination of cell division, cell hypertrophy and matrix secretion. By assuming that the ulna is essentially a cylinder with a partial sphere at either end it is possible to estimate the volume of each individual zone of cells, from camera lucida drawings of fixed rudiments (see Fig. 7) and, using cell density values from Table I, a calculation can be made of the number of cells present in each zone. Therefore it is possible to estimate how much of the overall growth of a rudiment (measured as increase in total volume) is due to cell division, cell hypertrophy or matrix secretion. Figure 25 shows these calculations in detail for in vivo growth and Table VIII presents similar data for in vitro growth and for growth in the presence of teratogenic drugs.

In all cases the percentage of the total increase in volume (i.e. growth) due to matrix secretion exceeded the sum of the increases in volume due to cell division plus cell hypertrophy. Cell division was always the smallest factor involved in the increase in volume (Table VIII). These results are consistant with the suggestion by Thorogood (1983) that matrix secretion may be the most important

factor in cartilage growth, and with the results of Biggers and Gwatkin (1964) when tibiotarsi were found to increase in length by approximately 90% of controls even after cell division was knocked out by X-irradiation.

Although direct comparison cannot be made between the relative roles of cell division, cell hypertrophy and matrix secretion *in vivo* and *in vitro*, due to the stages and time periods used, it would seem that the process of cell hypertrophy does not proceed as well *in vitro* as *in vivo* (Table VIII). Hypertrophic cell volume increases with age *in vivo* such that, by stage 34, the volume of the cell is greater than the volume of the ECM associated with it (ratio of ECM volume : cell volume = 41:59) (Table I). If this trend continued throughout development then if a stage 32 ulna was compared with a rudiment 3 days older - stage 36, it would be expected that the hypertrophic cell volume would increase until the ratio of ECM volume : cell volume was high on the cell volume side. When a stage 32 ulna is grown *in vitro* for 3 days, the hypertrophic cell volume does increase but the ratio of ECM volume : cell volume is only 47:53 (Table II), therefore, hypertrophy has not proceeded as *in vivo*. This observation may account for the fact that any increase in length, when rudiments are grown *in vitro*, is always smaller than the increase in length observed, over the same time period *in vivo* (see chapter I), and is supported by the findings of Biggers that when cell hypertrophy is experimentally prevented, very little increase in length occurs (Biggers, 1957).

Although growth and development may not proceed as well *in vitro* as *in vivo*, it is important to note that the pattern of changes in cellular parameters is similar and, therefore, *in vitro* culture acts as a good model for *in vivo* growth at the cellular level.

The general effect of teratogenic drug and collagenase treatment, at the gross morphological level was to reduce longitudinal expansion but increase the epiphyseal width when compared with non-treated controls (Table II-VII).

Teratogenic drugs always produced their greatest effect on the region of flattened cells - cells tended to round up but remained within flattened lacunae in discrete rows across the rudiment (fig. I0). This would be expected since these drugs only affect new collagen synthesis and assembly, therefore, the existing cartilage matrix should be unaffected. It is important to note that interference with the normal collagen biosynthetic pathway is sufficient to change the shape of the flattened cells and in some cases (e.g. ~~'dipyridyl) actually kill all the cells. (The collagen biosynthetic pathway will not be discussed here - for recent reviews see Prockop et al (I979) and Kleinman et al (I98I).

Although collagenase treatment also resulted in a degree of rounding up of flattened cells, the greatest effects were observed to be the loss of discrete rows of flattened cells and an increase in cell density in the rounded cell zones (Fig. I9 and 20, Table VII). Collagenase results in collagen fibre breakdown therefore it is probable that the loss of discrete rows of flattened cells is due to disruption of collagen fibres within the cartilage matrix. It is not known whether the increase in cell density in rounded cell zones is due to an increase in the rate of cell division or whether it is due to a requirement for non-disrupted collagen fibres for the separation of cells.

Fitton-Jackson (I97I) demonstrated that prolonged treatment with weak collagenase (2 days at a concentration I/I00 of that used here) resulted in a loss of the characteristic shape of the epiphyseal

ends of I2 day old tibiae with no decrease in length when compared with controls. A large proportion of a rudiment at this age is bone therefore no difference in size between treated and control would be expected. However, in a rudiment composed solely of cartilage, disruption of the matrix would result in a failure to increase in length by as much as controls. Treatment of I2 day old ulnae with 0.2% collagenase for I hour had no effect on the shape of the rudiment (data not shown) therefore, prolonged treatment would be necessary and, although Fitton-Jackson did not perform histological examination, it is possible that the loss in shape of the epiphyseal ends may occur in a similar way to that described in this chapter.

It seems surprising that when the relative contributions of cell division, cell hypertrophy and matrix secretion are examined, matrix secretion appears to play a larger role when agents which disrupt collagen synthesis and assembly are added to rudiments (Table VIII). This result, however, may be misleading and may be caused by matrix disruption allowing matrix, at a lower density than normal to spread throughout the rudiment and, therefore appear to be present at a higher quantity when there is in fact less matrix present.

Collagen fibres in cartilage matrix are intimately associated with proteoglycan aggregates, therefore disruption of the collagen may result in total disruption of the cartilaginous matrix. From the results obtained using teratogenic drugs and collagenase it would appear that collagen fibres, either on their own or in conjunction with proteoglycans, are responsible for maintaining both the shape and spatial distribution of flattened cells. This would suggest that collagen is present in some sort of organised manner and this has

been found to be the case between cells in the flattened cell zone (Fig. 23).

Cartilage matrix in the rounded cell zone has no organised collagen fibres (Fig. 21) and it is proposed that as the process of cell hypertrophy progresses a wave of cell flattening moves out towards the epiphyseal ends; rounded cells come into contact with the organised collagen fibres and become flattened parallel to them (Fig. 26). This cell flattening may result in oriented matrix secretion which produces new, organised collagen fibres on which more rounded cells can flatten. In this way the process of cell flattening is self propogating and can be considered as being similar to the process of crystallisation. Such a process has been outlined in general terms by Alberts et al (1983) as a hypothetical model for changes in cell shape being controlled by the extracellular matrix.

In conclusion, the results presented above show that much of the increase in total rudiment volume observed during growth is due to the secretion of cartilage matrix. This matrix not only favours growth in a longitudinal direction (due to polarised matrix secretion from flattened cells) but also determines the shape of the chondrocytes and controls the change from rounded to flattened cell. Therefore, the cartilage matrix must be considered as one of the most important factors controlling cartilage morphogenesis.

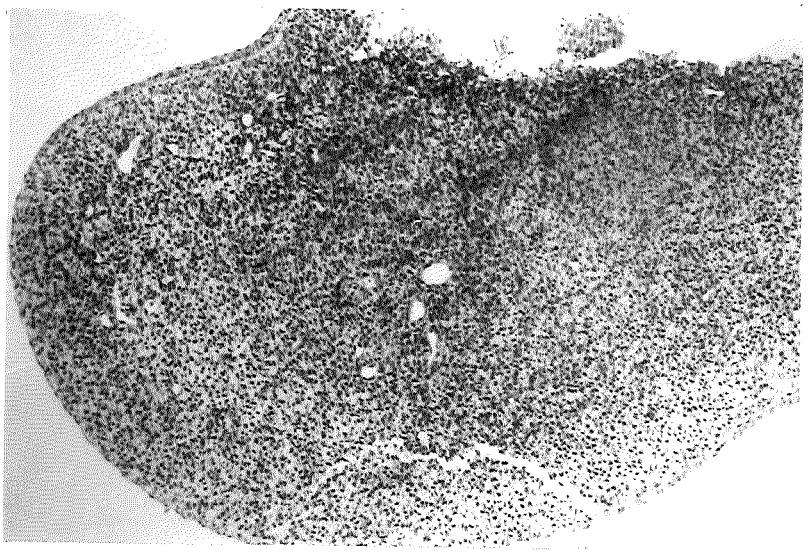
Lack of an organised collagenous matrix in the rounded cell zone may explain why these cells are rounded and why the zone enlarges by radial expansion but it does not explain why radial expansion does not spread throughout the whole limb. This would imply that something is acting as a barrier preventing radial expansion. The most likely candidate for this barrier would be the perichondrium and the role

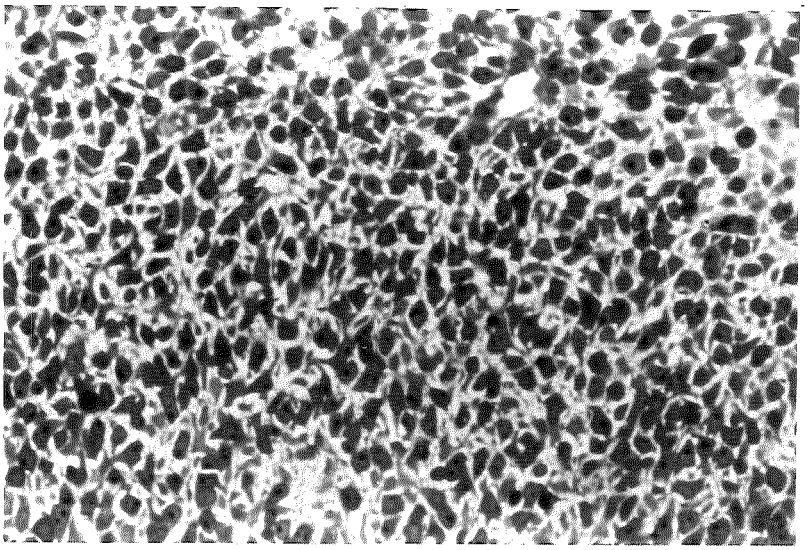
of the perichondrium as a morphogenetic factor will be considered in the following chapter.

Fig. I. Stage 24 wing. Cells in the centre of the wing appear to be at a higher density than peripheral cells but no apparent cellular orientation can be observed.
Tol. blue stain. Mag. x I25.

Fig. 2. Stage 25 ulnal condensation. The shape of the condensation can be clearly distinguished from the surrounding mesenchyme and the cells appear to be at a very high density. No metachronasia can be detected between the cells and no overt cellular orientation can be observed.
Tol. blue satin. Mag. x I25

Fig. 2a. Stage 25 humerus. Metachromatic extracellular matrix can be observed between cells. The cartilage cells appear to be orientated perpendicularly to the long axis of the wing.
Tol. blue stain. Mag. I25.





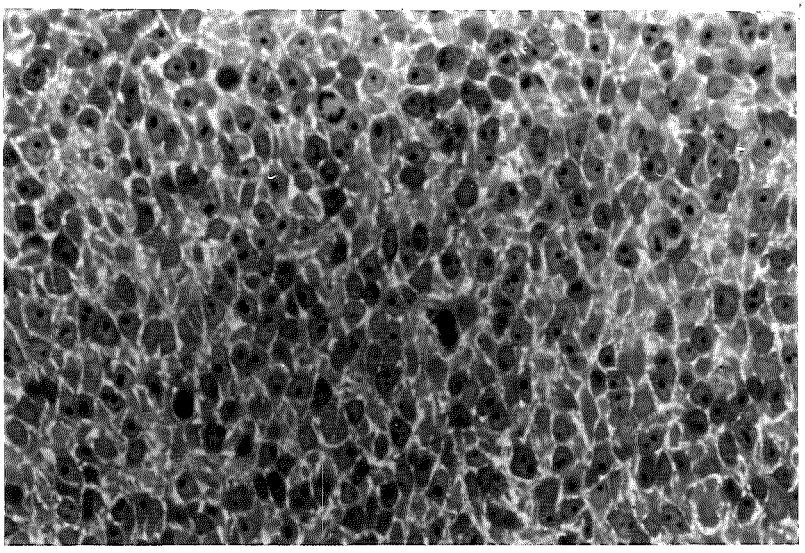
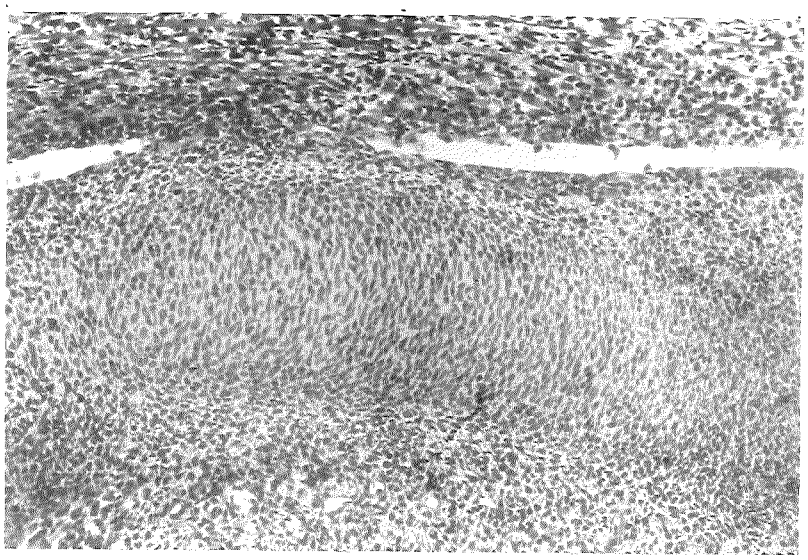
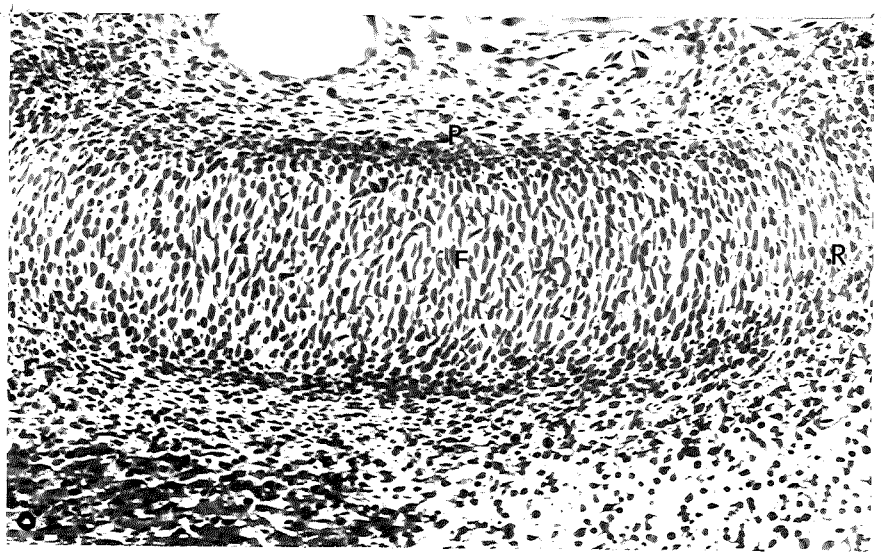


Fig. 3. Stage 26 ulna. The ulna at this stage is very similar to the stage 25 humerus in that cells are separated by a metachromatic matrix and have become orientated perpendicularly to the long axis of the wing.
Tol. blue stain. Mag. x 125.

Fig. 4. Stage 27 ulna. The rudiment consists primarily of one large zone of typically flattened chondrocytes with fewer rounded chondrocytes merging into the mesenchyme at either end. The first appearance of an early perichondrium can be observed at this stage but it is very loose and appears to be composed of polygonal shaped cells.
Tol. blue stain. Mag. x 100.
R = rounded, F = flattened, P = perichondrium.

Fig. 5. Stage 27 ulna, transverse section of the same rudiment as in Fig. 4. Centrally located chondrocytes appear polygonal in shape (suggesting that flattened cells are in fact disc-shaped) whilst perichondrial cells are crescent-shaped and are elongated around the central core of cartilage.
Tol. blue stain. Mag. x 200.
P = perichondrium.




P
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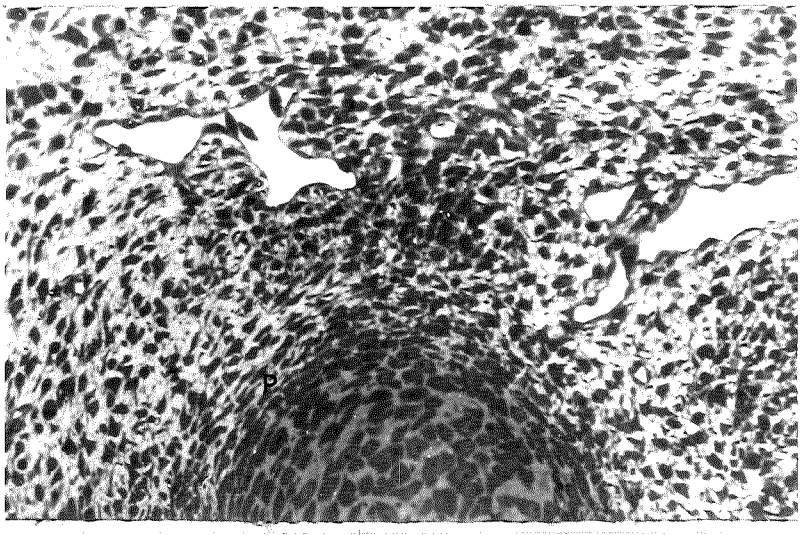
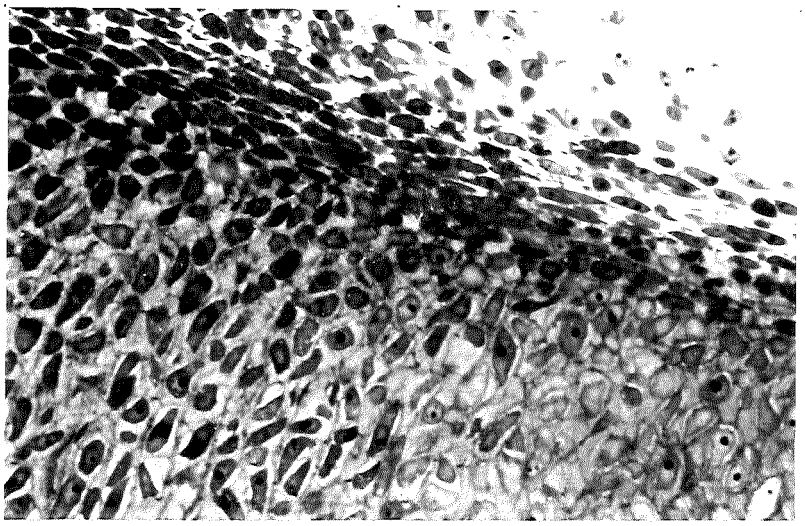
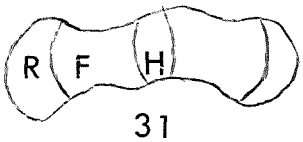

P

Fig. 6. Stage 32 ulna, hypertrophic cell region. The flattened chondrocytes swell up to form large spherically shaped hypertrophic cells which are still separated by extracellular matrix. As this occurs the weak perichondrium is exposed to considerable pressure which results in it becoming consolidated with the cells aligned longitudinally.
Tol. blue stain. Mag. x 320.

Fig. 7. Camera lucida drawings of histological sections of the developing ulna. Once 3 types of chondrocytes are present, the rudiment increases much more in length than in width e.g. between stage 31-stage 34 the increase in length observed is 202% whilst the increase in width of the diaphysis is only 27%. Examination of sectioned material allows comparison of the cellular zones at each stage.
R = rounded cell, F = flattened cell, H = hypertrophic cell.





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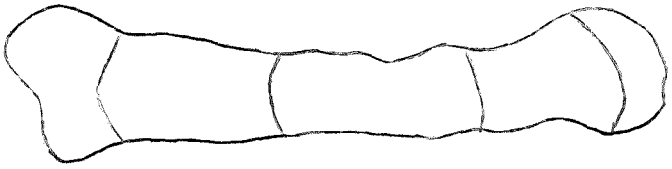

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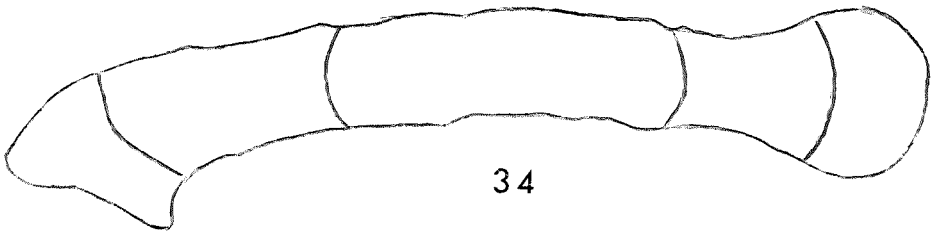

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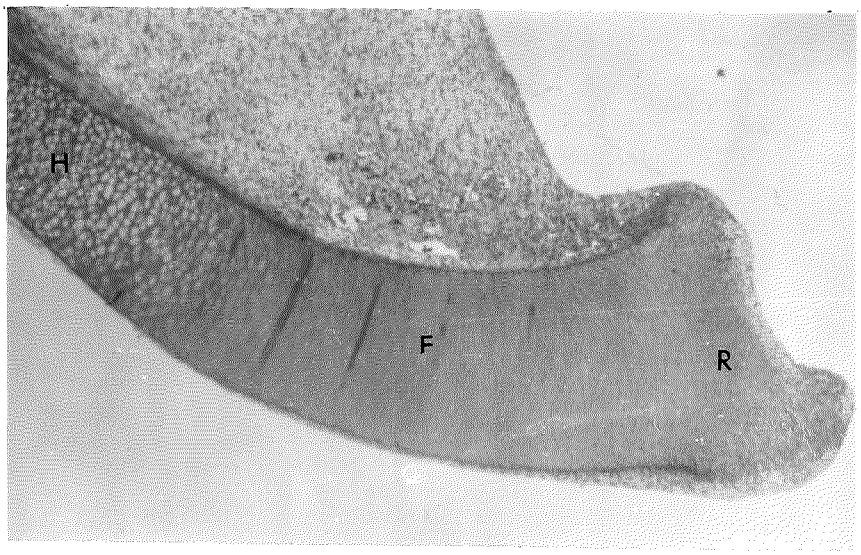
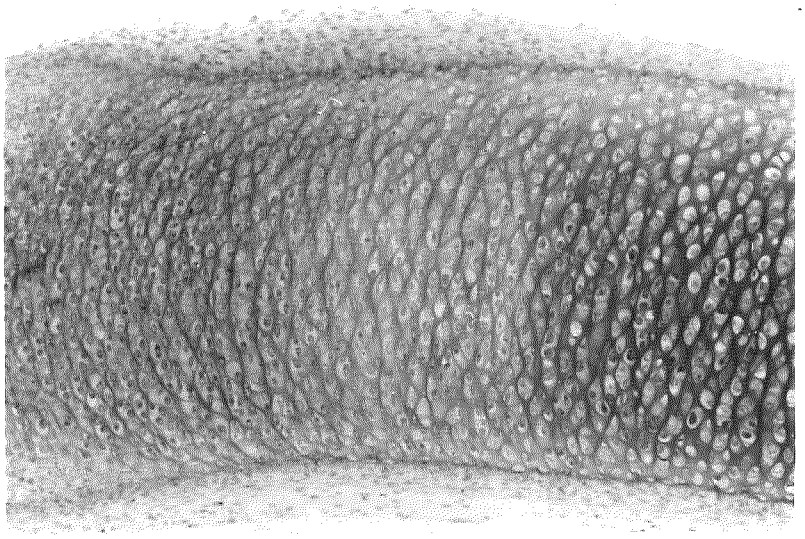
Fig. 8. Stage 32 ulna plus 3 days of culture, proximal half. Sub-periosteal bone extends over the whole of the hypertrophic cell zone but no invasion of blood vessels has occurred. The epiphysis appears asymmetrical which indicates that the olecranon has partially developed.
Tol. blue stain. Mag. x 40.
R = rounded, F = flattened, H = hypertrophic.

Fig. 9. Stage 32 ulna plus 1mg/ml LACA. Flattened cells are no longer surrounded by a flattened-shaped lacuna, many have appeared to round up.
Tol. blue stain. Mag. x 300.

Fig. 10. Stage 32 ulna plus 5mg/ml LACA. Cells within the flattened cell zone have rounded up but have retained some flattened lacunae. Flattened zone cells are no longer distinguishable from rounded zone cells.
Tol. blue stain. Mag. x 125.


H
F
R



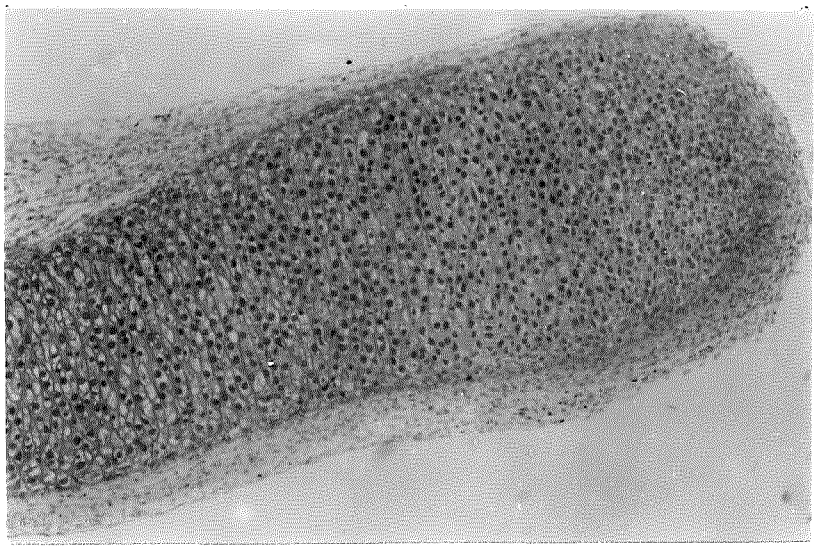
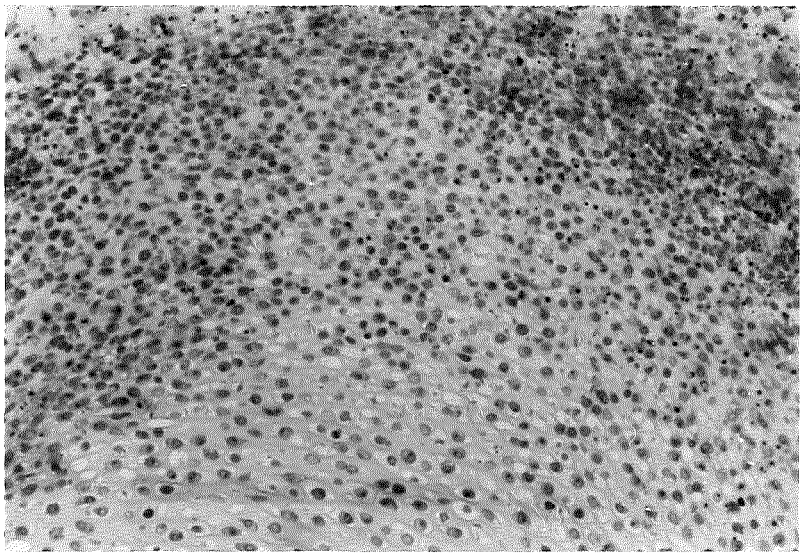
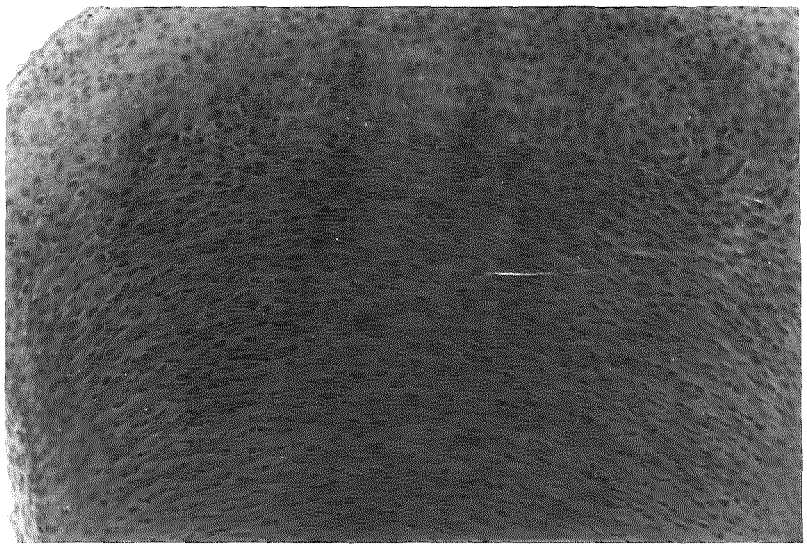


Fig. 11. Stage 32 ulna plus 10mg/ml LACA. Many cells appear dead and have pycnotic nuclei.
Tol. blue stain. Mag. x 125.

Fig. 12. Stage 32 ulna exposed to 1mg/ml LACA and allowed to recover. The distribution of the cellular zones and the shapes of the cells are similar to that found in controls.
Tol. blue stain. Mag. x 40.

Fig. 13. Stage 32 ulna plus 1mg/ml BAPN. The rudiment contains many dead cells, some with empty lacunae. Note the longitudinal orientation of hypertrophic cells.
Tol. blue stain. Mag. x 40.
H = hypertrophic cells.





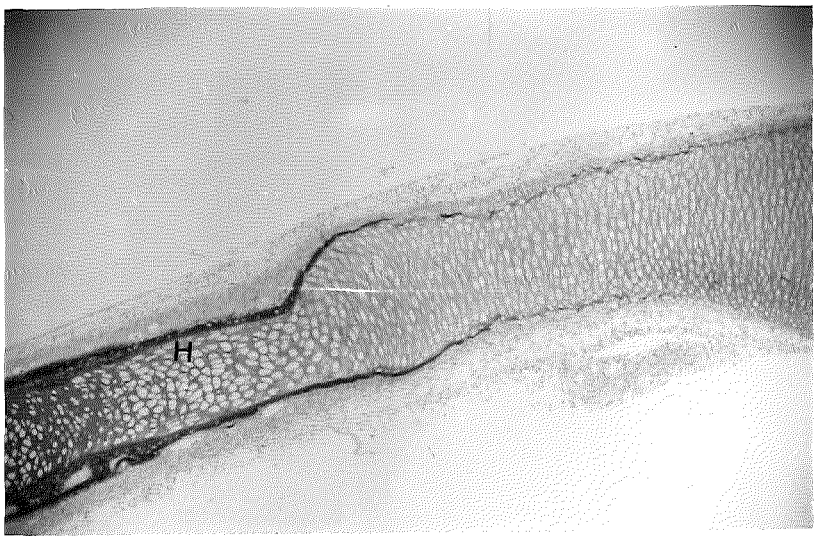
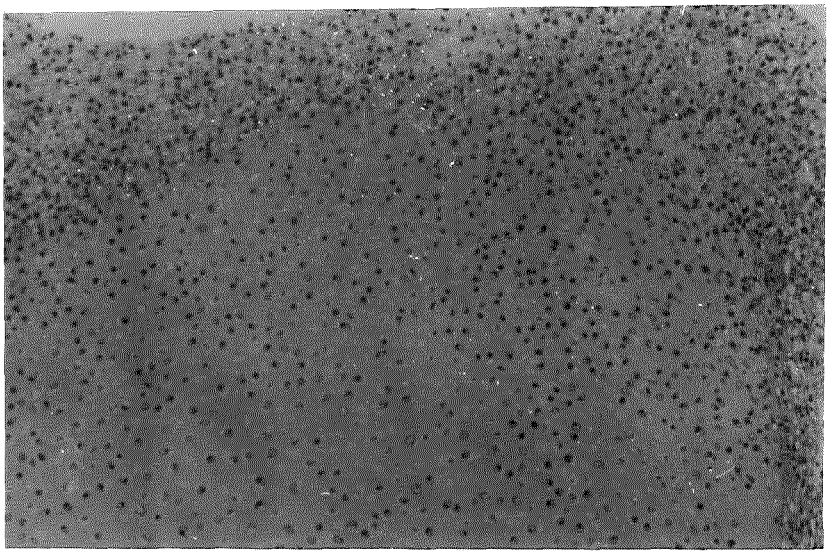
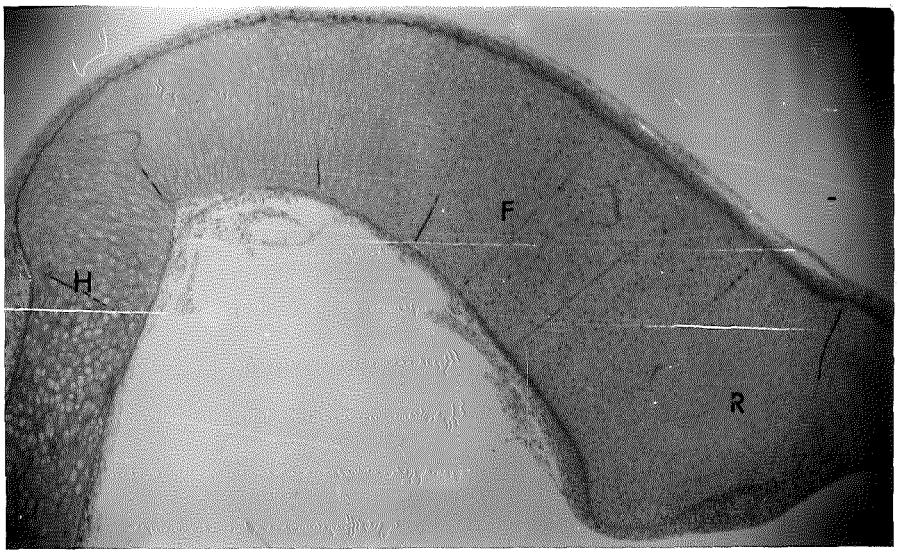

H

Fig. 14. Stage 32 ulnae plus 5mg/ml BAPN. Many cells appear dead and have pycnotic nuclei.
Tol. blue stain. Mag. x 125.

Fig. 15. Stage 32 ulna exposed to 1mg/ml BAPN and allowed to recover. Notice that the flattened cell zone is extremely small, hypertrophic cells are aligned longitudinally and the rudiment is no longer straight.
Tol. blue stain. Mag. x 40.
R = rounded, F = flattened, H = hypertrophic.

Fig. 16. Stage 32 ulna plus 1mg/ml [illegible]. Many cells contain pycnotic nuclei, some flattened zone cells appear rounded and virtually no perichondrium is present.
Tol. blue stain. Mag. x 125.
R = rounded, F = flattened, H = hypertrophic.




F
H
R

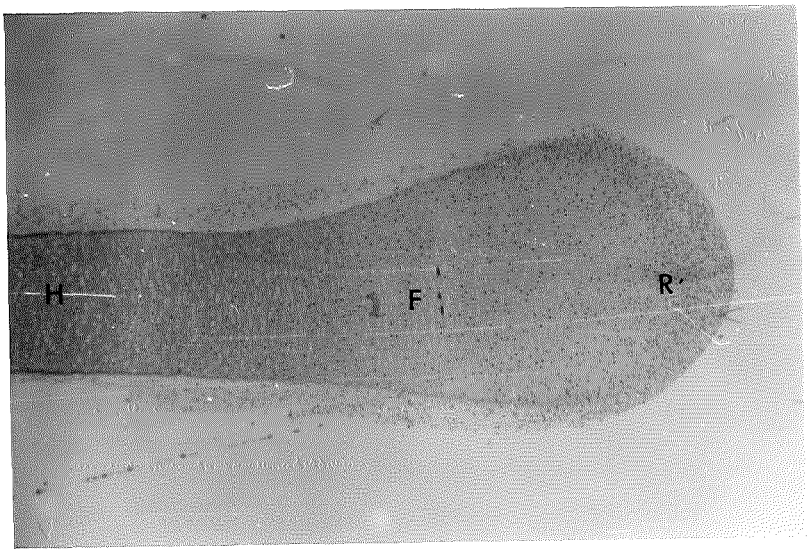
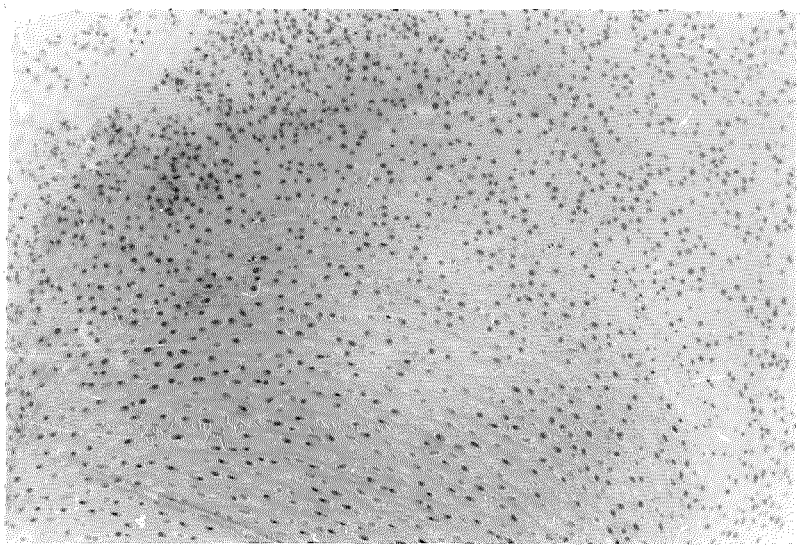
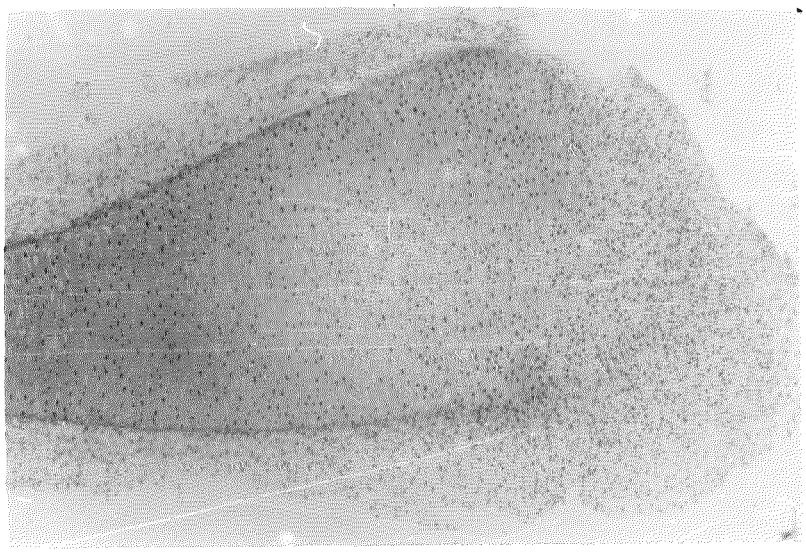

H
F
R

Fig. I7. Stage 32 ulna plus 5ng/ml $\alpha\alpha'$. Many cells appear dead and contain pycnotic nuclei.
Tol. blue stain. Mag. x I25.

Fig. I8. Stage 32 ulna exposed to Img/ml $\alpha\alpha'$ and allowed to recover. The rudiment appears very similar to Fig. I6, many cells are dead and no perichondrium is seen.
Tol. blue stain. Mag. x I25.

Fig. I9. Stage 30 ulna exposed to collagenase for 45 minutes, proximal half. The rounded cell zone is composed of a layer of densely packed cells sandwiched between two regions of rounded cells at a slightly lower density. Flattened zone cells are no longer arranged in discrete rows across the rudiment.
Tol. blue stain. Mag. x I25.
R = rounded, F = flattened.





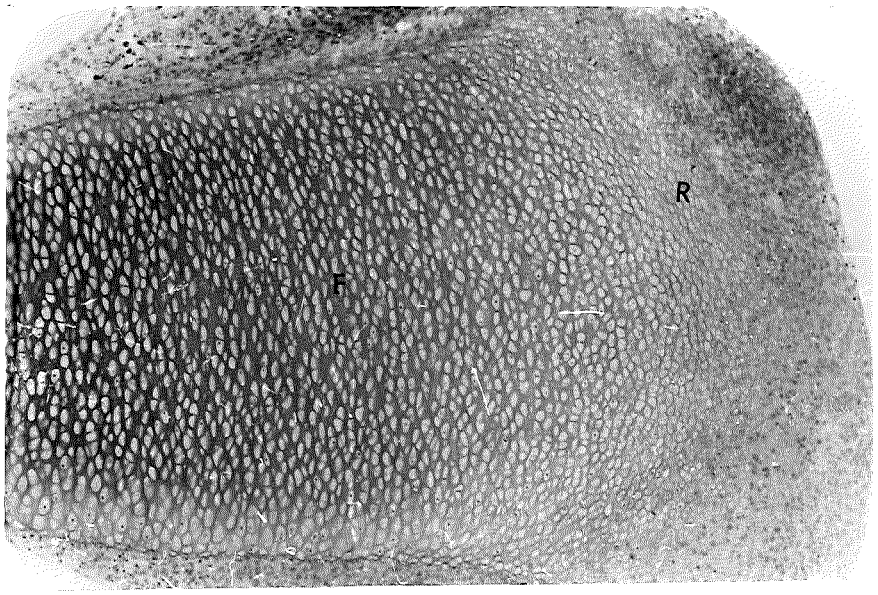
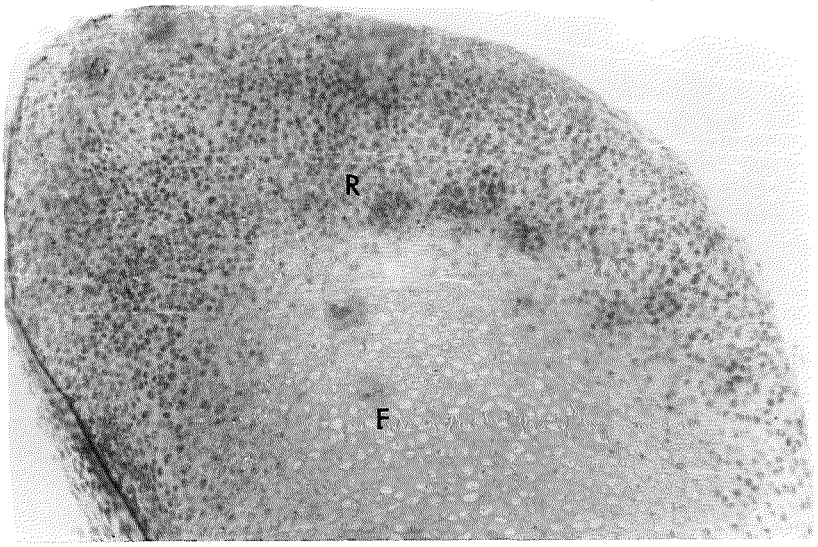

R
F

Fig. 20. Stage 32 ulna exposed to collagenase for 60 minutes, distal half. The rounded cell zone appears similar to that in Fig. 19, flattened cells have lost their discrete rows and some are almost in touch with each other. The perichondrium surrounding these zones is very thick and the cells do not appear elongated.
Tol. blue stain. Mag. x 125.
R = rounded, F = flattened.

Fig. 21. Electron micrograph of stage 32 rounded cells. The cells appear to be small and round in shape separated by a granular ECM.
Uranyl acetate/lead citrate stain. Mag. x 30,000.

Fig. 21a. Higher power of ECM between rounded cells to show sparcity of collagen fibres and lack of organisation.
Uranyl acetate/lead citrate stain. Mag. x 40,500
All sections are longitudinal unless stated otherwise.


R
F

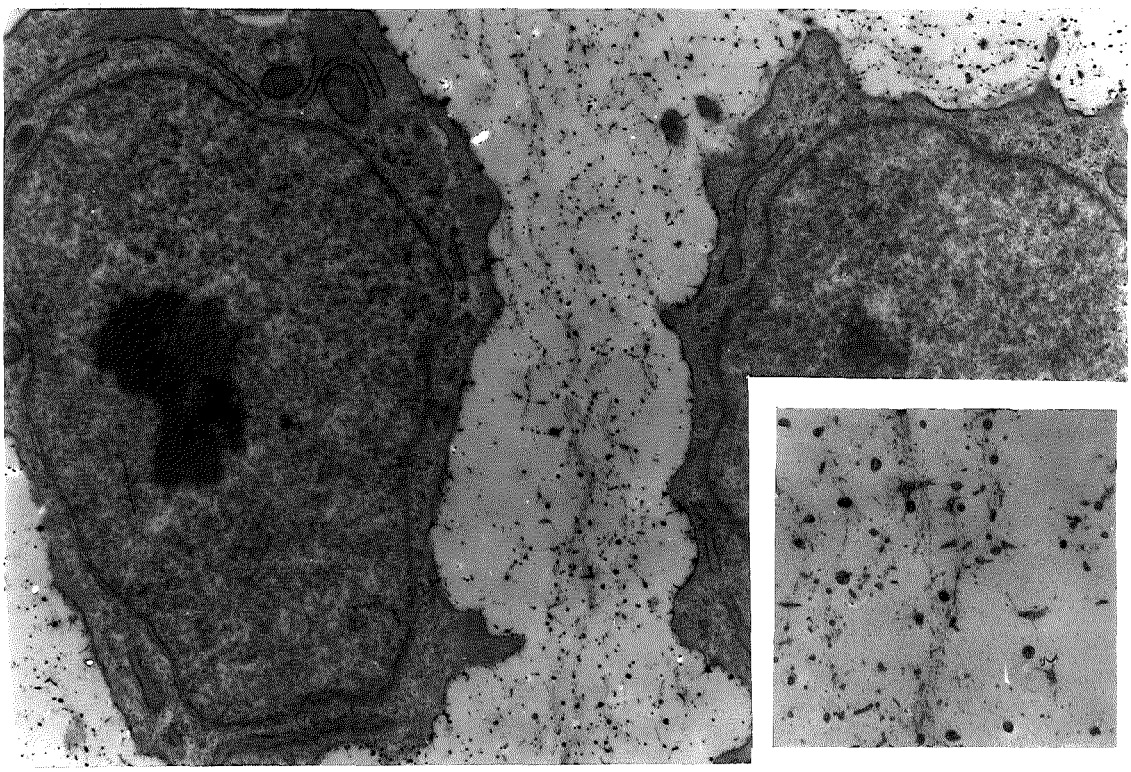
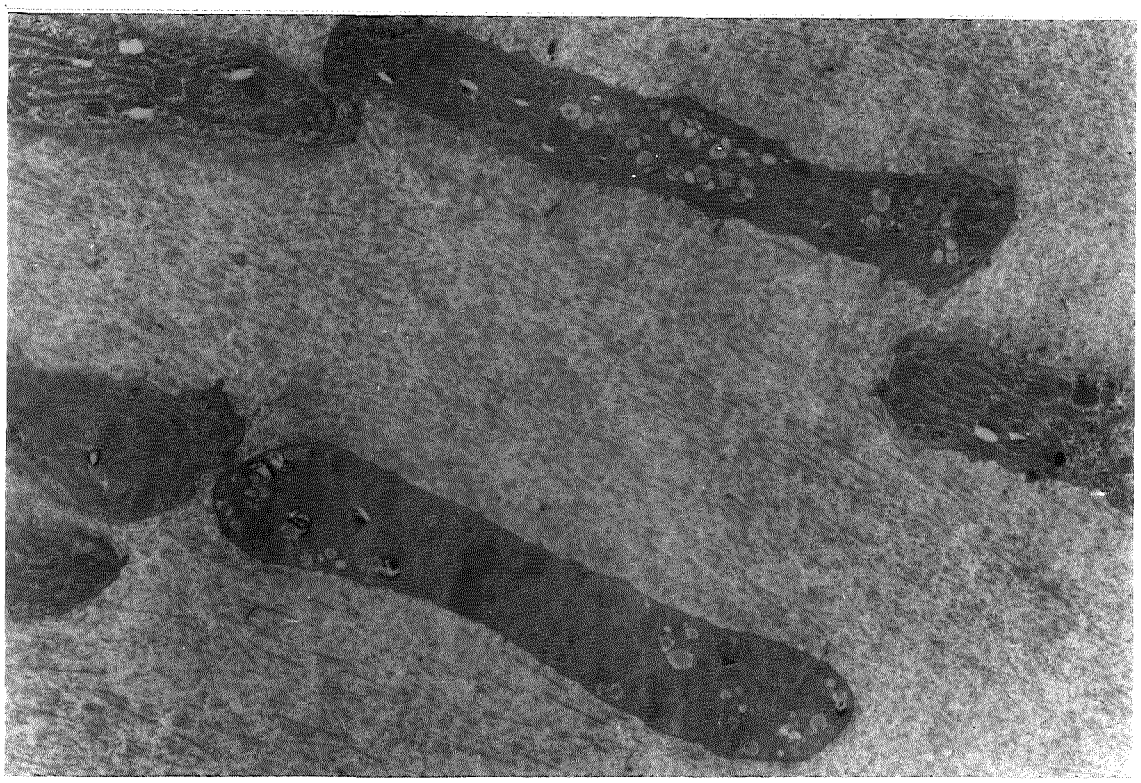


Fig. 22. Electron micrograph of stage 32 flattened cells. The cells are elongated, contain an overt nucleus and are separated by a fibrillar ECM.
Uranyl acetate/lead citrate stain. Mag. x 10,000

Fig. 23. Higher power of ECM between flattened cells to show the fibrillar material arranged parallel to the long axis of the cell. This fibrillar material has been identified at even higher magnification to be collagen fibres.
Uranyl acetate/lead citrate stain. Mag. x 35,000.



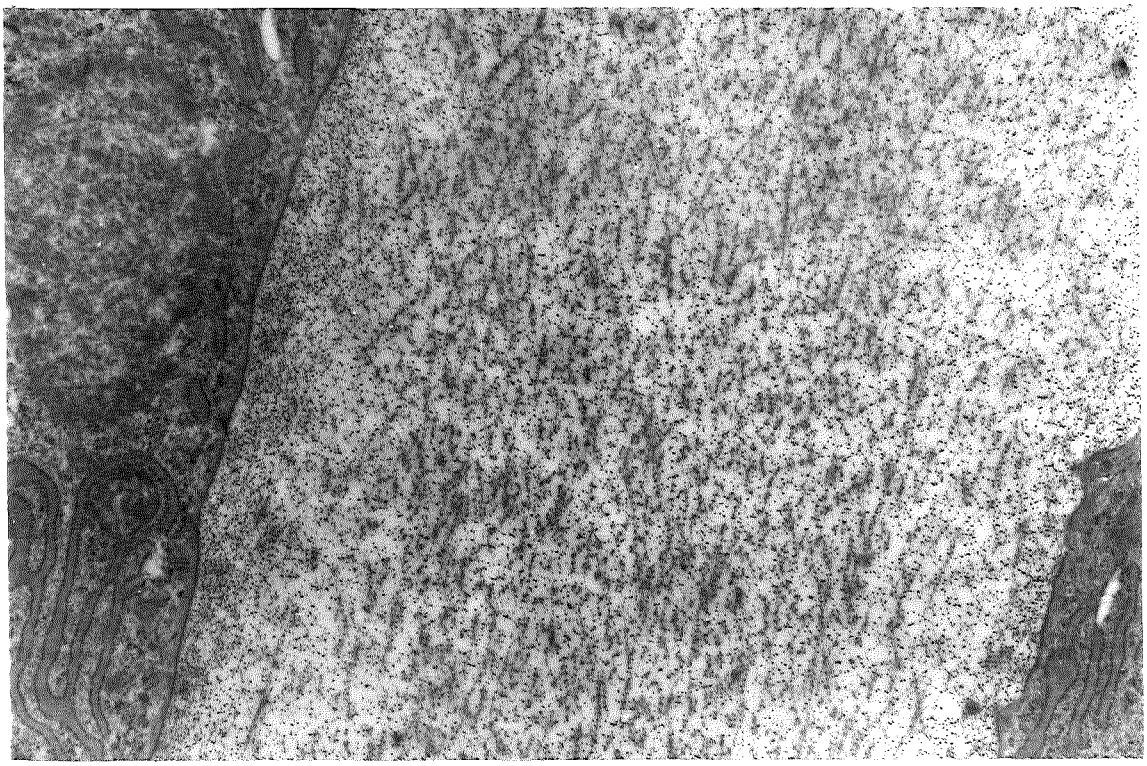
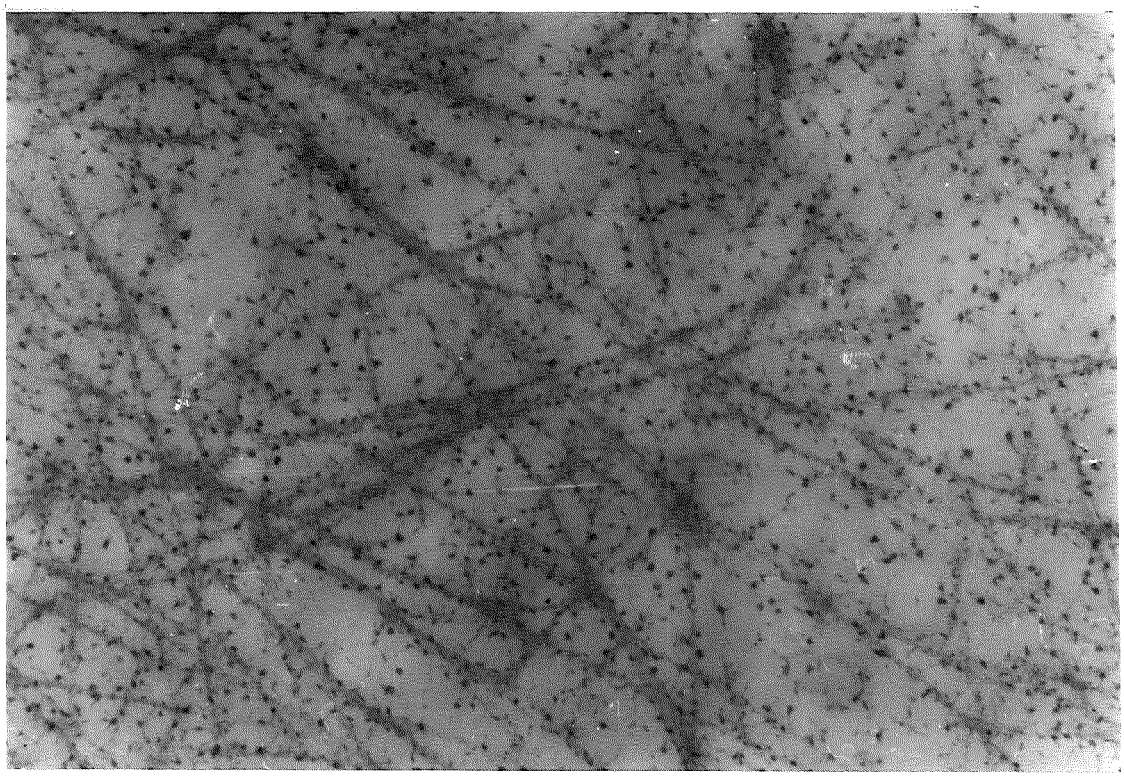
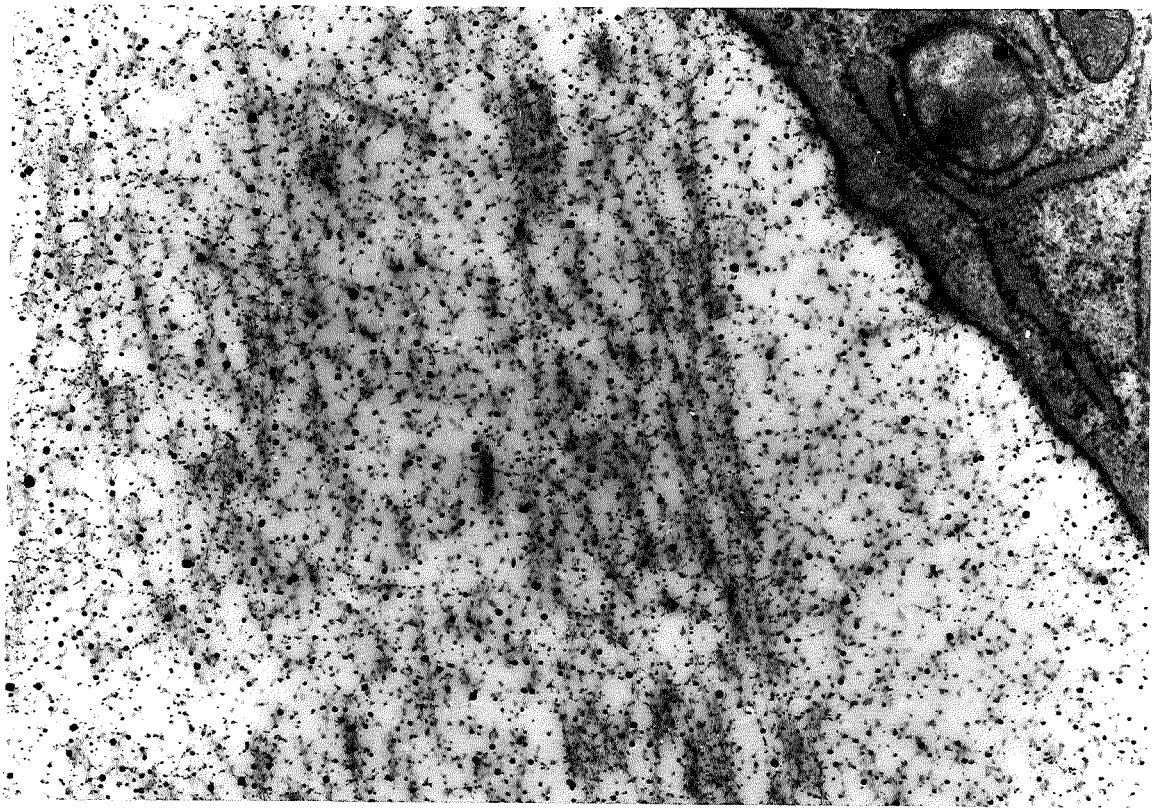


Fig. 23a. Transverse section of the area seen in Fig. 23. The collagen fibres appear to be arranged into a meshwork. Uranyl acetate/lead citrate stain. Mag. x 40,500.

Fig. 24. Electron micrograph of stage 32 hypertrophic cells. The cells are large and spherical and are widely separated by a fibrillar ECM. No ECM is seen within the lacunae and the fibres appear to be arranged circumferentially around the cell. Uranyl acetate/lead citrate stain. Mag. x 30,000.





Total volume of rudiment at stage 3I = 67 x $10^{-3}mm^3$

Total volume of rudiment at stage 34 = 393 x $10^{-3}mm^3$

Total increase in volume = 326 x $10^{-3}mm^3$

Total increase in volume = increase in volume due to cell volume + increase in volume due to matrix secretion

Total volume of cells at stage 3I = 23 x $10^{-3}mm^3$

Total volume of cells at stage 34 = 134 x $10^{-3}mm^3$

Total increase in cell volume = 141 x $10^{-3}mm^3$

% of total increase in volume due to increase in cell volume

$= \frac{141}{326} \times 100 = \underline{43.25\%}$

% of total increase in volume due to increase in volume of matrix

$= \frac{185}{326} \times 100 = \underline{56.75\%}$

From labelling index data every rounded cell divides but only 10% of flattened cells divide, therefore

Total number of proliferating cells at stage 3I = 51520+5236=56756 cells

Total number of cells at stage 3I = 107058

Total number of cells at stage 34 = 319030

Total number of cells produced = 211972

Since these cells must arise from the initial population of proliferating cells = proliferating cells have to increase by $\frac{211972}{56756}$ = 3.735 fold

Total volume of proliferating cells at stage 3I = 6.6 x $10^{-3}mm^3$

Final volume of proliferating cells = 24.54 x $10^{-3}mm^3$

Total increase in volume due to new cell production = 17.94 x $10^{-3}mm^3$

% of total increase in volume due to new cell production

$= \frac{17.94}{326} = 100 = \underline{5.5\%}$

Total increase in cell volume = increase in volume due to new cell production + increase in volume due to process of cell hypertrophy.

% of total increase in volume due to process of cell hypertrophy

$= \frac{141 - 17.94}{326} \times 100 = \underline{37.75\%}$

Relative % of increase in rudiment volume due to

New cell production 5.5%

Cell hypertrophy 37.75%

Matrix secretion 56.75%

Fig. 25. The roles played bycell division, cell hypertrophy and matrix secretion in the growth of non-cultured ulnae between stage 3I - stage 34. The data used in this calculation are obtained from Table I.

Table I Changes in the mean cell densities, mean cell volumes and the mean volumes of ECM/cell in the chick embryo ulna during *in vivo* development.

Stage	Cell density ($10^6/mm^3$)	Cell volume ($10^{-9}mm^3$)	Total vol. of zones ($10^{-3}mm^3$)	No. of cells/zone	Vol. of ECM/cell ($10^{-9}mm^3$)	% ratio ECM:cell
31						
Round.	1.84	97	28	51520	446	81:19
Flat.	1.54	309	34	52360	344	52:48
Hyp.	0.64	539	5	3178	1007	66:34
32						
Round.	1.39	111	35	48615	609	85:15
Flat.	1.13	359	83	93928	525	60:40
Hyp.	0.30	1570	27	8172	1738	52:48
33						
Round.	1.59	115	47	74871	514	82:18
Flat.	1.16	407	84	97789	452	52:48
Hyp.	0.28	1739	34	9373	1888	52:48
34						
Round.	1.44	118	117	168313	577	83:17
Flat.	0.84	561	147	122915	635	53:47
Hyp.	0.22	2711	129	27802	1928	41:59

Ulnae were removed from the embryo and fixed immediately. Values were calculated from 3 central sections, 10 µm apart, from each of 4 different rudiments for each stage.

In each case the value is rounded up to the nearest whole number.

Standard deviations are omitted for clarity - for cell densities they ranged from 3-10%.

Table II The cellular parameters of stage 32 ulnae cultured continiously in the presence of teratogenic drugs.

Treatment	Cell density*	Cell volume*	volume of ECM/cell**	% ratio ECM:cell	% increase in length+
Control					105
Round.	1.259	135	659	83:17	
Flat.	0.690	561	888	61:39	
Hyp.	0.163	3261	2853	47:53	
L-1mg/ml					47
Round.	1.162	193 [1]	668	78:22	
Flat.	0.661	571	942 [1]	62:38	
Hyp.	0.104	5610 [1]	4012 [1]	42:58	
L-5mg/ml					25
Round.	0.502	432 [1]	1561 [1]	78:22	
Flat.	0.330	438 [2]	2594 [1]	86:14	
Hyp.	0.098	4521 [1]	5646 [1]	56:44	
B-1mg/ml					46
Round.	0.922	256 [1]	828 [1]	76:24	
Flat.	0.581	680 [1]	1042 [1]	61:39	
Hyp.	0.094	4739 [1]	5909 [1]	55:45	
∝-1mg/ml					29
Round.	1.052	144	809 [1]	85:15	
Flat.	0.836	289 [2]	907	76:24	
Hyp.	0.242	1219 [2]	2909	70:30	

Ulnae were grown on top of Millipore filters either in control medium or in the presence of drugs for 3 days. Mean values were calculated from 3 central sections, 10 /um apart, from 6 different rudiments for each treatment.

Values are rounded up to the nearest whole number, and standard deviations are omitted for clarity.
L = L-azetidine - 2 - carboxylic acid.
B = B-aminoproprionitrile fumerate.
∝ = ∝∝' dipyridyl.
Concentrations of L greater than 5mg/ml, B greater than 1mg/ml and greater than 1mg/ml all killed the rudiment, therefore, values for these treatments are not included.

*Values were calculated from the equations used in Table I.

**Units = 10^{-9} mm^3 ($/um^3$) calculated from the equation

$$\text{Vol. of ECM/cell} = \frac{\text{Unit volume} - (\text{cell density} \times \text{cell volume})}{\text{cell density.}}$$

+Rudiments were measured immediately and at the end of the 3 day culture period using a calibrated eyepiece graticule.

Statistical analysis was performed using Student's t-test. Zones in treated rudiments were compared with equivalent zones in cultured controls. Significance was taken at the 5% level.

1 = significant, $P < 0.05$. (increase) 2 = significant (decrease)

Table III The cellular parameters of stage 32 ulnae cultured for 1 day in the presence of teratogenic drugs followed by 2 days in control medium.

Treatment	Cell density	Cell volume	Volume of ECM/cell	% ratio ECM:cell	% increase in length
Control					105
Round.	1.259	135	659	83:17	
Flat.	0.690	561	888	61:39	
Hyp.	0.163	3261	2853	47:53	
L-Img/ml					96
Round.	0.989	210[1]	801[1]	79:21	
Flat.	0.720	396[1]	993[1]	71:29	
Hyp.	0.124	4092[1]	3994[1]	49:51	
B-Img/ml					97
Round.	1.255	139	657	83:17	
Flat.	0.699	356[2]	1074[1]	75:25	
Hyp.	0.084	7347[1]	4585[1]	38:62	
α-Img/ml					31
Round.	1.373	95[2]	633	87:13	
Flat.	0.865	212[2]	1086[1]	84:16	
Hyp.	0.237	1193[2]	3032[1]	72:28	

Ulnae were cultured in the presence of a teratogenic drug for 1 day, washed twice in control medium, and then cultured in control medium for a further 2 days.

Values were calculated and presented as in Table II.

Statistical analysis was performed using Student's t-test.

1 = significant, $p<0.05$. (increase) 2 = Significant (decrease)

Table IV The cellular configuration of stage 32 ulnae cultured continuously in the presence of teratogenic drugs.

Treatment	Total volume of zones*	Number of cells/zone**	Number of cells along the long axis of each zone+	Number of cells across the transverse axis of each zone+
Control			(478)	
Round.	I99	250448	64/45	83/57
Flat.	48I	33I724	86/87	44/37
Hyp.	256	40979	I96	22
L-Img/ml [1]			(422)	
Round.	302	350752	48/36	II3/75
Flat.	545	360383	70/68	68/57
Hyp.	5I8	53922	202	2I
L-5mg/ml[a] [2]			(326)	
Proximal	256	I06579	I06	43/39
Distal	I69	70308	94	32/27
Hyp.	II3	II074	I26	I8
B-Img/ml			(4II)	
Round.	247	227900	67/37	82/7I
Flat.	5I0	296895I	84/73	47/53
Hyp.	I34	I2625	I50	I8
∝-Img/ml [2]			(265)	
Round.	II6	I22I24	45/35	49/38
Flat.	303	250307	64/48	33/28
Hyp.	78	I89I5	73	20

Values were calculated from the same sections as used in Table II.

*Units = 10^{-3} mm^3. Values were calculated directly from camera lucida drawings of fixed and sectioned rudiments (see Fig. 7). Volumes presented show total values for proximal plus distal zones.

**No. of cells/zone = vol. of zone x cell density. (see Table II).

+Values for proximal zones are given first. Numbers in parentheses represent the total number of cells along the longitudinal axis.

[a]Flattened regions in rudiments treated with LACA at 5mg/ml contained rounded cells, therefore the values presented here represent total values for rounded plus flattened zones in proximal and distal halves of the rudiment.

Statistical analysis was performed using Student's t-test.

1= significant, $P<0.05$. (increase) 2 = significant (decrease)

Table V The cellular configuration of stage 32 ulnae cultured for I day in the presence of teratogenic drugs followed by 2 days in control medium.

Treatment	Total volume of zones*	Number of cells/zone*	Number of cells along the long axis of each zone*	Number of cells across the transverse axis of each zone*
Control			(478)	
Round.	I99	250448	64/45	83/57
Flat.	48I	33I724	86/87	44/37
Hyp.	256	40979	I96	22
L-Img/ml[1]			(455)	
Round.	2I0	207I96	4I/4I	9I/6I
Flat.	62I	447502	85/82	62/53
Hyp.	425	52673	206	I7
B-Img/ml			(384)	
Round.	297	372I45	5I/48	85/75
Flat./Round.	543	379872	28+59/I4+66[a]	54/47
Hyp.	I07	9027	II8	I8
α-Img/ml[2]			(259)	
Round.	7I	9696I	5I/4I	47/39
Flat.	I5I	237027	6I/5I	33/25
Hyp.	70	I6609	55	22

Values were calculated from the same sections as used in Table III.

*Values were calculated and presented as in Table IV.

[a]Treatment with BAPN at Img/ml for I day resulted in a flattened region which contained both flattened and rounded cells. The number of flattened cells end to end are presented first.

Statistical analysis was performed using Student's t-test.

1 = Significant, $P < 0.05$. (increase) 2 = Significant (decrease)

Table VI The effect of collagenase on the growth of ulnae cultured *in vitro*.

Treatment	% increase in epiphyseal width* Day I	Day 2	Total	% increase in length*
St. 30 control	26/I7**	7/I0	35/28	88
I5 min.	50/29	I9/5	78/36	84
30 min.	55/37	I5/I6	78/59	79
45 min.	72/46	I3/5	94/53	69
60 min.	65/35	8/I4	78/54	63
St. 32 control	20/I7	8/5	30/23	82
I5 min.	40/22	5/7	47/30	78
30 min.	46/27	II/8	62/37	74
45 min.	45/22	I4/I2	66/37	68
60 min.	64/48	II/4	82/54	65

*Rudiments were measured daily using a calibrated eyepiece graticule. Values were calculated from at least I0 rudiments for each treatment time.

Standard deviations are omitted for clarity but ranged from 4-I4%.

**Proximal values are presented first.

Table VII The effect of 0.2% collagenase on the cellular parameters and cellular configuration of stage 30 and stage 32 ulnae.

Treatment	Cell density*	Cell volume*	Vol. of ECM/cell*	Number of cells along the long axis of each zone+	Number of cells across the transverse axis of each zone+
Stage 30 control				(256)	
Round.	I.62I	85	532	42/33	47/37
Flat.	I.223	345	473	72/55	35/28
Hyp.	0.5I8	I29I	640	54	2I
Stage 30 45 min.				(252)	
Round.	I.975	9I	4I5	46/26	66/49 [1]
Flat.	0.932	3I7	756	66/59	40/33
Hyp.	0.409	I792	653	55	23
Stage 32 control				(377)	
Round.	I.554	II6	528	65/35	80/5I [1]
Flat.	0.92I	529	557	94/75	54/37
Hyp.	0.243	2389	I726	I08	23
Stage 32 60 min.				(3I5) [2]	
Round.	I.667	I52	448	5I/34	93/52 [1]
Flat.	0.747	802	536	55/64	45/37
Hyp.	0.20I	2920	2055	III	23

Ulnae were treated with 0.2% collagenase for the time periods shown, they were then washed twice in control medium and cultured on top of Millipore filter rafts for I day, fixed and sectioned.

Values were calculated from 4 central sections, I0 µm apart, from 6 different rudiments for each treatment.

*Values were calculated from the equations used - Table I

+Values are presented as in Table IV.

Statistical analysis was performed using Student's t-test.

1= Significant, P 0.05. (increase)

2= Significant (decrease)

Table VIII The roles played by cell division, cell hypertrophy and matrix secretion in the growth of ulnae.

Treatment	% of total increase in volume* Cell division	Cell hypertrophy	Matrix secretion	% increase in volume**
Stage 31-34 in vivo	5.50	37.75	56.75	----
Stage 32 + 3 days	7.91	30.21	61.88	100
L-1mg/ml + 3 days	6.88	36.06	57.05	154
B-1mg/ml + 3 days	6.64	29.22	64.13	94
L-1mg/ml 1 day + 2 days	6.78	27.81	65.41	140
B-1mg/ml 1 day + 2 days	10.39	14.70	74.91	101

*Values are means of calculations carried out on 2 central sections, 10 um apart, from 4 different rudiments for each treatment. Values were calculated as shown in Figure 25. All measurements were taken from camera lucida drawings of fixed specimens.

**The increase in volume observed in stage 32 ulnae cultured in control medium for 3 days was taken as 100%, - all the other increases in volume were correlated with this.

C H A P T E R T H R E E

THE ROLE OF THE PERICHONDRIUM IN CARTILAGE MORPHOGENESIS

Introduction

Two questions arise from the data presented in the previous chapter:

i) if a long bone rudiment is designed mainly for an increase in length, what controls the increase observed?

ii) although the flattened and hypertrophic cell zones primarily enhance longitudinal growth, what limits the small increase in width observed in these zones?

The most obvious answer must be that some external factor is constraining the expansion in both axes, - the best candidate for this external factor has been suggested by Wolpert (1982) and by Thorogood (1983) to be the perichondrium.

Surprisingly little is known about the role of the perichondrium in cartilage development even though it was first implicated by Carey in 1922. Whilst studying the development of the embryonic pig femur, Carey observed that until a recognisable perichondrium formed the femur increased more in width than in length, however, after formation, the increase in length for exceeded any increase in width.

Since Carey's study, the perichondrium has been considered more as a means of allowing appositional growth to occur rather than a morphogenetic entity, however, as mentioned in the General Introduction, very little experimental evidence has been presented to support this proposed function.

During development, the perichondrium in the diaphyseal region gradually changes into a bilayered periosteum and this structure has been shown to be important in the control of length in the growing bone (Crilly, 1972; Haughton and Dekel, 1979).

The object of this chapter is to determine if the perichondrium

plays a similar role on the developing cartilaginous rudiment and also to see if the perichondrium determines the shape of the rudiment in any way.

Materials and Methods

Ulnae were dissected free from the wings of embryos aged between stage 30-34 and either fixed immediately for E.M. examination, as described in the General Methods, or cultured under various conditions:

i) Cultured intact for I day with part, or all, of their perichondrium removed. Partial removal was performed by carefully stripping away the perichondrium from around the flattened cell region at one epiphyseal end; total removal was accomplished by repeatedly rolling the rudiment on a piece of sterile, dry Millipore filter. 24 rudiments were used for each experiment.

ii) Cultured as whole or half rudiments, up to 3 days, after one small nick had been made through the perichondrium, at various levels, by means of irredectomy scissors. In all, a total of 80 ulnae were nicked in this experiment.

iii) Cultured for up to 3 days after being cut into 2, 4 or 6 equal pieces by irredectomy knives. A total of 210 ulnae were used in this experiment - 10 for each cut at each stage between stage 30 - stage 34 and 10 for each cut at stages 36 and 37, i.e. 30 ulnae from each stage were looked at.

iv) Stage 34/35 ulnae were cultured intact but with a cube of cells within the flattened cell zone rotated through 90°. In all, 16 rudiments were used.

Results

I Ultrastructure of the perichondrium

The first appearance of a perichondrial structure was at stage 27 in the ulna when the cells appeared polygonal in longitudinal section but crescent shaped when viewed in transverse section (see chapter 2). There was no apparent cell contact between these cells and the extracellular matrix (ECM) separating them stained metachromatically with toluidine blue. No evidence of a perichondrium could be seen at the epiphyseal ends.

At a later stage, stage 29/30, the perichondrial cells around the centre of the flattened cell zone were elongated in a direction parallel to the long axis of the rudiment, just as the process of cell hypertrophy begins, but still there was no cell contact. At this stage the perichondrial cells and their ECM remained virtually indistinguishable from the central chondrocytes (Fig. I). At the presumptive epiphyseal ends the differentiating chondroblasts merged imperceptively with the mesenchyme (Fig. 2).

At stage 3I when central chondrocytes are clearly hypertrophic a more distinct perichondrium was observed around the centre of the diaphysis. The perichondrium was several cells thick, the cells were very elongated and some small cell-cell contacts could be observed (Fig. 3). (The nature of these junctions has not yet been defined but they may be "tight" junctions). It should be noted that very little ECM was present between perichondrial cells at this stage. Elongation of perichondrial cells appears to be a progressive phenomenon in that some cells surrounding the flattened cell zone have also elongated by stage 3I, however, these cells merely overlap and do not exhibit cell contacts (Fig. 4). At the epiphyseal ends of a stage 3I ulna, chondroblasts could be distinguished from

mesenchyme cells by their more uniform shape, but still no distinct barrier could be observed (Fig. 5).

By stage 32 the perichondrium at the level of hypertrophic cells was a very distinct, tight structure with many areas of extensive cell-cell contacts (these contacts are believed to be tight junctions) (Fig. 6). No ECM could be observed between these cells at this stage. At the flattened cell region, the perichondrium was beginning to show some cell contacts producing a loosely bound structure 5-10 cells thick (Fig. 7). As can be seen, cartilage cells nearest the perichondrium appear either spherical or elongated parallel with the perichondrium, which might imply appositional growth. There was still little evidence for a perichondrial structure surrounding rounded cells at stage 32 (Fig. 8).

The perichondrium began to change into a periosteum in the centre of the diaphysis at stage 32 but no morphological change was immediately apparent until stage 33 when perichondrial cells closest to the cartilage appeared to round up (Fig. 9). The cells retained extensive cell-cell contacts and osteoid was deposited between these cells and the cartilage (Fig. 9). At this stage no ECM was observed between the periosteal cells but at higher magnification coated pits were seen which were presumably present for the secretion of ECM (Fig. 9a). The structure of the perichondrium surrounding the flattened cell region was now very tight with many extensive cell-cell contacts (Fig. 10). (This structure was very similar to the perichondrium surrounding the centre of the diaphysis at the earlier stage 32). In addition, the perichondrium surrounding the rounded cell region was also becoming more consolidated with cells just beginning to overlap each other but without obvious cell-cell contacts (Fig. 11).

The central region of a stage 34 ulna was very difficult to cut

and photographs of this region show large deposits of osteoid between the cartilage and the periosteum (Fig. I2). There would appear to be some alignment of the collagen fibres, at the periphery of the cartilage, so that they lie parallel to the long axis of the rudiment. This directionality of the collagen fibres can be seen more clearly at higher magnification (Fig. I2a). At a level a small distance away from the centre of a stage 34 ulna, the beginnings of a bilayered periosteum could now be seen adjacent to the hypertrophic cells. This periosteum consisted of an inner layer of rounded, more tightly packed (possibly osteogenic), cells and an outer layer of very elongated fibroblast-like cells (Fig. I3).

The fibroblast-like cells were arranged in layers separated by strips of a very electron-dense material (Fig. I4). At higher magnification the fibroblast-like cells appeared to be in contact not only by cell-cell contacts but also by what appeared to be glycosaminoglycan strands which extended from the strips of electron-dense material to a "glycocalyx" on the opposite cell surface (Fig. I4a). In contrast, no extracellular material was observed between the cells of the inner layer of the periosteum (Fig. I5). The nature of the electron dense material is unknown but it is possible that these strips correspond to the white fibrous material described in Fell's light microscopical study of chondrogenesis (Fell, I925). It is possible that this material is composed of densely packed rows of collagen fibres intermingled with elastin fibres although this has yet to be histochemically confirmed.

The perichondrium surrounding the flattened cell region at stage 34 was still similar to that of stage 33 and no true perichondrium had as yet formed over the rounded cell region. However, the perichondrial cells of this region were more clearly overlapping and

a definite barrier between cartilage and mesenchyme was evident (Fig. 16).

2) Removal of the perichondrium

When the whole perichondrium was removed from a stage 32 ulna and the rudiment was cultured overnight, the morphogenesis of the rudiment changed considerably. Instead of a typical long bone rudiment, an "S" shaped element was produced in greater than 90% of the cases. In these cases bending of the rudiment always occured in a region between the diaphysis and epiphysis - the diaphysis itself remained straight. The overall length of the rudiment was, on average, 16% greater than that of controls (Table I).

Histologically the rudiment appeared almost normal (Fig. 17). It should be noticed that the hypertrophic cell zone occupied almost all of the straight portion of the diaphysis. A similar result was obtained if ulnae were used from any stage between stage 31-34, but if they were used from stage 36 embryos, or older, no S shape distortion was produced.

In the case of partial removal of the perichondrium, the rudiment retained it's shape during culture. There was an average excess increase in length of 11% when compared with controls (Table I) but histologically the rudiment appeared normal.

3) The effect of cutting through the perichondrium into the cartilage

When a small incision was made, with irredectomy scissors, through the perichondrium of intact ulnae at the region of rounded/flattened cells so that the incision just pierced the cartilage, no change was observed in the morphology of the rudiment during a 3 day culture period (Table II).

However, if the rudiment was cut into 2 pieces either before or after the incision was made then a swelling appeared at the region of the incision within 24 hours in 55% of the cases (Table II).

This swelling was found to contain normal staining cartilage (Fig. 18). At higher magnification (Fig. 19), the swelling contained rounded and flattened cells with flattened cells orientated in the direction of the swelling. The entire swelling appeared to be enclosed within a very thin perichondrial sheath (Fig.19), and no evidence of an increased mitotic index could be found. If two incisions were made in this region, a swelling was only rarely observed (Table II).

If the incision was made into the hypertrophic cell region no swelling was observed even if the rudiment was cut in two (Table II).

4) The effect of cutting ulnae into pieces

When stage 31, or younger, ulnae were cut into 2, 4 or 6 equal sized pieces and the increase in length of each piece was measured over a 3 day culture period, then the sum of the increase in length of the pieces was always less than that observed in an intact rudiment, in fact very little growth occurred at all (Table III).

However, if ulnae aged between stage 32-34 were cut into 2 or 4 pieces the overall increase in length of these pieces always exceeded that for the intact rudiment. The excess ranged from 5-12% depending on how many pieces were cut (Table III). It was noticed that portions containing the epiphyses increased in length more than diaphyseal portions. If stage 32-34 ulnae were cut into 6 pieces the increase in length was less than that of intact rudiments (Table III).

If pieces were cut from stage 36, or older, ulnae diaphyseal portions which contained bone at this stage, hardly increased in length at all, therefore, the overall increase in length was always less than for intact rudiments (Table III).

Two further observations arose from these experiments, the first was that the process of cell hypertrophy was tissue autonomous. When portions cut from the diaphyseal region, which might be expected

to contain flattened plus hypertrophic cells, or in some cases only flattened cells, were cultured for 3 days, the cut end containing flattened cells was found to expand greatly in width. At first, it was thought that a new epiphysis had formed but histological examination showed the swelling to be due to cell hypertrophy (Fig. 20).

The second observation was that if the cut pieces were held too tightly during transfer to the culture system then some amorphous material protruded from the cut end. To investigate this, rudiments between stage 31-32 were cut into individual zones and gently squashed. (It was found that the best method of squashing was to lay a piece of glass coverslip over the piece of rudiment and gently apply pressure by tapping the glass with the point of blunt watchmakers forceps). It was found that only the hypertrophic cell region could be expelled from the surrounding perichondrium/periosteum. This piece of cartilage was totally free of connective tissue (Fig. 21), and this procedure was used in later experiments to isolate hypertrophic cells. When rounded or flattened cell zones were squashed, much of the perichondrium remained attached to the cartilage (Fig. 22).

5) Rotation of flattened cells

When a cube of flattened cells was rotated through 90° and the rudiment was cultured for 2 days, preliminary findings indicated that the cube became oblong shaped and distorted the shape of the epiphysis (Fig. 23). Appropriate controls, i.e. mock operations, or rotation through 180° and 360°, always remained cube-shaped. Histological sectioning of the rotated tissue proved difficult and often resulted in the cube being torn from the rudiment, however, examination of some sections implied that the flattened cells had not re-orientated.

Discussion

The results presented in this chapter describing the ultrastructure of the perichondrium are in total agreement with the light microscopical observations of Fell (1925). Prior to cell hypertrophy there is no definite perichondrium. At stage 27, there is a barrier of polygonal cells between flattened chondrocytes, in the centre of the rudiment, and the surrounding mesenchyme but these polygonal cells closely resemble chondrocytes and in fact have a similarly staining ECM (see chapter II). As cell hypertrophy begins, at stage 30, cells in the barrier become elongated in a direction parallel to the long axis of the rudiment, and the structure can now be regarded as an early perichondrium (Fig. 1). However, it should be noted that the elongated cells remain similar in character, and in their ECM, to the chondrocytes. No distinct perichondrium can be observed at the rounded cell regions where chondroblasts and mesenchyme cells are virtually indistinguishable (Fig. 2).

As cell hypertrophy becomes more pronounced, perichondrial cells become more elongated and begin to exhibit cell-cell contacts so that by stage 32, a very distinct, tight structure has formed around the hypertrophic cell zone (Figs. 3 and 6). This consolidation of the perichondrium may be a mechanical property and may be a function of the dilation caused by the process of cell hypertrophy and the continued accumulation of ECM. Once the perichondrium has become consolidated, very little increase in measurable width, as proposed by Carey (1922), or in the number of cells across the width of the hypertrophic zone is observed (see Table V, chapter I).

The consolidation of the perichondrium is a progressive process in that it spreads outwards from the hypertrophic zone to the flattened cell zones. However, the perichondrium surrounding the flattened

cell zones never exhibits a high degree of cell-cell contacts until the flattened cells begin to hypertrophy (Fig. 10). The flattened cell perichondrium is usually composed of layers of overlapping cells (Figs. 4 and 7) which would allow limited radial expansion of this zone. This expansion is necessary since the flattened cell zone is continually advancing into areas which had previously been composed of rounded cells.

The rounded cell zone is continuously expanding in a radial manner due to non-polarised ECM secretion and cell division. This expansion occurs freely and may be aided by the lack of a perichondrium between the cartilage and the surrounding mesenchyme (Figs. 2, 5 and 8). The expansion is only constrained when rounded cells, closest to the flattened cell zone, begin to flatten and, therefore, become surrounded by a loose perichondrium (as in Fig. 4). The first appearance of a perichondrial structure around rounded cells, at the tip of the ulna, is observed at stage 33 when cells between the cartilage and the mesenchyme begin to overlap (Fig. 11) and this structure also consolidates with age (Fig. 16).

The transformation from a perichondrium into a periosteum, at stage 32/33, is not the main point of study here but it seems to involve both the rounding up of cells in the inner layer of the perichondrium (Fig. 9), and a build up of aligned collagen fibres on the inner surface of the perichondrium (Fig. 12). These inner layer cells are believed to be osteogenic (Fell, 1925) but how they round up and how they become osteogenic is unknown. There is virtually no ECM between cells in the inner layer of the periosteum (Fig. 15) but by stage 34, long, electron-dense strips appear between cells in the outer fibroblastic layer (Fig. 13 and 14). These electron-dense strips appear at the same time and in the same place as the "delicate white fibres" described by Fell (1925). Although

no biochemical assay has as yet been performed, this ECM is thought to be composed of collagen fibres, glycosaminoglycans and elastin fibres, which may explain why the periosteum seems to spring back whenever it is cut (e.g. see Crilly, 1972). It is important to note that the periosteum only extends as far as the hypertrophic cell zone and only enlarges at the same rate as the process of hypertrophy.

Experimental results presented in this chapter indicate that the main function of the perichondrium is to aid in the production of a typical long bone morphology by means of constraining expansion both longitudinally and radially.

The overgrowth observed when part of the perichondrium was removed (Table I) is very similar to that observed when a circumferential incision was made into the periosteum of a 20 day old chicken radius (Crilly, 1972), or when part of the periosteum was stripped from the femur of a juvenile rat (Haughton and Dekel, 1979). The production of an S shaped rudiment when the whole of the perichondrium was removed (Fig. 17) not only implies that the perichondrium is important in morphogenesis but also that the cartilage itself in the diaphysis is relatively firm whilst that in the epiphysis is soft.

Further evidence for a difference in consistancy between cartilage from different regions comes from the observation that when a nick was made, through the perichondrium, at the epiphyseal region of a stage 32 ulna a swelling was observed (Fig. 18), but if the nick was made in the diaphyseal region, no swelling appeared (Table II). As shown in chapter II, rounded cell cartilage ECM (epiphyseal) is granular rather than fibrillar and very little collagen is present, whereas the ECM in the flattened or hypertrophic

cell region (diaphyseal) is very fibrillar with proteoglycan aggregates attached to the collagen. It would be expected, therefore, that epiphyseal cartilage would have a greater swelling pressure of proteoglycans when exposed to culture medium and the cartilage would swell out through the hole. Older epiphyseal cartilage contains more collagen fibres with aggregates attached and this may explain why no swelling was ever found when stage 36, or older, rudiments were nicked (data not shown).

It is not known why a rudiment had to be cut in two before any swelling was observed but two possibilites exist : the first is that cutting the rudiment allows water to enter and it is the build-up of hydrostatic pressure, in the soft, malleable epiphyseal cartilage which forces the cartilage out through the nick; the second possibility is that cutting the rudiment in two and/or cutting through the perichondrium induces excess matrix secretion, therefore, matrix builds up and gets forced out through the nick. (Cartilage is held under pressure within a rudiment and the release of this pressure is known to have effects on both cell division and ECM secretion - Rodan *et al* (1975), it is possible that cutting the rudiment causes release of this tension).

The observation that hypertrophic cartilage does not flow out through a nick may simply be a reflection of the solidity of the matrix, but it could also be due to the fact that the perichondium at the hypertrophic region is a very distinct, very cellular structure which may rapidly heal preventing formation of a swelling.

It has long been known that if one epiphyseal growth plate of a bone is damaged the other growth plate may compensate for it (Reidy *et al*, 1947; Hall-Cragg and Lawrence, 1969), therefore,

if a nick is made into one epiphysis of an intact rudiment, the rudiment may show compensatory growth which would not allow pressure to build up and no swelling would be produced.

The results obtained when ulnae were cut into pieces suggest that overgrowth may be a stage dependant phenomenon, but this is not necessarily the case and it is possible that the trauma of cutting stage 30 ulnae into pieces may be sufficient to kill the pieces, therefore, no growth at all is observed (Table III). A similar explaination is probably the case when stage 32 ulnae are cut into 6 pieces. However, when stage 32 ulnae are cut into 2 or 4 pieces each portion does increase in length and the total increase in length observed is always greater than that for control ulnae from the same embryo (Table III). The most imporatant observation from this set of results was that when the flattened cell zone was isolated, the flattened cells progressed to hypertrophy, therefore, the process is autonomous. This hypertrophy was somewhat different from that *in vivo* in that it involved both longitudinal and radial expansion. Cell hypertrophy *in vivo* is primarily in a longitudinal direction and it could be the case that this directionality is forced upon the cells. The distinct, tight perichondrial structure found around the hypertrophic cell zone is probably ideal for this function.

The preliminary results obtained when a cube of flattened cells was rotated through 90° implies that directionality of growth in the flattened cell region is intrinsic to that zone and is not influenced greatly by the perichondrium. If the perichondrium did control the direction of growth in the flattened cell zone then it would be expected that the rotated cells would re-orientate and the shape of the epiphysis would not change.

The overall conclusion from the above results is that the structure of the perichondrium lends itself to the function of constraining circumferential expansion whilst favouring longitudinal expansion. It is proposed that at an early stage, when no hypertrophic cells are present and the cartilage is fairly "soft", there is no need for a distinct perichondrium and increases in width can occur as well as increases in length. As chondrocytes undergo hypertrophy a more distinct structure is required which facilitates directionality to the growth process. No such restraint is required at the rounded cell zone which continues to increase in width. Formation of the periosteum at stage 32/33 produces even more constraint in the diaphysis and it is interesting to note that a spurt of growth is observed at this time (see chapter I). Also, if collagen and elastin fibres are present in the periosteal ECM this would produce tension in the periosteum and since it is known that periosteal tension restricts growth (Crilly, 1972), increases in length may be controlled in this way.

The idea of the perichondrium acting as a constraining sheath has been outlined briefly by Wolpert (1982) when he introduced the hypothesis of "directed dilation". Directed dilation suggests that embryonic cartilage is a visco-elastic fluid which exerts pressure on the perichondrium during growth. This pressure is resisted by the perichondrium, but where the pressure exerted is greater than the resistance, as at the rounded/flattened region, a bulge appears. In a long bone rudiment, perichondrial resistance is "high" in the diaphysis and "low" at the epiphysis, therefore, a narrow rod with a bulge at either end is produced.

Directed dilation is not thought to be restricted to long bone morphogenesis and probably plays a role in the morphogenesis

of every cartilaginous element. Support for this comes from 2 other sources:

a) in Meckel"s cartilage, which is a long narrow rod consisting almost entirely of one type of chondrocyte, a uniform perichondrial structure of very elongated cells with many cell-cell contacts is observed. This structure is similar to that surrounding a long bone diaphysis and, therefore, would favour growth primarily in a longitudinal direction so that a long narrow rod is produced.

b) the thickness of the perichondrium surrounding the scleral cartilage of the eye can be experimentally thickened or thinned depending on how much radial pressure is expended upon it (Coulombre and Coulombre, 1957). When the eye is expanded, i.e. high radial pressure, the perichondrium is a very thin structure of elongated cells similar to the perichondrium surrounding the rounded/flattened cell zone. When the eye is made to collapse, i.e. low radial pressure, the perichondrium is a much thicker structure.

The data presented in this chapter supports, but also extends, the hypothesis of directed dilation in that the perichondrium can be thought to play a role in the morphogenesis of a long bone rudiment in 4 ways.

i) it produces directionality in the process of cell hypertrophy (by "high" resistance) thereby favouring longitudinal growth;

ii) it allows for a bulge at either end of the rudiment (by "low" resistance) thereby producing the primitive epiphysis;

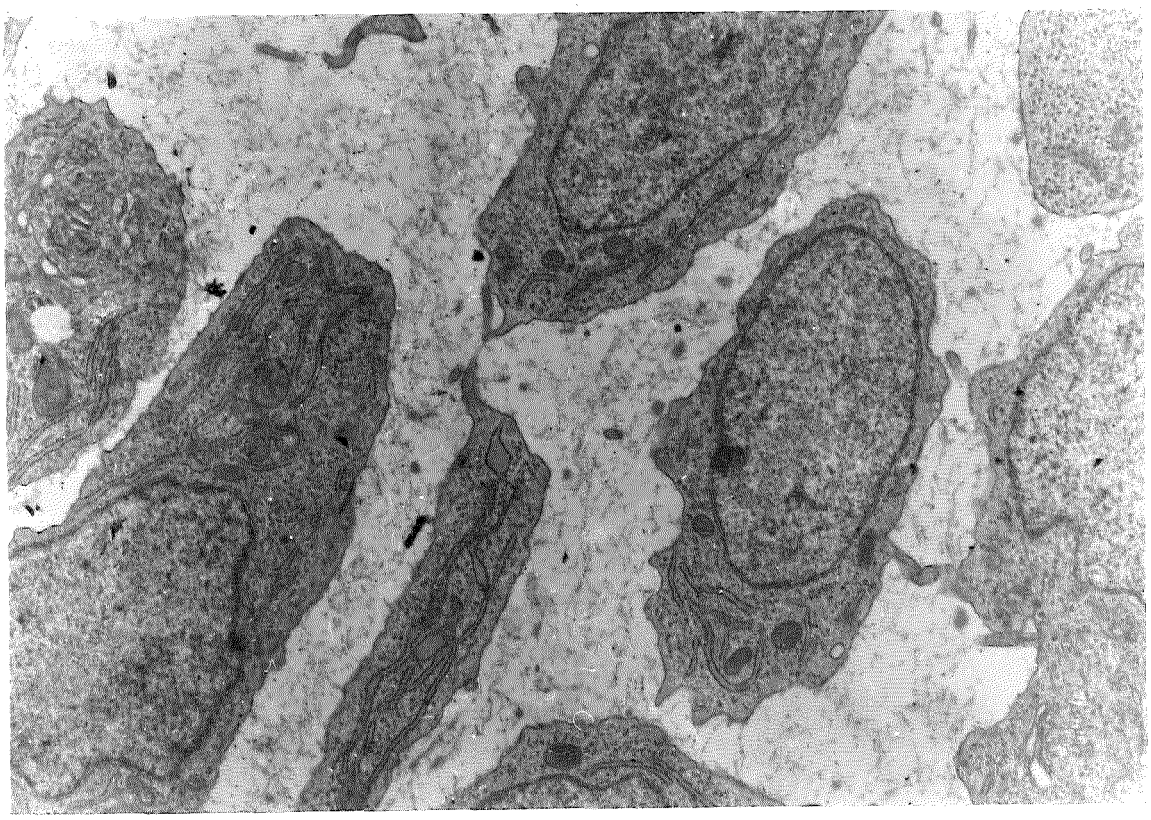
iii) it plays a role in controlling the extent of the increase in length observed during growth and

iv) it controls the final width of the rudiment by gradually consolidating and preventing further increase.

In conclusion, the results presented here suggest that the perichondrium does in fact play a fundamental role in the final morphogenesis of a long bone rudiment. However, the structure of the perichondrium appears to be dictated by the internal distribution of the chondrocytes and should, therefore, be considered as an essential but secondary morphogenetic factor.

Fig. 1. Stage 30, formation of the early perichondrium around the hypertrophic cell zone. The perichondrial cells appear elongated but remain similar in their staining properties and ED. to the more rounder chondrocytes.
Uranyl acetate/lead citrate stain. Mag. x 8,400.
All Electron Micrographs are from longitudinal sections.

Fig. 2. Stage 30, junction of rounded cells and mesenchyme. No barrier is observed between chondroblasts and mesenchyme at the "epiphyseal ends". The cells appear similar and cannot be distinguished from each other.
Uranyl acetate/lead citrate stain. Mag. x 5,000.
M = mesenchyme, C = cartilage.



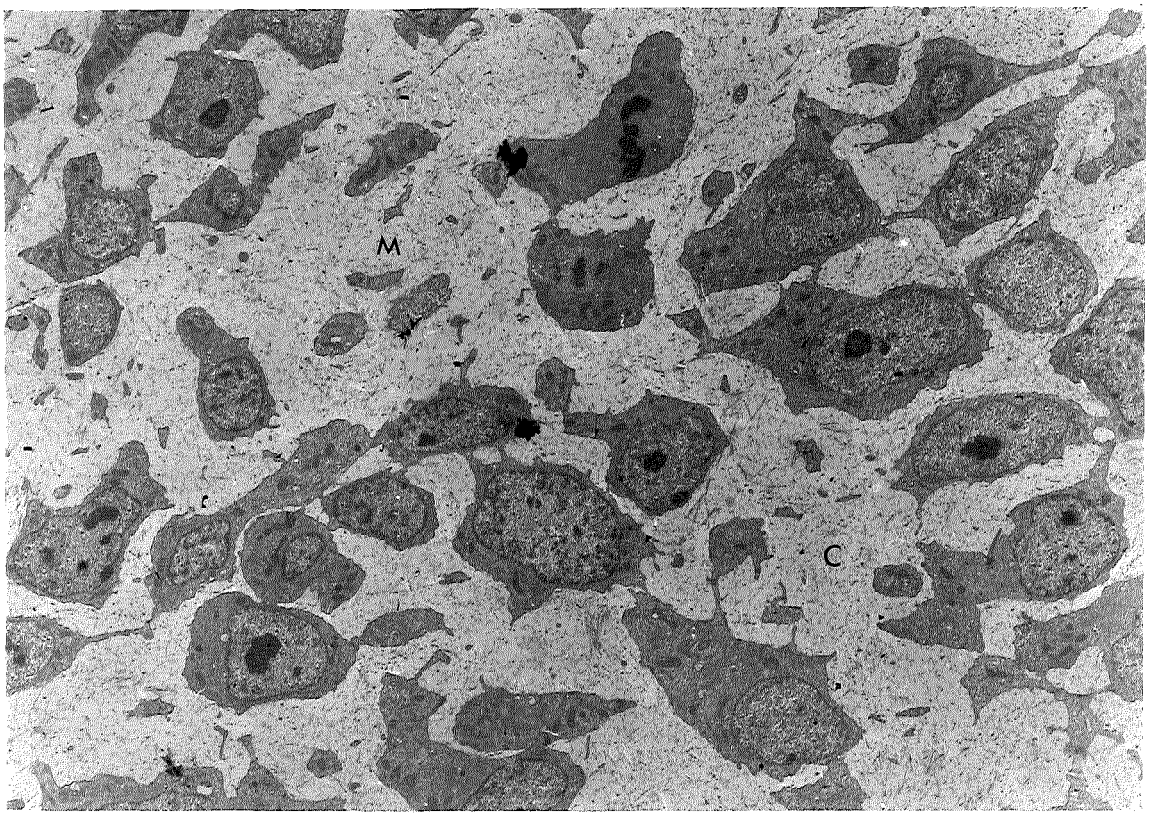
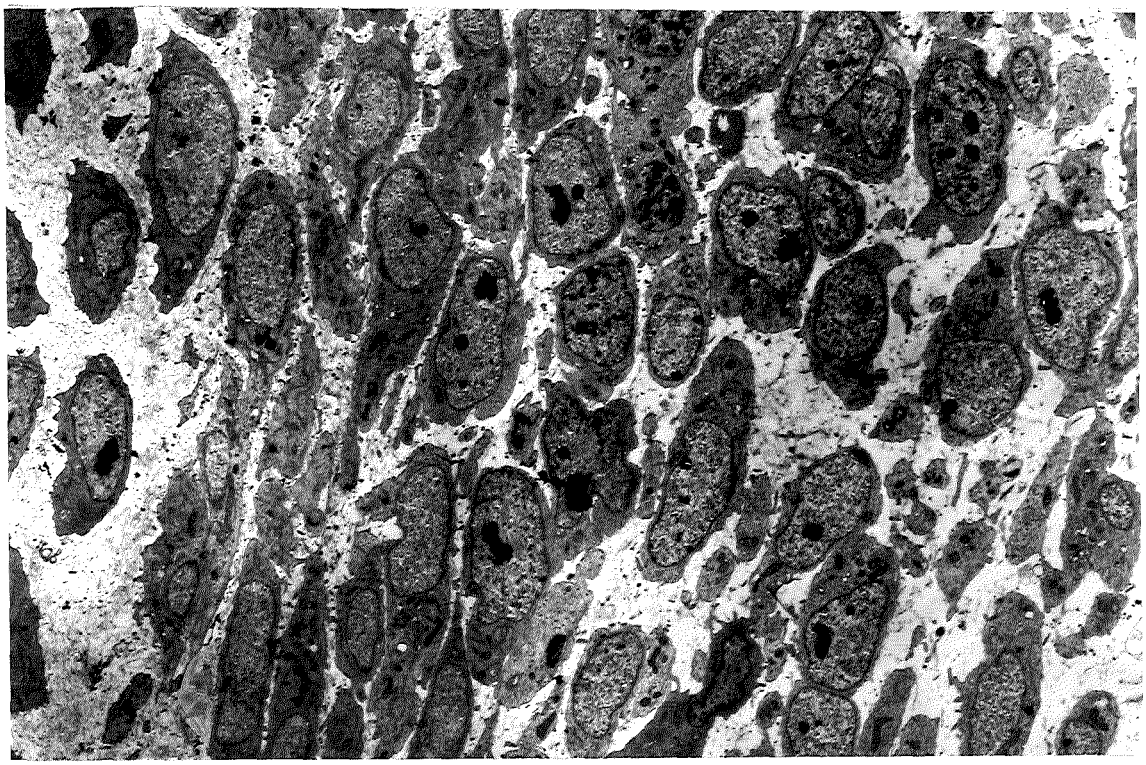

M
C

Fig. 3. Stage 3I, perichondrium surrounding the hypertrophic cell zone. A distinct barrier has formed between the chondrocytes and the mesenchyme. The perichondrial cells are very elongated, close together and exhibit some cell-cell contacts.
Uranyl acetate/lead citrate stain. Mag. x 4,500.

Fig. 4. Stage 3I, perichondrium surrounding the flattened cells zone. The perichondrial cells are elongated but merely overlap and present little resistance to radial expansion. Note that flattened chondrocytes nearest the perichondrium appears polygonal, these cells are in fact elongated circumferentially around the rudiment.
Uranyl acetate/lead citrate stain. Mag. x 4,000.



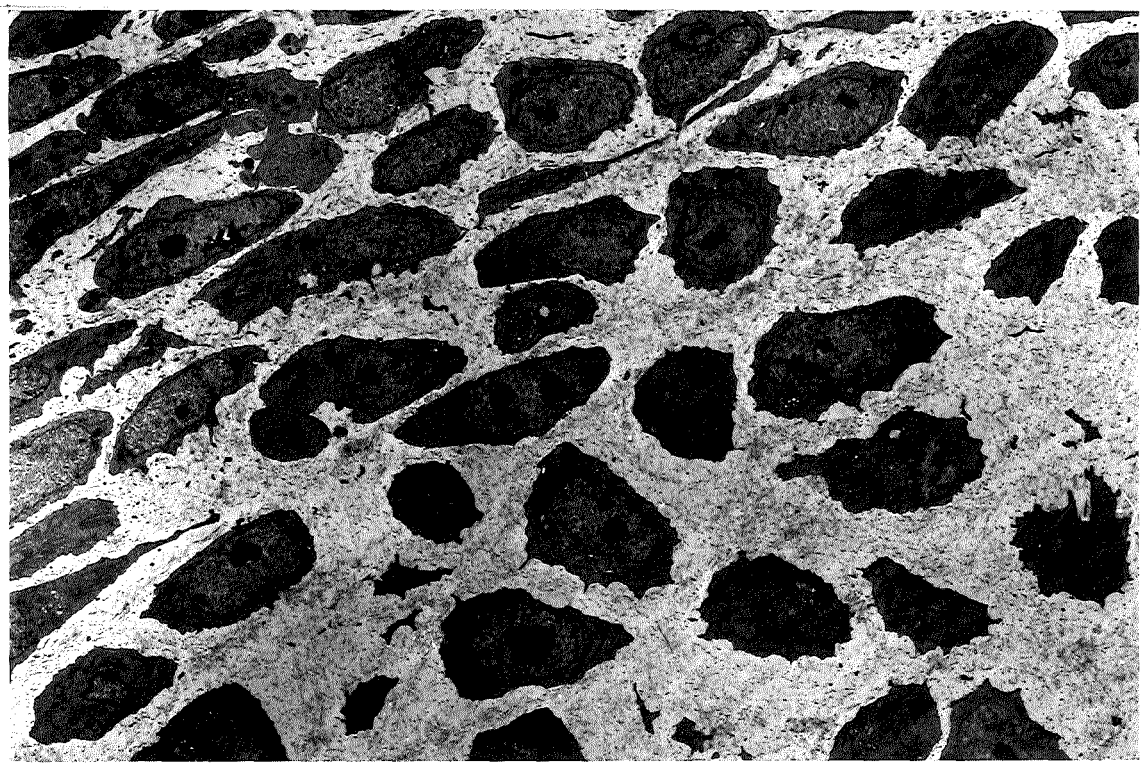
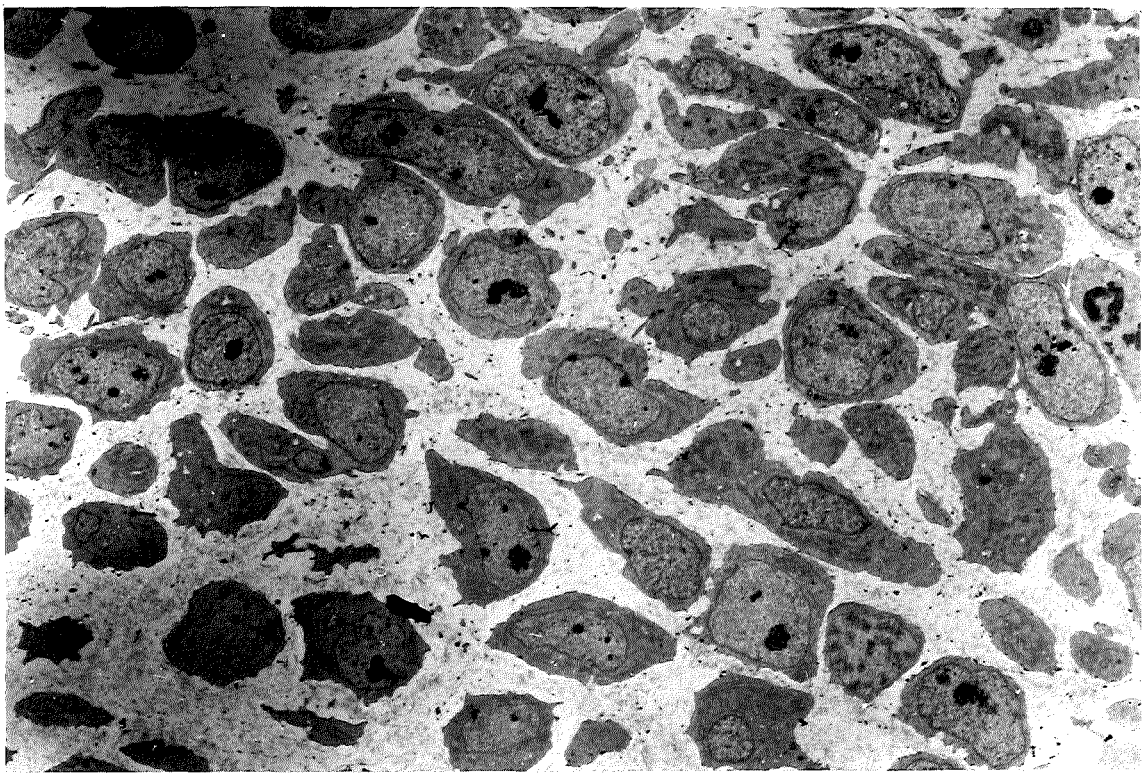


Fig. 5. Stage 32, junction of rounded cells and mesenchyme. Cartilage cells appear more uniformly round or polygonal than the mesenchyme but still no definite barrier is present. Uranyl acetate/lead citrate stain. Mag. 4,500.

Fig. 6. Stage 32, perichondrium surrounding the hypertrophic cell zone. Perichondrial cells are elongated and show areas of extensive cell-cell contacts (tight junctions). No LCH can be observed between the cells at this stage. Uranyl acetate/lead citrate stain. Mag. x 5,000.



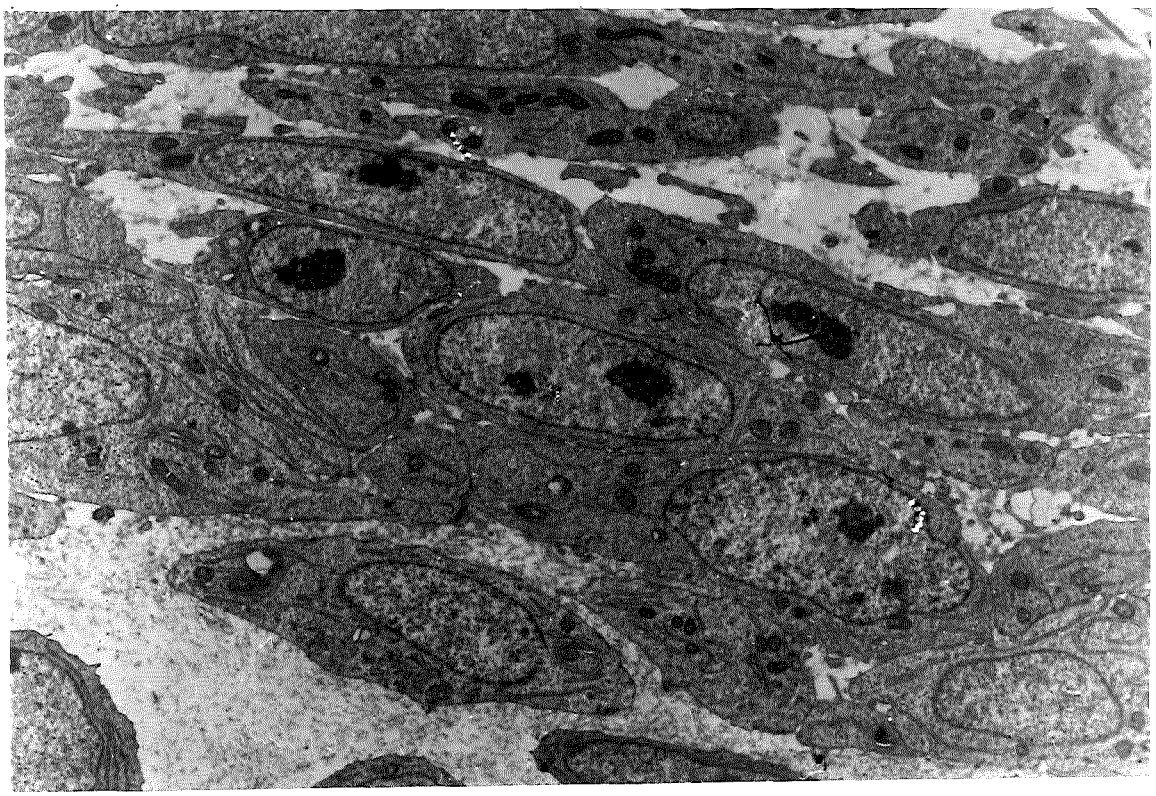


Fig. 7. Stage 32, perichondrium surrounding the flattened cell zone. The perichondrium consists of several layers of overlapping cells with some cell-cell contact. Cells in the inner layer appear closer together.
Uranyl acetate/lead citrate stain. Mag. x 4,500.

Fig. 8. Stage 32, junction of rounded cells and mesenchyme. No clear distinction can be observed between the cartilage and mesenchyme. However, some cells are flattening in a region which may become the future perichondrium.
Uranyl acetate/lead citrate stain. Mag. x 5,000.

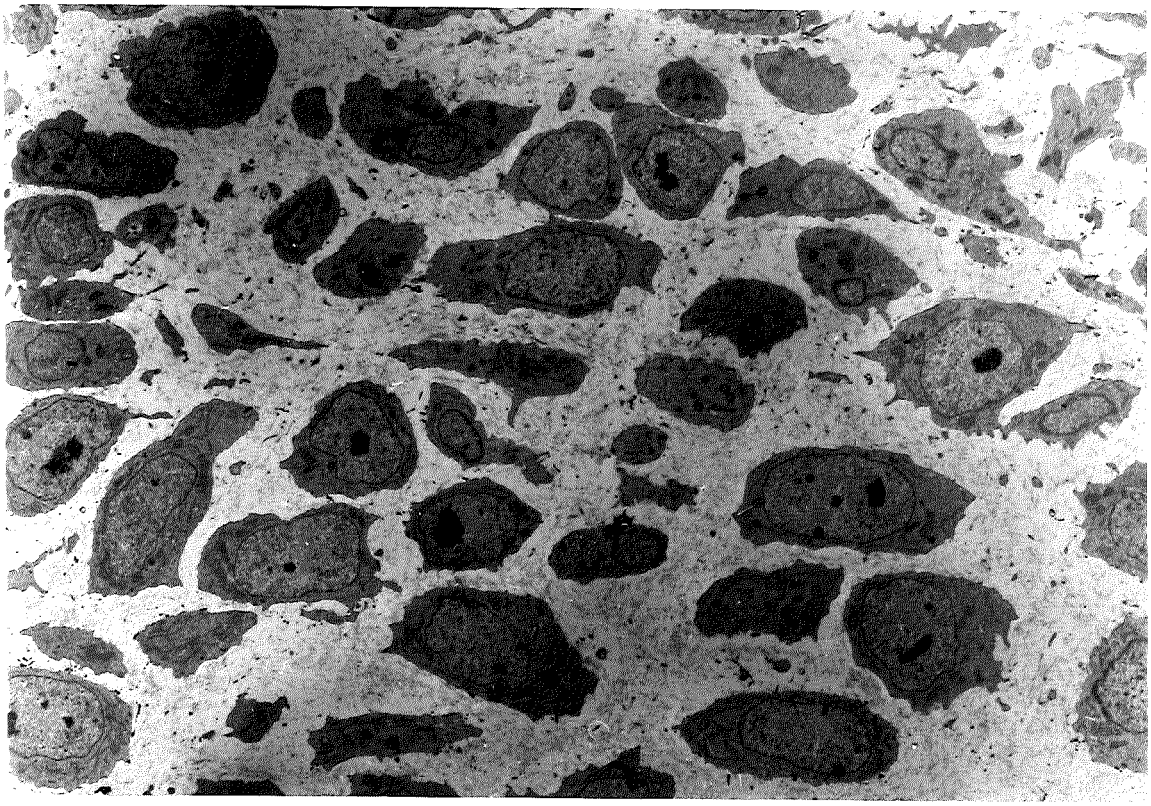
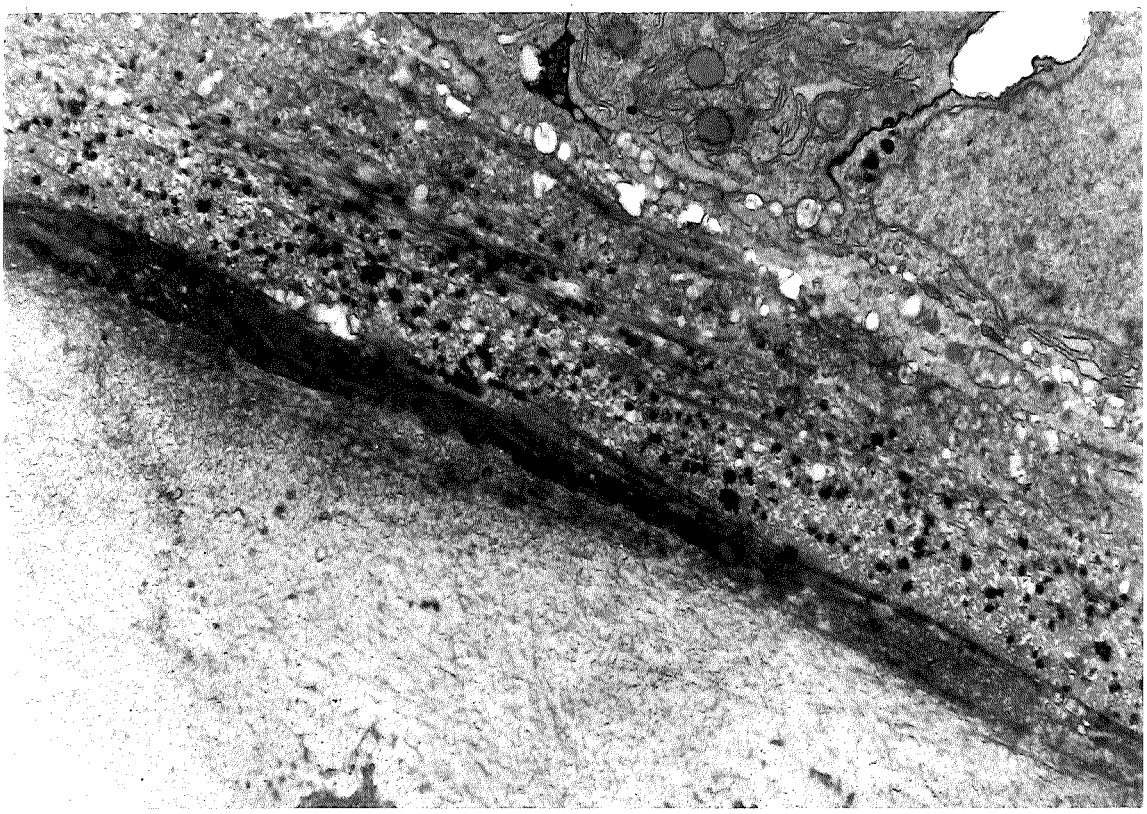


Fig. 9. Stage 33, early formation of the periosteum around hypertrophic cells. Periosteal cells closest to the cartilage appear round but retain extensive cell-cell contacts. These cells are separated from the cartilage by layers of orientated collagen fibres with deposits of osteoid intermingled between them. No ECM. can be seen between the cells.
Uranyl acetate/lead citrate stain. Mag. x 8,400.

Fig. 9a. Higher magnification of Fig. 9. The cell membranes show very close apposition and no evidence of ECM, can be observed, a coated pit is clearly visible which implies that ECM production is, or soon will be, occurring.
Uranyl acetate/lead citrate stain. Mag. 63,000.



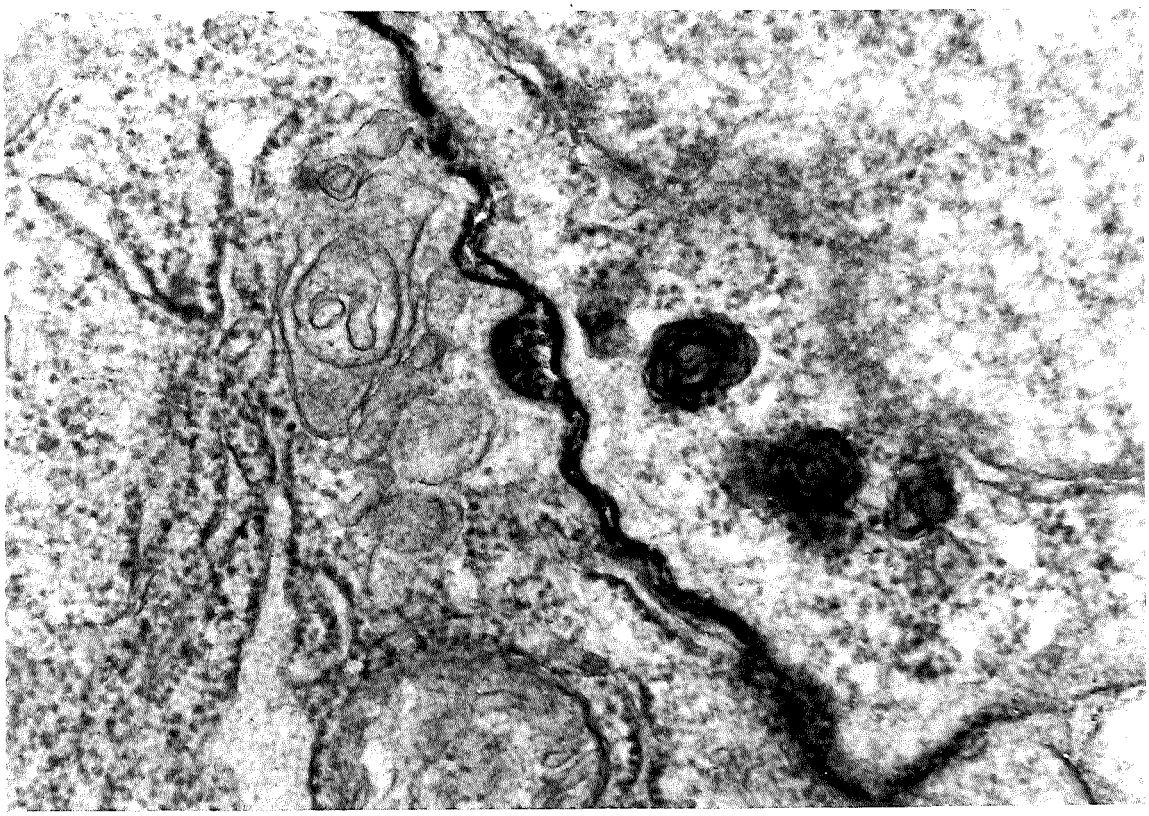
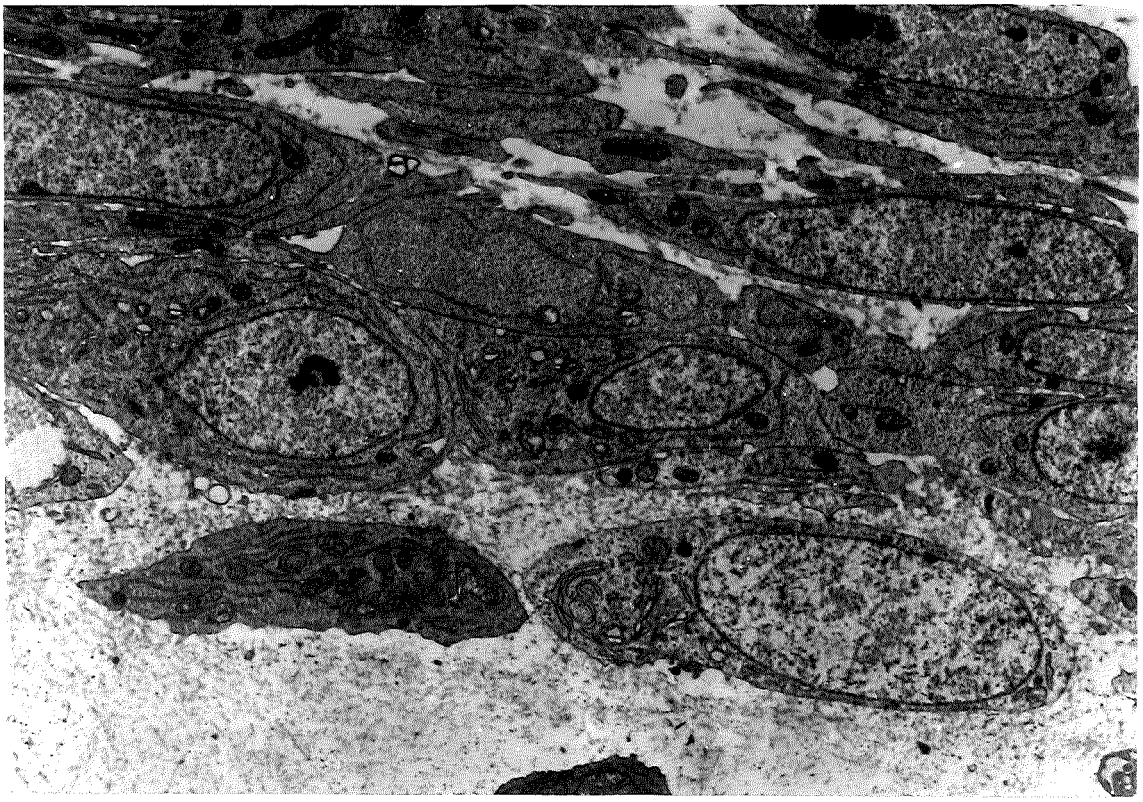


Fig. 10. Stage 33, perichondrium surrounding the flattened cell zone. The perichondrium is now tight and consists of elongated cells exhibiting extensive cell-cell contacts. No ECM can be seen between the cells.
Uranyl acetate/lead citrate stain. Mag. x 5,000.

Fig. 11. Stage 33, perichondrium surrounding the rounded cell zone. Although there is no obvious cell-cell contact, a distinct layer of overlapping cells separates the cartilage from the mesenchyme.
Uranyl acetate/lead citrate stain. Mag. x 5,000.



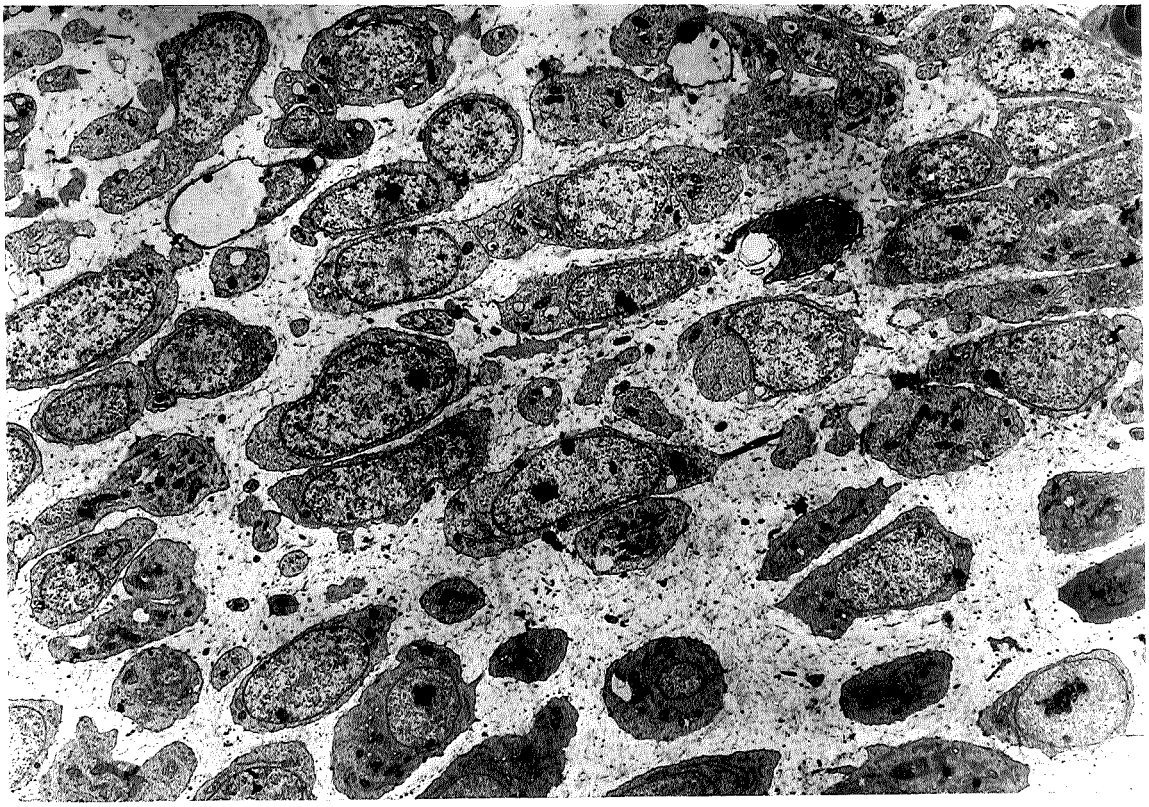
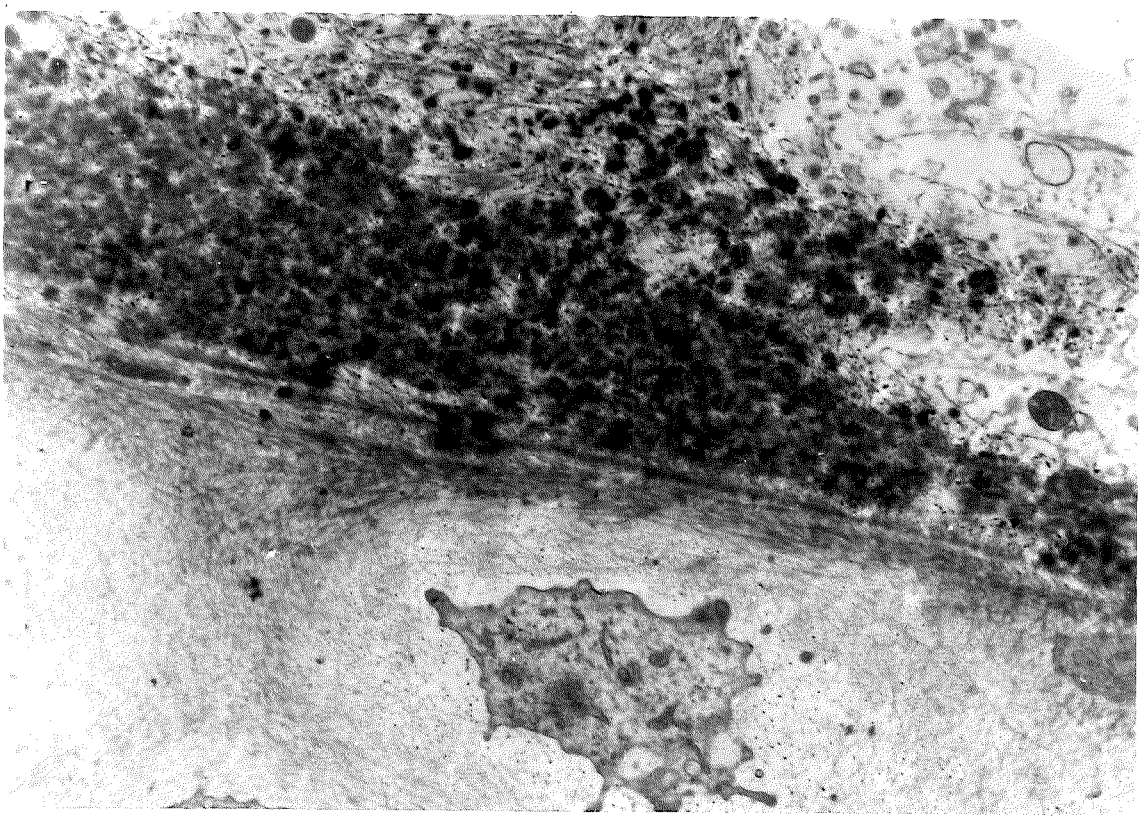


Fig. 12. Stage 34, midshaft on the dorsal aspect of the diaphysis. Perichondral cells are separated from the cartilage by a thick layer of osteoid deposition. Note the presence of fibrous material on both sides of the osteoid. The hypertrophic cell layer appears relatively fibre-free.
Uranyl acetate/lead citrate stain. Mag. x 7,500.

Fig. 12a. At higher magnification, the fibrous material next to the cartilage appears to be aligned parallel to the long axis of the rudiment. This fibrous material is thought to be collagen fibres.
Uranyl acetate/lead citrate stain. Mag. x 40,500.



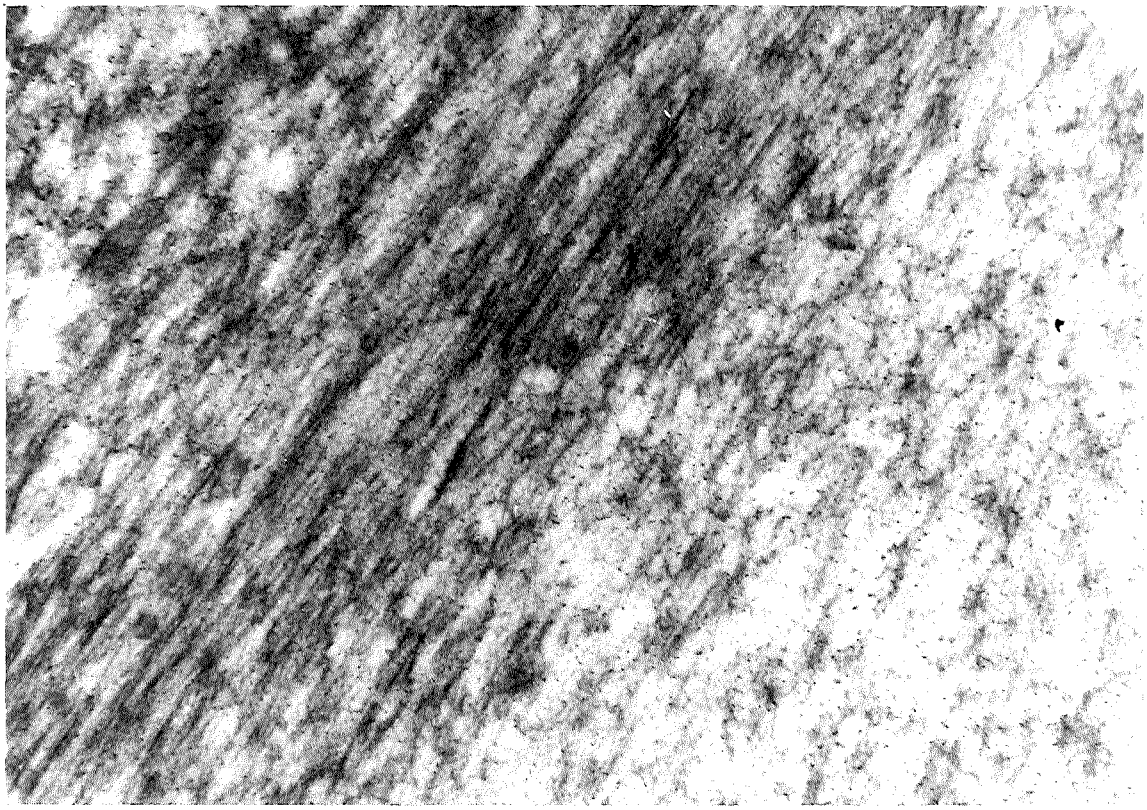
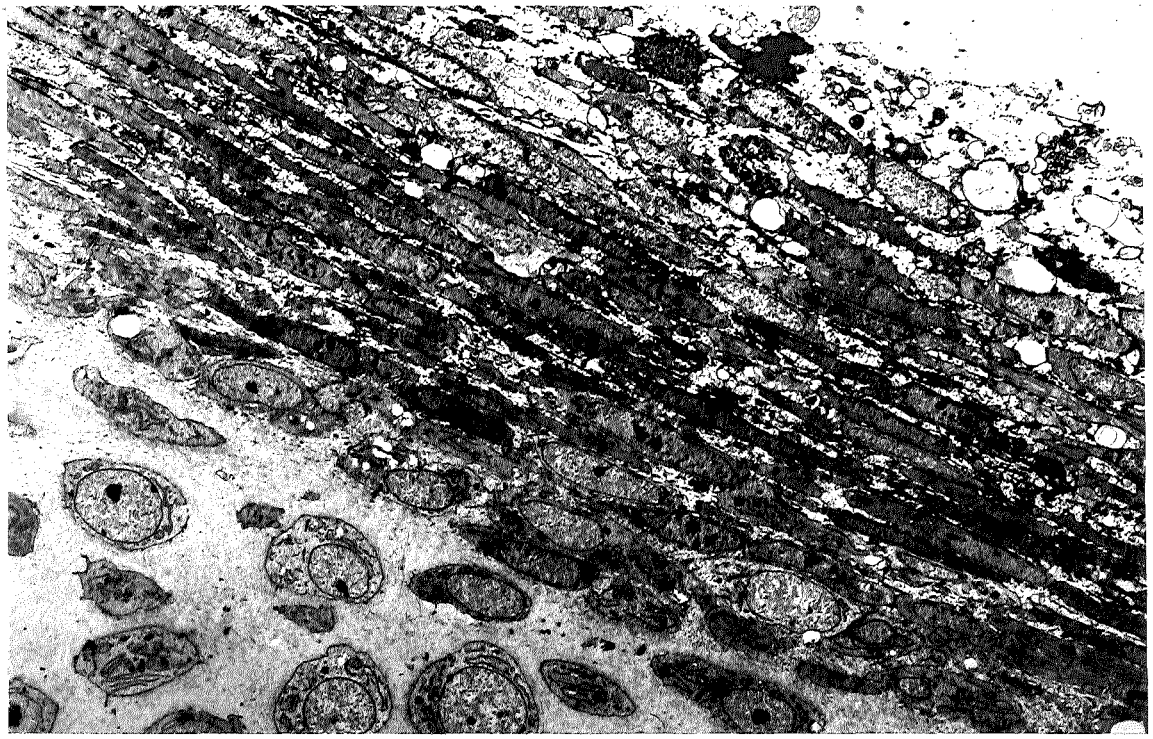


Fig. I3. Stage 3+, periosteum surrounding the hypertrophic cell zone. The periosteum appears bilayered with an inner layer of rounded, closely packed, cells and an outer layer of very elongated fibroblastic cells.
Uranyl acetate/lead citrate stain. Mag. x 4,000.

Fig. I4. Stage 3+, outer layer of the periosteum. The very elongated cells show some areas of cell-cell contact but also show large areas where the cells are separated by strips of electron-dense material.
Uranyl acetate/lead citrate stain. Mag. x I0,000.



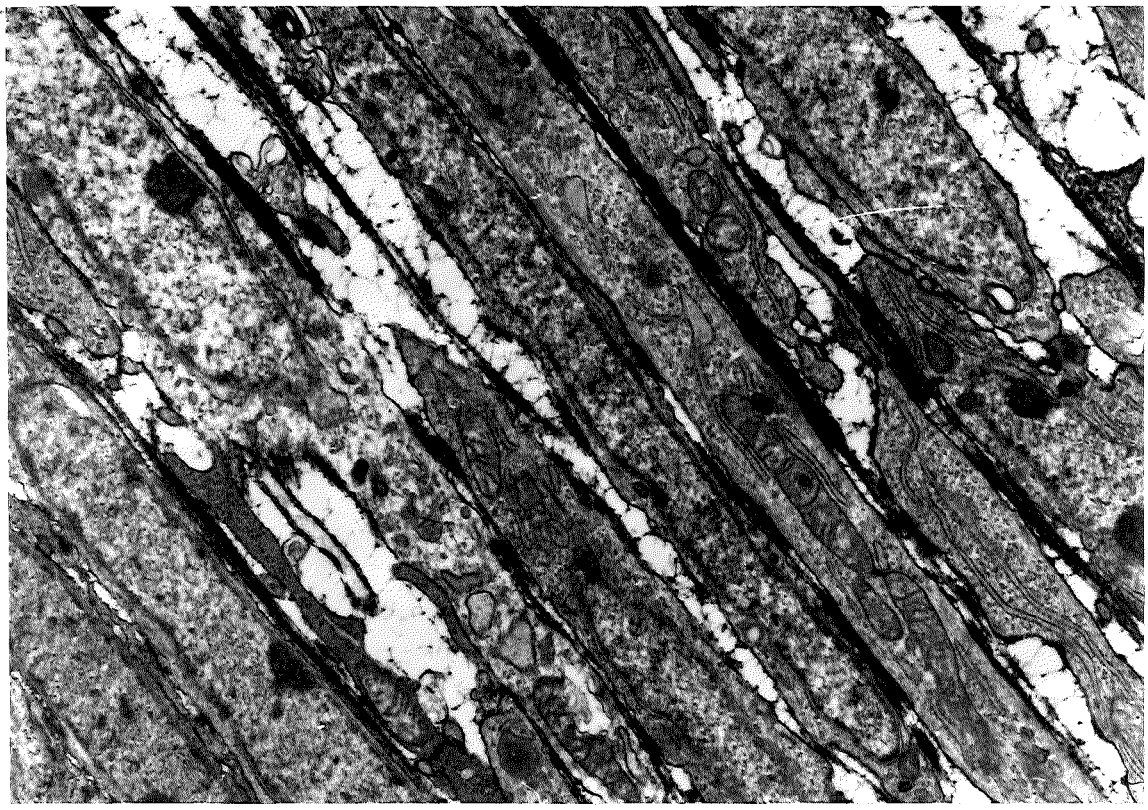
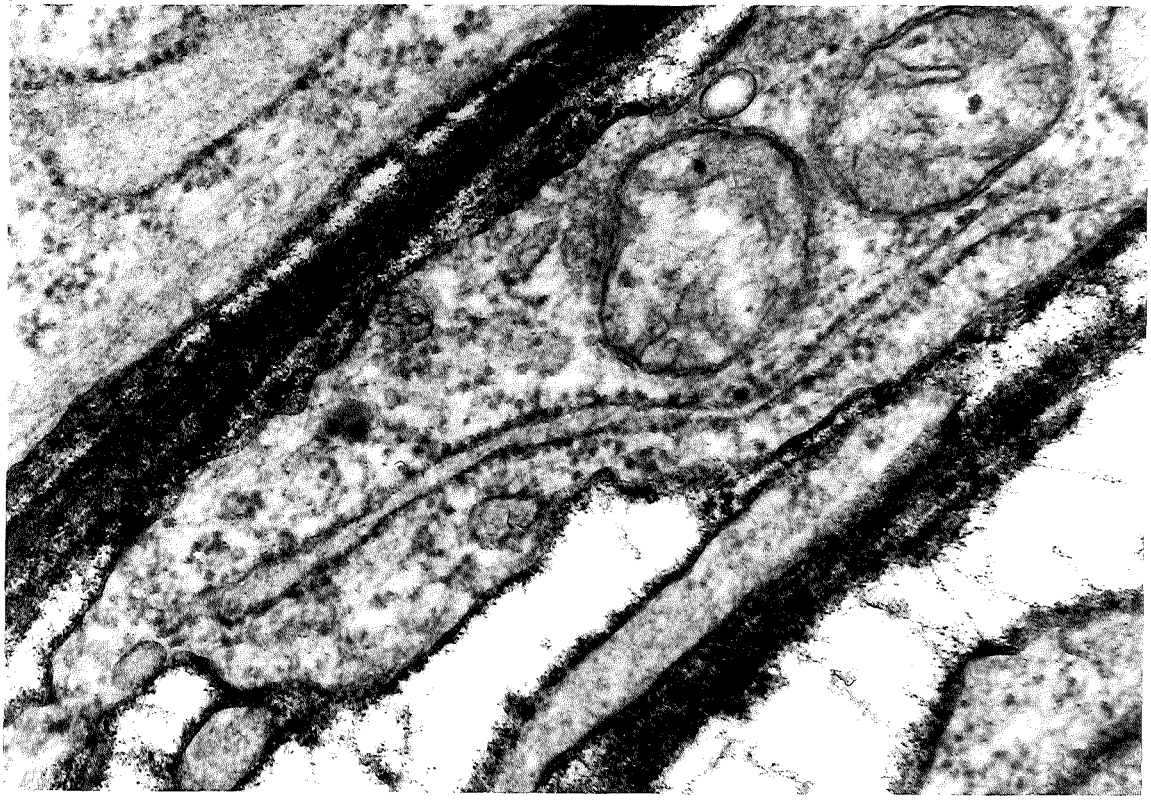


Fig. 14a. Higher magnification of the electron-dense strips between outer layer cells. Small strands of what are thought to be glycosaminoglycans extended from the electron-dense strips to a glycocalyx on the opposite cell surface. Note that many ribosomes are present suggesting that the cells are biologically active.
Uranyl acetate/lead citrate stain. Mag. x 63,000.

Fig. 15. Stage 3½, inner layer of the periosteum. The cells are rounder than outer layer cells and exhibit extensive cell-cell contacts with very little ECM between them. Note that there appears to be a slight build-up of aligned collagen fibres within the cartilage.
Uranyl acetate/lead citrate stain. Mag. x 10,000.



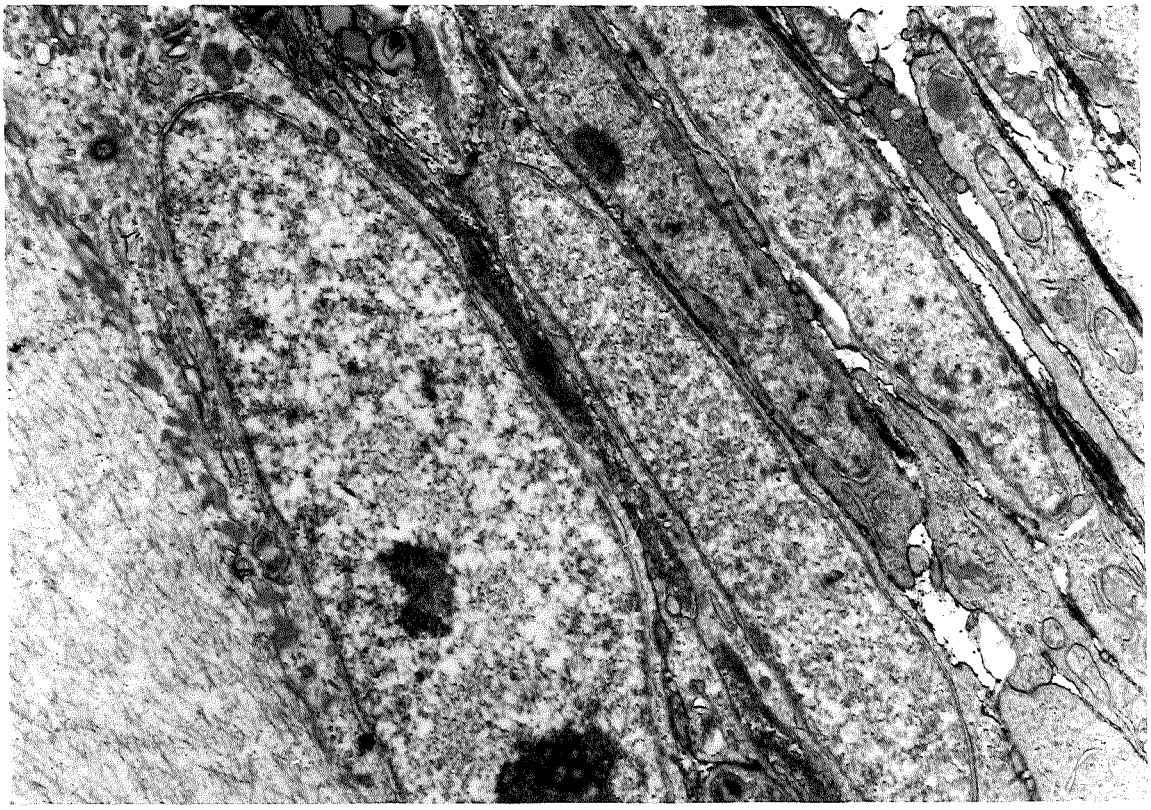
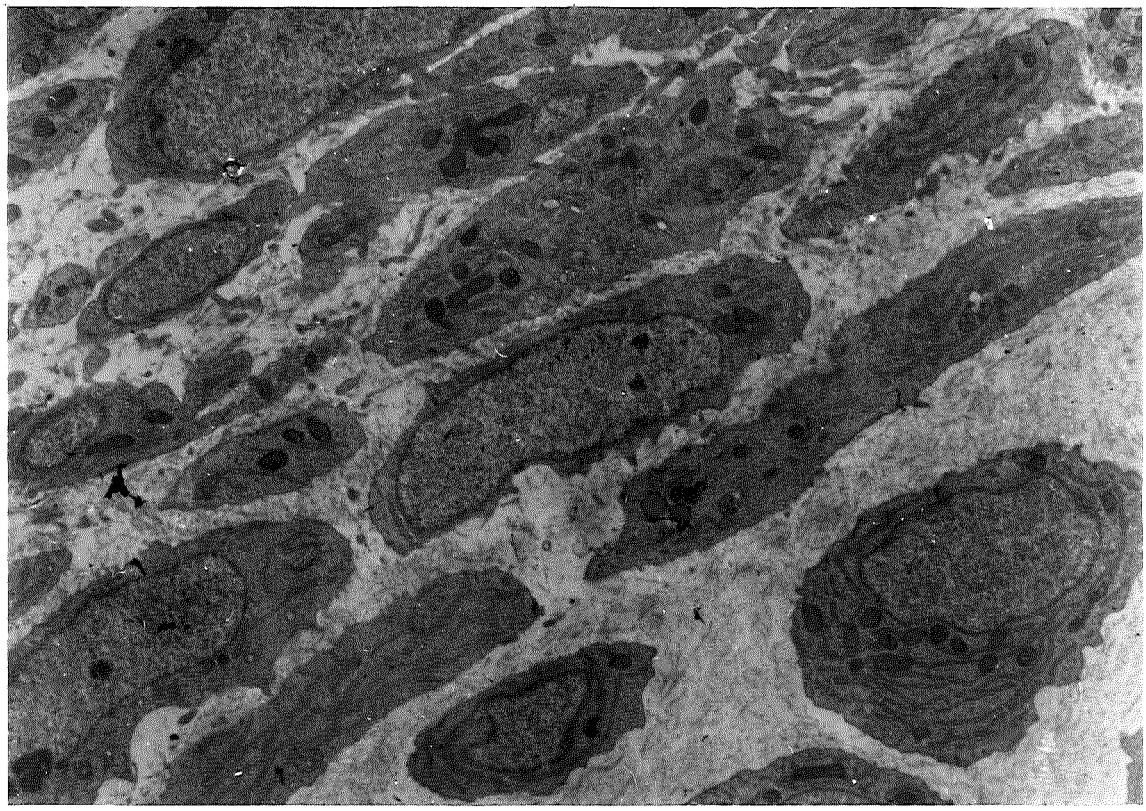


Fig. I6. Stage 34, perichondrium surrounding the rounded cell zone. The perichondrium is composed of overlapping cells, 4-5 cells thick, and a clear distribution can be made between cartilage and mesenchyme.
Uranyl acetate/lead citrate stain. Mag. x 5,000.

Fig. I7. Stage 32 ulna. The perichondrium was removed by rolling the rudiment on dry Millipore filter and the rudiment was cultured for 24 hours. Note that the diaphysis has remained straight whilst the epiphyseal ends have bent round forming an S shape.
Tol. blue stain. Mag. x 40.
E = epiphyseal region, D = diaphyseal region.



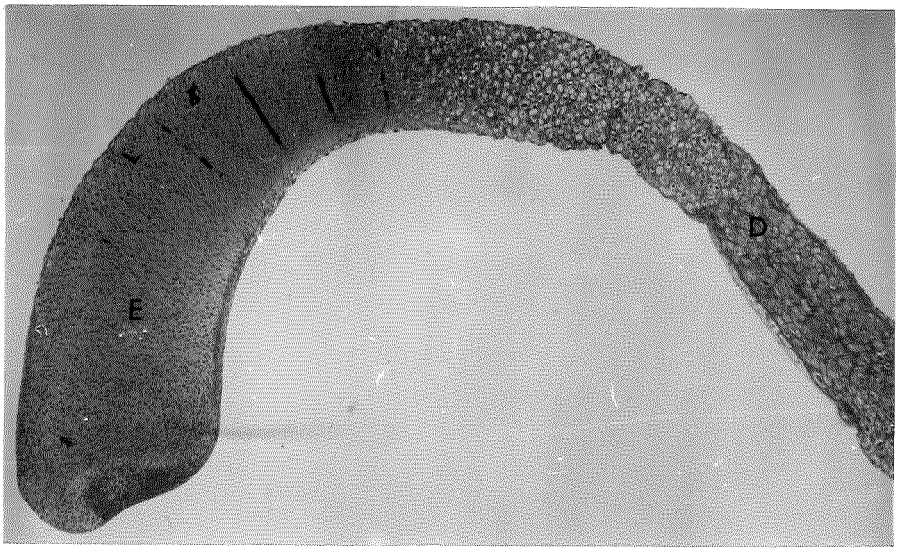
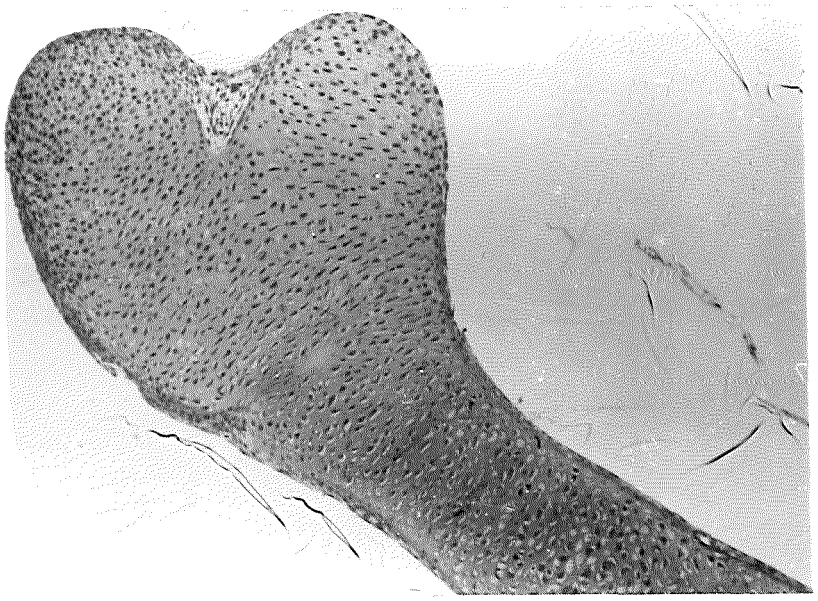
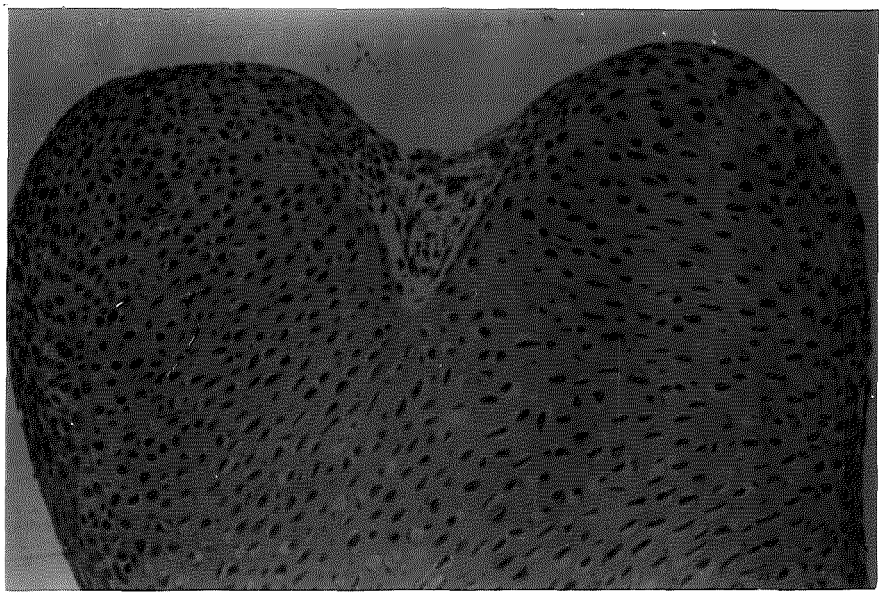

E
D

Fig. 18. Stage 32 ulna, proximal half. A small nick was inserted into the cartilage, through the perichondrium, by means of irredectomy scissors and the rudiment was cultured overnight. The resultant swelling is seen to be full of normal staining cartilage. Tol. blue stain. Mag. x 50.

Fig. 19. Higher power of Fig. 2. Note that the constituent cartilage cells appear normal and are orientated in the direction of the swelling. The entire swelling is covered by a thin perichondrium. Tol. blue stain. Mag. x 125.

Fig. 20. The effect of isolating the flattened cell zone from a stage 32 ulna. All the cells undergo hypertrophy. Cells in the cut end nearest the hypertrophic zone are constrained by a tight perichondrium, therefore, hypertrophy is directed. However, cells in the cut end nearest the rounded cell zone have no constraint and hypertrophy is radial.





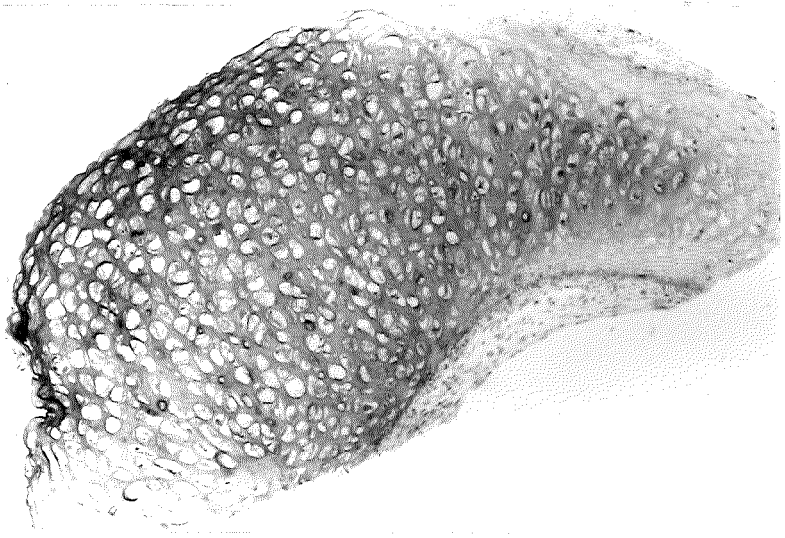
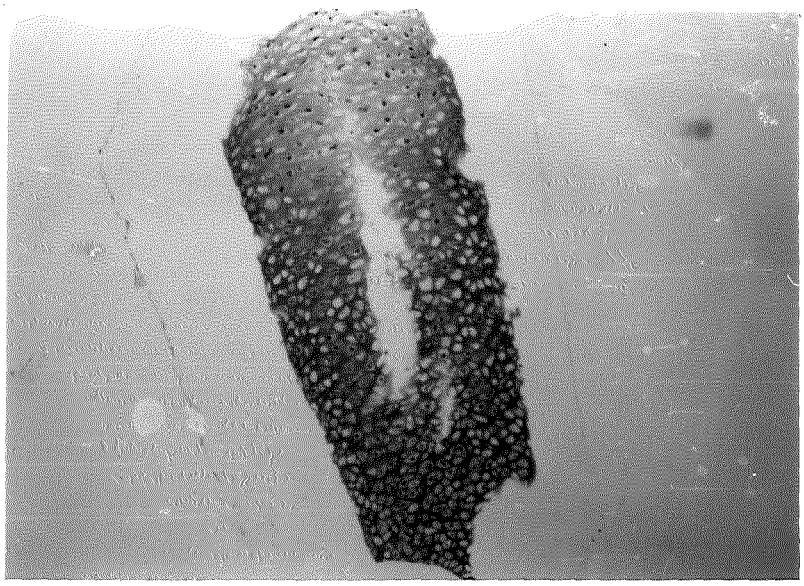
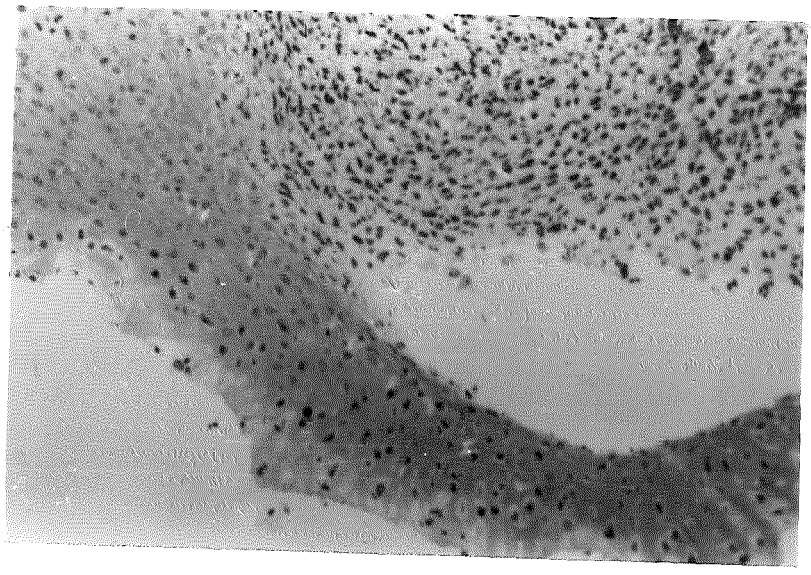


Fig. 21. The effect of gently squashing the diaphysis of a stage 32 ulna. The hypertrophic cell zone is expelled totally free of any connective tissue. The tear in the centre is due to the stress caused by squashing.
Tol. blue stain. Mag. x 40.

Fig. 22. The effect of squashing the epiphyseal region of a stage 32 ulna. The perichondrium remains attached to the cartilage cells.
Tol. blue stain. Mag. x 40.





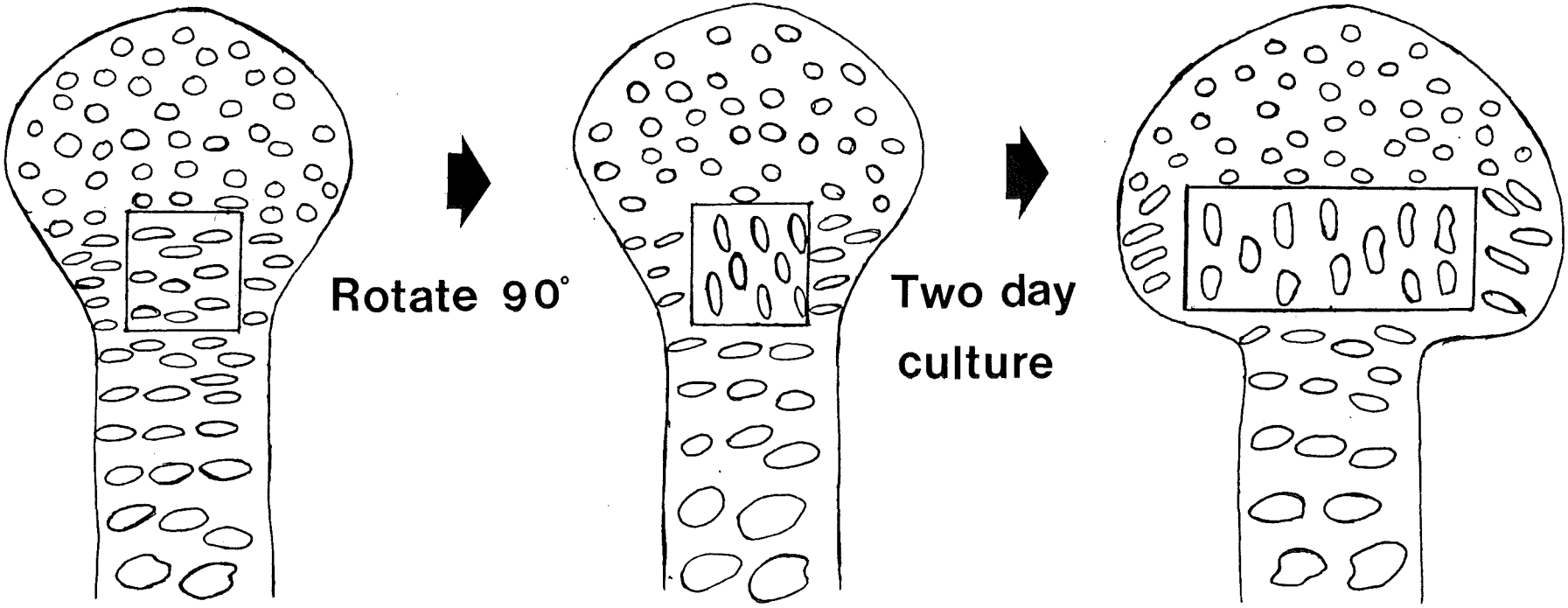


Fig. 23. Diagramatic representation of the effect of rotating a cube of flattened cells through 90°. By 2 days of culture the cube had become a rectangle, the cells had retained their orientation and the epiphysis became distorted.

Table I The effect of removal of the perichondrium from stage 32 ulnae.

Treatment	Length (mm) Day 0	Day I	% increase*
Control	2.93 ± 0.I0	4.20 ± 0.II	43 ± 3
Total removal	2.9I ± 0.09	4.63 ± 0.I3	59 ± 4
Partial removal	2.94 ± 0.I0	4.53 ± 0.I2	54 ± 3

*Ulnae were cultured overnight on Millipore filter rafts.

Measurements were made on living specimens using a calibrated eyepiece graticule.
Statistical analysis was performed using Student's t-test. Treated rudiments were compared with controls.

I = significant, $P < 0.05$.

Table II The effect of making a small incision into the cartilage of developing ulnae.

Treatment	Number	Position of nick	No. of swellings	%
Intact	20	Epiphyseal region	0	0
Cut in two	40	Epiphyseal region	22	55
Cut in two	IO	Diaphyseal region	0	0
Cut in two	IO	2 in epiphyseal region	I	IO

Rudiments were removed from embryos aged between stage 32 - stage 34 and a small incision was made, with irredectomy scissors in the epiphyseal region at a point close to the junction between rounded and flattened cells. The incision was judged deep enough to cut through the perichondrium and just pierce the cartilage itself.

Rudiments were cultured for up to 3 days, but if a swelling did appear, it did so within 24 hours of the incision.

Stage 30 ulnae proved too small to make an accurate incision into without damaging the cartilage too much.

Stage 36, or older, ulnae produced no swelling no matter what position the incision was made in.

Table III The effect of cutting developing ulnae into 2, 4 or 6 pieces.

Treatment	% increase in length*
Stage 30	
Control	109 ± 4
Cut into 2	32 ± 8 [2]
Cut into 4	21 ± 4 [2]
Cut into 6	0
Stage 32	
Control	103 ± 6
Cut into 2	108 ± 5
Cut into 4	115 ± 5 [1]
Cut into 6	65 ± 8 [2]
Stage 36	
Control	81 ± 9
Cut into 2	60 ± 7 [2]
Cut into 4	53 ± 6 [2]
Cut into 6	26 ± 8 [2]

*Ulnae were cultured on top of Millipore filter rafts for 3 days and were measured using a calibrated eyepiece graticule. Statistical analysis was carried out using Student's t-test. Treated ulnae were compared with their appropriate control only if an increase in length occurred.

1 = significant, $P < 0.05$. (increase) 2 = significant (decrease)

C H A P T E R F O U R

THE RELATIONSHIP BETWEEN CELL SHAPE AND CARTILAGE DIFFERENTIATION

Introduction

The expression of a cartilage phenotype by determined limb bud mesenchyme *in vitro* has been shown by several authors to be a density dependant phenomenon (Umansky, 1966; Caplan, 1970; Ahrens *et al*, 1977). If cells are plated out below confluence they become fibroblastic and fail to secrete a cartilaginous matrix, whereas cells plated at high densities remain rounded or polygonal, and secrete a metachromatic extracellular matrix (ECM) which stains positively with alcian blue at pH 1.0 (Lev and Spicer, 1964). One interpretation has been that a high cell density facilitates cell-cell contacts and it is these "histogenic interactions" which are responsible for chondrogenesis (Solursh, Ahrens and Reiter, 1978). It is also thought that the requirement of a high cell density *in vitro* may be similar to the increase in cell density (the prechondrogenic condensation) observed at the region of the presumptive cartilage rudiment *in vivo* (Gould *et al*, 1972; Thorogood and Hinchliffe, 1975).

An important observation has been that during the process of chondrogenesis, both *in vivo* and *in vitro*, presumptive chondroblasts are always present in a rounded cell shape (Gould *et al*, 1972; Thorogood and Hinchliffe, 1975; Solursh *et al*, 1978). In addition, cell shape has also been shown to play a fundemental role in the control of proliferation *in vitro* of a number of cell types (Folkman and Moscona, 1978), and a rounded cell shape has recently been shown to maintain phenotypic expression in already differentiated human chondrocytes (Glowacki, Trepman and Folkman, personal communication, now in print, 1983). The shape of cells in culture can be changed by altering the adhesivity of the substratum. Cells will readily attach, spread and flatten on an adhesive substratum whereas, on a less adhesive surface, cells attach less, spread little and take

up a rounded configuration. Folkman and his colleagues have used the plastic poly(2-hydroxyethyl methacrylate) - poly(HEMA) which, when coated onto petri-dishes at various concentrations, alters cell shape in a seqential manner (Folkman and Moscona, I978). At high concentrations it is non-adhesive to cells but on dilution with ethanol and subsequent polymerisation it gradually becomes more adhesive allowing cells to attach and exhibit various degrees of cell spreading. The mechanism of attachment in this system is unclear. However, the results obtained imply that cells with different morphologies in culture behave differently and that the in vitro shape of a cell may determine the phenotype expressed.

This chapter has two main aims -

i) to investigate the role of cell shape in the differentiation of early limb mesenchyme into cartilage in vitro, and

ii) to determine whether cells which have similar phenotypes but different morphologies in vivo, e.g. rounded, flattened and hypertrophic chondrocytes of long bone rudiments, also behave differently in vitro.

Materials and Methods

i) Cell cultures

a) Limb mesenchyme

Chick limb mesenchyme was obtained from wings of $3\frac{1}{2}$-4 day old embryos (stage 23/24), dissociated as in General Methods and plated out at 2×10^5 cells/35mm tissue culture dish. The cultures were maintained in Hams FI2 containing I0% foetal calf serum plus 5% chick serum.

b) Chondrocytes

Ulnae were removed from embryos aged 7/8 days and I6 days

(stage 32-34 and stage 42). Individual cell zones, rounded, flattened and hypertrophic, were isolated and dissociated as described in the General Methods. Cells were plated out in tissue culture dishes at high ($2xIO^5$ cells/IO μl drop), medium ($5xIO^4$ cells/IO μl drop) and low ($2xIO^5$ cells/35 mm dish) cell densities.

Chondrocytes from stage 32 Meckel's cartilage and wrist elements were also plated out, at the same densities, for comparison. Meckel's cartilage is composed almost totally of flattened chondrocytes whereas wrist elements contain mainly rounded chondrocytes.

All cultures were maintained in complete Hams FI2 medium with either foetal calf or chick serum for 7-IO days when they were fixed as described in the General Methods.

ii) Treatment of culture dishes

Stock solutions of poly(HEMA) were made up by dissolving 6g of poly(HEMA) powder (Hydron Labs. Inc., New Jersey) in 50 ml of 95% ethanol and gently rotating the mixture overnight at 37°C. The solution was clarified by centrifugation at 2500 r.p.m. for 30 min. and the supernatent was harvested. This I2% stock solution was diluted with 95% ethanol until dilutions of $6xIO^{-3}$ and $8xIO^{-3}$ were obtained. 35mm tissue culture dishes were then coated with 0.2ml of either of these dilutions and allowed to dry at 37°C for at least 2 days. Once dry, stage 23 limb mesenchyme or stage 32 rounded chondrocytes were plated onto the dishes.

iii) Incorporation of radioisotopes

Medium was removed from limb mesenchyme cultures after 2 days and replaced by fresh medium containing either ^{3}H-thymidine (2 μCi/ml) or $Na_2\,^{35}SO_4$ (2 uCi/ml) for I6 hours. After the labelling period cultures were processed for incorporation of isotope as described in the General Methods. Radioisotope incorporation was

determined for both normal tissue culture plastic and poly(HEMA) coated dishes.

Results

I Cell shape and cartilage differentiation.

i) Cell shape

When stage 23 limb mesenchyme cells were plated out (at $2xIO^{5}$ cells/dish) onto normal tissue culture dishes, many cells became flattened and fibroblastic by I day of culture (Fig. I). Cells plated onto dishes coated with a $6xIO^{-3}$ dilution of poly(HEMA), attached but only partially spread (Fig. 2). At the slightly higher concentration of $8xIO^{-3}$ poly(HEMA), the cells attached but remained rounded (Fig. 3). It must be noted that in all cases, on normal tissue culture plastic and on poly(HEMA), many cells failed to attach at all.

ii) ^{3}H-thymidine incorporation

When the degree of cell division was assessed, by ^{3}H-thymidine incorporation, it was found that rounded cells incorporated much less label than flattened cells. Cells grown on $6xIO^{-3}$ poly(HEMA) and $8xIO^{-3}$ poly(HEMA) exhibited a 46% and 57% reduction respectively in the amount of ^{3}H-thymidine incorporated when compared with cells grown on normal tissue culture plastic (Fig. 4).

iii) Production of sulphated glycosaminoglycans.

The amount of sulphated GAG's produced by the cells, under the various conditions, had to be evaluated in 2 ways,

a) those deposited as an extracellular matrix and

b) those released into the culture medium in a soluble form.

a) Cells grown on poly (HEMA) were found to produce more sulphur-containing ECM than cells grown on tissue culture plastic - the

greater the concentration of poly (HEMA), i.e. the rounder the cell, the more sulphated matrix produced (Fig. 5).

b) Cells grown on poly (HEMA) were also found to secrete more cetylpyridinium chloride-precipitable $^{35}SO_4$ into the medium than cells grown on tissue culture plastic (Fig. 6).

Occasionally, rounded chondrocytes from stage 32 epiphyses were also grown on poly (HEMA). Preliminary findings were that growing chondrocytes on poly(HEMA) resulted in a larger and more deeply stained halo around the cells when stained with alcian blue (pH I.0) and toluidine blue. This suggested that maintaining the already differentiated chondrocytes in a rounded configuration also increased the amount of sulphated ECM produced, but since this result was very similar to that obtained by Glowacki et al (I983) the experiment was not taken further.

2 Chondrocyte cell culture

i) Stage 32-34 cells

a) Rounded (epiphyseal) cells

After 24 hours high density cell cultures of rounded chondrocytes produced a central sheet of cartilage surrounded by layers of fibroblasts at the periphery (Figs. 7 and 8). The central sheet consisted of tightly packed, rounded cells separated by a refractile ECM which in histological section stained metachromatically with toluidine blue (Fig. 9). During a 7 day culture period the size of the central cartilage mass did not increase much but the layers of peripheral fibroblasts did expand greatly in a radial fashion. Clusters, or nodules, of chondrocytes were occasionally found amongst the fibroblastic outgrowths closest to the central mass (Fig. I0).

When plated at medium density the cells formed areas of cartilage (cartilage nodules) interspaced by fibroblast-like cells (Fig. II)

by 3 days of culture. The appearance of these cultures hardly changed during the culture period.

Low density cultures contained rounded cells during the first day of culture (Fig. I2) but these rapidly became fibroblastic by 3-4 days so that by 7 days the entire culture was fibroblastic (Fig. I3). Occasionally, giant cells were found amongst the fibroblasts (Fig. I4), especially in areas of low cell density, but the nature of these cells is unknown.

Foetal calf serum was always used in subsequent experiments because cells, particularly at low density, were found to contain large vacuoles if grown in I0% chick serum (data not shown). The nature of these vacuoles is unknown but they may be full of lipid droplets as observed in other systems - see Discussion.

b) Flattened cells

By I-2 days in vitro, high density cultures of flattened cells produced a sheet of rounded/polygonal cells interspaced by a refractile ECM (Fig. I5). The peripheral cells of these cultures were also polygonal (Fig. I6) and no fibroblast-like cells were observed until 4-5 days when a few began to appear (Fig. I7).

The behaviour of cells in medium and low density cultures was similar to those in high density culture in that the cells remained polygonal, even if totally isolated (Fig. I8). Once again fibroblast-like cells did not appear until at least 4 days. Giant cells, similar to those in low density rounded cell cultures, also appeared at this time.

c) Hypertrophic cells

Very few viable hypertrophic cells were obtained from young ulnae, therefore, only medium and low cell density cultures were set up. In both cases the cells remained large and polygonal

throughout the culture period (Fig. I9). Individual hypertrophic cells were different in that some were surrounded by a refractile ECM whilst others were not, also, some cells contained small vacuoles (Fig. 20). No fibroblasts were present after 7 days. Occasionally giant cells were found, but the most remarkable observation was that small groups of hypertrophic cells grew into large groups of hypertrophic cells during the culture period (Fig. 2I), i.e. hypertrophic cells were dividing and giving rise to more hypertrophic cells.

d) Meckel's chondrocytes

Cells from Meckel's cartilage behaved in a similar manner to flattened cells no matter what density the cells were plated out at (Fig. 22).

e) Wrist chondrocytes

Cells from wrist elements behaved in a similar manner to rounded epiphyseal cells no matter what density the cells were plated out at (Fig. 23), however, no giant cells were observed even after I0 days of culture.

ii) Stage 42 cells

On the whole, all 3 types of long bone chondrocytes behaved similarly to the equivalent cell type from stage 32 ulnae (Fig. 24) but some differences were observed. Fibroblast-like cells were not observed in rounded cell cultures until 2-3 days, nor in flattened cell cultures until 6-7 days. Also, very few giant cells were observed.

Discussion

The general conclusion from the results presented in this chapter is that the expression of a cartilage phenotype *in vitro*

is largely dependant on the morphology of the cell in culture. If a differentiated chondrocyte, or a determined mesenchyme cell, is maintained in a rounded configuration, whether by plating at a high cell density or by plating onto poly(HEMA), it will secrete what appears to be a cartilage ECM. (Since an assay for cartilage specific type II collagen has not been performed it is not possible to state that the matrix produced is definitely cartilage). However, if the cell is allowed to spread and become fibroblastic it will lose, or not attain, the cartilage phenotype. These results are consistent with the findings of several authors, e.g. Umansky (1966), Caplan (1970), Muller et al (1977), Solursh et al (1982), Glowacki et al (1983) and have already been published in part - Archer, Rooney and Wolpert (1982).

Studies on differentiated chondrocytes suggest that each type of chondrocytes within a long bone rudiment essentially behaves in a similar manner (Levenson, 1969). As has already been mentioned, the shape of a chondrocyte in vitro is determined by the initial plating density, therefore, what role, if any, does chondrocyte morphology play in vivo? Chondrocyte morphology in vivo can be regarded simply as a reflection of the stage of maturation attained by the cell. The results presented here show that the stage of maturation attained by a chondrocyte in vivo determines the timing of dedifferentiation in vitro. Therefore, the morphology of a chondrocyte in vivo determines at what time, if at all, that cell will dedifferentiate in vitro. It should be noted that, under the inverted microscope, dedifferentiation can only be observed at the periphery of a high density culture (Fig. 7 and 8) which suggest that dedifferentiation will only occur if a chondrocyte is allowed to flatten and assume a fibroblast-like morphology. If this is so

then dedifferentiation should occur anywhere throughout low cell density cultures (Fig. I3).

When plated at low density, the least mature chondrocyte, the rounded cell, dedifferentiates during the first I-2 days (Fig. I3) whereas chondrocytes from the flattened cell population take at least 4 days (Fig. I8) and the most mature chondrocytes, the hypertrophic cells, do not dedifferentiate at all (Fig. I9). These times are similar to the time-course of dedifferentiation, of chondrocytes derived from embryonic mouse long bone rudiments, presented by Grundmann et al (I980), however, these authors state that mouse hypertrophic cells die off by 4 days of culture and this is clearly not the case in chick hypertrophic cells (Fig. I9). In fact, chick embryo hypertrophic cells were found to survive and divide for atleast 7 days (Fig. 2I) - as has also been reported by Levenson (I969).

It is interesting to note that chondrocytes from Meckel's cartilage, which are primarily flattened in vivo, behaved identically to flattened cells from long bone rudiments when grown in vitro (Figs. I8 and 22). The growth characteristics of Meckel's chondrocytes outlined here are similar to those reported by Levenson (I969).

Similarly, wrist cells, which closely resemble long bone rudiment rounded cells, were found to behave almost identically to epiphyseal cells in vitro (e.g. Figs. 7 and 23). The only difference noted between these cultures was that wrist cells failed to produce giant cells. It is tempting to associate the production of giant cells by rounded and flattened cells in vitro (Fig. I4) with the process of maturation in vivo, whereby large, i.e. hypertrophic, cells are produced. Hypertrophic cells in vitro also form giant

cells but whether this is due to the lack of an *in vivo* growth constraint allowing cells to continue increasing in size or is due to some property of the culture system is not known. Cultures were not maintained for longer than I0 days in these experiments simply because the important morphogenetic events observed in long bone rudiments *in vivo* occur within a short time period.

The observation that chick cells grow abnormally in medium containing certain types of sera appears to be widespread, e.g. chick cells cannot be grown in medium containing mouse serum (they produce many lipid vacuoles), therefore, horse or foetal calf serum is normally used instead (Parker *et al*, I980).

Two major points concerning chondrogenesis, *in vitro* and *in vivo*, arise from the results using poly(HEMA) coated dishes :-

i) "Histogenic interactions" as proposed by Solursh *et al* (I978), may not be a prerequisite for chondrogenesis *in vitro*. Individual mesenchyme cells, when maintained in a rounded configuration, secrete sulphated ECM without coming into contact with any other cell (Figs. 3, 5 and 6). This result has already been published (Archer *et al*, I982) and a similar conclusion has recently been reached independantly by Solursh *et al* (I982), who also found that the initiation of cartilage specific type II collagen is also dependant on cell shape.

ii) The observation that when pre-chondrogenic mesenchyme cells flatten they become fibroblastic and secrete less sulphated ECM then rounded cells (Figs. 5 and 6) may help to explain why perichondrial cells, which initially were part of the condensation (see chapter 3), do not secrete cartilage matrix. Gould *et al* (I974) have shown that, during the initial stages of matrix secretion, centrifugal forces flatten the outer cells of the condensation

to form, eventually, the perichondrium. Thus the flattening and elongation of the cell may switch off the expression of the cartilage phenotype and instead induce the secretion of type I and type III collagens. This phenomenon has also been shown to occur in mature chondrocyte cultures where peripheral cells are fibroblast-like and secrete collagen type I (Muller et al, 1977), and this presumably is what is occuring during the chondrocyte dedifferentiation process mentioned above. Also, cells which spread on tissue culture plastic show a higher degree of ^{3}H-thymidine incorporation (Fig. 4, also see Folkman and Moscona, 1978) and this is reflected in the fact that perichondrial cells have a higher labelling index than chondrocytes (see chapter 6).

If the hypothesis that rounded cells promote high matrix secretion but low cell division, and flattened cells show the reverse situation is extended to cover differentiation in vivo then problems arise. An apparent anomaly arises in that chondrocytes in the flattened cell region secrete much matrix (and may be important in longitudinal growth - chapter 2) and have a lower mitotic index than rounded cells (chapter 1). However, if flattened chondrocytes are looked at in both longitudinal and transverse sections, it is found that they are in fact disc-shaped and are not similar to fibroblast-like cells. Also, chondrocytes in vivo are held under pressure and this may play some role in the proliferation of these cells (Rodan et al, 1975). It must be noted, therefore, that flattened chondrocytes in vivo are totally different from flattened cells in vitro and that chondrogenesis and differentiation in vitro may differ considerably from chondrogenesis and differentation in vivo. (This topic will be covered in more detail in the following chapter).

The finding that the maintenance of a rounded cell shape in vitro

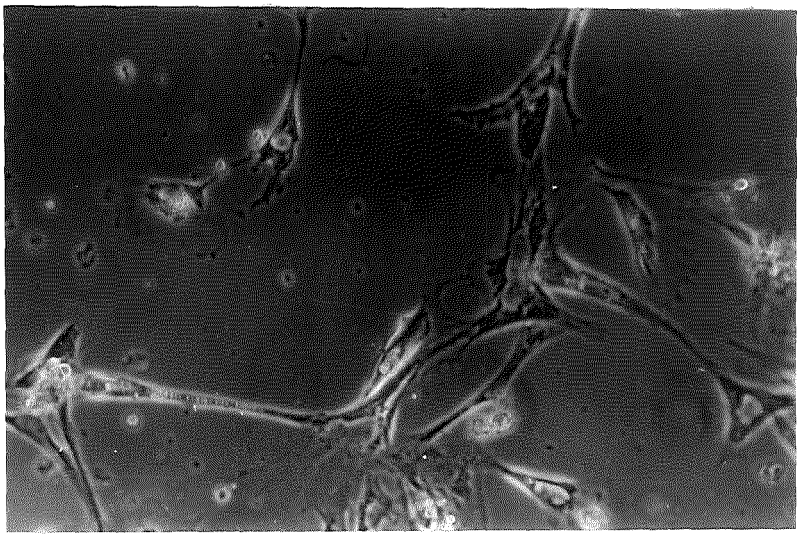
is conducive to matrix secretion but diminishes cell division has been shown by several authors, but the conclusion has not always been the same, e.g. Bjornsson and Heinegard (I98I) and Rifas *et al* (I982) both showed that chondrocytes grown in the absence of serum secreted sulphated ECM but did not divide. Bjornsson and Heinegard concluded that glycosaminoglycan synthesis did not require serum but DNA synthesis did, whilst Rifas *et al* concluded that they had specially selected a population of pure chondrocytes. However, both these observations can be explained by the fact that cells grown in the absence of serum attach but do not spread (just as on poly(HEMA)) and it may be that the shape of the cell was the controlling factor in the results obtained.

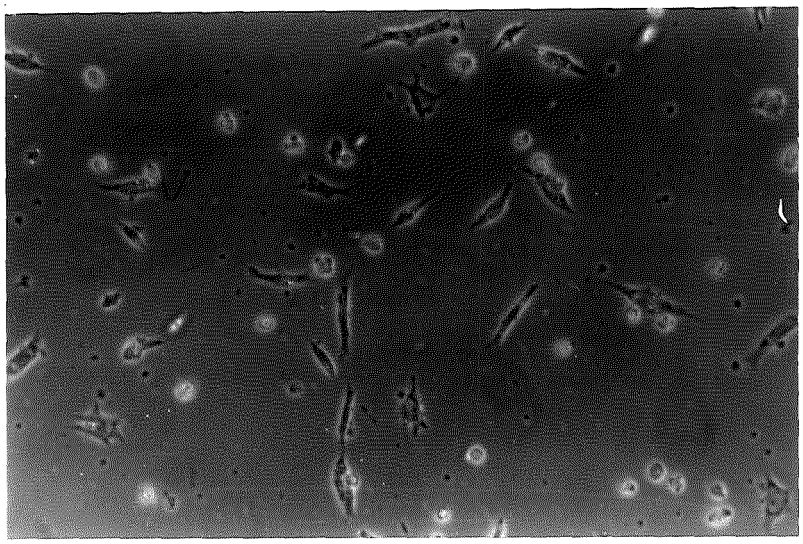
In conclusion, *in vitro* culture of chondrocytes with different morphologies *in vivo* has shown that each type of cell behaves quite similarly to each other in culture and that the *in vivo* morphology only controls the timing of dedifferentiation. Also, if a differentiated chondrocyte, or an undifferentiated mesenchyme cell, is maintained in a rounded cell configuration, the production of sulphated ECM, and thus presumably cartilage matrix, is increased. This increase is observed even when cells are cultured at low densities so that cell-cell contacts do not occur, therefore, histogenic interactions may not be a prerequisite for cartilage differentiation.

Fig. 1. Stage 23 limb mesenchyme cells on normal tissue culture plastic. The cells appear very elongated and typically fibroblastic. Living culture - phase contrast. Mag. x 200.

Fig. 2. Stage 23 limb mesenchyme cells on a $6xI0^{-3}$ dilution of poly(HEMA). The cells appear slightly elongated and bipolar. Living culture - phase contrast. Mag. x 200.

Fig. 3. Stage 23 limb mesenchyme cells on a $8xI0^{-3}$ dilution of poly(HEMA). The cells have attached but have remained round and have not spread.
Living culture - phase contrast. Mag. x 200.





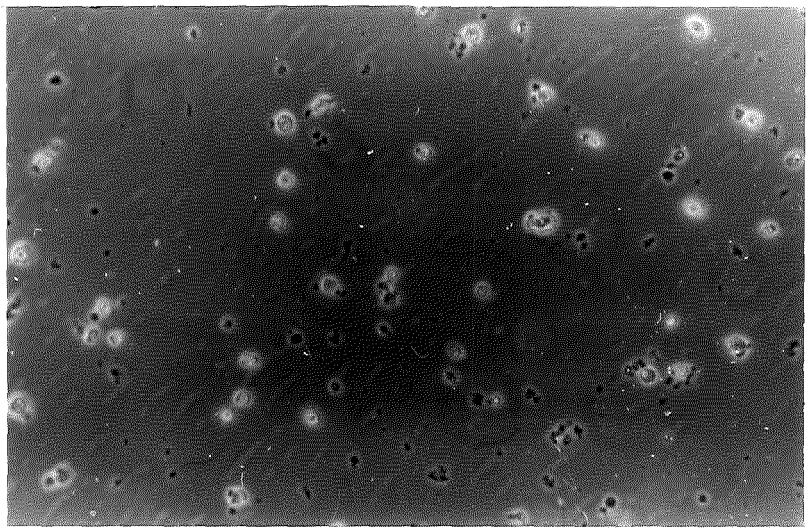
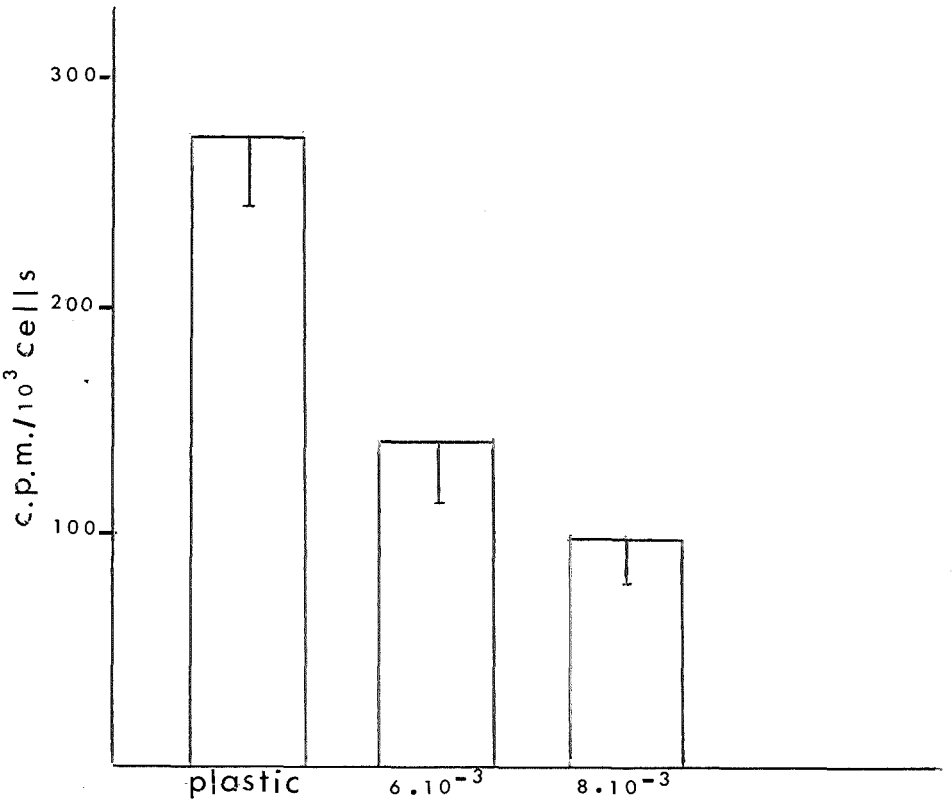


Fig. 4. The incorporation of ^{3}H-thymidine into limb mesenchyme cells grown on plastic and poly(HEMA) after 64 hours in culture. t-Test analysis shows significant differences between thymidine incorporation into cells grown on plastic, poly(HEMA) 6×10^{-3} ($P < 0.05$) and poly(HEMA) 8×10^{-3} ($P < 0.02$).

Fig. 5. The incorporation of $^{35}SO_4^{2-}$ into hyaluronidase-sensitive material deposited by limb mesenchyme cells after 64 hours in vitro. Cells plated on poly(HEMA) showed a 13% and 112% increase in labelled material deposited over cells grown on plastic, but only those plated on poly(HEMA) 8×10^{-3} showed a significant difference after t-test analysis ($P < 0.02$).


300
200
100
c.p.m./10^3 cells
plastic
6.10^{-3}
8.10^{-3}

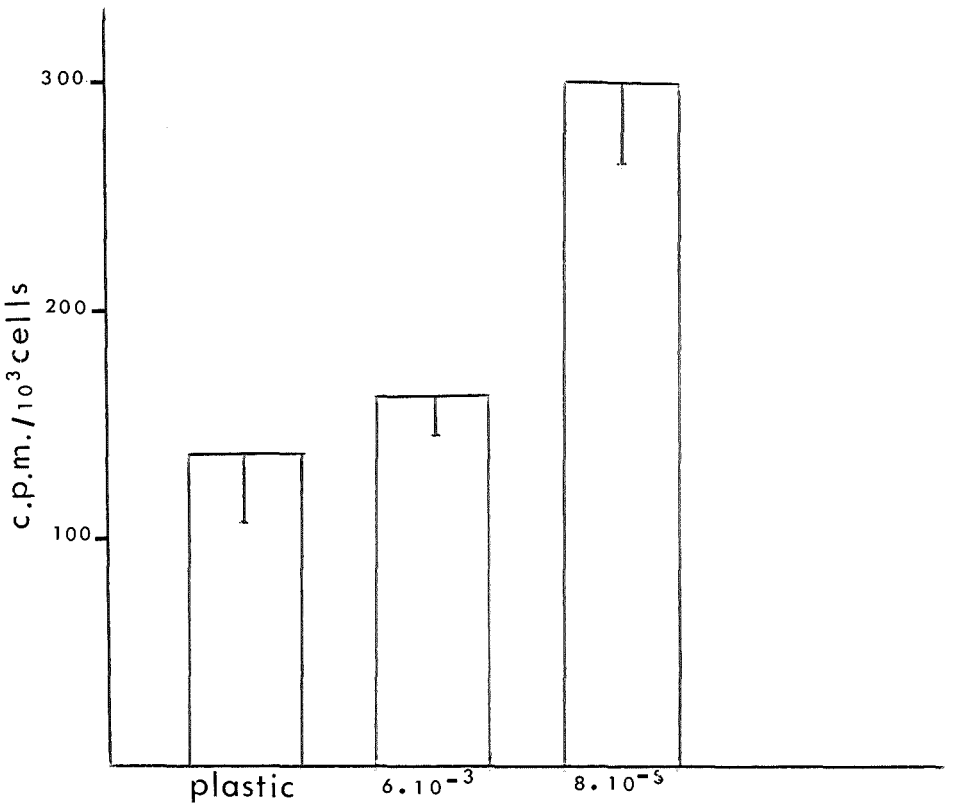
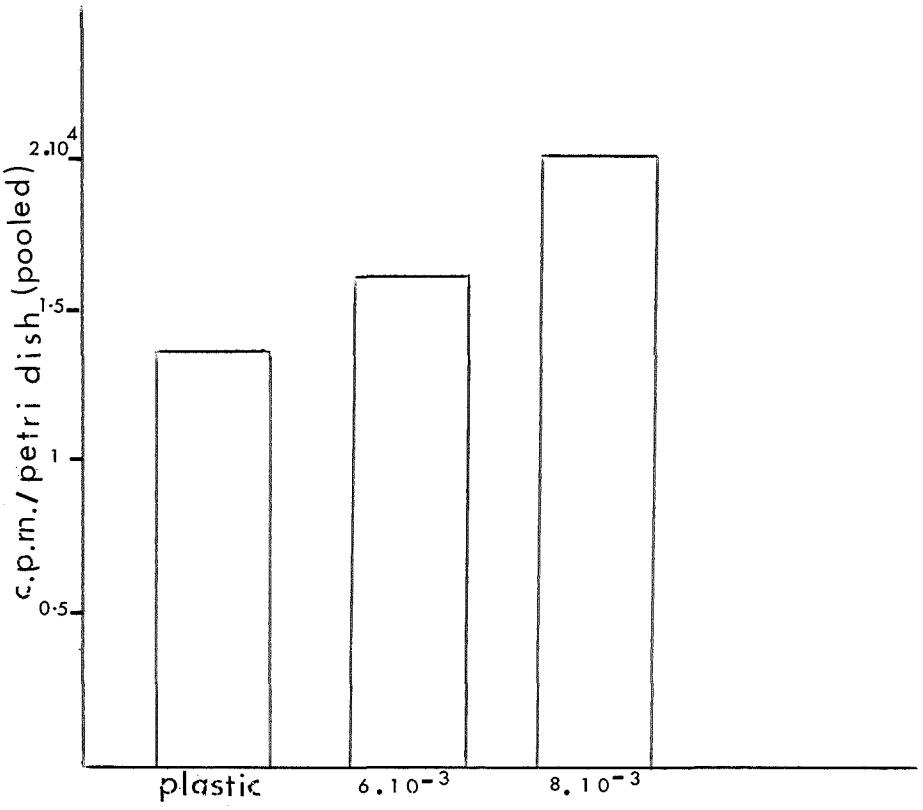

300
200
100
c.p.m./10^3 cells
plastic
6.10^{-3}
8.10^{-3}

Fig. 6. Cetylpyridinium chloride-precipitable $^{35}SO_4^{2-}$ in the culture medium from limb mesenchyme cells after 64 hours *in vitro*. In the analysis, medium from three Petri dishes in each experimental group was pooled and a 1ml sample from each group analysed. Again, cells grown on poly(HEMA) showed an increase in precipitable material (19% and 48%) over mesoblasts cultured on plastic.

Fig. 7. Stage 32 rounded chondrocytes, centre of 1 day old high density culture. The cells appear rounded, tightly packed and separated by a refractile ECM.
Living culture - phase contrast. Mag. x 125.


c.p.m./petri dish (pooled)
2.10^4
1·5
1
0·5
plastic
6.10^-3
8.10^-3

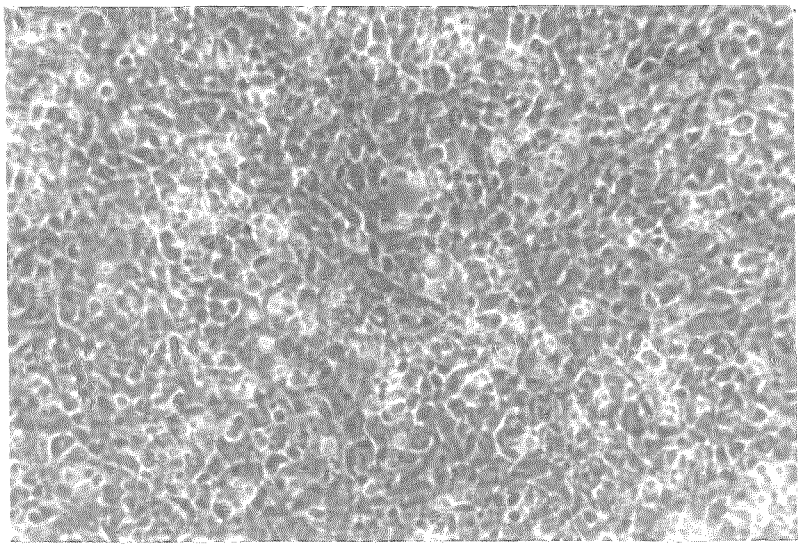
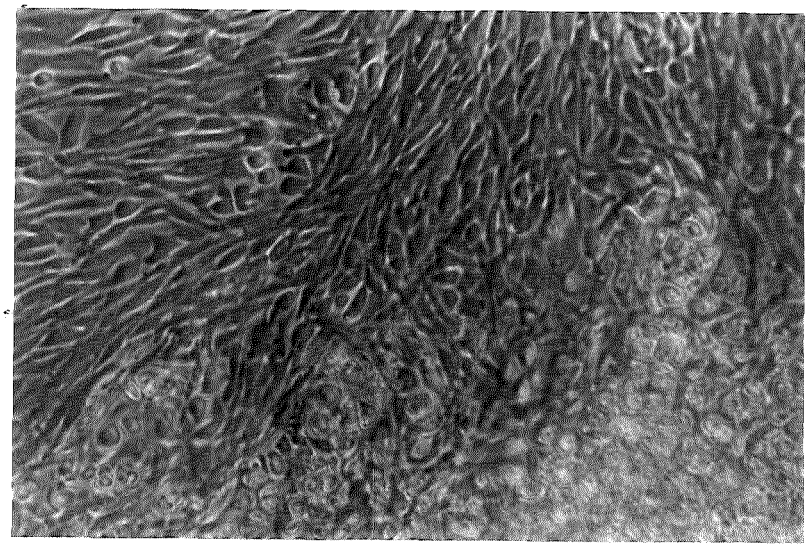
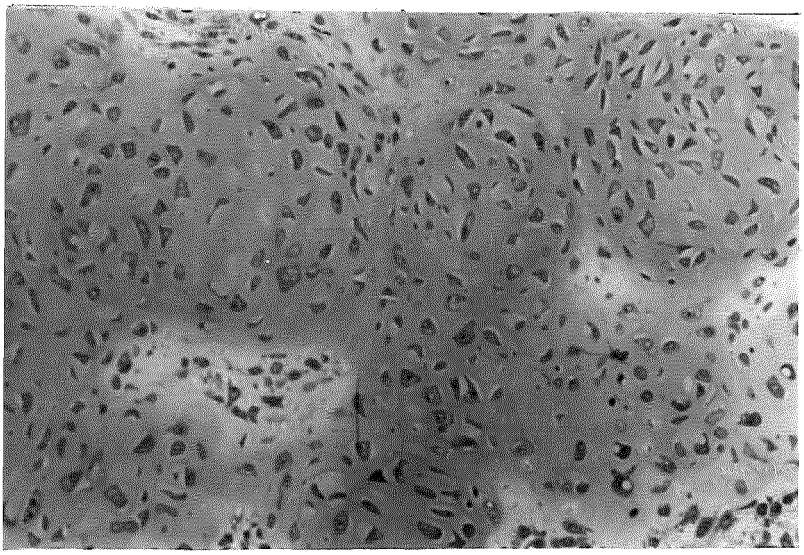


Fig. 8. Stage 32 rounded chondrocytes, periphery of a I day old high density culture. Rounded cells appear to be surrounded by layers of fibroblast-like cells growing out in a radial direction.
Living culture - phase contrast. Mag. x I25.

Fig. 9. Stage 32 rounded chondrocytes, centre of 3 day old high density culture. The cells appear as typical chondrocytes separated by a metachromatic ECM.
Tol. blue stain. Mag. x I25.

Fig. I0. Stage 32 rounded chondrocytes, periphery of 7 day old high density culture. A group of cells separated by a refractile ECM, a cartilage nodule, appears to be surrounded by fibroblast-like cells. Note that the fibroblastic cells are aligned circumferentially around the nodule.
Living culture - phase contrast. Mag. x 250.





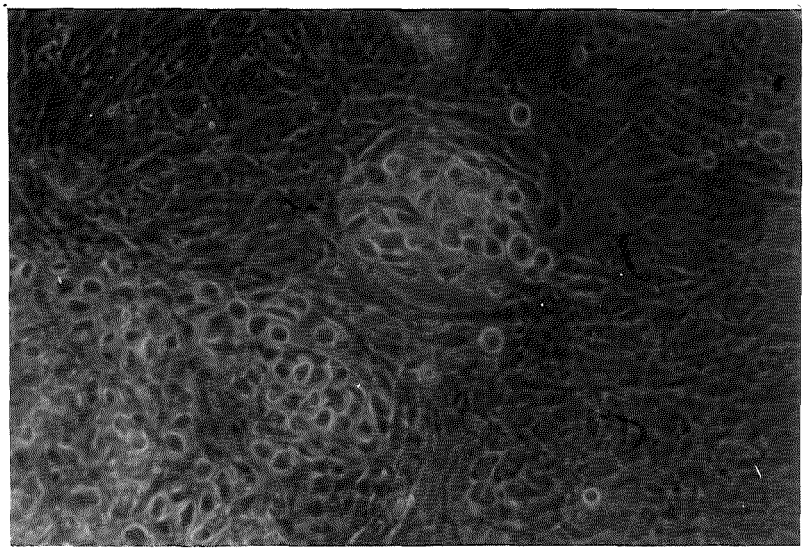
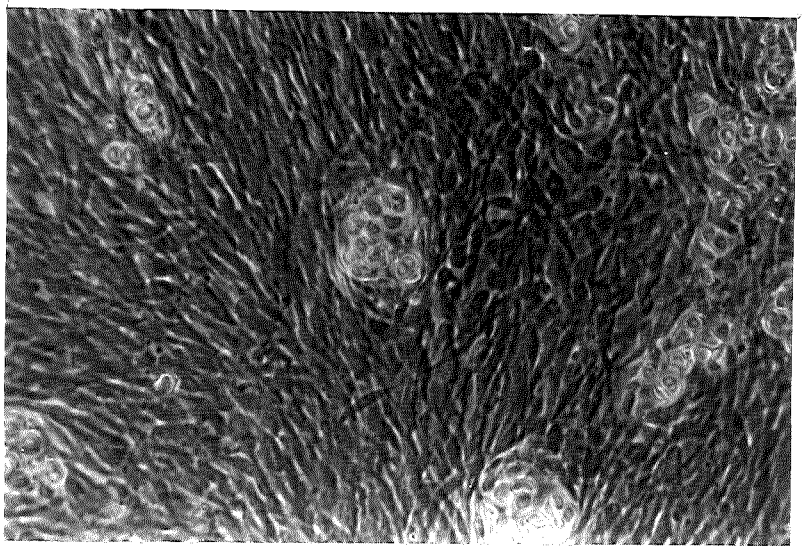
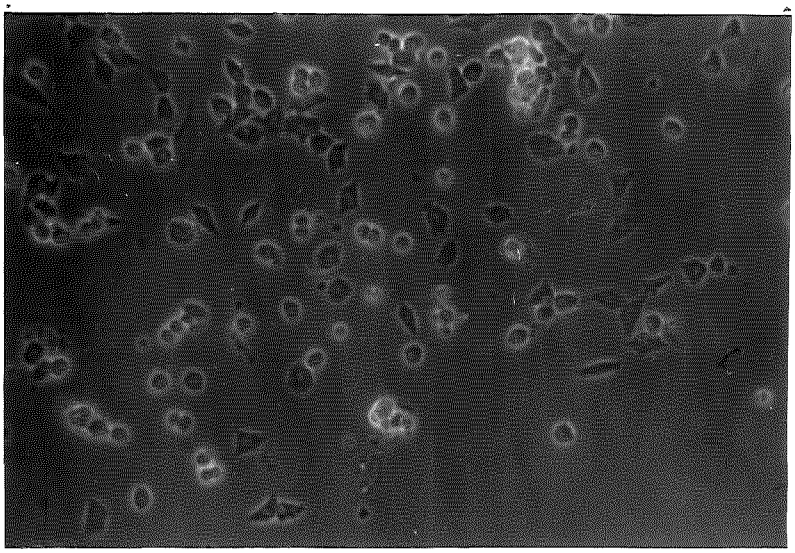


Fig. 11. Stage 32 rounded chondrocytes, 3 day old medium density culture. The culture is composed of many cartilage nodules interspaced by fibroblastic cells.
Living culture - phase contrast. Mag. x 125.

Fig. 12. Stage 32 rounded chondrocytes, 1 day old low density culture. The cells appear separated and round in shape but no refractile ECM. can be observed.
Living culture - phase contrast. Mag. x 250.

Fig. 13. Stage 32 rounded chondrocytes, 7 day old low density culture. Almost every cell has become elongated and fibroblastic.
Living culture - phase contrast. Mag. x 250.





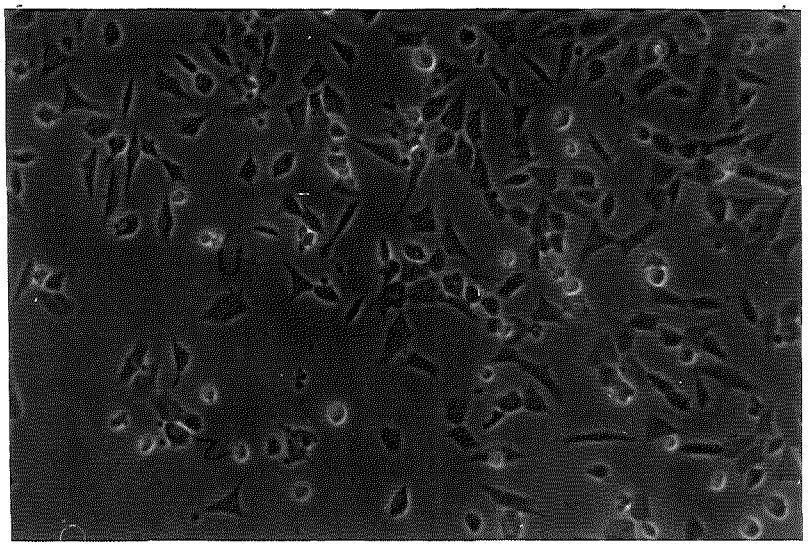
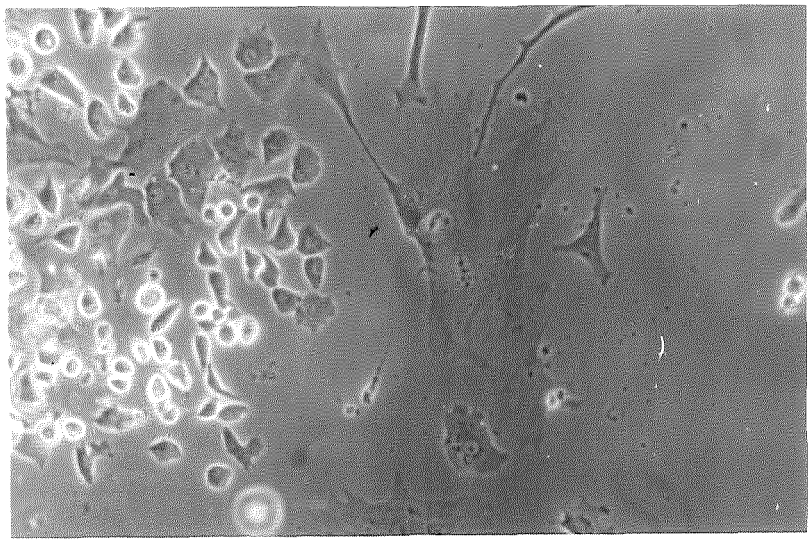
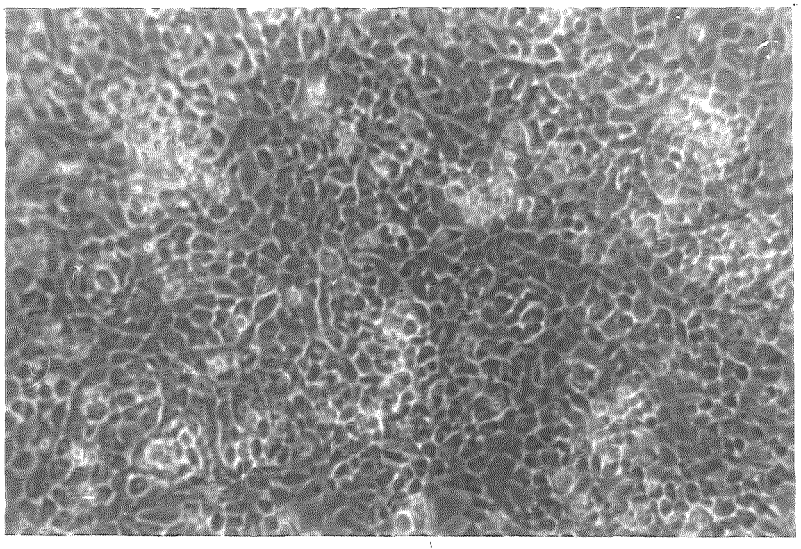


Fig. I4. Stage 32 rounded chondrocytes, 7 day old low density culture. Occasionally giant cells can be seen intermingled with the fibroblasts. These cells do not appear for at least 4-5 days of culture.
Living culture - phase contrast. Mag. x 250.

Fig. I5. Stage 32, flattened chondrocytes, centre of 2 day old high density culture. The cells appear rounded, tightly packed and separated by a refractile ECM.
Living culture - phase contrast. Mag. x I25.

Fig. I6. Stage 32 flattened chondrocytes, periphery of 2 day old high density culture. The cells are no longer tightly packed but they still retain their rounded shape and occasionally ECM can be observed.
Living culture - phase contrast. Mag. x I25.





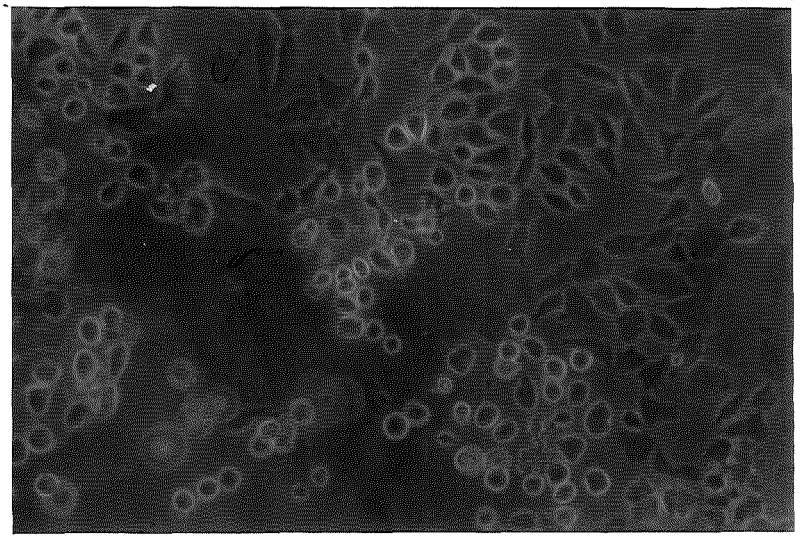
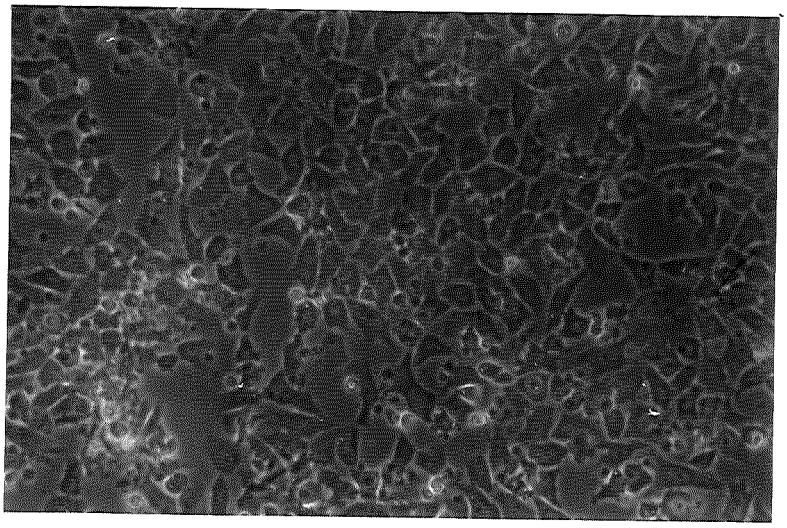
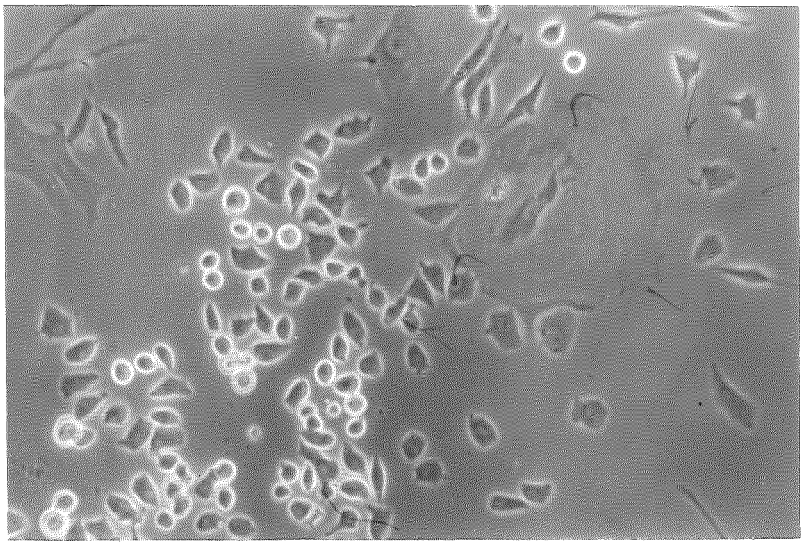


Fig. I7. Stage 32 flattened chondrocytes, periphery of 5 day old high density culture. Many of the cells have retained their rounded cell shape but a few fibroblast-like cells have now appeared. Living culture - phase contrast. Mag. x I25.

Fig. I8. Stage 32 flattened chondrocytes, 3 day old low density culture. Even when totally isolated the cells retain their rounded shape and appear to be capable of dividing to produce new cells retaining the rounded cell shape.
Living culture - phase contrast. Mag. x 250.

Fig. I9. Stage 32 hypertrophic chondrocytes, 3 day old low density culture. The cells have retained their polygonal shape even when not in contact with other cells.
Living culture - phase contrast. Mag. x I25.





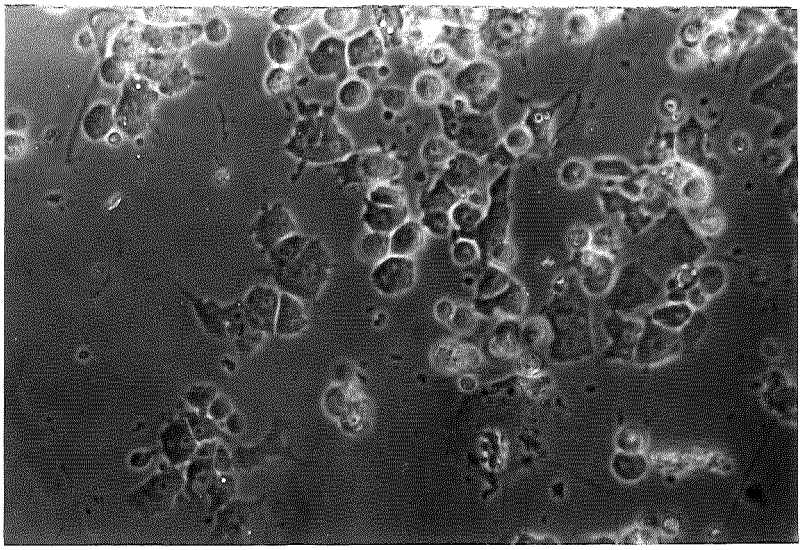
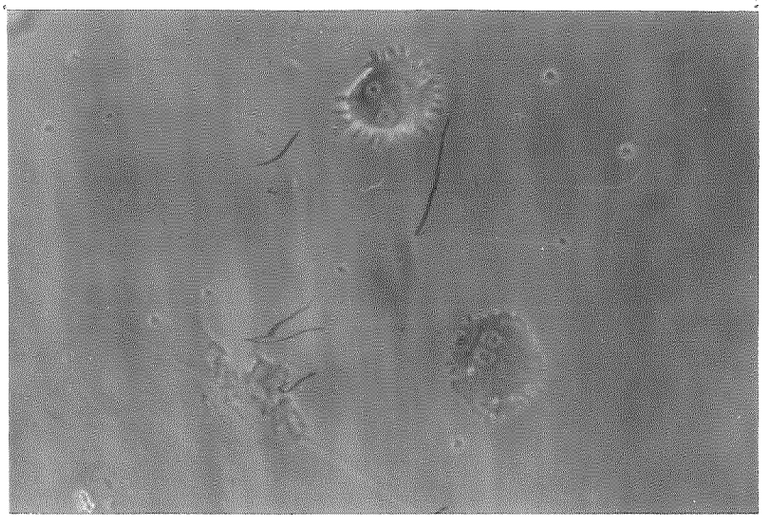
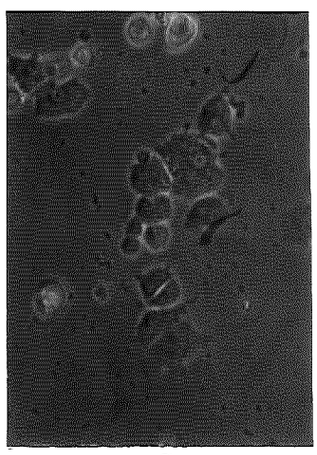


Fig. 20. Stage 32 hypertrophic chondrocytes, 3 day old low density culture. Occasionally hypertrophic cells appeared which contained vacuoles.
Living culture - phase contrast. Mag. x 250.

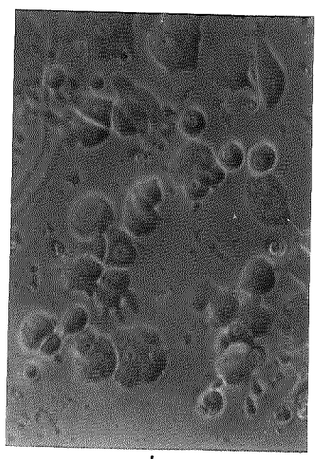
Fig. 21. Stage 32 hypertrophic chondrocytes. During the culture period, small colonies of cells were found to grow into larger colonies suggesting that the hypertrophic cells were dividing.
a = 3 day culture, b = 7 day culture.
Living culture - phase contrast. Mag. x 125.

Fig. 22. Stage 32, Meckel's chondrocytes, periphery of 2 day old high density culture. Some cells are no longer tightly packed but they remain rounded. This observation is identical to that in flattened chondrocyte cultures - see Fig. 16.
Living culture - phase contrast. Mag. x 125.





a



b

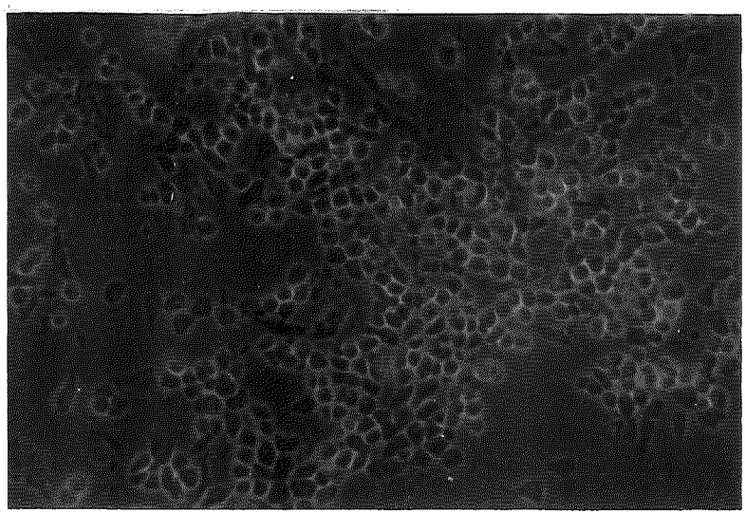
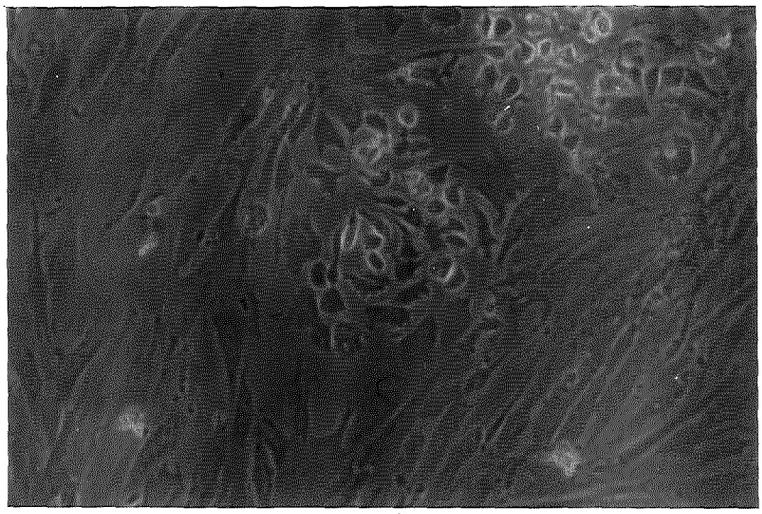
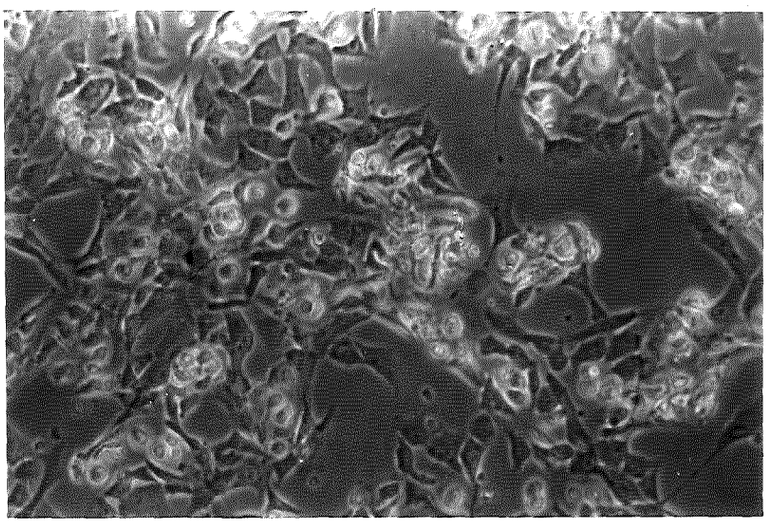


Fig. 23. Stage 32 wrist chondrocytes, periphery of 3 day old high density culture. Occasionally cartilage nodules can be observed intermingled with fibroblasts at the edges of a tightly packed sheet of polygonal cells. This observation is identical to that found in rounded chondrocyte cultures - see Fig. 10. Living culture - phase contrast. Mag. x 125.

Fig. 24. Stage 42 rounded chondrocytes, periphery of a 5 day old low density culture. Some cells have become fibroblastic but the majority have remained polygonal and are surrounded by a refractile PCM. In a similarly aged stage 32 culture most of the cells would be fibroblastic by this time, this difference reflects the increase in time required by older cells to dedifferentiate. Living culture - phase contrast. Mag. x 125.





C H A P T E R F I V E

NON-SPECIFIC MORPHOGENESIS OF CARTILAGE *IN VITRO*

Introduction

Cartilage elements in an embryo are present in several forms which range from sheets in the sclera through the rod-like form of Meckel's cartilage to the rudiments of the future long bones of the limb and the complex 3-dimensional structures of the vertebrae prior to ossification.

Several investigators have found that, under appropriate conditions, committed chondrogenic tissue can be removed from an embryo and grown in non-chondrogenic sites, or in culture to produce an element similar in shape to that which would have formed *in vivo* (Murray and Huxley, 1925; Fell, 1956; Weiss and Moscona, 1958 and Iten and Murphy, 1980). These results have given rise to the phenomenon of "tissue-specific" morphogenesis. The shapes which form are not absolutely identical to those *in vivo* and most authors agree that a balance must exist between intrinsic (genetic) and extrinsic (local environmental) factors within the embryo, however, Weiss and Moscona (1958) suggest that morphogenesis is due solely to intrinsic properties.

These authors took dissociated chick embryo mesenchyme from two separate sources which, *in vivo*, give rise to cartilage of very different morphologies, and cultured them at high density *in vitro*. Periocular mesenchyme gave rise to flat sheets of cartilage similar to the sheet of scleral cartilage which surrounds the retina *in vivo*. In contrast, limb mesenchyme gave rise to whorls of cartilage *in vitro* which they considered analogous to the initial cartilage formation in long bone rudiments.

The experiments of Weiss and Moscona have been repeated and expanded here using the micromass technique of Ahrens *et al* (1977), however, the results here have not been interpretated as "tissue-specific"

morphogenesis but rather in terms of density-dependant morphogenesis at the time of plating. In addition, the cartilage form was found to be highly dependant on the presence of non-chondrogenic cells.

Materials and Methods

Culture

Limb mesenchyme was obtained from the wing and leg buds of stage 23 or 24 embryos. Whole limb buds were dissociated by trypsin digestion (0.1% in PBS for 10 minutes at 37°C) with frequent agitation. Any cell clumps which remained were broken up by gentle aspiration with a pipette.

Periocular mesenchyme was obtained from the developing eyes of 7 day old chick, or quail, embryos as described by Weiss and Moscona. Briefly, the eyes were removed to sterile PBS, punctured and left for approximately 15 minutes. This latter proceedure facilitated the separation of the periocular mesenchyme which was then dissected free with forceps and dissociated as described above but for 20 minutes.

Chick heart fibroblasts were also harvested. Hearts were shredded into small pieces and trypsinised for 15 minutes with frequent agitation.

Mature epiphyseal and scleral chondrocytes were obtained from 8 and 9 day old embryos and dissociated in trypsin and collagenase as in the General methods.

Each type of cell was inoculated at medium and high density in a manner similar to the Micromass technique described by Ahrens et al (1977) - see General methods (medium density cultures were plated out at 5×10^4 cell/10 ul drop, i.e. 5×10^6 cells/ml; high density cultures were plated out at 2×10^5 cells/10 ul drop, i.e. 2×10^7 cells/ml - in each case cells were plated at 4 drops/dish).

All cells were plated out in complete Hams F-12 medium in the following combinations (All cells were chick unless otherwise stated):

High density scleral mesenchyme

Medium density scleral mesenchyme

High density limb mesenchyme

Medium density limb mesenchyme

High density scleral mesenchyme/chick heart fibroblasts (3:1)

High density limb mesenchyme/chick heart fibroblasts (3:1)

High density limb mesenchyme/quail scleral mesenchyme (1:1)

High density epiphyseal chondrocytes

Medium density epiphyseal chondrocytes

High density scleral chondrocytes

Medium density scleral chondrocytes

In all cases a minimum of 15 cultures were analysed for each combination after 4 or 5 days of culture.

Microscopy and Histology

Cultures were examined daily and photographed using an inverted microscope. Samples were fixed and stained with alcian blue as described in General methods. Thick (2 µm) araldite sections were cut and stained with toluidine blue.

Immunofluorescence

Cartilage specific type II collagen was localised in cell cultures by indirect immunofluorescence (von der Mark et al, 1976). Rabbit antiserum to chick type II collagen was kindly donated by Dr. G. Shellswell, ARC Meat Research Institute, Long Ashton, Bristol.

For extracellular matrix (ECM), cultures were washed 3 times in PBS prior to the application of antibody solutions. Intracellular

localisation of type II collagen synthesising cells was performed by prior fixation of cultures in buffered 2% formal saline. Cultures were incubated with antiserum (immunoabsorbed rabbit anti-chicken II) at room temperature (30 minutes), washed 3 times in PBS and overlaid with fluorescein isothiocyanate (FITC) - conjugated goat anti-rabbit globulin (Wellcome labs, England) (30 minutes) at a 1:15 dilution. After 2 further washes in PBS, the cultures were mounted in glycerol and viewed with a Zeiss photomicroscope II equipped with a U.V. Light source for fluorescence microscopy.

Results

1) Scleral mesenchyme

At a high density ($2x10^5$ cells/drop) scleral mesenchyme formed a homogenous sheet of polygonally-shaped cells many of which showed evidence of a refractive ECM after 2 days in culture (Fig. 1). Histologically it was observed that most of the cells were polymorphic and surrounded by a faintly staining ECM with outgrowths of fibroblast-like cells at the periphery of the cultures (Fig. 2). Apart from the outgrowths, both the cells and the ECM in these cultures stained positive with the antibody to type II collagen (Fig. 3).

Medium density cultures ($5x10^4$ cells/drop) behaved, initially, in a similar manner to high density cultures. However, discrete islands, or nodules, of cartilage separated by fibroblastic cells were apparant after 4 days (Fig. 4). Unlike the cartilage nodules, the fibroblastic regions showed no evidence of intra or extracellular fluorescence when stained with collagen type II antibody (not shown).

2) Limb mesenchyme

The behaviour of high density limb mesenchyme cultures has been

extensively covered by Solursh and his colleagues (see review by Solursh, I980). Initially (I-2 days) the cells formed concentric aggregates with the centre of most aggregates acting as a focus for cartilage formation which was detectable, by alcian blue and toluidine blue staining, at 3 days (Fig. 5). The foci of cartilage enlarged to form whorled nodules which by day 5 had begun to coalesce (Fig. 6). Immunofluorescent labelling to type II collagen was found predominately within the nodular regions (Fig. 7) and is consistent with the findings of Solursh (I982).

In contrast, medium density limb mesenchyme cultures rarely became chondrogenic. Numerous fibroblasts were present by 2 days and these rapidly overgrew the entire culture (Fig. 8). Some small areas remained polymorphic but these did not exhibit a refractile ECM and, invariably the whole culture was fibroblastic after 5 days in vitro. No marked fluorescence of type II collagen was observed (not shown). In addition, these cultures showed a complete absence of metachromasia upon staining with toluidine blue and reacted negatively with alcian blue at pH I.

3) Chondrocytes

High density scleral and high density epiphyseal chondrocytes both gave rise to flat sheets of cartilage which were very similar in appearance (Figs. 9 and I0). When plated at medium densities, both types of chondrocytes produced discrete nodules of cartilage separated by non-chondrogenic fibroblast-like cells (Figs. II and I2). The histological appearance of these cultures are reminiscent of the morphology observed in cultures of limb bud mesenchyme (cf. Fig. 6) and to a lesser degree of medium density scleral mesenchyme (Fig. 4).

4) Mixed cell cultures

Chondrogenesis was dramatically reduced in both high density

scleral mesenchyme/chick heart fibroblast and high density limb mesenchyme/chick heart fibroblast cultures when compared with non-mixed controls (Fig. I3 and I4). In the former case small areas containing polygonal cells surrounded by a refractile matrix were occasionally observed (Fig. I3). However, in the limb mesenchyme culture, the characteristic aggregation of cells was abolished and as a result overt chondrogenesis did not occur. (A similar result was obtained with limb mesenchyme and flank mesenchyme).

Equal mixtures of chick limb mesenchyme with quail scleral mesenchyme resulted in substantial chondrogenesis and nodule production (Fig. I5). Histological examination for the quail nucleolar marker, wing Feulgen's stain, suggested that the cartilaginous areas were composed mainly of quail cells but the nucleolar staining was poor and, therefore, the result was inconclusive. However, it was observed that cartilage production occurred in the absence of any cellular aggregation.

Combinations of epiphyseal or scleral chondrocytes with chick heart fibroblasts resulted in indistinguishable cellular arrangements with both sets of cultures exhibiting substantial areas of chondrogenic tissue separated by fibroblast tracts (Fig. I6 and I7).

Discussion

The results in this chapter confirm the distinct patterns of chondrogenesis produced by mesenchyme from different locations in the embryo. The "whorled" cartilage nodules observed in limb bud mesenchyme cultures are undoubtedly similar to those described by Weiss and Moscana (I956) as are the cartilage sheets formed from scleral or periocular mesenchyme. More interestingly, the types of

chondrogenesis observed seem to reflect the behaviour patterns of the mesenchyme prior to matrix secretion, i.e. limb bud mesenchyme formed numerous concentric aggregates in which the foci normally becomes chondrogenic Ahrens et al, (I977) whereas scleral mesenchyme remained as a sheet of rounded/polygonal cells from plating to the onset of overt chondrogenesis.

A central question is whether these cellular arrangements represent a morphogenetic response to an intrinsic property of the cell or whether the cellular arrangement produced - can be influenced by environmental factors. For instance, can scleral mesenchyme, under appropriate conditions, ever form whorled aggregates or limb mesenchyme form a flat sheet of cartilage? The answer is yes since Ahrens et al (I979) have shown that central core limb mesenchyme cultured at high density forms a flat sheet of cartilage and the above results show that scleral mesenchyme is capable of forming whorls. The two systems, however, are not directly comparable for the following reasons.

Scleral mesenchyme, from 7 day old embryos is comprised of a homogenous population of cells all of which are determined to become chondrogenic in vivo and give rise to a cup of cartilage surrounding the eye. (Scleral cartilage appears between 8-9 days in vivo). These cells are in close association with the basement membrane of the retinal pigmented epithelium whose presence is required for their determination (Newsome, I976). Therefore, once determined, plating at high density onto a flat surface such as tissue culture plastic merely mimics the surface of the PRE, so that, when the chondrogenic phenotype is expressed, a flat cartilage sheet will result (Fig. I).

In contrast, stage 23-24 limb bud mesenchyme comprises of a number of cell types which include presumptive chondroblasts, myoblasts, vascular elements and undetermined mesoblasts. In fact Lewis (I977)

has calculated that as few as 5% of the cells in the chick wing bud will give rise to cartilage. Therefore, unlike scleral mesenchyme cultures, cartilage differentiation from limb mesenchyme in vitro must occur in close association with a large majority of non-chondrogenic cell types. (However, if the central core regions of stage 24 wing buds, which presumably have a high proportion of pre-chondrogenic cells, are cultured under Micromass conditions, virtually a flat sheet of cartilage is produced (Ahrens et al, 1979)).

The whole issue of tissue specific morphogenesis as proposed by Weiss and Moscona (1958) revolves around the similarity between the concentric cell aggregates and subsequent cartilage nodules observed in limb mesenchyme cultures and the concentric cellular orientation seen in early long bone rudiments when viewed in transverse section (Gould et al, 1974). The crucial question is are these two events homologous or is the similarity coincidental?

Ahrens et al (1977) demonstrated that limb bud mesenchyme from stages 20-24 will form aggregates and subsequent cartilage nodules when cultured under Micromass conditions. An important point is that the electron microscope evidence of these authors shows convincingly that aggregation occurs in the absence of matrix secretion (Ahrens et al, 1979; see also Ede, 1980; Solursh et al, 1982). Aggregation in vitro is believed to come about by active cell migration of peripheral cells towards a central, "founder" cell (Ede and Agerbak, 1968; Ede et al, 1977; Ede 1983) in a manner similar to the aggregation phase of the slime mould (Ede, 1983). However, as discussed in the General Introduction, there is no direct evidence for such centripetal movement in the limb and other evidence points against it (Gould et al, 1972).

What does seem clear is that the concentric arrangement of core cells in the limb is only observed after matrix secretion has

begun (Gould et al, 1978 and Chapter 2). This obviously contrasts with *in vitro* aggregation which occurs in the absence of matrix secretion (Solursh *et al*, 1982). It seems likely, therefore, that these two superficially similar cell arrangements are fundamentally different and should be considered separately:

i) *In vitro* morphogenesis - The differentiation of mesenchyme into actively secreting chondroblasts has been shown to be a density-dependant phenomenon (Umansky, 1966; Caplan, 1970; Ahrens *et al*, 1977) and only cultures plated at above confluence progress to chondrify substantially. Solursh *et al* (1978) have proposed a developmental sequence of events in limb mesenchyme chondrogenesis *in vitro* in which the aggregation process brought about necessary "histogenic interations" which in turn increased levels of cAMP and resulted in overt chondrogenesis. The net result of this was that, in a given culture, there were evenly spaced and evenly-sized roundish nodules of cartilage. More recently we have suggested that maintainance of a rounded configuration is sufficient to induce chondrogenesis in limb mesenchyme cells (Archer *et al*, 1982; also see Solursh *et al*, 1982). The results presented in this chapter will therefore be considered as changes in cell shape.

The formation of cartilaginous sheets by Micromass cultures of scleral mesenchyme, epiphyseal or scleral chondrocytes can be explained by considering each culture as a population of homogenous cells which, due to the high density involved, remain in a rounded, or polygonal, configuration. However, when the cell density at inoculation is reduced by such an extent that cells can flatten enough to become fibroblastic, then the cartilage sheet morphology is lost. Instead, groups of cells cluster thereby remaining rounded and therefore form cartilage nodules which are interspersed by non-chondrogenic fibroblast-

like cells. Cells which flatten and become fibroblastic will not become chondrogenic.

A similar result is obtained if scleral mesenchyme is diluted with chick heart fibroblasts or if limb mesenchyme, which already contains several non-chondrogenic cells, are plated out even at Micromass densities. However, if limb mesenchyme is diluted further with chick heart fibroblasts then no aggregation or cartilage differentiation occurs. This result implies that the presence of a very high concentration of non-chondrogenic cells can prevent aggregation and therefore prevent chondrogenesis. Thus it would seem that the aggregation process is an important differentiative step rather than a morphogenetic step.

ii) In vivo morphogenesis - There are a number of contrasting differences which must be considered when comparing in vitro and in vivo morphogenesis.

a) The 3-dimensional nature of morphogenesis in situ. This is particularly relevant to structures such as long bone rudiments, Meckel's cartilage and vertebrae.

b) The diversity in the biochemical and physical nature of cartilage. Early cartilage can be viewed essentially as a visco-elastic fluid which becomes more solid as it matures e.g. from the proliferative to the hypertrophic zones in long bone rudiments (Wolpert, 1982).

c) The presence of a multilayered perichondrium which has been shown to play a role in the early morphogenesis of cartilaginous rudiments (see earlier chapter, also see Carey, 1922; Wolpert, 1982; Archer et al, 1983).

d) Cell orientations. Unlike in vitro morphogenesis, chondrogenic cells in vivo assume distinct orientations which may be related to the overall shape of the cartilaginous element. Therefore, cylindrical

elements possessing longitudinal growth programmes have cells orientated at right angles to the long axis of the element as are found in the diaphyses of the long bone rudiments (Fell, 1925; Wolpert, 1978), and also in Meckel's cartilage (Jacobson and Fell, 1934).

As suggested in an earlier chapter, oriented matrix secretion may occur along the long axis of these flattened cells and this would facilitate longitudinal growth. It should be noted that flattening of chondrocytes within long bone rudiments bears no relation to cell flattening which occurs in tissue culture.

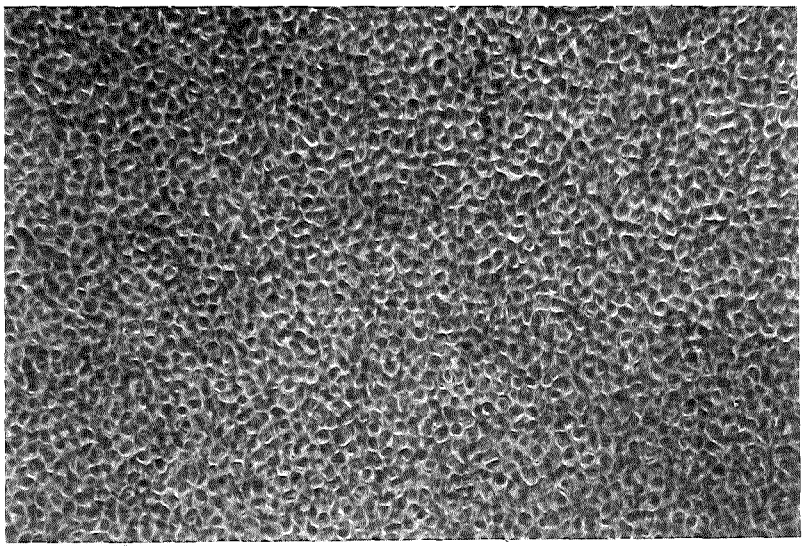
In contrast, both round-shaped cartilage structures such as epiphyses and sheet-like cartilages such as the sclera possess rounded chondrocytes which might secrete matrix in a non-polarised manner.

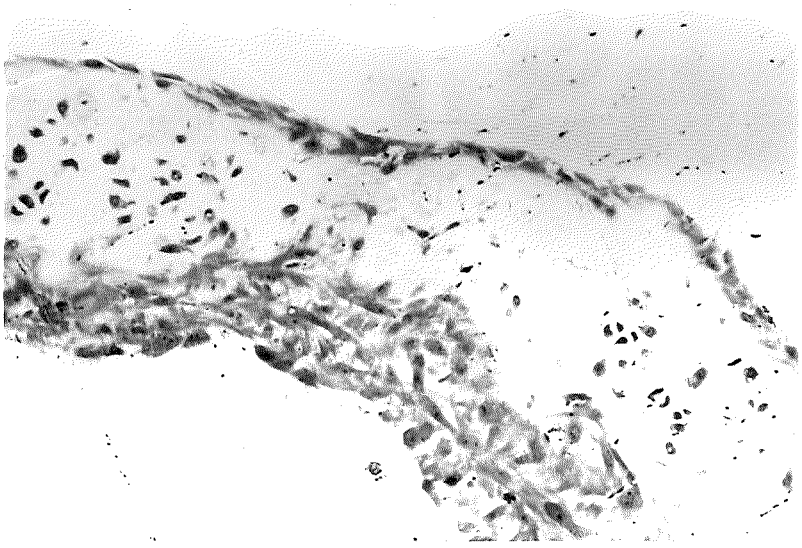
Viewed in these terms, cell shape plays crucial yet quite separate roles in the morphogenesis of cartilage in vivo and in vitro. In the latter case, the inoculation density of the cells at plating, which ultimately controls cell shape, together with the number of cells "committed" to the chondrogenic phenotype will be the major factors determining morphogenesis and, therefore, it is not necessarily determined on a tissue-specific basis.

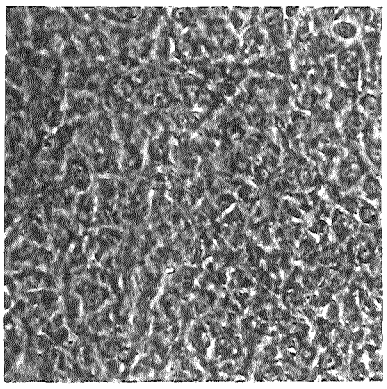
Fig. 1. High density scleral mesenchyme. By 2 days in culture a flat sheet of tightly packed polygonal cells, separated by a refractile ECM, is observed.
Living culture - phase contrast. Mag. x 125.

Fig. 2. High density scleral mesenchyme. When histological sections are cut perpendicularly to the surface of the dish, the culture is found to contain polygonal cells separated by a metachromatic ECM.
Tol. blue stain. Mag. x 125.

Fig. 3. High density scleral mesenchyme. When exposed to anti-type II collagen anti-body, both the cells and the ECM stain positively, indicating the presence of cartilage matrix.
Fluorescence microscopy. Mag. x 125.







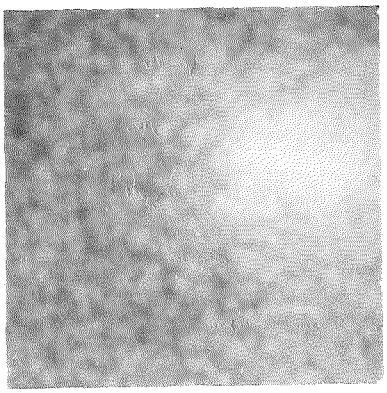
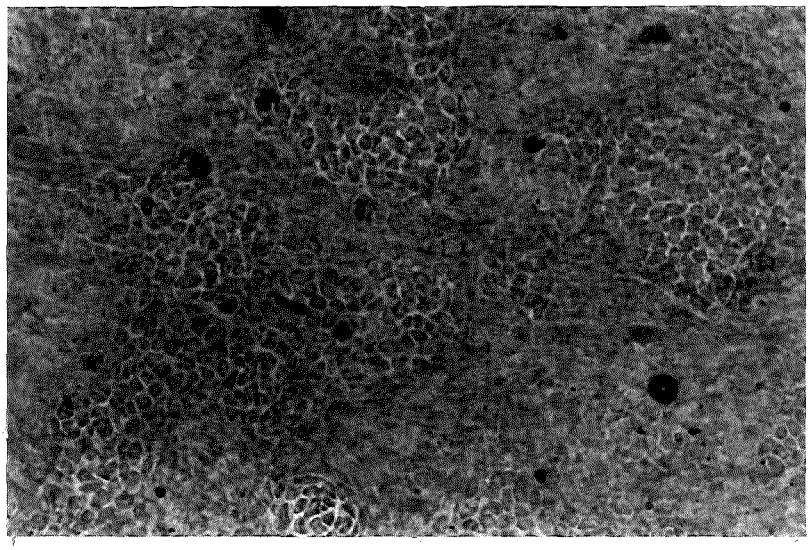
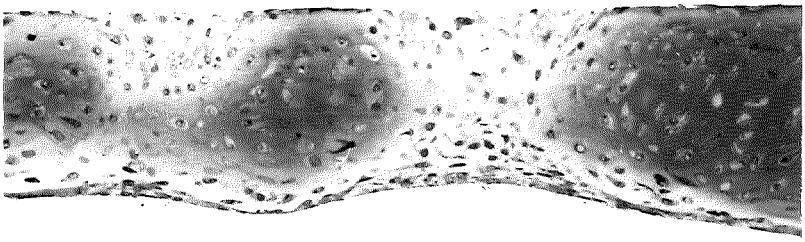


Fig. 4. Medium density scleral mesenchyme. By 4 days the culture is composed of cartilage nodules separated by fibroblastic cells.
Fixed culture - phase contrast. Mag. x 125.

Fig. 5. High density limb mesenchyme. When viewed in perpendicular section, a 3 day old culture consists of nodules of cartilage separated by fibroblastic cells.
Tol. blue stain. Mag. x 125.

Fig. 6. High density limb mesenchyme. Cartilage nodules increase in size during culture so that by 5 days they appear close together and begin to coalesce.
Fixed culture - phase contrast. Mag. x 125.





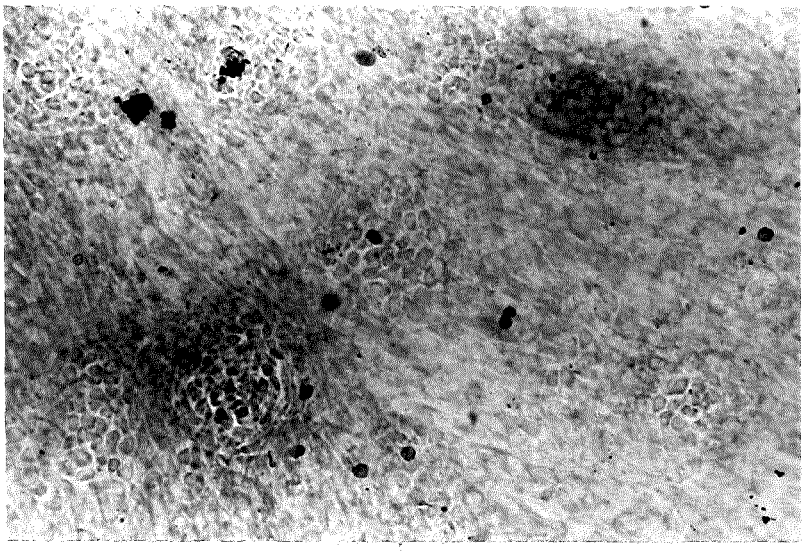
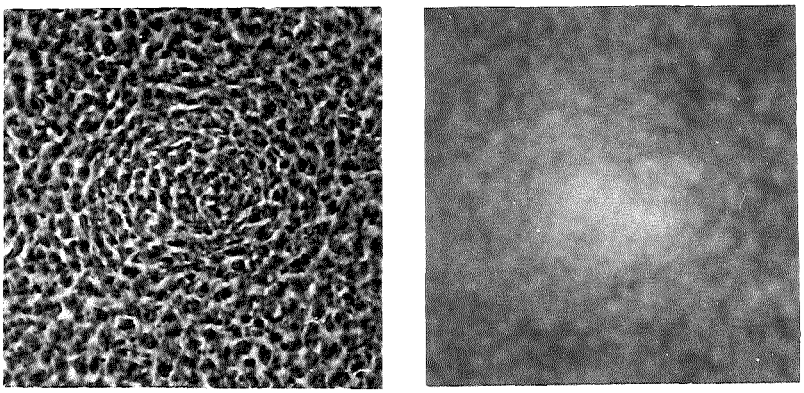
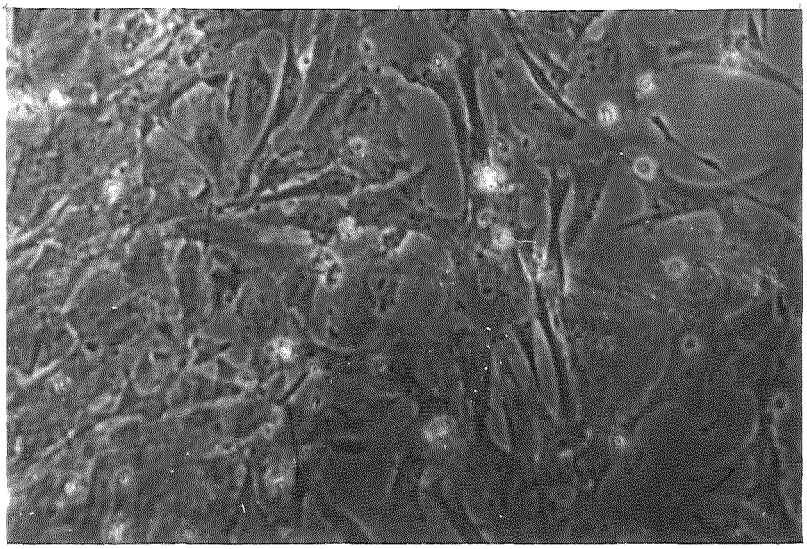


Fig. 7. High density limb mesenchyme. When exposed to anti-type II collagen antibody, cells in the nodular regions stained positively. Intermodular regions failed to stain significantly. Fluorescence microscopy. Mag. x 125.

Fig. 8. Medium density limb mesenchyme. By 5 days, the culture is almost entirely fibroblastic with very few chondrogenic areas. Living culture - phase contrast. Mag. x 300.

Fig. 9. High density scleral chondrocytes. By 3 days, the culture consists of a flat sheet of polygonal cells separated by a metachromatic matrix.
Tol. blue stain. Mag. x 125.





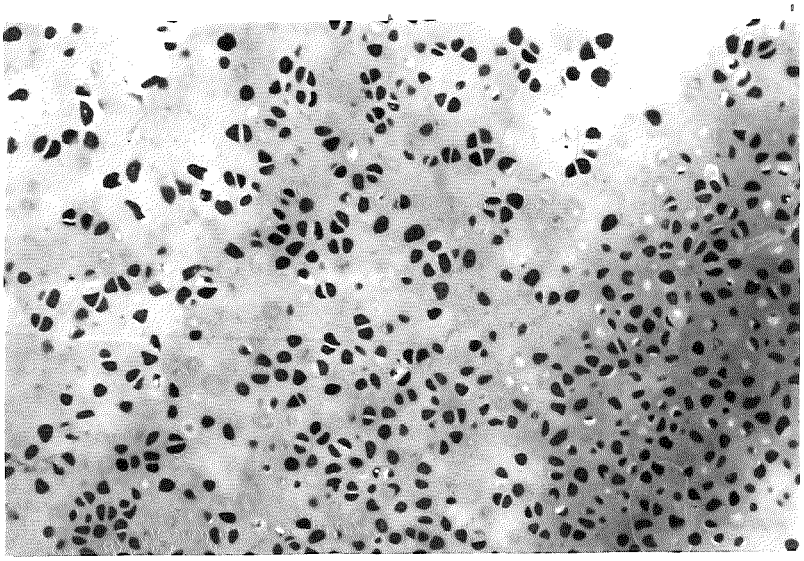
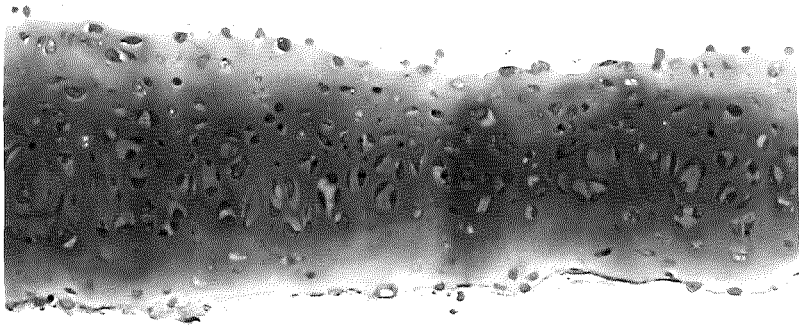


Fig. 10. High density epiphyseal chondrocytes. In perpendicular section, a 3 day old culture, consists of a strip of polygonal cells separated by a metachromatic ECM.
Tol. blue stain. Mag. x 125.

Fig. 11. Medium density scleral chondrocytes. By 3 days the culture consists of cartilage nodules interspaced by fibroblastic cells.
Living culture - phase contrast. Mag. x 125.



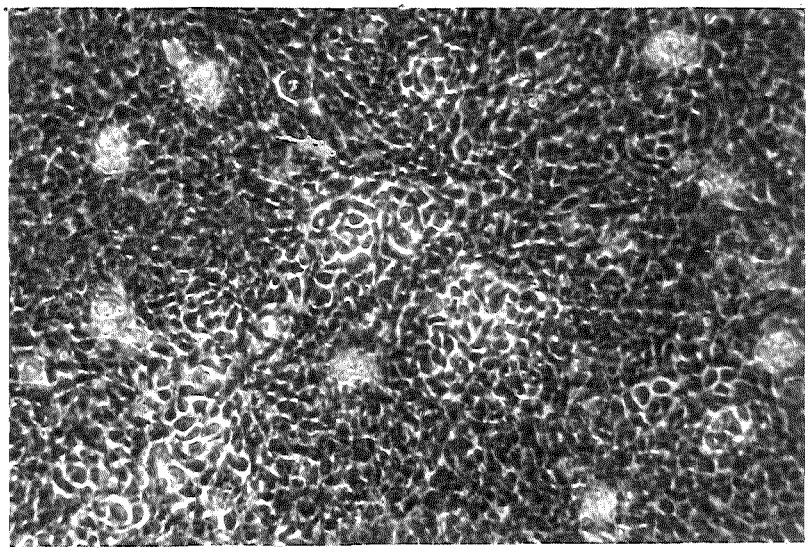
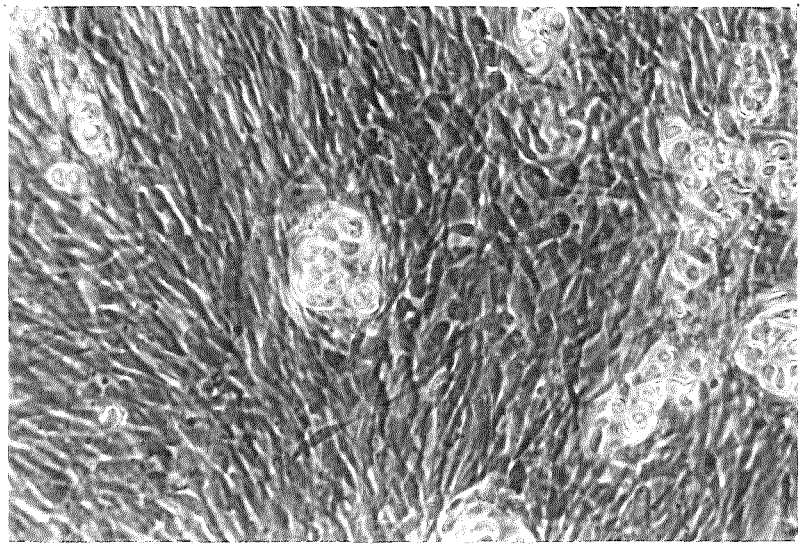


Fig. I2. Medium density epiphyseal chondrocytes. By 3 days the culture consists of cartilage nodules interspaced with fibroblastic cells and is similar to Fig. II.
Living culture - phase contrast. Mag. x I25.

Fig. I3. High density scleral mesenchyme/chick heart fibroblasts. Chondrogenesis is reduced and cartilage appears to be confined to small clumps interspaced with large areas of fibroblastic cells.
Tol. blue stain. Mag. x I25.



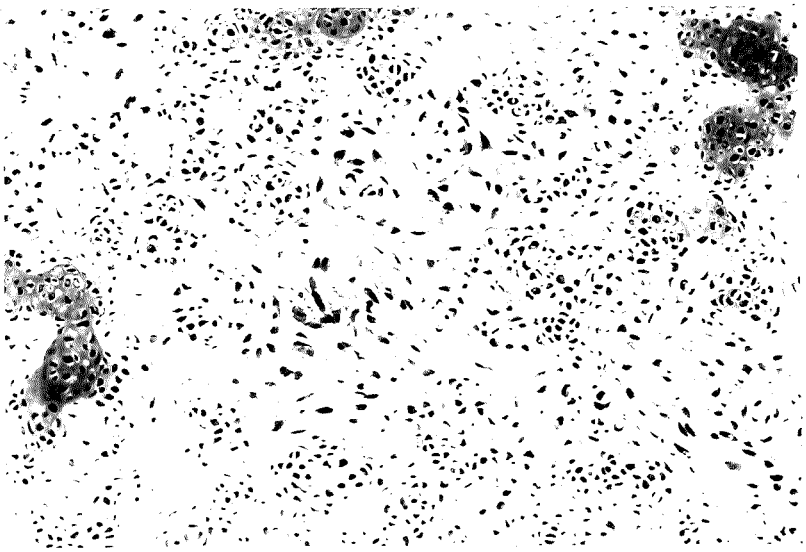
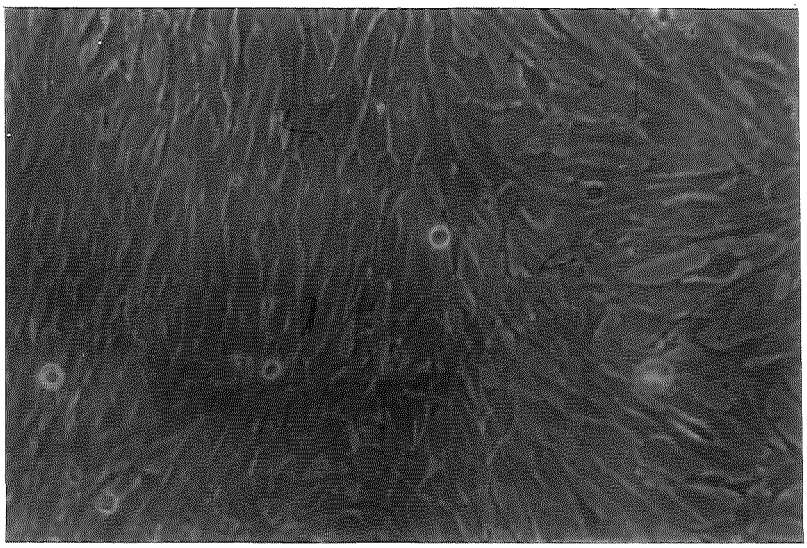


Fig. I4. High density limb mesenchyme/chick heart fibroblasts. Chondrogenesis is inhibited and the culture is composed mainly of fibroblasts.
Living culture - phase contrast. Mag. x 300.

Fig. I5. High density limb mesenchyme/quail scleral mesenchyme. By 3 days, a pattern similar to that in normal mesenchyme cultures is produced. Cartilage nodules are found interspaced with fibroblastic cells. Nodules are similar in size to those of limb mesenchyme.
Living culture - phase contrast. Mag. x I25.



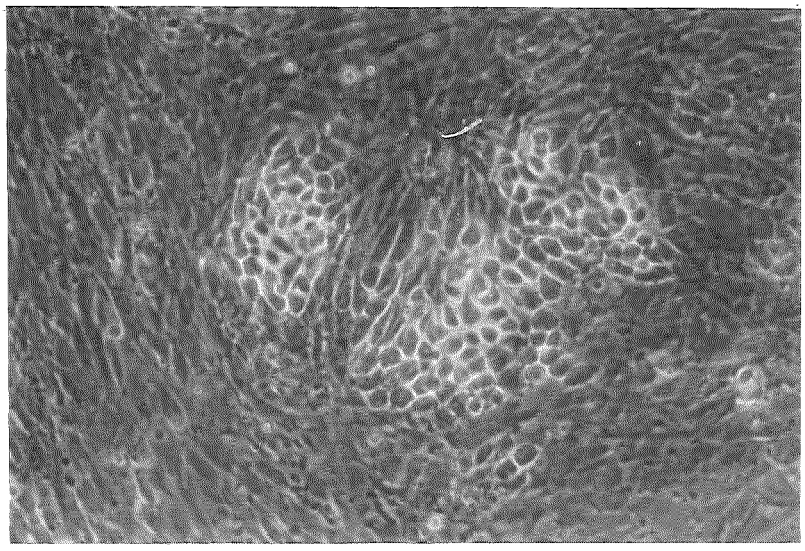
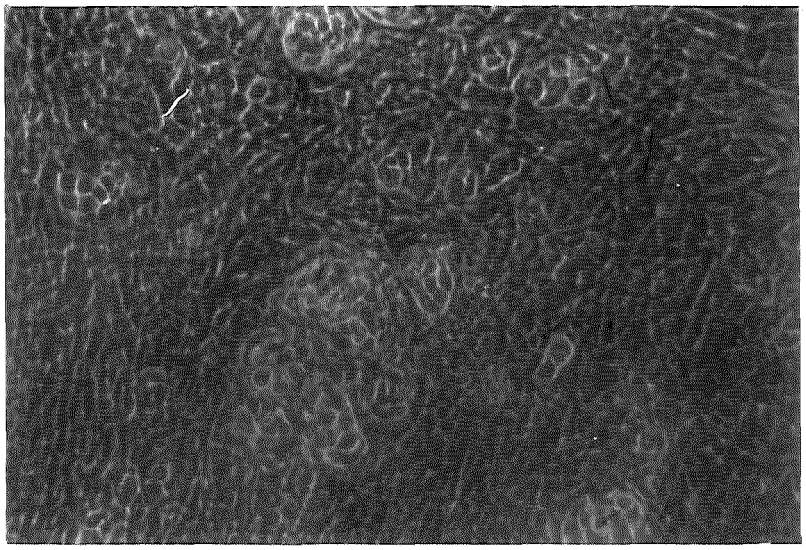
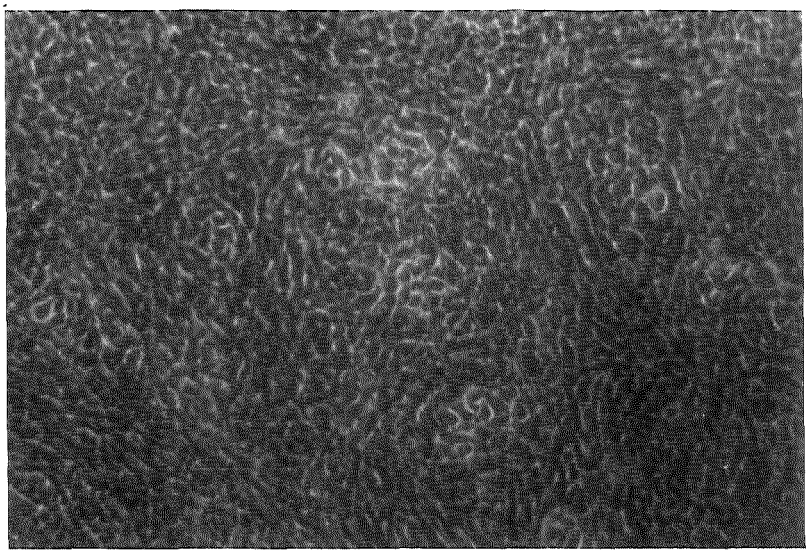


Fig. I6. High density scleral chondrocytes/chick heart fibroblasts. By 3 days, the culture resembles a medium density scleral mesenchyme culture (Fig. II) in that cartilage nodules are produced. Living culture - phase contrast. Mag. x I25.

Fig. I7. High density epiphyseal chondrocytes/chick heart fibroblasts. By 3 days the culture is very similar to that in Fig. I6. Living culture - phase contrast. Mag. x I25.





C H A P T E R S I X

CELL KINETICS OF CARTILAGE

LONG BONE RUDIMENTS

Introduction

Data already presented have shown that cell shape plays an important role in the degree of cell division exhibited by cells *in vitro* (chapter 4), and preliminary studies suggest that the location and degree of cell division within an intact long bone rudiment may also be determined by cell shape (chapter I). Cell division has been studied in many systems and the duration of the cell cycle has been calculated for various cell types but very few studies have concentrated on embryonic chondrocytes. Murison (I972) cultured chondrocytes from the vertebral trunks of I0 day old chick embryos and calculated a cell cycle time of approximately I8 hours. An important finding was that the cell cycle time was similar whether it was calculated from cells exposed to a short pulse of tritiated thymidine (producing a Frequency of Labelled Mitosis curve - FLM) or from cells cultured continuously in the presence of the label.

Very little has been published concerning the cell cycle of cells within a long bone rudiment either *in vivo* or in organ culture. The most closely related work has been performed by Kember (I972, I973, I978) when he studied the cell kinetics of chondrocytes within the epiphyseal growth plates of young rats. The rates of proliferation of the entire growth plates have been calculated but the cell cycle times of the individual chondrocytes within the growth plates have not been clearly stated.

The objects of this chapter are as follows:

i) to determine the cell cycle times of chondrocytes within an intact long bone rudiment in organ culture,

ii) to examine the relationship between cell shape and division rate or cell cycle time within an intact rudiment and

iii) to determine the cell cycle time of chondrocytes freed from the

the rounded cell zone and grown in cell culture to give an indication of how closely cell culture studies can be related to organ culture studies.

Materials and Methods

Ulnae were removed from stage 33 embryos and maintained in culture as described in the General Methods. After I hour, the "cold" medium was replaced with medium containing ^{3}H-thymidine at a concentration of I5 /uCi/ml. Rudiments were incubated continuously in medium containing ^{3}H-thymidine for up to 28 hours. Samples were removed every 4 hours, fixed, sectioned and processed for autoradiography as described in the General Methods. A mean labelling index (L.I.) was obtained for each time period by counting the total number of cells/cellular zone and the number of cells within each zone which had incorporated ^{3}H-thymidine. Cell counts were performed on 3 central I /um sections, each I0 /um apart, from 3 different rudiments for each lebelling period.

Cell cultures were obtained by isolating the rounded cell zones from stage 33 ulnae and dissociating them as described in the General Methods. Cells were plated onto 35 mm tissue culture dishes at a concentration of $2xI0^{5}$ cell/dish and cultured in Hams F-I2 medium for I day. After I day the "cold" medium was replaced with fresh medium containing ^{3}H-thymidine at a concentration of I /uCi/ml. Cells were cultured continuously in the presence of ^{3}H-thymidine for up to 28 hours. Samples were removed at 2 hour intervals, fixed, exposed to nuclear emulsion and processed for autoradiography as described in the General Methods. The labelling index was obtained by counting the number of lebelled nuclei in at least 500 cells in each of 3 dishes for each time period.

Results

I) Organ culture

i) Rounded cells

The labelling indices of proximal and distal rounded cells within an intact rudiment over a 28 hour period are shown in Figure I. Distal rounded cells tend to have a higher L.I. than proximal cells with a maximum of approximately 40% after 28 hours. In both zones, the accumulation of labelled cells increased almost linearly for about I2-I4 hours when it began to plateau. According to Murison (I972) the cell cycle time can be taken as the breakpoint of the ascending curve and the plateau - in both zones this occurs between I2-I6 hours and can be centred on I5±I hours (Fig. I). The L.I. and the number of cells in each zone are shown in Table I.

ii) Flattened cells

The L.I.'s of proximal and distal flattened cells within an intact rudiment over a 28 hour period are shown in Figure 2. Proximal flattened cells tend to have a higher L.I. than distal cells with a maximum of approximately 20% after 28 hours. In both zones, the accumulation of labelled cells increased linearly for about I6-I8 hours when it began to plateau. If the breakpoint of the curve is taken as an estimate of the cell cycle time then a figure of approximately I8±I hours is obtained (Fig. 2). Again, the actual number of cells counted is shown in Table I.

iii) Hypertrophic cells

No labelled hypertrophic cells were observed in stage 33 ulnae until at least I2 hours of culture. The cells are at the boundary between the flattened and hypertrophic cell zones (Fig. 3) and are probably more accurately described as hypertrophying flattened cells. Over a 28 hour time period only about 3% of hypertrophic cells showed

any label, therefore, no cell cycle time could be obtained. The L.I. and the number of cells counted are shown in Table I.

iv) The intact rudiment

An estimate of the L.I. of the intact rudiment can be obtained by expressing the total number of labelled cells as a percentage of the total number of cells found in each section (Table I). (The number of labelled cells in each cellular zone can be calculated by multiplying the L.I. of that zone by the number of cells within it). The curve produced (Fig. 4) is of a similar pattern to that found in rounded and flattened cell zones and shows a breakpoint at approximately I6±I hours.

II) Cell culture

The L.I. of cells freed from the rounded cell zone and grown in cell culture is shown in Figure 5. As in the above cases there is an almost linear accumulation of labelled cells for about I6-I8 hours when the increase slows down. If the breakpoint of the curve is looked at, a cell cycle time of I6±I hours is obtained. It is noticable that the maximum percentage of labelled nuclei is only 83% after 28 hours; a I00% L.I. was never observed.

Discussion

The cell cycle times presented in this chapter are all calculated from graphs of labelling indices of continuously labelled cells. Cell cycle times in most other systems have been evaluated using FLM curves (for example see Quastler and Sherman, I959; Steel and Haines, I97I and Gilbert, I972) which involve exposing cells, or tissues, to a short pulse of label followed by culture in unlabelled medium. However, FLM studies were not utilised in this system for several reasons:

i) In the case of a long bone rudiment, rounded cells divide but

they also mature into flattened cells. This implies that the amount of label incorporated into the rounded cell zone during the pulse would not only be diluted by cell division but a proportion would continuously be lost from the zone entirely making accurate studies of cell division within the zone impossible.

ii) Recent studies have stated that in certain cases FLM curves give inaccurate values of the cell cycle (Hudson and Hahn, I977; Hamilton and Dobbin, I983).

iii) Murison (I972) showed that continuous labelling curves gave a similar value of cell cycle time in chondrocytes as FLM curves and therefore, if only the total cell cycle time, and not the times of the individual cell cycle phases, is required the simpler continuous labelling curve is sufficient.

The results presented in this chapter show that a higher proportion of rounded cells are capable of incorporating ^{3}H-thymidine than flattened cells. It seems surprising that a maximum L.I. of only 40% is obtained within the rounded cell zones whilst a L.I. of 20% is observed within the flattened cell zones (Fig. I and 2) but it must be remembered that rounded cells are continuously maturing into flattened cells and therefore, the L.I. of the rounded cells (Fig. I) is artificially low whilst the L.I. of the flattened cells (Fig. 2) is made artificially high. If a stage 33 ulna is labelled for a short period of time, e.g. 2 hours, the L.I. of the flattened cells is found to be less than I0% (see Table XIII, chapter I) which implies that very soon after removal from the embryo, only about I0% of the initial flattened cell population is dividing. Over a 24 hour culture period the number of cells in the proximal flattened cell zone of a I/um section increases from 938-I6I3 cells (Table I), an increase of approximately 650 cells.

However, if only 10% of the existing flattened cell population is dividing (assuming there is only one complete cell cycle of 18 hours during the 24 hour study period) this implies that only about 100 of these cells are derived from existing flattened cells, the remaining 550 cells must arise from maturing rounded cells.

The data in Table I shows an increase in proximal rounded cell number, in a 1 um section, from 1193-1892, approximately 700 cells, during the culture period. If a further 550 flattened cells have also been produced from rounded cells this implies that the total number of new cells produced by the proximal rounded cell zone is closer to 1250 during the culture period. If this is the case, then the initial rounded cell population, of approximately 1200 cells, has doubled during the 24 hour study period. Assuming that there is only one cell cycle of 15 hours during the culture period, this implies that every rounded cell has divided. Therefore, the actual L.I. of the proximal rounded cell zone should be closer to 100%, and the L.I. of the flattened cell zone should be closer to 10% over the 24 hour period. Similar calculations can be obtained for distal rounded and flattened cell zones.

This is the first report of differences in the cell cycle times of chondrocytes within different zones of the same long bone rudiment. Rounded cells have a mean cell cycle time of 15±1 hours whilst flattened cells have a cell cycle time of 18±1 hours (Figs. 1 and 2). Although significant in that it does occur, it would perhaps be expected since flattened cells are more mature (and therefore older) than rounded cells and evidence suggests that the proliferative ability of chondrocytes decreases with age e.g. Walker and Kember, (1972) have shown that, during maturation of the proximal tibial growth plate, the rate of chondrocyte proliferation and the L.I. of the chondrocytes

is reduced by at least 50%. Also, chondrocytes from vertebrae of neonatal rats have a mean cell cycle time of 22 hours (Dixon, 1971) whilst chondrocytes from 6 week old rat tibial epiphyses have a cell cycle time of 55 hours (Walker and Kember, 1972) however, it is not known how much of this difference is due to location rather than age.

No cell cycle time could be obtained for hypertrophic cells since only about 3% of the cells incorporated label by 28 hours (Table I). It is unlikely that these labelled hypertrophic cells were originally labelled flattened cells since histological examination shows that the distribution of labelled flattened cells is not uniform. There is a higher percentage of labelled flattened cells close to the junction with the rounded cell zone than there is close to the hypertrophic cell zone - even after 16 hours of incubation (Fig. 3). There is a distinct gap of at least 10 cell diameters with absolutely no label between the last labelled flattened cell and the first labelled hypertrophic cell. This would not be expected if the labelled hypertrophic cells were originally labelled flattened cells (Fig. 3). This observation implies that the onset of cell hypertrophy may be associated with a phase of DNA synthesis.

The non-uniform distribution of label within the flattened cell zones suggest that as a flattened cell matures it withdraws from the cell cycle. Since it is only the most mature flattened cell which becomes hypertrophic and since the first labelled hypertrophic cell does not appear for at least 12 hours (Table I) this implies that the time taken for a mature, non-dividing flattened cell to become hypertrophic is at least 12 hours. How long a flattened cell is capable of dividing before it fully matures

and what causes it to stop dividing once mature are not known.

When the entire rudiment, as a whole, is considered a mean cell cycle time of approximately I6 hours is obtained (Fig. 4). This figure fits remarkably well with the data obtained by counting the number of cells within a I/um section of an ulna (see Table VI, chapter I). The time period between stage 30 - stage 32 (6/6½ days - 7 days) is approximately I6 hours (Hamburger and Hamilton, I95I) and during this time the number of cells within the sectioned ulna increases from I896-3733, an increase of approximately I00%. Similarly, the time period between stage 3I - stage 33 (6½/7 days - 7½ days) is also about I6 hours and during this time the total number of cells increases from 2472-5I96, once again, an increase of approximately I00%.

The L.I. of the perichondrium was not determined but it was observed that a uniform intense label was found throughout the perichondrium. Figure 3 shows that the perichondrium was heavily labelled even at areas where no cartilage was labelled.

When rounded cells were freed from their extracellular matrix and grown in cell culture a mean cell cycle time of I6±I hours was obtained (Fig. 5). This value is similar to the cell cycle time of I5±I hours obtained for rounded cells within an intact rudiment and is close to the cell cycle time of I8 hours recorded by Murison (I972) using chick embryo vertebral cartilage. Also, the maximum L.I. of 83% is similar to that of 82% recorded for vertebral cartilage. A L.I. of I00% was never obtained, but this is not unusual and may be explained by damage to cells during the dissociation process or by some of the cells maturing into "flattened" chondrocytes (not flattened, fibroblastic cells) and withdrawing from the cell cycle.

Murison (1972) cultured his cells for 3 days prior to labelling and found two cell types produced - polygonal cells, surrounded by a metachromatic extracellular matrix, and fibroblastic cells. However, he noticed that the cell cycle time was identical whether the cell was polygonal or fibroblastic and this would imply that the dedifferentiation event occurring in rounded cell cultures during the first 1-2 days (see chapter 4) can be ignored for the purpose of this study.

The general conclusions from this chapter are as follows:

i) every rounded cell is capable of dividing whilst only about 10% of the flattened cell population can, or does, divide,

ii) rounded cells have a shorter cell cycle time than flattened cells,

iii) the process of cell hypertrophy may require a phase of DNA synthesis,

iv) the mean cell doubling time within an intact rudiment is approximately 16 hours, and

v) the cell cycle time of rounded cells is similar whether the cell is grown in organ or cell culture.

Fig. 1. Labelling indices of stage 33 rounded cell zones.

■ — — ■ Proximal

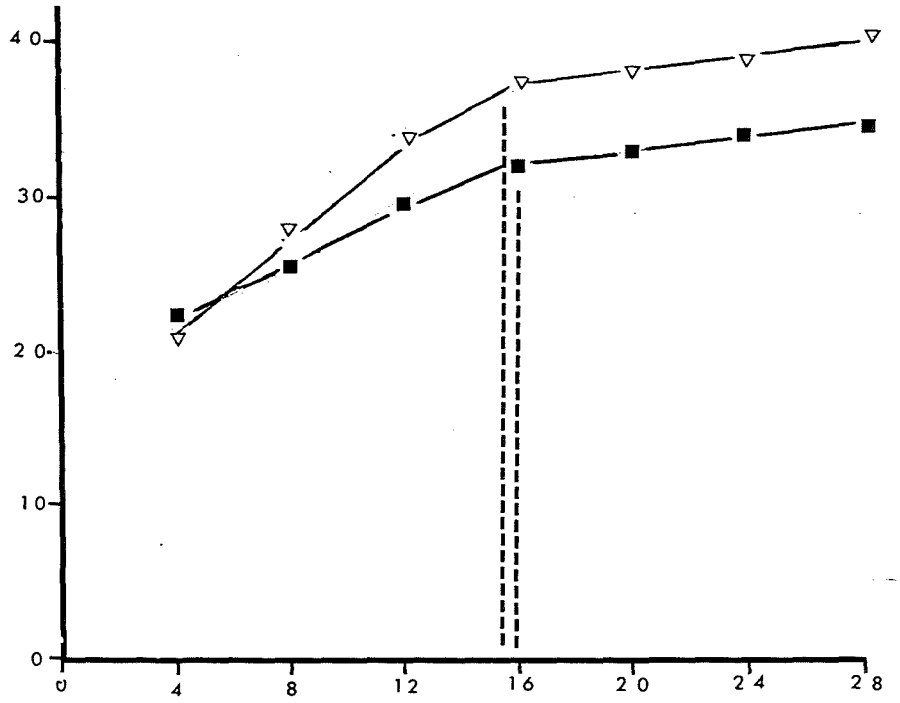
▽ — — ▽ Distal

Fig. 2. Labelling indices of stage 33 flattened cell zones.

■ — — ■ Proximal

▽ — — ▽ Distal

Labelling
index (%)



Labelling
index (%)

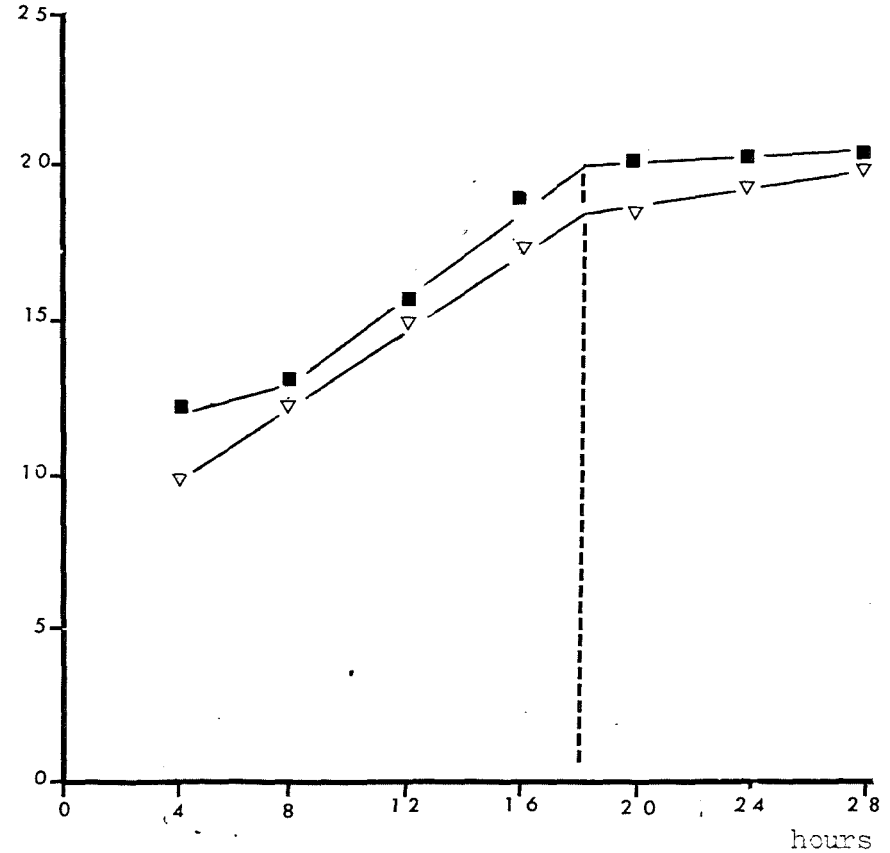
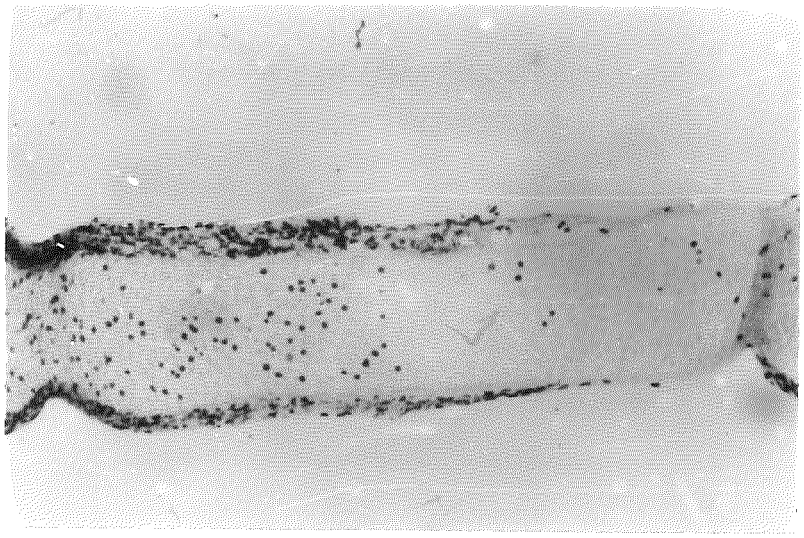


Fig. 3. Stage 33 hypertrophic cell zone exposed to ^{3}H-thymidine for 16 hours. The only hypertrophic cells which incorporate label are those closest to the flattened cell zone. Note the distance between labelled flattened and hypertrophic cells. Also, note that the perichondrium is heavily labelled throughout it's length.
Tol. blue stain. Mag. x 125.

Fig. 4. Labelling index of stage 33 ulnae.



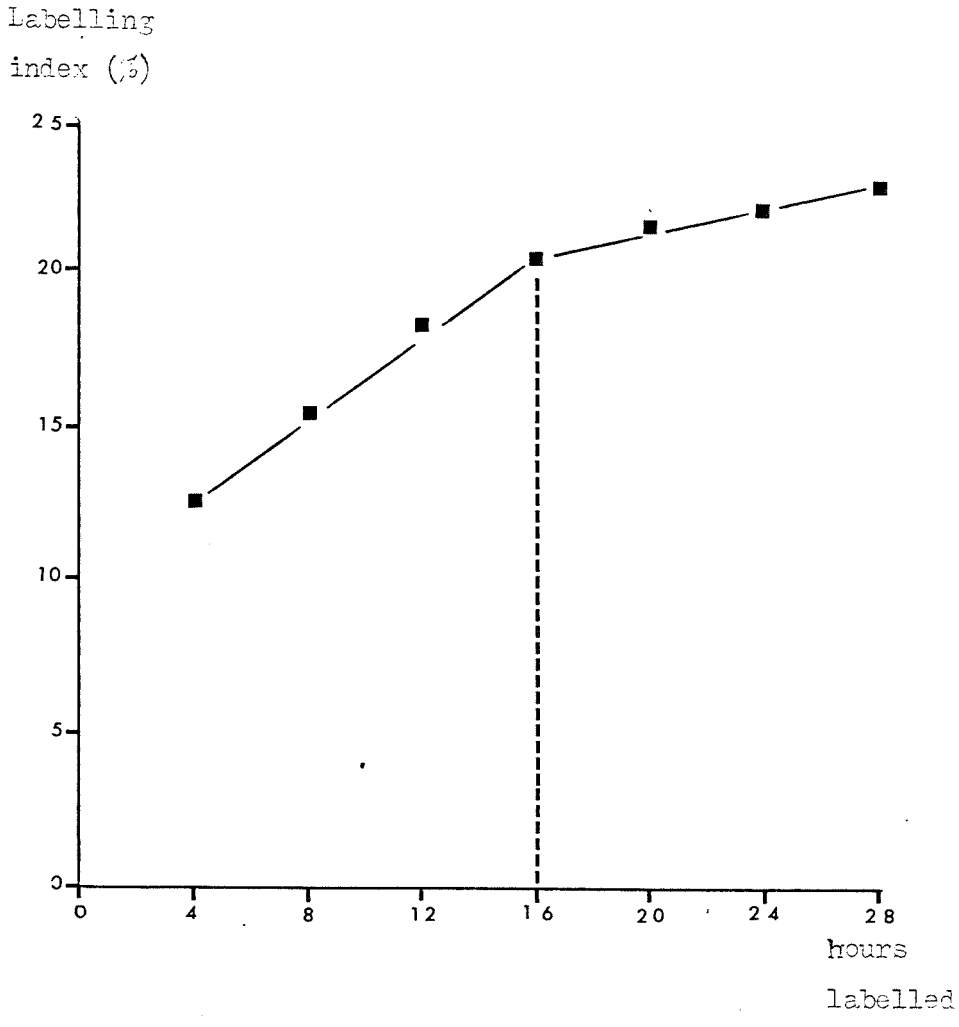

Labelling
index (%)
25
20
15
10
5
0
0
4
8
12
16
20
24
28
hours
labelled

Fig. 5. Labelling index of stage 33 rounded cells dissociated and grown in cell culture.

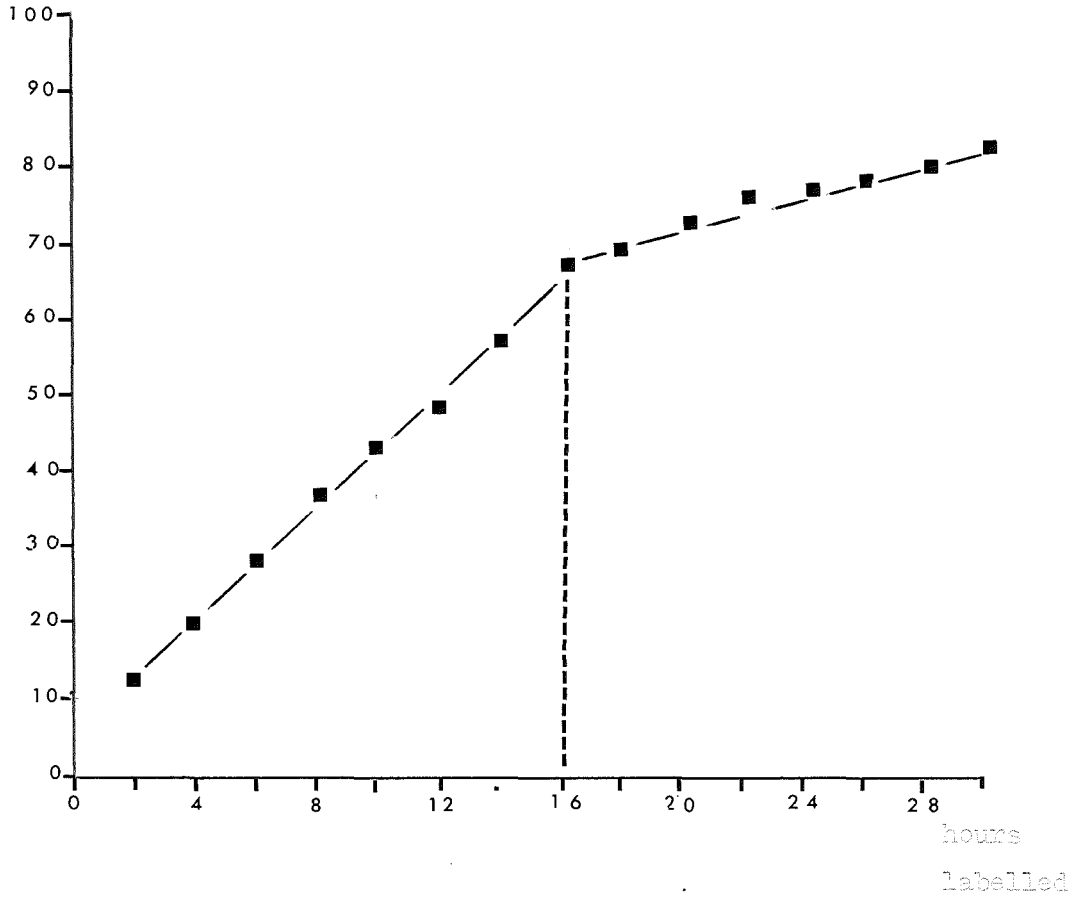


Table I Mean labelling indices observed in the histological zones of a stage 33 ulnae.

Zone (P- D)	Labelling index+ (Total no. cells) Hours labelled 4	8	I2	I6	20	24	28
Round.	2I.96 (II93)	25.42 (I38I)	29.55 (I462)	32.02 (I593)	33.59 (I676)	34.04 (I757)	34.4I (I892)
Flat.	I2.26 (938)	I3.36 (I078)	I5.83 (II94)	I8.93 (I305)	I9.88 (I403)	20.08 (I524)	20.I5 (I6I3)
Hyp.	0 (I27I)	0 (I395)	0.I3 (I482)	0.57 (I590)	0.72 (I665)	2.I9 (I784)	3.3I (I902)
Flat.	I0.II (9I0)	I2.45 (940)	I4.99 (I0I4)	I7.36 (I083)	I8.56 (II64)	I9.64 (I232)	20.03 (I298)
Round.	20.86 (858)	27.92 (985)	34.05 (I063)	37.30 (II66)	38.37 (II99)	39.43 (I263)	40.I8 (I349)
Total	I2.59 (5I70)	I5.35 (5779)	I8.29 (62I5)	20.52 (6697)	2I.53 (7I07)	22.26 (7560)	22.86 (8054)

+Values are presented as a percentage of the total number of cells. Values were calculated from 3 central I/um sections, I0/um apart, from 3 different rudiments for each labelling period.

Standard deviations are omitted for clarity but ranged from 3-I3%.

G E N E R A L C O N C L U S I O N

A typical cartilage long bone rudiment consists of 3 types of chondrocytes, rounded, flattened and hypertrophic, arranged in 5 cellular zones such that along the proximo-distal axis the sequence is rounded, flattened, hypertrophic, flattened and rounded. The main aims of this thesis were to determine how each cellular zone was set up and maintained, and to investigate the role played by each cell type in the growth and morphogenesis of a long bone rudiment. The rudiment studied here was the chick embryo ulna from it's appearance at 5 days of incubation until just prior to cartilage erosion at 9 days, i.e. it was studied between stage 25 - stage 34 (Hamburger and Hamilton, 1951).

At stage 25 the ulnal condensation appeared as a tightly packed mass of cells, at a cell density higher than that of the surrounding mesenchyme, but with no apparent cellular orientation. Several reports state that if the nucleus - golgi-body axis is locked at a definite cellular orientation is present at this stage, however, mouse and chick condensation cells appear to have their nucleus - golgi-body axes pointing in different directions (Holmes and Trelstad, 1980; Ede et al, 1977) and the actual role played by such an orientation is, as yet, unclear.

The first appearance of overt cellular orientation in the ulna was at stage 26. The condensation was oval-shaped and the cells within it were no longer closely packed but were separated by a metachromatic ECM and appeared to be aligned in a direction at right angles to the long axis of the wing, making them appear somewhat flattened. This orientation was never observed without ECM secretion and supports the suggestion of Gould et al (1974) that cartilage cell orientation is always associated with ECM secretion.

Almost every cell in the stage 26 ulna was flattened and the short time interval, about 6 hours, between stage 25 - stage 26 suggests that the flattened cell may be the first type of chondrocyte produced. Although no direct experimental evidence is available to indicate how these cells become flattened, it may be purely mechanical. Carey (1922) stated that prior to the formation of a perichondrium, the embryonic pig femur increased more in width than in length. If the same is true for the ulna then the increase in width between stage 25-26 may stretch the cells causing them to flatten at right angles to the long axis of the wing. The increase in width may be due to the MPH secreted by the cells.

At stage 28 the ulna consisted of a cigar-shaped tissue containing two cell types, flattened chondrocytes in the centre and regions of fewer, rounded cells at either end. These rounded cells were presumably cells at the ends of the oval-shaped condensation which did not get stretched when the condensation increased in width. However, the rounded cells merged into the mesenchyme and the possibility that some were actually derived from the mesenchyme cannot be ruled out.

No true perichondrium was observed at stage 28, therefore, the rudiment could continue to increase in width and no epiphyseal swellings were observed. However, there was a barrier of polygonal shaped cells between central flattened cells and the mesenchyme. These polygonal shaped cells were, in fact, elongated cells arranged circumferentially around the rudiment and were the earliest indication of a perichondrial structure.

It was interesting to note that if the number of cells which constitu[illegible] the perichondrial structure, in longitudinal section,

was added to the number of flattened cells across the diameter of the rudiment, at stage 28, the sum was exactly the same number as that found across the diameter of a stage 26 condensation. This implied that the early perichondrium might form from cells at the periphery of the condensation and gave even more support to the suggestion of Gould et al (1974) that the perichondrium formed as a result of peripheral cells becoming stretched due to pressure generated by ECM secretion by central cells.

The third cell type, the hypertrophic cell, did not appear until stage 30 when flattened cells, in the centre of the rudiment, began to enlarge and degenerate. Experimental evidence suggested that the process of cell hypertrophy did not come about due to any environmental factor but was, rather, an autonomous property of flattened cells. If the flattened cell zone was removed to organ culture the cells within it proceeded to hypertrophy.

In brief, the flattened cell zone appeared at stage 26, probably as a result of cells becoming stretched due to an increase in width brought about by ECM secretion; rounded cells might also be present at stage 26 but did not appear in any number until stage 28, the regions these cells were found in did not increase much in width at stage 26, therefore, the cells did not become stretched, and; hypertrophic cells appeared at stage 30 as a result of an autonomous property of flattened cells.

During development, each cellular zone continually increased in size but no zone became depleted, therefore, how was each zone maintained? The hypertrophic cell zone got larger simply because flattened cells were continually undergoing hypertrophy. Autoradiographic evidence suggested that cell division was confined mainly to the rounded cell zones, therefore, these zones were

maintained by new cell production. The flattened cell zone continually increased in size due to the maturation of rounded cells, therefore, the problem which had to be answered was how did a rounded cell become flattened? Ultrastructural studies have shown that the ECM between flattened cells contain oriented collagen fibres. These collagen fibres were polarised in a direction parallel with the long axis of the flattened cell. The rounded cell zone, on the other hand, contained little collagen and the ECM was totally unorganised. It is proposed that, as a wave of cell flattening moves out from the centre of the rudiment, rounded cells come into contact with the organised collagen meshwork and flatten onto it, this cell flattening induces further polarised collagen secretion which in turn causes more rounded cells to flatten. In this way the process is self-propagating and can be considered as a form of crystallisation. Such a process has recently been proposed, in general terms, by Alberts et al (1983) and some experimental evidence is available to support it. When stage 32 ulnae were grown in the presence of L-azetidine-2-carboxylic acid (LACA), a drug which causes abnormal collagen secretion, the gross effect was that the rudiment was shorter and fatter than controls. At the histological level it was observed that the flattened cell zone was no longer present, instead rounded cells were found within flattened lacunae. This result suggests that unless collagen is present, and is being secreted, in an organised manner, there is no further cell flattening and already flattened cells revert back to being rounded cells. It is interesting to note that the process of cell hypertrophy also ceases in these rudiments, therefore an organised collagenous ECM not only influences cell shape it also plays a role

in the normal growth and development of the rudiment.

Once the 5 cellular zones are set up, growth and morphogenesis result in the production of a rudiment which has a long, narrow diaphysis with epiphyseal swellings at either end. How does this shape come about?

The first appearance of a true perichondrium was observed around the central region of the stage 30 diaphysis just as the process of cell hypertrophy was beginning. Cell hypertrophy involved a large increase in volume which resulted in the cells, which had previously been arranged circumferentially around the rudiment, becoming elongated in a direction which was parallel to the long axis of the rudiment. These elongated cells overlapped and provided a limited resistance to radial expansion causing the diaphysis to increase in width at a rate lower than at the epiphyseal ends where no such constraining barrier was observed. Thus it was from this stage onwards that the typical long bone morphology of a narrow diaphysis with epiphyseal swellings, was produced.

Further cell hypertrophy consolidated the perichondrium such that by stage 32 the perichondrium surrounding the hypertrophic cell zone was a very distinct, very tight structure with many extensive cell-cell contacts, believed to be tight junctions. The width of the hypertrophic cell zone ceased to increase from stage 32 (as measured in the number of cells across the diameter) and it was at this stage that a sudden, large increase in length was observed in the developing ulna. The consolidation of the perichondrium appeared to be a progressive phenomenon in that the flattened cell zone at stage 32 was surrounded by a perichondrium of overlapping cells. Just as at stage 30, these overlapping cells allowed limited radial expansion which was necessary since the flattened cell zone

was continually spreading into areas which had previously been occupied by rounded cells. No perichondrial structure was observed around the rounded cell zone and radial expansion proceeded.

The ulna at stage 32 was essentially a perfect minature of the adult bone but changes still took place at the cellular level which had influences upon it's growth and morphogenesis. The perichondrium around the centre of the diaphysis transformed into a bilayered periosteum at stage 33. This structure consisted of an inner layer of rounded, possible osteogenic, cells which exhibited extensive cell-cell contacts and had little ECM, and an outer layer of very elongated fibroblastic cells which showed both areas of cell-cell contact and areas where the cells were separated by strips of electron-dense material. These electron-dense strips are thought to contain collagen fibres, elastin fibres and glycosaminoglycans and appear similar in nature and location to the "delicate white fibres" described by Fell (1925). The perichondrium around the flattened cell zone gradually became more distinct and an overlapping structure was found around the cell zone where radial expansion slowed down.

The perichondrium/periosteum is thought to influence the rate at which longitudinal growth proceeds by exerting tension on the growing cartilage in a manner similar to that of the periosteum of the chicken radius as shown by Crilly (1972). If part of the perichondrium was removed from an ulna aged between stage 32 - stage 34, an overgrowth was always found when compared with controls. It was observed that when the perichondrium/periosteum was cut it appeared to spring back and it is possible that the electron-dense strips between cells in the outer layer, which are thought to contain collagen and elastin fibres, are responsible for this tension.

As a long bone rudiment developed, it not only changed shape, it also grew larger. Cartilage morphogenesis and growth are closely associated and come about by a combination of cell division, cell hypertrophy and the secretion of ECM. In any study of cartilage morphogenesis it is essential therefore to determine what role each of these parameters play.

By assuming that a rudiment was essentially a long cylinder with a partial sphere at either end it was possible to obtain estimates for the volume of the rudiment at various stages. Also, using camera lucida drawings of histological sections, it was possible to obtain values for the volume of each cell type, the volume of ECM associated with each cell type, the volume of each individual cellular zone and an estimate of the total number of cells in each zone. A calculation of the relative percentage of growth (i.e. increase in volume) due to each parameter could then be estimated. When this was performed it was found that cell division accounted for 5.5%, cell hypertrophy accounted for 37.75% and matrix secretion accounted for 56.75% of the total increase in volume. It would appear therefore that ECM secretion plays the largest role in the growth of an ulna.

The small amount of growth provided for by cell division may be expected since cell division can be knocked out by X-irradiation and the rudiment continues to increase in length by up to 90% of controls (Biggers and Gwatkin, 1964).

Although cell hypertrophy was not the largest factor involved in cartilage morphogenesis, it seems likely that it may be the main factor involved in the increase in length observed. Cell hypertrophy *in vivo* involved a large increase in volume which was normally reflected as an increase in the short axis of the flattened cell.

However, when isolated flattened cell zones underwent hypertrophy it occurred in a radial direction. This implies that, *in vivo*, the process of cell hypertrophy was directed into a longitudinal direction. It was not coincidental that as soon as cell hypertrophy began a perichondrium formed which constrained radial expansion and enhanced longitudinal growth. The main function of the perichondrium at this region, therefore, was to constrain radial expansion in the diaphysis and thereby direct the dilation caused by cell hypertrophy into longitudinal growth. This constraining effect of the perichondrium is the basis for the hypothesis of "directed dilation" as proposed by Wolpert (1982).

The finding that ECM secretion was the main factor involved in cartilage growth and morphogenesis was perhaps not surprising since every cartilage cell is capable of secreting ECM. Although ECM secretion may influence morphogenesis in every zone it's main point of action appeared to be at the flattened cell zone where it determined both cell shape and the directionality of growth. The role of the flattened cell ECM in determining cell shape has already been discussed and appears to involve polarised secretion of matrix. Preliminary observations from experiments where a cube of flattened cells were rotated through 90° suggest that the ECM continues to be secreted and organised parallel to the long axis of the flattened cell with the result that during the culture period the cube transformed into a rectangle. This result suggests that the secretion of ECM in a polarised manner by flattened cells *in vivo* is more conducive to an increase in length rather than an increase in width.

Although the above results answered the initial aims of this thesis, various other morphogenetic problems were investigated, these included the role of cell shape during chondrogenesis and

a comparison of *in vivo* and *in vitro* chondrogenesis.

During development, rounded chondrocytes gradually mature into flattened and then hypertrophic chondrocytes, do these differences in shape reflect differences in any other cell property? This problem was investigated in both the intact rudiment and when the cells were dissociated and grown in cell culture. Autoradiographic studies of the intact rudiment, using ^{3}H-thymidine, demonstrated that cell division was confined mainly to the rounded cell zone. Over a 24 hour culture period, almost every rounded cell was capable of dividing whilst only about 10% of flattened cells took up the label. Very few hypertrophic cells were found to incorporate label and the location of those which did suggested that the label was taken up just prior to the cells undergoing hypertrophy implying that DNA synthesis may be a pre-requisite for cell hypertrophy. When cell kinetic studies, similar to those of Kember (1972, 1973, 1978), were performed, it was found that differences in cell shape also reflected differences in cell cycle times. Rounded cells had a mean cell cycle time of approximately 15 hours whilst flattened cells had a mean cell cycle time of approximately 18 hours. Therefore, it would appear that the change in cell shape from rounded to flattened not only caused approximately 90% of the flattened cells to withdraw from the cell cycle, it also caused the cell cycle time to increase.

Withdrawal from the cell cycle appeared to be a function intrinsic to the rudiment since rounded, flattened and hypertrophic cells all divided when grown in cell culture. With increasing time in culture it was found that some cells de-differentiate into fibroblastic cells which ceased to secrete a cartilage-specific ECM, however, the only difference observed between cells from the different zones was in the time taken for de-differentiation to begin. This

result implied that rounded cells were the most immature cells since, when grown at low cell density, ($2x10^5$ cells/tissue culture dish) they de-differentiate within 1-2 days whereas flattened cells took at least 4 days. Hypertrophic cells did not de-differentiate to any extent.

When rounded or flattened cells were grown at a high cell density, ($2x10^5$ cells/10 μl drop - the Micromass system of Ahrens et al, 1977) de-differentiation was only observed in peripheral cells. Central cells which were closely packed and polygonal in shape continued to secrete a metachromatic ECM. This observation has been interpretated as being a density-dependant phenomenon in that chondrogenesis will only proceed if the cells are plated out above confluence. Similar observations have been reported in differentiating cartilage (Umansky, 1966; Caplan, 1970; Ahrens et al, 1977) and has led to the suggestion that a high cell density in vitro allows "histogenic interactions" to occur. The pre-cartilage condensation is also composed of closely packed cells and Solursh et al (1978) have suggested that histogenic interactions are necessary for chondrogenesis in vivo to proceed. However, Glowacki et al (1983) have demonstrated that cartilage specific ECM secretion in already differentiated chondrocytes could be influenced by cell shape and that no cell-cell contact was required. Therefore, the possibility that the initial stages in chondrogenesis could also proceed without histogenic interactions was investigated.

When stage 23 non-chondrogenic limb mesenchyme cells were grown on a semi-adhesive substratum, poly(HEMA) at a low cell density ($2x10^5$ cells/dish) they attached, remained rounded and never came into contact with other cells. The results from this study have already been published (Archer et al, 1982) and the general conclusion was that maintaining the cells in a rounded cell configuration was

conducive to the secretion of a sulphated ECM and, thus, presumably cartilage. Therefore, histogenic interactions are not necessarily a pre-requisite for chondrogenesis.

If stage 23 cells were cultured on normal tissue culture dishes at a low cell density, they became fibroblastic and secreted less sulphated ECM than control cells - this process may be similar to the de-differentiation step observed in differentiated chondrocytes. When stage 23 cells were grown at a higher density ($5x10^4$ cells/10 µl drop) the culture was found to produce areas of chondrogenic and non-chondrogenic tissue. When stained with alcian blue (pH 1.0) the chondrogenic areas were found to consist of "cartilage nodules" containing polygonal chondrocytes separated by ECM and surrounded by cells elongated in a whorled fashion. These nodules appeared very similar to the whorled appearance of early cartilage rudiments, when viewed in transverse section, *in vitro*.

Several authors have stated that chondrogenesis *in vitro* is similar to chondrogenesis *in vivo* (Ede *et al*, 1977; Ahrens *et al*, 1978) and Weiss and Moscona (1958) in particular have suggested that the pattern of chondrogenesis observed is due to intrinsic properties of the constituent chondrocytes. Weiss and Moscona dissociated and cultured committed pre-chondrogenic mesenchyme from the limb and from the sclera of the eye. Their results demonstrated that limb mesenchyme always formed whorled cartilage whilst scleral mesenchyme always formed a flat sheet. These experiments were repeated in this thesis, using the Micromass technique, and the results were confirmed, however, when the experiments were expanded, by mixing cell types or plating at low densities, the idea of an intrinsic cell property could no longer be substantiated. If scleral mesenchyme was plated out at a medium density ($5x10^4$ cells/10 µl drop) or mixed with heart fibroblasts,

cartilage clumps and nodules were produced. In contrast, if only the central core region of the limb mesenchyme was plated out, at high density (2×10^5 cells/10 μl drop) a flat sheet of cartilage was produced with no whorling (Ahrens *et al*, 1979). These results implied that the patterns of chondrogenesis observed were dependant on the initial plating density or the cell type plated out and was not an intrinsic property.

Further evidence became available from the above studies to suggest that chondrogenesis *in vivo* and *in vitro* were two different mechanisms. The production of a cartilage nodule *in vitro* required an aggregation of cells prior to ECM secretion, (Ahrens *et al*, 1977) therefore, the whorled pattern formed independantly of ECM secretion. On the other hand, whorling *in vivo* was only observed after ECM secretion had begun and is probably dependant upon it. It seems likely, therefore, that chondrogenesis *in vitro* bears only superficial similarity to chondrogenesis *in vivo*.

The results presented in this conclusion have answered the main aims of this thesis, i.e. how is each cellular zone set up and maintained, how is the final long bone morphology produced and what role do the parameters cell division, cell hypertrophy and ECM secretion play in cartilage growth and morphogenesis. In addition, some insights into the dependance between cell shape and the expression of a cartilage phenotype, and the relationship between chondrogenesis *in vivo* and *in vitro* have also been gained. However, since this thesis has dealt almost entirely with cartilage after the secretion of ECM had begun, no attempt has been made to discover why or how the initial condensation formed, and since there are conflicting reports on this formation this system is now ripe for further investigation.

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