The Effect of Muscle Paralysis on the Development of the Skeleton of the Chick Embryo

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In The Name Of God
The Beneficient, The Merciful

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SUMMARY

In order to study the effects of paralysis on the development of the skeletal system in the chick embryo, embryos were paralysed by periodical administration of decamethonium bromide at 6, 8 and 10 days of incubation and were compared with controls to which saline was administered.

1. Experimental embryos showed retardation of the developmental stages of Hamburger & Hamilton (1951) and were of smaller size and weight.

2. Whole mount clearing showed that experimental embryos had scoliosis, cartilaginous or even bony fusion of cervical vertebrae, the knee joint and some other joints, distortion of the pubis and scapula, and failure of chondrification of the patella and the plantar tarsal sesamoid bone.

3. Linear measurement showed reduction in length of long bones, e.g. femur, tibia, humerus, ulnar and radius and lower and upper beaks in experimental embryos. The upper beak showed a greater reduction than the lower.

4. Volumetric measurement of the tibia showed a reduction in all tissues in experimental embryos and a marked reduction in cartilage formation and resorption between the 13th and 14th days of incubation.

5. Timings of onset of invasion by the osteogenic bud, of ossification assessed histologically and of calcification assessed by alizarin staining were similar between control and experimental embryos.

6. Histological and scanning electron microscopical study of the tibia revealed no changes between the two groups until the 11th day of incubation. From 12 days onwards:-

   i) less periosteal bone formation occurred in the experimental embryos.

   ii) cartilage resorption was not progressing in the experimental embryos at the extent of their corresponding controls

   iii) blood vessels in the Haversian canals were larger in diameter in the experimental embryos.

   iv) the tibiofibular interosseous ligament and fibular crest of the tibia regressed in experimental embryos.

7. Muscle fibres were atrophied and lost their orderly arrangement in the experimental embryos.

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

NORMAL DEVELOPMENT OF AVIAN LONG BONES

For greater understanding of bone development many investigators have studied the embryonic chick skeletal system, particularly that of the limbs. Fell (1925) presented a detailed optical microscopic description of limb development in the embryonic chick which in a purely descriptive sense, has not been surpassed since, but amongst the more recently published work controversial points do exist. In this section it is intended to review the literature in this field. Special points about the development of long bones, particularly the tibia, will be taken up later when these events are of particular relevance to this project.

Cartilage model

The embryonic chick limb arises as a local thickening in the somatopleure. When this prechondral mass of mesenchyme cells is first formed, the cells are still undifferentiated (Fell, 1925). The cells at the centre of the developing limb are the first to exhibit traces of differentiation into chondroblasts and they become distinguishable from the surrounding peripheral region. The cells become elongated in a direction at right angles to the long axis of the cell mass, while at the same time narrow intercellular spaces appear due to the secretion and accumulation of extracellular material. Towards the extremities of the limb bud, the cells are smaller and more

irregularly arranged. Enveloping the shaft is a distinct layer of oblong cells, which is the rudimentary perichondrium. These cells are continuous with the peripheral mesenchyme.

The next step in development is characterised by the formation of three zones of cells. The cells in and around the central region of the diaphysis enlarge and cease to divide (hypertrophic zone). As growth proceeds the hypertrophied cells increase to nearly three times their original size. The second zone lies on either side of the first and consists of longitudinally flattened, plate like cells many of which are in mitosis. The third zone is the epiphysial region which is composed of round or polyhedral cells undergoing very active proliferation. Streeter (1949) describes similar zonation in the cartilage model of the human embryonic humerus.

The timing of onset of hypertrophic cell formation in the mid portion of the cartilage model is important and has been reported by several investigators for the embryonic chick tibial diaphysis. The earliest time of onset of hypertrophy of central cells in the tibial and fibular rudiment was reported by Archer and Tarcliffe (1983) to be Stage 27 according to the morphological stages described by Hamburger and Hamilton (1951). Von der Mark et al. (1976a) observed hypertrophic cells in the mid diaphyseal region of tibia in the embryonic chick at Stage 29(6.5 days) as did Abu-Hijleh (1987). Lutfi (1974) reported that hypertrophic cartilage cells were seen in the mid diaphyseal zone of the

tibia at day 7 of incubation (approximately Stage 30) and Lansdown (1968) revealed that cells in the mid diaphysis of tibia were undergoing hypertrophy at day 8 of incubation (approximately stage 32).

Perichondrium to periosteum

Morphological changes are observed in the overlying perichondrium of the developing cartilage model of the embryonic chick tibia, concomitant with the establishment of the three different cell zones. The timing of these changes is again a matter of controversy between different investigators.

Lansdown (1968) has reported that the perichondrium at late day 7 of incubation (Stage 31 approx.) showed two layers while Scott-Savage and Hall (1979); Osdoby and Caplan (1981) and Abu-Hijleh (1987) have described this at Stage 29 while Archer and Tarcliffe (1983) reported a distinct bilayering of the perichondrium at Stage 27. At this time according to previous investigators differentiation of the perichondrium into the bilayered periosteum occurs. The new bilayered periosteum consists of a dense, outer layer of fusiform or spindle-shaped cells which secrete closely packed, mainly longitudinally directed collagen fibres. This layer forms the fibrous layer of periosteum, which is very diffuse towards the extremities of the cartilage model and gradually fades into the general connective tissue of the limb. The inner layer

of the periosteum usually consists of two to three layers of closely packed, round to oval shaped cells. This is also called the osteoblastic layer. The two layers are fairly sharply defined from one another and from the underlying cartilage only in the mid diaphyseal region of the shaft of the long bone (Fell, 1925). The differentiation of the perichondrium into the periosteum advances towards the epiphysis of the long bone as development proceeds.

The potential of the periosteum

Fell (1932) recognised the potential of the osteoblastic layer of periosteum for bone formation. In her experiment periosteum was removed from the middle third of various long bones of 6 day chick embryos and cultured in vitro. It was found that periosteum in its earliest stages of development is able to form bone even when deprived of its normal association with cartilage and of a blood and nerve supply. Mareel (1967) claimed that the hypertrophic cartilage of a transversely divided 9 day embryonic chick tibia was able to induce the differentiation of osteoblasts when placed against the fibrous periosteum of a second intact 9 day tibia. He observed the accumulation of cells at the contact zone and termed them "contact cells", believing them to be young osteoblasts on the basis of their morphology. However, he did not report any of the features taken as diagnostic for the identification of osteoblasts, such as the presence of

alkaline phosphatase, or osteoid, or osseous extracellular matrix. This conclusion was, therefore, considered unreliable by Scott-Savage and Hall (1980) who in a study concerning the differentiative ability of the tibial periosteum from the embryonic chick, attempted to show experimentally that only the inner layer of the periosteum is osteogenic. Thus, they repeated the original experiment of Mareel (1967), again using the same morphological criteria to establish the identity of the cells at the contact zone. It was found that the fibrous layer of the periosteum underwent hyperplasia to form fibroblast-like cells at the contact site, due to close contact between this layer and the hypertrophied cartilage. The contact of the graft with the outer fibrous layer of the periosteum was not sufficient to allow additional osteoprogenitor cells to accumulate or to allow those present to differentiate into osteoblasts or to deposit ostoid and, therefore, they concluded that the outer layer of the periosteum was not osteogenic. This experiment relies totally on the assumption that hypertrophic cartilage brings about osteoblastic differentiation in the periosteum.

The onset of ossification

The bones of the embryonic chick limbs, in particular the tibia, have provided much useful information about the onset of ossification.

A survey of the available literature reveals several opinions about the timing of the onset of ossification in the chick embryo. Ossification of the chick tibia, for example, is described as commencing between 6-9 days of incubation (Stages 28-34). This wide range of opinions may reflect differences in the methods used to determine the onset of ossification as well as some differences in the strain of chicks used. Based on information obtained from Alizarin-stained whole mounts, the various investigators have reported the time of onset of ossification as follows:

Rumpler (1962) reported that the first centres of ossification were observed at 8 days about stage 32 of incubation in limb bones, e.g. humerus, ulna, radius, femur, tibia and fibula. Holder (1978) reported that ossification in the embryonic chick tibia began at around Stage 32-33 or 8 days. Mortimer (1970) found that bone first became visible in the long bones of the limbs and that this occurred at about Stage 31-32, i.e. about 7-7.5 days.

Lansdown (1968) established the onset of ossification at about 8 days of incubation in the embryonic chick tibia, using classical histological techniques. Lutfi (1974) using the same kind of techniques reported the first appearance of a thin collar of bone ensheathing the hypertrophic region in the middle portion of the cartilage model of the tibia at about day 7 of incubation. Hall (1970) used histochemical methods involving Alcian blue-chlorantine Fast Red (Lison, 1954); Masson Trichrome

(Pantin,1962); 0.01% Toluidine Blue (Ham and Harris, 1950); Periodic Acid-Schiff Reagent (Pearse, 1960), and observed the time of onset of ossification of the tibia at about 7 days of incubation. Scott-Savage and Hall (1979) used the osteoid-specific staining method described by Ralis and Ralis (1975) to detect osteoid and found that the freshly secreted, unmineralised product of fully-differentiated osteoblasts (ostoid) was first detected adjacent to the hypertrophic cartilage at Stage 30, in the embryonic chick tibial diaphysis. Osdeby and Caplan (1981) with the aid of histochemistry with alizarin red S reported that the initial appearance of calcium in the developing chick limb (tibia) can be observed at Stage 33. Archer and Tarcliffe (1983) used histological methods and observed the onset of osteogenesis which occurred at the same time in both tibia and fibula of the embryonic chick adjacent to hypertrophic cartilage at the mid diaphysis between Stages 31-32. Gaytan et al. (1987) also reported the formation of the osseous ring of collar bone in the mid diaphysis of tibia of the embryonic chick at 9 days of incubation. Abu-Hijleh (1987) has observed the first sign of periosteal ossification at Stage 30 (7 days) in the mid diaphyseal portion of the embryonic tibia. Von der Mark et al. (1976, a & b) and Von der Mark and Von der Mark (1977) used a more sophisticated technique to study the onset of ossification on the embryonic chick tibia diaphysis. They prepared antibodies specific to chick type I and II collagens and

used them to localise these collagen types in the developing tibia. They revealed that the first evidence of the presence of type I collagen, usually associated with bone, could be observed at Stage 31 in the perichondrial sheath surrounding the mid diaphyseal area of the tibia. In a later report they described the same process happening as early as Stage 28. Osdoby and Caplan (1981) have shown that alkaline phosphatase activity, a feature commonly associated with osteoblasts, could be detected at late Stage 28 and early 29 or 5.5 to 6 days of incubation.

Decker (1966) has used E.M. techniques and reported that the middle third of the cartilaginous rudiment of the tibia is surrounded by a narrow ring of bone by the tenth embryonic day and Dillaman et al. (1979), also using E.M. techniques, has reported that mineralisation of the tibia begins at about 8 days of incubation.

In summary, the onset of ossification in the tibia has been reported by different methods as follows:

1. Alizarin red stained whole mounts 7 to 8 days, Stages 31 to 33.
2. Classical histology techniques and histochemical methods 7-8 days, Stages 31 to 33.
3. Immunofluorescence technique to localise type I collagen, Stages 28-31.
4. Alkaline phosphatase activity, Stages 28 and 29.
5. Electron microscopy, 8-10 days, Stages 33-35.

Further development of periosteum

As development proceeds, the cells of the inner layer of the periosteum continue to deposit osteoid and soon a thin compact cylinder of collar bone is formed around the mid-diaphyseal portion of the shaft of the developing long bone. This central bony cylinder increases in thickness by the deposition of fresh matrix on its outer surface by neighbouring osteoblasts and also by fusion of younger, peripheral lamellae, until a fairly stout layer of bone is formed around the middle region of cartilage. At this stage, the osteoblastic layer becomes somewhat diffuse (Fell, 1925).

Dillaman et al. (1979) using scanning electron microscopy, have confirmed and extended the original definitive account of this developmental process by Fell (1925). Both authors describe the transition from a single cylinder of bone surrounding the cartilage model to one of several layers by the formation of columnar radial trabeculae on the outer surface which support a second mineralised cylinder.

Fell (1925) observed that the diameter of the diaphysis became larger at the extremities than at its middle, where cell division ceases early. Thus, the fibroblastic layer of the periosteum becomes drawn away from the cartilage model in its mid-portion, due to the enlargement of the ends of diaphysis and in longitudinal section appears as two almost straight lines on either side

of the somewhat hour-glass shaped cartilage. The osteoblasts cease to form a compact layer around this middle part of the cartilage and became scattered in the enlarged space between the inner periosteal collar of bone and the fibroblastic layer of the periosteum.

The vascularisation of the osteoblastic layer

The osteoblastic layer becomes richly vascularised, and the osteoblasts become broken up into intervascular groups, which give rise to short irregular trabeculae of bone fused at one end with the central cylinder and having a tendency to radiate from the original centre of ossification. Through the ordered deposition of the trabeculae in rows which parallel the long axis of the bone and their fusion into ridges, canals are formed and Haversian systems are initiated (Fell, 1932). This was illustrated by scanning electron microscopy by Dillaman et al. (1979). As the vessels increase in length and diameter, and the space intervening between the fibroblastic layer and the inner tube of bone widens, the transverse trabeculae become correspondingly longer, and at the same time a second series of laminae are formed concentric with the circumference of the cartilage. A series of incomplete tubes is thus gradually built up around the blood vessels. As vascular channels form on the exterior of the mineralised cylinder, ridges develop and so another cylinder is laid down (Dillaman et al., 1979).

The onset of invasion of the osteogenic bud

The onset of invasion of the osteogenic bud signals the initiation of an important developmental event in the long bone, leading to the formation of the marrow cavity by resorption of uncalcified cartilage. Streeter (1949) adopted the presence of invasion of the osteogenic bud in the humerus as an arbitrary boundary-line, marking the end of his terminal developmental horizon xxiii in man. He classified the specimen having an osteogenic bud as fetus rather than embryo. The timing of invasion of the tibia in the avian embryo has been variously quoted as taking place between Stage 34-36 or 10 and 11 days of incubation inclusive (Lutfi, 1971; Von der Mark et al., 1976a; Dillaman et al., 1979; Archer & Tarcliffe, 1983; Morrison, 1984; Gaytan et al., 1987). This invasion takes place over a very short time range - about 2 days. This range may reflect a variety of factors: biological variation between and within the species studied, differences between methods in determining the actual time of onset of invasion. This apparent constancy in timing is not limited to avian embryos. Streeter (1949) reported constancy in timing of the onset of osteogenic bud invasion in the human embryo and Johnson (1980) found similarly in the mouse. This constant timing of invasion of the osteogenic bud in a given species of vertebrates, suggests that it may be a genetically programmed event. Secretion of specific macromolecules by the chondrocytes of the hypertrophic zone

has been suggested as a causative factor (Floyd et al., 1987; Cowell et al., 1987).

The sequence of events in the resorption of the cartilage model in the avian embryo is very similar to that in a mammalian embryo, but unlike the mammal it is not replaced by bony trabeculae and does not calcify prior to erosion and resorption (Wolbach & Hegsted, 1952). Invasion of uncalcified cartilage is relatively rare and occurs in few situations. One situation is the resorption of the uncalcified cartilage model of long bones in the avian embryo during the formation of the marrow cavity. Another situation is during the penetration of capillaries into the lacunae of degenerating, hypertrophic chondrocytes in the calcification of the epiphyseal plate in growing rats as reported by Schenk et al. (1967). Pathological resorption of uncalcified articular cartilage in rheumatoid arthritis has also been reported by Sorrell and Weiss (1980).

Mechanism of invasion of the osteogenic bud

The invading osteogenic bud is made up of blood vessels and accompanying cells. Erosion begins in the middle region of the cartilage model. The blood vessels of the cancellous bone at intervals burst through the central osseous cylinder carrying with them osteoblasts and strands of connective tissue, and make their way into the substance of cartilage, the matrix of which appears to dissolve before their passage. This is the description of Fell (1925) and implied that the blood vessels were the driving

force of the invasion. Cameron (1961) and Schenk et al. (1967) suggested that the capillary extensions (cytoplasmic processes of endothelial cells) themselves may actively resorb uncalcified cartilage and therefore may be the driving force.

However, Dodds (1932) stated that connective tissue cells are seen to advance ahead of the endothelial cells of blood vessels, forming a thin investment for the advancing capillary loop. Silvestrini et al. (1979), Sorrell and Weiss (1980) and Howlett (1980) have reported that the accompanying cells of the blood vessels are responsible for the actual resorption of the uncalcified cartilage. They also recognised the importance of blood vessels in the formation of the marrow cavity, although the evidence was not direct. For example, Sorrell and Weiss (1980) reported that they had not observed endothelial cells in contact with the cartilage but that these were always separated by a few layers of cells. They suggested, therefore, that endothelial cells may not be directly responsible for cartilage resorption.

Fell (1929) in her pioneering work on the organ culture of explanted chick embryo femora reached the conclusion that progress beyond the periosteal collar stage in vivo depended upon the invasion of the developing marrow cavity by blood vessels. Her explanted specimens were able to continue their anatomical development on the same general lines as in the normal limb, but did not develop a

marrow cavity. Thus, the cells of the periosteal collar were regarded as being excluded from the marrow cavity by the layer of bone which they have laid down.

This view was not shared with Johnson (1980) who illustrated the role of the cells of the osteogenic bud of periosteal origin, in the development of the marrow cavity. He cultured mouse limb buds aged 12 to 17 days and observed that in those explanted at 15 days or later where the centre of the rudiment cartilage had broken down leaving only a few strands of matrix indicating the site of the original cartilage cells, a marrow cavity developed. He concluded that even in the absence of a blood supply, long bones could develop a marrow cavity and suggested that there may be two types of invasion: one by mesenchymal cells and the other by blood vessels. In vivo both types of invasion take place while in culture there are no blood vessels to invade.

In summary, it would seem that there are two possible mechanisms for resorption of cartilage: (i) the blood vessels are directly involved in the resorption of the cartilage matrix and (ii) the blood vessels are indirectly involved and accompanying cells erode the matrix while the blood vessels sprout and grow into the spaces. The evidence in favour of the second possibility, as mentioned, is greater than the first.

Type of cell responsible for cartilage resorption

In the light microscopic studies of Fell (1925) and

Lutfi (1974) concerning the resorption of avian embryonic cartilage, the cells responsible for cartilage resorption were not clearly identified, but were assumed to be mononuclear cells, either of vascular or of connective tissue origin.

Dodds (1932), Schenk et al. (1967) and Howlett (1980) have suggested that different cell types may be involved in the resorption of uncalcified and calcified cartilage. They proposed that calcified cartilage was removed by multi-nuclear cells, and uncalcified cartilage by mononuclear cells. The studies of Silvestrini et al. (1979) and Sorrel and Weiss (1980) are in agreement with the concept of mononuclear involvement in uncalcified cartilage removal.

Silvestrini et al. (1979) claim to have observed one mononuclear cell type involved in the resorption of uncalcified cartilage in the embryonic chick tibia which they call clear cells. They report that multinucleated osteoclasts are primarily seen in contact with bone and are only rarely seen in contact with uncalcified cartilage, a view supported by Sorrel and Weiss (1980), who studied the cartilage and marrow interface, suggesting that resorption always occurs in this area. They were also in complete agreement with Fell (1925) and Lutfi (1971) that there was no internal dissolution of cartilage. In the light of their histological and transmission E.M. study they noted that two cell types were found to be principally associated

with the cartilage-marrow interface: the more frequent are cells containing extensive amounts of rough endoplasmic reticulum and the remainder are cells containing many vesicles. They concluded that the removal of uncalcified cartilage is mediated by mononuclear cells which invade the cartilage model. The predominant cell type has the characteristics of a protein synthetic cell and as such could be secreting chondrolytic enzymes. The second type, consisting of vesiculated cells, is possibly the same type of cell in a different state of activity. The former may be macrophages in an actively secreting state and the latter may be macrophages in an actively phagocytic state.

Sorrel and Weiss (1980) did not provide direct evidence for resorptive action by the mononuclear cells, but on the basis of the following observations they believed these cells to be responsible for cartilage resorption: (i) mononuclear cells were the predominant cell types seen at the cartilage-marrow interface except for restricted regions (e.g. region of endochondral bone formation or acellular regions); (ii) these cells were intimately associated with cartilage, often sending small processes into the matrix; (iii) they had the appearance of invasive cells and occupied lacunae near the interface; (iv) there were changes in cartilage matrix near these cells: the amount of sulfated material is reduced and collagenous fibres may assume an amorphous texture; (v) few other cells were present.

Endochondral ossification

There are not many studies which have been done to investigate the process of endochondral ossification in birds which is somewhat limited. It does occur in the vertebrae (Lillie, 1952) in the cartilage bones of the skull (Wolbach & Hegsted, 1952) and in a limited area of long bones (Fell, 1925). The cartilage model of the developing long bone of the chick embryo is invaded by the osteogenic bud in the mid-diaphyseal region, but unlike the situation which exists in mammals the cartilage is resorbed and excavated without being replaced by bone except in the epiphysis and the distal one-sixth of the diaphysis (Fell, 1925; Wolbach and Hegsted, 1952; Romanoff, 1960). The timing of onset of endochondral ossification in long bones is a matter of controversy, for example endochondral ossification of the chick tibia being reported to begin at Stage 39 (13 days of incubation) (Von der Mark et al., 1976), at the end of embryonic life (Fell, 1925; Lutfi, 1974) and even 7 days after hatching (Wolbach and Hegsted, 1952).

Ossification of the epiphysis in the avian long bone

Wolbach and Hegsted (1952) studied chickens from 18 to 32 days after hatching and described three zones in the avian epiphysis. These are articular, hyaline and growth plate cartilage. This arrangement essentially is the same as that found in mammals, except that in mammals the

epiphysis undergoes ossification independently of the diaphysis by a secondary centre of ossification. The epiphysis of the avian long bone does not ossify independently of the diaphysis but as a continuation of it. Wolbach and Hegsted (1952) have demonstrated that the hyaline zone of the avian epiphysis is penetrated by blood vessels and accompanying cells from the diaphysis via the growth plate. Kalayjian and Cooper (1972) and Floyd et al. (1987) in their studies of guinea pig and mouse epiphysis, showed that epiphyseal bone was formed after the osteogenic bud invaded the cartilage model from the overlying perichondrium. Howlett (1979, 1980) has reported many similarities between the growth plate of birds and mammals, such as the cytological sequence of cartilaginous cells from the resting, through proliferating, to maturation zones and cytoplasmic dense bodies in all the zones more particularly in the latter portion of the proliferating and the prehypertrophic chondrocytic zone. The invading vessels and cells which penetrate the growth plate of avian long bone are reported to branch and anastomose with one another and are thought to be responsible for the eventual removal of the cartilaginous epiphysis (Wolbach and Hegsted, 1952).

Factors regulating or controlling ossification

Isolated portions of the osteoblastic layer of periosteum are able to form mineral in culture, free from the influence of cartilage, nerve and blood vessels (Fell,

1932). To clarify and expand on this observation, two types of "in vivo" experiments were designed by Holder (1978). The first involved transplanting a piece of cartilage from a 6 day (Stage 28) long bone (i.e. cartilage model plus its intact perichondrium and a small amount of surrounding mesenchyme) into the wing of a chick embryo 2 days younger (Stage 24). Theoretically, the implant would normally ossify after a further 2 days (8 days), but if the onset of ossification was initiated by some influence outwith the cartilage, then the implant would begin osteogenesis not according to its own programme but to a new one implemented by the younger experimental host embryo. Holder found that the graft began to ossify before the corresponding bones of the host and concluded that ossification in the graft is programmed by that stage.

Secondly, Holder then carried out truncation experiments to determine whether ossification in the region near the epiphysis occurred at the expected time in the absence of any influences from the ossification centres of the extirpated central diaphyseal regions. It was thought that this might clarify whether ossification occurring proximal and distal to the initial central collar was due to programming along the complete length of the cartilage element or to a sequential induction of bone formation after the initial onset. These experiments were performed as early as Stage 24, which is only three stages after the forearm region has been defined (Summerbell, 1974), and it

is the stage when the first signs of cartilage differentiation occur in this region (Searls, 1965; Gould, et al., 1974). The results of this experiment showed that ossification did take place normally in the remainder of the limb when the central diaphysis was removed. Holder thus concluded that the osteoblasts along the shaft are programmed to produce bone matrix at specific times in limb development and there is no sequential induction, or wave of competence emanating from the initial ossification site. In this experiment the bones were truncated well before any visible changes in the central area of the diaphysis.

These experiments, however, do not establish exactly when or how the osteoblasts become programmed to begin bone matrix secretion. Holder (1978) considered there to be two possible alternatives: (i) the future osteoblasts themselves may be programmed in the young wing-bud in the same way as are the muscle and cartilage cells and may subsequently differentiate independently; (ii) osteoblasts may be induced to begin bone formation by a localised interaction with the adjacent cartilage cells, once cartilage differentiation has begun. The latter alternative suggests that the onset of ossification may be controlled by some signal passing from the cartilage cells to the adjacent periochondrial cells to promote the formation of the inner osteogenic layer. This would enable the limbs to programme osteogenesis by an indirect mechanism.

There is a close temporal and spatial relationship

between the differentiating perichondrium and the hypertrophying cartilage cells in the mid-diaphyseal region of a long bone. This has led to an inference that the enlargement of chondrocytes in this region of the cartilage model, may induce osteogenic expression in the perichondral cells.

1) Evidence in favour of independence of osteoblasts

Osdaby and Caplan (1981), by observing alkaline phosphatase activity in the perichondrium at Stage 28 and in the light of the immunofluorescent localisation of type I collagen by Von der Mark et al.(1976b), suggested that initial osteogenic activity does not require chondrocytic hypertrophy. This experiment rests on the results of investigations which reveal that the first changes in the cartilage model were observed at Stage 29 (Von der Mark, 1976a; Scott-Savage and Hall, 1979; Osdaby and Caplan, 1981), but as previously mentioned the first hypertrophic chondrocytes in the mid-diaphyseal region of tibia were observed at Stage 27 by Archer and Tarcliffe (1983).

Most recently, Caplan (1988) from a series of studies of the embryonic chick tibia concluded that osteogenic progenitor cells are observed before the formation of the cartilage model, as a layer of four to six cells, referred to as stacked cells, around a prechondrogenic core of undifferentiated cells. These osteoprogenitor cells give rise to all of the newly forming bone. Importantly, this

newly forming bone arises outside and in a manner similar to the intermembranous bone formation seen in the vault of the skull. Indeed, the cartilage model is replaced not by bone but by vascular and marrow tissue. The interplay between the osteogenic collar and the chondrogenic core provides an environment which stimulates the further differentiation of the cartilage core into hypertrophic cartilage and eventually leads to this core being replaced by vascular and marrow tissue. There is an intimate relationship between the osteogenic cells and vasculature which is obligatory for active bone formation. He stated that bone formation in long bones, such as the tibia, as well as in the vault of the skull, seems to proceed in a similar manner with vascular tissue interaction being the most important aspect of successful osteogenesis, as opposed to the presence or interaction of cartilage.

2) Evidence in favour of interaction between hypertrophic chondrocytes and osteoblasts and capillaries

Hall (1982) maintains that the control over the initial onset of osteogenesis resides not in the cells forming the bone, but in the adjacent tissue with which they interact. Hunziker et al. (1984) studied the epiphyseal plate and observed that all its cells together with their associated matrix compartment were morphologically and by implication, functionally intact at the ultrastructural level. They suggested that mineralisation and vascular invasion are controlled not by

haphazard changes in local environmental conditions but, at least in part, by the chondrocytes themselves.

Floyd et al. (1987) examined the sequence of events that is involved in the formation of the secondary centre of ossification in the distal femoral epiphysis of the CDI mouse. They observed that the hypertrophy of chondrocytes and vascular invasion happened before mineralisation of cartilage matrix. Their observation suggests that the hypertrophic chondrocytes may be synthesising a factor that is chemotactic for endothelial cells and secreting it into extracellular matrix, thus inducing capillaries to grow into the cartilaginous epiphysis toward the hypertrophic chondrocytes.

Boden et al. (1987), on the other hand, reported that in patients who have metatrophic dwarfism (a rare heritable skeletal dysplasia that is thought to result from a defect in endochondral ossification), the maturation of proliferating chondrocytes into hypertrophic cells was aborted. Capillary invasion did not occur normally and mineralisation of cartilage matrix and bone formation did not take place. Observations, on the role of chondrocytes in secretion of growth factor (Klagsbrun & Smith 1980; Lobb et al., 1986), improved techniques involving high pressure freezing, freeze substitutional fixation and low temperature embedding to study the lowermost hypertrophic chondrocytes in the epiphyseal plate (Hunziker et al., 1982; 1983; 1984) and secretion of type X collagen only in

the matrix of the lower hypertrophic zone (Grant et al., 1985; Schmid & Linsenmayer 1985) have led Cowell et al. (1987) to infer that the zone of so-called degenerating chondrocytes does not exist. Hypertrophic chondrocytes appear to be highly differentiated cells, with their own characteristic ultrastructural morphology. They synthesise and secrete into extracellular matrix a set of macromolecules that are different from those that are produced by proliferating chondrocytes. Some of the macromolecules that are produced by the chondrocytes of the lower hypertrophic zone may be directly involved in the processes of mineralisation of the matrix, capillary invasion, and formation of bone on calcified cartilage.

In summary, it can be said that it appears that the onset of ossification may not be under the control of one specific factor. It probably involves both genetic and epigenetic influences.

Longitudinal growth

In mammals the process by which the long bones continue to grow in length up to adolescence is similar to that of the replacement of embryonic cartilage models by bone, with the exception that the epiphyseal cartilage disk and its surrounding structure become organised and orientated to perform a specific function. This requires a mechanism which is termed the growth apparatus by McLean and Urist (1968). The epiphyseal face of the epiphyseal plate is made up of cartilage matrix, within which

cartilage cells are embedded. These cells continue to divide and mature and are arranged in columns. The cartilage cells become larger as they near the diaphyseal front. The matrix of this area becomes reduced to thin longitudinal and transverse septa because of the enlargement of the lacunar spaces. As the hypertrophic cells merge into the final zone, the hyaline matrix becomes increasingly calcified resulting in the restricted diffusion of nutrients and ensuing death of hypertrophic chondrocytes (Jee, 1988). Between the columns of cells the vertically orientated matrix becomes fully calcified, whereas the transverse septa fail to calcify. In the meantime, the dying hypertrophic cartilage cells are replaced by proliferation and maturation of the cells on the epiphyseal surface of the plate, and the whole process continues until growth in length of the bone is complete (Vaughan, 1981).

The histology of the avian growth cartilage has been described by Fell (1925) and Wolbach and Hegsted (1952), who stated that the flat cells (proliferative) and hypertrophic zones are present as in mammals. Lutfi (1970) studied the growth cartilage in chickens and described a similar pattern of columns as in mammals. However, unlike mammals, in which mitotic figures in the proliferative zone are limited to the upper end of the cell column, they are randomly scattered through most of the depth of the columns. He also described the location and behaviour of

stem cells in the chicken. These stem cells responsible for replenishing the cartilage are situated in the perichondrium and also within the cartilage canals in the epiphysis.

The endochondral system in birds differs from that of mammals in that there is no distinct limit between growth plate and the metaphysis (Kember and Kirkwood, 1987).

The longitudinal growth of bone depends on the activities of individual chondrocytes of the growth plate (Hunziker et al., 1987) and it is generally accepted that longitudinal growth of a long bone is dependent upon an intact growth cartilage (Haas, 1945; Ring, 1955a,b). However, Selye (1934) reported that if the distal end of the femur is removed in rats during the first few weeks of life, a new growth cartilage is formed and the growth in length is resumed.

In the chick embryo, as described previously, endochondral ossification in the long bone does not begin until around hatching, and until then the bony shaft is produced through periosteal ossification which keeps pace in longitudinal direction with cartilage growth and hypertrophy. The cartilage is the obvious pacemaker for the lengthening bony shaft (Lutfi, 1974).

Lutfi (1970) found that the fibula in the chicken continued to grow in length after excision of the upper cartilaginous epiphysis and adjacent proliferative zone, and he observed that the periosteum and proximal end of the fibular stump became connected by a thick fibrous tissue

bundle to the proximal tibial epiphysis. Thus, Lutfi (1974) concluded from these observations that an important role of growth cartilage may be to stretch the periosteum and thereby induce deposition of subperiosteal bone at the ends of the shaft.

Thorp (1988) estimated the rate of longitudinal bone growth and measured the thickness of the growth plate in the femur, tibia and tarsometatarsus of different groups of domestic fowl and reported a positive linear relation between the two.

EFFECT OF IMMOBILISATION ON LONG BONES OF ADULT MAMMALS

Loss of bone mass and reduction in longitudinal and appositional growth have been reported in mammals, including man, after immobilisation and resultant lack of muscle activity caused by weightlessness, fixation in plaster, long term bed rest, peripheral nerve injury and the use of drugs. Factors suggested as responsible for this include sudden diminution or cessation of the rate of blood flow through a bone, increased pressure within the bone which initiates extra osteonal activity, reduction of oxygen causing reduction in osteoblast activity and lack of mechanical stimuli to activate skeletal cells.

Weightlessness

Jee et al. (1983) reported the effects of weightlessness during space flight on the metaphyses of rat long bones. These involved a reduced mass of mineralised tissue and an increased fat content of the bone marrow. They considered that the most obvious factor, which may have contributed to the skeletal changes, is mechanical unloading. Simmons et al. (1983) in a similar study concluded that weight-bearing bones are at greater risk than the non weight-bearing bones. Periosteal bone loss in the diaphyses of tibiae and femurs in male Wistar rats placed in space for 18.5 days was reported by Wronski and Morey (1983). They also observed that the most significant changes were in the tibia which is normally subjected to

the greatest mechanical stress.

Fixation in plaster and long term bed rest

Loss of bone mass in man after bed rest or immobilisation in a cast has been reported on extensively (Albright et al., 1941; Minaire et al., 1974; Vaughan, 1981). Loss of long bone thickness in the rat after immobilisation has been reported by Gedalia et al., (1970); Mattson (1972) and in the rabbit by Semb (1969) and Hardt (1972). Uhthoff and Jaworski (1978) have reported bone loss in response to long term immobilisation in the dog and Kazarian and Vongierke (1969) in the monkey. Progressive loss of bone during hibernation in the bat has been reported by Whalen et al. (1972).

Peripheral nerve injury

The effect of division of peripheral nerves on longitudinal bone growth has been studied by a number of authors.

Combes et al. (1960) have reported decreased growth of the foot after sciatic nerve injury resulting from an injection needle in children and the same finding was reported by Curtis and Tucker (1960) when they studied premature children with a similar injury.

Gillespie (1954) has studied pelvic limbs of kittens and rats paralysed by nerve root section, or by peripheral nerve section and reported no change in longitudinal growth of bones but significant reduction in the weight of bones

of the lower limb. He carried out lumbar sympathectomy, in the kittens and observed that this did not affect the bony changes and concluded that these changes cannot be primarily due to a diminution of the blood supply, since an improvement in this did not prevent their occurrence. He postulated that the bone changes are due to the secondary loss of muscular activity rather than to direct result of nerve injury. Selye and Bajusz (1958) who sectioned the femoral, obturator and sciatic nerves in the pelvic region of albino rats reported no significant reduction in the length of the tibia. Graces and Santandreu (1988) have reported reduction in length of metatarsal bones but no changes in the length of tibia in rats paralysed by sciatic nerve sectioning. Schoutens et al. (1988) have studied the effect of sciatic nerve sectioning in rats and reported no changes in the length and volume of tibia and femur. Ring (1961) even reported that longitudinal growth of tibia was stimulated after sectioning of both peroneal nerves and by division of ventral nerve roots supplying the pelvic limb of the rabbit, but that it was always lighter than its fellow. Ring (1957, 1958, 1961) and Ring and Ward (1958) reported that the immediate effect of the neural lesions of poliomyelitis is to produce increased length of the bones of the affected limb and that this lengthening happens during the first year and may persist during the second year.

The foregoing authors who have observed no changes

after immediate nerve injury considered that the peripheral nervous system has no direct effect on bone growth, but that it could act through other factors, e.g. immobilisation and muscular inactivity following nerve injury. However, Troupp (1961) divided the motor roots supplying the pelvic limb in rabbits and observed reduction in longitudinal growth of femur and tibia and found that the more motor roots of the lumbar region which were cut, the greater was the diminution of femoral growth and to a lesser extent tibial growth. In his experiment only one animal was assessed at each time.

Effect of blood flow on bone activity

The importance of blood flow for the activity of osteogenic cells is well known. Increased blood flow is thought to stimulate osteonal remodelling which is essential for the formation of healthy bone (Little, 1973) and to stimulate longitudinal bone growth (Goetz et al, 1955; Grace and Santandreu, 1988). Ring (1961) suggested that the increased length of long bone immediately after nerve injury may be the result of hyperemia. Bone loss and reduction in new bone formation has been thought to be the result of impaired blood circulation during severe muscle inactivity (Little, 1973).

Many clinicians have, therefore, tried to increase the blood flow to bone in order to prevent bone loss or to increase the length of long bones. In an attempt to stimulate the growth of a polio shortened extremity, Harris

(1930) performed a lumbar sympathectomy in a ten year old girl as a result of which the leg length increased by 0.5 inches relative to other limb. Other authors, including Bisgard (1933) and Fahey (1936), failed to achieve significant growth stimulation after sympathectomy even though there was an increase in the temperature of the extremity. However, Goetz et al. (1955) claimed that increased blood supply by sympathectomy stimulated longitudinal bone growth by 2.5% 30 days and 4.25% 80 days post operation in rabbits.

Effect of mechanical stress on bone and cartilage

Murray (1936) stated that intermittent pressure leads to an increase in the total quantity of bone present, and constant pressure leads to a decrease. It is now known that the response to cyclic mechanical stimulation of various cell types, e.g. myoblasts, fibroblasts and periosteal progenitor cells is increased DNA synthesis and cell division (Curtis and Seehar, 1978; Hall, 1979, 1985). The forces to which most skeletal elements are exposed are derived from respiration, blood flow or locomotion, all of which produce cycles, often of very short duration, of alternating tension and compression (Lanyon et al., 1975; Goodship et al., 1979). This intermittancy of mechanical stress is the physiological stress to which cartilage and bone respond (Hall, 1985).

Bassett (1971) claims that from the data available

the stimulus for bone formation and destruction involved in bone remodelling appears to be largely electrical in nature. He suggests that the direct effects of functional processes on bone cells are of minor importance but that control of remodelling is largely dependent on an indirect effect when mechanical energy is transduced to electrical energy by the bone matrix and vasculature.

Electro Mechanical Property of Bone and Cartilage

Research carried out on the electromechanical properties of bone over the past three decades have established the piezoelectric (generation of electrical potential by mechanical stress) nature of bone and have identified collagen as the generating source in bone (Hastings and Mahmud, 1988). Currey (1968) claimed that the transduction of mechanical to electrical energy is the most likely mechanism behind the control of osseous cell activity involved in bone growth and remodelling and Hall (1975b) reported that mechanical effects on cartilage and bone may be mediated by altered electrical activity. Cartilage has also been shown to respond to changes in electrical activity (Currey, 1968; Gjelsuik, 1973a,b; Hall, 1983, 1985).

Electrical energy has been used to directly stimulate bone healing. Spadaro (1977) reviewed 119 publications directly concerning the electrical stimulation of bone growth, fracture healing and bone regeneration. About 95% showed positive results despite an extraordinarily wide

selection of experimental techniques and models.

Lavine et al. (1977), Becker et al. (1977), Masuriek and Eriksson (1977), Inoue et al. (1977) and Connolly et al.,(1977) applied constant direct current to non-union fractures, congenital pseudoarthrosis and fresh fractures. All reported that electricity in its various forms does stimulate osteogenesis. Goh et al.(1988) reported that electrical stimulation increased new bone formation and also increased breaking strength and resistance to bending in fracture healing in rabbits. Borsalino et al. (1989) used low frequency pulsing electromagnetic fields to stimulate union of bone in patients with femoral intertrochantric osteotomy and reported that this significantly increased osteotomy healing.

Transduction of mechanical forces to biochemical signals in chondrocytes

Uchida et al. (1988) claimed that mechanical forces transduce to biochemical signals in chondrocyte cells. Long-term exposure of chondrocytes to mechanical loading caused increased synthesis of matrix. They concluded that mechanical stress can alter bone growth by modulating the metabolism of growth of cartilage cells.

EFFECT OF IMMOBILISATION ON THE DEVELOPMENT OF THE MUSCULO-SKELETAL SYSTEM OF CHICK EMBRYO

As mentioned previously, muscular activity as an extrinsic or environmental factor is able to interfere with the form and size of bone and cartilage. It is often possible to evaluate the role of an element in a system by experimentally witholding it from the system and observing the resultant effects. Three different general methods have been used by various authors in order to immobilise chick embryos and to study the effect of immobilisation on the development of synovial joints, muscles, bones and tendons. These methods are:

a) culturing the embryonic limb bud in vitro; b) grafting the limb bud to the chorioallantoic membrane, and c) administration of neuromuscular blocking agents.

General features of immobilised chick embryos

Murray and Drachman (1969) injected 7 day chick embryos with botulinum toxin and studied them at day 19 of incubation. They reported that the paralysed embryos were smaller and lighter in weight than the control embryos. Their sizes were equal to untreated 10 day embryos, otherwise they had matured normally for their age and had no bizarre malformations. The paralysed embryos showed severe fixation of many joints, and protrusion of the tip of the lower jaw . The facial part of the skull was narrowed while the cranial part was of normal width.

Skeletal muscles were strikingly shrunken and fatty. The skin, feathers, subcutaneous tissue and internal organs were unremarkable and the yolk sac was partially retracted into the abdominal cavity, as occurs normally on the 19th day of incubation.

Sullivan (1966, 1967, 1971, 1974) has studied the development of paralysed chick embryos caused by administration of different paralysing agents and has found that the rate of development was retarded. A number of embryos exhibited deformation in the shape of the body or abnormal posture of the limbs. The neck was markedly bent to the right and immobilised, while the trunk often had a compressed appearance associated with the normal curvature of the vertebral column. The embryos were stunted and the lower jaw was also protruded.

Retardation in growth, distortion of the body, protruded lower beak, significant reduction of body weight and subcutaneous inflammation are the features which were observed by Persson (1983) when he immobilised the chick embryo by injection of decamethonium iodide into the air sac, a technique first used by Hall (1975a). He did not observe any obvious malformation of the trunk or limbs externally. Dogra (1987) paralysed the chick embryo using the same method as Hall (1975a) and Persson (1983) and has reported protrusion of the cerebral hemispheres through the malformed skull bones, retarded development of feather germs over the area of subcutaneous oedema in addition to

retardation in growth, protrusion of lower jaw and reduction in weight.

Effects of immobilisation on the development of specific bones

By grafting the chick limb bud on to the chorio-allantoic membrane on the fourth day of incubation, it was found that in the limb bud the femur and patellar rudiments could undergo self differentiation to full femur and patella both anatomically and histologically (Murray and Huxley, 1925). The general shape of bone was determined basically by intrinsic genetic factors and extrinsic factors such as movement produced only minor sculpturing (Murray and Selby, 1930).

Niven (1933) studied the development of the patella in vivo and in vitro, and found that in culture of the whole patellar mesenchyme cartilage appeared in all cases at the normal stage (11 days) and resembled that seen in normal development, but that its articular surface did not appear, even after 7 weeks culture. When he cultured the knee joint region plus the ends of the adjacent long bones from 7 and 9 day embryos, the patella did not differentiate. This was considered to be due to the large size of the explants which prevented adequate nutrition of the tissue.

Hamburger and Waugh (1940) studied the development of the skeleton in the chick embryo by transplanting denervated and partially denervated limbs into the coelomic

cavity, or into the umbilical cord and found that hypodactyly and hypophalangy were the most common abnormalities in the transplants and were occasionally combined with the absence of the fibula or ulna. The girdles (shoulder and pelvic) were also more poorly developed than the remainder of the limb, particularly the dorsal girdle elements, i.e. the scapula and the ilium which were disproportionately small. Morphogenesis of skeletal elements in the absence of innervation was normal in a high percentage of cases. Chondrification and the first phase of ossification proceeded normally in the absence of innervation, but skeletal growth of transplants was reduced by approximately 20% which might be due to either an inadequate establishment of vascularisation of the transplants or to deficiencies in their innervation.

Drachman and Sokoloff (1966) paralysed chick embryos between 6 and 8 days of incubation by administration of drugs such as decamethonium bromide and botulinum toxin and also by spinal cord extirpation. They reported that the bones of the pelvic limb matured normally in the treated embryos, as judged by the degree of ossification, vascularisation and marrow invasion, although they were shorter than normal. The plantar tarsal sesamoid was absent in 37% of embryos but a small, separate patella was present in 5 of the 6 embryos at the appropriate position within the quadriceps tendon. They concluded that skeletal muscle contractions were essential during embryonic

development for the appearance of the plantar tarsal sesamoid cartilage, but were not necessary for differentiation of the patellar cartilage and bones. The tarsal sesamoid normally appears in cartilage form at 10 days of incubation (Drachman & Sokoloff, 1966) and the patella at 10 days (Niven, 1933).

Sullivan (1966) paralysed chick embryos from 5 days of incubation and studied their vertebral columns between days 9 and 13 by routine histological methods and reported that the thoracic vertebra of most of the paralysed embryos were found to have been rotated about their axis. Sullivan (1971, 1973, 1974, 1975) treated chick embryos with different paralysing agents and studied them with whole mount alizarin red S staining and observed a series of bony abnormalities including: fusion and asymmetries of cervical vertebrae and wry neck, abnormal curvatures or rotation of the vertebral column in the trunk region, distortion of clavicle in the form of sharp kink instead of normal curvature, on one or both sides, distortion of posterior ribs and narrowing of intervening spaces and abnormal bending of scapula. He concluded that the overall appearance of the paralysed embryos suggested that they had been affected by mechanical pressure related to growth within the confined space of the amniotic cavity and therefore an important role of muscular activity during development may be to counteract mechanical forces that would otherwise deform the embryo.

Murray and Drachman (1969) studied the role of

movement in the development of joints and related structures in the head and neck in the chick embryo. They injected botulinum toxin directly into the chorioallantoic circulation from 7 days of incubation onwards and studied the embryos at 19 days of incubation. They reported that although the general appearance of the head and neck was remarkably normal in the experimental embryos, the skeletal elements had certain distorted details, e.g., the retroarticular cartilage process of Meckel's cartilage extended caudally in the experimental embryos rather than being angled dorsally as normal, several skeletal prominences which normally give attachment to muscle were either missing or grossly distorted in the experimental embryos, and anatomical relationships between skeletal elements were distorted. They concluded that these distortions were attributed to (a) failure of skeletal muscle to exert their normal moulding influence on the skeleton and (b) fixed retention of certain malpositions resulting from growth of the embryo within a confined space, unopposed by muscular activity.

Bradley (1970) cultured limb buds from chick embryos at stage 20 on the chorioallantoic membrane and concluded by histological examination that chondrification and the first phase of ossification proceeded normally in all skeletal elements in the absence of innervation.

Hall (1975a) paralysed embryonic chicks from day 10 of incubation and studied them at the 18th day of

incubation. He observed 50% reduction in the growth of the tibia. He did not observe any gross abnormalities and the histological appearance of the tibia was normal.

Effects of immobilisation on the development of the musculature

Hunt (1932), Eastlick (1943), Eastlick and Wortham (1947) and Bradley (1970) have studied muscle differentiation in cultured chick limb buds. They reported that the muscle underwent initial differentiation, but that they could not invariably maintain this independency. Gradual accumulation of fat within the muscle cells led to their degeneration. It was concluded that nerve supply is necessary for continuation of muscular normal development, for multiplication of myofibrillae and for the organisation of the fibres into typical muscle bundles.

Reduction of muscle mass is the feature described by all workers who studied the effect of immobilisation in vivo by administering paralysing agents to the chick embryo (Sullivan, 1966, 1967; Drachman, 1964; Drachman and Sokoloff, 1966; Murray and Drachman, 1969; Hall, 1975a). Most also reported reduction in individual fibre diameter in transverse section, replacement of muscle by fatty (adipose) tissue, increase in the number of sarcolemmal nuclei, with alignment, clumping and pyknosis of nuclei, phagocytosis of degenerated muscle fibres, lack of striation and poorly organised bundles.

Effect of immobilisation on development of synovial joints

Fell and Canti (1934) studied the development of the avian knee joint in vitro and reported that when a whole limb was explanted, a joint developed, but eventually disappeared by secondary (cartilaginous) fusion. Hamburger and Waugh (1940) claimed that in their transplanted limbs of chick embryos joint formation was perfect and complete. However, in the majority of cases, in addition to fusion, deficiencies in the sculptural details of articulating surfaces were common, condyles were smoother and grooves were shallower than normal. Mitrovic (1982), who studied the role of movement in development of the joint cavity by administration of a paralysing drug and by organ grafts reported that the first stages of development of a cavity were not inhibited but did not progress and were rapidly invaded by blood vessels and loose connective tissue and finally disappeared. The bones forming the joint fused together, with fibrous or cartilaginous tissue.

Lelkes (1958) cultured knee joints of 6-7 day old chick embryos. Some of the specimens were subjected to passive movement while the others served as controls. He concluded that in control explantations, where no movement was applied, the undifferentiated intra-articular tissue generally chondrified leading to complete cartilaginous fusion across the joint. An articular cavity was sometimes formed in the manipulated explants. He suggested that proper differentiation of a joint depended on its

undergoing movement.

Drachman and Coulombre (1962) administered d-tubocurarine to the chick embryo and noted resultant ankylosis of the joints of the foot. Drachman and Sokoloff (1966) observed failure of joint cavity formation in their paralysed chick embryos and fusion by fibrous and cartilaginous tissue.

Absence of joint cavities and fibrous, cartilaginous or bony fusion of articular elements were found by Murray and Drachman (1969) in their study of paralysed chick embryos.

All these authors agreed that full development of joint cavities did not occur in the absence of movement. This view has since been confirmed by later studies (Bradley, 1970; Drachman et al., 1976; Ruano-Gil et al., 1978, 1985; Persson, 1983; Llusa-Perez et al., 1988; Valojerdy and Scothorne, 1989).

METHODS OF IMMOBILISATION

As already indicated, three general methods have been employed to eliminate movements in the embryonic chick.

i) organ culture of the limb bud in vitro

ii) grafting the limb bud to the chorioallantoic membrane

iii) administration of neuromuscular blocking agents in ovo.

Both organ culture and chorioallantoic membrane grafting have disadvantages, such as, alteration of the environment, impossibility of studying embryo for long periods of time and stage by stage, and traumatic effects due to handling. Therefore, it seems that the injection of neuromuscular blocking agents is the preferable method of inducing total muscular paralysis.

Three variations of this method have been used since it was first introduced by Drachman and Coulombre (1962).

a) Infusion of the drug into the chorioallantoic blood vessels (Drachman and Coulombre, 1962; Drachman and Sokoloff, 1966).

b) Periodically applying the drug directly onto the chorioallantoic membrane (Murray and Smiles, 1965; Sullivan, 1966, 1974; Mitrovic, 1982).

c) Single injection of the drug into the air sac (Hall, 1975; Beckham et al., 1977; Persson, 1983; Dogra, 1987).

EMBRYONIC MOVEMENT IN THE NORMAL CHICK EMBRYO

Commencement of embryonic movement in the chick has been well documented to occur as early as 3.5 - 4 days of incubation with a slight flexure of the head and neck, caused by contraction within somites (Kuo, 1932a, 1932b, 1938, 1939; Orr and Windle, 1934; Windle and Orr, 1934; Hamburger, 1963; Hamburger and Balaban, 1963; Hamburger et al., 1965). Trunk movement appears after head movement at the end of day 4 of incubation, this movement being of two kinds: a) the flexion and extension of the cervical and caudal regions, and b) the twisting of the body to sides (Kuo, 1932a). Oral movement begins at the 6th day and movements of eyeball on the eighth or ninth day of incubation (Kuo, 1932a). Kuo (1932a,b, 1938, 1939) has also reported that movement of the root of the limbs commences at the end of the 4th day but he did not observe extension and flexion of the distal portions of the limbs before the seventh day of incubation, when he reported the movement of the digits to be dependent on the remainder of the limb, but at 8 days the digits moved independently of each other. On the other hand, Beckham et al. (1977) could not detect movements of pelvic limb digits in the majority of their specimens until the 10th day of incubation. Rapid flexion and extension of the pelvic limb at the ankle joint was observed by Dogra (1987) in 10 day old chick embryos and this extended to the digits at later stages. Progressive increase in phasic activity in the chick

embryo, simultaneous with development of the large joints of the pelvic limbs from 8 days onwards, was reported by Llusa-Perez et al. (1988). Phasic nature of embryonic movements have been reported to occupy 80% of time in embryos at 13 days. This situation lasted until day 17, before it declined (Hamburger and Balban, 1963; Hamburger et al., 1965; Llusa-Perez et al., 1988).

AIMS OF THE STUDY

From the foregoing review of literature on the role of movement on the development of the skeletal system in the chick embryo it was clear that there were few publications devoted to this topic. Most previous authors had mainly investigated the effects on the development of joints. Some skeletal abnormalities had also been reported, but in little detail. In addition there were also disagreements in the literature regarding the timing of some events such as chondrocyte hypertrophy, periosteal ossification, osteogenic bud invasion, etc., in the normal sequence of skeletal development.

This study was designed, therefore, to investigate the possible effect of paralysis of the skeletal muscle on the development of the skeleton in the chick embryo and to compare this with the normal situation. Particular points considered worthy of investigation included the timing of ossification and of invasion of the osteogenic bud, rates of longitudinal growth of long bones, volumetric composition of developing bones and the architecture of developing bone.

CHAPTER TWO

MATERIALS AND METHODS

PARALYSIS

General - specimens, incubation, handling, etc.

Fertile Ross Brown eggs were obtained from Ross Poultry, Aberdeenshire, and incubated from day zero in a forced draught type incubator (Westernette) which provided hourly rotation of eggs and maintained a humid atmosphere at 37 - 38°C. At day 3 of incubation the eggs were transferred to a Western Static incubator at 38°C and the eggs were allowed to settle for a period of 20 minutes. The eggs were removed one at a time from the static incubator and candled; infertile ones were discarded. In the case of fertile eggs, the position of the air sac was marked with a pencil and the position of the embryo was marked by a 2cm x 1cm rectangle pencilled on the shell. The egg was then placed into a Crokwell which allowed the egg to be kept in the same horizontal position as it had been in the static incubator.

Opening a window

After wiping the shell overlying the air sac and embryo with Hibitane in alcohol, a burr-hole about 1.5mm in diameter was made on the air sac using a dental drill. The rectangle over the embryo was gently grazed by means of a fine grinding wheel, fitted to a dental drill. The egg was again wiped with Hibitane alcohol to remove the saw dust and a very small portion of the rectangle was removed with fine forceps to expose the shell membrane. A small tear

was made in this with a sterile needle avoiding damage to underlying structures, and at the same time the embryo and its membranes were dropped away from the shell by applying gentle suction to the hole in the air sac. Once this was done the rectangular piece of shell and the shell membrane under it were removed using fine sterile forceps. The window and hole of the air sac were sealed with sellotape, and the eggs replaced in the static incubator.

Examination of movement

Embryonic movements were subsequently looked for, through the window, under a binocular microscope twice daily, from day 3 of incubation onwards, each egg being viewed for at least 3-4 minutes.

Method of paralysis

As described previously the method selected as the most suitable on the basis of previous reports was that of the single injection technique. However, the results obtained by this method were found to be very unsatisfactory. Consequently a method involving repeated dosage of a paralysing agent on to the chorioallantoic membrane was devised as described below. This gave very acceptable results. The details of the single injection technique and the results so obtained are included in appendix I. Possible explanations of variations in efficacy of different methods are discussed later.

Preparation of drug

A 0.2% solution of decamethonium bromide which is a post synaptic blocking agent was prepared in sterile saline at room temperature (1mg decamethonium bromide dissolved in 0.5ml sterile saline).

Method of administration

At day 6 of incubation, embryos were divided into two groups, 198 experimentals and 109 controls.

Each embryo of the experimental group received 0.5ml of decamethonium bromide solution and each control 0.5ml normal saline dropped onto the chorioallantoic membrane directly using a disposable 1ml syringe fitted with a sterile 26G (3/8) needle. To maintain the paralysis long term with a reasonable survival rate, the procedure was repeated at 8 and 10 days with 0.25ml of decamethonium bromide solution for paralysed embryos and 0.25ml normal saline for control embryos.

Examination for movement after administration of drug

The embryos were again inspected at regular twice daily intervals from half an hour after the first application of the drug. Experimental embryos which showed any signs of movement or which appeared dead, were discarded.

Sampling of embryos

Embryos were sacrificed at daily intervals from 8-20 days of incubation by rupturing the chorioallantoic membrane. By opening each egg into a petri dish, a final assessment of embryonic movement was made as the embryos were removed. If any sign of movement was seen within the experimental group, the embryos were treated as not totally paralysed and were discarded. The embryos were staged according to Hamburger & Hamilton (1951).

Weighing the embryos

Oedema fluid, which was present subcutaneously in experimental embryos was removed before weighing by making a small incision in the overlying skin. Embryos from both groups were dried on a clean tissue and weighed on an Electronic Reading Balance (IJBBOR ED-200).

CLEARED WHOLE MOUNTS

Staining/clearing

72 control and 66 experimental embryos were sacrificed in groups at daily intervals from 8 - 20 days, skinned, cleared and stained with alcian blue and alizarin red from the method of Kimmel and Trammel (1981). They were then stored in pure glycerine with crystals of thymol. Details of the method are contained in Appendix II.

Observation of centres of calcification

Using a Wild dissecting microscope the occurrence of centres of calcification and presence of skeletal abnormalities were investigated. To observe the skull it was found advantageous to section skulls mid-sagittally and examine them in lateral view.

Linear measurement

a) Long bones

In 47 experimental and 57 control embryos, the lengths of the entire femur, tibia, humerus, radius and ulna and the lengths of their calcified diaphyses were measured from day 11 onwards when red staining of alizarin red confirmed commencement of calcification of their diaphyses. The bones were placed flat in a shallow petri dish and a drawing was made using a Wild binocular microscope with a camera lucida attachment. The drawing of the entire bones and of their ossified diaphysis were measured with a planimeter. The magnification was calculated by placing a ruler beside the bone in the dish. The measurement was then checked by measuring the bone directly with the aid of a Vernier caliper with an error value of 0.02mm. Whenever the bone was flat there was very little difference between the two measurements. If a difference of more than 0.1mm was revealed the bone was repositioned, redrawn and remeasured. The results from control and paralysed embryos were then compared

statistically by multiple analysis of variance using statgraphic computer software.

b) Jaws

Upper and lower jaw lengths were also measured, in 16 experimental and 16 control embryos from 16-19 days of incubation, with the same Vernier caliper. The lower jaw was measured from the caudal end of angular to the rostral end of dentary. The upper jaw was measured from the caudal end of quadratojugal to the rostral end of premaxilla. To check these measurements the skulls were sectioned midsagittally and drawn and measured as described previously.

HISTOLOGY

A group of control and experimental embryos was selected for study of the histology of the tibia. 12 specimens including controls and experimentals were obtained at day 7, 8 and 9, and 4 from each group at daily intervals from day 10 to 15 inclusive. Right tibias were taken from the embryos by cutting through the lower end of the femur and the upper end of the metatarsus. These were fixed in Bouin's fixative and then placed in 70% ethanol for 1-2 hours. The specimens were then dehydrated through an ascending series of ethanols, cleared in amylacetate using an automatic tissue processor and embedded in paraffin wax. Serial transverse sections were cut on a Jung microtome at thicknesses of 6 um for tibias from embryos aged 6-9 days and the rest at 8 um. Sections were

stretched in a water bath, mounted on albuminised glass slides and dried for 24 hours in an oven at 37°C. The sections were dewaxed in xylene, hydrated and then stained. Three sets of 1 in 5 series from younger material (6-9 days) were mounted and stained with H & E, alcian blue and Masson's trichrome (Appendices III and IV respectively). One set of 1 in 5 series from the rest of the material was mounted and stained with Mayer's haemotoxylin and eosin (Appendix V). Finally they were dehydrated, cleared in xylene and mounted in Histomount.

The tibias of six embryos at early stages (2 controls at 6 days and 2 controls and 2 experimental at 7 days of incubation) were fixed in 5% glutaraldehyde and dehydrated through a series of acetone and propylene oxide and then embedded in pure Spurr's resin. Serial sections were then cut on a Reichert Jung Ultracut E ultra microtome at a thickness of 1.2 um, using 6mm wide glass knives of the Latta-Hartman type. Sections were then mounted on glass slides and stained with Azur blue II (details in Appendix VI).

MORPHOMETRIC MEASUREMENT

The serially sectioned tibias from 4 experimental and 4 control embryos of 11, 12, 13 and 14 days of age were used for a morphometric study following the method described by Gaytan, et al. (1987).

The following tissue compartments were defined on the transverse tibial sections:

The cartilage (CAR), the connective and vascular tissue invading the cartilaginous model (CVT) and the area enclosed by the perichondrium-periosteum (PER) that represented the total reference volume needed for the determination of the relative volumes of the other structures. At the mid-diaphyseal zone the area between cartilage and periosteum which was occupied by osseous trabeculae and intertrabecular blood vessels was called external space (EXT). Most of the cartilage at the mid diaphyseal zone had been resorbed by day 13 and it was difficult to distinguish between the connective and vascular tissue invading the cartilaginous model and the intertrabecular blood vessels of the external space. To solve this problem the tibia was divided into three different zones. Zones I and II corresponded to the proximal and distal epiphyseal ends, and zone III corresponded to the mid diaphyseal part of the bone (Fig. 1). The boundaries of these zones were defined as the last section in which there were remnants of cartilaginous tissue making it possible to distinguish between the external space and the invading connective and vascular tissue (Fig. 1). The number of sections per zone was recorded. A total number of 60 regularly spaced sections (20 selected sections per zone) in each tibia was used. These numbers were considered to be adequate because the variability among sections from each animal was similar to

that found when a greater number of sections was used. In zones I and II the volume enclosed by the perichondrium-periosteum (V_{PER}) was given as $V_{PER} = S_{PER}$ n.p were S_{PER} was the average surface area obtained by drawing the sections with a light microscope with camera lucida attachment and measured by a planimeter, n the number of sections in that zone and p the thickness of the sections.

The volume densities of the cartilage (V_{vCAR}), connective and vascular tissue (V_{vCVT}) and external space (V_{vEXT}) were obtained relative to the volume enclosed by the perichondrium/periosteum (V_{PER}). The absolute volumes of the cartilage (V_{CAR}), connective and vascular tissue (V_{CVT}) and the external space (V_{EXT}) were given as the product of their volume densities and V_{PER}.

In zone III the volume enclosed by the periosteum was found as described above. The average volume of the early cartilage (before it was invaded by the connective tissue and vascular tissue, V_{CAR-1}) was obtained by considering the mid diaphyseal zone as two truncated cones, each half of this figure being generated by a straight line (Fig. 2b) rotating around the longitudinal axis of the shaft. The equation of this line was obtained from the 11 day old embryos in the following way:

On serial sections of the mid diaphyseal zone the co-ordinate origin (0,0) was situated at the narrowest cartilage section. In each section situated distal to this point, the diameter of the section divided by 2 (Yi) and

the distance to the origin (Xi) was recorded. The straight line equation was then given as the regression line between Xi and Yi (Fig. 2a) and turned out to be (Y = 0.09x + 0.15) for control and (Y = 0.03x + 0.12) for experimental where x was the distance in millimetres between the sections delimiting zone III divided by 2 (see Fig. 2a) since the progression of the connective and vascular tissue towards both ends of bone was similar. By integration the volume of the truncated cones generated by these lines in rotating around the OX axis was:

for control

$$V = \pi \int X\ (0.09X + 0.15)^2\ dX = \pi(2.7X^3 + 13.5X^2 + 22.5X)10^{-3}$$

and for experimental

$$V = \pi \int X\ (0.03X + 0.12)^2\ dX = \pi(0.3X^3 + 3.6X^2 + 14.4X)10^{-3}$$

and therefore the volume of the two truncated cones which corresponded to the volume of the cartilage models before resorption was:

for controls

$$V_{CAR1} = 2\pi\ (2.7X^3 + 13.5X^2 + 22.5X)10^{-3}$$

and for experimentals

$$V_{CAR1} = 2\pi\ (0.3X^3 + 3.6X^2 + 14.4X)10^{-3}$$

The volume of the remaining cartilage (V_{CAR2}) was

calculated as the product of its volume density and V_{PER}.

The difference $V_{CAR1} - V_{CAR2}$ represented the volume of connective and vascular tissue (V_{CVT}) in zone III. The volume of external space (V_{EXT}) was given as:

$$V_{EXT} = V_{PER} - (V_{CVT} + V_{CAR2})$$

The values of these parameters for the whole tibia were found by summing the partial values for the volumes.

The volume of the cartilage resorbed in a given time (V_{CP}) was obtained as the increased (Δ) in the absolute volume of the connective and vascular tissue ($V_{CP} = \Delta V_{CVT}$) during that time.

The volume of cartilage formed in a given time (V_{CF}) was obtained from the increase in the cartilage volume (ΔV_{CAR}) and the volume of the cartilage resorbed (V_{CP}) during the same time ($V_{CF} = \Delta V_{CAR} + V_{CP}$). These increases were obtained for each animal with respect to the average values of the day before.

The values obtained for the control embryos were compared statistically with those for the experimental ones.

SCANNING ELECTRON MICROSCOPY

The left limbs were dissected from 3 control and 3 experimental embryos aged 12, 13, 14, 15 and 16 days and the tibias were obtained by trimming above the stifle joint and just below the tarsal joint. The tibias were fixed in 5% gluraraldehyde in Millonig's buffer (pH 7.4) for 24-48

hours. They were supported with agar and serially sectioned transversely at 400 u, and after processing were mounted in numerical order in a clockwise fashion onto aluminium stubs and coated with gold (see Appendix VI).

Care was taken while placing the tissue in agar so as to ensure that sections were cut in an exactly transverse plane. Difficulty was experienced in cutting paralysed material in which loose muscle did not adhere tightly to the bone. In these cases the bones were placed again in agar and re-sectioned.

The mid diaphyseal section was selected for scanning because it was the only one which could be identified and compared reliably in both groups. Photographs were taken at magnifications of x75, x350 and x 1000.

CHAPTER THREE

RESULTS

MOVEMENTS

Three types of movement were observed in the control embryos. These movements were observed to be unaffected by the administration of normal saline which took place on the 6, 8 and 10th days of incubation.

The types of movements were:

1. Contractions of the amnion and the swing movement of the embryo beginning at the end of the 4th day. These movements were not continuous, but of a phasic nature and were caused by the contraction of the smooth muscles of the amnion. There were active and inactive phases. The duration of the active phase increased until the 10th day, after which it began to decrease.

2. The chorionic vessels were also observed to engage in a pulsatile movement, in phase with the contraction of the heart. On the 11th day the head and neck region also started to exhibit movements, thought to be due to transmitted pulsations from the heart.

3. Embryonic movements proper began from the head and neck then progressed caudally. The lifting and bending movements of the head were observed on the 4th day, but at the later stages these two movements transformed into lateral turning of the head. The trunk movement appeared on the 5th day before any movement of the limbs. The trunk movements included

flexion and extension of the cephalic and caudal regions, and lateral twisting of the body which decreased in range at later stages.

The movements of the pectoral and the pelvic limbs began by the beginning of the 6th day at the root of the limb buds progressing distally. Flexion and extension of the stifle and tarsal joints began on the 8th day of incubation. These movements extended to the digits on the 10th day, first engaging in a group movement and later moving individually.

These embryonic movements were also phasic; phases of activity lasted for about 15 seconds, and were followed by longer phases of inactivity which lasted 25 to 30 seconds, during which the embryo showed no movements. The duration of the activity phase gradually increased and that of the inactivity phase decreased, but the duration of the activity phase began to decrease again at about 17th day. The amniotic movements were independent of the embryonic movements proper, but the movements of the limbs occasionally appeared to initiate the amniotic movement.

In experimental embryos, after the administration of the decamethonium bromide no movement was observed apart from those due to contraction of amnion and those of the chorionic vessels.

In 8 of a total of 150 embryos, some movements were exhibited after the first administration of the decamethonium bromide and these were discarded.

GENERAL FEATURES OF EXPERIMENTAL EMBRYOS

Experimental embryos showed retardation of the developmental stages of Hamburger and Hamilton (1951) after day 13 of incubation as follows:

Day of incubation	Control	Paralysed
13	38-39	37-38
14	39-40	37-38
15	40-41	38-39
16	41-42	38-39
17	42-43	39-40
18	43-44	39-41
19	44-45	39-41
20	44-45	40

Most of the experimental embryos showed subcutaneous oedema mostly in the abdominal region and pelvic limbs from day 10 onwards, and lack of feather germs in the oedematous regions.

Bent and rigid neck was obvious from day 12 onwards and was severe from day 15 onwards; the head was turned to the right. The rigidity of the wry neck was obvious when the experimental embryos were suspended by their feet immediately after removal from the egg. Experimental embryos showed abnormal positioning of digits either hyperflexion or hyperextension. In later stages paralysed

embryos appeared shorter and wider and the upper jaw was shorter than lower after 16 days of incubation which is the reverse of the normal situation. The experimental embryos were of smaller size and lower weight after removal of oedema than the control embryos. The statistical comparison of weight of the two groups is shown in Fig. 3. This revealed a significant reduction in weight of paralysed embryos from day 16 onwards.

SURVIVAL RATE

The control embryos showed 85% survival rate up to 20 days of incubation. Experimental embryos showed 84% survival rate up to 15 days of incubation. From 15 days onwards the mortality rate increased and they showed a survival rate of 53% out of 96 embryos, between 15-20 days.

MORPHOLOGICAL CHANGES IN EXPERIMENTAL EMBRYOS EXAMINED WITH WHOLE MOUNT CLEARING

Alcian blue and alizarin red stained cleared control and experimental embryos were compared and the following observations were made.

Vertebral column

The vertebral column in control embryos had its normal ventral curvature without any lateral flexion (Fig. 4a). The joints between the cervical vertebrae were seen clearly from day 10 (Figs. 4a and 5a). The arches of C_3,

C_4, C_7 and C_8 fused dorsally at 17 days of incubation and those of the rest at 18 days (Fig. 6a).

In experimental embryos the head turned to the right with bending of the neck which tended to be continued caudally, involving the vertebral column in the thoracic and even in the lumbar region, resulting in a combination of wry neck and scoliosis (Fig. 4b). The cervical vertebrae showed cartilaginous fusion across the joints after day 13 and by day 17 they showed bony fusion with most of them united into a continuous mass of bone (Fig. 5b). The arches of cervical vertebrae were not fused dorsally in cartilage at 18 days (Fig. 6b) and had not undergone bony fusion by 20 days.

Jaws

In control embryos the upper jaw was consistently longer than the lower jaw (Fig. 7a), but in experimental embryos the upper jaw was shorter than the lower jaw from day 16 onwards (Fig. 7b).

Ribs

In the control embryos, the vertebral ribs were well apart from each other and showed intervening synovial joints with the sternal ribs (Figs. 8a & 9a). Uncinate processes were situated obliquely caudal to the

corresponding vertebral ribs (Fig. 8a).

In the experimental embryos, on the other hand, the caudal vertebral ribs were close to each other in most cases due to the compressed appearance of the trunk and the joints between the sternal and vertebral ribs were fused in cartilage and distorted (Fig. 9b). Uncinate processes were either parallel with the shafts of the ribs or were not represented in either cartilage or bone (Figs. 8b & 9b).

Scapula

In control embryos the scapula had a slightly curved shape (Fig. 10a) and its caudal end did not reach the ilium (Fig. 8a). In the experimental embryos it was straight with a malformed cartilaginous caudal end (Fig. 10b) and in most cases it overlapped with the ilium (Fig. 8b).

Pelvic girdle

In controls the pubis was rod-shaped and slightly curved (Fig. 11a). In the experimental embryos the pubis was markedly malformed, sometimes C-shaped and sometimes L-shaped (Fig. 11b).

Pelvic Limbs

There were no morphological variations between the 2 groups regarding the bones, but the long bones of the

experimental embryos were noticeably shorter than the controls (Figs. 12a & b). In controls the articular cartilages were reciprocally curved (Fig. 13a), while they were flattened in the experimental embryos (Fig. 13b). The patella was present from 10 days onwards (Fig. 13a) as a cartilaginous model and the plantar tarsal sesamoid from 11 days (Fig. 14a), whereas the patella was not identifiable in all but 4 out of 47 experimental embryos (Fig. 13b) and the plantar tarsal sesamoid was not identifiable in any of them (Fig. 14b). There was bony fusion between the distal end of the femur and proximal end of the tibia and fibula in 19 and 20 days old experimental embryos (Fig. 13b).

LINEAR MEASUREMENT

1. Long bones

Linear measurements are shown with 95% confidence intervals for factor means for the entire femur, tibia, humerus, ulna and radius in Figures 15 to 19 and for their calcified diaphyses in Figurs 20 to 24.

All bones showed an increase in length of the their full and of their calcified diaphyseal length during the period studied. Compared with control embryos the experimental embryos showed a reduction in full length and lengths of the calcified diaphyses of all these bones from 11 days onwards, but these changes did not become significant for all these bones at the same time. The ages when significant differences in length were found are summarised in the following table.

Bone	Age of significant change	
	Full length	Calcified diaphysis
Femur	15	15
Tibia	14	15
Humerus	15	15
Ulna	15	15
Radius	16	16

The amount of reduction in the full length of these long bones and the length of their calcified diaphyses in

the experimental embryos at 19 days are shown in the following table.

Bone	Reduction at 19 days of age (%) Full length	Length of calcified diaphyses
Femur	25	33
Tibia	27	33
Humerus	20	27
Ulna	30	25
Radius	27	25

2. Jaws

The lengths of the lower and upper jaws in control and experimental embryos are shown with 95% confidence intervals for factor means in Figs. 25 and 26 respectively.

The lower jaw was shorter in paralysed embryos, but not significantly, whereas the upper jaw had significantly shortened in experimental embryos from 16 days of incubation. By 20 days incubation the lower jaw was 10% shorter in the paralysed whereas the upper jaw was 25% shorter. The greater shortening of the upper jaw accounts for the difference in protrusion, i.e. in paralysed embryos the lower jaw protruded rostral to the upper from 16 days onwards.

CALCIFICATION

The timing of onset of calcification was studied in alcian blue and alizarin red stained and cleared preparations in control and experimental embryos from 8 to 20 days of incubation. At days 8 and 9 there were no signs of calcification. At day 10 some bones showed a pale "ring" of perichondral ossification which was completely red stained at day 11. A similar pattern was exhibited subsequently by other bones.

1. **Skull**

The first signs of calcification in 20 selected bones of the skull were compared in control and experimental embryos. The selected bones included elements of the vault and base of the cranium and of the face.

A. Control

Times of appearance of centres of calcification are shown in figures 27 and 28. All the bones showed one day period of variability in the commencement of calcification.

B. Experimental

Times of appearance of centres of calcification are shown in figures 29 and 30. 11 bones showed one day delay

in commencement of calcification whereas the remaining 9 behaved similarly to the controls. Those bones exhibiting the delay in onset of calcification belonged to all different regions of the skull. The period of variability between different embryos was one day for 3 bones, two days for 8 bones and three days for 9 bones.

2. **Vertebrae**

i) Cervical

A. Control

Calcification times are shown in figure 31. The typical cervical vertebrae number 3-14 showed one centre of calcification for the body, two for the arch and two for costal processes. The atlas showed one centre in the region of intercentrum and two centres in the neural arch and the axis showed one centre for body and two for neural arch. Number 15 and 16 had false ribs rather than costal processes (see later). The commencement of calcification between different control embryos had a one day period of variability in all centres except that of arches of C_6 to C_{12} inclusive which showed a two day variability period.

B. Experimental

Calcification times are shown in figure 32. Compared with controls the experimental embryos showed a two day delay in calcification of the body of the axis and the

arches of C_6 and C_{16}. The arches of atlas and axis and the costal processes of the remaining cervical vertebrae did not show a delay and the remaining centres showed a one day delay in commencement of calcification. The period of variability between the experimental embryos was two days for most centres except for the body of the atlas and the axis which were one day and body of C_{12} to C_{16} which was three days. In most cases, therefore, it lasted for about one day longer than in the controls.

ii) Thoracic, lumbar, sacral and caudal

A. Control

Times of onset of calcification are shown in figure 33. The centres present for each vertebra consisted of one for body, and two for the arch and in addition two for transverse processes in vertebrae 4-9 of the lumbar, sacral and caudal series. All centres showed a period of one days variability in the time of onset of calcification.

B. Experimental

Times of onset of calcification are shown in figure 34. Onset of calcification was delayed by one day in the case of most centres and was not found at all in the 16th element in the lumbar, sacral and caudal group. The time of onset was again slightly more variable occurring in many cases over a period of two days rather than one.

3. **Ribs and sternum**

A. Control

Times of onset of calcification are shown in figure 35. Single centres for all vertebral ribs, sternal ribs and for the false ribs 1 and 2 related to the most caudal 2 cervical vertebrae were found to appear during embryonic life. Four uncinate processes for the most caudal false rib and the first 3 vertebral ribs were found to ossify variably from day 18 onwards. In the sternum centres appeared one for the body and the paired centres for the processes which are termed the cranial centres and the caudal centres. Other than those for the uncinate processes all others except that for sternal rib 5 exhibited a one day period of variability in the time of onset of calcification.

B. Experimental

Times of onset of calcification are shown in figure 36. Sternal ribs 2, 3 and 4, all vertebral ribs and the caudal centre of the sternum showed one day delay in commencement of calcification. The uncinate processes did not show calcification during the period studied. Sternal ribs 2, 3 and 4 and the body and cranial centre of the sternum showed two days of variability between embryos in the time of onset of calcification, the remainder exhibiting a one day period.

4. **Pectoral girdle and limb**

A. Control

Single centres of calcification were found in 13 bones. Their times of onset of calcification are shown in figure 37. The variability in the time of commencement of calcification was one day in all cases.

B. Experimental

Times of onset of calcification are shown in figure 38. There were no changes in the times of 6 bones. 5 bones showed a delay of one day. The phalanx of the minor digit showed a three day delay and the phalanx of the alular digit did not show a centre of calcification by the end of the period studied. Variability for most of the bones was two days except for 2 bones which were one day and for the distal phalanx of major digit in which it was three days.

5. **Pelvic girdle and limb**

A. Control

Single centres of calcification were found in 24 bones. Their times of onset of calcification are shown in figure 39. All centres exhibited a period of one day's variability in onset of calcification.

B. Experimental

Times of onset of calcification are shown in figure 40. 14 bones showed no change in time of commencement of calcification and period of delay of one day was found in 6 bones. The second, third and fourth phalanges of 4th digit did not show signs of calcification, thus exhibiting as delay of at least two days. The variability in time of onset between the experimental embryos for all centres, except one, was two days.

DEVELOPMENT OF THE TIBIA

1. Light Microscopy

i) Chondrification and ossification

A. Controls

Early on the 6th day of incubation the cells at the centre of the shaft of the tibial rudiment became elongated in a direction at right angles to the long axis of the cell mass, and narrow intercellular spaces appeared denoting that the formation of cartilage matrix has commenced. Towards its extremities the developing cartilage was still composed of undifferentiated mesenchymal cells. In the centre the demarcation between the developing cartilage and surrounding tissue was fairly sharp where the first indications of the perichondrium were beginning to appear (Fig. 41). Late on the 6th day of incubation the transverse elongation of cells had spread to the ends of the diaphyseal portion of the shaft. At the centre of the shaft some polyhedral cells were present. The perichondrium was now bilayered, enveloping the middle region of cartilage. The outer layer had fusiform shaped cells while the inner layer had oval or rounded cells. The two layers were sharply defined in the middle region both from one another and from the cartilage (Fig. 42). The perichondrium was still uniform in structure around the ends of the cartilage rudiment.

At 7 days some polyhedral cells in the mid-diaphyseal region enlarged and ceased to divide. They showed no linear or columnar arrangement and some showed degenerative changes (Fig. 43). At either side of these cells towards the epiphyses were longitudinally flattened cells. In the middle region of diaphysis the two distinct layers of perichondrium were obvious and were showing the first signs of ossification with formation of a delicate, undulating ring composed of fine interwoven fibres (Figs. 43 and 44).

At 8 days, the mid-diaphyseal region of the tibia showed one layer of ossified tissue surrounding the cartilage composed of hypertrophic chondrocytes. Delicate deposition of new ossified tissue among the peripheral osteoblasts appeared (Fig. 45).

A second layer of ossified tissue had been laid down at 9 days but this was not yet a complete ring (Fig. 46).

Two layers of ossified tissue had almost surrounded the mid-diaphyseal hypertrophic cartilage of tibia at 10 days. The first layer had become thicker and many osteoblasts had been trapped in its substance, becoming osteocytes. Some bone spicules connected the two layers of bone together (Fig. 47).

More bone had been deposited in the mid-diaphyseal region at 11 days and resorption of cartilage had started after the periosteal collar of bone had been eroded by the osteogenic bud (Fig. 48).

At 12 days, two to three layers of bone were observed in the mid-diaphyseal region and these were thicker than at

11 days. Their interconnections had increased in thickness and number. The first indications of Haversian canals were seen (Fig. 49a). The formation of the fibular crest on the lateral side of the proximal end of the tibia with its attachment to the interosseous tibiofibular ligament could be seen (Fig. 50a).

Four to five layers of bone with increased number of interconnections were observed at 14 days (Fig. 51a). The fibular crest and the interosseous tibiofibular ligament were well developed (Fig. 52a).

B. Experimental

Until 11 days no differences were found in the paralysed embryos.

At 12 days the tibia showed two to three layers of bone at the mid-diaphyseal region, fewer than controls (Fig. 49b). Only a small fibular crest was observed with its attachment to the interosseous tibiofibular ligament (Fig. 50b).

At 14 days, two to three layers of bone only were observed in the mid-diaphyseal region (Fig.51b). The fibular crest and interosseous tibiofibular ligament were not obvious (Fig. 52b).

ii) Resorption of cartilage and development of marrow cavity

A. Controls

Until 10 days of incubation no signs of cartilage resorption were seen in the tibia (Fig. 47).

At 11 days of incubation cartilage resorption had commenced in the mid-diaphyseal region but remnants of unresorbed hypertrophied cartilage were observed in some sections (Fig. 48). Resorption had progressed towards both ends of the bone although there were no signs of cartilage resorption in the extremities. Rapid progress of resorption of cartilage towards the ends of bone had occurred at 12 days and two to four resorption sites were usually observed in the transverse sections of the proximal and distal ends of the tibia (Figs. 50a and 53a). At this stage the marrow cavity was well formed in the central region (Fig. 49a).

At 14 days of incubation four to five resorption sites could be seen at the proximal and distal end of the bone (Fig. 52a). The marrow cavity was now well formed in the central region and also contained a large central blood vessel (Fig. 51a).

B. Experimental

Until 10 days of incubation no signs of cartilage resorption were seen. At 11 days cartilage resorption had

commenced in the mid-diaphyseal region and the osteogenic bud had progressed towards both ends of bone but not to the same extent as in control embryos. The remnants of cartilage with hypertrophic cells were greater in quantity than in control embryos. No signs of resorption were found in the extremities. At day 12, the marrow cavity and remnants of cartilage could be seen in the mid-diaphyseal region (Fig. 49b), but the progress of resorption towards the ends was not as rapid as in control embryos. Indeed it had extended very little and no signs of resorption were observed at both extremities (Figs. 50b & 53b).

In the mid-diaphyseal region the marrow cavity was well formed at 14 days (Fig. 51b) but had not reached the ends of bone (Fig. 52b). In some embryos remnants of hypertrophic cartilage persisted in the mid-diaphyseal region (Fig. 51b). Progress in the spread of the cartilage resorption was very slow and in some cases there were no major differences in extent between day 12 and 14.

2. **Scanning electron microscopy**

Examination of the section from the middle of the diaphysis of the tibia in control and experimental embryos from day 12 to 16 by scanning electron microscopy revealed the following results.

A. Control

Day 12 (Figs. 54a,b,c)

3 to 5 layers of periosteal bone were seen in an asymmetrical pattern, the maximum number of layers being at the caudolateral aspect of the bone. Numerous dense and compact interconnecting spicules (bony trabeculae) connected the layers of the bone together. Many blood vessels were present in the intertrabecular spaces, surrounded by loose connective tissue. The marrow cavity was well formed.

Day 13 (Figs. 55a,b,c)

4 to 6 layers of periosteal bone occurred in an asymmetrical pattern, with most layers being in the caudo lateral part of tibia. Interconnecting spicules had increased in number. Compact bony trabeculae which had formed the Haversian canals surrounded the blood vessels and the connective tissue around them.

Day 14 (Figs. 56a,b,c)

4 to 6 layers of periosteal bone in more symmetrical pattern were observed. Again the rather thick region was on the caudolateral side of the tibia. The marrow cavity was well formed and a large blood vessel was seen in its

centre. Interconnecting bony spicules were mostly united and Haversian canals were more obvious. Blood vessels in the Haversian canals were rather smaller in diameter than at 13 days and were surrounded by loose connective tissue.

Day 15 (Figs. 57a,b,c)

5 to 6 layers of periosteal bone were observed in all specimens which were studied arranged in a relatively symmetrical pattern. The innermost layer was becoming resorbed and only remnants of it were present. Interconnecting spicules were dense and compact, and had a smooth surface. They united the adjacent layers of bone and surrounded blood vessels with loose connective tissue around them forming Haversian canals. The marrow cavity was well formed and a large central blood vessel was always seen within it.

Day 16 (Figs. 58a,b,c)

5 to 6 layers of periosteal bone were still seen in a fairly symmetrical pattern. The innermost layer of bone had been resorbed and interconnecting spicules had their free ends projecting into the marrow cavity. Bony trabeculae showed a very compact structure. Blood vessels of small diameter were seen within Haversian canals, surrounded by loose connective tissue. The marrow cavity continued to show a large blood vessel in its centre.

B. Experimental

Day 12 (Figs. 59a,b,c)

2 to 4 layers of periosteal bone had been laid down in the same asymmetrical fashion as in the controls and numerous interconnecting spicules connected the layers of bone together. The structure of the trabeculae, the marrow cavity and developing Haversian canals were similar in appearance to those of the control embryos.

Day 13 (Figs. 60a,b,c)

3 to 5 layers of periosteal bone were present, tending to be slightly less than in the control embryos. Otherwise the appearance was similar. A well formed marrow cavity with a relatively small central blood vessel within it was observed. The bony trabeculae appeared dense. Blood vessels in the Haversian canals were surrounded by loose connective tissue.

Day 14 (Figs. 61a,b,c)

3 to 5 layers of periosteal bone were present in the asymmetrical pattern of the controls, but no obvious progress from day 13 was recognised. Interconnecting bony spicules seemed rather thicker than in controls, and blood vessels in the Haversian canals were of slightly larger

diameter than in controls. The marrow cavity was well formed but the blood vessel inside it appeared smaller in diameter than the corresponding vessel in the controls.

Day 15 (Figs. 62a,b,c)

4 to 6 layers of periosteal bone had been laid down still in asymmetrical fashion. The innermost layer showed the first indication of resorption. Marrow cavity was well formed and blood vessel was seen among its contents. This was smaller in diameter than in the corresponding controls. Bony trabeculae were less compact with a slightly roughened surface. Blood vessels in the Haversian canals were of larger diameter than those of the controls.

Day 16 (Figs. 63a,b,c)

5 to 7 layers of periosteal bone were laid down still in a slightly asymmetrical pattern. No obvious progress was observed in the resorption of the innermost layers since day 15. Interconnecting spicules which connected the layers of periosteal bone together were less compact and had rougher surfaces than in controls. Blood vessels in the Haversian canals were larger in diameter while those in marrow cavity were of smaller diameter than the corresponding vessels in controls.

3. Morphometric measurement of the volume of tibia

A. Control

By day 11 the mid-diaphyseal zone of the tibia had been invaded by connective and vascular tissue though resorption was minimal but by day 12 most of the cartilaginous core had been resorbed.

The volume enclosed by the periosteum-perichondrium increased during the whole period under investigation, 5.62 times, showing highly significant increase between day 11 and 12, significant increase between day 12 and 13 and very highly significant increase between day 13 and 14 (Fig. 64 & Table 1). The volume of cartilage increased from day 11 onwards and significantly between day 13 and 14. The volume of cartilage increased between day 11 and 14 3.68 times (Fig. 65 & Table 2). Only the increase from day 13 to 14 was significant (Table 2). The volume of the invading connective and vascular tissue increased significantly between day 11 and 12 and very highly significantly between day 13 and 14. Between days 11 and 14 it increased by a factor of 17.62 times (Fig. 66 & Table 3). The external space volume showed a highly significant increase between day 11 and 12 and between day 13 and 14 and a very highly significant increase between day 12 and 13. Between days 11 and 14 it increased 9 times (Fig. 67 & Table 4).

Between 11 and 14 days the cartilage volume density decreased by a factor of 1.51 (Fig. 68). The external

space volume density increased 1.67 times (Fig. 69) and the connective and vascular tissue volume density increased 3.16 times (Fig. 70). No level of significance was detected between two individual days for the volume densities.

The volume of cartilage formed per day increased markedly between day 13 and 14 (Fig. 71) and the volume of cartilage resorbed per day also increased markedly between day 13 and 14 (Fig. 72).

B. Experimental

By day 11 the mid diaphyseal zone had been initially invaded by the connective and vascular tissue, though resorption was minimal up to 14 days.

The volume enclosed by the perichondrium-periosteum increased during the whole period under investigation (2.25 times) (Fig. 64 and table 1) but not significantly between any 2 consecutive days. The volume of cartilage increased 1.91 fold throughout the period but not significantly and there was no increase between day 13 and 14 (Fig. 65 & Table 2). The volume of connective and vascular tissue showed an increase of 8.24 times between 11 and 14 days but this was not significant between any consecutive days (Fig. 66 & Table 3). The volume of external space increased 2.74 times showing a significant increase between day 11 and 12 but no significant increase thereafter (Fig. 67 & Table 4).

Between day 11 and 14 the cartilage volume density decreased 1.17 times (Fig. 68), the external space volume density increased 1.22 times (Fig. 69) and the connective and vascular tissue increased 3.66 times (Fig. 70) none of these was significant.

Regarding formation of cartilage per day there was a small increase between day 12 and 13 followed by a rapid decrease between day 13 and 14 (Fig. 71) and the volume of cartilage resorbed per day decreased in the whole period under investigation and almost to zero between day 13 and 14 (Fig. 72).

C. Comparison of control and experimental

1. Volume enclosed by perichondrium-periosteum

Differences in total volume were apparent at 11 days and were significant at 12 days and very highly significant at 14 days (Fig. 64 & Table 1).

2. Volume of cartilage

There were differences between the two groups which were highly significant at 14 days (Fig. 65 & Table 2).

3. Volume of connective and vascular tissue

In experimental embryos a lower volume was obvious at 12 days and this became very highly significant at day 14 (Fig. 66 & Table 3).

4. Volume of external space

Reduction in the volume of external space in experimental embryos was significant at 13 days and very highly significant at 14 days (Fig. 67 & Table 4).

5. Volume density of cartilage

The cartilage volume density in both groups decreased but there was a greater decrease in controls than experimental. This difference was significant at 14 days (Fig. 68).

6. Volume density of external space

The volume density of external space increased in both groups but not much in the experimental group especially between day 12 and 14. The difference between control and experimental embryos was significant at 14 days (Fig. 69).

7. Volume density of connective and vascular tissue

There was an increase of volume density of connective and vascular tissue in both groups but this was less in the experimental. However, no significant differences were detected between the two groups (Fig. 70).

8. Formation of cartilage

Formation of cartilage decreased in the experimental embryos between day 13 and 14 when it markedly increased in the controls (Fig. 71).

9. Resorption of cartilage

Resorption of cartilage decreased in the experimental embryos especially between day 13 and 14, although it markedly increased in the controls (Fig. 72).

Table 1

Comparison of volume enclosed by periosteum-perichondrium in control and experimental chick embryos.

AGE (DAY)	CONTROL MEAN ± S.D.	experimental MEAN ± S.D.	D.F.	T-TEST	P
11	1.9 ± 0.4	1.5 ± 0.3	6	1.673	N.S.
12	3.1 ± 0.4[b]	2.2 ± 0.5	6	2.707	0.05
13	5.0 ± 1.0[a]	3.1 ± 1.2	6	2.335	N.S.
14	10.9 ± 0.9[c]	3.3 ± 1.0	6	11.337	0.001

a = significant

b = highly significant

c = very highly significant from previous day

Table 2

Comparison of volume of cartilage of the tibia in control and experimental chick embryos.

AGE (DAY)	CONTROL MEAN ± S.D.	experimental MEAN ± S.D.	D.F.	T-TEST	P
11	1.4 ± 0.3	1.1 ± 0.3	6	1.611	N.S.
12	2.0 ± 0.4	1.4 ± 0.3	6	2.424	N.S.
13	3.0 ± 0.7	1.9 ± 0.7	6	2.126	N.S.
14	5.2 ± 1.2[a]	1.9 ± 0.4	6	5.080	0.01

a = significant from previous day

Table 3

Comparison of volume of the connective and vascular tissue invading the tibia of chick embryo in control and experimental chick embryos.

AGE (DAY)	CONTROL MEAN ± S.D.	experimental MEAN ± S.D.	D.F.	T-TEST	P
11	0.08 ± 0.04	0.04 ± 0.02	6	1.535	N.S.
12	0.26 ± 0.13[a]	0.17 ± 0.11	6	1.080	N.S.
13	0.45 ± 0.16	0.28 ± 0.21	6	2.181	N.S.
14	1.41 ± 0.19[c]	0.33 ± 0.13	6	9.272	0.001

a = significant

c = very highly significant from previous day

Table 4

Comparison of volume of external space in control and experimental chick embryos.

AGE (DAY)	CONTROL MEAN ± S.D.	experimental MEAN ± S.D.	D.F.	T-TEST	P
11	0.5 ± 0.1	0.4 ± 0.1	6	1.937	N.S.
12	0.8 ± 0.1[b]	0.6 ± 0.2[a]	6	2.029	N.S.
13	1.5 ± 0.2[c]	0.9 ± 0.4	6	2.841	0.05
14	4.5 ± 0.8[b]	1.0 ± 0.4	6	7.423	0.001

a = significant

b = highly significant

c = very highly significant from previous day

NOTE ON THE FEATURES OF MUSCLE ATROPHY

Reduction in the size of muscle bulk in the limbs was obvious by day 12 of incubation in the experimental embryos. Muscle fibres had lost their regular arrangement and the sarcolemmal nuclei seemed rather greater in number (Figs. 73a & b). Muscle fibres became replaced by loose connective and adipose tissue. White blood cells which were rounded in shape with pseudopodia and fatty droplets were numerous among muscle fibres. The endomysium and perimysium could not be distinguished in most places. Numerous white blood cells also could be detected around blood vessels (Figs. 74a,b & 75a,b).

CHAPTER FOUR

DISCUSSION

Technique of paralysis

From the review of the literature it was understood that the best method of paralysing would be the injection of the decamethonium bromide into the air sac as used by Hall (1975a) at 10 days of incubation and Persson (1983) and Dogra (1987) at 8 days of incubation. When the same method was applied as to the 6 day chick embryos described in Appendix I it proved not to be a satisfactory method. Three possibilities may account for this:

i) the solution of the decamethonium bromide evaporated before absorption by the embryo.

ii) the shell membrane is not permeable enough at this stage and the solution therefore cannot pass through.

iii) the chorio-allantoic blood vessels were not grown enough at 6th day of incubation to come in contact with the shell membrane over the air sac.

The third possibility seems to be the most likely cause of unsuccessful paralysis. Therefore, the method of periodic dropping of the decamethonium bromide solution onto the chorioallantoic membrane was used and complete long term paralysis of skeletal muscle was achieved by three administrations of the drug at 6, 8 and 10 days of incubation.

Decamethanium bromide is a post synaptic blocking agent which depolarises the muscle membrane at the end plate junction and has been reported to be very efficient in inducing paralysis in the chick embryo, allowing

survival over a long period of embryonic development (Drachman and Sokoloff, 1966). The present study supports this statement.

In this study, experimental embryos showed retardation of development, were of smaller size and reduced in weight and were markedly oedematous after administration of decamethonium bromide. These findings support Drachman and Sokoloff (1966), Murray and Drachman (1969), and Dogra (1987). The oedema which was observed may be the result of one or both of the following possibilities:

1. Diminished venous return due to loss of skeletal muscle tone.
2. Peripheral vasodilatation from decamethonium inducing release of histamine; decamethonium releases detectable amounts of histamine only when administered in very high doses (Koelle, 1975).

Morphological changes in experimental embryos

The embryos were studied as cleared whole mounts which had the advantage of allowing a large number of specimens to be compared and the shape and relationships of each bone to be easily determined.

i) **Vertebral column**

Some union of vertabrae takes place normally in parts of the vertebral column in the domestic fowl in the

lumbosacral and thoracic regions. Although the actual fusions take place some time after hatching (Hogg, 1982) it is unclear what the actual structure of these joints is in the embryonic period. To avoid any misinterpretation as to what fusion may be attributed purely to paralysis, particular attention was paid to the cervical region in which the vertebrae remain unfused.

Sullivan (1966) has reported cartilaginous fusion of cervical vertebrae in paralysed embryos as young as stage 35 (10 days) by histological methods. He also reported bony fusion of cervical vertebrae at the 17th day of incubation in cleared whole mount paralysed embryos (Sullivan, 1974). In the present study the cartilaginous fusion was identifiable at 13 days of incubation in all cervical vertebrae and progressed to bony fusion at 17 days. Differences in the timing of cartilaginous fusion with the previous author could be due to the differences in the methods used.

The two halves of the vertebral arches usually fused together dorsally at about 17 and 18 days of incubation in control embryos while in the experimental embryos this fusion failed to occur even at the 20th day of incubation. The whole vertebral column in the experimental embryos was abnormally curved and showed a combination of wry neck and scoliosis confirming the findings of Sullivan (1971, 1973, 1974). The head was turned to the right and followed by "S" shaped scoliosis. As it will be discussed later, this

distortion may come from the forces of the contraction of the amniotic membrane having compressed the embryo.

Murray and Drachman (1969) considered that some of the abnormalities present in their specimens were due to a fixed retention of embryonic posture, with the neck anteflexed and the head tucked under the right wing. This explanation, however, may not explain all of the cases of occurrence of bent neck. Hamburger and Oppenheim (1967) stated that the tucking of the head under the right wing in preparation for hatching takes place at about the 17th day in normal chick embryos. In the present study, it was observed that the experimental embryos showed scoliosis prior to 17 days, suggesting that this is not a retention of the normal embryonic posture.

ii) **Jaws**

The normal appearance of the jaws in the chick embryo is that the upper is longer than the lower. Protrusion of the lower jaw beyond the upper has been reported in the absence of movement by others. Murray and Drachman (1969) reported protrusion of the lower jaw in 19 day old paralysed embryos. They measured the upper and the lower jaws and reported that the upper jaws were shorter and the lower jaws longer in the paralysed than in normal embryos. They suggested three possible explanations: (i) an abnormally long mandible; (ii) an abnormally short upper jaw, and (iii) malposition of the skeletal elements favouring a forward position of the mandible. They

considered that all three above factors contributed to the protrusion of the mandible. However, they were unable to suggest by which mechanism one single influence (muscle paralysis) could produce elongation of one part (lower jaw) and shortening of another (upper jaw). Hall (1975a) and Dogra (1987) have also reported protrusion of the lower jaw but without measurement. Persson (1983) reported shortening of the upper jaw and Cameron (1986) studied the effects of shell-less culture on the development of the tibia in the chick embryo and again reported retarded growth of the upper jaw without presenting any measurements.

In the course of the present study it was clear that in experimental embryos from 16 days onwards the upper jaw is shorter than the lower. Measurements of both the upper and the lower jaws confirmed that both were retarded in their growth in the experimental embryos. However, the retardation in the upper jaw (about 25%) was markedly greater than that of the lower jaw (about 10%). Although this does explain the nature of the abnormality which occurs, it does also raise a new question: why the effect is greater on the upper jaw. It is therefore necessary in the future for both the upper and lower jaws and the bones in the base of the skull which articulate with them to be examined in greater detail histologically and for measurements of constituent bones to be made individually rather than collectively as was done in the present study.

iii) **Ribs**

In control embryos ribs were evenly spaced and showed well formed synovial joints between vertebral and sternal ribs. The uncinate processes were situated obliquely caudal to the corresponding vertebral ribs. In the experimental embryos, on the other hand, the caudal vertebral ribs were close to each other in most cases due to the compressed appearance of the trunk. The same deformity was reported by Sullivan (1974). The joints between the sternal and vertebral ribs became fused in cartilage and were distorted. This fusion was not reported previously. It perhaps is due to the paralysis of the intercostal and the other muscles which normally attach to the ribs.

Uncinate processes were either parallel with the shafts of the ribs or were not represented in either cartilage or bone in the experimental embryos. This abnormality is also related to paralysis of skeletal muscle because uncinate processes are the site of the external intercostal muscle attachements.

iv) **Scapula and pelvic girdle**

The scapula in control embryos had a slight ventral concavity and its caudal end was well apart from the ilium. Bent and distorted scapulae were reported by Sullivan (1974) in paralysed chick embryos treated with decamethonium bromide, but in the present study the osseous

scapula was straight and had lost its ventral concavity perhaps due to the mechanical pressure of amniotic membrane on the dorsal aspect of the embryo. The distal cartilaginous part of the scapula was malformed and in most of the cases overlapped the ilium. This was always accompanied by the scoliosis of the vertebral column and resulting forward displacement of the pelvic girdle, so that the embryo had a stunted appearance and a short body.

The pubis was markedly malformed in the experimental embryos, sometimes C or L-shaped. This was not previously reported and is because of the same caudocranial compression of embryo mentioned above.

The compressed appearance of most of the paralysed embryos, and the bony anomalies such as twisted vertebral columns, pelvic girdle jammed up against the thorax with the consequent pushing together of the ribs and distortion of the cartilaginous end of the scapula and deformed pubis can be explained as being due to mechanical forces pressing on the embryo. Sullivan (1974) reported that during the second half of incubation, the growing embryo is cramped in the confines of the amniotic cavity, and would be subject to compression and consequent deformation of shape unless it had some means of counteracting the pressures upon it. As the administration of decamethonium bromide prevents the contraction of skeletal muscle the embryo loses the means to oppose the environmental pressure which mostly comes from the contractions of the amniotic membrane in which the smooth muscle is unaffected by decamethonium bromide and

keeps contracting normally. Murray (1936) has stated that the intrinsic factors which direct the growth of the shaft of the bones tend to cause it to become curved in the normal manner but that extrinsic mechanical factors may override the intrinsic factors if they become sufficiently strong. It is probable, of course, that the extrinsic factors which normally act in the developing limb are such as to favour the development of the normal form.

v) **Pelvic limb**

Bones showed no obvious morphological variations between the control and experimental embryos. The articular cartilages were reciprocally curved in the control embryos, whereas they became flattened in the experimental embryos, confirming the previous report of Hamburger and Waugh (1940).

1. Patella

In the control embryos in the present study the cartilaginous patella was observed for the first time at the 10th day of incubation about stage 35 which is in agreement with Drachman and Sokoloff (1966) and Abu-Hijleh (1987) but is one day earlier than reported by Niven (1933). On the other hand, in the experimental embryos the patella was not distinguished at all in 43 out of 47 embryos over that age and in the 4 embryos in which it was recognisable it was very small in size. This finding is in

agreement with Niven (1933) who reported the absence of the patella when he cultured the whole knee joint, and disagrees with Drachman and Sokoloff (1966) who reported that five out of six of their experimental embryos paralysed with decamethonium bromide showed small patellas. The possible explanation for their finding is because of administration of decamethonium bromide at 8 days of incubation rather than 6 days which is after the initiation of the mesenchymal condensation for the patella. This was reported to occur at about stage 30 (6.5 to 7 days of incubation) by O'Rahilly and Gardner (1956) and Abu-Hijleh (1987).

2. Plantar tarsal sesamoid

The plantar tarsal sesamoid was observed as a cartilaginous condensation at 11 days in the control embryos which confirms the findings of Drachman and Sokoloff (1966). It was absent in all the experimental embryos studied. This finding disagrees with Drachman and Sokoloff (1966) who reported the absence of the plantar tarsal sesmoid in only four out of their eleven embryos perhaps for the same reason discussed above for patella, although there is no timing of mesenchymal condensation of the plantar tarsal sesamoid available in the present literature so it is difficult to draw this conclusion.

3. Knee joint

Cartilaginous fusion of the knee joints in the

absence of movement has repeatedly been reported by Fell and Canti (1934), Drachman and Coulombre (1962), Drachman and Sokoloff (1966), Murray and Drachman (1969, Bradley (1970), Drachman et al. (1976), Ruano Gil et al. (1978, 1985) and Mitrovic (1982).

In the present study cartilaginous fusion of the distal end of femur and proximal end of tibia and fibula was observed which became bony at later stages of development before hatching in the experimental embryos. This confirms the previous reports by other workers and also it may explain the cartilaginous and bony fusion of the cervical vertebrae. As mentioned above, and fusion of skeletal elements in the absence of movement has been reported. Lelkes (1958) showed that cartilaginous fusion could be prevented in the chick embryo knee joint cultures if each was subjected to movement several times daily by means of a fine glass rod.

Whether the formation of a joint cavity is genetically determined and the initial stages of the joint formation are under control of intrinsic factors or extrinsic such as movement, is not possible for this investigation to answer, as it is not known whether this union is pre-cavitation or occurs secondarily after initial formation of a joint cavity. The only statement which can be made here is that in the absence of movement the fusion between the skeletal elements in the sites of the developing articulation is distinguishable. Further

investigation is needed particularly at early stages of embryonic development under normal and experimental environments to study the initial stages of joint formation.

Longitudinal growth of certain bones

Measurements of five long bones which ossify perichondrally and upper and lower jaws, formed mainly by intramembranously ossifying bones in the control embryos showed continuous growth in their length from 11-20 days of incubation. The experimental embryos also showed a continuous longitudinal growth but this was much less than in controls. The amount of this reduction at day 19 of incubation for the full length of the long bones and the length of their calcified diaphyses was about 20 to 33 per cent of the length of the corresponding bones in the control embryos and for the membral bones of the jaws was about 10 to 25 percent.

There would appear to be no previous comprehensive study on the measurement of the bones in the chick embryo under the influence of muscular inactivity. Hall (1975a) reported 50% reduction in the normal growth of the tibia in the paralysed chick embryo by using the dry weight of the tibia as a factor to determine growth.

The reduced growth of the bones which was observed in the present study was not a result of malformation of the skeletal tissue but was apparently due to a reduction in the normal process of bone formation. As will be discussed

later the normal growth processes were occurring more slowly in the experimental embryos.

Comparable results are produced in other experimental situations. Troupp (1961) reported reduction in length of femur and tibia in the rabbit after motor root and peripheral nerve section. Reduction in the length of the limbs involved in poliomyelitis is well documented although some reports have claimed increase in the length in the first year but the end result is a reduction in length (Ring, 1957, 1958, 1961). Therefore, it can be concluded that muscle activity is essential for normal growth of the skeleton and in the absence of such activity longitudinal growth will slow down although it will not cease completely.

Development of tibia

1. **Qualitative changes**

For closer observation on skeletal development the tibia was chosen and histological and S.E.M. studies were carried out in control and experimental embryos.

In the control embryos at the beginning of the 6th day of incubation (Stage 28) the cells in the centre of tibial rudiment started to secrete cartilage matrix and the demarcation between cartilage and surrounding tissue was fairly sharp in that region where the first indications of the perichondrium were beginning to appear. Late on the 6th day of incubation (Stage 29), at the centre of the

shaft of the tibial rudiment some chondrocytes began to enlarge. The time of this finding confirms Von der Mark et al.,(1976a) and Abu-Hijleh (1987) and is one day in advance of that described by Lutfi (1974) and Lansdown (1968). At the same time as hypertrophy of the chondrocytes in the centre of the shaft of the tibial rudiment commenced the perichondrium enveloping the middle region of cartilage model showed two morphologically distinct layers. This is in agreement with the findings of Scot-Savage and Hall (1979), Osdoby and Caplan (1981) and Abu-Hijleh (1987). It also falls between the findings of Lansdown (1968) and Archer & Tarcliffe (1983).

In the control embryos in the middle region of the tibial diaphysis the two distinct layers of perichondrium were obvious and at the 7th day of incubation (Stages 30-31) showed the first signs of ossification with formation of delicate, undulating rings composed of fine interwoven fibres. This confirms the findings of those workers who also used histological methods (Mortimer, 1970; Lutfi, 1974; Von der Mark et al., 1976a; Hall, 1970; Scott-Savage and Hall, 1979; Abu-Hijleh, 1987). Osdoby and Caplan (1981) detected alkaline phosphatase activity, a feature commonly associated with osteoblast activity, at 5.5 to 6 days of incubation (Stage 28) and Von der Mark et al. (1976a,b) detected collagen type I at 6 and 7 days of incubation (Stage 29 and 31). The stage 31 confirms the findings of this study while stage 29 does not, perhaps on

account of the differences in method used. In the present study the hypertrophy of chondrocytes (Stage 29) was observed earlier than the initiation of ossification (Stage 31) and this finding supports the suggestion of the importance of hypertrophic cartilage for bone formation (Hall, 1982; Hunziker et al., 1984; Floyd et al., 1987).

Bone development continued by addition of several new periosteal bone layers surrounding the cartilage model by the formation of columnar radial trabeculae on the outer surface which support a second mineralised cylinder according to the descriptions of Fell (1925) and Dillaman et al. (1979).

Up to the 11th day of incubation (Stage 35 to 36) no differences were found in the experimental embryos regarding tibial development and even the timing of events were similar to those of the control embryos. Therefore, it can be concluded that movement has no role over the transition of perichondrium to periosteum or the initiation of ossification. This is in agreement with the statement of Hamburger and Waugh (1940) that chondrification and the first phases of ossification proceed normally in the absence of innervation when they transplanted denervated and partially denervated limbs into the coelomic cavity and into the umbilical cord in the chick embryo.

The histological and electron microscopic study revealed that after the 11th day of incubation in experimental embryos the tibia showed fewer layers of periosteal bone than in controls.

The asymmetrical pattern of periosteal bone which was seen in the 12 and 13 day control embryos with more bone present in the caudolateral aspect of tibia was remodelled in the later stages of development and by day 16 it showed a complete symmetrical pattern, while this asymmetrical pattern continued up to the end of the period under investigation in experimental embryos.

While resorption of the innermost layer of periosteal bone was nearly completed at 16 days in control material it was still almost intact in the experimental embryos, suggesting that the process of resorption had been slowed down.

In the experimental material, therefore, the amount of bone laid down was reduced as judged by the number of layers of the periosteal bone. The physical and structural appearance of this bone was also different and it was observed about 15-16 days that the trabeculae appeared less well formed and that they exhibited rough surfaces and a less compact appearance than in controls. By alizarin staining it was found that some bones of the experimental embryos did not stain sharply. Loss of calcium content may be a possible explanation as Schoutens et al. (1988) observed in the tibia of paraplegic rats.

As to whether the structural appearance of these bones observed in the present study is a direct effect of the paralysis or an indirect effect requires more information such as, histochemical investigation to

evaluate calcium level of the trabeculae, transmission electron microscopy of developing trabeculae to investigate the nature of rough surfaces and radiographical examination of the bones to investigate density of different bones.

In the present study it was observed from 14 days onwards in the experimental embryos that the blood vessels in Haversian canals were of wider diameter than those in control embryos. The size of the actual Haversian canals did not show any obvious changes between the two groups of embryos and the enlarged blood vessels seemed to have pushed the loose connective tissue around them to the walls of Haversian canals. The other change which was noticeable in the experimental embryos was a reduction in diameter of the blood vessels in the marrow cavity. Possible explanation for enlargement of blood vessels in the Haversian canals may be the release of histamine which has been reported to occur after administration of high doses of decamethonium (Koelle, 1975). Further investigation is needed to confirm this possibility such as, measurement of the level of histamine after administration of different doses of decamethonium and also measurement of the diameter of the blood vessels within bone and in its surroundings. The difference in the size of blood vessels in the marrow cavity is perhaps a direct result of muscular inactivity. Little (1973) suggested the main effect of the muscles on marrow blood vessels is that contraction blocks the venous outflow, causing vascular engorgement in the marrow cavity which ceases as the muscles become inactive.

At day 12 both control and experimental embryos showed the existence of a fibular crest at the proximal end of the lateral aspect of tibia. This was rather smaller at the 12th day of incubation in experimentals while it continued to grow in control embryos and had disappeared in the experimental embryos by 14 days. As mentioned the articular cartilages were flattened in experimental embryos rather than curved as in control embryos. These findings suggest that muscular activity has some influence upon the detail of final shape of bones. This is in agreement with the conclusion of Lanyon (1980) that the general shape of bones is predetermined but that many of their dimensions and characteristic features, particularly the size and position of their crests and tuberosities are dependent upon the presence of activity of an associated musculature both during growth and after maturity.

In control embryos invasion of the osteogenic bud to the diaphysis of the tibia was found to occur at the 11th day of incubation (Stages 36-37) agreeing with Lutfi (1971), Von der Mark et al. (1976a), Dillaman et al. (1979), Archer & Tarcliffe. (1983). Experimental embryos also showed the same pattern and timing of osteogenic bud invasion to the mid-diaphyseal region of the tibial shaft suggesting that the invasion of blood vessels into the diaphyseal shaft of bones is not under the control of environmental factors such as movement and may be a genetically programmed event as Johnson (1980) has claimed.

Whether the hypertrophic chondrocytes play a role in it as suggested by Floyd et al. (1987) and Cowell et al. (1987) cannot be confirmed here, but it can be said that hypertrophy of chondrocytes was observed 4 days earlier than initiation of invasion by the osteogenic bud.

Following invasion of blood vessels the resorption of cartilage started in the mid-diaphyseal region and progressed towards both ends of the bone. By day 14 in controls the progress of resorption of cartilage had reached the proximal and distal ends of the bone and the marrow cavity was well formed. The initial stages of cartilage resorption occurred in the experimental embryos as in controls. Later the progress of the resorption became slower and remnants of cartilage could be seen in the marrow cavity even on the 14th day of incubation. No signs of resorption were found in the ends of bone even in 14 day old embryos. This suggests a marked reduction in cartilage resorptive activity and this will be discussed further later.

2. **Quantitative changes**

After the qualitative changes in the tibia between the control and experimental embryos had been assessed it was decided to quantify these changes and the volumetric composition of the bone was measured during its development.

In the control embryos the absolute volume of cartilage, as well as the volume of the other tissues

enclosed by the perichondrium-periosteum, increased throughout the period of study. The volume of cartilage formed and resorbed per day increased too. The experimental embryos presented a retarded growth of all tissues enclosed by the periochondrium-periosteum and also a marked reduction in cartilage growth and cartilage resorption when compared with the control embryos of the same age, confirming what was seen in the histological study of the developing tibia. There are two possible explanations for this. Firstly, that the immobilisation inhibited the activity of the resorptive cells and secondly, that it had an effect on the timing of invasion by the connective and vascular tissue. The initiation of invasion had already been found to occur at the same time in experimental and control embryos and, therefore, it can be concluded that the paralysis does not have any effect on this event as discussed previously. The only possibility which remains is that after initial invasion the process of resorption had been markedly slowed down or in other words the cells are much less active than in the normal situation. This needs further investigation on the ultrastructure of the resorptive cells in order to study their activity more closely. Possible explanation of slow down of their activity will be further discussed later.

In summary, the experimental embryos failed to achieve normal growth as follows:

i) Reduction in longitudinal growth of bone.

ii) Reduction in circumferential growth of bone.

iii) Reduction in remodelling of bone.

iv) Reduction in cartilage and bone resorption.

v) Reduction in cartilage formation

Three possible mechanisms could be responsible for these differences.

1. Interference with blood supply

According to Little (1973) severe muscular inactivity results in impairment of blood supply of bone and this in turn results in reduction in supply of oxygen, etc. (Anderson, 1980), leading to reduction in osteoblast activity.

2. Lack of electrical stimuli for bone and cartilage cells

Bassett (1971) has claimed that the stimulus for bone formation and destruction involved in remodelling appears to be largely electrical in nature. Currey (1968) reported that the transduction of mechanical to electrical energy is the most likely mechanism behind the control of osseous cell activity involved in bone growth and remodelling and Hall (1975b) reported that mechanical effects on cartilage and bone may be mediated by altered electrical activity.

The mechanical force of locomotion is cyclic (Lanyon et al., 1975; Goodship et al., 1979) and it is the physiological stress to which cartilage and bone respond (Hall, 1985). In experimental embryos this mechanical

force is absent and therefore there is no electrical stimulation and it can be concluded that this may be one of the factors which slows down the growth of cartilage and bone.

3. Lack of biochemical signals

As Uchida et al. (1988) have reported mechanical forces transduce to biochemical signals in chondrocytes. They concluded that mechanical stress can alter bone growth by modulating the metabolism of growth of cartilage cells. Muscle paralysis results in lack of such biochemical signals.

Although it seems that the three above factors may contribute to the retardation of growth of the skeletal system in the experimental chick embryos it is the second, lack of electrical stimuli due to the inactivity of skeletal muscle which has most evidence in its favour . Therefore, for further investigation to assess the importance level of this factor some kind of electrical stimulation could be applied to the paralysed embryos. One possibility could be by positioning the embryo several times a day in an electromagnetic field. Recently, Borsalino et al. (1989) have described the use of low frequency pulsing electromagnetic fields to stimulate union of bone in patients with femoral intertrochantric osteotomy and reported that this significantly increases osteotomy healing.

Calcification

In the present study the timing of calcification in control embryos was established for the majority of the skeletal system including membral and chondral bones. At days 8 and 9 there were no signs of calcification. At day 10 some bones showed a pale ring of perichondral ossification which was completely red stained at day 11. A similar pattern was exhibited subsequently by other bones. All the bones which were studied with only one exception showed a one day period of variability in the time of commencement of calcification.

Schinz and Zangerl (1937) studied the time of calcification in the whole skeleton of the chick embryo by alizarin red staining. The findings of this study are very similar to their findings, or in some cases one day later.

A comprehensive study of ossification of the whole skeleton in the chick embryo was performed by Rumpler (1962). The results in the present study are 1 to 3 days later than his findings in the majority of cases. Other studies done by Erdmann (1940) and Jollie (1957) with alizarin red staining investigated the commencement of bone formation in the avian head skeleton alone. Here the timing quoted by Erdmann (1940) are about 2 to 3 days earlier than the findings of this study and those of Jollie (1957) about one day earlier. None of the previous authors have described staging criteria for their embryos, so that it is difficult to fully explain the differences in their

findings. The method employed by previous authors was purely alizarin red staining instead of the alcian blue and alizarin red used in the present study and the blue staining by alcian blue may mask the earliest signs of calcification accounting also for the period of variability which is sometimes up to 3 days.

No published work was found giving data on timing of calcification of the skeleton during muscle paralysis. In the present study the timing of commencement of calcification in the experimental embryos was established. No major changes were found between the two groups. The experimental embryos lagged about one day behind the controls for some bones and the period of variability in onset was rather longer. This was not considered as a major difference. Some of the experimental embryos were stained very palely because of loss of calcium content of skeleton as discussed previously or did not clear as well as the control embryo because of technical problems, e.g. they were very fragile and this created difficulties in skinning or eviscerating them. Therefore, it was concluded that there were no significant differences between the control and experimental embryos regarding the commencement of calcification according to age despite the fact that the experimental embryos lagged behind the controls even to the extent of 4 to 5 stages, when based on external morphological features. The timing of calcification was therefore concluded to be a genetically programmed event unaffected by muscle paralysis.

Effect of the paralysis on skeletal muscle

In this study the experimental embryos showed obvious reduction of bulk of the muscles of the limbs from about day 12 onwards compared with the controls. The muscle fibres lost their arrangement and were replaced by loose connective tissue and adipose tissue. These findings are in agreement with the findings of those authors who studied the degenerating muscle fibres in the cultured and the paralysed limbs of chick embryos (Hunt, 1932; Eastlick, 1943; Eastlick and Wortham, 1947; Sullivan, 1966, 1967; Drachman, 1964; Drachman and Sokoloff, 1966; Hall, 1975a). Therefore, it is concluded that movement is necessary for the normal development of musculature in the chick embryo.

From the present study and previous studies it appears that, in the chick embryo at least, muscular activity is essential for normal development of the skeleton, joints and musculature. It seems to play a role in enabling the cells of the bone and the cartilage to maintain their activity at a maximum for normal growth of these tissues. It also enables the embryo to counteract mechanical pressures which otherwise distort its overall shape as well as that of many of its skeletal elements.

APPENDICES

APPENDIX I:

Single injection method described by Hall (1975)

i) Method of injection

After wiping the shell overlying the air sac with a solution of Hibitane in alcohol, a burr-hole of about 1.5mm diameter was made on the upper aspect of the air sac using a dental drill. The eggs were then divided into 2 groups (20 experimental and 10 control). The experimental group were injected into the air sac with 0.5ml of sterile saline containing 1mg of the Decamethonium bromide and the control with 0.5ml sterile saline through the burr-hole using a disposable 1ml syringe fitted with a sterile 26G needle. The burr-hole was sealed with sellotape and the injected eggs were returned to the static incubator.

ii) Result

The movements of drug injected embryos were compared with that of saline injected embryos and it was found that both groups had similar movements.

iii) Variation in dosage

The procedure was then repeated in 3 more batches of eggs - by applying 2mg, 3mg and 4mg of drug in 0.5ml sterile saline for experimental groups of embryos. Again, no signs of immobilisation were observed.

iv) Conclusion

It was felt that as the single injection technique did not achieve paralysis in the regimes used it was not a satisfactory method for this investigation.

APPENDIX II:

Clearing of specimens with staining of cartilage and bone with alcian blue and alizarin red (Kimmel and Trammel, 1981).

Staining solution:

Alcian blue, 0.14% in 70% ethanol 2ml

Alizarin red, 0.12% in 95% ethanol 4ml

Glacial acetic acid 2ml

70% ethanol 50ml

1. Paralysed and normal control embryos were skinned, rinsed in running water and stained with the above staining solution for 42 hours changing solution with fresh stain after 24 hours.
2. The staining solution was decanted and replaced with acetone for 3-4 days.
3. Transferred to 90% ethanol for 12-24 hours.
4. Treated with 1% KOH from 6 hours to 24 hours.
   The paralysed embryos being fragile, were treated with 0.5% KOH. This made control of maceration and differentiation of tissue easier to regulate. Early stages of normal embryos were also treated in this way.
5. Embryos were then cleared in increased concentrations of glycerine 20%, 40%, etc. in 1% KOH (fragile ones were treated with 0.5% KOH).
6. Specimens were stored in pure glycerine containing crystals of thymol.

APPENDIX III:

Processing for wax histology -

Bouin's fixative

	75%	picric acid
	25%	formalin
+	5%	glacial acetic acid added prior to use

Staining with Mayer's haematoxylin and eosin

1)	Xylene	1-2 hours to remove wax from sections.
2)	Absolute ethanol	2 minutes
3)	Absolute ethanol	2 minutes
4)	90% ethanol	2 minutes
5)	70% ethanol	2 minutes
6)	Running water	2 minutes
7)	Mayer's haematoxylin	5 minutes
8)	Wash in running water until clear	
9)	Eosin	15-30 seconds
10)	Wash in water	2 minutes
11)	70% ethanol	1 minute
12)	90% ethanol	1 minute
13)	Absolute ethanol	0.5 minutes
14)	Absolute ethanol	0.5 minutes
15)	Xylene	5 minutes

APPENDIX IV:

Processing for wax histology -

Bouin's fixative

75% picric acid

25% formalin

5% glacialacetic acid added prior to use

Staining with Alcian blue and Mayer's haematoxylin and eosin

1)	De waxed in xylene	1-2 hours
2)	Absolute ethanol	2 minutes
3)	Absolute ethanol	2 minutes
4)	90% ethanol	2 minutes
5)	70% ethanol	2 minutes
6)	Running water	2 minutes
7)	Alcian blue pH 2.5	10 minutes
8)	Running water	2 minutes
9)	Mayer's haematoxylin	4-5 minutes
10)	Blue in running water	
11)	Eosin	2 minutes
12)	Washed in water	2 changes
13)	70% ethanol	1 minute
14)	90% ethanol	1 minute
15)	Absolute ethanol	0.5 minute
16)	Absolute ethanol	0.5 minute
17)	Xylene	5 minutes
18)	Slides then mounted in Histomount	

APPENDIX V:

Processing for wax histology -

Bouin's fixative

75%	picric acid
25%	formalin
5%	glacial acetic acid added prior to use

Masson staining

1)	Dewaxed in xylene	1-2 hours
2)	Absolute ethanol	2 minutes
3)	Absolute ethanol	2 minutes
4)	90% ethanol	2 minutes
5)	70% ethanol	2 minutes
6)	Running water	2 minutes
7)	Weigert's haematoxylin	15-20 minutes
8)	Wash in water	2 changes
9)	Differentiate in 1% acid alcohol until only nuclei is visible	
10)	Blue in Scott's (80 magnesium sulphate and 14 sodium bicarbonate)	
11)	Wash in water	2 changes
12)	2% ponceau fuchsin in 1% acetic acid	1.5 minutes
13)	Rapid wash in water	
14)	3% light green in 2% acetic acid	3 minutes
15)	Wash in water	2 changes
16)	70% ethanol	1 minute
17)	90% ethanol	1 minute
18)	Absolute ethanol	0.5 minute
19)	Absolute ethanol	0.5 minute
20)	Xylene	5 minutes

Slides then mounted in Histomount

APPENDIX VI:

Processing for semi-thin histology.

Buffered glutaraldehyde fixative

To make 5% glutaraldehyde solution:-

20 mls glutaraldehyde 25% w/v (Taab)

80 mls Millonig's buffer solution D, pH 7.2-7.4

3 mls Solution C

stored in fridge at $0^{o}C$ and used cold.

Phosphate buffered osmium tetroxide fixative

0.5 gms	osmium tetroxide
45 mls	Millonig's buffer solution D
5 mls	Solution C

This is made up in a dark coloured bottle and allowed to dissolve at room temperature overnight, and thereafter stored in fridge at $0^{o}C$.

Millonig's buffer

Solution A - 2.26% sodium dihydrogen phosphate

Solution B - 2.52% sodium hydroxide

Solution C - 5.4% glucose

Solution D - 41.5 mls A + 8.5 mls B, pH adjusted to 7.2 - 7.4.

Post fixation carried out as follows:-

1. Immersion in 1% osmium tetroxide, 55-60 minutes.
2. Washed in several changes Millonig's buffer, 0.5 hours.
3. 50% acetone: 2 hours
4. 70% acetone: 3-6 hours
5. 90% acetone: 18 hours
6. 100% acetone: 4 changes over 72 hour period
7. 1st propylene oxide - 2 hours
8. 2nd propylene oxide - 2 hours
9. Propylene oxide/Spurr's resin 1:1 mixture, 12 hours.
10. Propylene oxide/Spurr's resin 1:3 mixture, 18 hours.
11. Pure Spurr's resin - 3 changes into fresh resin taken over 7 days during which specimens were subjected to periods of time under vacuum.

Vacuum impregnation:-

Specimens were kept in their bottles of pure resin in a vacuum embedding over at 60°C for 3 periods of partial vacuum, each lasting 15-30 minutes, returning to atmospheric pressure at the end of each cycle. The process was repeated for each batch of pure resin. At 60°C, the resin is much less viscous and this, along with the partial vacuum, greatly improved resin penetration and therefore section quality.

Resin polymerisation:-

Specimens were embedded in fresh Spurr's resin, polymerisation began overnight at 30°C rising thereafter to 60°C for 16 hours.

Azur blue II stain- prepared as follows:-

Solution A - 1% Azur II	60 mls
Solution B - 1.9% sodium borate	40 mls

Mix together and filter several times.

1. Sections were dried.
2. Stained with Azur blue II 10-20 sec.
3. Washed with running water
4. Xylene 5 mins.

The sections were then mounted in Histomount.

APPENDIX VII:

Processing of the tibia for scanning electron microscopy.

1) Fixation in 5% glutaraldehyde in Millonig's buffer (pH 7.4) for 24-48 hours.
2) 3 changes of Millonig's buffer (pH 7.4)
3) Support tibia with agar
4) Tibiae were cut transversely at 400 u
5) Prepared material was washed with Millonig's buffer (pH 7.4)
6) Postfixed in Osmium tetroxide for 1 hour
7) Dehydrate through 70%, 90% and three absolute acetones
8) Specimen dried in critical point drier
9) Mounted serially, in proximodistal sequence, onto aluminium stubs and coated with gold in Polaron S.E.M. Coating unit E5000 for 5 minutes set at 1.2kV and 30MA.
10) Scanned stubs in the Jeol JSMT-300 scanning electron microscope

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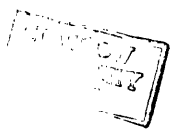

The Effect of Muscle Paralysis on the Development of the Skeleton of the Chick Embryo

By

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A Thesis Presented for the degree of Doctor of Philosophy in the Faculty of Medicine, University of Glasgow

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Fig. 1

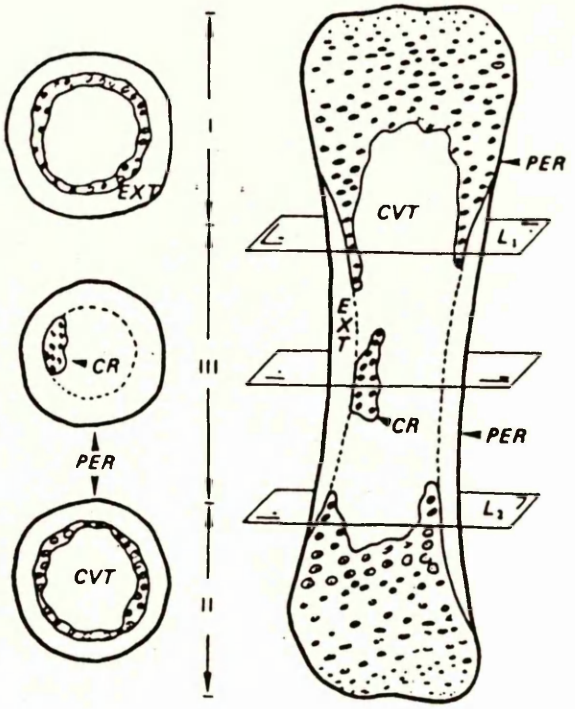
Schematic drawing of longitudinal and transverse sections of the tibia. Zones I, II and III are indicated. L1 and L2 correspond to the limits of Zone III.
PER = perichondrium-periosteum
CVT = connective and vascular tissue
CR = cartilage remnants
EXT = external space

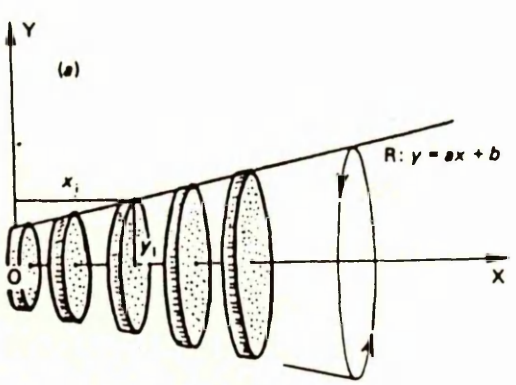
From Gaytan et at. (1987)

Fig. 2 (a-b)

Schematic drawings of the geometrical model: (a) The radius (Yi) and the distance to the coordinate origin (Xi) were measured in each cartilage slice from 11 days old embryos. Xi was given as the product of the number of sections between this point and the origin (0,0) and the average thickness of the sections. The regression line between Yi and Xi was R, which has a general equation $y = ax + b$. The truncated cone was generated by this line in rotating around the OX axis. (b) Schematic drawing of a longitudinal section of the tibia after resorption of the mid-shaft cartilage. The truncated cone section corresponds to the cartilage volume before resorption and is occupied by the connective and vascular tissue and the cartilage remnants.

From Gaytan et al. (1987)





(b)

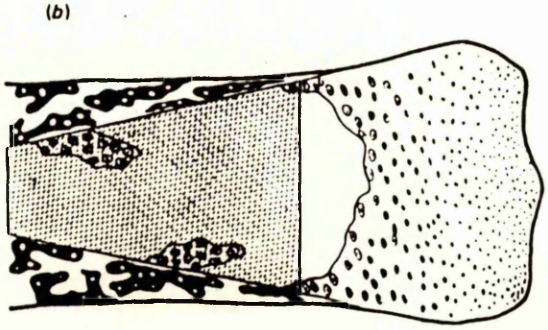


Fig. 3

Statistical comparison between the weight of the control ● and experimental O embryos showing significant reduction in the weight of the experimental embryos from 16 days onwards.

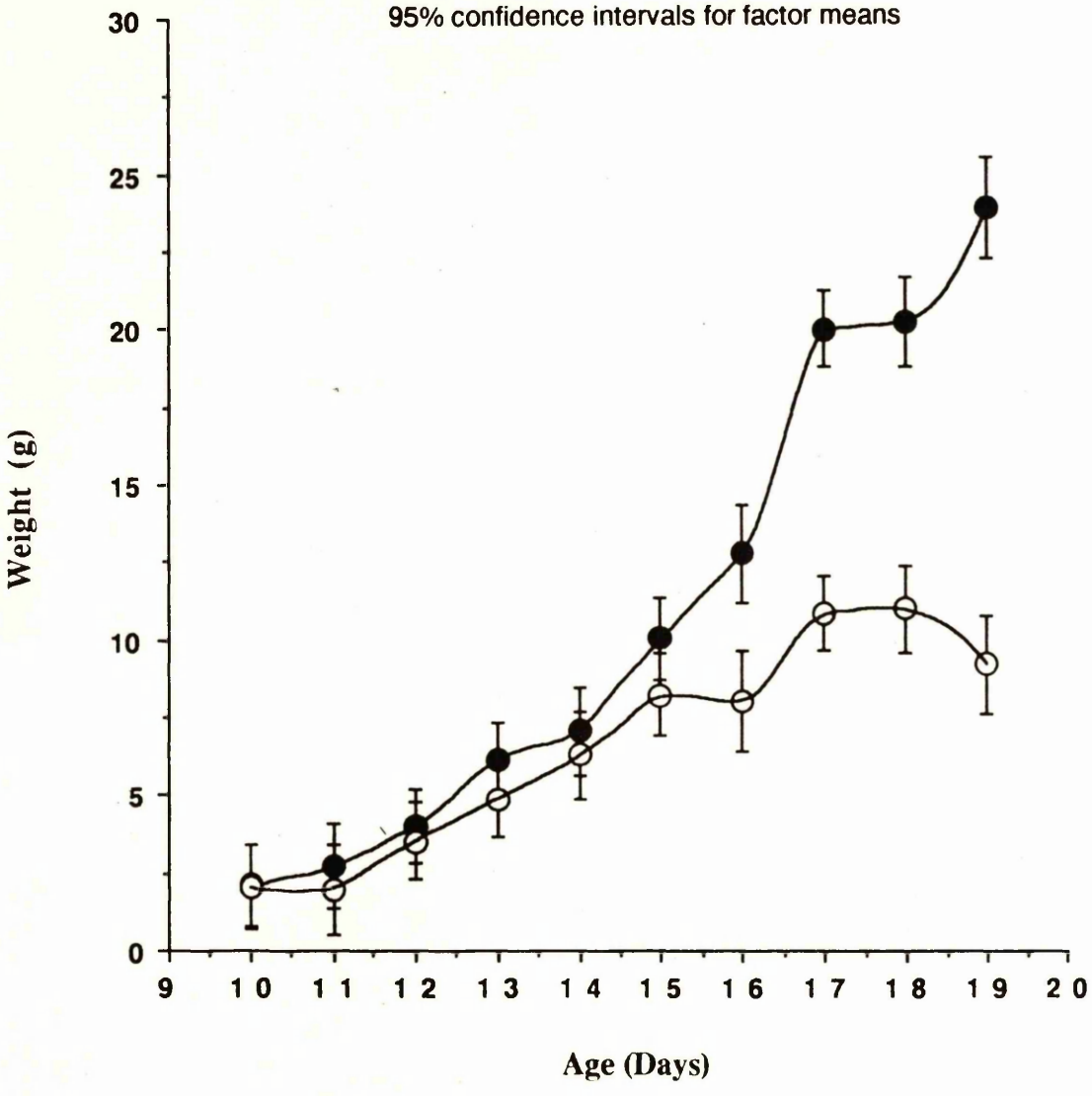

95% confidence intervals for factor means
Weight (g)
30
25
20
15
10
5
0
9
10
11
12
13
14
15
16
17
18
19
20
Age (Days)

Fig. 4a

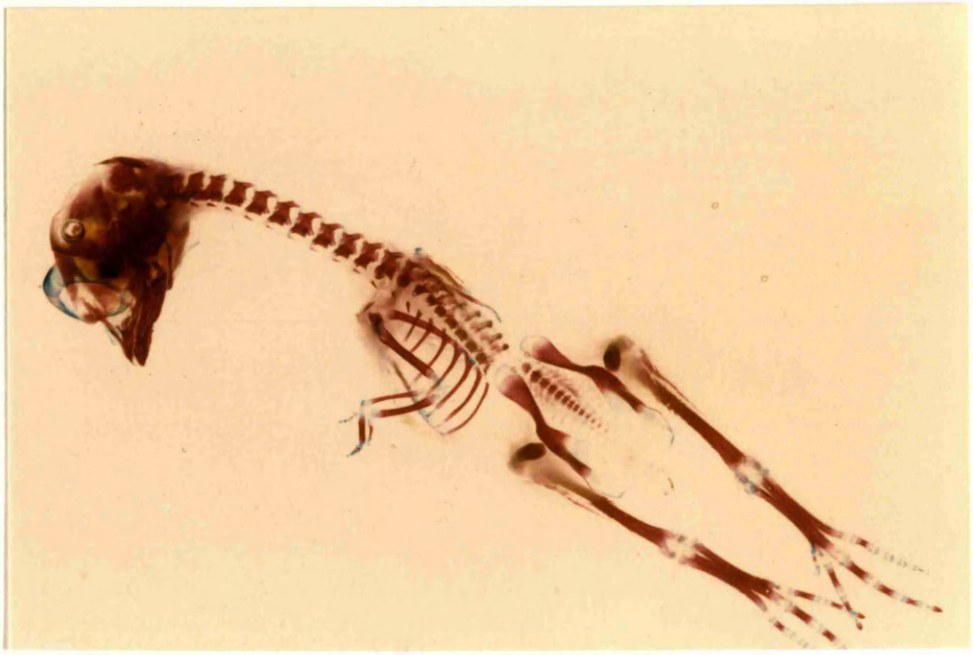
Dorsal view of a control embryo. 18 days. The vertebral column has its normal ventral curvature without any lateral flexion.

Alizarin red and alcian blue. x0.8

Fig. 4b

Dorsal view of an experimental embryo. 17 days. The embryo is small in size, distorted in shape and of stunted appearance. The vertebral column exhibits marked scoliosis.

Alizarin red and alcian blue. x3



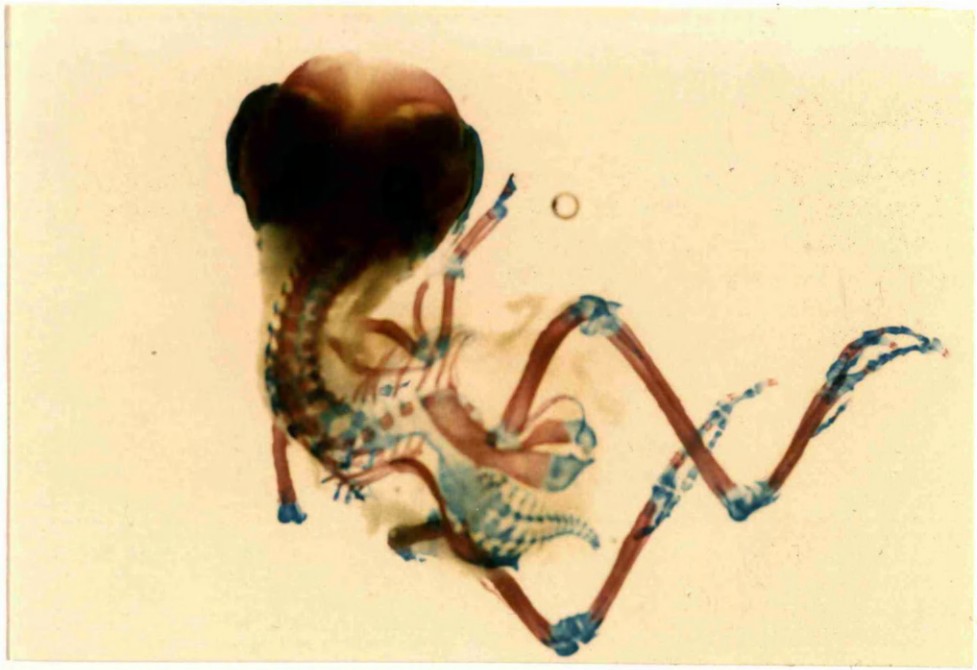


Fig. 5a

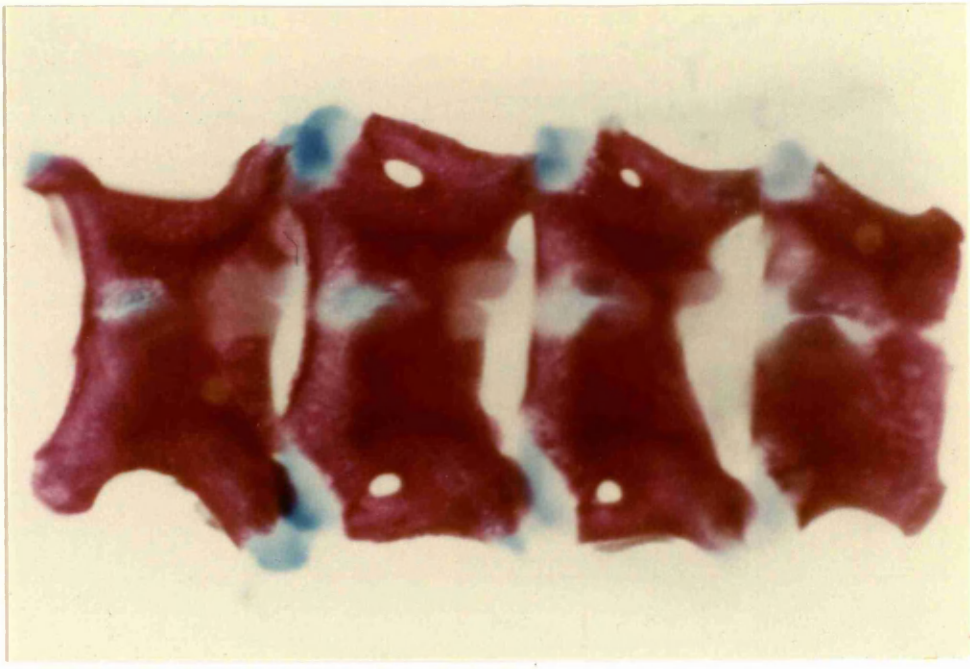
Four cervical vertebrae of a control embryo. 18 days.
The joint spaces between the vertebral bodies can be seen.

Alizarin red and alcian blue. x12.5

Fig. 5b

Four cervical vertebrae of an experimental embryo. 18 days.
The bony fusion of vertebral bodies can be seen.

Alizarin red and alcian blue. x12.5



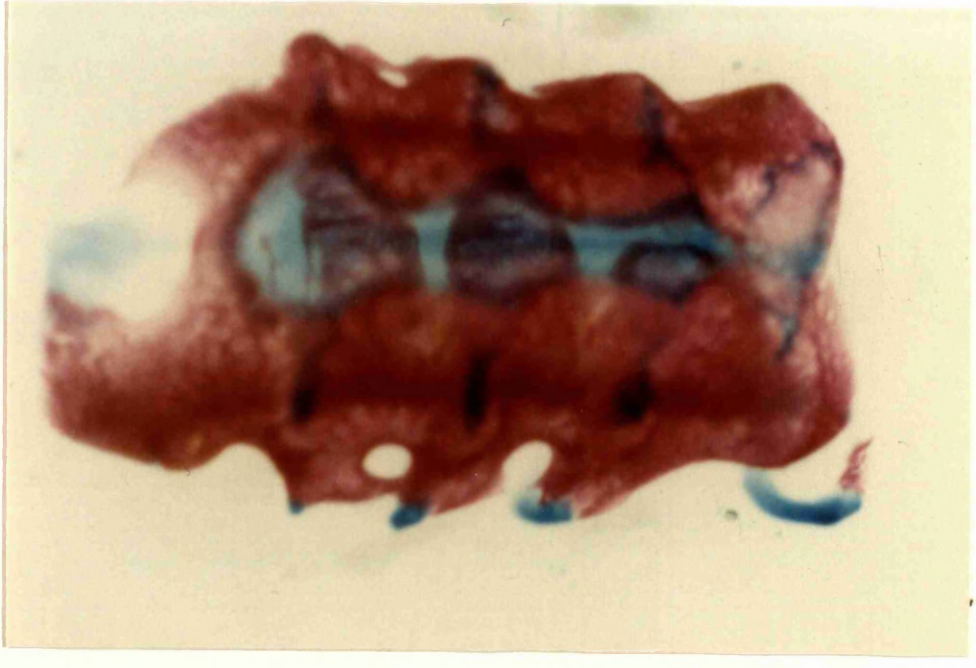


Fig. 6a

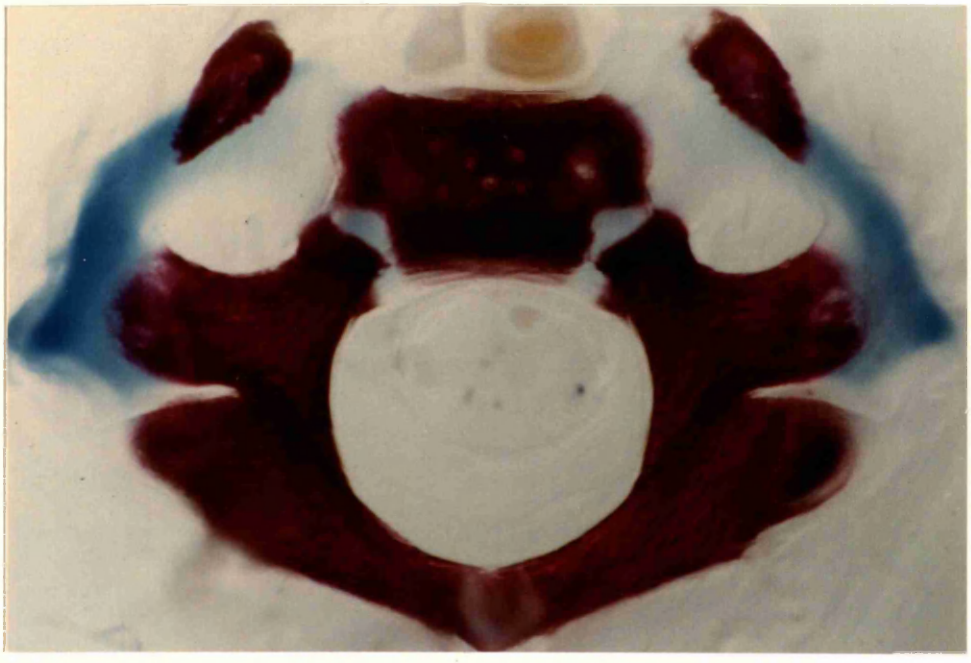
A single cerical vertebra (C3) of a control embryo . 18 days.
Dorsal fusion within the vertebral arch has occurred.

Alizarin red and alcian blue. x6

Fig. 6b

Two fused cervical vertebrae (C3 and C4) of an experimental embryo. 18 days.
The vertebrae are of small size and the arches are not completely formed of cartilage dorsally.

Alizarin red and alcian blue. x6



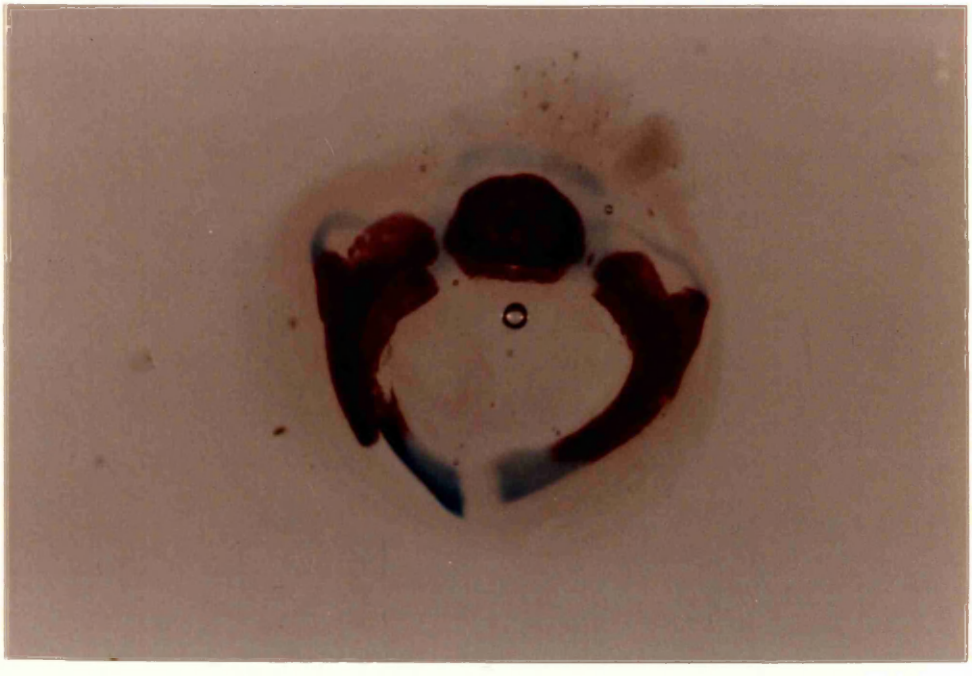


Fig. 7a

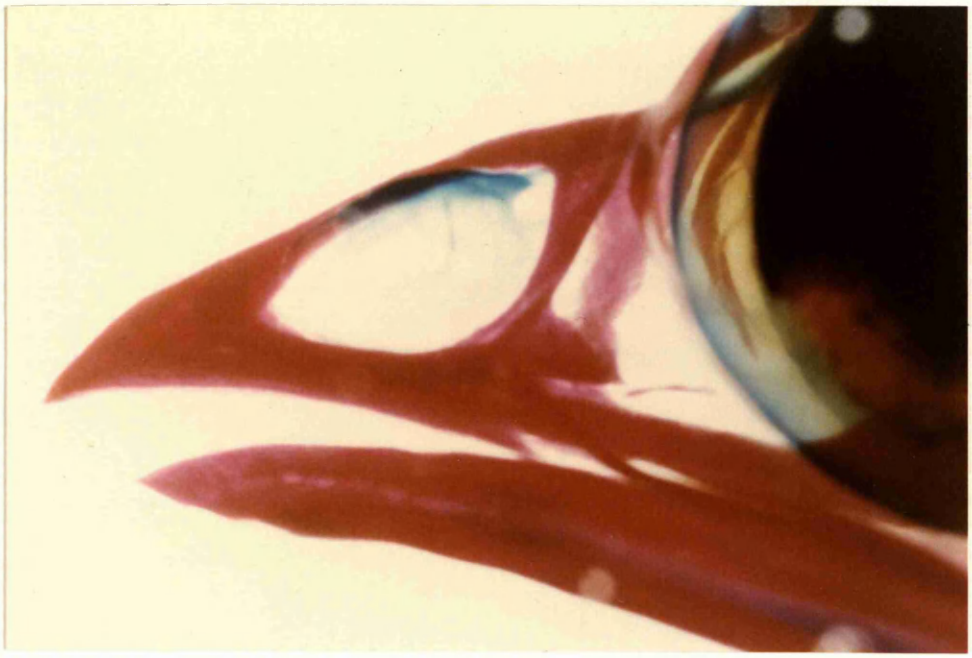
Left lateral view of jaws of a control embryo. 18 days.
Upper jaw is longer than lower.

Alizarin red and alcian blue. x8

Fig. 7b

Left lateral view of jaws of an experimental embryo. 18 days.
Upper jaw is shorter than lower and lower is protruded beyond the upper jaw.

Alizarin red and alcian blue. x12.5



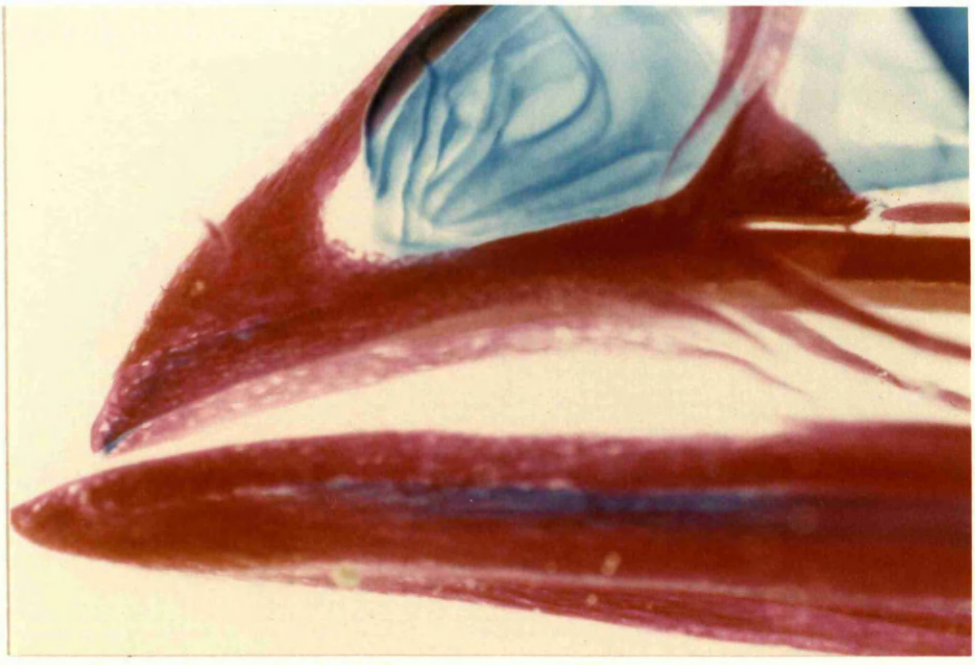


Fig. 8a

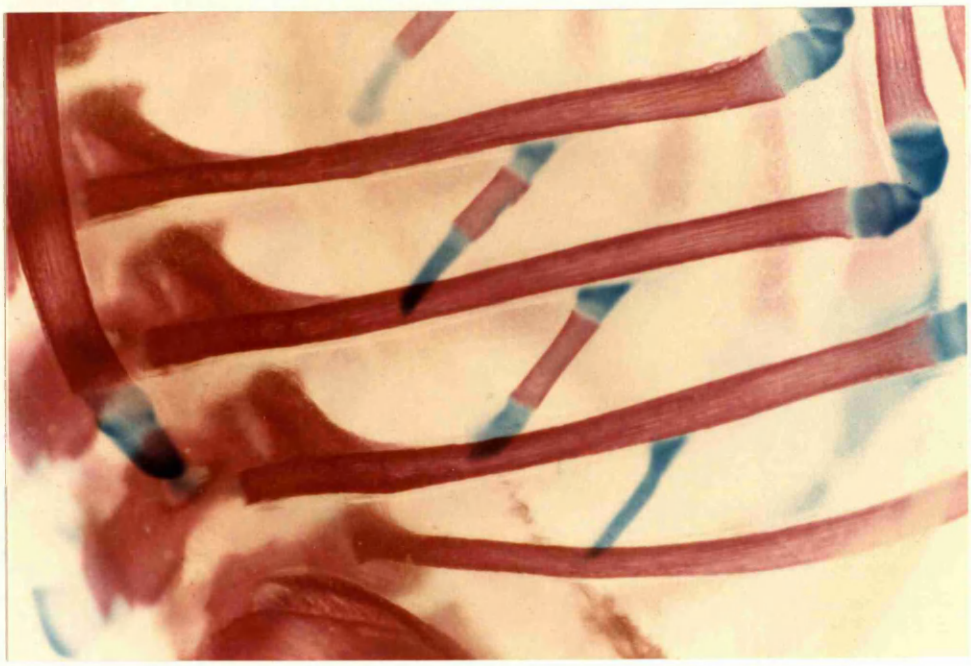
Dorsolateral view of a control embryo. 19 days. Uncinate processes are present between the vertebral ribs and ribs are evenly spaced. An intervening gap between the scapula and ilium is present.

Alizarin red and alcian blue. x10

Fig. 8b

Dorsolateral view of an experimental embryo. 20 days.
The pelvic girdle has pushed the two caudal vertebral ribs closely together and the ilium and scapula overlap. Uncinate processes are not represented in either cartilage or bone.

Alizarin red and alcian blue. x10



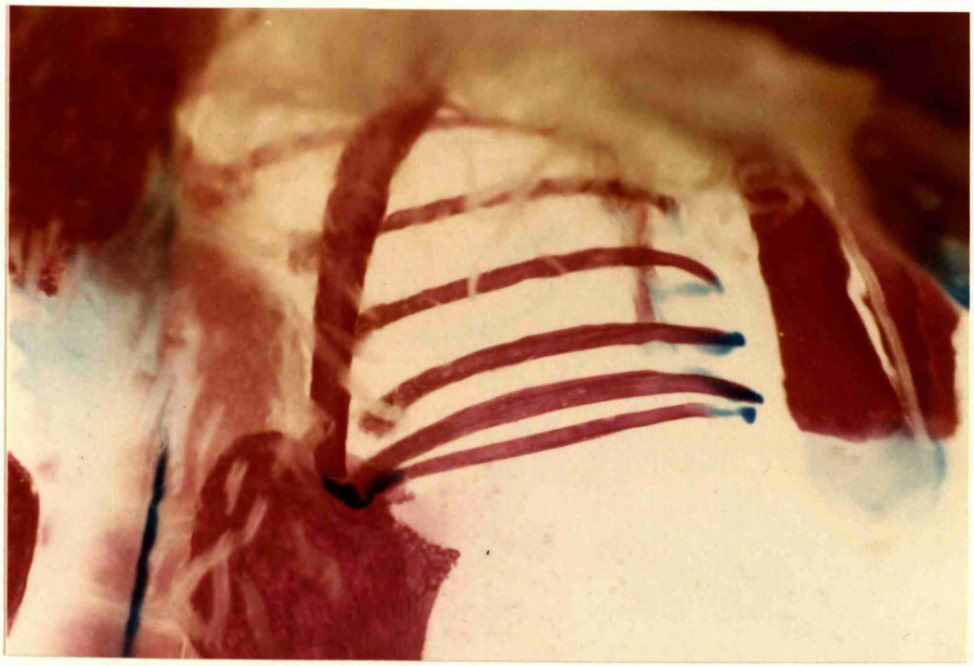


Fig. 9a

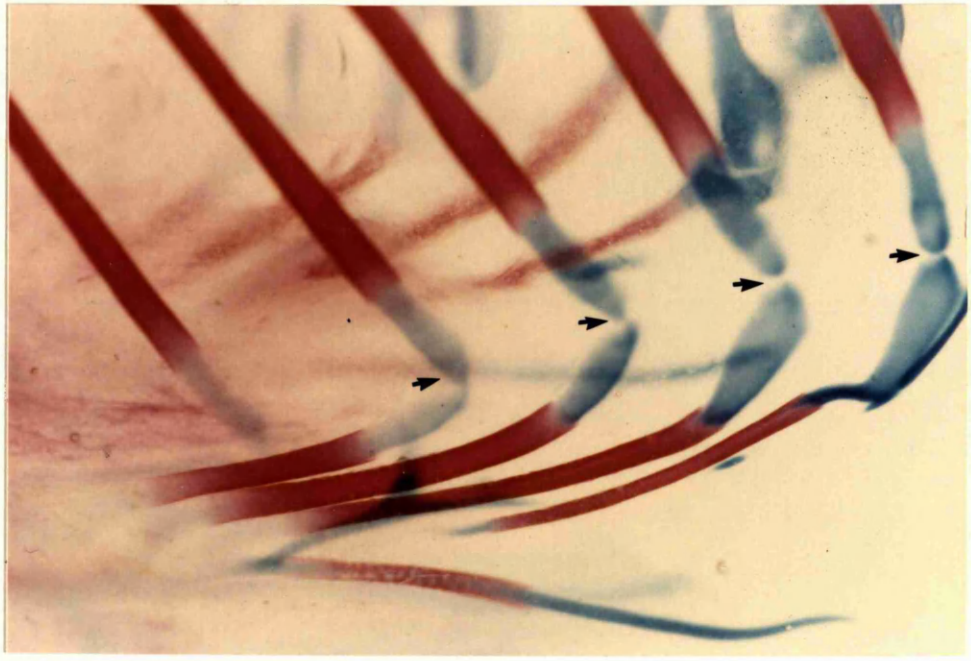
Lateral view of a control embryo. 17 days. Synovial joints between vertebral and sternal ribs (arrows) are represented by clear spaces.

Alizarin red and alcian blue. x10

Fig. 9b

Lateral view of an experimental embryo 18 days. The vertebral and sternal ribs are fused in cartilage (arrows) and are distorted. Cartilaginous uncinate processes (U) are present with the most caudal one being almost parallel with the body of the corresponding rib.

Alizarin red and alcian blue. x10



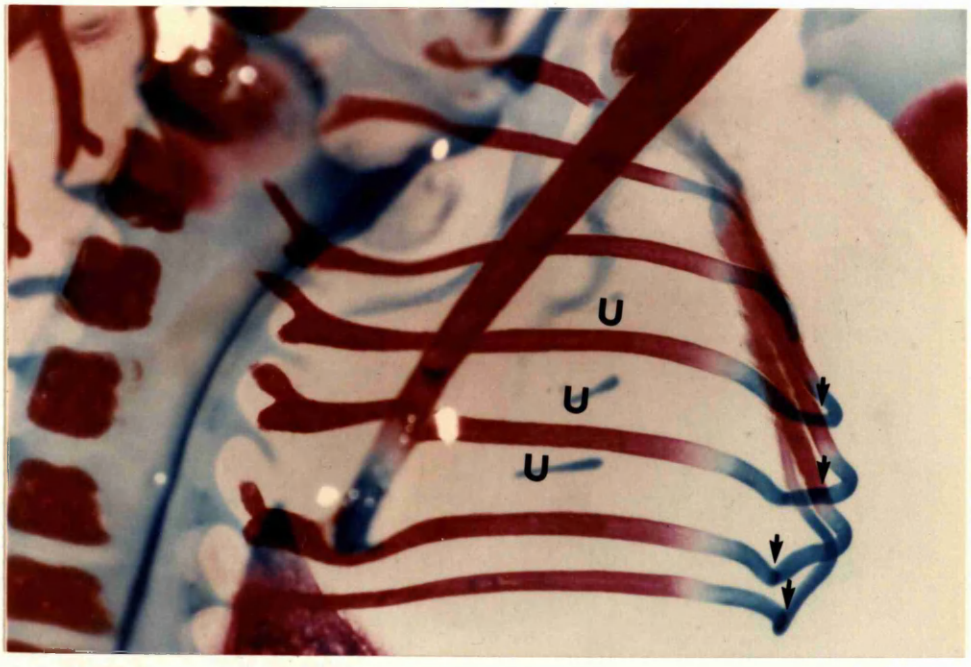

U
U
U

Fig. 10a

Left lateral view of a control embryo. 18 days. The scapula (S) is slightly curved.

Alizarin red and alcian blue. x10

Fig. 10b

Left lateral view of an experimental embryo. 18 days.
The scapula (S) has lost its normal curve and its cartilaginous caudal end is distorted. Bony fusion between cervical vertebrae can be seen.

Alizarin red and alcian blue. x10


S

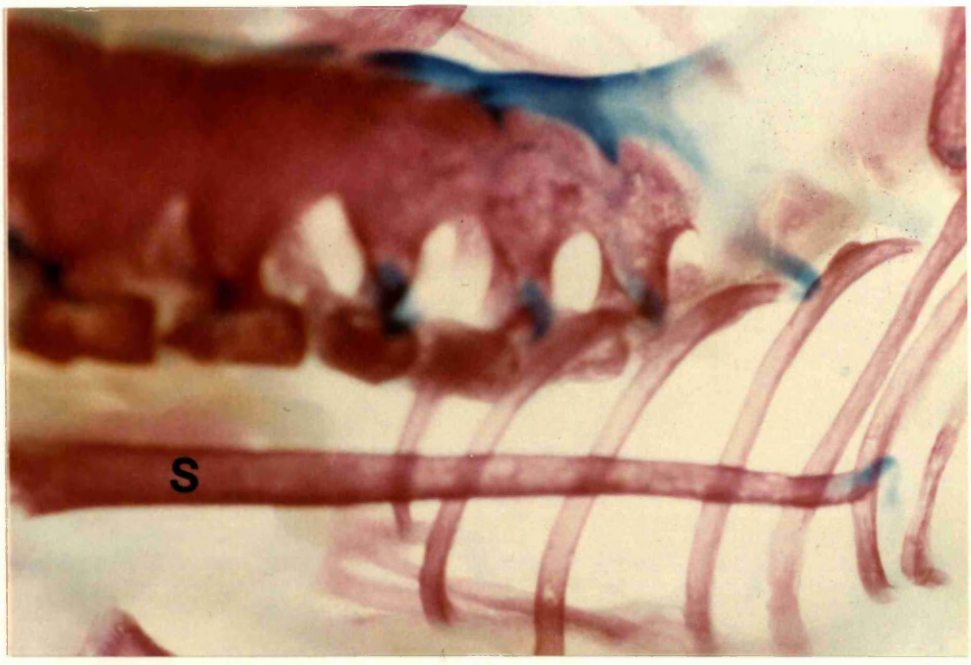

S

Fig. 11a

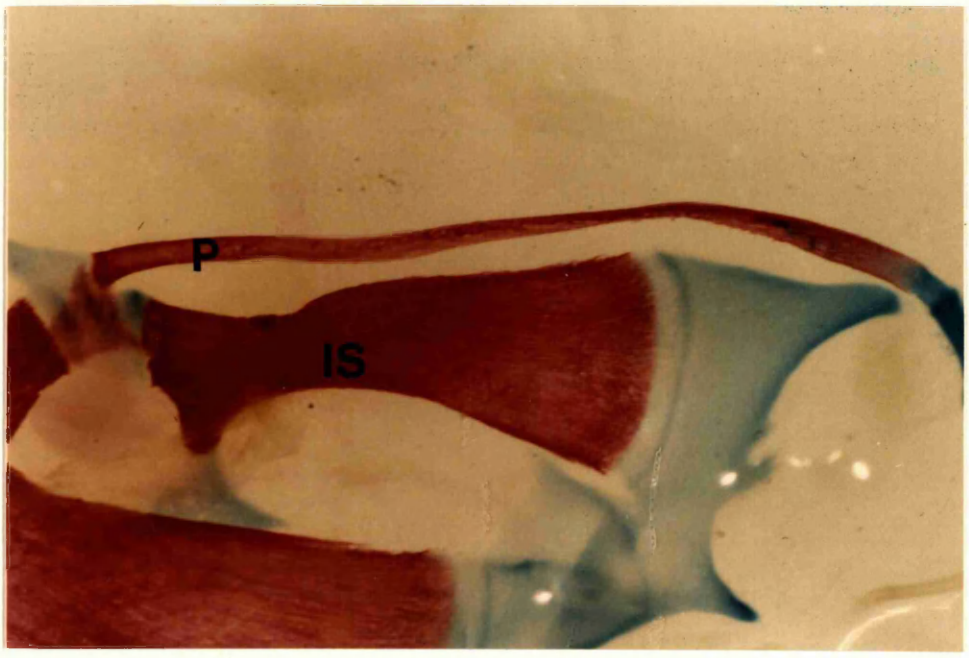
Pelvis of a control embryo. 18 days.
The rod-shaped pubis (P) extended caudally parallel with the ischium (IS) and is gently curved.

Alizarin red and alcian blue. x8

Fig. 11b

Pelvis of an experimental embryo 18 days.
The pubis (P) is markedly distorted caudally.

Alizarin red and alcian blue. x12.5


P
IS

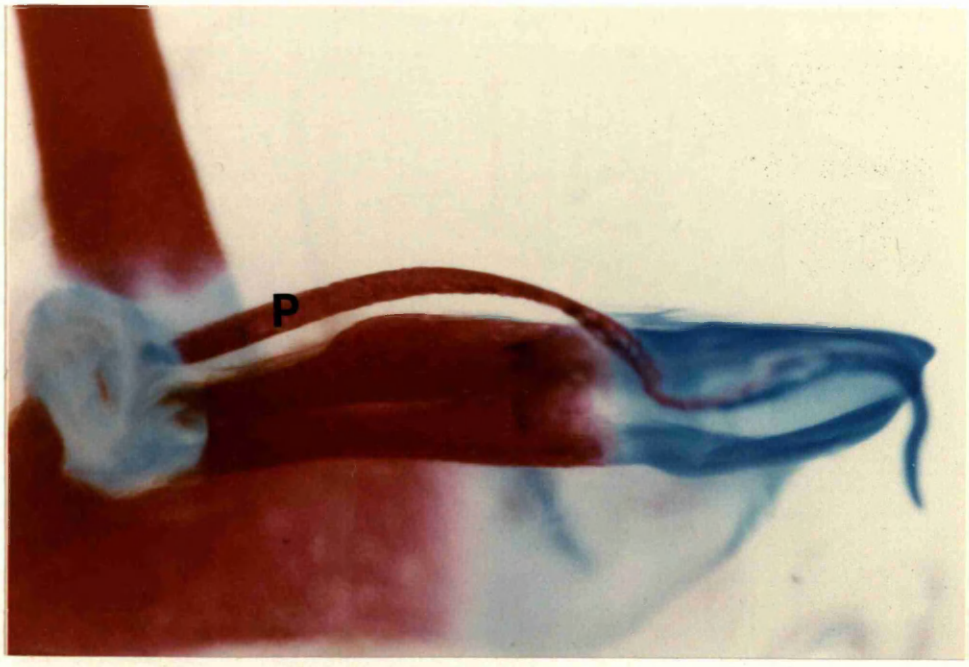

P

Fig. 12a

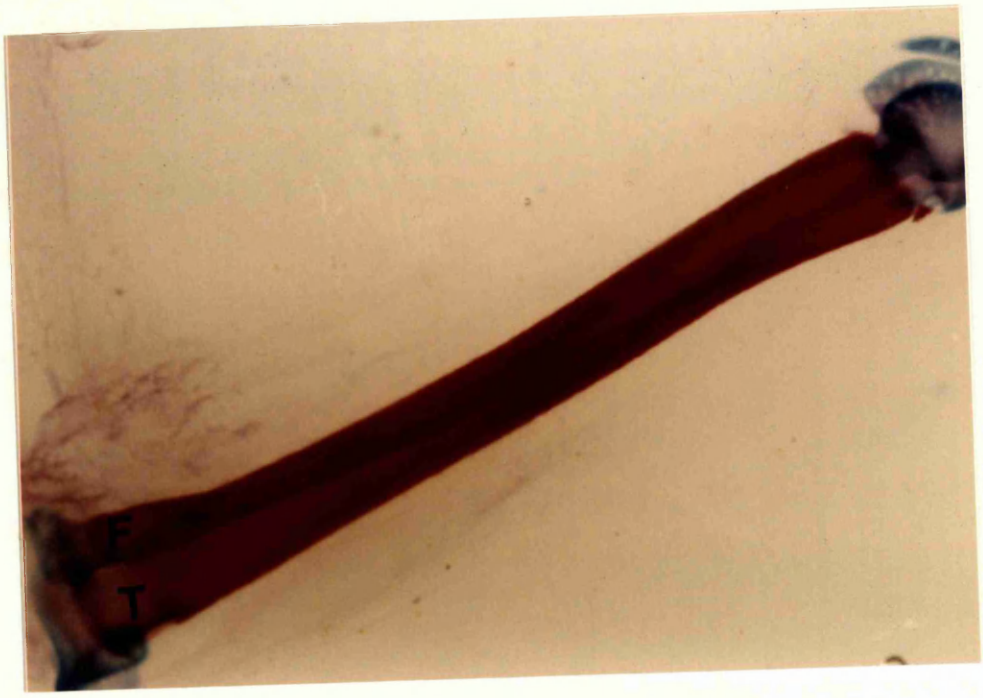
Lateral view of the tibia of a control embryo. 17 days.
Red is calcified bone, blue is cartilage.
T = tibia
F = fibula

Alizarin red and alcian blue. x6.3

Fig. 12b

Lateral view of the tibia of an experimental embryo 18 days.
Red is calcified bone, blue is cartilage.
T = tibia
F = fibula

Alizarin red and alcian blue. x22


F
T

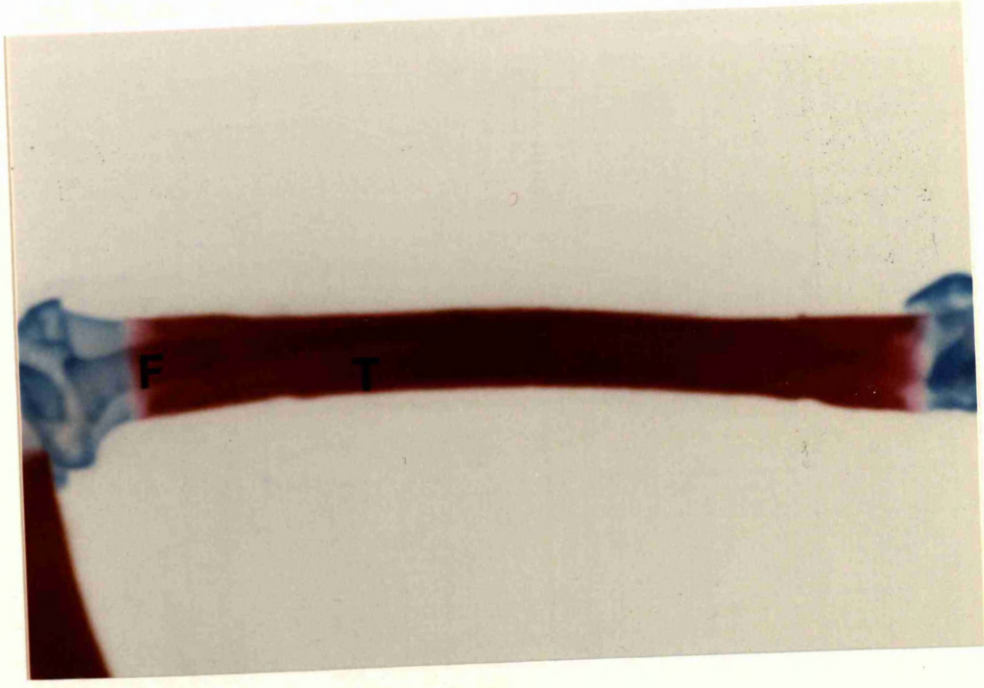

F
T

Fig. 13a

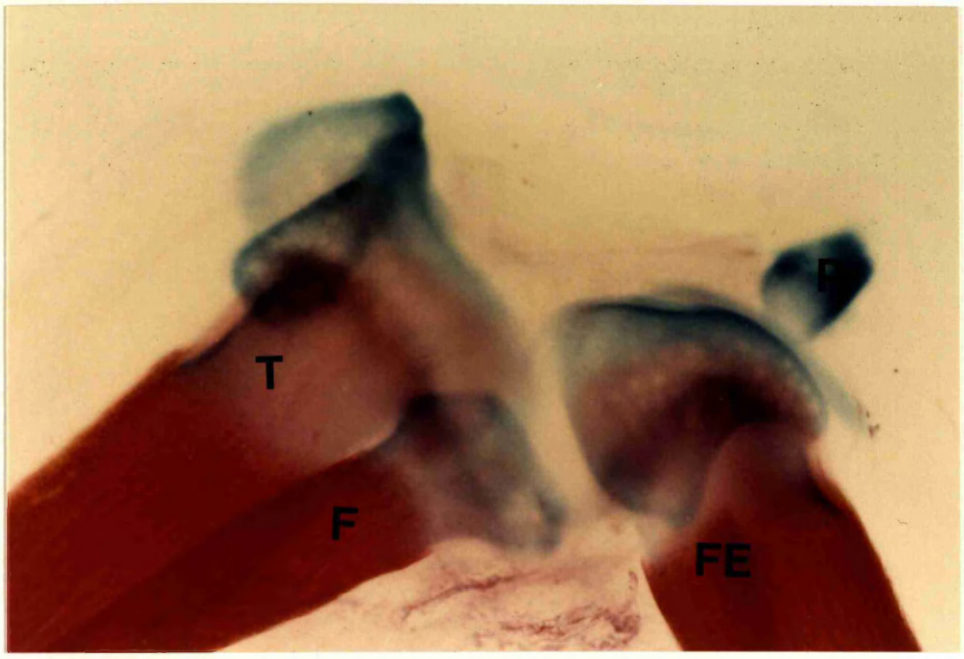
Lateral view of knee joint of a control embryo. 17 days.
The joint cavity between femur (FE) and fibula (F) and tibial (T) and the patella (P) is present. Articular surfaces are reciprocally curved.

Alizarin red and alcian blue. x14.5

Fig. 13b

Lateral view of knee joint of an experimental embryo. 20 days.
Bony fusion between the femur (FE) and fibula (F) and tibia (T) is seen. Patella is not visible. The articular surfaces are relatively flat.

Alizarin red and alcian blue. x14.5


T
F
FE

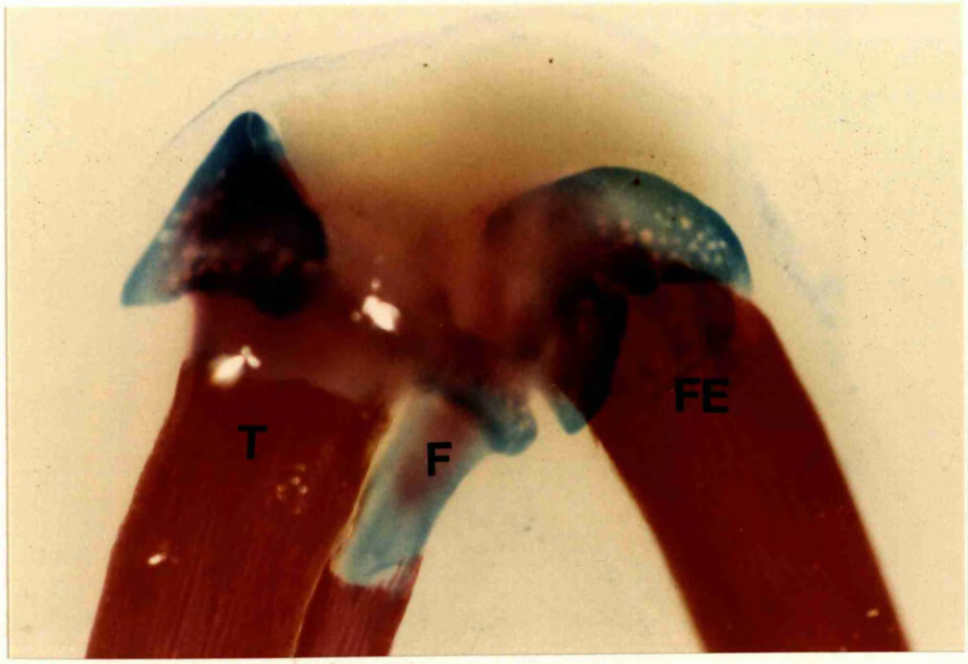

T
F
FE

Fig. 14a

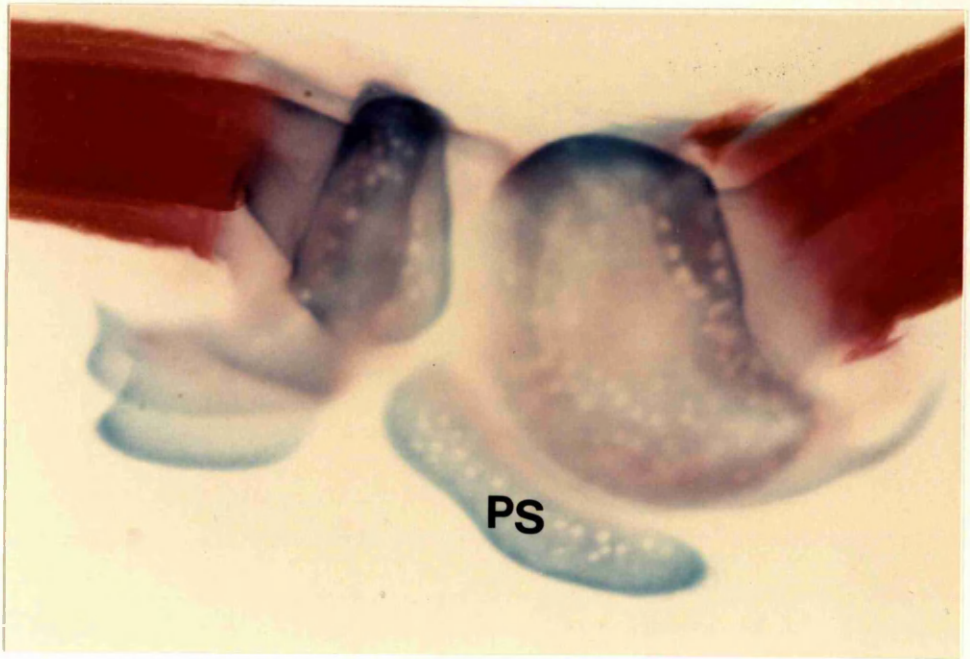
Lateral view of tarsal joint of a control embryo. 18 days.
The plantar tarsal sesamoid (PS) is present in cartilage.

Alizarin red and alcian blue. x15

Fig. 14b

Lateral view of tarsal joint of an experimental embryo. 19 days.
The plantar tarsal sesamoid is not identifiable.

Alizarin red and alcian blue. x15


PS

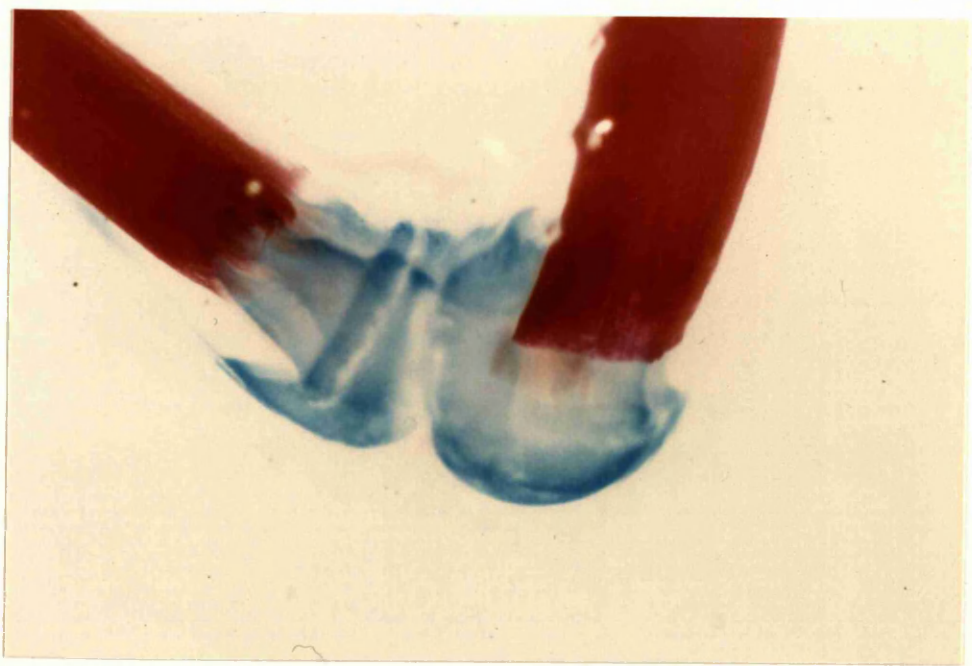


Fig. 15

Statistical comparison between the full length of femur of the control ● and experimental ○ embryos. Significant reduction of the full length of the femur is shown from 15th day onwards for experimental embryos.

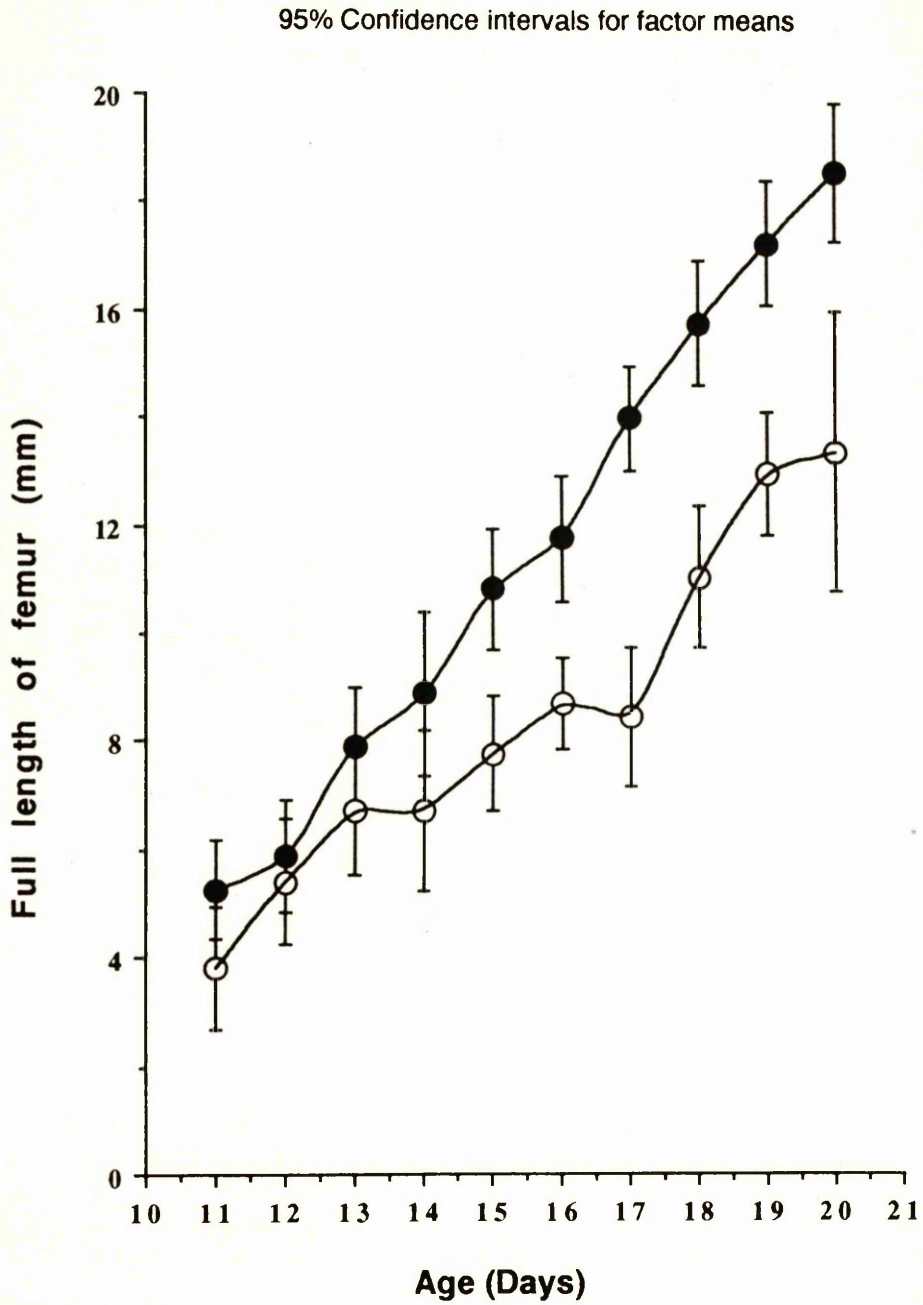

95% Confidence intervals for factor means
Full length of femur (mm)
20
16
12
8
4
0
10
11
12
13
14
15
16
17
18
19
20
21
Age (Days)

Fig. 16

Statistical comparison between the full length of tibia of the control ● and experimental ○ embryos. Significant reduction of the full length of the tibia is shown from 14th day onwards for experimental embryos.

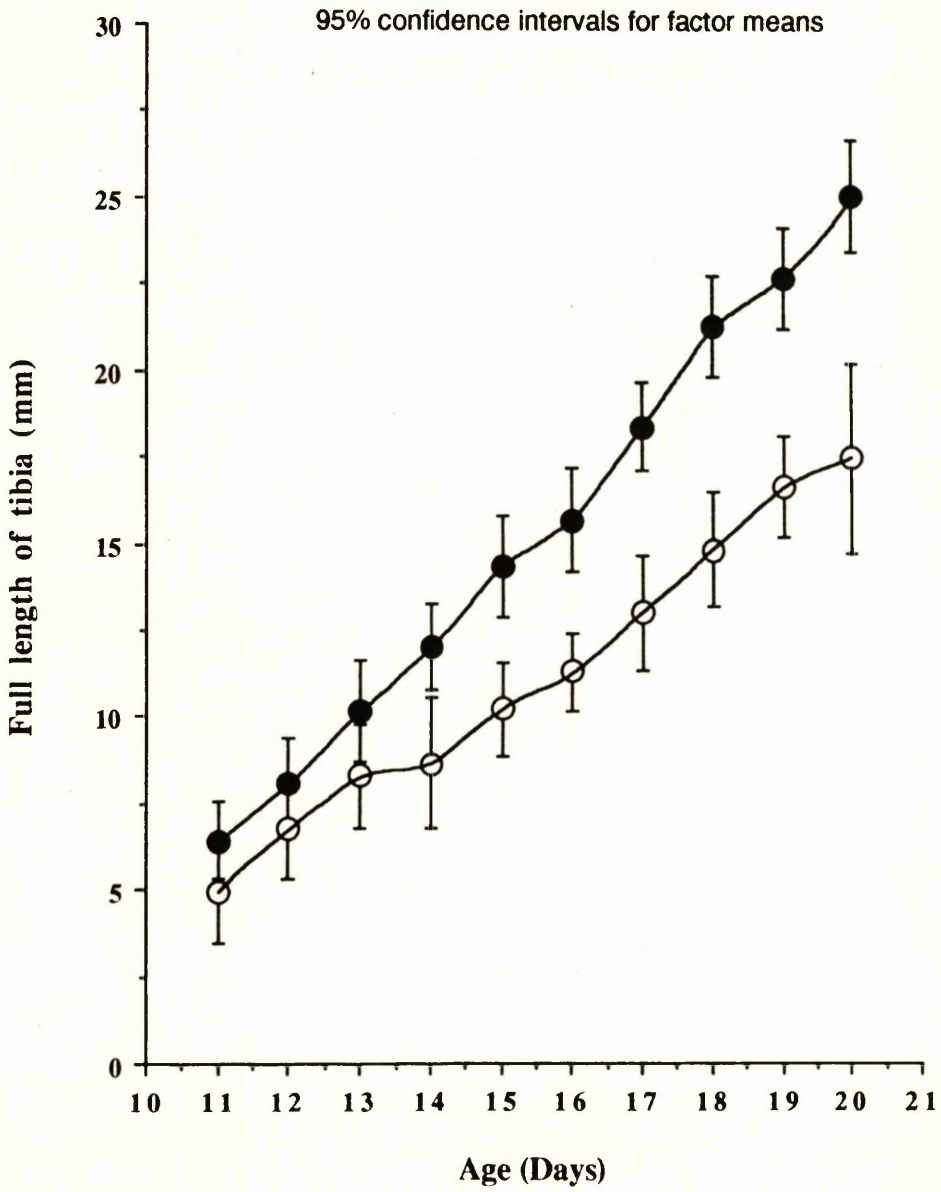

95% confidence intervals for factor means
Full length of tibia (mm)
30
25
20
15
10
5
0
10
11
12
13
14
15
16
17
18
19
20
21
Age (Days)

Fig. 17

Statistical comparison between the full length of humerus of the control ● and experimental O embryos. Significant reduction of the full length of the humerus is shown from 15th day onwards for experimental embryos.

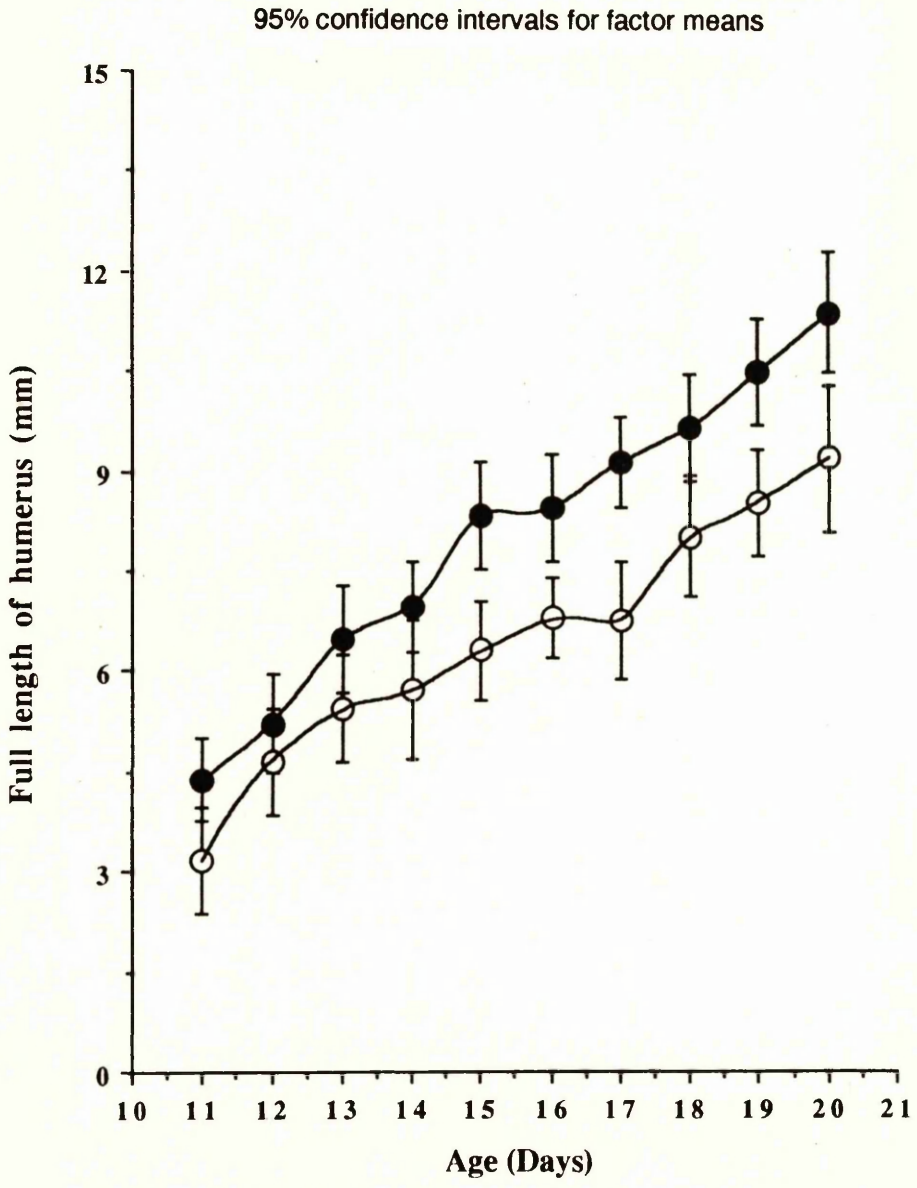

95% confidence intervals for factor means
Full length of humerus (mm)
15
12
9
6
3
0
10
11
12
13
14
15
16
17
18
19
20
21
Age (Days)

Fig. 18

Statistical comparison between the full length of ulna of the control ● and experimental ○ embryos. Significant reduction of the full length of the ulna is shown from 15th day onwards for experimental embryos.

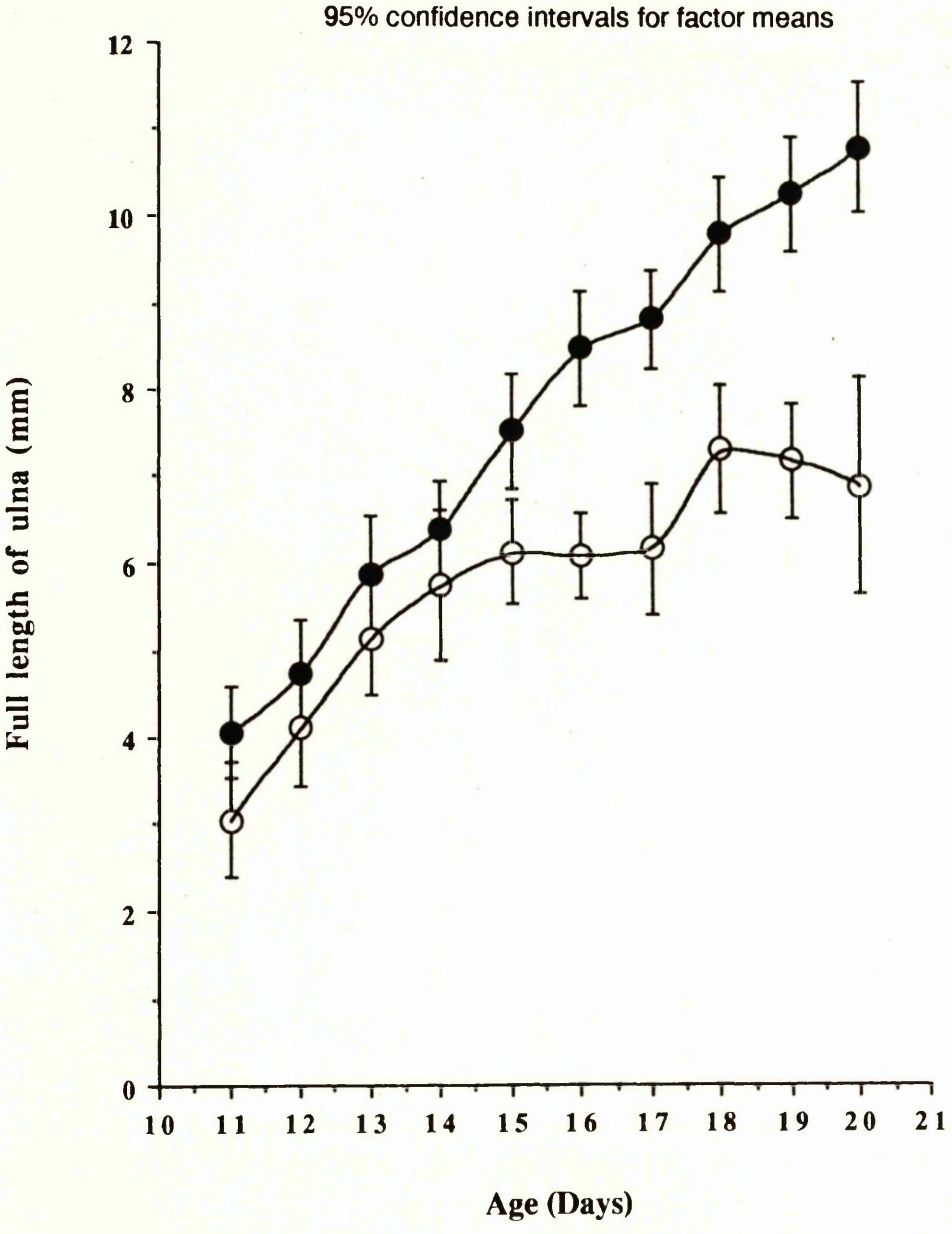

95% confidence intervals for factor means
Full length of ulna (mm)
12
10
8
6
4
2
0
10
11
12
13
14
15
16
17
18
19
20
21
Age (Days)

Fig. 19

Statistical comparison between the full length of radius of the control ● and experimental O embryos. Significant reduction of the full length of the radius is shown from 16th day onwards for experimental embryos.

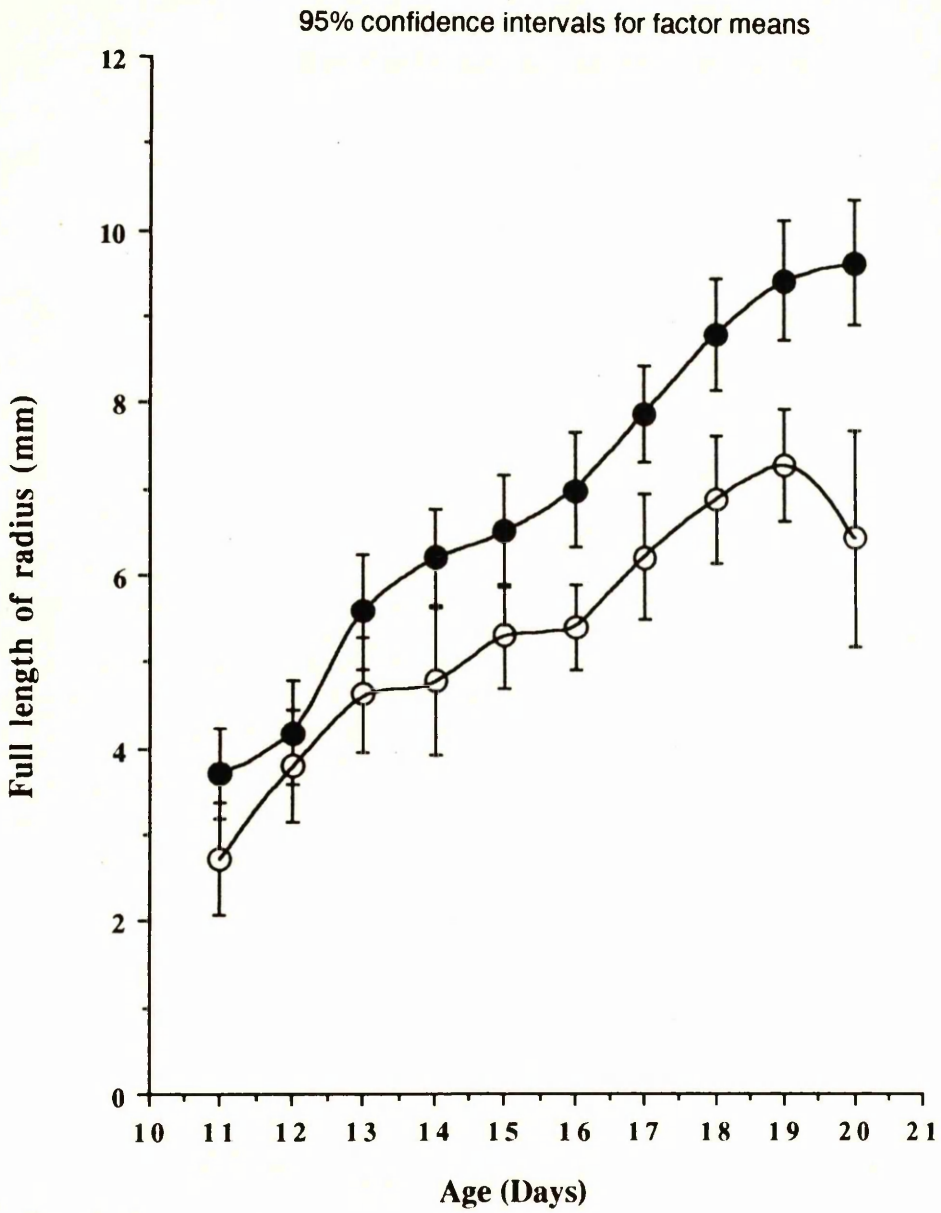

95% confidence intervals for factor means
Full length of radius (mm)
12
10
8
6
4
2
0
10
11
12
13
14
15
16
17
18
19
20
21
Age (Days)

Fig. 20

Statistical comparison between the length of calcified diaphysis of femur of the control ● and experimental O embryos. Significant reduction of length of calcified diaphysis of femur is shown from 15th day of incubation onwards for experimental embryos.

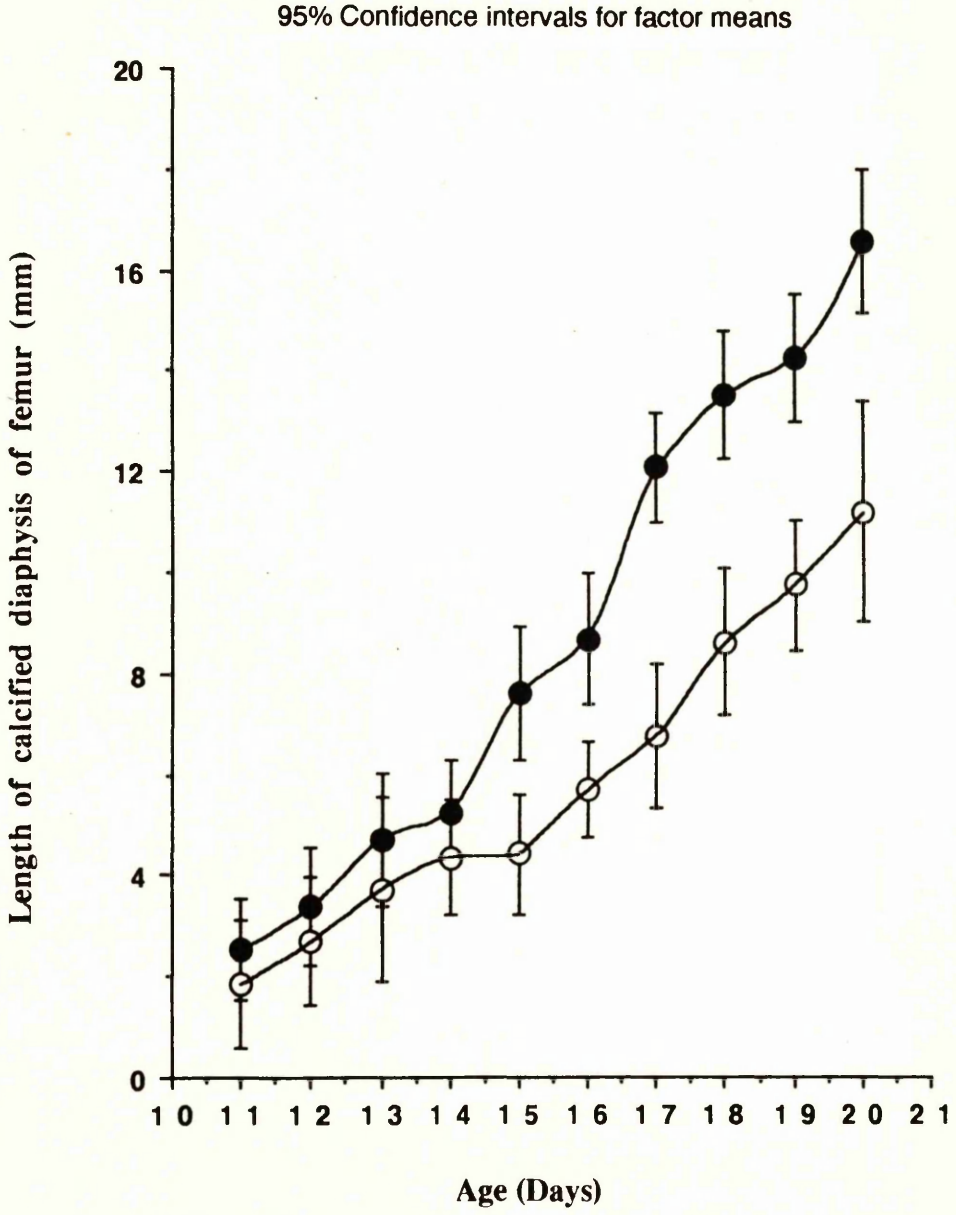

95% Confidence intervals for factor means
Length of calcified diaphysis of femur (mm)
20
16
12
8
4
0
10 11 12 13 14 15 16 17 18 19 20 21
Age (Days)

Fig. 21

Statistical comparison between the length of calcified diaphysis of tibia of the control ● and experimental ○ embryos. Significant reduction of length of calcified diaphysis of tibia is shown from 15th day of incubation onwards for experimental embryos.

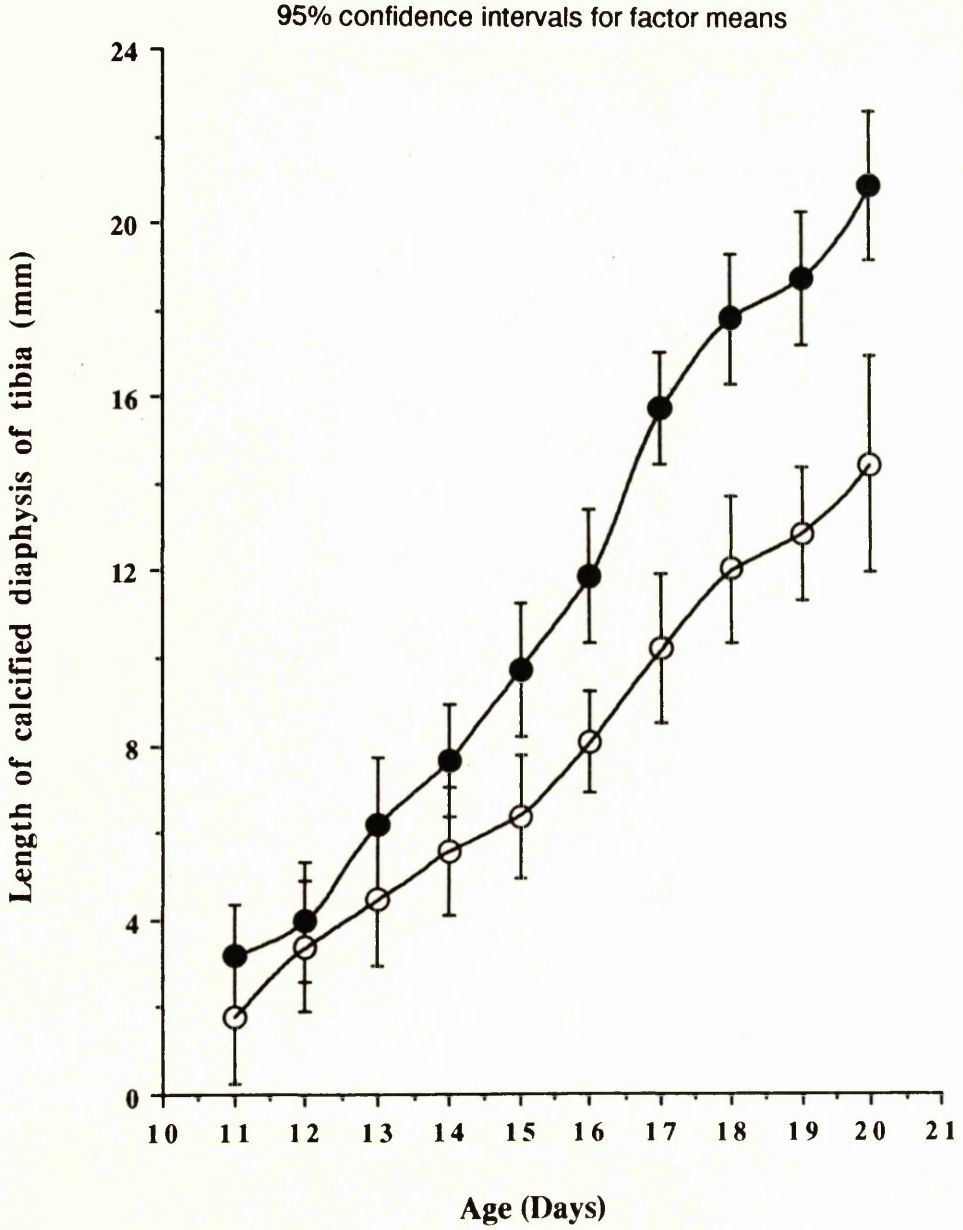

95% confidence intervals for factor means
Length of calcified diaphysis of tibia (mm)
24
20
16
12
8
4
0
10
11
12
13
14
15
16
17
18
19
20
21
Age (Days)

Fig. 22

Statistical comparison between the length of calcified diaphysis of humerus of the control ● and experimental O embryos. Significant reduction of length of calcified diaphysis of humerus is shown from 17th day of incubation onwards for experimental embryos.

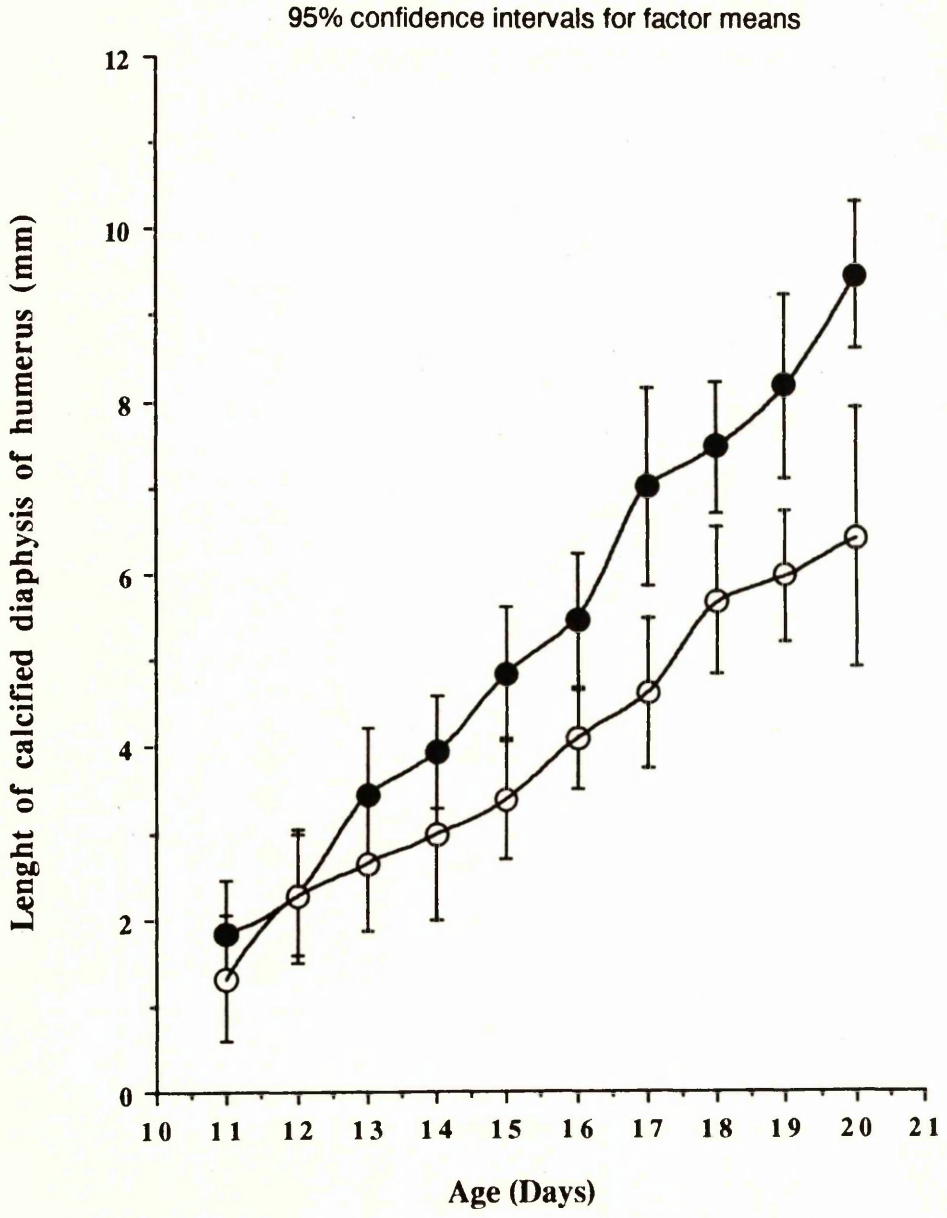

95% confidence intervals for factor means
Lenght of calcified diaphysis of humerus (mm)
0
2
4
6
8
10
12
10
11
12
13
14
15
16
17
18
19
20
21
Age (Days)

Fig. 23

Statistical comparison between the length of calcified diaphysis of ulna of the control ● and experimental O embryos. Significant reduction of length of calcified diaphysis of ulna is shown from 15th day of incubation onwards for experimental embryos.

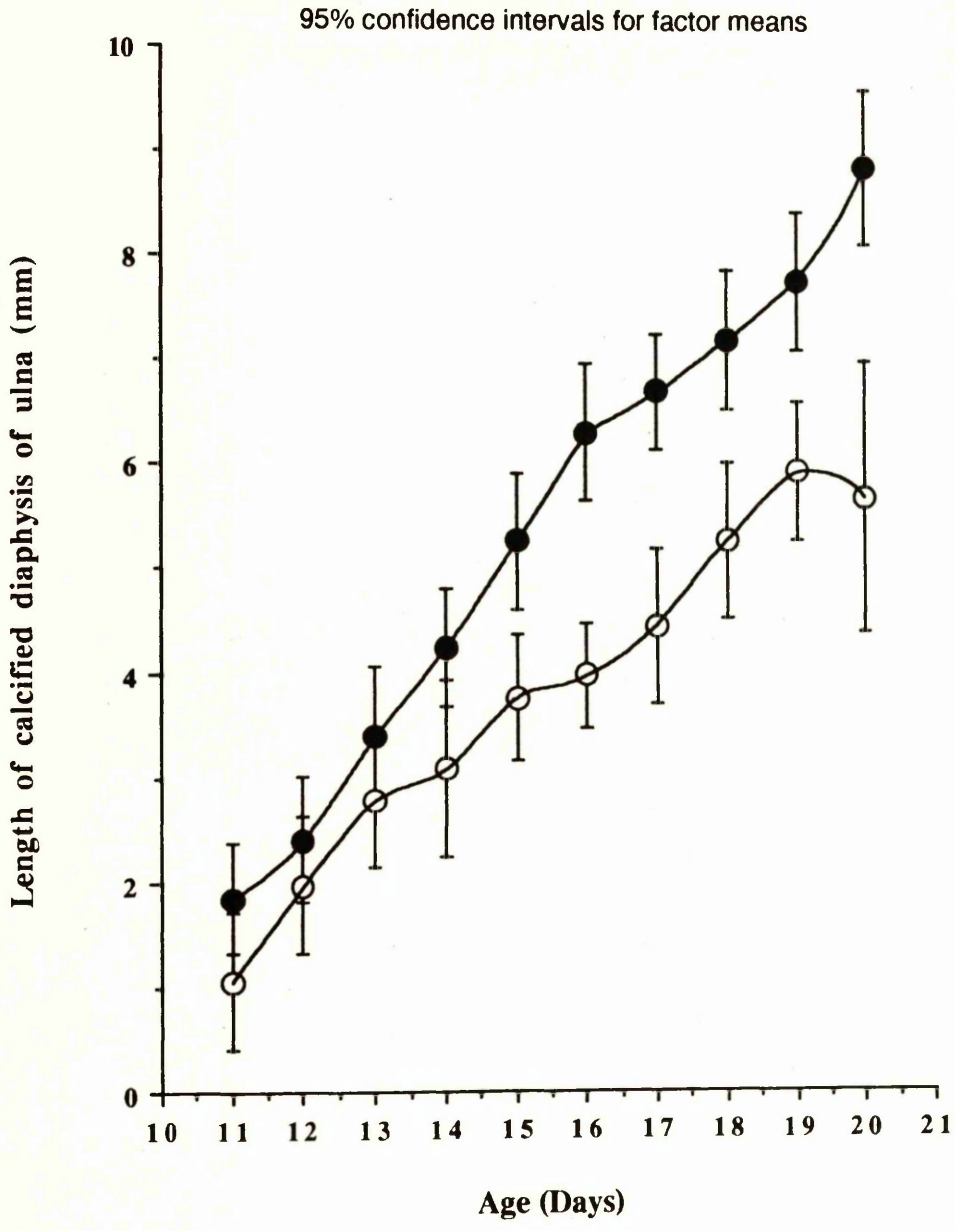

95% confidence intervals for factor means
Length of calcified diaphysis of ulna (mm)
10
8
6
4
2
0
10
11
12
13
14
15
16
17
18
19
20
21
Age (Days)

Fig. 24

Statistical comparison between the length of calcified diaphysis of radius of the control ● and experimental O embryos. Significant reduction of length of calcified diaphysis of radius is shown from 16th day of incubation onwards for experimental embryos.

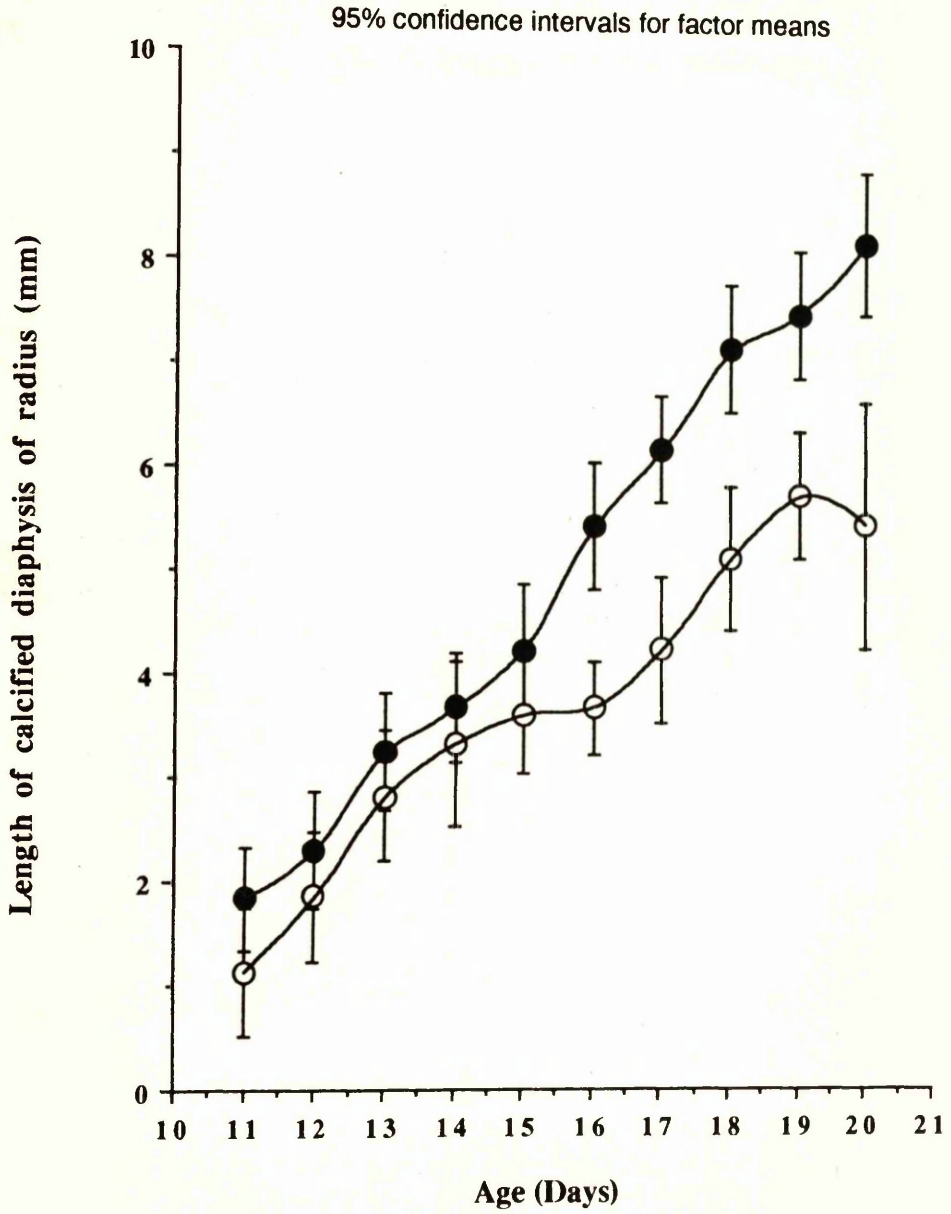

95% confidence intervals for factor means
Length of calcified diaphysis of radius (mm)
10
8
6
4
2
0
10
11
12
13
14
15
16
17
18
19
20
21
Age (Days)

Fig. 25

Statistical comparison between the length of lower jaw in control ● and experimental ○ embryos. No significant differences are shown between the two groups.

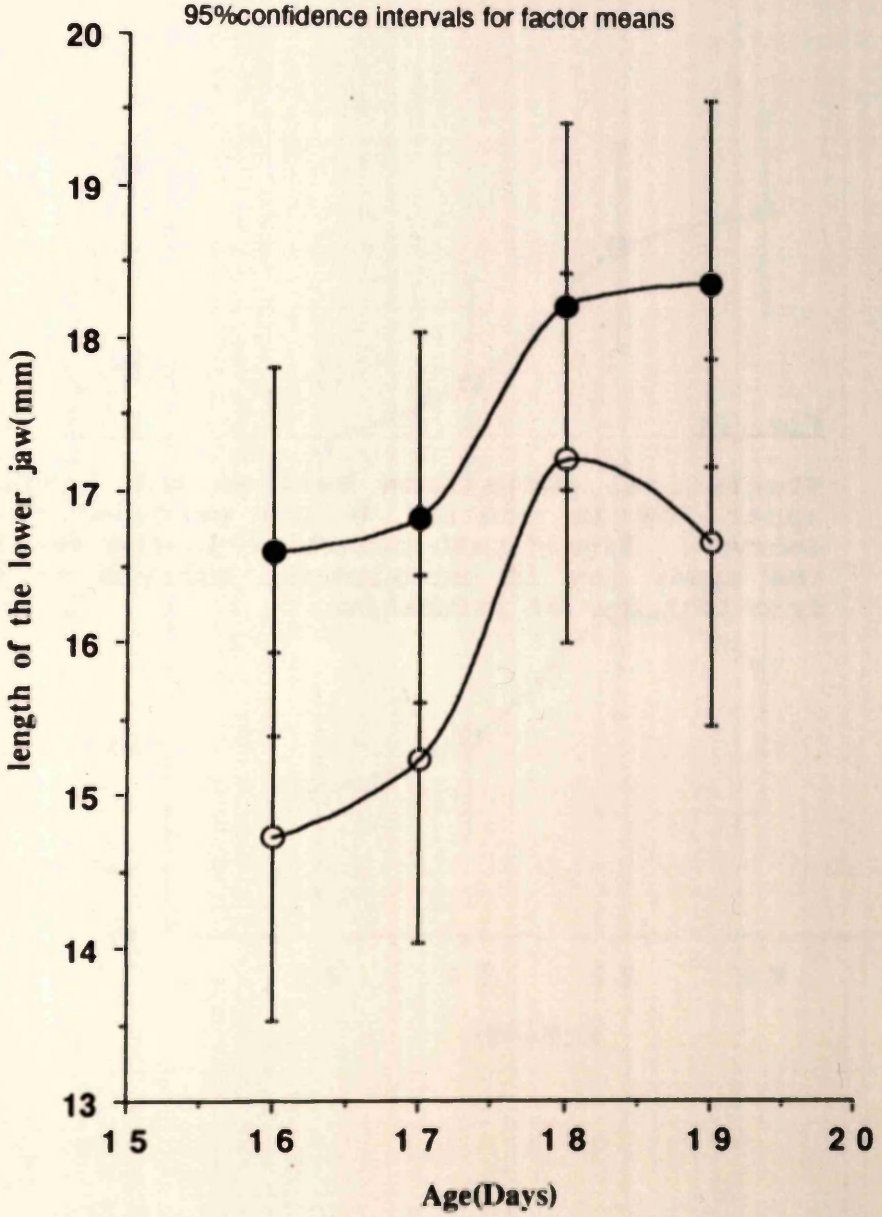

95%confidence intervals for factor means
20
19
18
17
16
15
14
13
length of the lower jaw(mm)
15
16
17
18
19
20
Age(Days)

Fig. 26

Statistical comparison between the length of upper jaw in control ● and experimental ○ embryos. Significant reduction in the length of the upper jaw in experimental embryos is seen from 16th day of incubation.

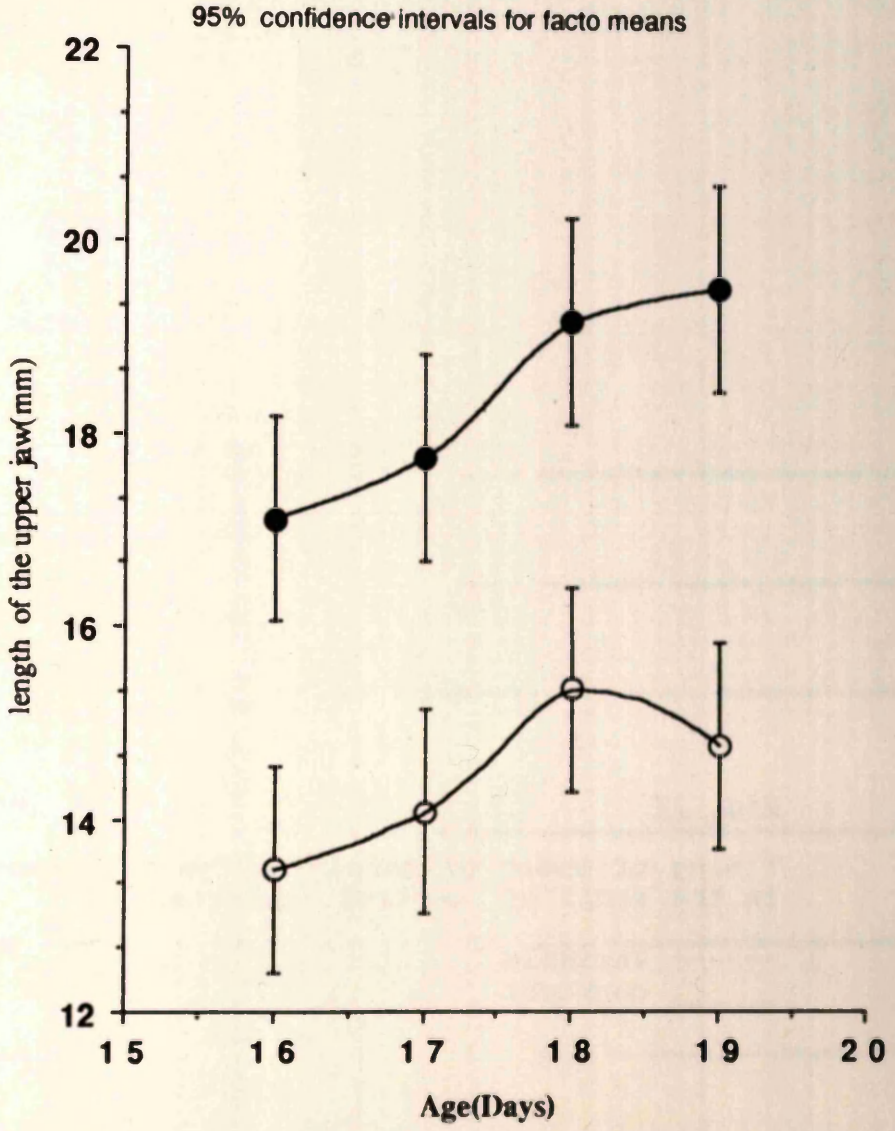

95% confidence intervals for facto means
22
20
18
16
14
12
length of the upper jaw(mm)
15
16
17
18
19
20
Age(Days)

Fig. 27

Timing of onset of calcification of bones in the skull of control embryos.

------ variable
_____ constant

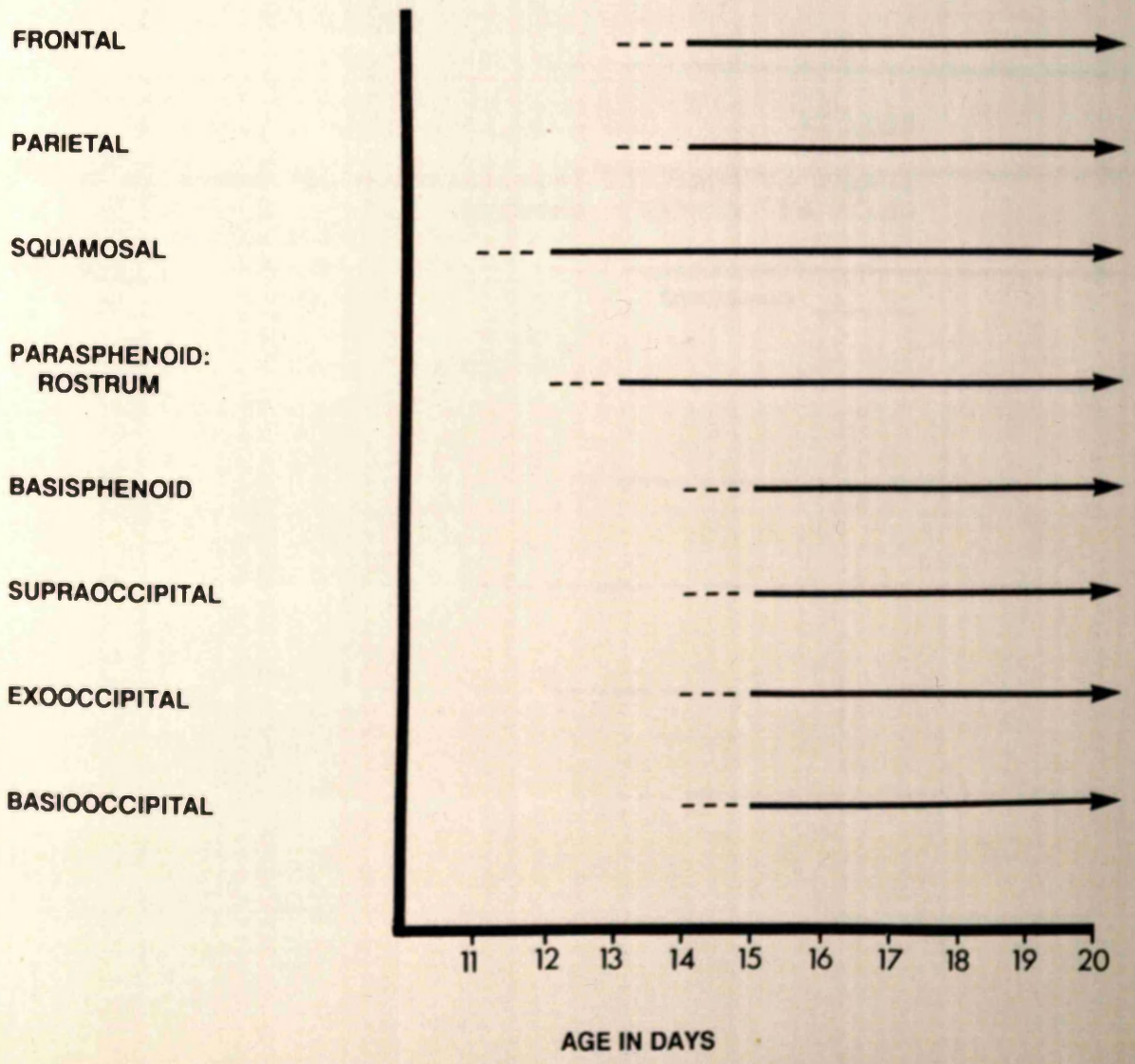

FRONTAL
PARIETAL
SQUAMOSAL
PARASPHENOID:
ROSTRUM
BASISPHENOID
SUPRAOCCIPITAL
EXOOCCIPITAL
BASIOOCCIPITAL
11
12
13
14
15
16
17
18
19
20
AGE IN DAYS

Fig. 28

Timing of onset of calcification of bones in the skull of control embryos.

------ variable
_____ constant

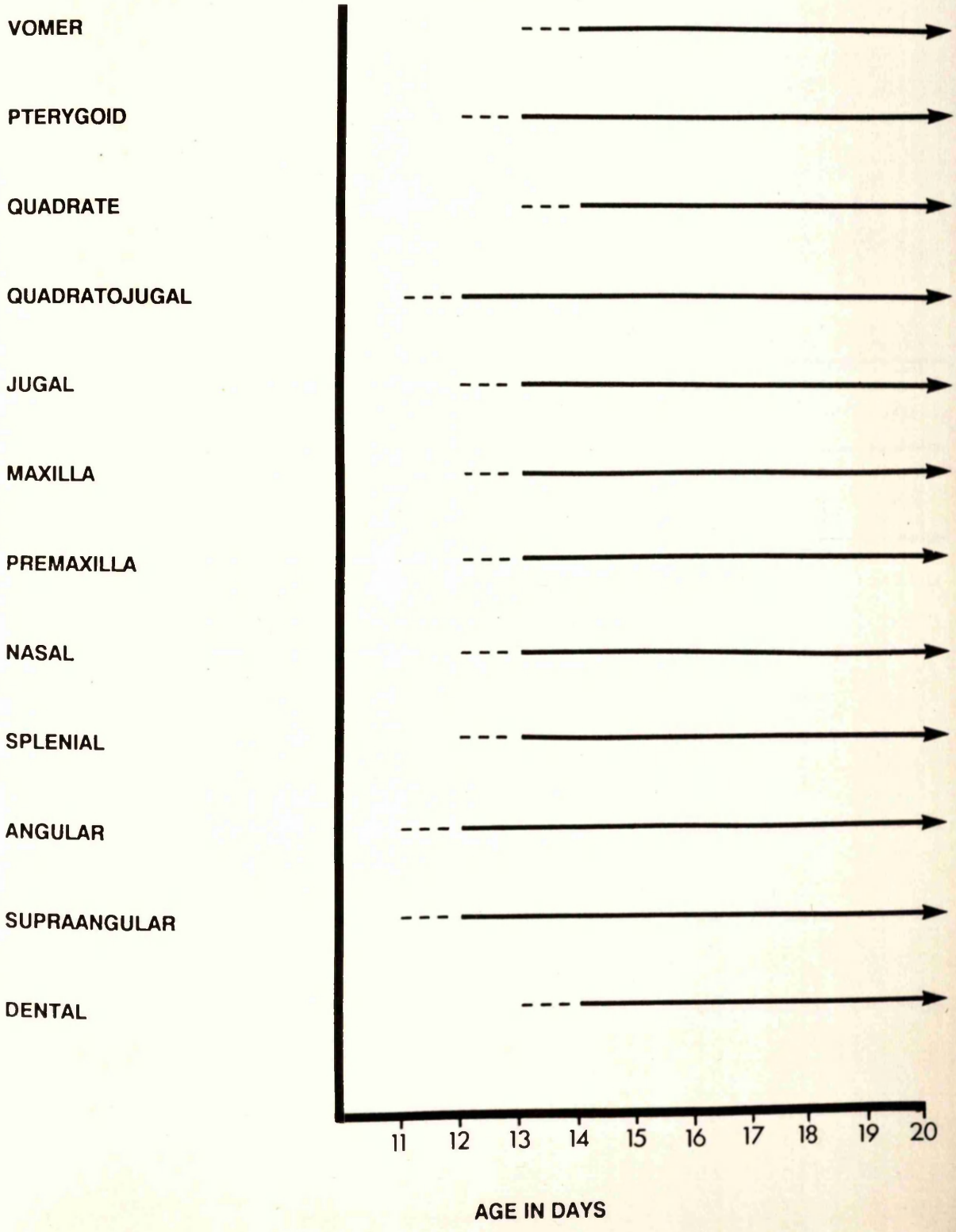

VOMER
PTERYGOID
QUADRATE
QUADRATOJUGAL
JUGAL
MAXILLA
PREMAXILLA
NASAL
SPLENIAL
ANGULAR
SUPRAANGULAR
DENTAL
11
12
13
14
15
16
17
18
19
20
AGE IN DAYS

Fig. 29

Timing of onset of calcification of bones in the skull of experimental embryos.

------ variable
_____ constant

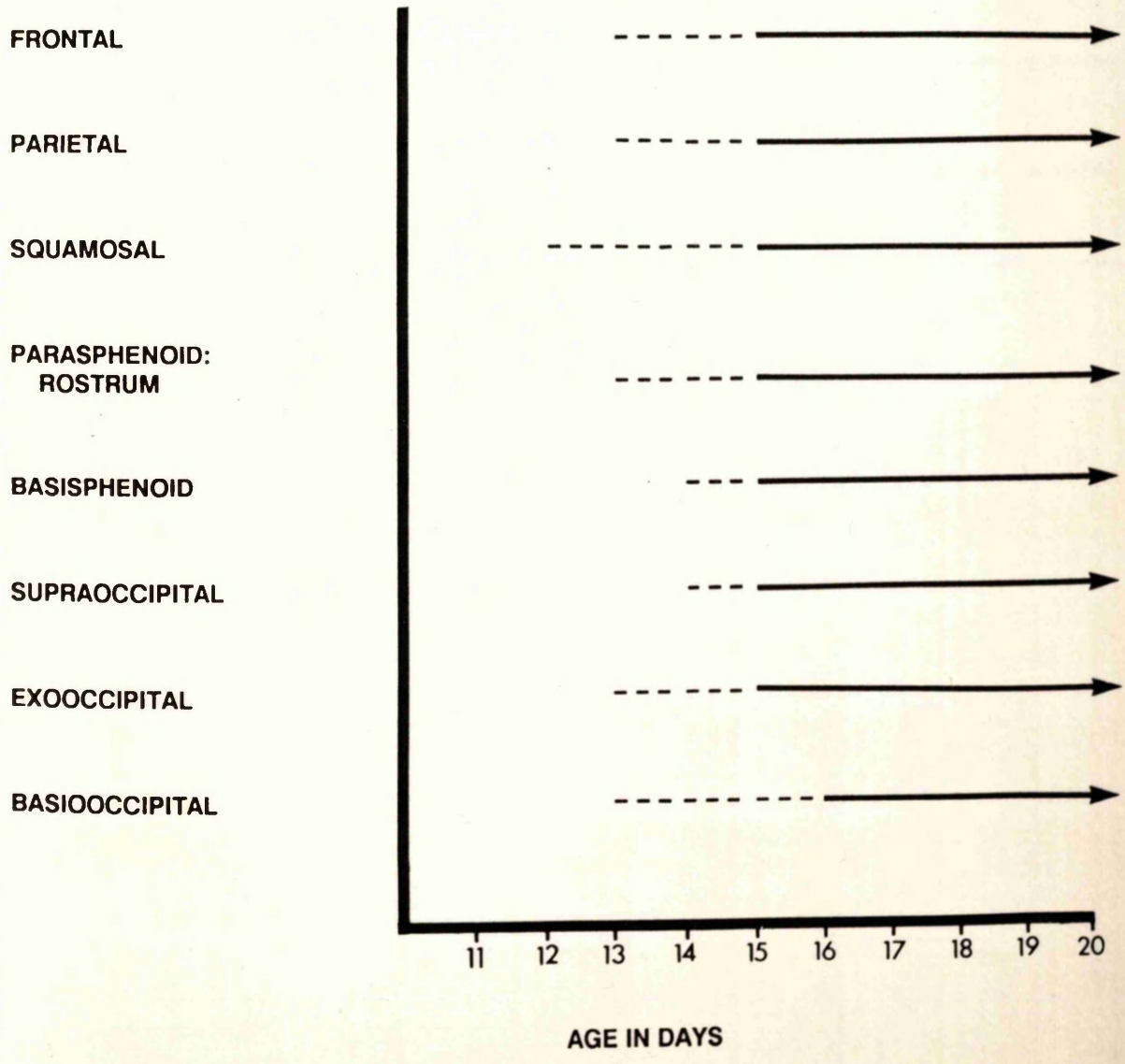

FRONTAL
PARIETAL
SQUAMOSAL
PARASPHENOID:
ROSTRUM
BASISPHENOID
SUPRAOCCIPITAL
EXOOCCIPITAL
BASIOOCCIPITAL
11
12
13
14
15
16
17
18
19
20
AGE IN DAYS

Fig. 30

Timing of onset of calcification of bones in the skull of experimental embryos.

------ variable
_____ constant

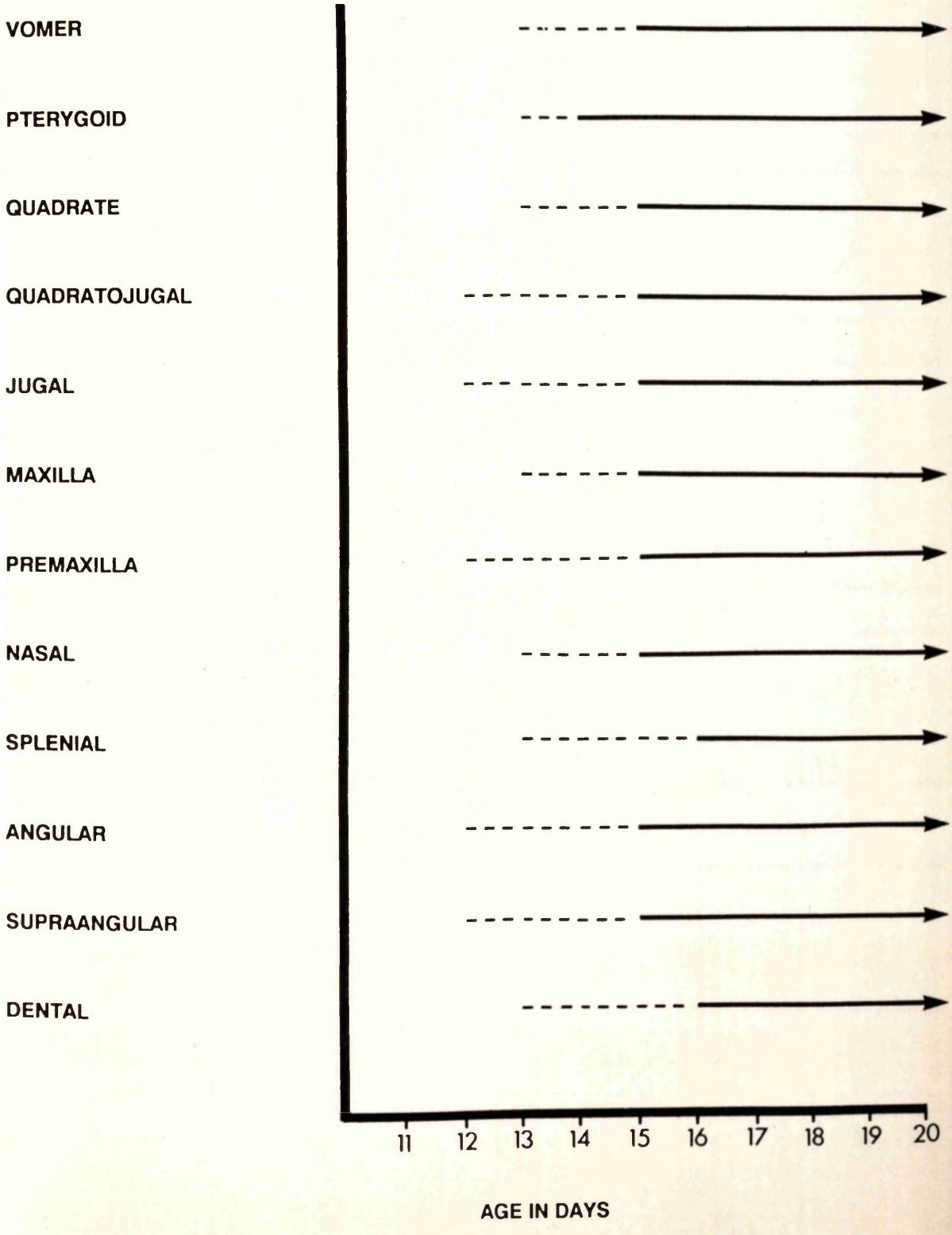

VOMER
PTERYGOID
QUADRATE
QUADRATOJUGAL
JUGAL
MAXILLA
PREMAXILLA
NASAL
SPLENIAL
ANGULAR
SUPRAANGULAR
DENTAL
11
12
13
14
15
16
17
18
19
20
AGE IN DAYS

Fig. 31

Timing of onset of calcification of the cervical vertebrae of control embryos.

------ variable
_____ constant

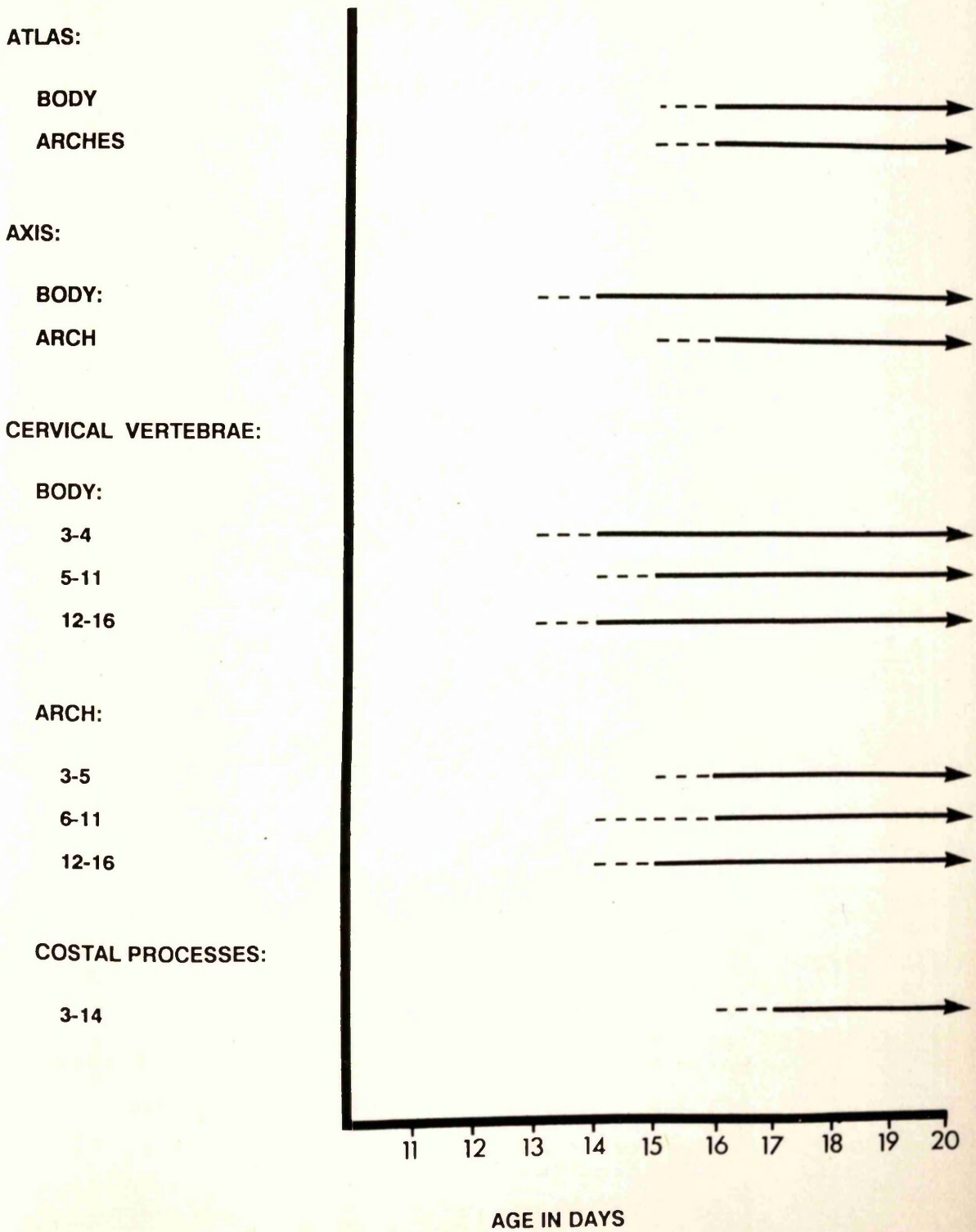

ATLAS:
BODY
ARCHES
AXIS:
BODY:
ARCH
CERVICAL VERTEBRAE:
BODY:
3-4
5-11
12-16
ARCH:
3-5
6-11
12-16
COSTAL PROCESSES:
3-14
11
12
13
14
15
16
17
18
19
20
AGE IN DAYS

Fig. 32

Timing of onset of calcification of the cervical vertebrae of experimental embryos.

------ variable
_____ constant

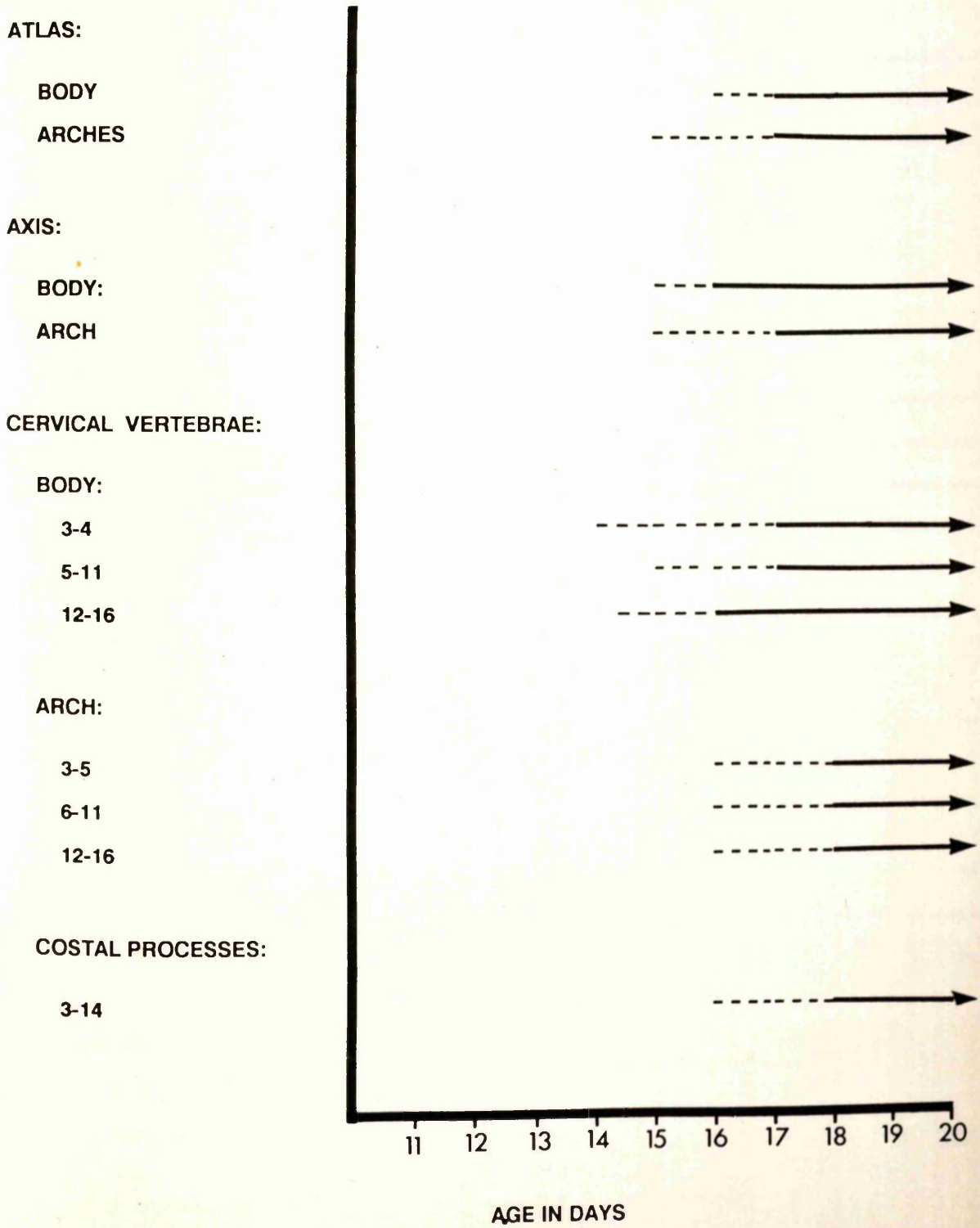

ATLAS:
BODY
ARCHES
AXIS:
BODY:
ARCH
CERVICAL VERTEBRAE:
BODY:
3-4
5-11
12-16
ARCH:
3-5
6-11
12-16
COSTAL PROCESSES:
3-14
11
12
13
14
15
16
17
18
19
20
AGE IN DAYS

Fig. 33

Timing of onset of calcification of the thoracic and lumbar , sacral and caudal vertebrae of control embryos.

------ variable
_____ constant

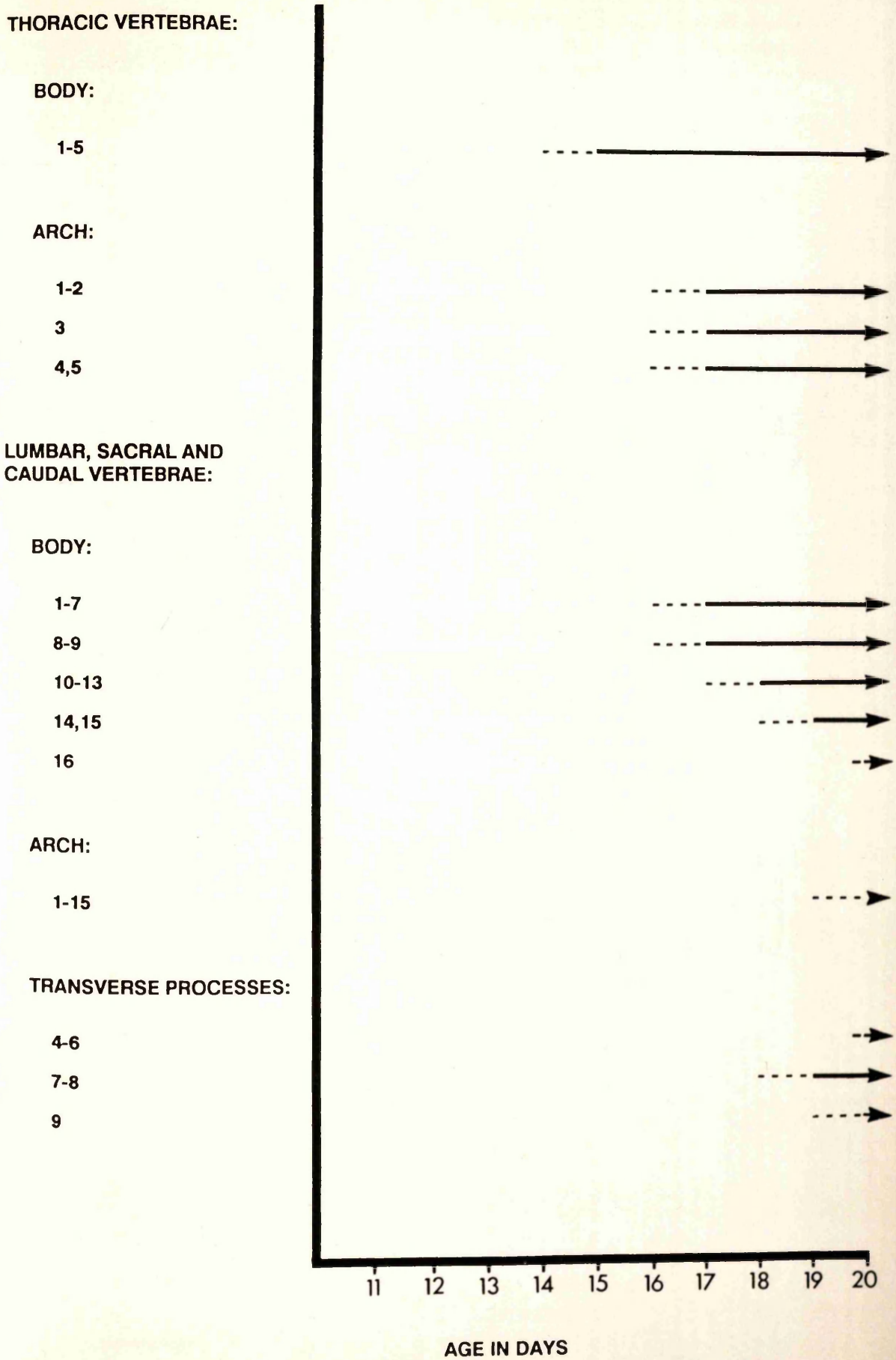

THORACIC VERTEBRAE:
BODY:
1-5
ARCH:
1-2
3
4,5
LUMBAR, SACRAL AND CAUDAL VERTEBRAE:
BODY:
1-7
8-9
10-13
14,15
16
ARCH:
1-15
TRANSVERSE PROCESSES:
4-6
7-8
9
11
12
13
14
15
16
17
18
19
20
AGE IN DAYS

Fig. 34

Timing of onset of calcification of the thoracic and lumbar, sacral and caudal vertebrae of experimental embryos.

------ variable
_____ constant

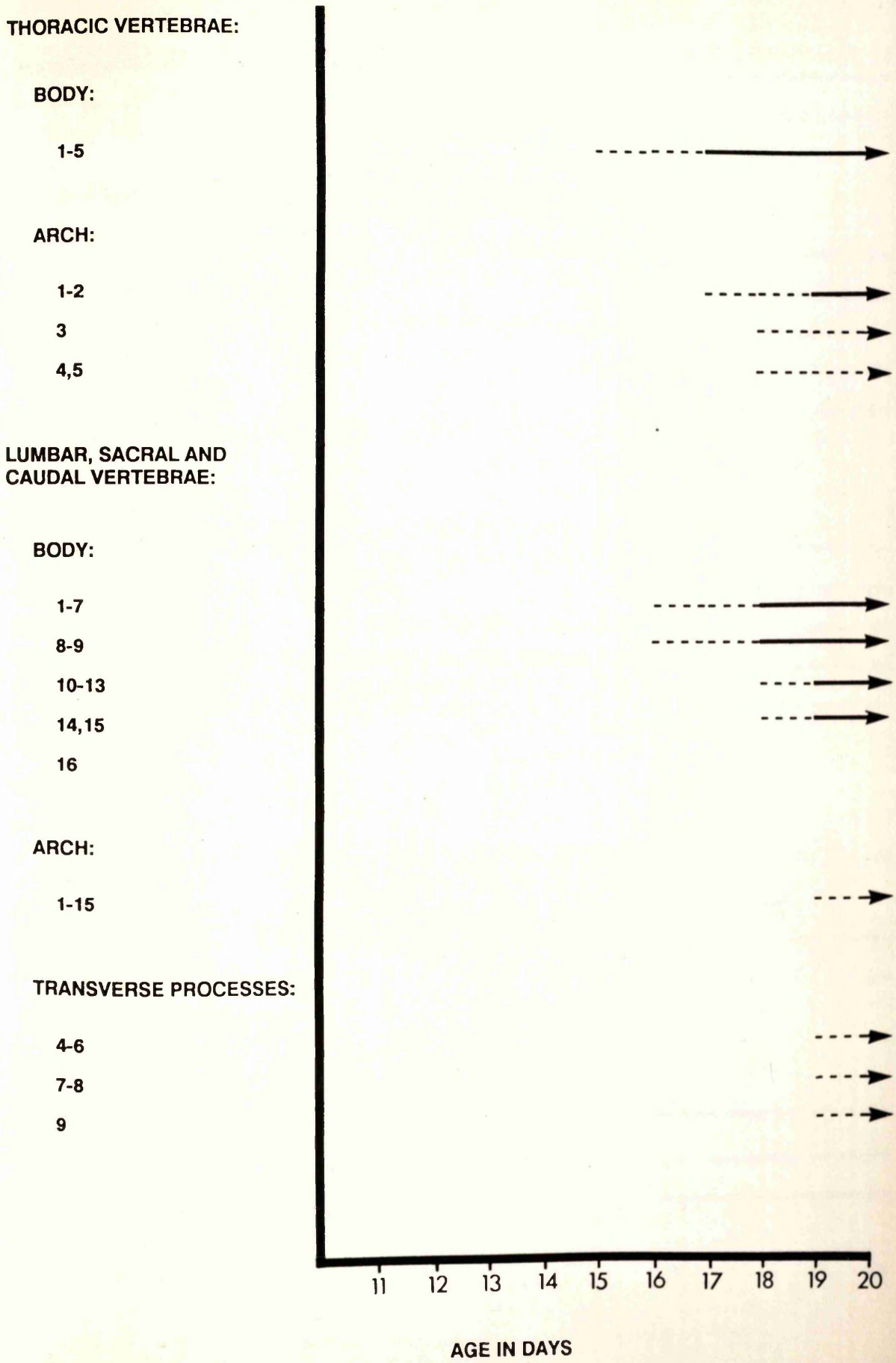

THORACIC VERTEBRAE:
BODY:
1-5
ARCH:
1-2
3
4,5
LUMBAR, SACRAL AND CAUDAL VERTEBRAE:
BODY:
1-7
8-9
10-13
14,15
16
ARCH:
1-15
TRANSVERSE PROCESSES:
4-6
7-8
9
11
12
13
14
15
16
17
18
19
20
AGE IN DAYS

Fig. 35

Timing of onset of calcification of the ribs and sternum of control embryos.

------ variable
_____ constant

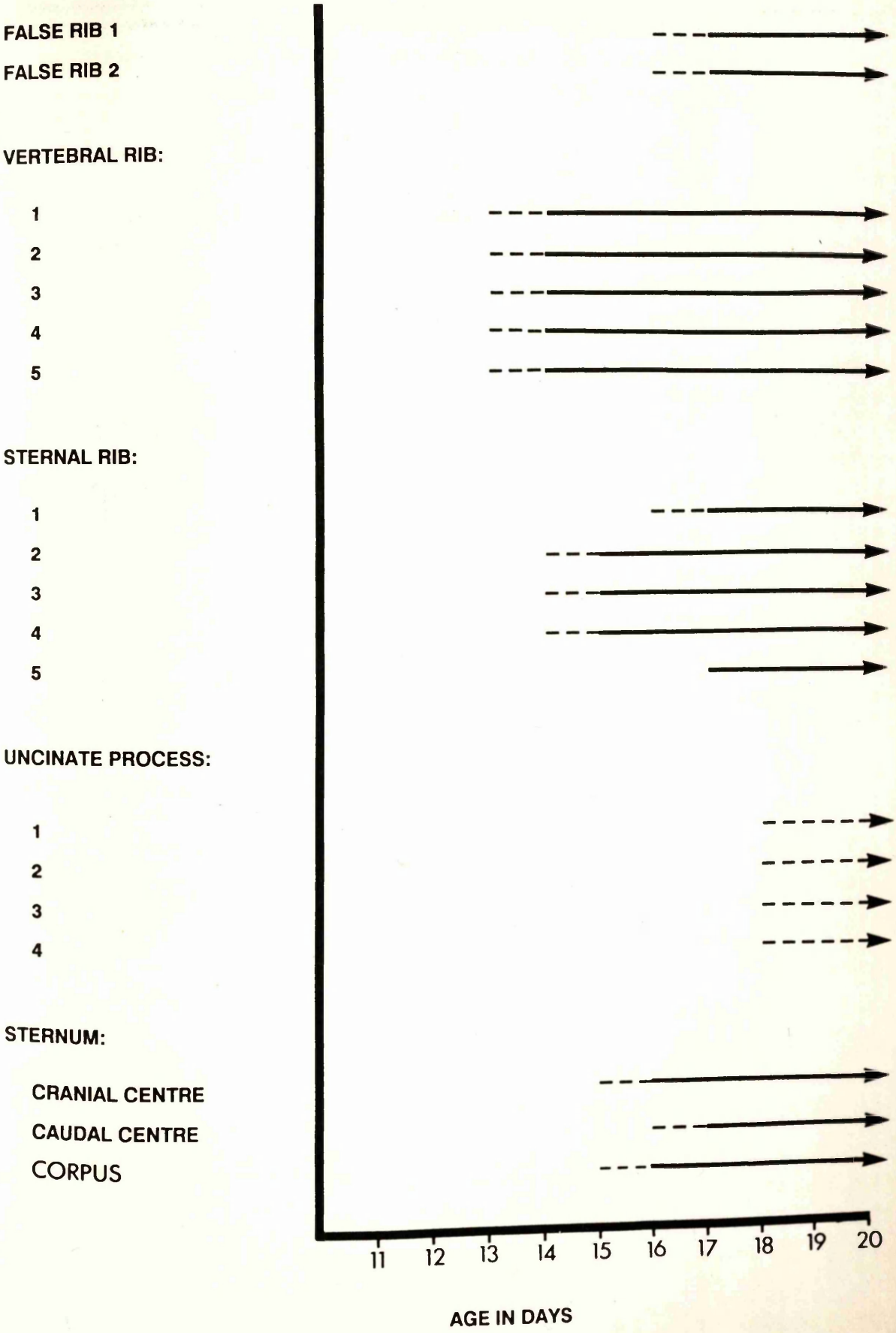

FALSE RIB 1
FALSE RIB 2
VERTEBRAL RIB:
1
2
3
4
5
STERNAL RIB:
1
2
3
4
5
UNCINATE PROCESS:
1
2
3
4
STERNUM:
CRANIAL CENTRE
CAUDAL CENTRE
CORPUS
11
12
13
14
15
16
17
18
19
20
AGE IN DAYS

Fig. 36

Timing of onset of calcification of the ribs and sternum of experimental embryos.

------ variable
_____ constant

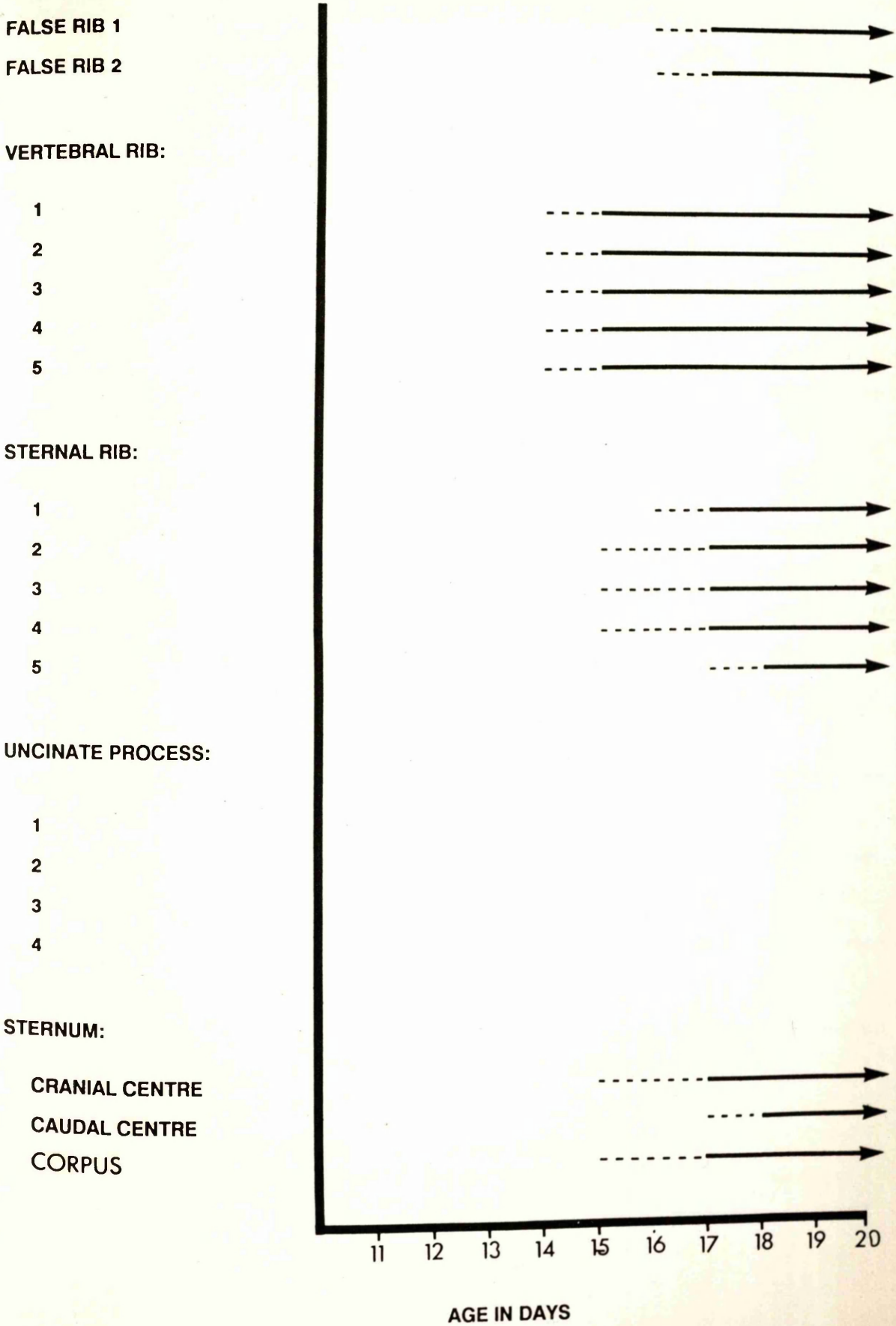
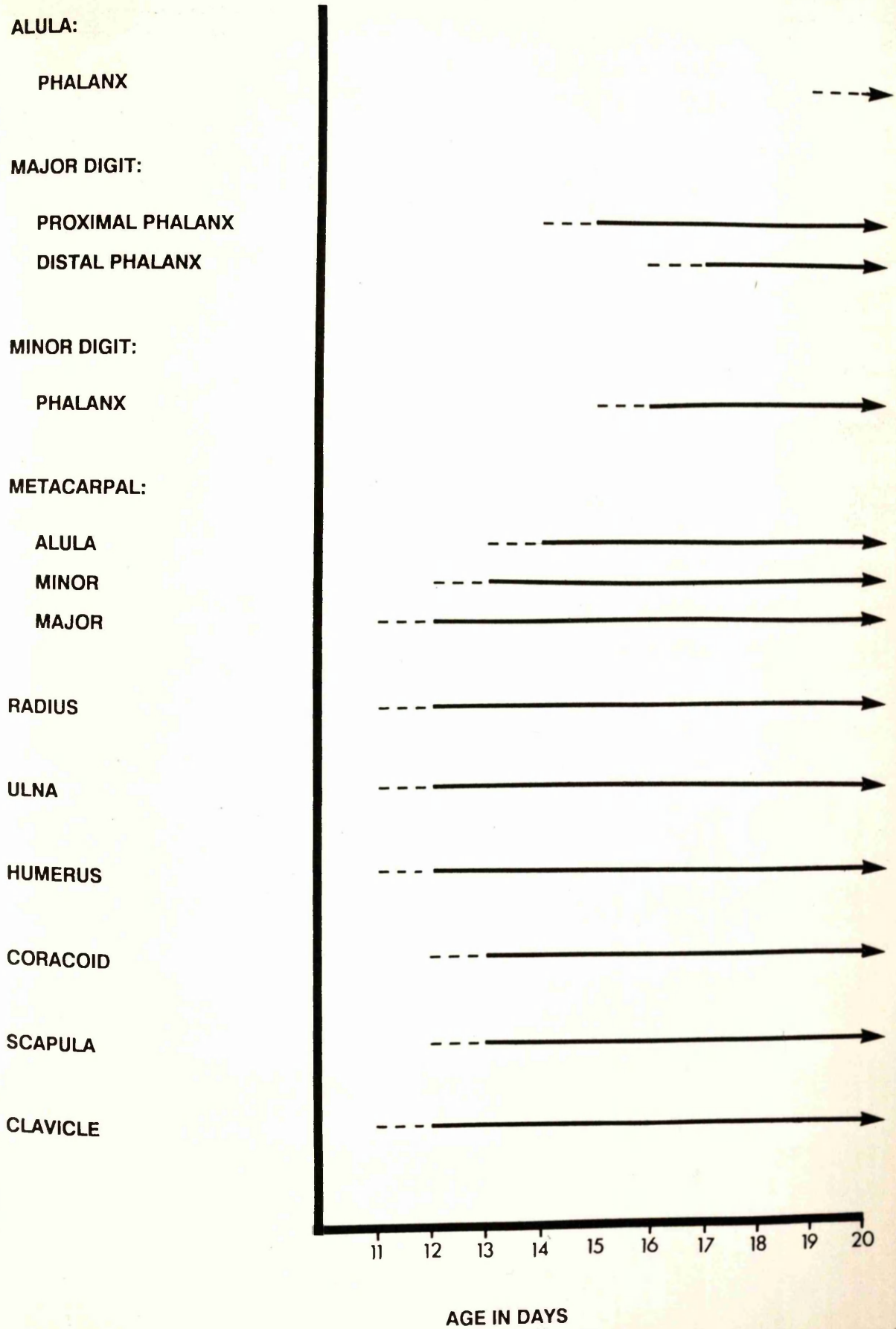

FALSE RIB 1
FALSE RIB 2
VERTEBRAL RIB:
1
2
3
4
5
STERNAL RIB:
1
2
3
4
5
UNCINATE PROCESS:
1
2
3
4
STERNUM:
CRANIAL CENTRE
CAUDAL CENTRE
CORPUS
11
12
13
14
15
16
17
18
19
20
AGE IN DAYS

Fig. 37

Timing of onset of calcification of bones in the pectoral girdle and limb of control embryos.

------ variable
_____ constant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DISTAL PHALANX
MINOR DIGIT:
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METACARPAL:
ALULA
MINOR
MAJOR
RADIUS
ULNA
HUMERUS
CORACOID
SCAPULA
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12
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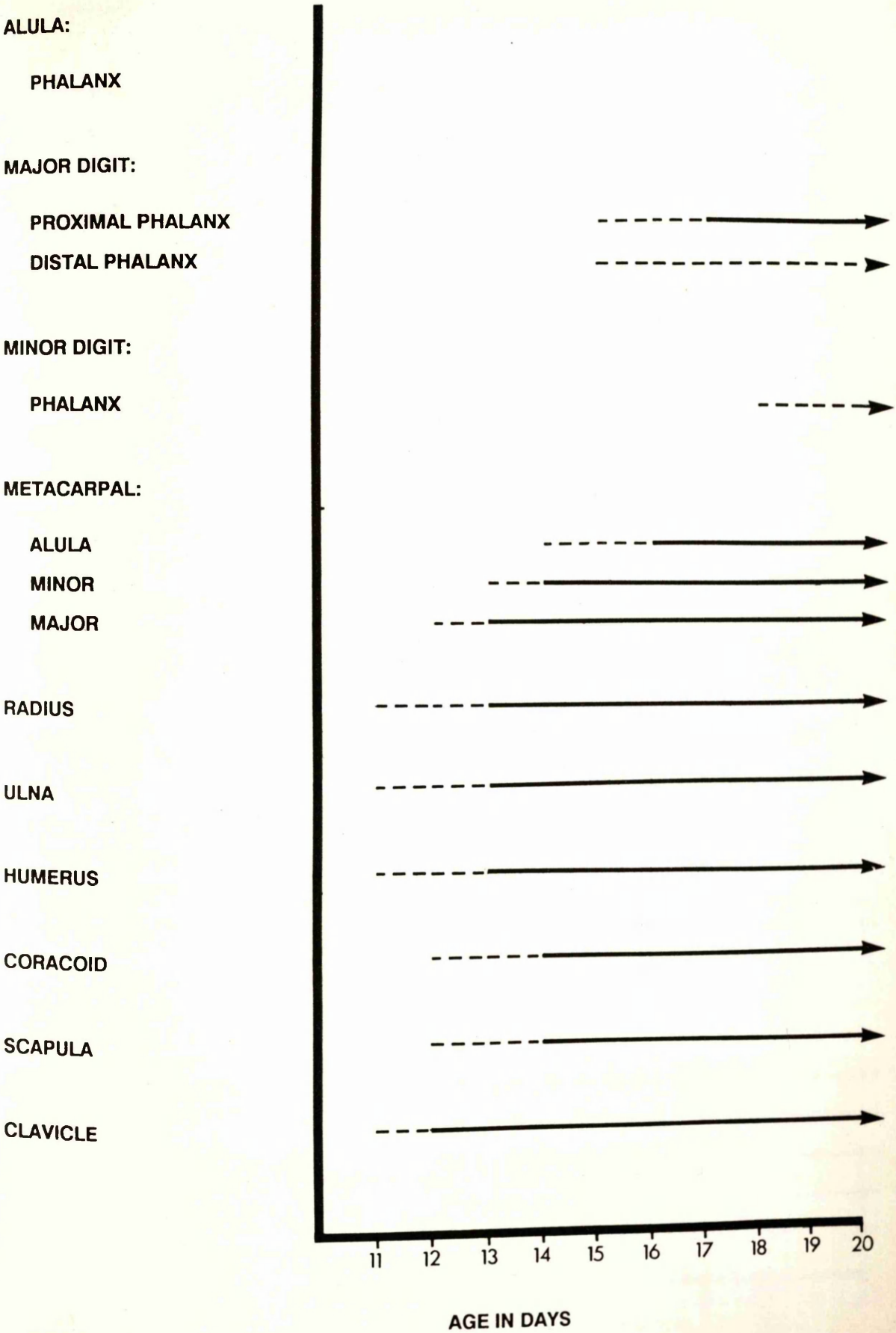
19
20
AGE IN DAYS

Fig. 38

Timing of onset of calcification of bones in the pectoral girdle and limb of the experimental embryos.

------ variable
_____ constant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RADIUS
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CORACOID
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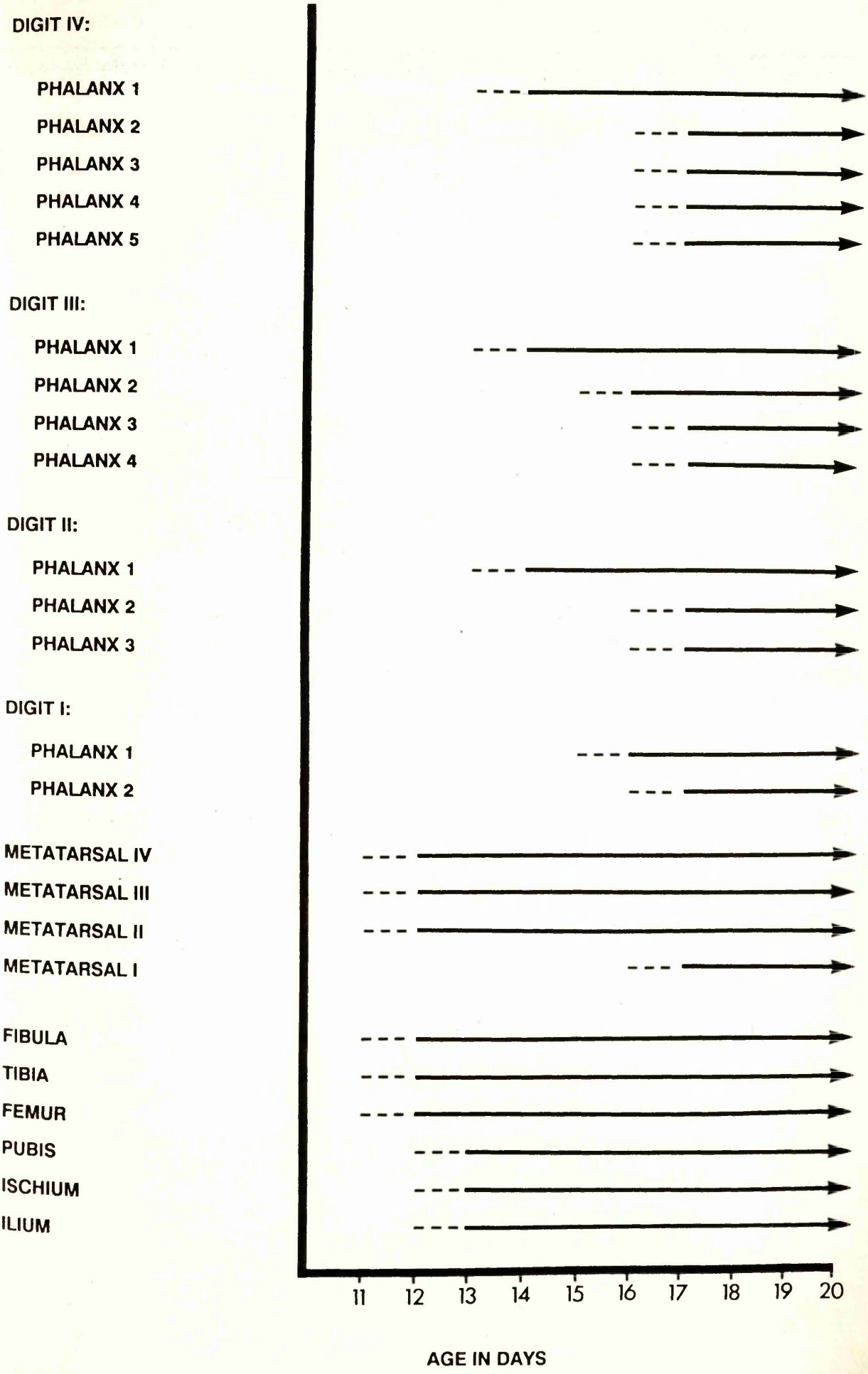
AGE IN DAYS

Fig. 39

Timing of onset of calcification of bones in the pelvic girdle and limb of the control embryos.

------ variable
_____ constant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DIGIT II:
PHALANX 1
PHALANX 2
PHALANX 3
DIGIT I:
PHALANX 1
PHALANX 2
METATARSAL IV
METATARSAL III
METATARSAL II
METATARSAL I
FIBULA
TIBIA
FEMUR
PUBIS
ISCHIUM
ILIUM
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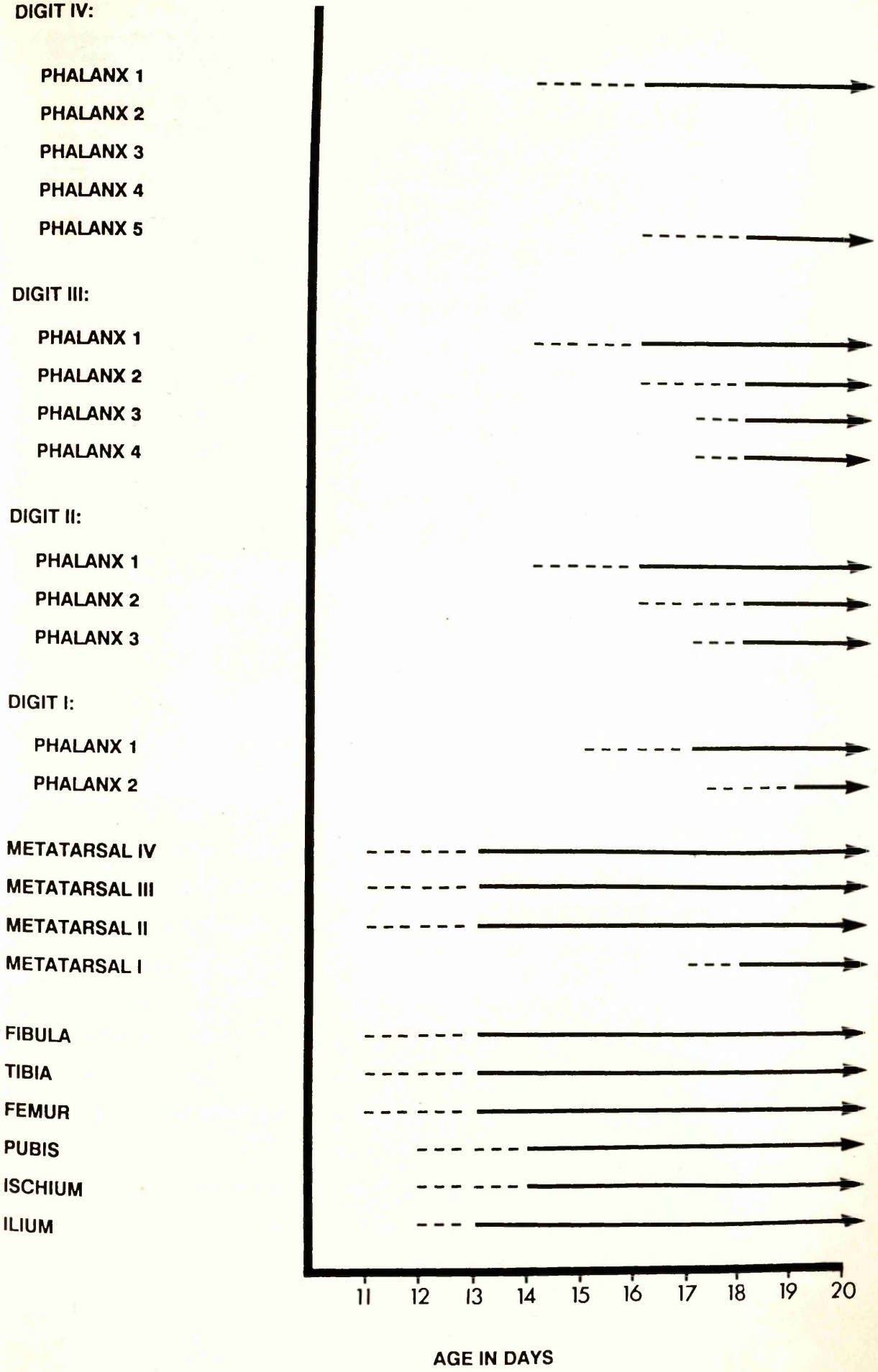
AGE IN DAYS

Fig. 40

Timing of onset of calcification of bones in the pelvic girdle and limb of the experimental embryos.

------ variable
_____ constant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DIGIT II:
PHALANX 1
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DIGIT I:
PHALANX 1
PHALANX 2
METATARSAL IV
METATARSAL III
METATARSAL II
METATARSAL I
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TIBIA
FEMUR
PUBIS
ISCHIUM
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AGE IN DAYS

Fig 41

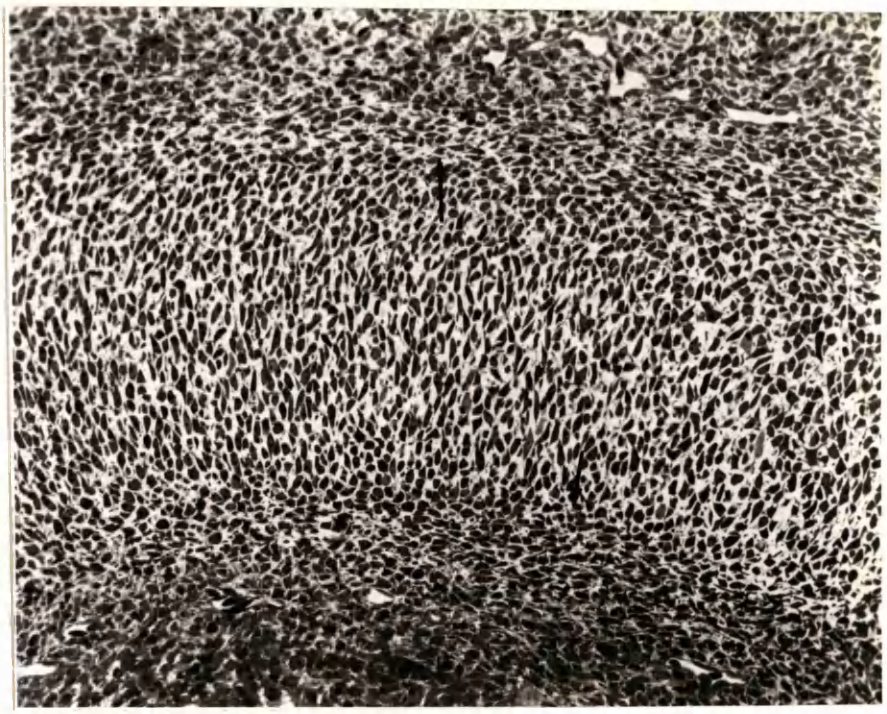
A longitudinal semithin section of the tibial rudiment of control embryo. 6 days (early). The cells in the centre are elongated in a direction at right angles to the long axis of the cell mass. The demarcation between the developing cartilage and surrounding tissue (arrows) is fairly sharp.

Azur blue II. x213

Fig. 42

A longitudinal semithin section of the tibial rudiment of control embryo. 6 days (late). Some polyhedral cells (arrows) are present in the centre of the shaft. A bilayered perichondrium (P) is present around this central portion of the diaphysis.

Azur blue II. x238



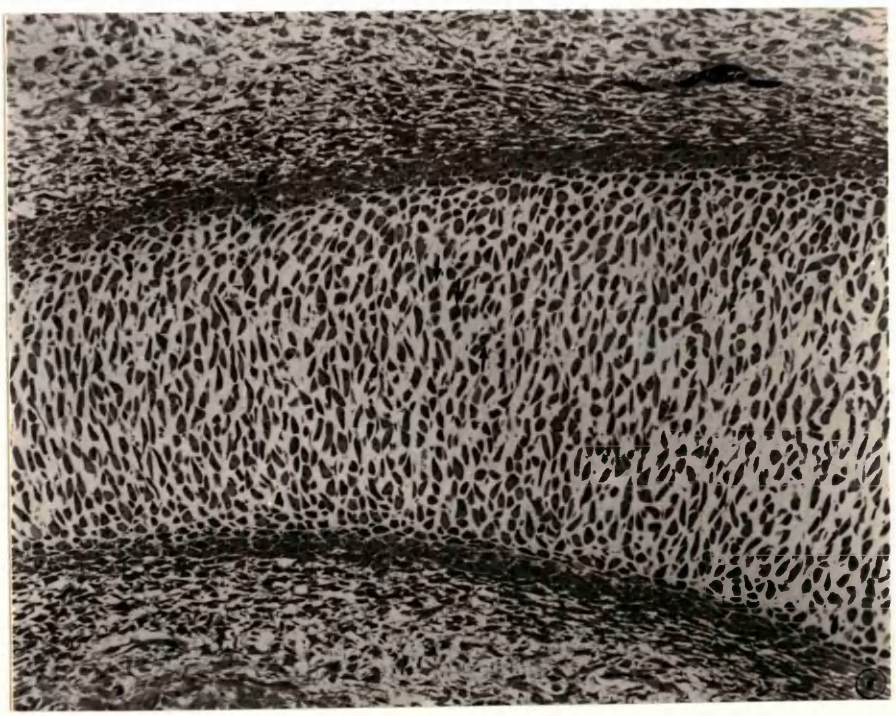


Fig. 43

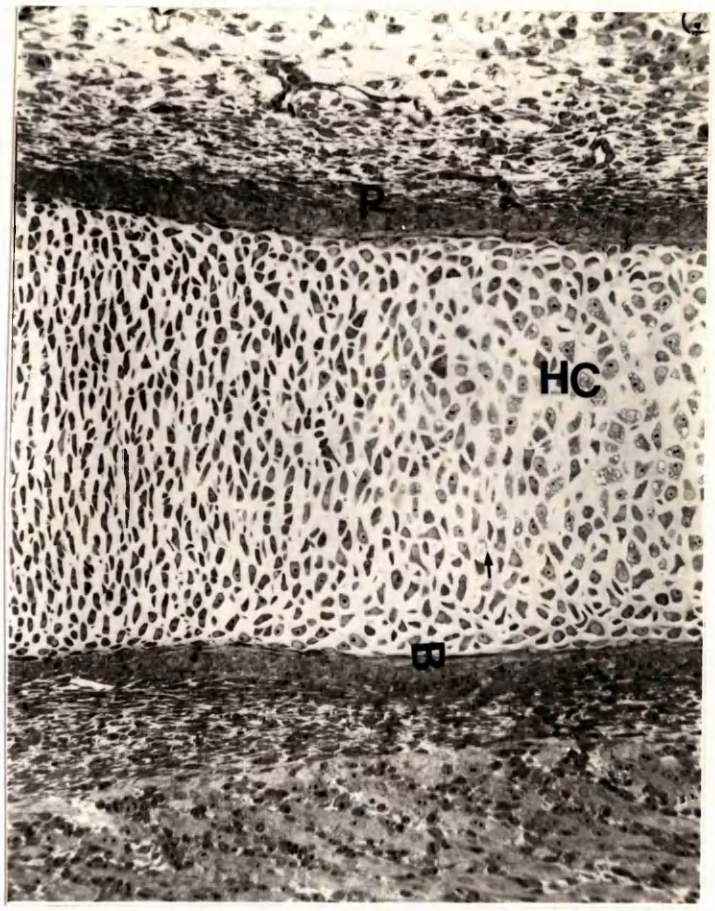
A longitudinal semithin section through the diaphysis of tibia of a control embryo. 7 days. Hypertrophic chondrocytes (HC) can be seen with some empty lacunae (arrow). Longitudinally flattened cells can be seen in the left side of the picture. Two distinct layers of perichondrium (P) are present and a delicate layer of bone (B) can be seen between the cartilage and perichondrium.

Azur blue II. x200

Fig. 44

Transverse section through the mid diaphyseal region of the tibia of control embryo. 7 days. Hypertrophy of chondrocytes in this region is marked. The cartilage is completely surrounded by a ring of woven bone (arrows).

H & E and alcian blue. x200


P
HC
B

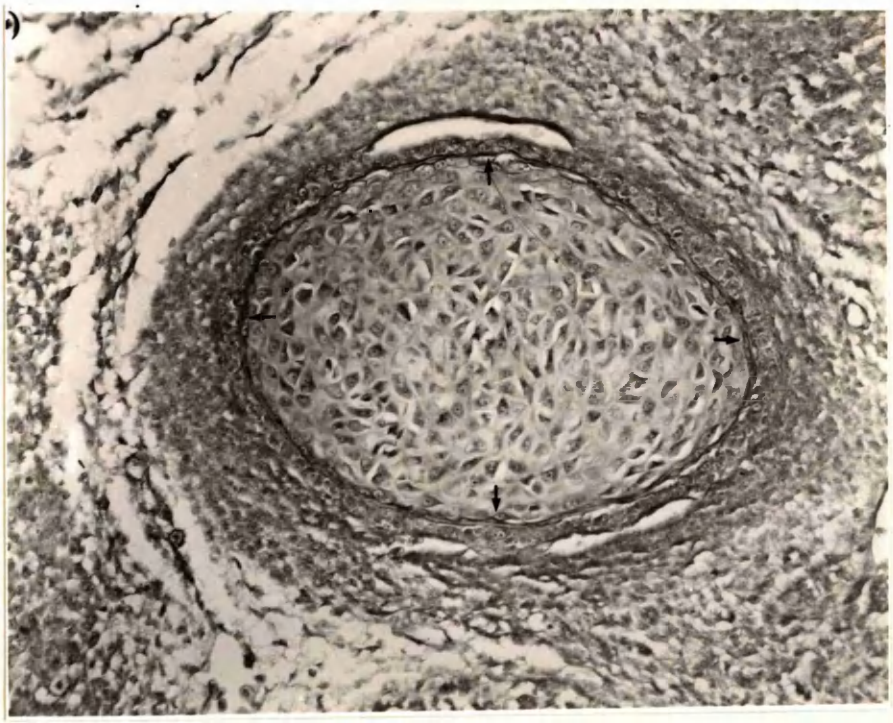


Fig. 45

Transverse wax section through the mid-diaphyseal region of the tibia of control embryo. 8 days. Hypertrophic chondrocytes are surrounded by a ring of woven bone. Initiation of second layer (arrow) can be seen.

H & E and alcian blue. x200

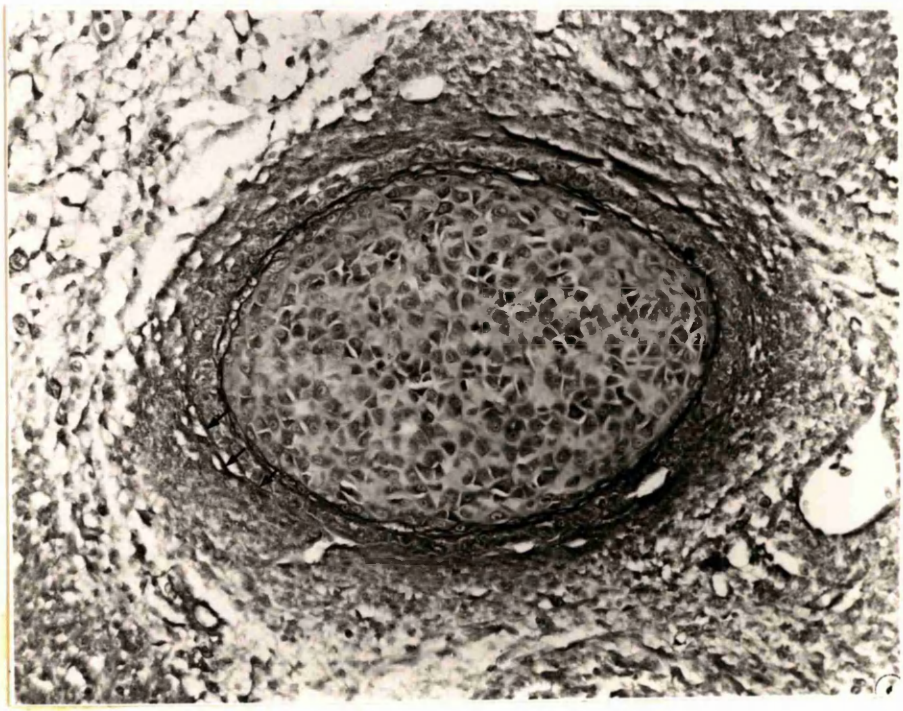


Fig. 46

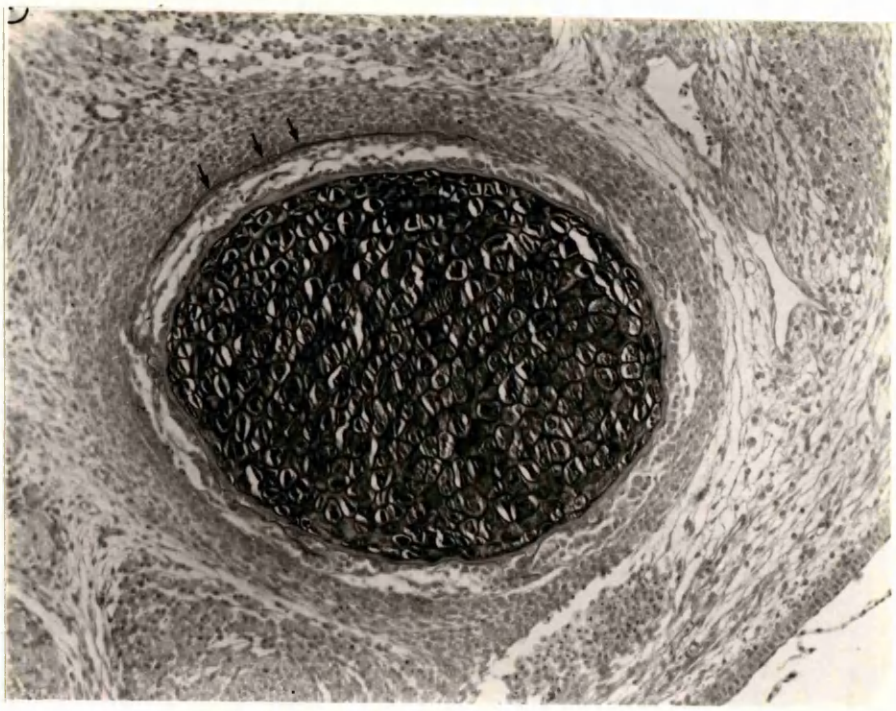
Transverse wax section through the mid-diaphyseal region of the tibia of control embryo. 9 days. Cartilage consists of markedly hypertrophic chondrocytes and is surrounded by a layer of woven bone in which a second layer has started to be laid down (arrows).

H & E and alcian blue. x158

Fig. 47

Transverse wax section through the mid-diaphyseal region of the tibia of control embryo. 10 days. Two layers of bone surround the hypertrophic chondrocytes of the cartilage model. The inner layer has thickened and a few interconnecting spicules can be seen (arrows).

H & E. x128



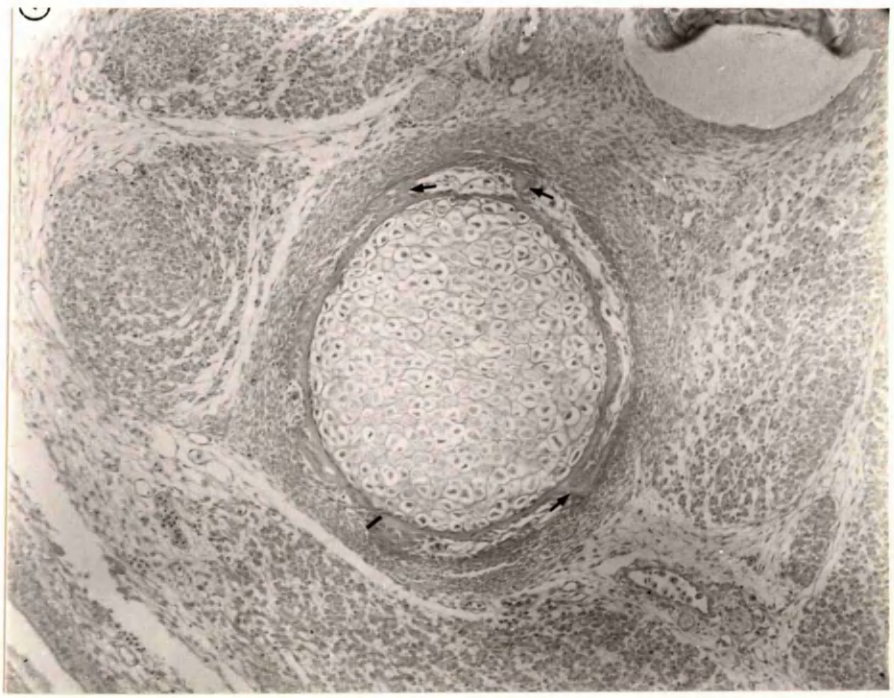


Fig. 48

Transverse wax section through the mid diaphyseal region of the tibia of control embryos. 11 days. Two layers of bone are present with the commencement of the third layer (arrows). The interconnecting spicules (S) are more numerous than the day before, some cartilage has been resorbed by invading connective and vascular tissue (CVT) and remnants of hypertrophic cartilage (CR) with many empty lacunae can be seen.

H & E. x110

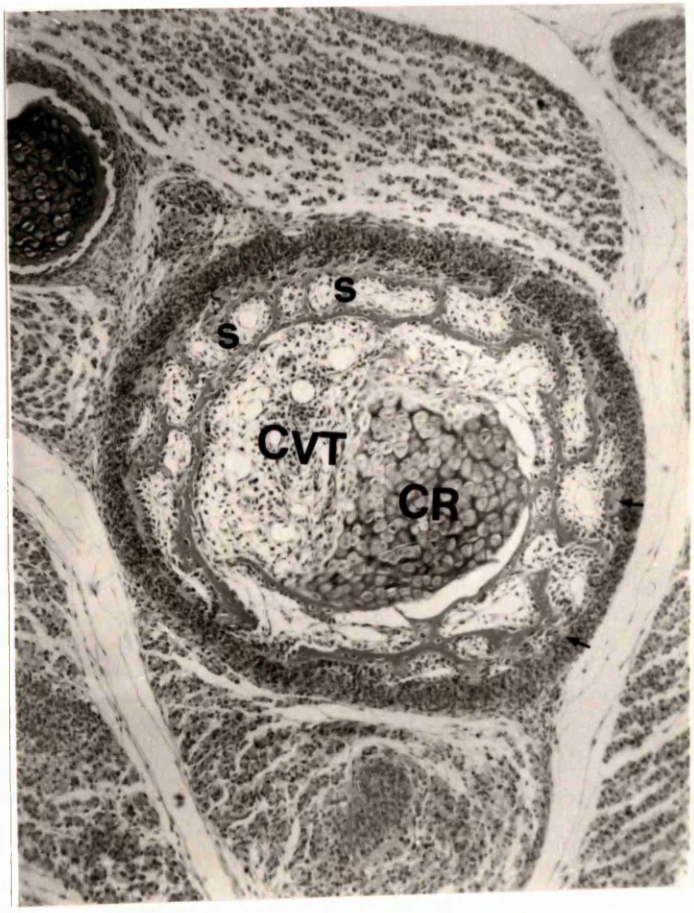

S
S
CVT
CR

Fig. 49a

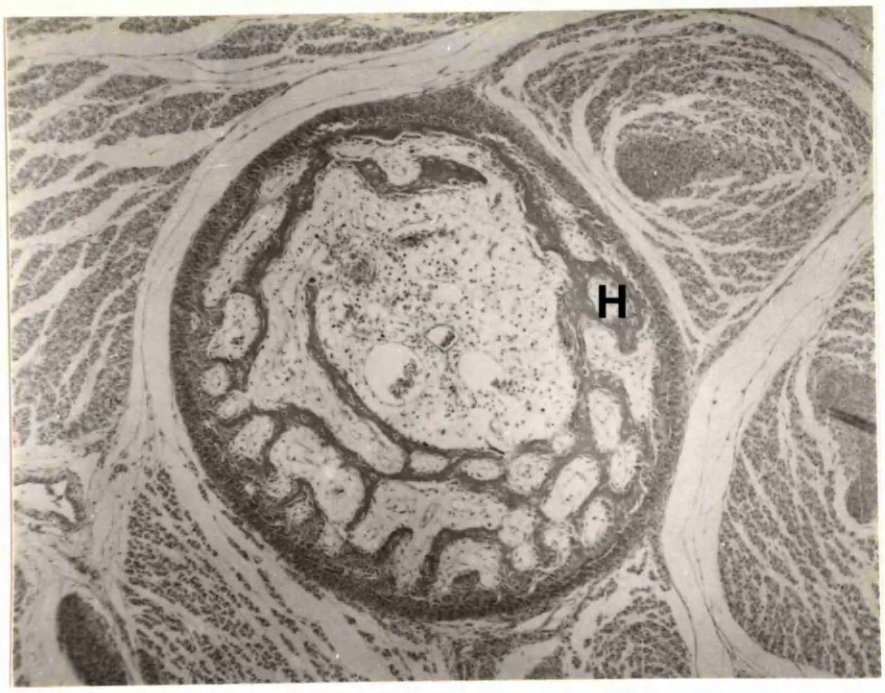
Transverse wax section through the mid diaphyseal region of the tibia of a control embryo. 12 days. Up to four layers of periosteal bone in a symmetric fashion surround the developing marrow cavity, the lateral part of the bone is thicker than the remainder. Interconnecting spicules are greater in number than in the previous day. The first indications of formation of Haversian canals (H) are seen. All the cartilage model in this region has been resorbed.

H & E. x90

Fig. 49b

Transverse wax section through the mid diaphyseal region of the tibia of an experimental embryo. 12 days.
Two layers of periosteal bone with the third starting to be laid down, surround the marrow cavity. Remnants of cartilage formed by hypertrophic cells (CR) can be seen. Compared with the above figure lesser interconnecting spicules of bone can be seen.

H & E. x90


H

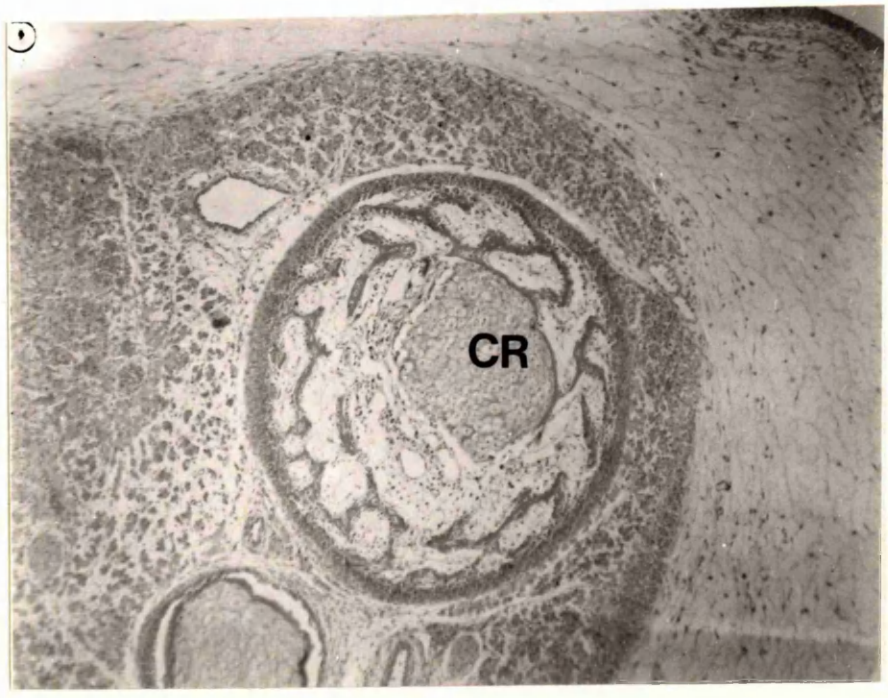

CR

Fig. 50a

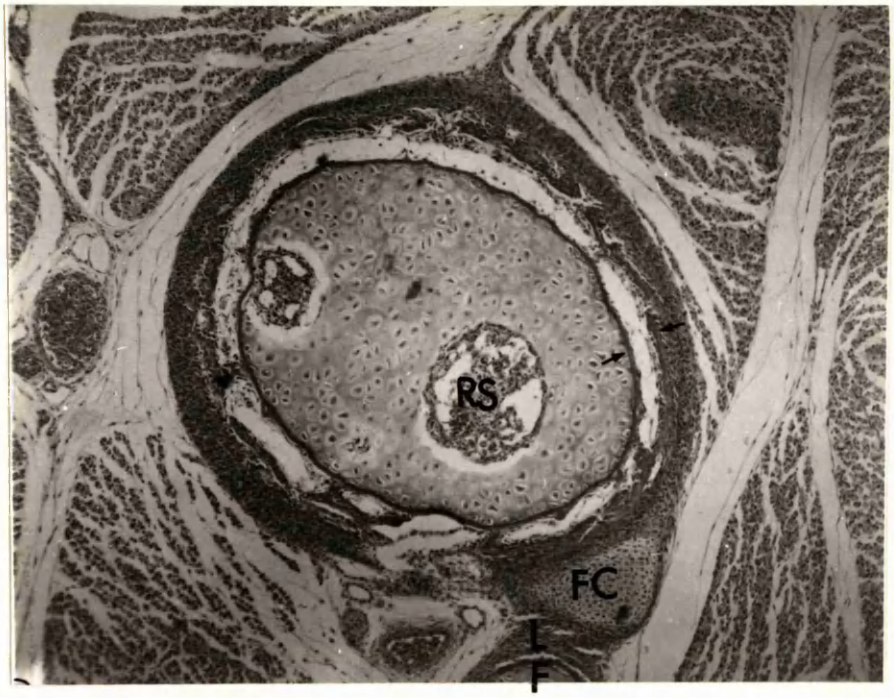
Transverse wax section through the proximal end of tibial diaphysis of control embryo. 12 days. Two resorption sites (RS) are present in the cartilage model. Two delicate layers of bone (arrows) surround the hypertrophic cells of the cartilage. On the lateral side of tibia the projection of fibular crest (FC) can be seen with its attachment to a well formed tibiofibular interosseous ligament (L), attaching to the fibula (F).

H & E. X90

Fig. 50b

Transverse wax section through the proximal end of tibial diaphysis of experimental embryo. 12 days.
Almost two complete layers of delicate bone surrounding the cartilage model which shows no signs of resorption but many hypertrophic chondrocytes. A small projection on the lateral side represents the fibular crest which is attached to a weak interosseous tibiofibular ligament (L), linking it to the fibula (F).

H & E. x90


RS
FC
L
F

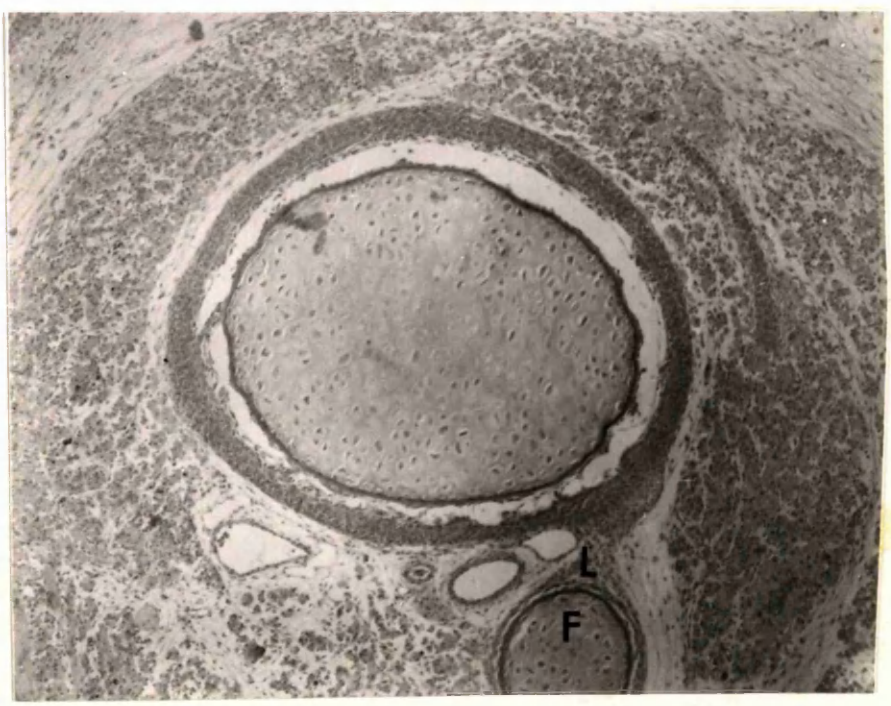

L
F

Fig. 51a

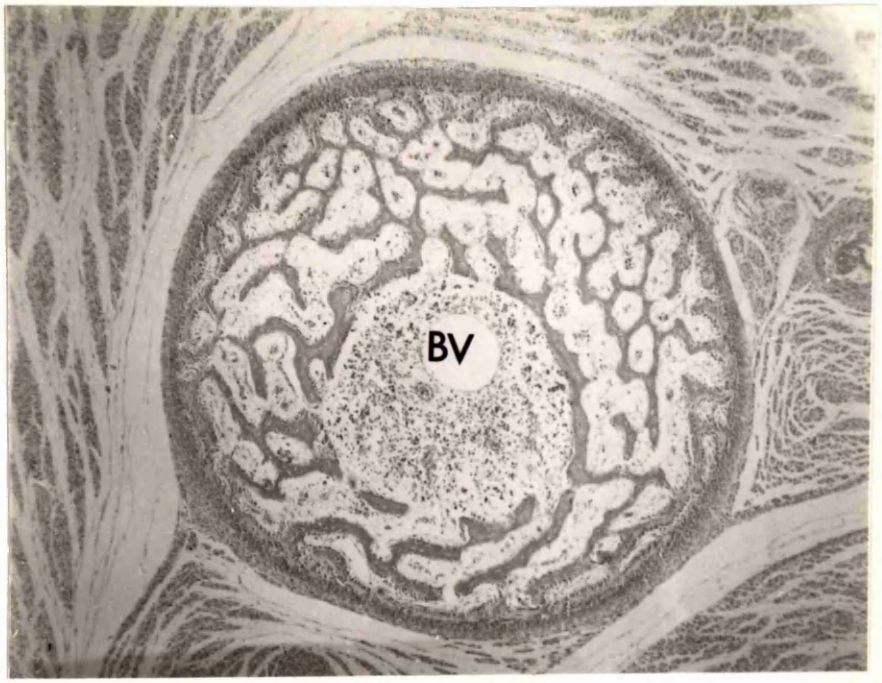
Transverse wax section through the mid diaphyseal region of the tibia of a control embryo. 14 days. Up to five concentric layers of bone are distributed asymmetrically, the maximum number of layers lying caudo-laterally. Numerous interconnecting spicules are present and a large blood vessel (BV) is present in the marrow cavity.

H & E. x75

Fig. 51b

Transverse wax section through the mid diaphyseal region of the tibia of an experimental embryo. 14 days.
A maximum of four layers of bone are distributed asymmetrically being greater in number caudolaterally. The interconnecting spicules are fewer in number than in the control. Remnants of unresorbed cartilage (C) are present in the marrow cavity.

H & E. x75


BV

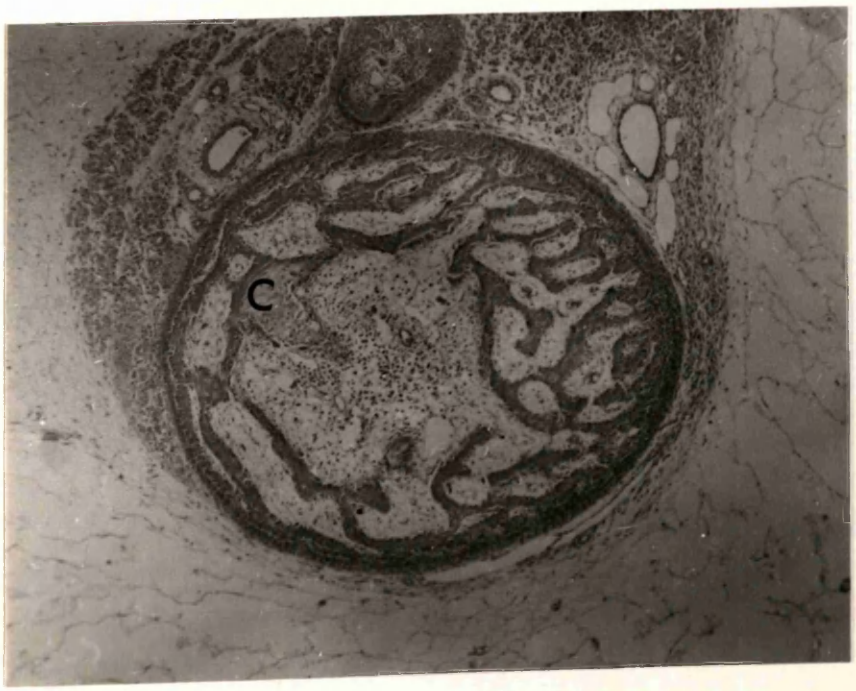

C

Fig. 52a

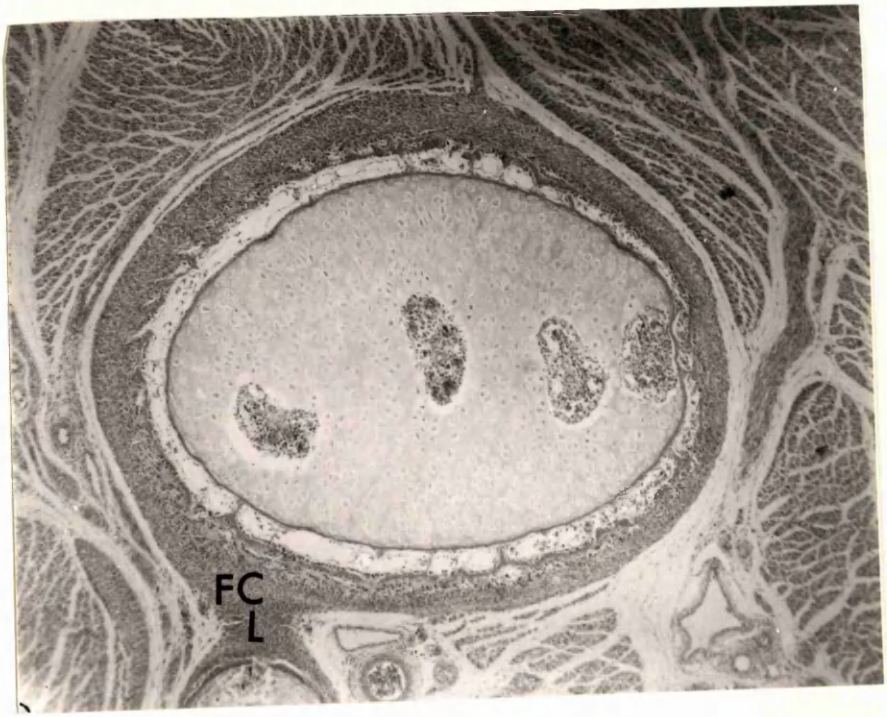
Transverse wax section through the proximal end of tibial diaphysis of a control embryo. 14 days. Four sites of cartilage resorption are present at the level of the proximal end of the fibular crest (FC) attached by the interosseous tibiofibular ligament (L) to the fibula. At this level the cartilage model is surrounded by just two layers of delicate bone.

H & E. x90

Fig. 52b

Transverse wax section through the proximal end of tibial diaphysis of an experimental embryo. 14 days.
No sign of cartilage resorption and no signs of the fibular crest and interosseous tibiofibular ligament can be seen.

H & E. x90


FC
L

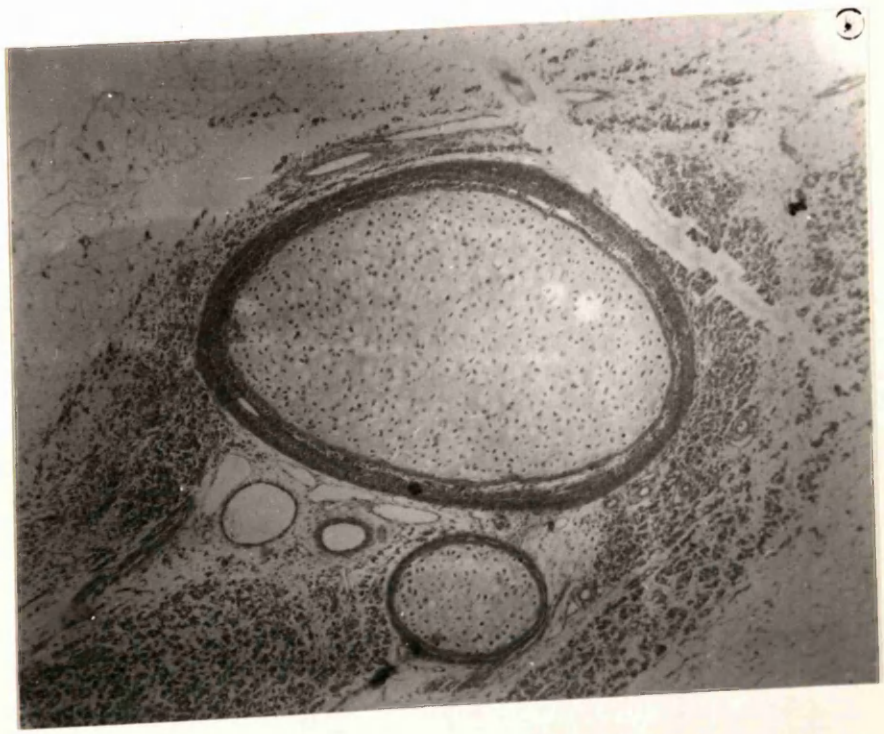


Fig. 53a

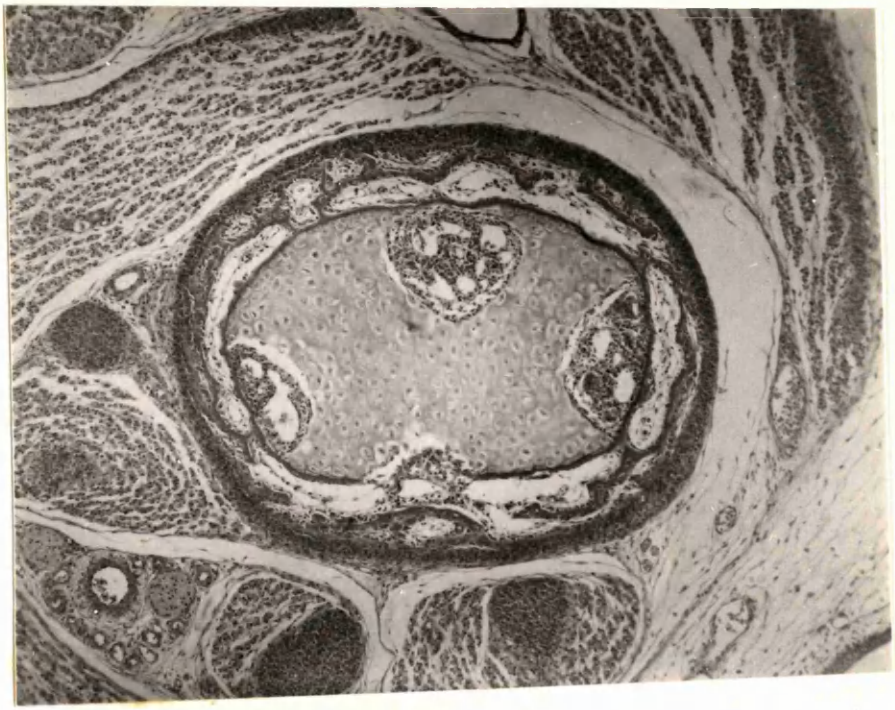
Transverse wax section through distal end of diaphysis of the tibia of control embryo. 12 days.
Two to three layers of bone surround the cartilage model in which four sites of resorption are present.

H & E. x90

Fig. 53b

Transverse wax section through distal end of diaphysis of the tibia of experimental embryo. 12 days.
One or two layers of bone surrounding the cartilage model with no signs of resorption.

H & E. x90



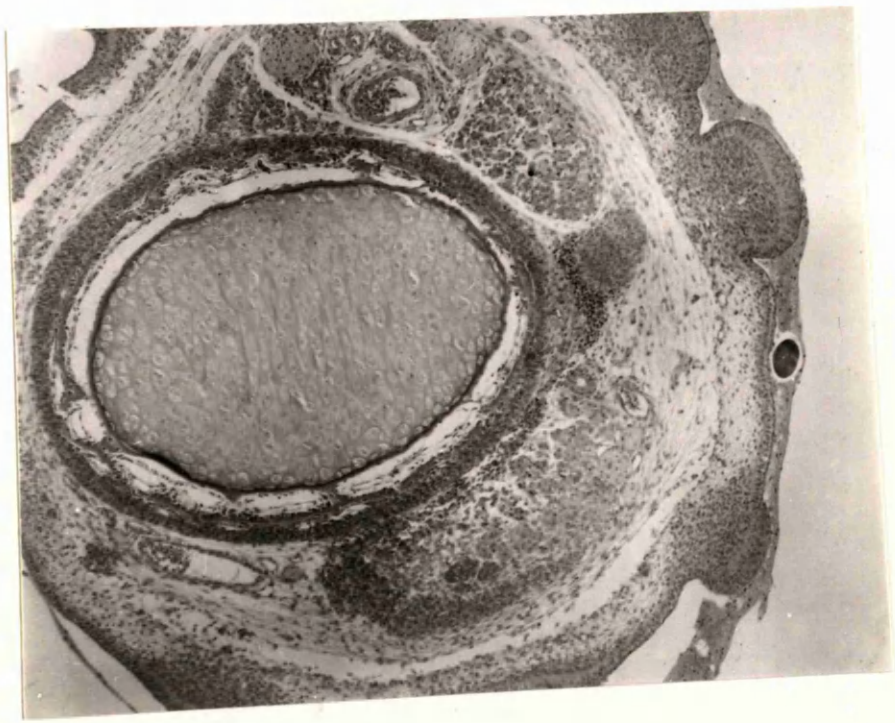


Fig. 54

Section of mid-diaphysis of tibia from control embryo. 12 days.

a) Up to 5 layers of periosteal bone are present in an asymmetrical pattern with interconnecting spicules connecting them together. The marrow cavity is well formed as a result of the cartilage having been resorbed.

SEM x75

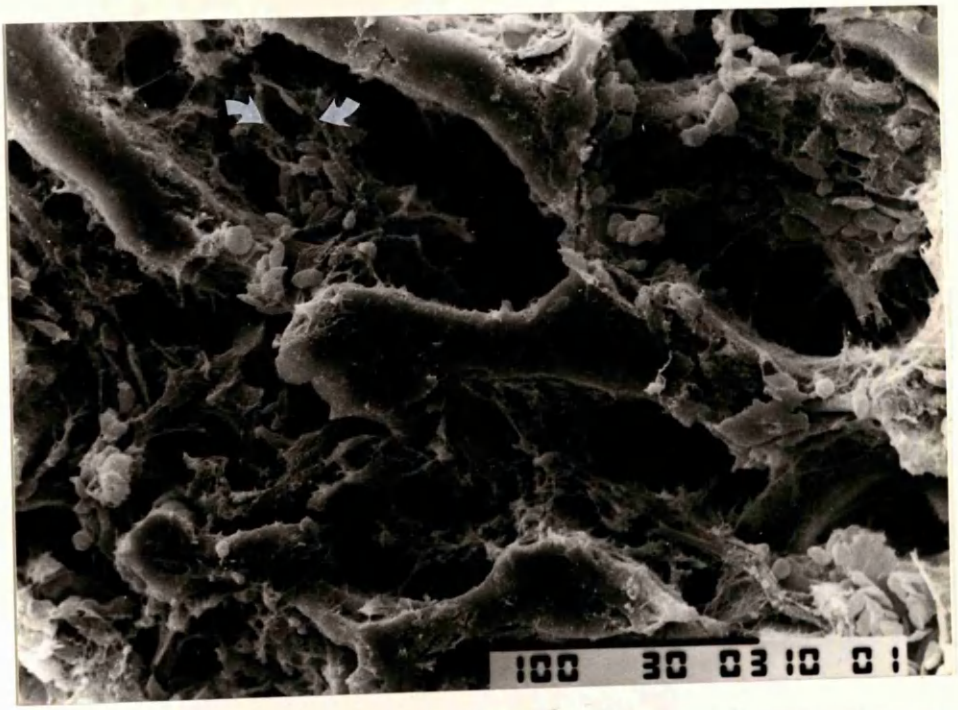
b) Trabeculae of bone are well formed and appear dense. Red blood cells (rbc) can be seen at the edge of blood vessel (arrows) in the developing Haversian canals.

SEM x350

c) A blood vessel (bv) present in a Haversian canal (arrows) contains red blood cells (rbc) and is surrounded by loose connective tissue.

SEM x1000


100 30 0309 10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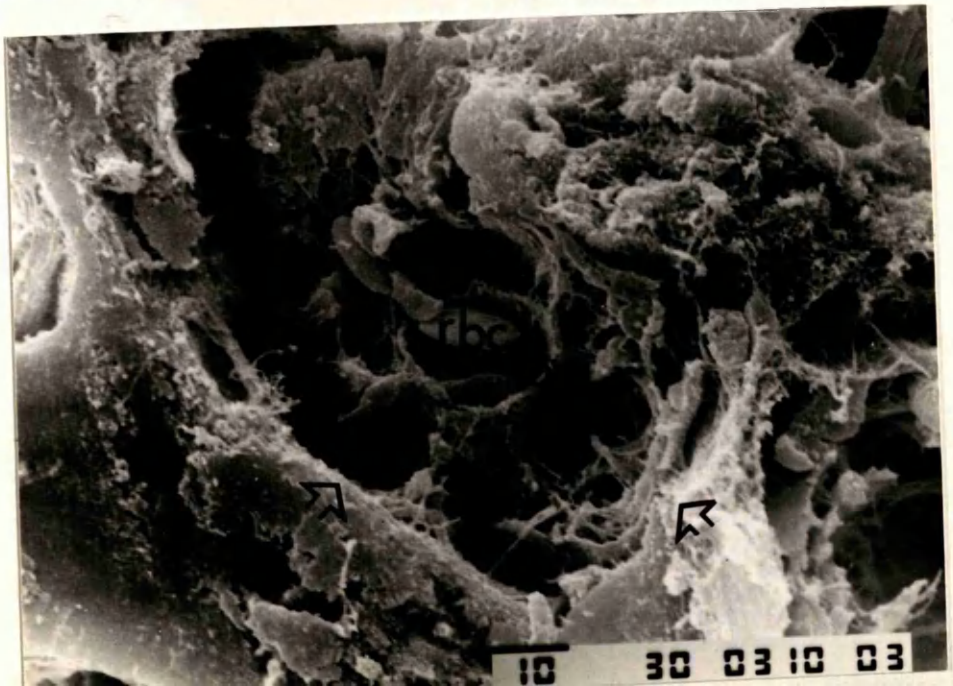

10 30 0310 03

Fig. 55

Section of mid-diaphyis of tibia from control embryo. 13 days.

a) A maximum of 6 layers of periosteal bone is present with numerous interconnecting spicules in an asymmetrical pattern. The marrow cavity is well formed.

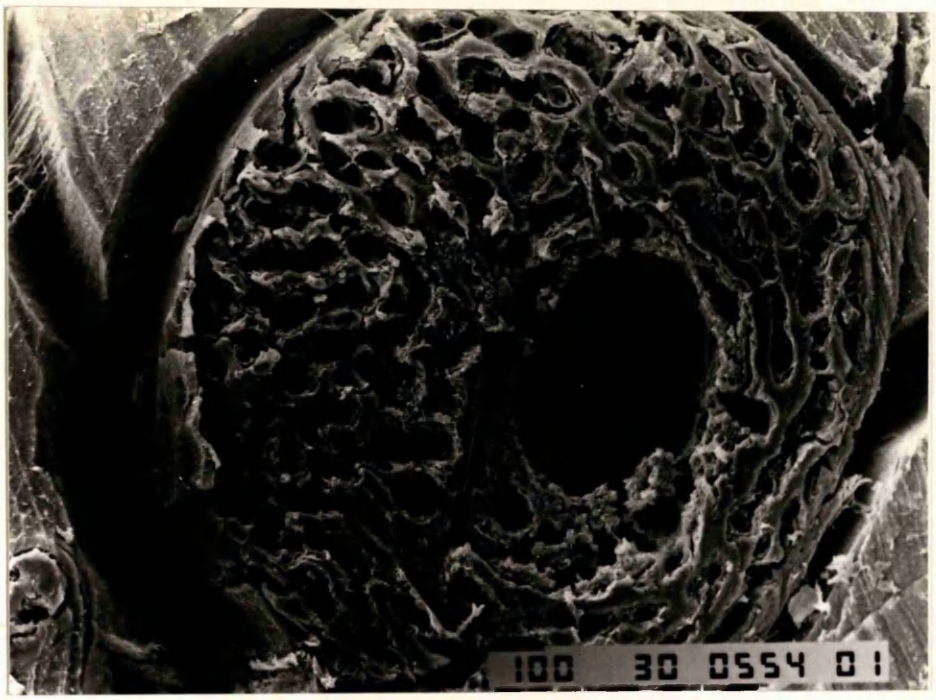
SEM x75

b) Compact bony trabeculae with numerous blood vessels and loose connective tissue filling the spaces between them can be seen. The development of Haversian canals (arrows) is recognisable.

SEM x350

c) A blood vessel (bv) containing numerous red blood cells (rbc) surrounded by loose connective tissue is seen in a Haversian canal (arrows).

SEM x1000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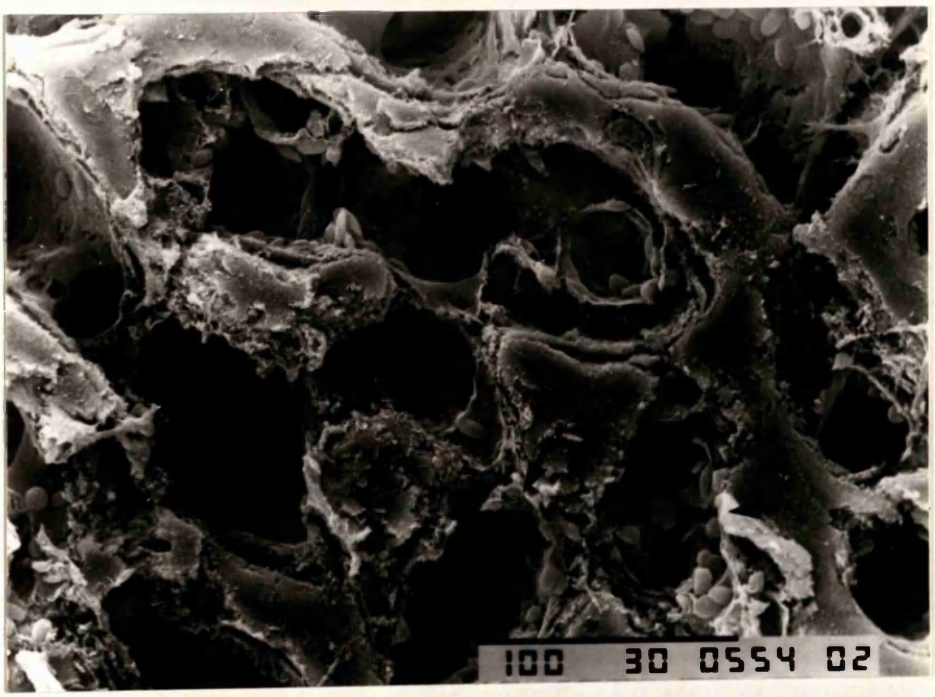
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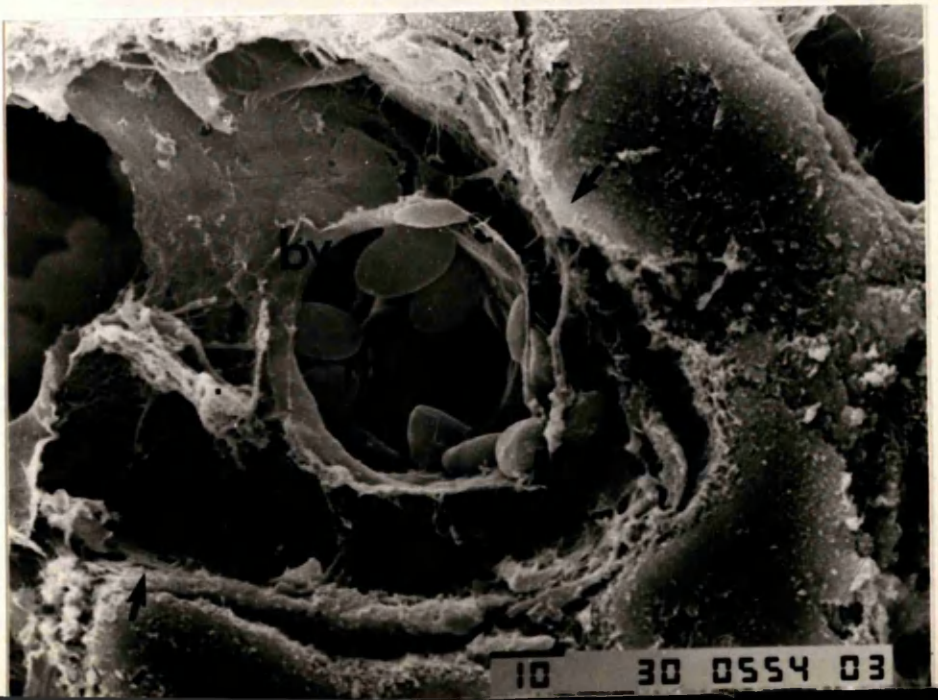

10 30 0554 03

Fig. 56

Section of mid-diaphysis of tibia from control embryo. 14 days.

a) A maximum of 5 to 6 layers of periosteal bone with a rather more symmetrical pattern is now present. A large blood vessel (bv) can be seen in the marrow cavity.

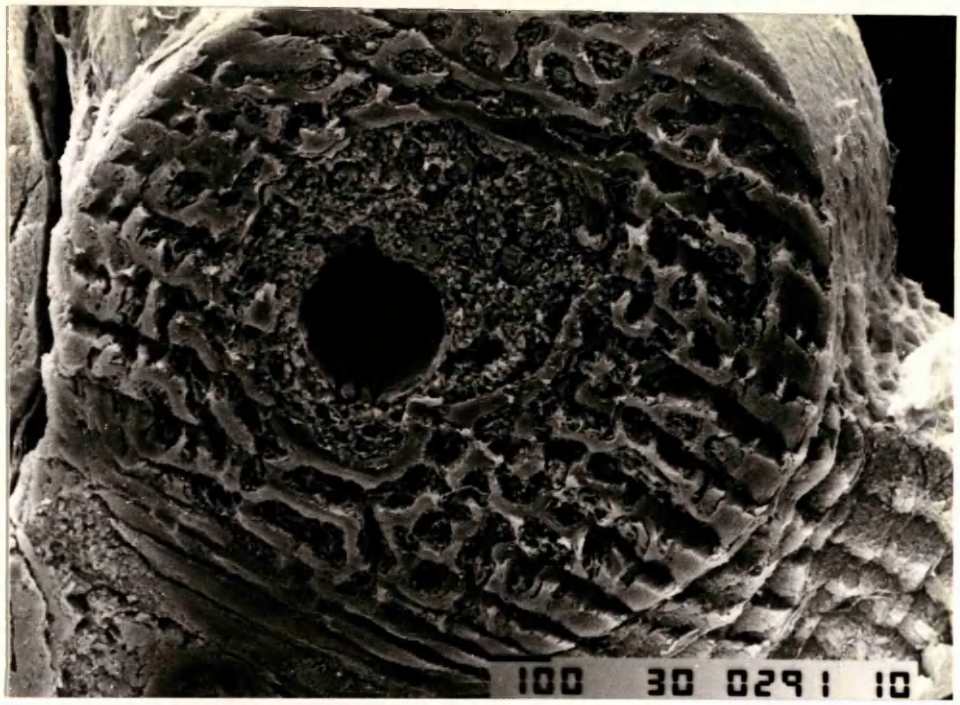
SEM x75

b) Trabeculae have a dense appearance. The intertrabecular spaces are filled with loose connective tissue.

SEM x350

c) A blood vessel (bv) is present in a Haversian canal (arrows) surrounded by bony trabeculae and loose connective tissue. Osteocytes (oc) can be seen in the substance of bone.

SEM x1000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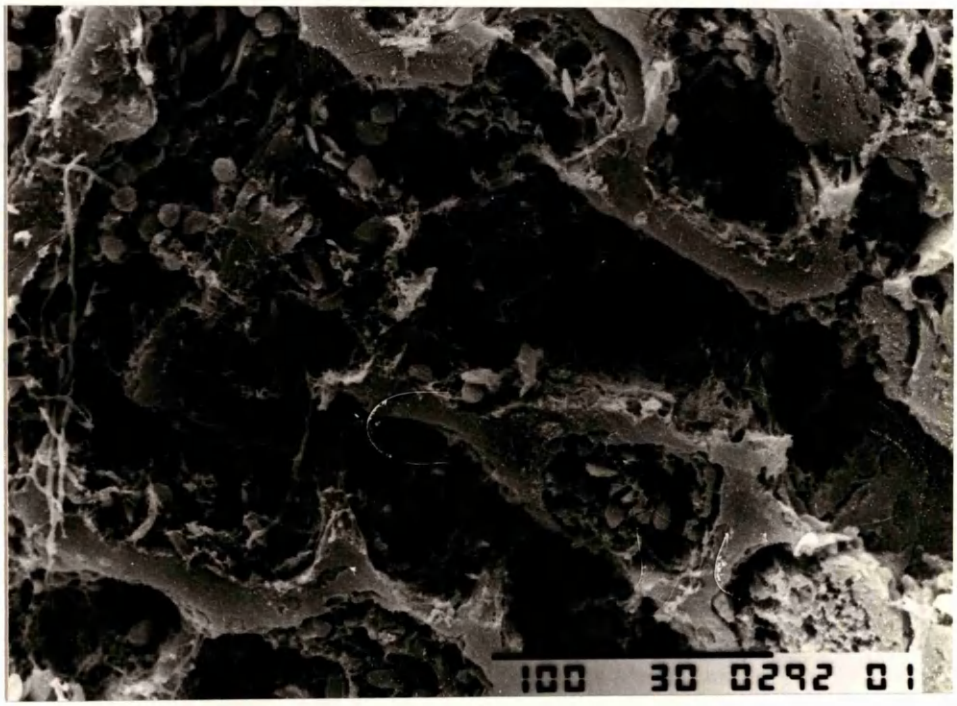
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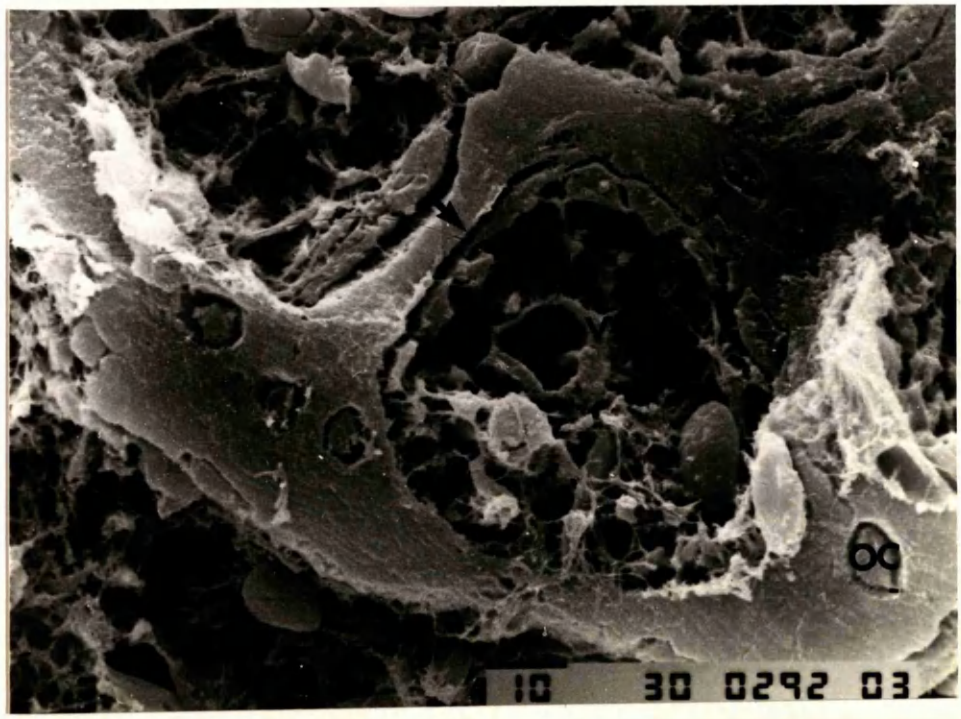

10 30 0292 03

Fig. 57

Section of mid-diaphysis of tibia from control embryo. 15 days.

a) 7-8 layers of periosteal bone are present with numerous interconnecting spicules. Haversian canals are well formed as is the marrow cavity which contains a large blood vessel (bv). A nutrient artery (na) can be seen opening into it. Remnants of the innermost layer (arrows) which has been resorbed are present.

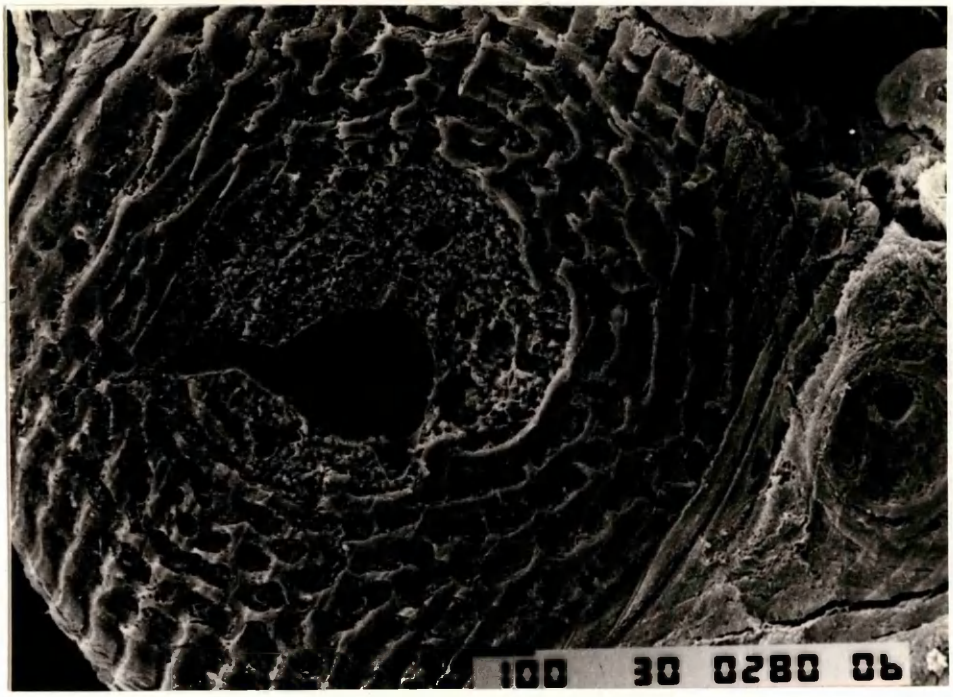
SEM x75

b) Dense and compact bony trabeculae are clearly defined and surround blood vessels (bv) with loose connective tissue around them in Haversian canals (arrows).

SEM x350

c) A blood vessel in a Haversian canal is surrounded by loose connective tissue.

SEM x1000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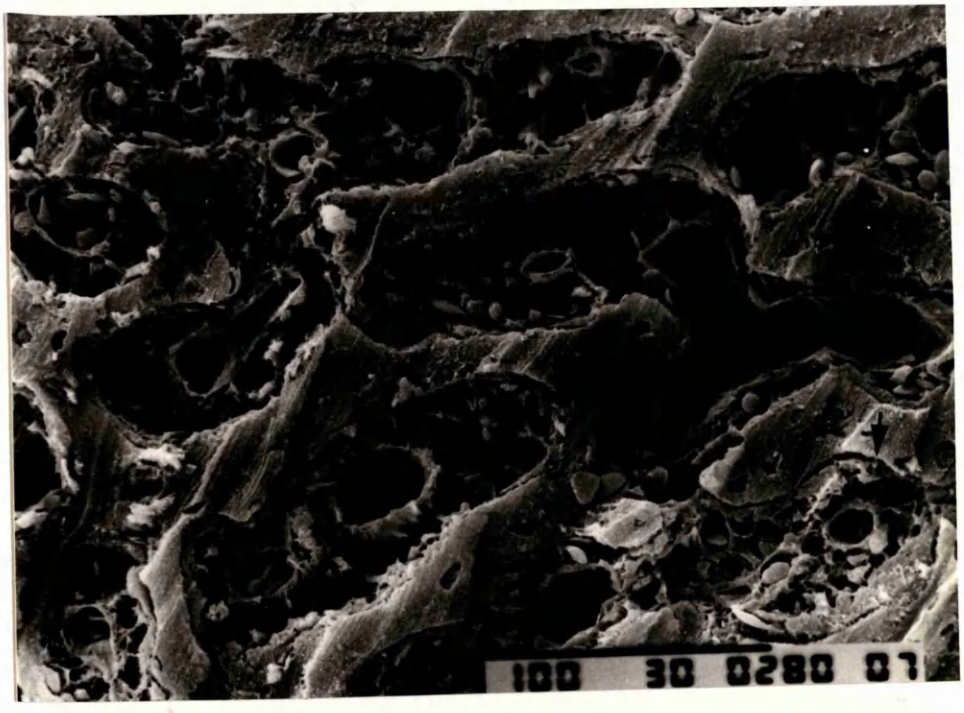

100 30 0280 07


30 0280 08

Fig. 58

Section of mid-diaphysis of tibia from control embryo. 16 days.

a)A maximum of 7 layers of periosteal bone can be seen. The innermost layer of bone has been resorbed and free ends of bony spicules can be seen at the edge of the well formed marrow cavity. A large central blood vessel (bv) is in the marrow cavity.

SEM x75

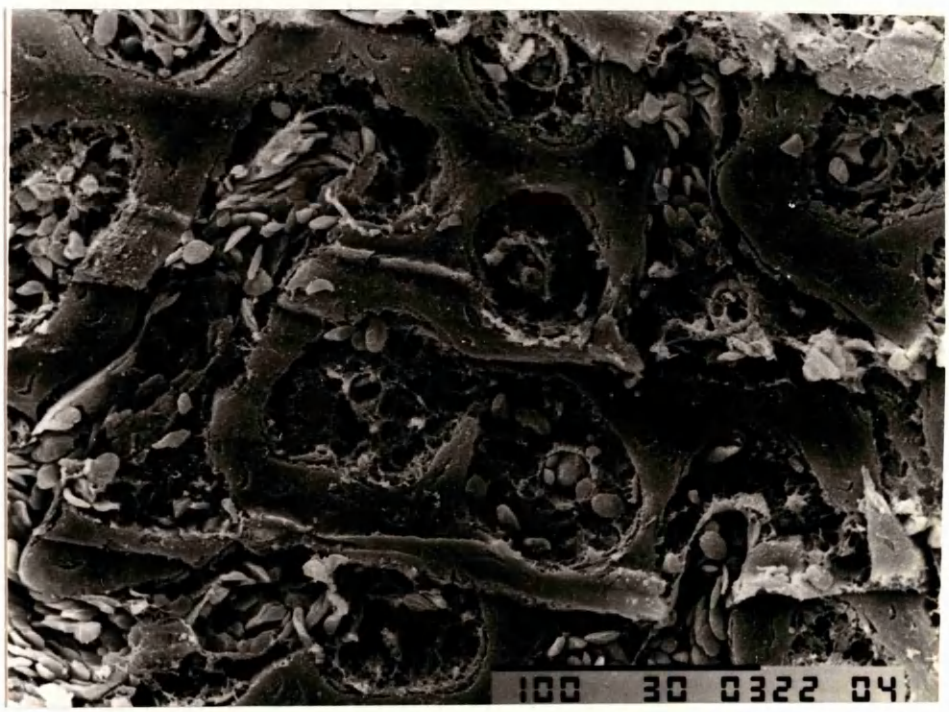
b) Bony trabeculae have a dense appearance with osteocytes trapped in their substance.

SEM x350

c) Two blood vessels (bv) can be seen in the Haversian canals surrounded by loose connective tissue. A few red blood cells (rbc) can also be seen.

SEM x1000


100 30 0322 02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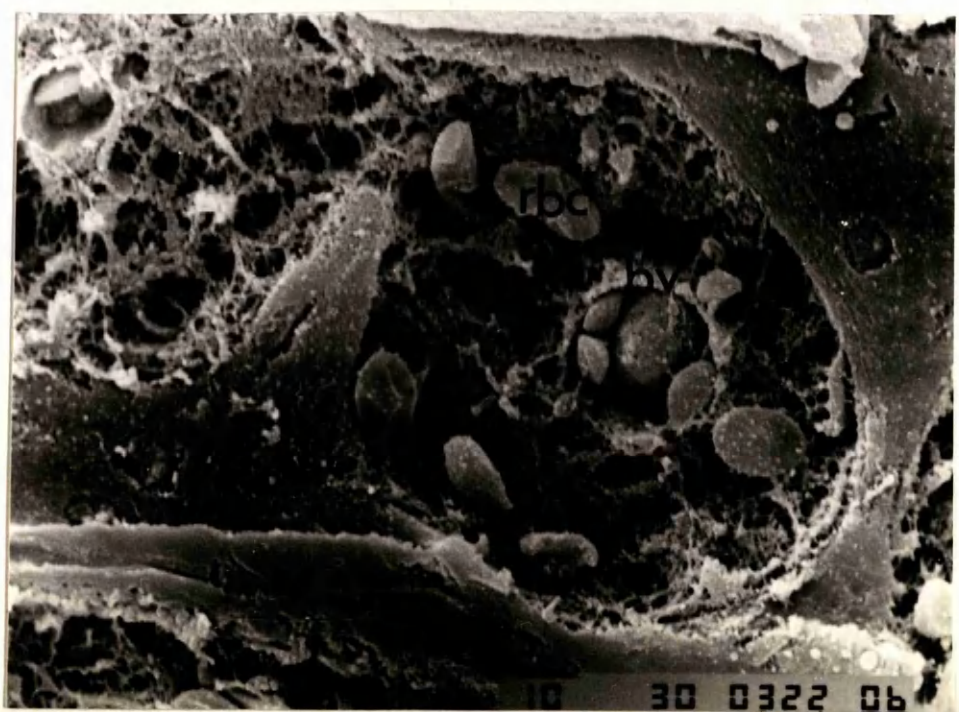

rbc
10 30 0322 06

Fig. 59

Section of mid-diaphysis of tibia from experimental embryo. 12 days.

a) A maximum of 4 layers of periosteal bone are present, arranged in an asymmetrical pattern with the caudolateral aspect of the tibia having the greatest number. The marrow cavity is well formed and a fairly large vessel (bv) can be seen within it. Interconnecting bony spicules have joined the layers of bone together.

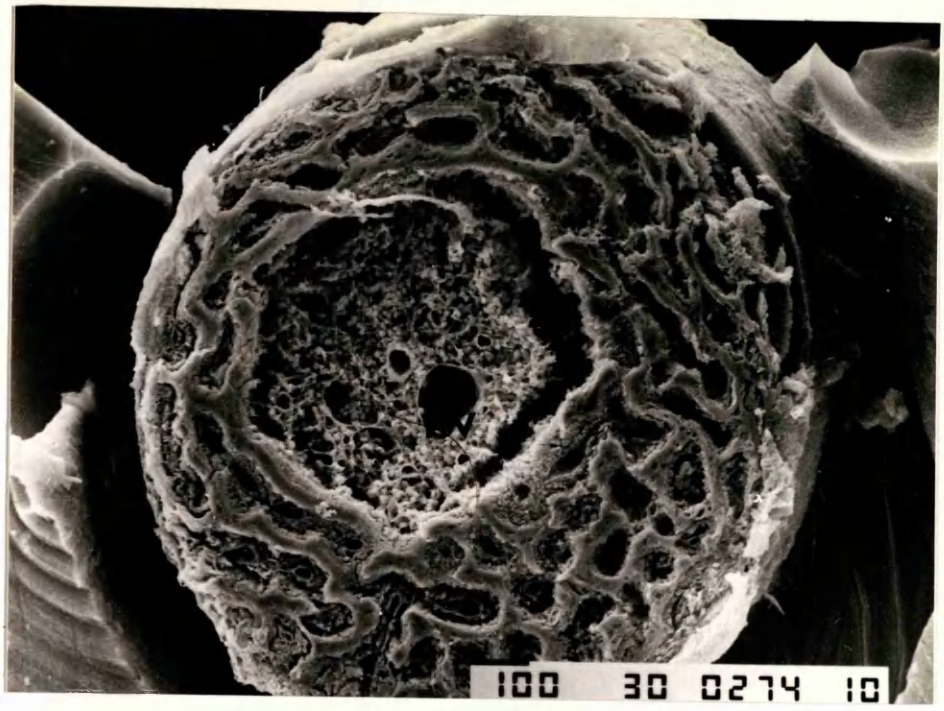
SEM x75

b) Bony trabeculae are of dense appearance with many osteocytes in their substance. Haversian canals (arrows) are well formed and blood vessels (bv) are surrounded by loose connective tissue.

SEM x350

c) A blood vessel (bv) in a Haversian canal is surrounded by connective tissue.

SEM x1000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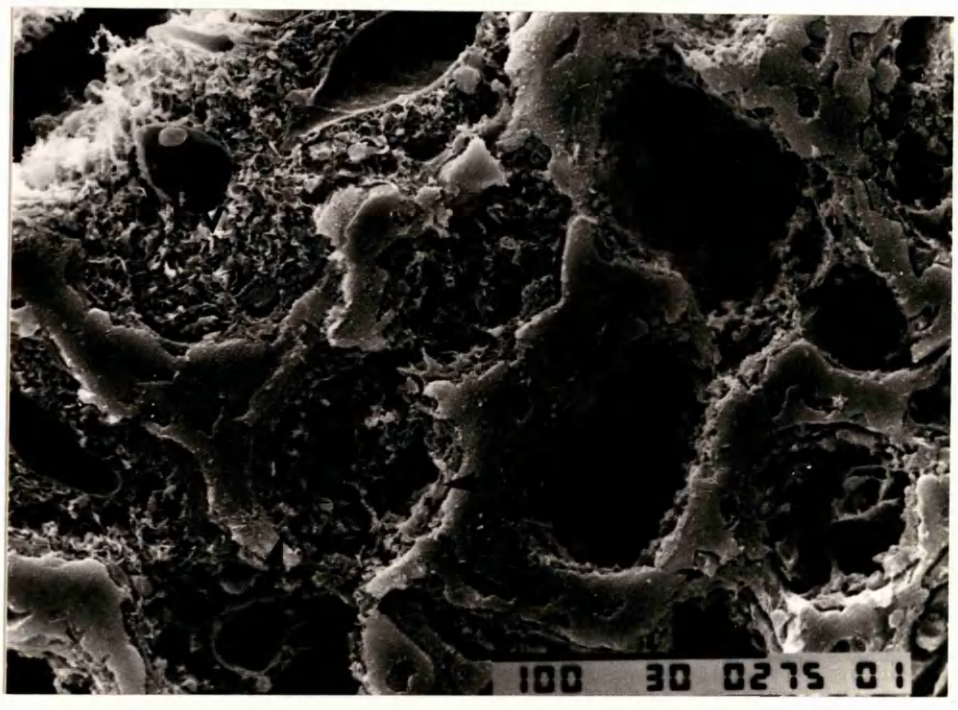
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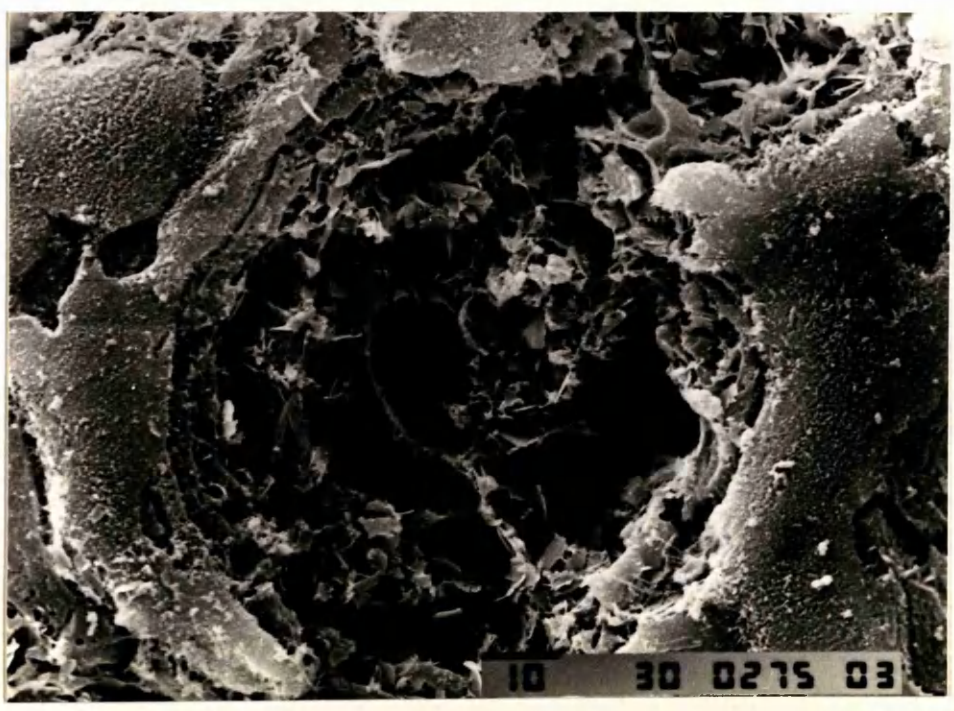

10 30 0275 03

Fig. 60

Section of mid-diaphysis of tibia from experimental embryo. 13 days.

a) A maximum of 5 layers of periosteal bone is present in an asymmetrical fashion with numerous interconnecting bony spicules. The marrow cavity is well formed and a large blood vessel (bv) can be seen in its centre.

SEM x75

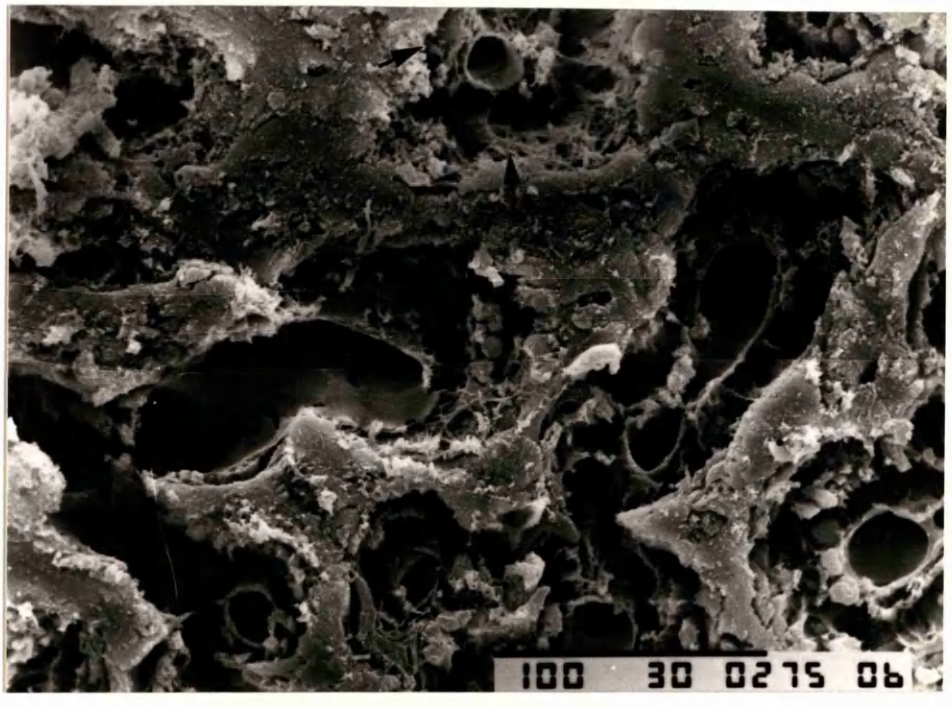
b) Bony trabeculae are of dense appearance and well formed Haversian canals can be seen (arrows).

SEM x350

c) A blood vessel (bv) in a Haversian canal (arrows) is surrounded by loose connective tissue.

SEM x1000


100 30 0275 05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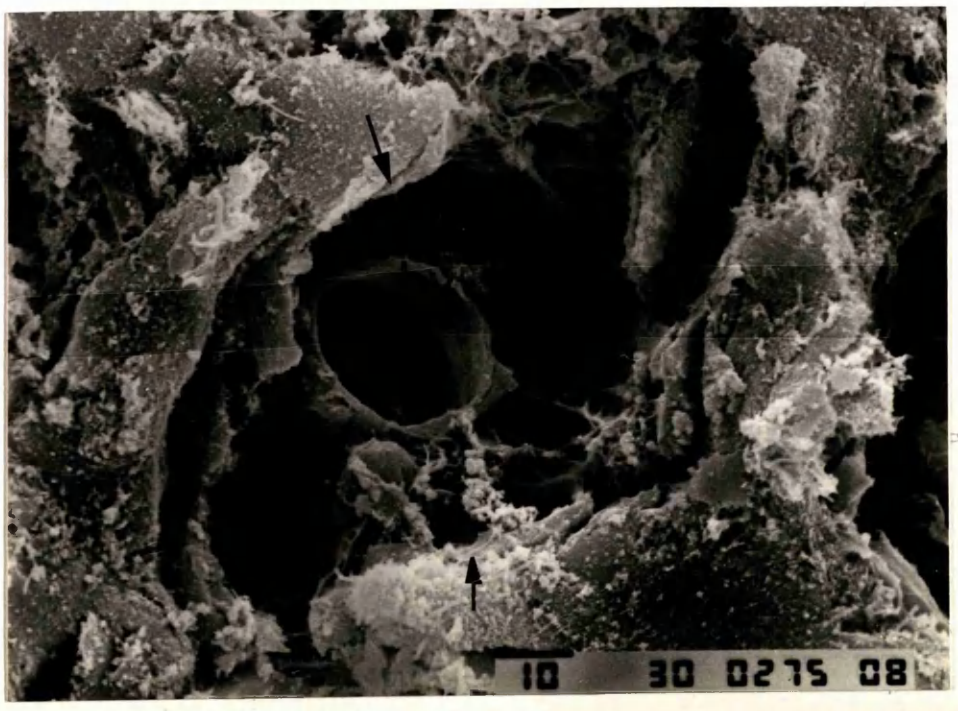

10 30 0275 08

Fig. 61

Section of mid-diaphysis of tibia from experimental embryo. 14 days.

a) A maximum of 4 layers of periosteal bone are present in an asymmetrical pattern. The marrow cavity is well formed and blood vessels are seen within it.

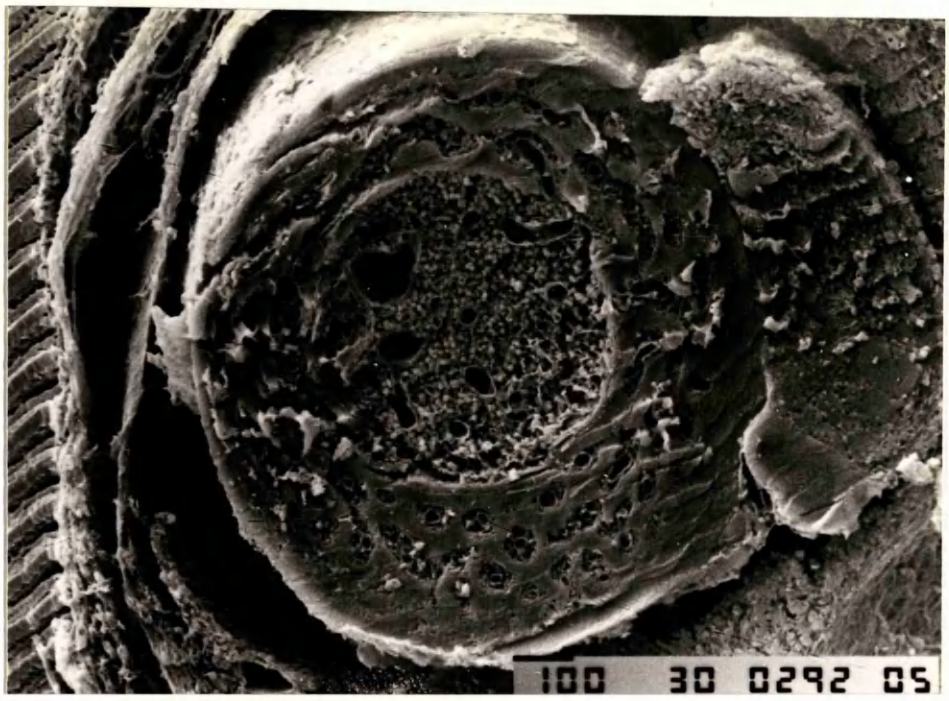
SEM x75

b) Bony trabeculae are dense and thicker than in controls. Blood vessels (bv) in well defined Haversian canals (arrows) are surrounded by connective tissue.

SEM x350

c) A dilated blood vessel (bv) can be seen in a Haversian canal (arrow). The vessel is rather wider than those of corresponding controls.

SEM x 1000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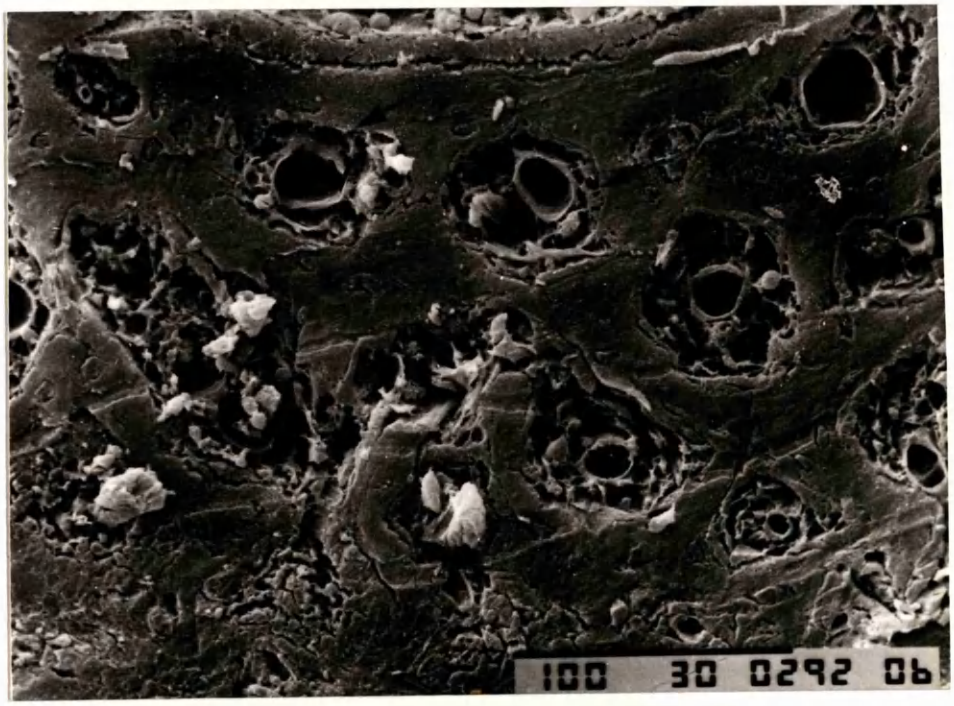
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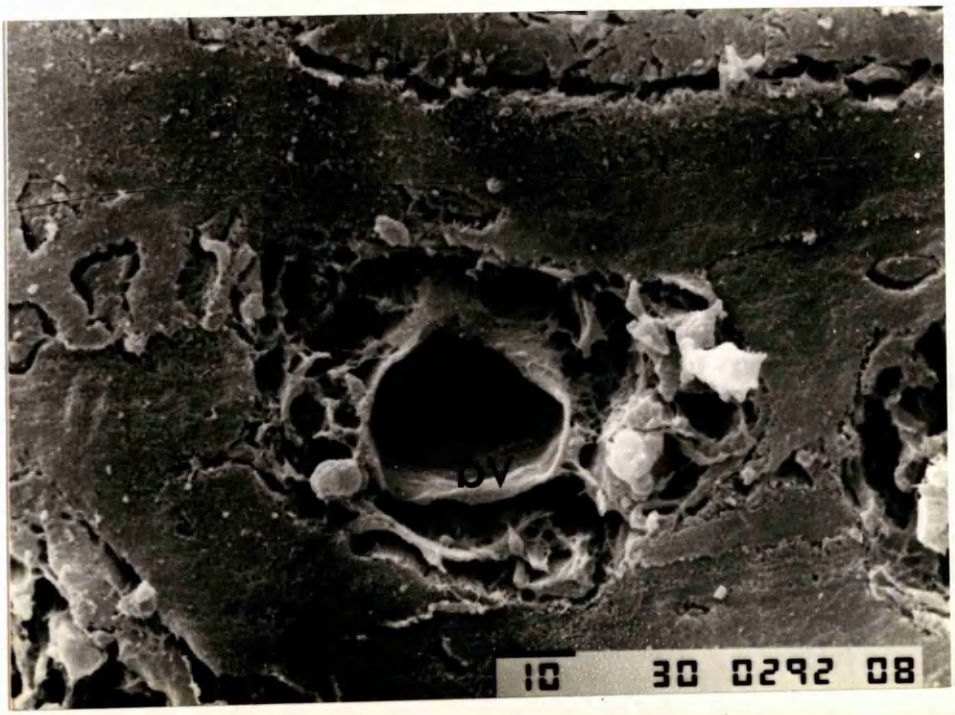

10 30 0292 08

Fig. 62

Section of mid-diaphysis of tibia from experimental embryo. 15 days.

a) Up to 6 layers of periosteal bone in an asymmetrical pattern are present. The innermost layer with little sign of resorption (arrows) is almost intact and surrounds the well formed marrow cavity. A blood vessel (bv) is seen in the marrow cavity which is noticeably smaller than that in controls. The diameter of tibia is smaller than that of the control.

SEM x75

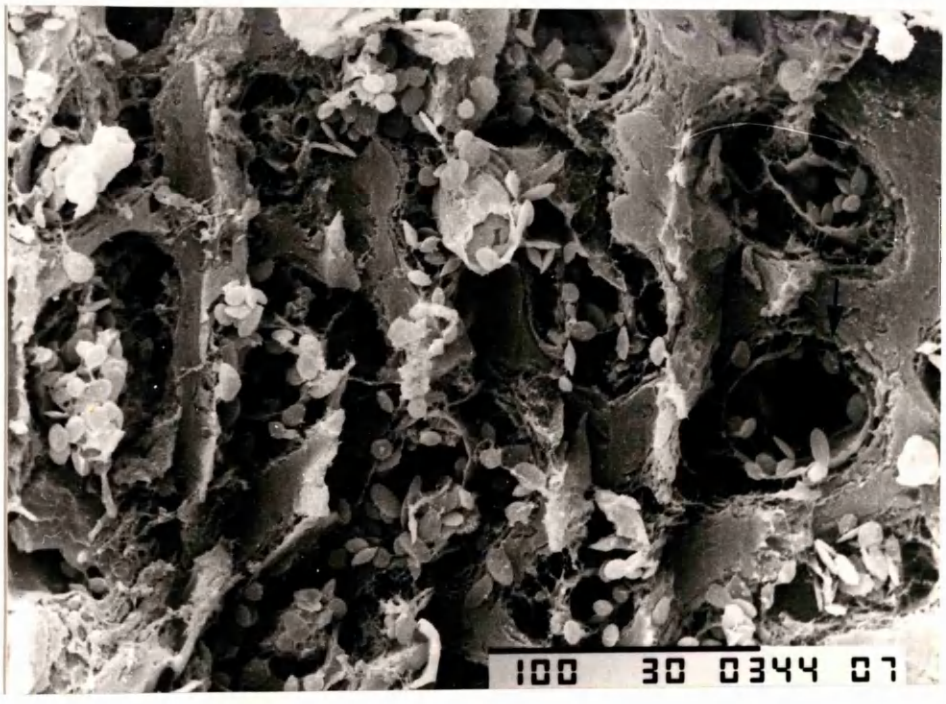
b) Bony trabeculae and well formed Haversian canals (arrows) can be seen.

SEM x350

c) A blood vessel (bv) in the Haversian canal can be seen which is dilated and is pushing the connective tissue around it close to the wall of the canal. Bony trabeculae are less compact and present rough surfaces (arrows).

SEM x1000


100 30 0344 06


100 30 0344 07

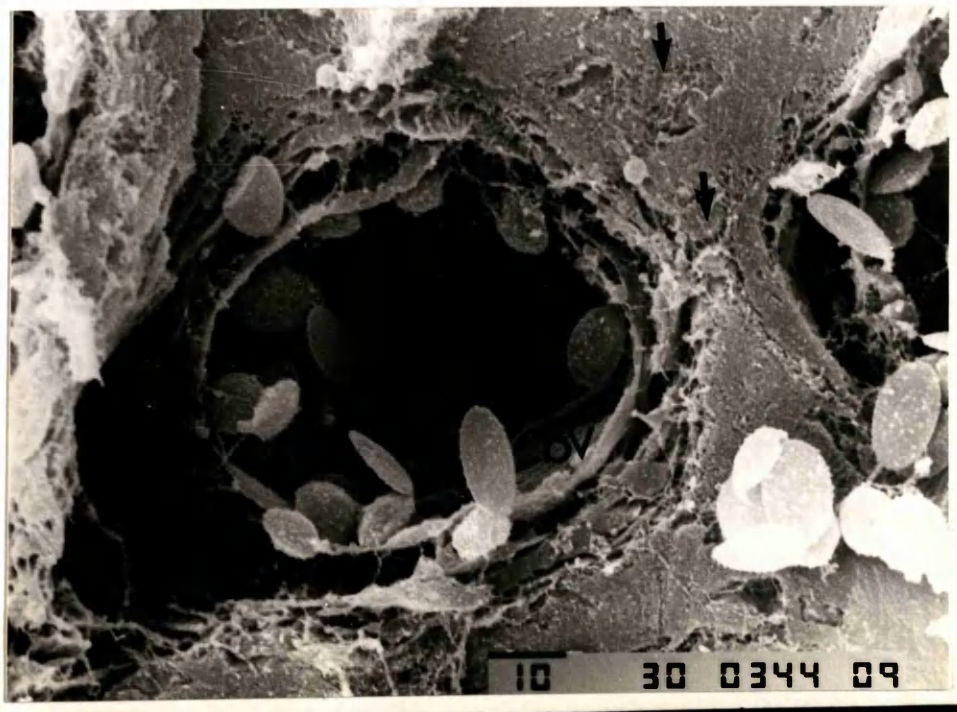

OV
10 30 0344 09

Fig. 63

Section of mid-diaphysis of tibia from experimental embryo. 16 days.

a) A maximum of 7 layers of periosteal bone have been laid down in an asymmetrical pattern. The marrow cavity is well formed and small blood vessels (bv) can be seen among its content. Resorption sites (arrows) in the innermost layer are seen.

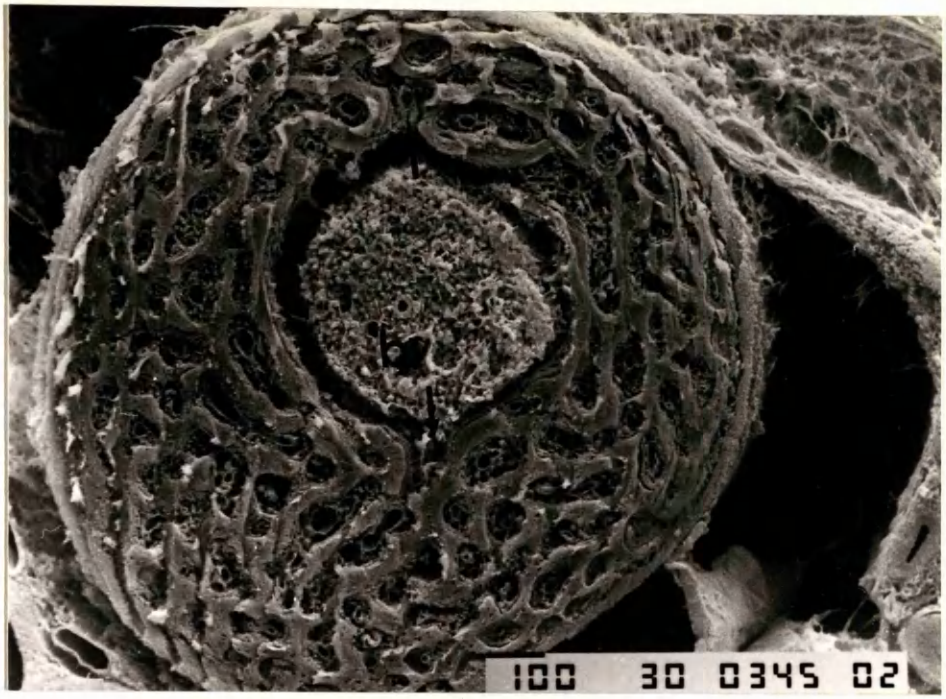
SEM x75

b) The bony trabeculae are less compact and have rougher surfaces than in controls. Blood vessels fill most of the intertrabecular spaces.

SEM x350

c) A blood vessel of large diameter can be seen in a Haversian canal. Bony trabeculae is less compact with a rough surface.

SEM x1000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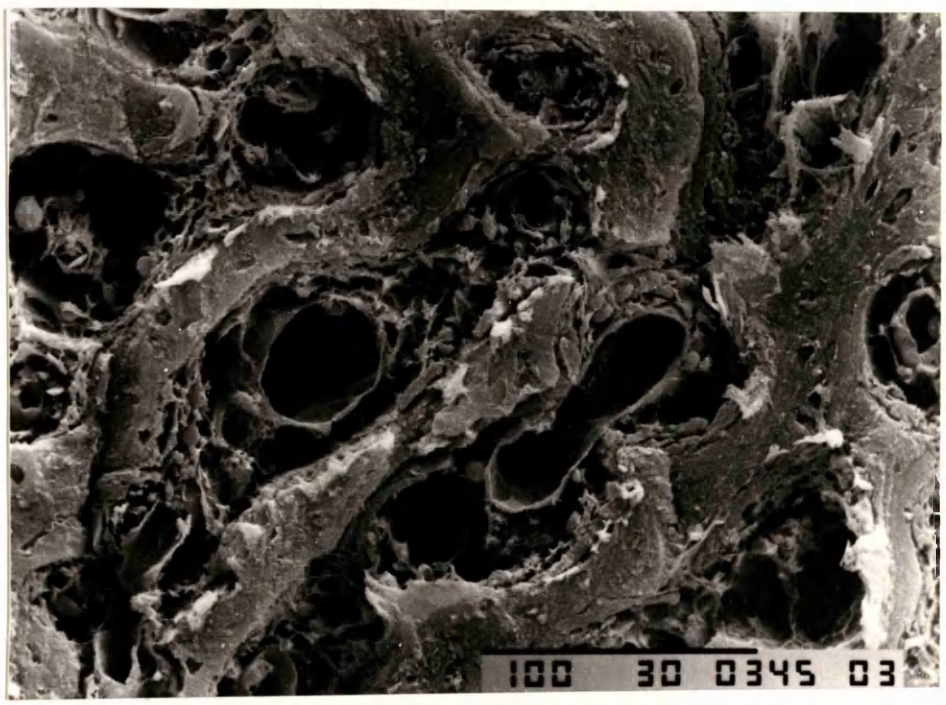

100 30 0345 03

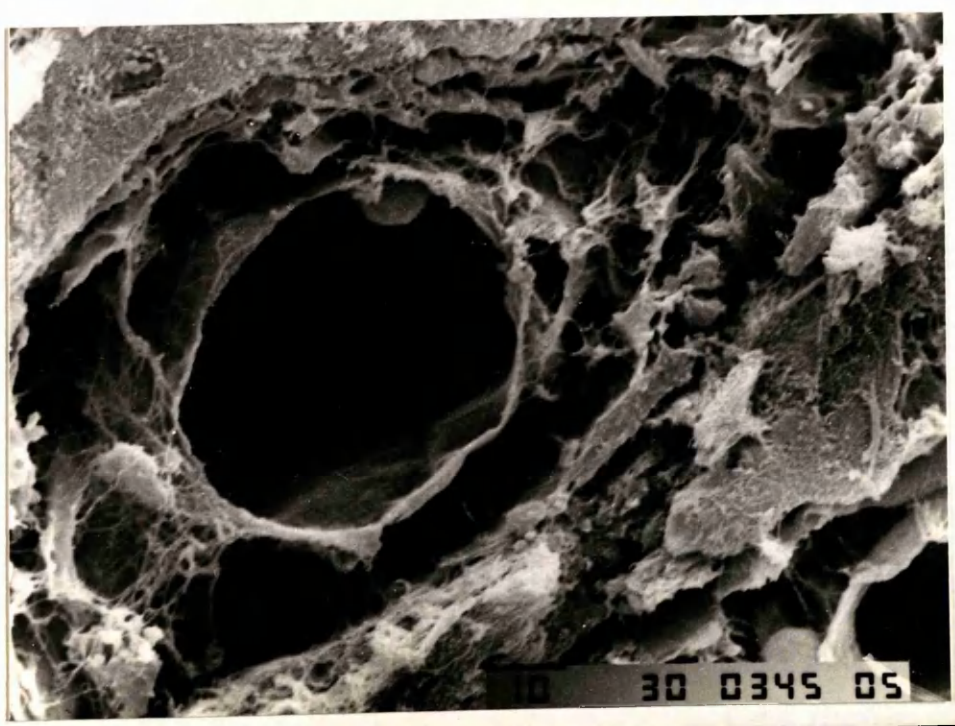
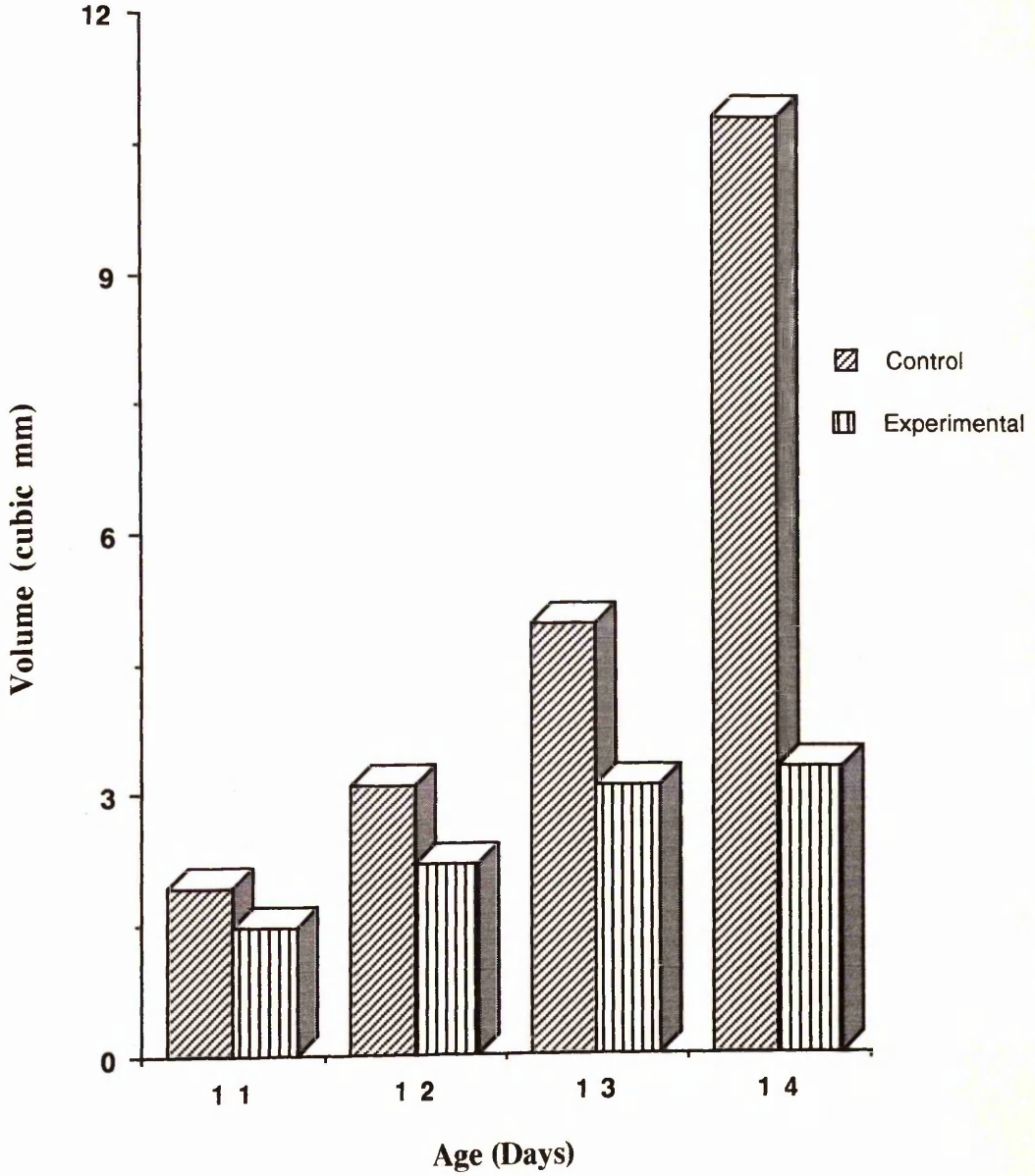

10 30 0345 05

Fig. 64

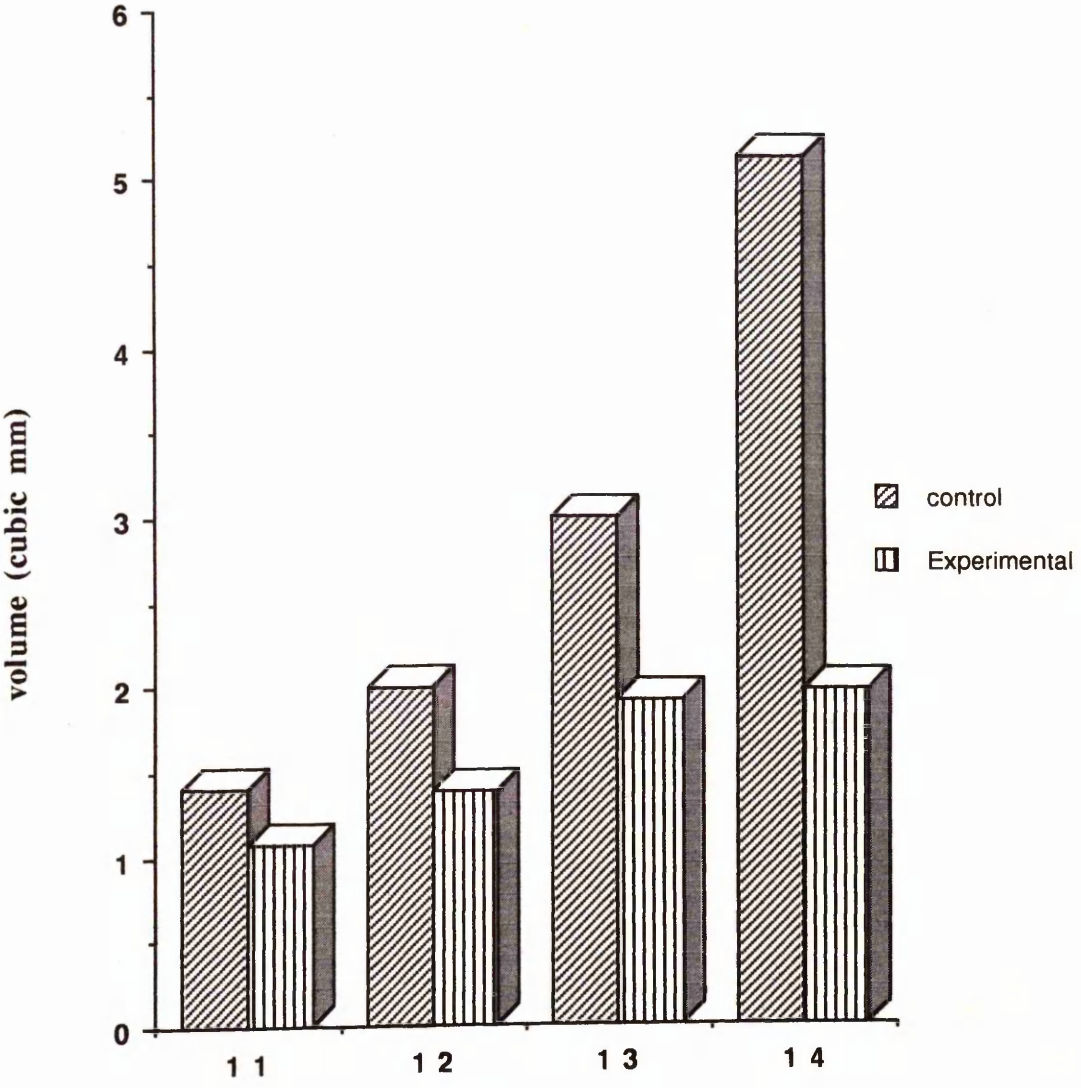
A histogram showing the volume enclosed by periosteum-perichondrium in control and experimental embryos between 11th and 14th days of incubation.



Volume enclosed by periosteum-perichondrium

Fig. 65

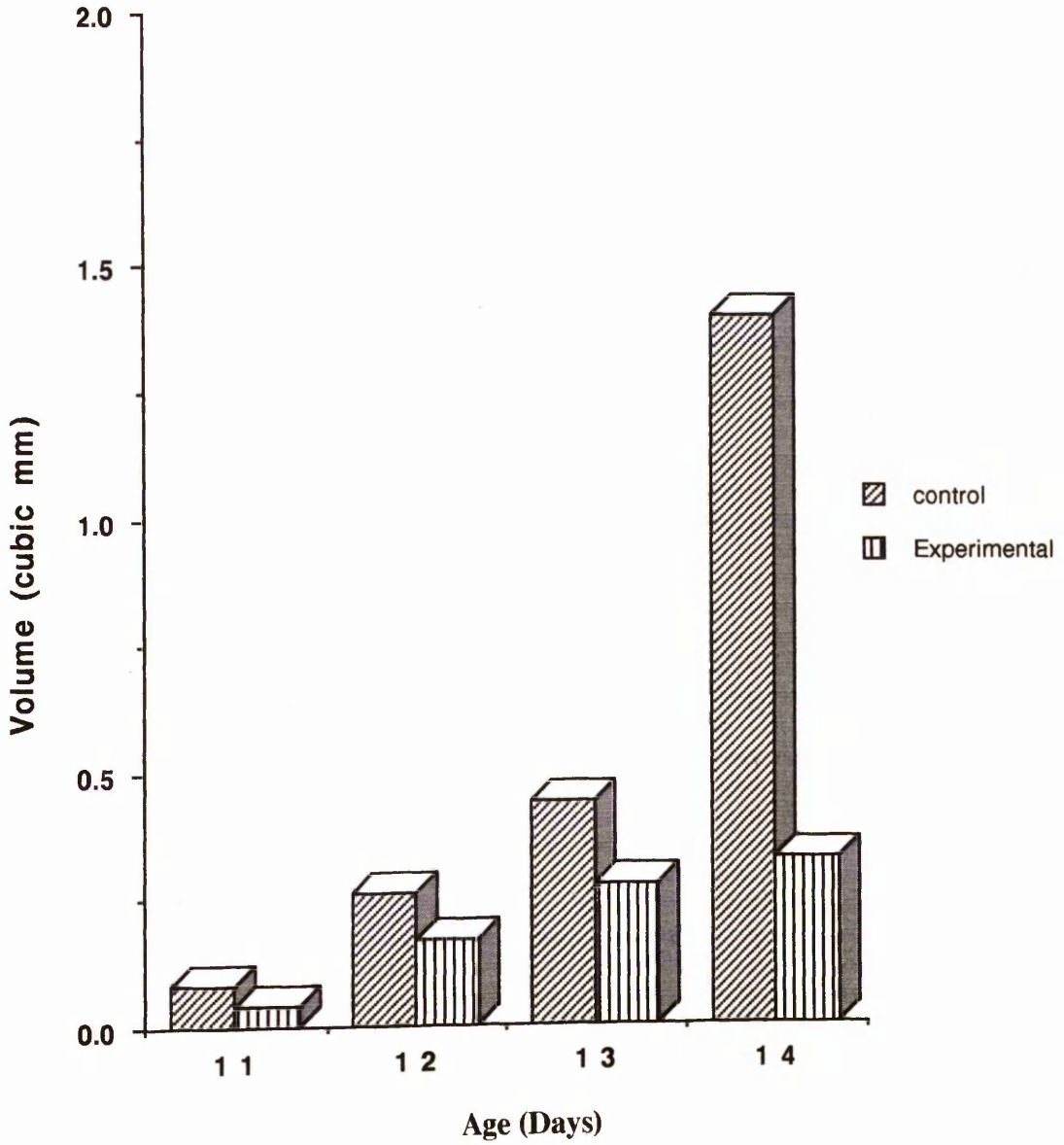
A histogram showing the volume of cartilage in control and experimental embryos between 11th and 14th days of incubation.



Volume of cartilage

Fig. 66

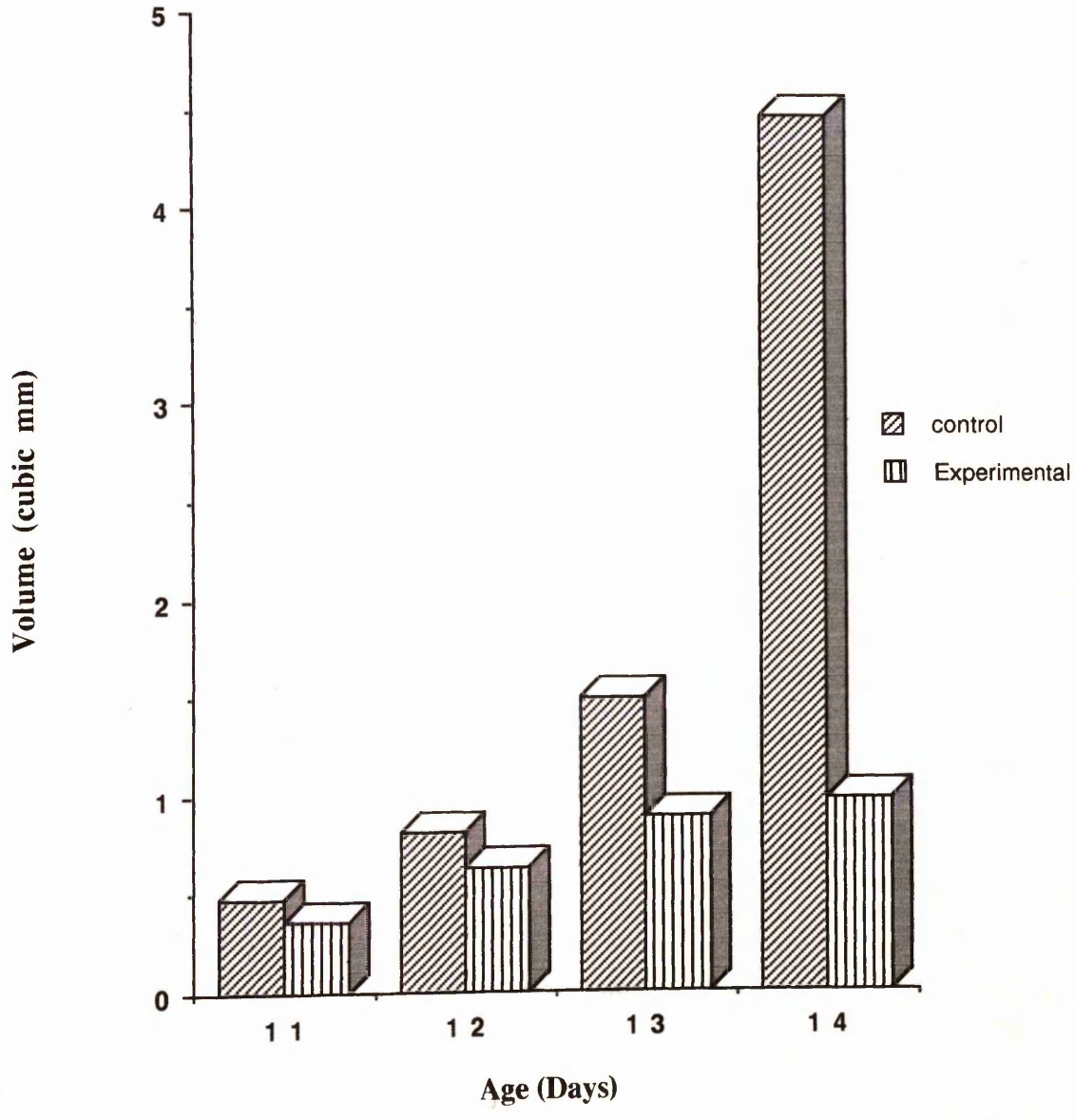
A histogram showing the volume of connective and vascular tissue in control and experimental embryos between 11th and 14th days of incubation.



Volume of connective and vascular tissue

Fig. 67

A histogram showing the volume of external space in control and experimental embryos between 11th and 14th days of incubation.



Volume of external space (collar bone)

Fig. 68

Statistical comparison between the cartilage volume density of control ● and experimental ○ embryos.

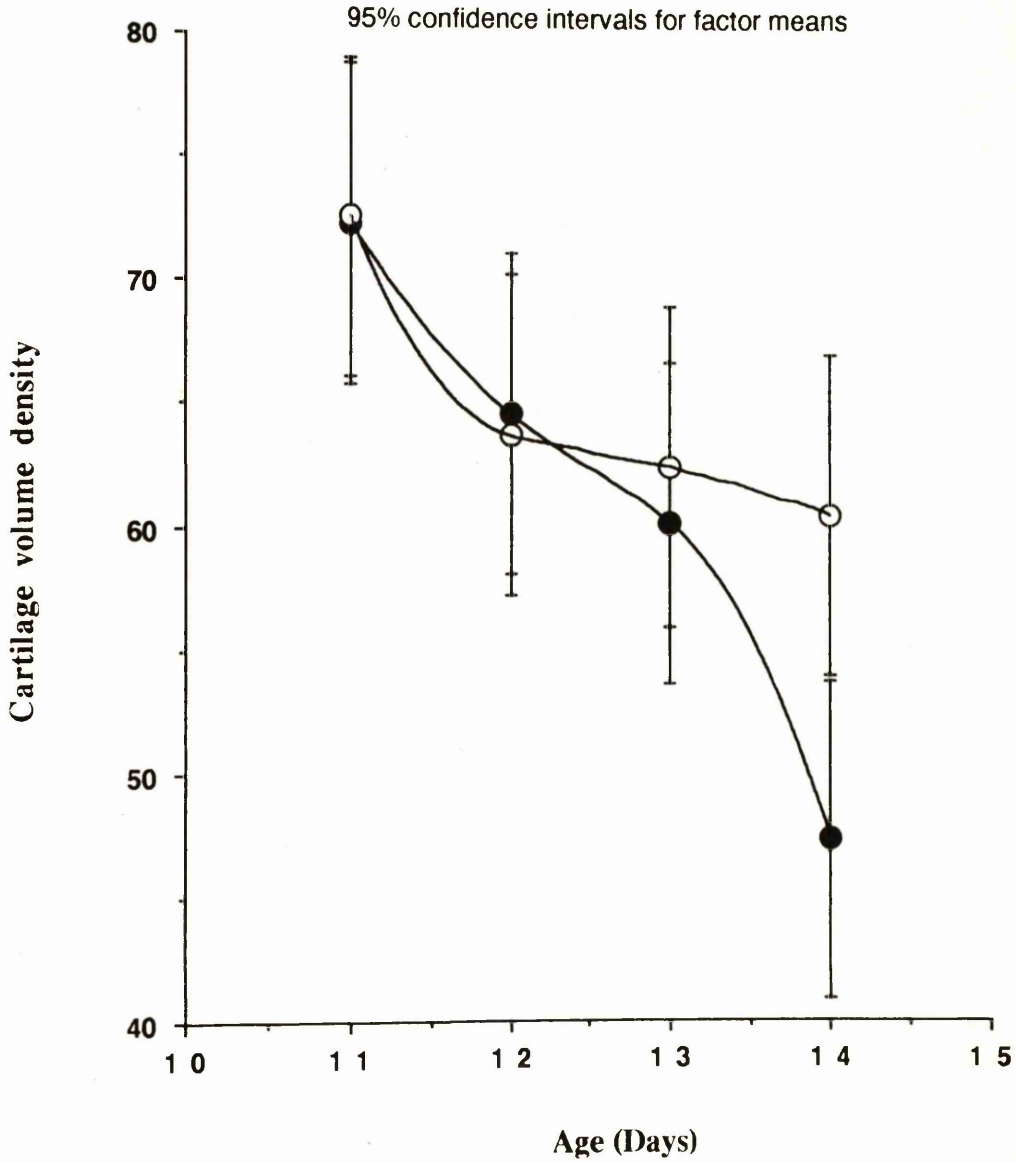

95% confidence intervals for factor means
80
70
60
50
40
Cartilage volume density
10
11
12
13
14
15
Age (Days)

Fig. 69

Statistical comparison between the external space volume density of control ● and experimental ○ embryos.

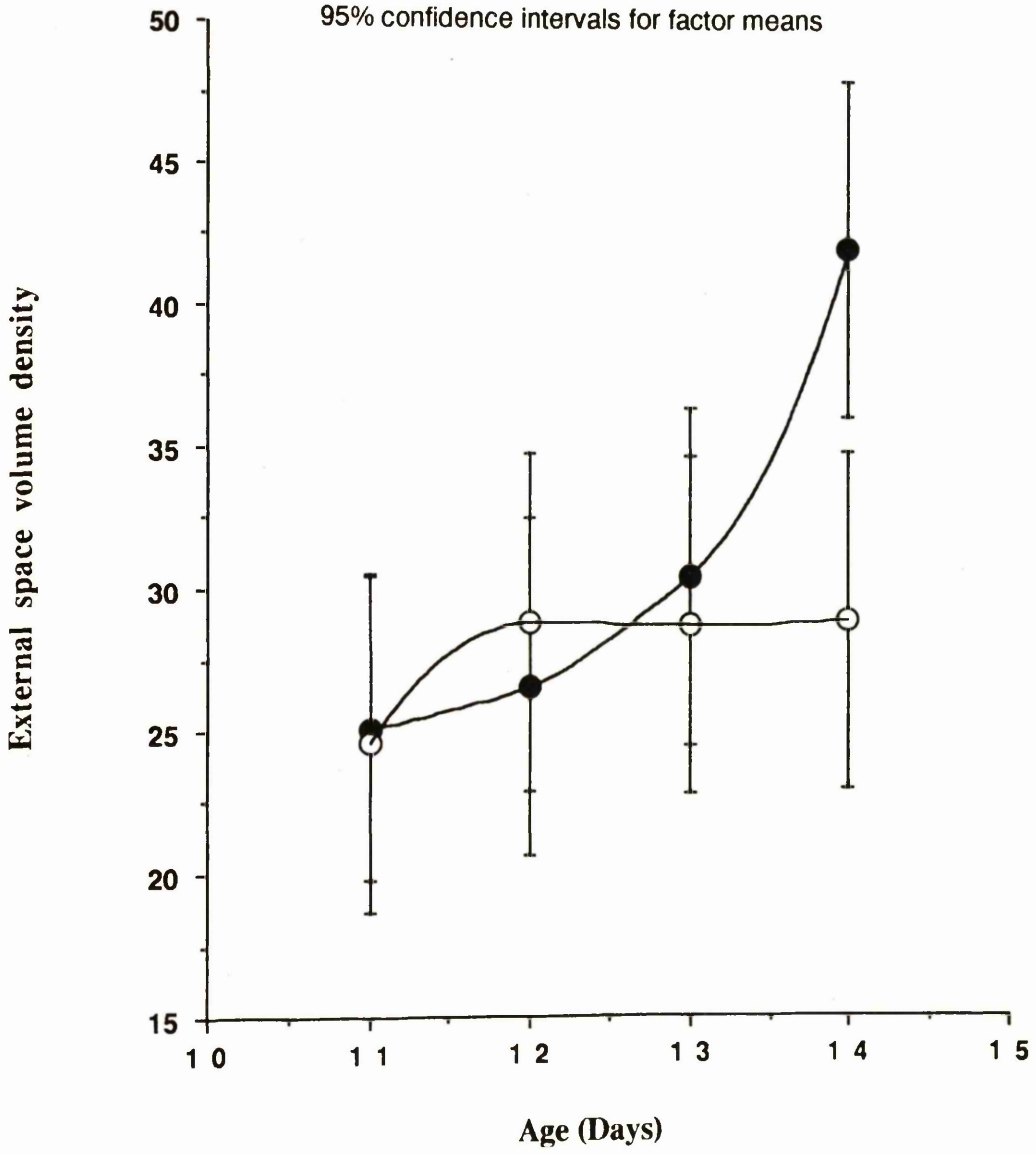

95% confidence intervals for factor means
External space volume density
50
45
40
35
30
25
20
15
10
11
12
13
14
15
Age (Days)

Fig. 70

Statistical comparison between the connective and vascular tissue volume density of the control ● and experimental O embryos. There are no significant differences between the two groups.

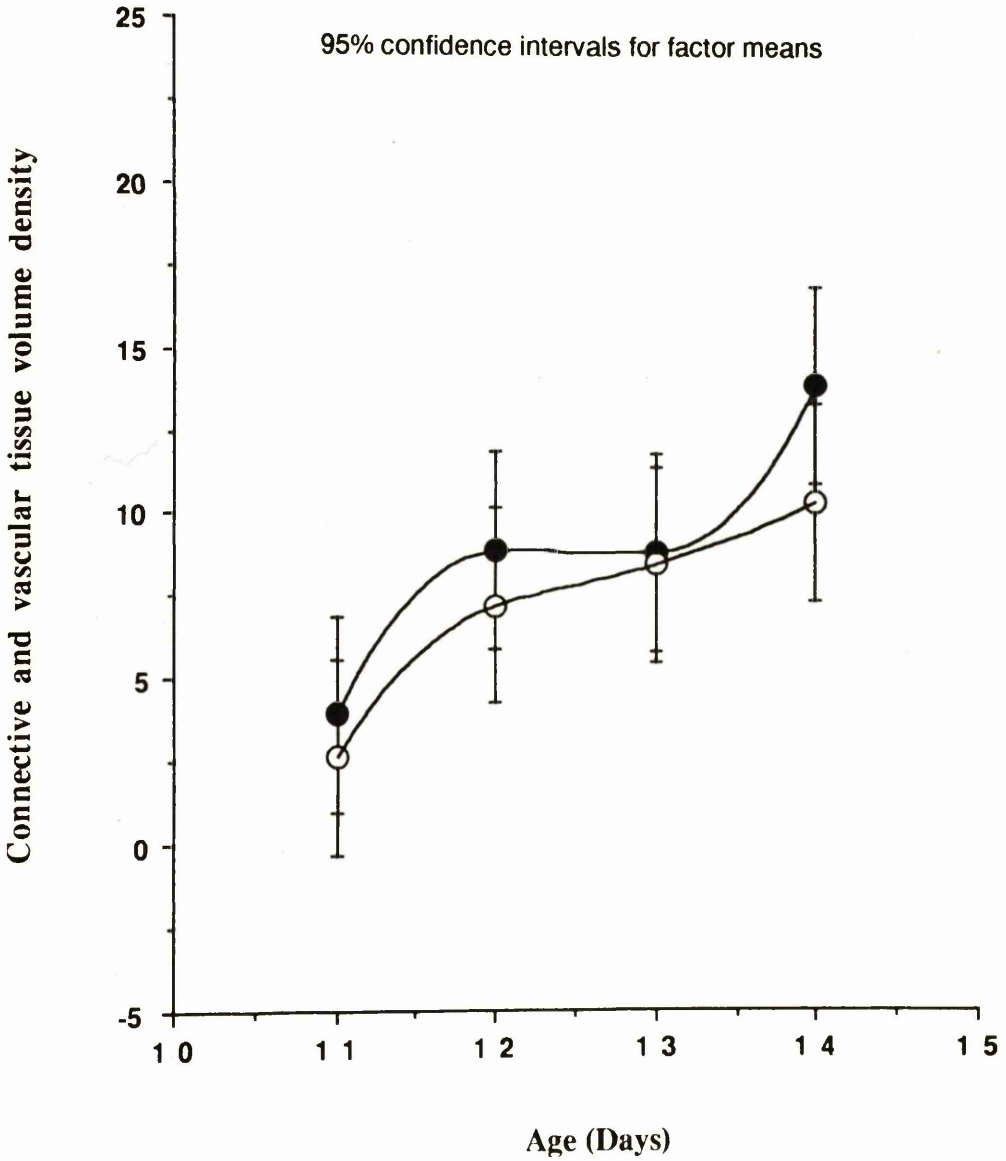
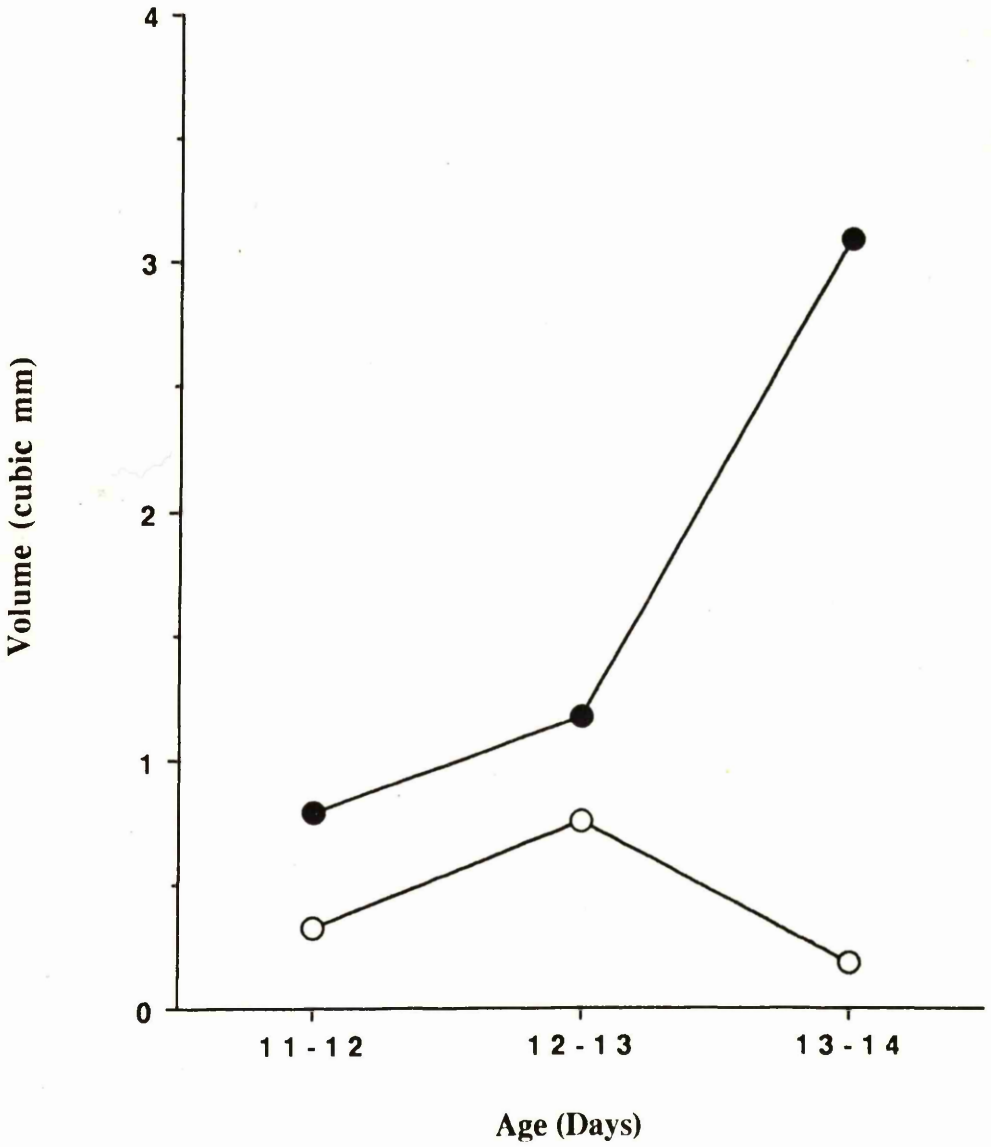

95% confidence intervals for factor means
Connective and vascular tissue volume density
25
20
15
10
5
0
-5
10
11
12
13
14
15
Age (Days)

Fig. 71

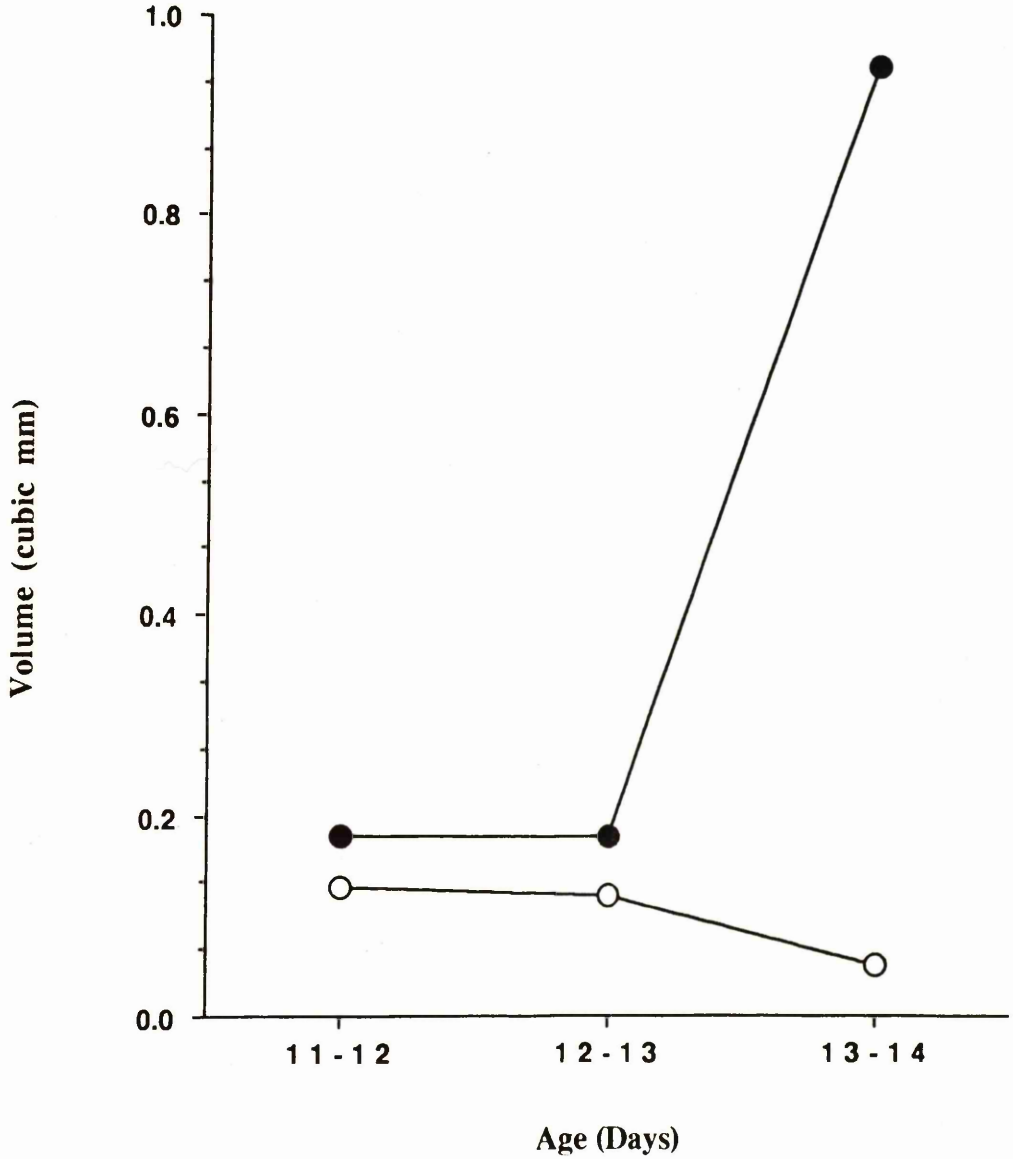
A graph showing the volumes of the formation of cartilage in control ● and experimental O embryos per day from 11th to 14th days of incubation.



Formation of cartilage

Fig. 72

A graph showing the volume of cartilage resorbed per day in control ● and experimental ○ embryos from 11th to 14th days of incubation.



Resorption of cartilage

Fig. 73a

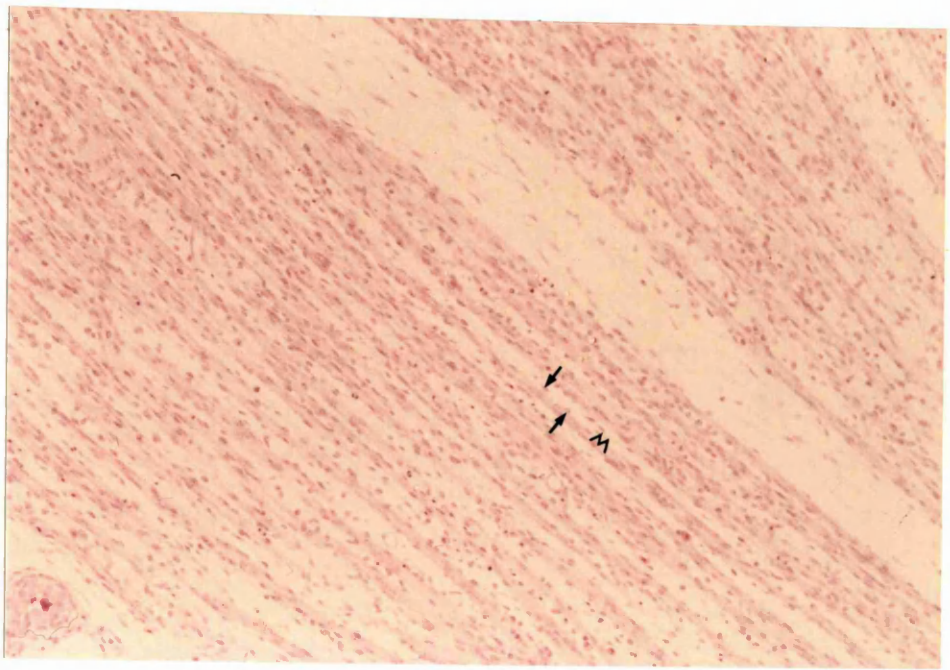
Longitudinal wax section through gastrocnemius muscle of control embryo. 12 days.
Individual muscle fibres (M) are elongated and contain many nuclei (arrows).

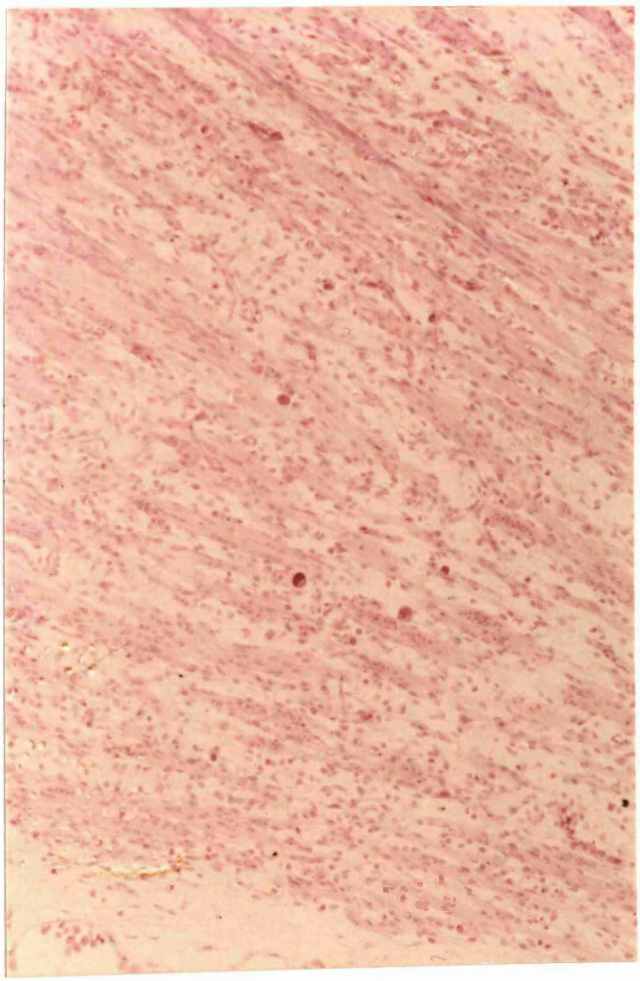
H & E x115

Fig. 73b

Longitudinal wax section through gastrocnemius muscle of experimental embryo. 12 days.
Muscle fibres have lost their regular arrangement.

H & E x115

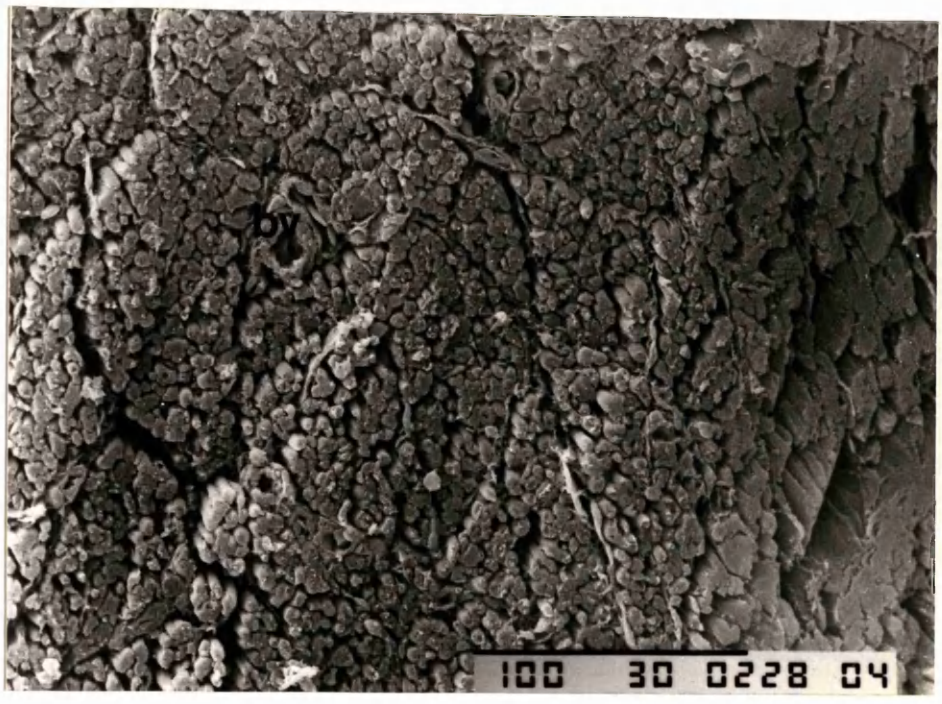




Figs. 74a & b

Transverse section through gastrocnemius muscle from control embryo. 14 days. Muscle fibres are arranged in bundles. Several blood vessels (bv) can be identified among the muscle fibres.

SEM a) x370 b) x2100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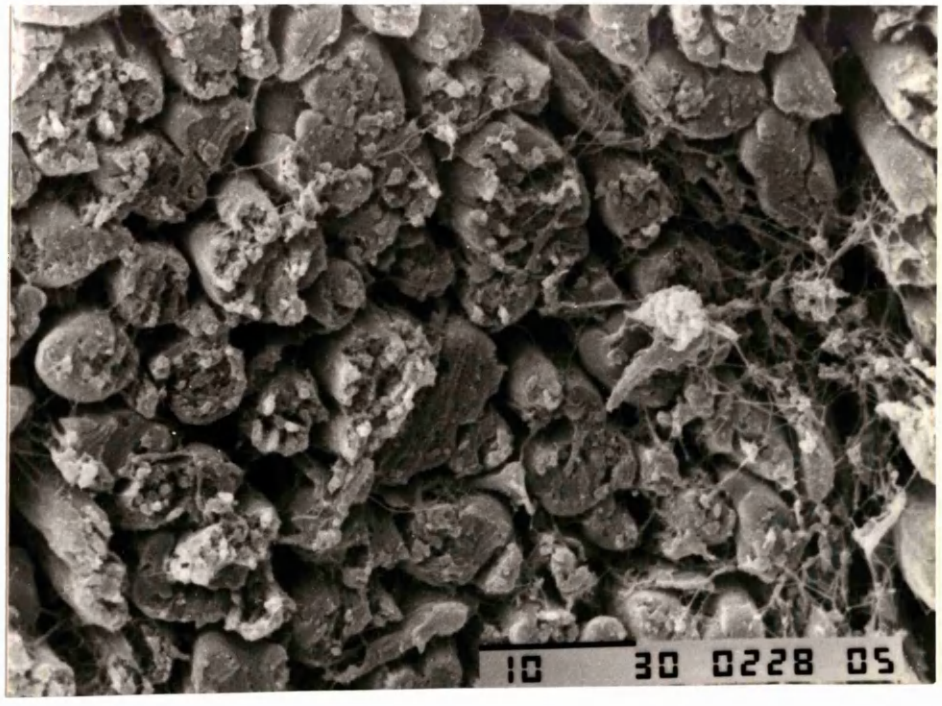
10 30 0228 05

Figs. 75a & b

Transverse section through gastrocnemius muscle from experimental embryo. 14 days.
Muscle fibres have lost their regular arrangement and adhere to each other. Many white blood cells (wbc) and fat droplets (f) are present among the fibres and around the blood vessels (bv).

SEM a) x370 b) x2100