Effect of Paralysis of Skeletal Muscles on the Development of Synovial Joints in the Chick Embryo

By

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In the name of Allah
The Beneficient, The Merciful

And to Him belongs praise in the heavens and the earth and at nightfall and when you are at midday.

He brings forth the living from the dead and brings forth the dead from the living, and gives life to the earth after its death, and thus shall you brought forth.

And one of His signs is that He created you from dust, then Lo! you are mortals (who) Scatter.

Verses 18-20
Chapter 30
Holy Quran

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SUMMARY

1. The effects of immobilization on the development of the knee and 3rd metatarsophalangeal joints and associated structures were studied in a staged series of chick embryos, paralysed by administration of decamethonium bromide from 6 days of incubation onwards. Control embryos were treated with normal saline.
2. Control and experimental material was studied in cleared whole mounts, stained with Alcian blue - Alizarin Red; in serial wax sections; by semi-thin histology and by transmission electron microscopy.
3. The significance of cell death in joint development.

   i. Dead cells were similarly distributed in the joint interzone, and at the same developmental stages, in paralysed and control embryos. Their occurrence is therefore independent of movement.

   ii. Dead cells were not seen in the region of the developing femoro-patellar joint in either control or paralysed embryos.

   iii. Dead cells were not seen along the cruciate ligaments (after Stage 32) or around tibialis anterior tendon in paralysed embryos, unlike controls.

   iv. Dead cells were more numerous in the interzone of the M-P joint of paralysed embryos, than in controls. Despite this, initial formation of the synovial cavity was very limited in paralysed embryos. Cell death is

not therefore a sufficient cause of cavity formation, for which movement is also required. A similar conclusion is drawn from the knee joint, although, for technical reasons, the numbers of dead cells were not counted or compared statistically in this joint.

v. Cell death is not solely responsible for sculpturing of articular surfaces and intra-articular structures.

vi. The cytological features of cell death, as seen by electron microscopy, in interzones of both normal and paralysed joints, were more like those described for apoptosis than for necrotic cell death.

4. In the paralysed knee and M-P joints, the interzone became progressively narrower and its surviving cells differentiated into cartilage cells (M-P joint) or into cartilage cells or fibroblasts (knee joint), leading to cartilaginous or fibrous fusion.

5. Although the development of the knee, femoro-patellar and M-P joints showed a common general pattern, there were some differences in detail, in respect of the appearance of a 3-layered interzone, and the extent of development of blood vessels in the interzone.

6. The patellar cartilage did not develop at all in the majority of paralysed embryos. In all normal embryos, and in paralysed embryos when it did appear, it developed posteriorly to the quadriceps tendon rather than within it.

7. The intra-articular structures of the knee joint developed initially in situ from the mesenchyme of the interzone, independently of movement, but their maintenance and further development was impaired in paralysed chicks and was therefore dependant upon movement.

8. The synovial mesenchyme developed similarly, in normal and paralysed chicks, from general mesenchyme, cut off from the periarticular mesenchyme by the development of the joint capsule. In paralysed chicks it later became more vascular and showed infiltration by white blood cells.

   The infra-patellar adipose tissue appeared earlier in paralysed than in control chicks.

9. The tendons of tibialis anterior and ambiens muscles initially developed similarly in paralysed and normal chicks, but in the absence of movement they degenerated and their synovial sheaths did not develop.

10. Paralysed skeletal muscles underwent disuse atrophy and were largely replaced by adipose tissue and fibrous connective tissue.

CHAPTER ONE

INTRODUCTION

GENERAL INTRODUCTION

The general features of the development of synovial joints are now well established:

1. The scleroblastema of condensed mesenchyme, appears as a continuous mass which is not initially demarcated clearly from the surrounding myogenic tissue. Centres of chondrification develop in this mesenchymal core and rapidly extend to delineate individual skeletal elements.
2. Each element becomes limited by a compact layer of undifferentiated cells, the perichondrium; this layer proliferates to produce cartilage cells which contribute to growth of the cartilage models by surface accretion.
3. The ends of the two skeletal elements are united by a densely cellular and avascular interzone which does not undergo any change into cartilage.
4. The joint interzone develops into a three layered structure: a chondrogenic layer covering each articular surface and a loose intermediate layer. The two chondrogenic layers are continuous peripherally with the adjoining perichondrium and contribute to the growth of the epiphysial cartilage, whereas the intermediate layer merges with the general mesenchyme of the limb, which is vascularized at the periphery of the interzone. A condensation of the mesenchyme forms the fibrous capsule, of the future joint, which is continuous with the perichondrium of the skeletal elements and encloses a layer of vascular mesenchyme, the precursor of the synovial

membrane.

5. As the skeletal elements chondrify and in part ossify, the joint cavity and intra-articular structures, such as menisci and cruciate ligaments develop within the looser intermediate layer of the interzone.

While there is agreement on the general plan of development, there are still controversies on details of the mechanism of development of the synovial joint and its associated structures.

Many authors have considered intrinsic (genetic) factors as the initial cause for the development of the synovial joint, whereas others have suggested that extrinsic factors, such as muscular movement, may play a role.

This study was designed to assess the possible role of muscular movement in the development of the synovial joint and its associated structures in the chick embryos.

REVIEW OF LITERATURE

I. NORMAL DEVELOPMENT OF SYNOVIAL JOINTS IN BIRDS AND MAMMALS

The essential stages in the normal development of synovial joints in mammals and birds have been described by many authors during the last hundred years.

a) Mammalian synovial joints

Hepburn (1889) studied the development of diarthrodial joints in various mammals and concluded that the

contributing bones and the articular disc between them constituted a continuous tissue mass, and were derivatives of a common blastema, of which the articular disc was at first the undifferentiated part. The joint cavity appeared within the articular disc while the proximal and distal segments of the articular disc developed into the articular cartilage. The tissue at the circumference of the articular disc also developed into the synovial mesenchyme and the joint capsule.

Bardeen (1905) studied the development of the human skeleton and recognized, in general, that development of the skeleton of the limb passed through three stages: blastemal, chondrogenous and osseogenous. During the chondrogenous stage, cartilage elements of the limbs seemed at first to be connected at their articulating ends by a dense mass of tissue. Later the peripheral blastemal tissue at the joints transformed into a capsular ligament which was continuous with the perichondrium of the cartilage elements; within the joint most of the tissue became less dense and finally disappeared, leaving a joint cavity.

Whillis (1940) studied the development of the interphalangeal joints of the human and found that, at 30 mm, the two chondrifying elements were separated by joint disc, composed of relatively undifferentiated mesenchymal cells. The mesenchymal cells of the central part of the joint disc were flattened, very closely packed and disposed with their long axes in the line of the joint, whereas in

the peripheral part of the disc they were much looser. At 72 mm, the joint disc was thinner and the loose arrangement of the cells at the periphery was still more evident, so that cavities appeared among them, while the central part of the disc was still formed by a thin layer of flattened closely packed cells. At 125 mm, the joint disc between the cartilages had entirely disappeared and the two skeletal elements were united across the joint line by primitive cartilage or precartilage tissue. The matrix of the latter was apparently undergoing liquefaction. He did not explain the cause of the liquefaction.

McDermott (1943) studied the human knee joint and concluded that the articular cavity developed by disappearance of the cells from the substance of the dense blastemal interchondral disc between the tibia and the femur, with coalescence of several primary spaces.

Haines (1947) studied the development of the joints of the human limbs from the time of their first definition to the time of the appearance of the synovial cavities. He found that the joints first appeared as interzones which were formed from the remains of the skeletal blastema between the cartilage and passed through a three-layered stage, with two chondrogenous layers and an intermediate loose layer, and a stage where the intermediate layer broke down and the chondrogenous layers became fully chondrified. Condensation in the extra-blastemal tissue near the joint developed as the fibrous capsule and cut off a part of the

general mesenchyme to form the synovial mesenchyme, and a part of the perichondrium to form the intracapsular perichondrium. He claimed that the cavities were formed partly from the interzone and partly by liquefaction at the synovial mesenchyme. He also mentioned that the fibula did not enter into the formation of the knee joint by articulation with the femur except for a brief period when the fibula and the tibia were one blastemal mass, before there was any evidence of a joint space.

Gray and Gardner (1950) studied the development of the human knee joint and found that, in a 6 week embryo, the tibial and femoral cartilages were separated from each other by a dense and avascular, unchondrified blastemal zone, which passed through a typical three-layered stage (two chondrogenous layers and an intermediate loose layer) at 7½ weeks. The tissue peripheral to the interzone differentiated into synovial mesenchyme. But they claimed that cavities first appeared in the synovial mesenchyme before they appeared in the interzone. These cavities were at first irregular in outline and frequently contained scattered cells and strands of tissue. They did not explain how the cavity was formed. They also showed that the fibula did not enter into the formation of the knee joint.

O'Rahilly (1951) studied the exclusion of the fibula from the human knee joint during development and concluded that, at an early stage, a three-layered interzone was formed between the femur and the fibula, but within a few

days, this interzone became invaded by tibial tissue. He claimed that when the tibia as a whole failed to develop, the femoro-fibular interzone may cavitate. He also reported that interzones took part in the chondrification process but were not an indication of joint differentiation since (a) they were not essential for the occurrence of joint differentiation and (b) at certain sites they did not lead to joint differentiation. Both these points were exemplified in the embryonic knee.

Haines (1953) studied the early development of the human knee joint and concluded that the interzones for the medial and lateral condylar regions were separate from the time of their first appearance, and were not formed by the subdivision of one originally single interzone. He also reported that before chondrification, the fibular and femoral regions of the skeletal blastema were continuous, but soon after chondrification, the fibula was found to be unattached to either the femur or the tibia and did not articulate with the femur at any stage, but eventually formed an interzone with the tibia.

Gardner and Gray (1953) studied the development of the shoulder and acromioclavicular joints in human embryos and reported that the development of the shoulder joint was basically similar to that of the knee, hip and elbow joints. At embryos between 12 and 17 mm, the unchondrified blastema between the humerus and scapula was slightly denser than the surrounding mesenchyme. Although

capillaries were located at the periphery, the interzone itself was avascular. It was continuous with, and similar in structure to, the perichondria of the humerus and scapula which it connected. At 20 mm, a three-layered interzone was formed for the first time by loosening of the middle part of the interzone. At 22 mm embryos, capillaries had begun to penetrate the capsular condensation and were beginning to convert the tissue immediately adjacent to the interzone into synovial mesenchyme.

Finally, in 25 mm embryos, cavities were present for the first time in both the anterior and posterior parts of the joint. They also mentioned that development of the acromioclavicular joint was different from that of the shoulder joint in several respects. A three-layered interzone was not seen at any stage of development and a cavity first appeared between the disc and acromion at 49 mm. This was supported by Andersen (1963), but he claimed that a joint cavity was first seen between the disc and acromion at 45 mm, while cavity formation between the disc and the clavicle did not appear until 68 mm.

Gardner et al. (1959) studied the prenatal development of the skeleton and joints of the human foot and concluded that ossification first appeared in the metatarsals and distal phalanges early in the fetal period, and later in the proximal and middle phalanges. They also reported that homogeneous interzones were present in the joints of the foot by stage 20 to 21 mm. Cavitation began in a few joints

during the embryonic period, but the formation of a three-layered interzone, followed by cavitation, had begun in most joints during the early fetal period. Capsules and ligaments were also present as cellular condensations before cavity formation and in some cases, before the appearance of a three-layered interzone.

Detailed formation of the joint interzone has been described by Andersen (1961, 1962a,b, 1963, 1964) and Andersen and Bro-Rasmussen (1961) who studied the histogenesis of the knee, elbow, shoulder and acromio-clavicular joints in human fetuses, using histochemical techniques. At the site of the future joint, there was in early stages the so-called interzone, inserted between the adjoining cartilages. The interzone was homogeneous, consisting of densely packed cells with round to oval nuclei and sparse cytoplasm. At this stage of development, there was no vascularization of the peripheral part of the interzone, but vessels from the surrounding general mesenchyme approximated to the peripheral areas of the interzone. Then the cells of the interzone began to secrete a highly metachromatic and Alcian blue-staining intercellular substance, which separated the cells, especially those in the central portion of the interzone.

These metachromatic intercellular substances were believed to be chondroitin sulphate A or C or possibly both, and not hyaluronic acid. Thus, a three-layered interzone resulted, comprising two chondrogenous layers,

each of which served as an appositional growth zone for the adjacent cartilage, and which was continuous at the periphery of the joint with the ordinary perichondrium. Between the chondrogenous layers there was an avascular, looser intermediate layer which was continued into a cellular, metachromatic tissue at the periphery. This tissue gradually became vascularized and was named the blastemal synovial mesenchyme. Finally cavitation was begun in the centre of the interzone and gradually spread to the periphery of the joint.

Schenck (1965) studied the prenatal development of the knee and ankle joints of the rabbit and found, in early development, that the bones of the knee and ankle joints passed through the typical mesenchymal, precartilaginous and cartilaginous blastemal stages with the formation of a characteristic interzone at the site of the developing joint. A linking condensation developed before the formation of the three-layered interzone. Cavitation began in the areas where there was much loose connective tissue between the more dense joint structures. He also added that the articular cartilage covering the articulating joint surfaces of the adult knee and ankle showed an arrangement of cell columns and collagen bundles perpendicular to the most superficial, tangential layers of the articular cartilage, a pattern which he thought might be produced by in utero fetal movements.

Gardner and O'Rahilly (1968) studied the early development of the knee joint in staged human embryos and

found that, at stage 20 (18-23 mm), a homogeneous interzone became defined between the skeletal elements, and the three-layered interzone was established at stage 21 (22-24 mm). At stage 22 (23-25 mm) all the intra-articular structures were identified, although cavitation did not occur until stage 23 (27-31 mm).

Rooket (1979) in a study on the embryological congruity of the human hip joint found that at around 20 mm the dense zone between the pelvic and femoral blastemas became interspersed by a middle portion which was lighter in appearance. This resulted in a three-layered interzone. The middle layer was directly continuous with the surrounding mesenchyme, while the two outer layers were continuous with the perichondrium of the pelvic and femoral blastemas. The fibrous capsule was first identified in 20-25 mm embryos and the joint cavity first appeared in the central part of the interzone at around 25 mm, and then extended around the head and down the neck of femur. These cavities were crossed initially by cellular strands, which were subsequently broken down, it was claimed, by early movement within the joint.

Mitrovic (1978) studied the development of diarthrodial joints in the rat embryo and found that, at the beginning of the joint development, the site of the future joint appeared as a closely packed, homogeneous, avascular cellular tissue. Cell degeneration was observed 12 hours after differentiation of the joint tissue, and it

was postulated that this early cell necrosis might account for the loosening of the central part of the articular mesenchyme leading to differentiation of a three-layered interzone. At the time of joint cavitation, degenerating cells were also seen in the peripheral parts of the developing articular cavity and in some cases cavitation was immediately preceded, or accompanied by, the appearance of cell degeneration. He also claimed that the synovial mesenchyme formed from the general mesenchyme by an invasion of blood vessels into the mesenchymal tissue at the peripheral parts of the joints. However, these blood vessels did not invade the inter-cartilaginous portion of the joint at any stage of the development.

Watson et al. (1986) studied the prenatal development of the composite occipito-atlanto-axial synovial joint cavity in the dog and found the three-layered interzone was formed in the composite occipito-atlanto-axial joint at 19-22 mm (30-31 days), joint cavities opened at 27-32 mm (33-34 days), and the atlanto-occipital and atlanto-axial cavities first communicated at 48 mm (37 days of gestational age).

b. Avian synovial joints

Hepburn (1889) in his study of the development of synovial joints in birds, noted that the essential stages were similar to those in the human embryo. (see page 3).

Fell and Canti (1934) studied the development of the avian knee joint in vitro and described its normal early

histological development. The rudiment of the limb first appeared when the leg bud was about 1 mm in length and was represented by a diffuse condensation of mesoderm in the proximal part of the bud. In a 1.6 mm limb bud the mass of condensed mesoderm became Y-shaped. The tail of the Y, which was proximal, was destined to form the femur and pelvis, while the two arms gave rise to the tibia and fibula. Distally the tibia and fibula were separated by loose vascular mesoderm, but proximally this tissue gradually merged with an elongated patch of more densely packed cells lying in the femur region. This elongated ("opaque") patch appeared opaque in fresh specimens and was found to consist of numbers of opaque, degenerate cells. Cartilage matrix was first seen in a limb about 2.8 mm in length. The rudiments of the tibia and fibula were still directly continuous with that of the femur, whilst the knee joint region still consisted of a dense mass of undifferentiated mesoderm.

The first sign of the future knee joint appeared in a 3 mm limb bud. The articular boundary of each bone rudiment became faintly indicated in the knee joint region by the flattening of the terminal mesoderm cells and their orientation along curves which were roughly parallel with the future articular surfaces. There was still, however, direct continuity between the densely cellular ends of the three rudiments.

In a 4.3 mm limb bud, cartilage matrix was

comparatively abundant in the middle third of the femur, tibia and fibula, and chondrification spread into the densely cellular tissue at the extreme ends of the three rudiments, and a very young and immature type of cartilage matrix extended right across the line of the joint, so that the articular surface of the femur was still united to the articular surface of the tibia and fibula.

In 6-day limb bud, cartilage was well developed throughout the epiphysial region except in the lightly cellular mesoderm of the joint region where chondrification was still at an early stage. This dense chondrifying interzone showed signs of transverse division and became differentiated into a thick layer of highly cellular early cartilage covering the articular surfaces. The narrow space between the articular surfaces was occupied by rather loose tissue, which later disappeared.

O'Rahilly and Gardner (1956) studied the development of the knee joint in staged chick embryos and found (1) correlation between the external staging of the chick embryo and the internal development of the knee joint. (2) A close similarity between the morphogenesis of the chick knee and that of the human, in contrast to the marked differences in osteogenesis.

They observed that, at stage 26, the early cartilaginous blastema was segmented into three portions (femur, tibia, fibula), thereby indicating the site of the future knee joint as an interzone or intercartilaginous blastemal disc. This interzone was a dense, cellular

region which, at first was uniform or homogeneous in structure; the nuclei were closely packed and uniformly distributed, without special orientation. At stage 27, chondrification had begun in the cartilage elements which accentuated the homogeneous interzones and extended partly across the interzones. Cavitation had begun at stage 34, between the medial condyle of the femur and the medial meniscus and between the lateral condyle of the femur and the lateral meniscus, without the prior development of a typical three-layered interzone.

Gardner and O'Rahilly (1962) studied the development of the elbow joint in staged chick embryos and found that the general morphogenesis of the elbow joint in the chick was similar to that of the human. At stage 28, a diffuse, avascular cellular interzone was present between the humerus and the radius, and between the humerus and the ulna which was continuous with the perichondrium. A few, small, scattered vessels were visible at the periphery of the interzone. At stage 29, by loosening of the central portions of the humero-radial and humero-ulnar parts of the interzone, a three-layered interzone was established and cavitation began to develop in all parts of the interzone from stage 35 through stage 37. A capsular condensation and early vascular synovial tissue deep to those condensations were observed first at stage 37.

Henrikson and Cohen (1965) studied the development of the interphalangeal joint in staged chick embryos, using

light and electron microscope and found that at stage 35, the presumptive joint interzone was a cellular condensation lying between the opposing phalangeal cartilages. By stage 37, the joint interzone was considerably more compressed and clearly delineated as an orthochromatically staining region between the metachromatic cartilages. At the peripheral portion, this compact interzone was continuous with synovial mesenchyme. The interzone occasionally could be resolved into three layers, two compact cell layers forming the presumptive articular surfaces (chondrogenous layers) and the intermediate layer between them.

In some joints, a band, metachromatic by azure B staining, could be discerned both in the intermediate part of the interzone and laterally in the synovial mesenchyme. Ultrastructurally this material consisted of (a) a sharply defined and sometimes stellate arrangement, of aperiodic fibrillar material; (b) a less well defined, moderately electron dense fluffy substance, whose relation to the adjoining cells could not be ascertained; and (c) a membrane-bound element, possibly representing cytoplasmic processes from adjacent cells. Cells with mitochondria and free ribosomes, and a well-developed Golgi area were occasionally found within this metachromatic band. It could not be determined whether this interzonal metachromasia always preceded joint space formation or occurred independently.

The definitive joint space was first seen in the interzone of stages 37-41 embryos as a narrow cleft either

between or lateral to the embryonic phalanges. This early cleft contained small amounts of collagen, thin cytoplasmic tendrils extending from lining cells and free cells without any apparent contact with the cleft border. No degenerative phenomena were noticed. They also found blood vessels in the intermediate layer of the interzone and thought that they might play a role in cavity formation.

Mitrovic (1977) studied the development of the metatarso-phalangeal joint of the chick embryo and observed that, at the beginning of the fifth embryonic day (stage 26-27), the joint existed as a closely packed, homogeneous, avascular cellular tissue. Within 6 to 12 hours the cells became oriented with their long axes parallel to the cartilaginous skeletal epiphysis. Capsular condensation appeared 6 to 12 hours after that of the central intercartilaginous part of the articular mesenchyme. At day 7, degenerating cells were noticed in the central part of the interzone and it was thought that these degenerative cells might serve one (or both) of two purposes: i) to prevent chondrification of the interzone by getting rid of cells with chondroblastic potentialities; and ii) to provide for loosening of the medial part of the interzone, leading to differentiation of a three-layered embryonic joint at the ninth embryonic day.

At the 10th embryonic day (stage 35-37), cavitation began in the periphery of the joint, which in some cases was preceded and/or accompanied by degenerative cells.

These cells were thought to be implicated in some way in the clefting process and later to constitute a surface cell layer of articular cartilage. These early cavities often, but not always, contained well preserved cells, erythrocytes and a filamentous and microfibrillar material. Using histochemical methods, he also found an organic component, presumably mucopolysaccharide, in the primitive synovial fluid which might also account for tissue cleavage at the sites of its accumulation.

Abu-Hijleh (1987) studied the development of the chick knee joint and found that at stage 27, chondrification had begun in the shafts of the three blastemata and the cells of the distal end of the femoral blastema and the proximal ends of the tibia and fibular blastemata gradually merged into the interzone region which appeared as an area of continuity between the three blastemata. This interzone was a dense cellular, avascular structure and did not chondrify at any stage. At stage 32-33, a typical three-layered interzone was first distinguishable between the lateral condyle of femur and fibula but the first signs of cavitation appeared first at stage 31, at the periphery, between the lateral condyle of femur and the lateral meniscus. Once cavitation was initiated, foci of cavity formation appeared at various new sites in the joint, which eventually coalesced to form a continuous cavity.

He also mentioned that blood vessels invaded the part of the general mesenchyme which was adjacent to the blastemal interzone, and converted it to synovial

mesenchyme.

Graig et al (1987) described the morphogenesis of the development of the chick metatarsophalangeal joint. At stage 26-27, the metatarsophalanx was seen as a densely packed homogenous avascular tissue. At stage 28, the presumptive joint region first became histologically identifiable as a cellular condensation lying between the opposing metatarsal and phalangeal cartilage elements. During stages 35 and 37, the three-layered interzone was well defined and cavitation had begun at the periphery of the joint interzone.

II. REVIEW OF SPECIAL ASPECTS OF DEVELOPMENT

A. Cavity formation

Based on various studies of the normal development of the synovial joints in mammals and birds, different mechanisms for the formation of the joint space have been suggested.

(a) Mammalian synovial joints

Whillis (1940) studied the development of the human and rat synovial joints and found that the two elements in the joint were united for a time by a primitive cartilage and this chondrification of the joint disc was attributed to the counter-pressure of the two growing cartilaginous elements. He suggested that the solution of continuity in the later stages of joint formation was accomplished by "liquefaction" of the matrix of the primitive cartilage uniting the two bones, but he did not explain the cause of the "liquefaction". He also suggested that movement alone did not cause breakdown of the bond of union between the two cartilage elements, but that it might play an ancillary part in the process.

McDermott (1943) studied the development of the knee joint in the human and concluded that the first articular cavity developed by the disappearance of cells from the substance of the dense blastemal interchondral disc between the tibia and the femur at the anterior and the posterior aspect. These changes appeared to be in the nature of a rapid and complete dissolution of the cells in the area and he did not find cells in different stages of

disintegration.

Haines (1947) studied the development of the synovial joints of the human and found that cavitation first appeared at the periphery within the synovial mesenchyme with the process of "liquefaction". The synovial surface was ragged and strands of tissue floated out from the surface into the synovial cavity. Some of the cells in the interior of the cavity appeared to be dying, and the synovial fluid contained cellular debris. The remains of the liquefied tissue of the interzone at first covered the articular surfaces of the cartilages, but soon were reduced to form a thin fibrillar layer containing flattened cells, some of them pyknotic or reduced to debris, and before birth all the flattened cells disappeared.

Gray and Gardner (1950) studied the development of the human knee joint and found that the joint cavities first appeared at the peripheral part of the interzone within the synovial mesenchyme and then extended towards the middle of the interzone. They also described that, at 8 weeks embryo, a small space was present between the medial head of the gastrocnemius muscle and the femur and another in the infrapatellar region. At 9 weeks, definite and extensive cavities were present, mainly femoro-patellar and femoro-meniscal in location. However, they did not explain how the joint cavities were first developed.

Andersen and Bro-Rasmussen (1961) in their histochemical and histological studies of the joint in

human fetuses, disagreed with Whillis (1940), McDermott (1943) and Haines (1947), and said that the joint cavities started centrally in the intermediate layer of the interzone and gradually spread to the periphery of the joint. These cavities developed through the accumulation of fairly large quantities of chondroitin sulphates A or C (or possibly both) in the interzone, and were not preceded by any sign of "liquefaction" or of degenerative cells. These results were in disagreement with Schneck (1965) who studied the prenatal development of the knee and ankle joints of the rabbit and said that the early cavitation could be reliably ascertained only in areas where there was much loose connective tissue between the denser joint structures, which confirmed the findings of Whillis (1940), McDermott (1943) and Haines (1947).

Frommer (1964) studied the prenatal development of the mandibular joint in mice and concluded that the joint cavity was preceded and accompanied by an increased vascularity within the interzone.

O'Rahilly and Gardner (1978), in their review of the embryology of movable joints, quoted Wassilev's (1972) finding that the formation of the joint cavity was preceded by degenerative changes of mesenchymal cells in the central part of the interzone.

Rooket (1979) studied the embryology of the human hip joint and concluded that the joint cavity first appeared in the central part of the interzone at around 25 mm, due to the cell degeneration and the appearance of the movement

within the joint. These cavities extended around the head and down the neck of the femur and initially were crossed by cellular strands, which were subsequently broken down by early movement within the joint. He did not explain the detail of the cavity formation.

(b) Avian synovial joints

O'Rahilly and Gardner (1956) studied the development of the knee joint in staged chick embryos and found that cavitation had begun at stage 34, between the medial condyle of the femur and the medial meniscus and between the lateral condyle of the femur and the lateral meniscus, without a preceding typical three-layered interzone. Degenerative cells were also found within the interzone of only two embryos at stage 34 (out of a total of 122 embryos at various stages). They did not explain the significance of these degenerative cells nor the mechanisms of the cavity formation.

Henrikson and Cohen (1965) studied the development of the interphalangeal joint in staged chick embryos and found that at stages 37-41, a narrow cleft either between or lateral to the embryonic phalanges first appeared. This early cleft contained small amounts of collagen, thin cytoplasmic tendrils extending from lining cells and free cells without any apparent contact with the cleft border. No degenerative phenomena or signs of liquefaction were noticed. They also found blood vessels in the intermediate layer of the interzone and thought that they might play a

role in cavity formation.

Mitrovic (1977) studied the histogenesis and mechanism of joint clefting of the developing chick embryo up to the 15th day of incubation and found that a "first wave" of cell degeneration appeared 24 hours after differentiation of the joint tissue. He thought that this early cell necrosis might account for the loosening of the medial part of the interzone, leading to differentiation of a three-layered interzone. A "second wave" of cell degeneration was seen in the peripheral parts of the developing articular cavity. They were elongated, basophilic and electron-opaque cells which were closely arranged along the zone of tissue cleavage. He suggested that some of these dying cells rapidly disintegrated and were phagocytosed by the surrounding cells, and that after the joint cavity was fully differentiated, some of them remained to form a discontinuous cover for the articular surfaces. In addition to these observations, clear morphological and histoautoradiographic evidence was found for the presence of an organic component presumably mucopolysaccharide, in the primitive synovial fluid. Fluid secretion was also thought to account for tissue cleavage at the sites of its accumulation.

Mitrovic (1978) studied the development of the synovial joint in the rat and found the same result; and in his 1982 study on paralysed chick embryos, he claimed that intrinsic factors such as cell death might have played some

role in the cavitation process and that extrinsic factors, such as muscular movement, were important for the maintenance of the joint cavity.

Abu-Hijleh (1987) studied the development of the knee joint in the staged chick embryos and found within the interzone degenerative cells which were considered to represent apoptotic (programmed) cell death. The distribution and identity of apoptotic cell death in the interzone at each stage were correlated with time and the site of the first appearance of the joint cavities; signs of the cell death always preceded signs of the cavitation, (except in the femoro-patellar joint), and accompanied the process of cavitation, up to stage 37, when cavitation was complete. He also claimed that these cell deaths in the interzone were responsible for sculpturing of the articular surfaces and the intra-articular structures. He did not find any degenerative cells during cavitation in the femoro-patellar joint, which he therefore regarded as atypical.

Graig et al. (1987) studied the development of the chick metatarsophalangeal joint, using the specific antibodies to collagen I and II and keratan sulfate-containing proteoglycans (KSPG) and found that, at stage 28, collagen II and KSPG, were seen throughout the cartilage anlage matrix and across the presumptive joint region. It was proposed that a decrease in KSPG in the presumptive joint region at stages 28 and 30 might be involved in the mechanism for flattening of cells in the

formation of the interzone whereas a decrease in collagen II across the joint interzone region might provide an area of weakness, which might allow the production of cavitation through movements produced by the developing musculature.

B. **Development of the intra-articular structures**

The development of the intra-articular structures and fibrous capsule has been investigated in synovial joints of mammals and birds by several authors.

(a) Mammalian synovial joints

Some authors have claimed that intra-articular structures arose from the interzone e.g. Hepburn (1889), Bardeen (1905), McDermott (1943), Andersen (1961), Schenck (1965), Mitrovic (1978) and Rooket (1979). Others found that the intra-articular structures arose from the synovial mesenchyme e.g. Haines (1947), Gray and Gardner (1950).

Hepburn (1889) studied the development of the synovial joints in mammals and concluded that the circumference of the articular disc developed into the capsule of the joint and the intra-articular fibro-cartilages and ligaments were derived from the articular disc as a result of the modifications of the joint cavity.

Bardeen (1905) studied the development of the human skeleton and found, in embryos of about 20 mm, the tissue immediately surrounding the cartilages became greatly condensed into a definite perichondrium. The peripheral blastemal tissue of the joints became transformed into a capsular ligament, strengthened in front by the tendon of the quadriceps. Within the joint most of the tissue began to show signs of becoming less dense, but the menisci and the cruciate ligaments, like the ligaments of the capsule, were differentiated directly from the blastema.

McDermott (1943) studied the human knee joint and concluded that the menisci and the intra-articular ligaments, as well as the capsule, were differentiated directly from the blastema, which remained after the disappearance of cells resulting in the formation of the joint space.

Andersen (1961) also studied the human knee joint and found that at 23 mm, the intra-articular structures were developed in situ as a cellular condensation in the interzone.

Schenck (1965) studied the development of the knee and ankle joint of the rabbit and concluded that intra-articular structures were derived from the interzone.

Mitrovic (1978), in the rat, found that the peri-articular ligaments, capsular and synovial tissue, differentiated from the general mesenchyme that surrounded the joint interzone. But the glenoid processes, menisci and intra-articular ligaments differentiated directly from the cells that constituted the interzone.

Rooket (1979) in the human hip joint, found that ligamentum teres and the transverse acetabular ligament developed in situ and appeared to originate from the interzone.

On the other hand, Haines (1947) studying development of the human joints, concluded that the development of the fibrous capsules as condensations in the extra blastemal tissue near the joints, cut off a part of the general

mesenchyme to form the synovial mesenchyme, and a part of the perichondrium to form the intra-capsular perichondrium. The synovial mesenchyme gave rise to the more central parts of the synovial cavities, to the synovial and sulci-synovial tissues and to all intra-capsular structures, including ligaments, tendons and fibro-cartilages.

Gray and Gardner (1950) studied the prenatal development of the human knee joint and found that as the joint increased in size, and before a fibrous capsule was present, adjacent mesenchyme became intra-articular in position. The menisci and cruciate ligaments arose from the characteristically vascular synovial mesenchyme and appeared first at about 8 weeks of development. These structures appeared early, before cavity formation was present.

(b) Avian synovial joints

Hepburn (1889) studied the development of intraarticular structures in birds and found the same results as he had described in mammalian synovial joints.

O'Rahilly and Gardner (1956) studied the development of the knee joints in the chick embryo and concluded that extra blastemal tissue, the "synovial mesenchyme", became incorporated in the joint. This was generally regarded as the source of most of the intra-articular structures, including synovial tissue, cruciate ligaments, and menisci.

Abu-Hijleh (1987) studied the development of the knee joint in the chick embryo and concluded that the intra-

articular structures developed in situ from the middle layer of the interzone, which was in disagreement with O'Rahilly and Gardner (1956).

C. **Development of the synovial mesenchyme**

Haines (1947), Gray and Gardner (1950), Gardner and Gray (1953), O'Rahilly (1957), Mashuga (1964), Schenck (1965), Gardner and O'Rahilly (1968), Mitrovic (1978) and Abu-Hijleh (1987) have claimed that the synovial mesenchyme was formed from the part of the general mesenchyme at the periphery of the joint which was separated from the surrounding mesenchyme by dense cellular fibrous capsule. Mashuga (1964) also mentioned that differentiation of the synovial membrane from the synovial mesenchyme depended upon the appearance of the movement in the joint, and the intensity of vascularization of the synovial membrane grew with the increase of the dynamic load on the joint, and diminished in response to the conditions of partial or full immobilization of the joint. He also claimed that the synovial villi were not found in less mobile animals (amphibians, turtles). Several authors believed that the synovial mesenchyme was blastemal in origin. Andersen (1961, 1962, 1963, 1964) claimed that the synovial mesenchyme was laid down as the peripheral part of the interzone and was derived like the latter from the original skeletal blastema, and during further development was the only part of the interzone which became highly vascularized. Chondroitin sulphate A or C (possibly both)

but not hyaluronic acid appeared in the synovial tissue even before the vascularization. After vascularization, there were also PAS-positive substances in the intercellular substance of the synovial tissue. This vascular synovial tissue later was invaded by numerous histiocytes, whose origin he could not explain. He also observed mast cells within the synovial mesenchyme.

Wasilev (1972) quoted by O'Rahilly and Gardner (1978), in an electron microscopic and histochemical study of the development of the knee joint of the rat found that the synovial mesenchyme was blastemal in origin which was in agreement with Andersen (1961, 1962, 1963, 1964). He also found that, after the cavity formation, acid phosphatase, B glucuronidase, and ATPase were present in the synovial lining cells.

D. **Development of the blood vessels within the interzone**

Several authors such as Haines (1947), Gray and Gardner (1950), O'Rahilly (1957), Andersen (1961, 1962, 1963, 1964), Andersen and Bro-Rasmussen (1961), Mitrovic (1978) and Watson *et al*. (1986) in their studies on mammalian joints, O'Rahilly and Gardner (1956) and Abu-Hijleh (1987) in their studies on avian joints, observed invasion of the blood vessels into the periphery of the joint but not within the joint interzone, whereas some authors have considered that vessels were present within the interzone during development.

Frommer (1964) in the mandibular joint of mice observed blood vessels at the site of the future joint cavity and concluded that the process of cavitation was preceded and accompanied by an increased vascularity.

Henrikson and Cohen (1965), in the chick interphalangeal joint, found blood vessels within the interzone. They suggested that even if vessels do not play a direct role in synovial space formation, their presence may be necessary, in some as yet undefined fashion, for the initiation and sustenance of the articular space.

Mitrovic (1977) in the metatarsophalangeal joint of the chick embryo, observed invasion of the blood vessels within the interzone. These blood vessels were lined by a single continuous layer of endothelial cells and lacked basement membranes. On a few occasions the endothelium was fenestrated. Erythrocytes were frequently seen crossing the capillary walls by diapedesis. He mentioned that these blood vessels and free erythrocytes might be associated either with cell degeneration or the process of cavitation.

E. Development of the patella and the femoro-patellar joint

There are still controversial reports concerning the development of the patella and the femoro-patellar joint by various authors who have studied these structures in mammals and birds.

1. Development of the patella

(a) Mammalian synovial joints

Some authors e.g. Brooke (1937), Andersen (1961), Schenck (1965), and Doskocil (1985) claimed that the patella was developed independently of the quadriceps tendon, and that a secondary invasion of the tendon took place later. Other authors have considered the patella as an intra-tendinous sesamoid and claimed that the patella developed initially within the tendon, e.g. Bardeen (1905), Walmsley (1940) and Haines (1947).

Brooke (1937) studied the morphology and function of the patella and described that the patella developed behind the quadriceps tendon and independently of it. He also maintained that function also played no part either in its formation or its growth.

Andersen (1961) studied the histogenesis of the knee joint in the human and concluded that the primordium of the patella developed from the blastema, like the other bones of the knee joint, and appeared at stage 23 mm behind the extensor tendon which formed first, and that secondary invasion of the tendon took place later. He also found that the patellar primordium was separated from the femur by a typical three-layered interzone. Schenck (1965) studied the knee joint of the rabbit and found that the precartilaginous condensation of the patella developed behind the dense cellular condensation of the quadriceps tendon and patellar ligament. Posteriorly the patella was separated from the femur by a typical three-layered

interzone.

More recently Doskocil (1985) studied the formation of the human femoro-patellar joint and found that the anlage of the patella was associated at the very beginning of its development with the blastema of the lower end of the femur and separated from it at the early prechondral blastema stage. From the early stage, the primordium of the tendon of the rectus femoris ran over the anterior surface of the anlage of the patella. The patellar ligament also started from the anterior surface of the patella. The tendons of the vastus medialis and lateralis were inserted in connective tissue discs on either side of the patella, but the tendon of the vastus intermedius was inserted in the upper edge of the patella. In view of these findings, he concluded that the patella was not a sesamoid bone.

On the other hand, Bardeen (1905) studied the human knee joint and claimed that as the musculature became differentiated around the knee joint, a dense tendon for the quadriceps was formed in front of the knee joint and the patella became differentiated in it.

Walmsley (1940) studied the development of the human patella, and found that in the 20 mm embryo, a precartilaginous patella first appeared in the deeper part of the quadriceps mass at the level of the lower end of the femur. At 30 mm, the patella became cartilaginous and interrupted the continuity of the quadriceps mass except for a superficial layer which remained undifferentiated.

At 35-40 mm a typical articular disc was secondarily formed between the patella and the femoral condyles by the fusion of the patellar and femoral perichondria. This result was in agreement with Haines (1947) who studied the development of the synovial joint in the human and found that at 13 mm, the quadriceps passed to the tibia as a band of dense tissue quite distinct from the femur, and separated from it by a layer of loose uncondensed tissue. At 20 mm, the patella was differentiated as an aggregation of round cells in the quadriceps mass, and at 23 mm, it was more distinct.

(b) Avian synovial joints

Niven (1933) studied the development of the avian patella in vivo, and found that it was formed by mesenchyme in front of the femur, isolated from the surrounding tissue at the beginning of the 11th day. Patellar chondrogenesis began between the 11th and 12th days, and it was surrounded by a perichondrium at the end of the 12th day. O'Rahilly and Gardner (1956) studied the knee joint in the staged chick embryo and found that the ligamentum patellae formed before the patella and that the future patellar area was at first occupied by loose mesenchymal tissue, which subsequently appeared condensed in the location of the future patella. This condensation could be recognised at about the time of initial ossification of the long bones (stages 29-30) and prior to the onset of joint cavitation. The primordium of the patella, from the time of its appearance, was separated from the femur by loose vascular

tissue. At stage 36, the patella became chondrified. They suggested that the patella was a peri-articular rather than an intratendinous sesamoid bone.

More recently Abu-Hijleh (1987) studied the development of the knee joint in the chick embryo and found that the quadriceps tendon was inserted into the upper border of the patella, and the patellar ligament began from the anterior surface of the patellar anlage. He also found the patella was separated from the lower end of the femur by loose vascular mesenchymal tissue from the outset, and until cavity formation began. In view of these findings, he suggested that the patella was not an intra-tendinous sesamoid bone.

2. The development of the femoro-patellar joint

(a) Mammalian synovial joints

According to some authors, the patella was separated from the femur at the outset by much loose tissue (e.g. Haines, 1947; Gray and Gardner, 1950; Doskocil, 1985).

Others have found that the patella and femur were separated by a typical interzone (e.g. Walmsley, 1940; Andersen, 1961; Schenck, 1965).

Haines (1947) studied the development of the human joints, and found that the femoro-patellar joint differed from the more typical joints in that no dense interzone was found at any stage, so that the loose tissue which separated the femur from the patella at 32 mm, persisted as such from an early stage of development.

Gray and Gardner (1950) studied the development of the human knee joint and found that the patellar condensation appeared in front of the femur at 7.5 weeks and separated from the femur by loose mesenchymal tissue until cavity formation began, and the joint did not form in blastemal tissue nor did a typical interzone arise. The femoro-patellar joint cavity began to develop at the time when the patella became cartilaginous, at 10 weeks. They did not give any suggestion about the relation between the patella and the quadriceps tendon.

Doskocil (1985) studied the development of the femoro-patellar joint and found that the patella was separated from the femur by loose mesenchymal tissue. The joint cavity first developed by the expansion of the

extracellular spaces and destruction of the cells of the loose mesenchymal tissue between the patella and the femur.

On the other hand, Walmsley (1940) found that a typical articular disc was secondarily formed at stage 35-40 mm between the patella and the femoral condyles by the fusion of the patellar and femoral perichondria. The femoro-patellar synovial cavities developed in the articular disc in a manner typical of the diarthroses. These cavities first appeared on the medial side and then on the lateral side, probably reflecting the phylogenetically greater importance of the medial femoral condyle; they were completed by about stage 70 mm.

Andersen (1961) found that the interzone between the patella and femur passed through a typical three-layered stage and a cavity appeared within the interzone by the formation of large quantities of chondroitin sulphates A and C.

Schenck (1965) found that the femoro-patellar interzone passed through a typical three-layered stage and that the cavity was formed by loosening of the interzone.

(b) Avian synovial joints

O'Rahilly and Gardner (1956) found that the patella was separated from the femur by very loose and vascular tissue from the outset. The cavity was formed as an extension of the knee joint cavity at stage 36.

Abu-Hijleh (1987) found that the femoro-patellar cavity first appeared in the loose vascular mesenchymal tissue

between the femur and the patella at stage 36. He did not find a typical interzone between the patella and the femur, nor any sign of cell degeneration during cavitation.

F. **Development of the fat pad within the synovium**

(a) Mammalian synovial joints

McDermott (1943) studied the development of the human knee joint and found that, at a later stage of development - about 29 to 30 weeks - adipose tissue first appeared in the area of the future infra-patellar fat pad.

Gardner and O'Rahilly (1959), in their studies on the development of the human foot joints, found that the synovial membrane began to develop by the appearance of the capillaries in the loose mesenchymal tissue at the periphery of the joint interzone by the end of the embryonic period, but from 50 mm onwards, blood vessels were very numerous, at the periphery of certain joints especially the ankle, in the areas of future fatpad and synovial folds. They also found that folds were present at 60 mm. Synovial villi began to form and were fairly numerous from 83-85 mm, and fat had begun to develop at 106 mm.

Andersen (1961) studied the human knee joint and found the different types of cells within the synovial mesenchyme: 1) "Synovialocytes" in various stages of differentiation; 2) histiocytes; 3) mast cells; 4) fat cells; 5) the cellular components of the vessels of which the endothelial cells appeared to exert particular

metabolic activity as far as the terminal arterioles were concerned, and lastly, 6) sometimes primitive white blood cells. He also found a small accumulation of fat cells first appeared in the infra-patellar tissue at 145 mm crown-rump length, while there were scattered, delicate, collagenous fibres in the infra-patellar tissue around 80 mm.

(b) Avian synovial joints

O'Rahilly and Gardner (1956) studied the development of the knee joint in staged chick embryos and observed that the adipose tissue first appeared in the infra-patellar tissue and in the subsynovial tissue at the posterior part of the joint at stage 44 (18 days), but they gave no details. The detailed structure of the subsynovial adipose cells of the ankle joint in the chick after hatching was studied by Luckenbill and Cohen (1966) who found that they contained lipid droplets which stained with oil red 0 and were arranged in large, discrete, but contiguous globules. At the lipid droplet-cytoplasm interface a meshwork of fine filaments (7 to 10 nm wide) within an interfilamentous spacing of approximately 10 to 40 nm was observed. They claimed that such an association of lipid with a cytoplasmic filamentous meshwork had not been described in studies of mammalian fat depots. These adipose cells were also distinguished from the glandular adipose cells of mammalian brown fat by the same accumulation of large, contiguous lipid globules and the absence of numerous mitochondria.

G. **Comparative sequential development of chick knee and metatarsophalangeal joints** (according to previous investigators).

(a) Chick knee joint:

Structures	O'Rahilly & Gardner (1956) Stage	Abu-Hijleh (1987) Stage
Homogeneous interzone	26	27
Skeletal chondrification	27	27
Quadriceps femoris muscle	27	27
Tibialis anterior tendon	27	29
Ambiens tendon	29	30
Three-layered interzone	?	32-33
Patellar condensation	29-30	30 (not localised)
Diaphyseal ossification	28-29	30
Menisci	30	30
Cruciate ligaments	30	28
Embryonic movement	30	?
Foci of apoptosis localized between		
L.C.F. and L.M.	"pyknotic cells" found in 2/125 embryos	30-37
L.C.F. and fibula		31-37
L.C.T. and L.M.		31-36
M.C.F. and M.M.		31-36
M.C.T. and M.M.		31-36
Cavitation:		
L.C.F and L.M.	femoro-meniscal at stage 34	31 anterior
L.C.F. and fibula		34 post
L.C.T. and L.M.		33 post
M.C.F. and M.M.		32 post
M.C.F. and M.M.		33 post
Patellar peri-chondrium	36	35
Chondrification in patella	36	36
Synovial tissue	36	36
Synovial folds and villi	37	37
Cartilage canals	35 femur 36 tibia	35 femur 36 tibia
Infra-patellar fatpad	44	?

Embryos of both investigations have been staged according to Hamburger and Hamilton (1951).

L.C.F. = Lateral condyle of femur.

L.C.T. = Lateral condyle of tibia.

L.M. = Lateral meniscus.

M.C.F. = Medial condyle of femur

M.C.T. = Medial condyle of tibia.

M.M. = Medial meniscus.

(b) Chick metatarsophalangeal joint:

	Mitrovic (1977)	Graig et al.(1987)
Structures	Stage	Stage
Homogeneous interzone	26-27	26-27
Three-layered interzone	34	35-37
Degenerative cell death	30-31 first wave 34 second wave	?
Cavitation	35-37	35-37

All staging according to Hamburger and Hamilton (1951).

III. THE PHENOMENON OF CELL DEATH

A. General

Cell death has been classified by Wyllie et al. (1980, 1985) in two categories: necrosis (or physiological) and apoptosis (programmed cell death).

Necrosis can be identified by swelling of all compartments of the cell, following the rupture of membranes and destruction of organelles. It results classically from injury by agents such as toxins and ischaemia, affects cells in groups rather than singly, and evokes exudative inflammation when it develops in vivo. In contrast, apoptosis is characterized by condensation of the cell, with maintenance of the integrity of organelles and the formation of surface protuberances that separate as membrane-bound globules; in tissue, these are phagocytosed and digested by neighbouring cells, there being no associated inflammation.

Although the distinction between necrosis and apoptosis is a real one, and has proved valuable in our understanding of events both in normal development and in pathology, it is not an absolute distinction, because, for example:

(1) although the immediate cause of apoptosis seems to be endogenous (i.e. genetically determined), in many situations, to be described later, an external stimulus is involved.

(2) both processes show some changes in common e.g. the phagocytosis of dead or dying cells, and their digestion by lysosomal enzymes.

1. Necrosis

Wyllie et al. (1980, 1985) in their comprehensive review of cell death, described the morphology, mechanism and incidence of cell necrosis.

(a) Morphological changes: Early abnormalities, which are compatible either with recovery of an injured cell or with progression to necrosis, include marginal clumping of loosely textured nuclear chromatin, dilatation of the endoplasmic reticulum and mild dispersal of ribosomes. The subsequent evolution is accompanied by rupture of nuclear, organelle and plasma membranes, the appearance of matrix densities in mitochondria, and the dissolution of ribosomes and lysosomes; as the nucleus swells, the masses of clumped chromatin may become slightly dispersed, but they soon disappear altogether. The chromatin often initially appears fairly uniformly compacted (pyknosis), but with swelling of the nucleus and rupture of its membrane, the marginated chromatin masses may become evident as small discrete masses (karyorrhexis). All basophilia is then lost leaving a faintly stained nucleus (karyolysis). The swollen cytoplasm also loses its basophilia, and cell boundaries become indistinct. Typically a number of contiguous cells are affected, and exudative inflammation develops in the adjoining viable tissue. The debris is eventually ingested and degraded by

specialized phagocytic cells.

(b) Mechanism of cell necrosis: Different types of injury may produce an increase in cell membrane permeability, through which intracellular constituents escape, and normally excluded extracellular molecules enter.

At an early stage of dying, there is potassium loss and sodium entry, due to failure of the plasma membrane ATP-dependent sodium-potassium pump. The calcium entry is the more significant, as it is known to inhibit the membrane sodium-potassium ATPase, and thus calcium in abnormally high concentration might abruptly amplify the intracellular sodium gain and potassium loss. It appears more likely however that the primary site of action of the incoming calcium is on membrane lipids, activating phospholipase and thus initiating the dissolution of membranes which is observed morphologically.

(c) Incidence of necrosis: The occurrence of necrosis is invariably associated with a gross departure from physiological conditions such as severe hypoxia and ischaemia, major changes in environmental temperature and exposure to toxins.

2. Apoptosis

Physiological cell death, synonomous with the term programmed cell death, is a phenomenon which has been recognised since the early days of embryology but which was first emphasised by Glucksmann (1951) and described as

shrinkage necrosis by Kerr (1971) and finally called apoptosis by Kerr et al. (1972).

Glucksmann (1951) has reviewed the many examples of morphogenesis in which cell death is involved. He reported that cell death occurs regularly at certain developmental stages of all vertebrate embryos. He suggested a classification of normal cell death as follows:

i) Morphogenetic degeneration precedes changes in the form of epithelial organs, e.g. during the invagination of the optic cup, the formation of the crystalline lens, the olfactory pit, the neural tube, etc. They bring about the separation of rudiments such as that of the neural tube and the lens from the ectoderm. They reduce the excessive thickening of uniting edges such as those of the body wall and of the palatal folds.

ii) Histogenetic degenerations are related to the differentiation of tissues and organs e.g. the differentiation of the three cell layers of the frog tadpole retina, for instance, is accompanied by three waves of degeneration. Similar cell death of early neuroblasts is found in the spinal ganglia outside the limb regions. Sex differentiation of the individual involves the partial degeneration of the Mullerian or of the Wolffian ducts.

iii) Phylogenetic cell death is of two types: those which represent a vestigial organ such as the paraphysis or the second muscle stage in higher vertebrates, and those concerned with the regression of larval structures such as the conjunctival papilla, parts of the ganglia of branchial

nerves, of the pronephros and mesonephros.

Glucksmann (1951) also said that the process of dying in an individual cell might take from less than 1 hour to about 7 hours, when only a small proportion of a living tissue dies, but may be prolonged to days when numerous cells die simultaneously and their resorption is delayed, and the number of dead cells varies in individuals of approximately the same stage.

In addition to the occurrence of apoptosis during normal development, many workers quoted by Wyllie et al. (1980) described apoptosis as occurring in a number of healthy mature mammalian tissues, e.g. lymphoid germinal centres (Swartzendrucher and Congdon, 1963), thymic cortex (Lundin and Schelin, 1965; Van Haelst, 1967a), liver (Kerr, 1965, 1971), adrenal cortex (Wyllie et al., 1973a), prostate (Kerr and Searle, 1973), and small gut crypts (Searle et al., 1975; Potten, 1977). More recently Potten et al. (1988) found that apoptotic cells in the normal human breast were less clearly influenced by the stage of menstrual cycle but showed a significant decline with age.

As many authors e.g. Walker et al. (1988), Searle et al. (1975) and Zaretskaia (1988) have reported, apoptosis occurs spontaneously in growing neoplasms, and is increased in both neoplasms and rapidly proliferating normal cell populations by certain radiomimetic cancer-chemotherapeutic agents and ionizing radiation.

Walker et al. (1988) examined colonic biopsies from patients with melanosis coli and found that apoptotic bodies in the surface epithelium and lamina propria were increased.

Searle et al. (1975) observed that typical apoptosis took place continuously in the lining of the small intestinal crypts of normal mice, and in untreated mouse ascites tumours. Injection of the cancer-chemotherapeutic agents actinomycin D, mitomycin C, cytosine arabinoside and cycloheximide massively enhanced the rate of apoptosis.

Zaretskaia (1988) studied apoptosis of rectal cancer cells before and after irradiation and found that after irradiation, the number of apoptotic cells in the tumour tends to increase whereas morphologic patterns of apoptosis remain unchanged.

Several authors e.g. Kerr (1971), Shrek etal (1980), Walker (1987) and Savill et al. (1989) also found that apoptosis can be produced by an external minor stimuli and inflammation.

Kerr (1971) found that careful ligation of the hepatic artery could produce striking atrophy of the liver, with much apoptosis, while rapid occlusion of the blood supply produced hepatic cell necrosis. Shrek et al. (1980) also found that exposure to temperatures between 37^{o} and $43^{o}C$ produced apoptotic degeneration of lymphocytes, while higher temperatures produced necrotic degeneration.

Walker (1987) observed that after experimental duct ligation of the rat pancreas, acinar cells disappeared from

the distal gland within 5 days. Necrosis was responsible for minor cell loss during the first 24 hours, but most of the acinar cells were deleted by apoptosis. Wyllie et al. (1980, 1985) described the morphology, mechanism and cause of apoptosis, in their comprehensive reviews of cell death.

The main differences between necrosis and apoptosis are given in the following Table (1) from Wyllie et al. (1980).

Table 1: Comparison of morphological features of necrosis and apoptosis.

Feature	Necrosis	Apoptosis
Histological appearances	Usually affects tracts of contiguous cells.	Characteristically affects scattered individual cells.
	Eosinophilic "ghosting" of entire cells.	Affected cell represented by one or more roughly spherical cytoplasmic masses, some also containing basophilic particles of condensed chromatin They lie both in the intercellular space and within tissue cells.
	Exudative inflammation usually present.	Exudative inflammation absent.
Ultrastructural changes.		
Chromatin	Marginates in small, loosely textured aggregates; disappears eventually, when nuclear membrane destroyed.	Marginates in condensed, coarsely granular aggregates confluent over entire nucleus or localized to large crescentic caps.
Nucleolus	Evident as compact body until cytoplasm degradation advanced.	Disperses to shower of granules while cytoplasm structurally intact.
Nuclear membrane	Retains pore structures until cytoplasmic degradation advanced; eventually destroyed with other organelles.	Progressive convolution; resulting protuberances eventually separate. Pores retained adjacent to euchromatin, but lost next condensed chromatin. Eventually becomes discontinuous, so that dense chromatin masses lie among cytoplasmic organelles.

Table 1 (Contd).

Feature	Necrosis	Apoptosis
Cytoplasm	Swelling of all compartments followed by rupture of membranes and destruction of organelles. Mitochondrial matrix densities characteristic.	Condensation of cytosol (endoplasmic reticulum may dilate focally); structurally intact mitochondria and other organelles compacted together, protuberances from cell surface separate to form apoptotic bodies.

B. Cell death during embryonic development

Embryonic cell death occurs in the 5h post coitum mouse blastocyst on the surface of the inner cell mass or trophoblast cells and in the adjacent uterine epithelium, which appeared to be phagocytosed by trophoblast cells following loss of the zona pellucida (El-Shershaby and Hinchliffe, 1974).

Cell death has been found before and during the gastrulation process in the chick embryo, when some yolk endodermal cells degenerate. Dead cells are also found during the transformation of the neural tube, first into a groove and then into a tube, and in the course of differentiation of the retina and lens (Glucksmann, 1965). These are all cases where evagination or invagination is taking place. Another situation is where openings are temporarily closed by a solid mass of epithelial cells, e.g. in the formation of olfactory organs, or by membranes in the case of the pharynx and cloaca. Here, the cells blocking these openings break down by degeneration.

Dead cells are also found in the visceromotor neurons of the cervical region of the spinal cord (O'Connor and Wyttenbach, 1974) and in the spinal ganglia of the chick embryo (Pannese et al., 1976).

Dead cells have been found in the branchial arches of humans, rat and chick embryos (Menkes et al., 1965). By using vital staining with Nile blue, they identified dead cells undergoing phagocytosis in these regions. They concluded that these areas of physiological necrosis were

likely to play an important part in the normal morphogenesis of the cervical and cephalic regions.

Extensive dead cells were also observed in the epithelium of the palatine processes, following palatal fusion in the mouse embryos, by Farbman (1968) and in the human fetus, by Matthiessen and Andersen (1972). Dead epithelial cells were phagocytosed by neighbouring epithelial cells in the fusion line and by invading connective tissue cells.

Behnke (1963) found extensive cell death in the duodenal mucosa of the rat fetus during formation of the villi, mainly in the cells bordering the lumen, when stratified epithelium was transformed into a single layer of columnar cells.

Garcia-Porrero and Ojeda (1979) found physiological cell death during the early development of the retina in the chick embryo, and they observed that these cells were eliminated by phagocytosis.

Manasek (1969) found myocardial cell death in the embryonic chick ventricle and described how, following loss of intercellular junctions, the degenerating cells rounded up and were expelled into the large intercellular spaces. Disrupted myofibrils and large pools of glycogen remained visible in the increasingly electron-dense cytoplasm of these degenerating cells. Later on, dead muscle cells were engulfed by macrophages.

Ojead and Hurle (1975) studied the heart of the chick

embryo between stages 9 and 11 and found physiological cell death in the midline of endocardial tubes during the process of fusion of the two heart anlagen and suggested that cell death played an important role in the elimination of the medial septum.

Cell death in limb morphogenesis

Cell death also occurs at early stages of limb development, in the following areas:

(a) Apical ectodermal ridge - AER.

(b) Interdigital areas.

(c) In the anterior and posterior areas between the limb buds and the body wall (posterior necrotic zone - PNZ, anterior necrotic zone - ANZ).

(d) In the joint interzone.

(a) Apical ectodermal ridge - AER

Cell death has been observed in the chick AER by several authors e.g. Zwilling (1961), Jurand (1965), Hinchliffe and Ede (1967) and Todt and Fallon (1984, 1986).

Zwilling (1961) observed degenerating cells in the ectodermal ridge, especially at the pre- and postaxial ends which were becoming flat. He claimed that cell death was one feature of ridge regression which occurred in regions where there had been a cessation of outgrowth.

Recently, dead cells have been observed in AER by Todt and Fallon (1984, 1986) in both leg and wing buds of chick embryo. They found that ectodermal cell death was not evident until stage 20 in the leg bud ridge, but could be

seen at late stage 18 in the wing bud apical ectoderm. At earlier stages, ectodermal cell death was not associated with the loss of ridge morphology, which continued to develop even in the presence of cell death. Regression of the ridge did not begin until much later in development. They suggested a possible relationship between ectodermal cell death and the regression at the extreme ends of the avian apical ridge.

Cell death has also been observed in the AER of the mouse (Jurand, 1965; Mi laire and Rooze, 1983) and in the rat (Mi laire and Rooze, 1983). Mi laire and Rooze claimed that cell death in AER of the mouse and rat was programmed cell death and distributed at the pre- and post-axial portions. The preaxial necrotic area was much more widespread than the postaxial one and remained present during the entire predigital period.

(b) Interdigital areas

Kelley (1970) found that dead cells in the interdigital spaces of human embryos were phagocytosed by macrophages at the final stage of the formation of the digits.

Hinchliffe and Thorogood (1974) studied interdigital cell death in normal and talpid mutant chick embryos and observed that the digital plate of stage 32 normal limbs was characterized by massive necrosis of the interdigital tissue, but in talpid fore- and hind- limbs of stages 30-35, interdigital necrosis was absent and there was no

regression of the tissue between the digits. They suggested that the morphogenetic role of interdigital cell death was causing separation of the digits through shaping and remodelling the contours of the digital plate.

Fallon and Cameron (1977) revealed that massive cell death was responsible for the elimination of interdigital webs in reptiles.

(c) Anterior and posterior necrotic zones

Superficial mesenchymal necrotic zones on the anterior and posterior areas between the limb buds and the body wall in the normal chick embryos have been found by various authors e.g. Saunders et al. (1962), Gasseling and Saunders (1964), Saunders (1966), Hinchliffe and Ede (1967, 1973), and Hurle and Hinchliffe (1978). However, the role of these necrotic zones on the limb morphogenesis is open to question.

Saunders et al. (1962) suggested that the shaping of the wing in the chick embryos between the 3rd and 9th day was brought about by contributions of some constant areas of cell death. Such areas were to be found on the anterior and posterior margin of the wing bud and in the future interdigital tissue. One of the main necrotic areas occurred at the posterior junction of wing bud and body wall during stage 24, which contributed to separation of the prospective elbow and upper arm from the limb bud base. In subsequent work, however, Saunders (1966) was able to prevent the occurrence of necrosis in PNZ, by grafting

dorsal wing tissues between the mesoderm of the PNZ and the ectoderm overlying it posteriorly. This did not affect the shaping of the wing, which developed normally. Thus Saunders concluded that cell death in the PNZ was not essential for the shaping of the limb.

Zwilling (1961), on the basis of his studies on the normal development of the chick limb, suggested that the apical ectodermal ridge (AER) was dependent on the existence of an apical ectodermal maintenance factor (AEMF) in the underlying limb mesoderm. Gasseling and Saunders (1964) found experimentally that apical ectoderm flattened when underlaid by a graft of PNZ mesoderm. They suggested that the PNZ's have a role to play in limiting the AER length through limiting the area of mesoderm from which AEMF is available. This suggestion was supported by Hinchliffe and Ede (1967) who found that, in the talpid mutant, ANZ and PNZ were absent, and the AER showed progressive enlargement at 5-6 days. It was suggested that this progressive extension was due to an abnormal increase in the area of talpid mesoderm concerned in AEMF production or distribution as a result of the failure of cell death to occur in the mesoderm. They also concluded that the absence of cell death in the ANZ and PNZ of the talpid mutant, makes it clear that foci of cell death are under genetic control.

Gasseling and Saunders (1964) also suggested that the forelimb PNZ may have some responsibility for the pattern

of limb symmetry. When they grafted the PNZ preaxially in the wing bud rim they obtained immediately posterior to the graft a supernumerary wing tip of left-hand asymmetry, mirror-imaging the normal right hand.

(d) Cell death in the joint interzone

Cell death in the joint interzone, during early development, has been found by several authors i.e. Fell and Canti (1934), Saunders et al. (1962), Dawd and Hinchliffe (1971), Wassilev (1972), Hinchliffe and Thorogood (1974), Rajan and Merker (1975), Tencate, Freeman and Dickinson (1977), Rooket (1979), Mitrovic (1977, 1978, 1982), and Abu-Hijleh (1987).

Fell and Canti (1934) found an area of degeneration in the region of the prospective knee joint of the chick embryo and called this area the opaque patch, because of its opacity to transmitted light in the living embryo. However, they did attach significance to the opaque patch in development of the knee joint.

Similar areas were also found by Saunders et al. (1962) and Dawd and Hinchliffe (1971) at the elbow region. Saunders et al. (1962) claimed that the opaque patch at the elbow region of the chick embryo played an important role in the differentiation of the elbow joint.

Dawd and Hinchliffe (1971) investigated cell death in the opaque patch of central mesenchyme of the developing chick forelimb, and found that a focus of cell death appeared first at stages 23 and 24 and reached its maximum

extent at stages 24 and 25, at which time it separated the ulna and radial mesenchymal condensation. It then decreased in size to a small area separating the proximal parts of the radius and ulna and disappeared at stage 28 by phagocytic activity of the neighbouring mesenchymal cells, which transformed into macrophages. In view of these findings, they suggested that cell death might play a role in the separation of the radius and ulna. This view was supported by Hinchliffe and Thorogood (1974) who found fusion of the radius, ulna and tibia/fibula in the talpid mutant chick embryos, in which dead cells in the opaque patch of the central mesenchyme of the fore- and hind-limbs were absent or reduced.

Wassilev (1972) (quoted by O'Rahilly and Gardner (1978)) studied the development of the synovial cavity in the rat knee joint, and found that cavitation proceeded with degenerative changes of mesenchymal cells in the central part of the interzone.

Tencate, Freeman and Dickinson (1977) found programmed cell death during the development of sutural joints in the cranial vault in rat embryos and suggested that these degenerative cells were implicated in the development of the suture.

Rajan and Merker (1975), Rooket (1979) also found cell death in the joint interzone during early development.

Mitrovic (1977, 1978, 1982) found two waves of cell death in the metatarsophalangeal joints of rat and chick embryos and suggested that cell death might play a role in

the development of the joint cavity.

More recently Abu-Hijleh (1987) found that programmed cell death preceded cavity formation in the chick knee joint and was also responsible for sculpturing of the articular surfaces and the intra-articular structures.

C. **Cytology of cell death**

Several workers have studied the morphology of cell death in vertebrate embryos by light and transmission electron microscopy. Glucksmann (1951) made the first adequate classification, using the light microscope, of the various stages in the cytology of embryonic degenerative processes.

He distinguished three stages:-

1. The initial stage, which he called chromatopyknosis, consisted in the condensation of nuclear chromatin into larger granules and finally into a single mass. The non-chromatic material seemed to liquefy and to form confluent vacuoles.

2. These nuclear changes resulted in the appearance of a single chromatic mass sitting as a cap on the vacuole formed by the non-chromatic material. This stage was described as hyperchomatosis of the nuclear membrane. Both the nucleus and the cytoplasm shrank from the loss of fluid.

3. After gradual shrinkage a more chromatic granule persisted, which lost its affinity for nuclear stains, became Feulgen-negative, broke up and disappeared; this was chromatolysis. Glucksmann (1951) stated that the changes could take place in an isolated cell, or in a degenerating cell phagocytosed by a neighbouring cell. Observations of cell death, using both light and electron microscopes have been published by Behnke (1963), Weber (1964), Farbman (1968), Dawd and Hinchliffe (1971),

O'Connor and Wyttenbach (1974), El-Shershaby and Hinchliffe (1974), Ojeda and Hurle (1975), Mitrovic (1977, 1978), and, more recently, by Abu-Hijleh (1987).

Behnke (1963) observed extensive dense cytoplasmic bodies in the epithelium of foetal rat duodenum, during formation of the villi. These dense cytoplasmic bodies were surrounded by one or two unit membranes and contained mitochondria, electron opaque vesicles, ribosomes, rough and smooth surfaced endoplasmic reticulum and lamellated membranes (myelin figures). Generally, the cytoplasmic components in the bodies seemed to be in varying stages of disintegration. When the villi formed, and the stratified epithelium changed to the simple columnar type, dense bodies largely disappeared. He did not find actual processes of phagocytosis. Weber (1964), working on the tails of Xenopus tadpoles, reported that the first regressive changes were in the tail muscle, where the myofibrils lost their cross striations. The mitochondria were eroded and the sarcoplasm disintegrated and was shed into the intercellular space. Later, phagocytes, presumably differentiated from mesenchymal cells, were responsible for the digestion of debris from the muscle cells in "phagosomes" rich in acid phosphatase.

Farbman (1968) studied palatal fusion in mouse embryos, and found widespread evidence of cell death in the midline epithelial seam. Examination of these dead cells with the electron microscope revealed that they were

pleomorphic membrane structures, containing amorphous dense material and profiles of organelles. Mitochondria, endoplasmic reticulum, nucleus-like structures bounded by a double membrane, vesicles, glycogen-like particles, ribosome-like particles and myelin figures were also observed. Dead cells were often seen between viable epithelial cells; however, sometimes it appeared that they were phagocytosed by neighbouring epithelial cells in the fusion line and by invading connective tissue cells.

Dawd and Hinchliffe (1971) studied cell death in the opaque patch of the central mesenchyme of the developing chick limb using T.E.M. and cytochemical methods and observed that the marginal chromatin of the nucleus in the dead cells became denser than that of neighbouring cells. The cytoplasm was rounded up, and contained dense membranous material and cytoplasmic vacuoles. The mitochondria and endoplasmic reticulum were swollen and apparently deteriorating. In some cases the aggregation of many cytoplasmic granules formed parallel bands which in the later stages of the degeneration, deteriorated, and in which the cytoplasmic bodies were difficult to identify.

These dead cells were surrounded by arms of cytoplasm from one or two viable mesenchymal cells and engulfed by those mesenchymal cells. At an early stage of phagocytosis, the dead cells showed little sign of digestion and the nucleus and cytoplasm could frequently be distinguished within the mesenchymal cells. Acid phosphatase distribution was the same as in normal

mesenchyme cells, and was localized in the Golgi apparatus and in lysosome-like bodies near the Golgi apparatus but not in the ingested dead cell. At a later stage of phagocytosis, these phagocytic mesenchymal cells differentiated and became typical macrophages. This process was reflected in the altered appearance of their nuclei which became denser and more indented, and increased in cytoplasm and cytoplasmic organelles (mitochondria and endoplasmic reticulum). Acid phosphatase activity increased within the macrophages, where activity was localized within the digestive vacuoles (secondary lysosomes) containing the dead cells and within the Golgi apparatus and Golgi vesicles (primary lysosomes).

Finally, the dead cells were digested within phagocytic vacuoles by the acid hydrolases of the macrophages until no visible structures remained, apart from myelin figures. These appeared to be extruded from the macrophages into the intercellular space.

O'Connor and Wyttenbach (1974) studied the cytological events of dead cells in the visceromotor neurons of the cervical region of the chick embryo's spinal cord using the electron microscope, and described the initial set of degenerative changes to include a decrease in nuclear size, the clumping of chromatin beneath the nuclear envelope, an increase in electron opacity of the cells, the disappearance of Golgi bodies, and the disaggregation of polysomes. These events were followed by the loss of the

nuclear envelope and most of the endoplasmic reticulum, the appearance of bundles of filaments, and the formation of many ribosome crystals. Ribosome crystals were seen only in the dying cells. Their abundance might indicate a drastic reduction in RNA synthesis as one of the initial events which leads to the death of these neurons. They also observed that the degenerative neurons were finally subdivided and engulfed by cells of the normal glial population, and further breakdown of the cell fragments occurred in large phagocytic vesicles of the gliocytes.

El-Shershaby and Hinchliffe (1974) studied the dead cells in the mouse blastocyst and in the adjacent uterine epithelium, and observed that initially the dead cells were rounded, and at the ultrastructural level clearly showed the process of chromatopyknosis involving concentration of the chromatic material of the nucleus and loss of the nuclear membrane. The cytoplasm showed lipid accumulation, ribosome segregation, swelling of the endoplasmic reticulum and probably also of the Golgi body. At a later stage of deterioration, chromatin condensation was more advanced and the nucleus was fragmented and surrounded by a gap. The cytoplasm showed absence of endoplasmic reticulum and of ribosomes from certain regions, while in other areas they were segregated. Finally these degenerative cells were phagocytosed by neighbouring trophoblast cells.

Ojeda and Hurle (1975) studied the morphological changes of endocardial cell death during the formation of tubular heart of the chick embryo, and found that the dead

cells were rounded in shape and frequently retained connexions with adjacent cells. All cellular elements showed an increased electron density; the chromatin appeared dense and marginal; small vacuoles were found and the mitochondria and endoplasmic reticulum were swollen. In all cases the perinuclear cisterna was widened and often showed beaded structures. In some endocardial dead cells distinct crystalline structures were seen which looked like ribosome crystals; they were commonly arranged in parallel sheets and in some cases were associated with microtubules. Finally some dead cells showed glycogen pools which were not normal constituents of developing endocardial cells. They rarely observed phagocytosis in the endocardial cells.

Mitrovic (1977, 1978) also described some changes which occur in dead cells in metatarsophalangeal synovial joints, in both chick and rat embryos. He reported that these degenerating cells exhibited marked pyknosis, basophilic and vacuolization, under the light microscope. In electron micrographs, these cells showed considerably increased opacity and appeared to be profoundly altered, with marked cytoplasmic and nuclear retractions. They rapidly disintegrated and were phagocytosed by the surrounding cells.

More recently, Abu-Hijleh (1987) found that degenerative cell death preceded the development of the cavity in the chick's knee joint and considered it as an apoptotic cell death. He described the cytological events

of this finding as follows:

The first stage was identified by increased density of the nucleus, and aggregation of chromatin material subjacent to the nuclear membrane; the nuclear outline became very irregular, due to the beginning of nuclear shrinkage and to local areas of separation of the two layers of the nuclear membrane. The cytoplasm also appeared more dense due to compaction of its organelles, especially the free ribosomes. Most of the cytoplasmic organelles retained their integrity, but some mitochondria and parts of the endoplasmic reticulum, and of the Golgi apparatus, were swollen, leading to vacuolation of parts of the cytoplasm. At more advanced stages, the nucleus became rounded up, with its chromatic material condensed into discrete and densely stained areas within the nucleus and beneath the nuclear membrane. The initially localized separations of the two layers of the nuclear membrane increased in size and appeared as electron translucent vacuoles. Nuclei at this advanced stage of dying were surrounded by cytoplasm which showed deterioration of its organelles. Finally, apoptotic bodies were found either dispersed in the intercellular spaces, or phagocytosed by adjacent viable mesenchymal cells.

These studies on the cytology of cell death suggest that there is a common pattern of degenerative change in cytoplasm and nucleus. After the cell has died it is phagocytosed and later digested by a macrophage, which may have differentiated from a neighbouring cell of the same

type as the dead cell.

IV. EMBRYONIC MOVEMENT AND ITS ROLE IN JOINT DEVELOPMENT

It is well known that somatic movements begin early in development in many species; in the chick, for example, studies of spontaneous embryonic muscular activity have been made by Kuo (1932(1), 1932(2), 1938, 1939); Orr and Windle (1934); Windle and Orr (1934); Hamburger (1963); Hamburger and Balaban (1963) and Hamburger et al. (1965). They reported that embryonic movement in the chick began at 3½-4 days with a slight flexure of the head and neck, caused by contraction of several head somites. Trunk movements appeared after head movement and before movements of the limbs in the later hours of the 4th day of incubation. This movement was of two kinds (1) flexion and extension of the cephalic and caudal regions and (2) twisting of the body to the sides. Oral movement began on the 6th day; movements of the eyelids were found on the 7th day and movements of the eyeball on the 8th or 9th day of incubation.

Kuo (1932(1), 1932(2), 1938, 1939) also observed that movement at the root of the limbs had generally begun by the end of the 4th day; from the 7th day onwards, the legs were much longer and their movements became extension and flexion. He also observed that movements of the toes were not independent of the movements of the other parts of the leg and could be detected on the 7th day, but, on the 8th day, they moved independently of each other. Beckham et al

(1977) noted that the earliest movements in lower limb digits of normal chicks might occur on the 9th day of incubation, but in the majority of chicks lower limb and digital movements did not begin until the 10th day. Dogra (1987) observed that rapid flexion and extension of the lower limbs occurred at the ankle joint in 10 day old embryos and extended to the digits at later stages.

Llusa-Perez et al. (1988) recorded that the progressive increase in the phases of activity in the chick embryo, from day 8 onwards coincided with the period of commencement and formation of the large joints (day 8, Hamburger-Hamilton (H-H), Stage 34, for the hip; day 9, H-H, Stage 35, for the knee, and day 10, H-H, Stage 36, for the ankle), but that complete cavitation was found on days 10-11 of incubation for the hip and knee and on days 12-13 for the ankle, whereas the small joints such as metatarsophalangeal and interphalangeal showed incipient signs of cavitation that were completed on days 14-15.

Hamburger and Balaban (1963); Hamburger et al. (1965) and Llusa-Perez (1988) also recorded that the chick embryonic movements were phasic and occupied 80% of the embryo's time at 13 days. This peak was maintained up to day 17 and subsequently declined.

Various authors e.g. Murray and Selby (1930), Fell and Canti (1934), Hamburger and Waugh (1940), Lelkes (1958), Drachman and Sokoloff (1966), Sullivan (1966), Murray and Drachman (1969), Drachman et al. (1976), Rauno-Gil et al.

(1978), Mitrovic (1982) and Persson (1983) have studied the effect of the chick embryonic movement on the development of the synovial joints and its associated structures using one of the three following experimental methods:

i) culturing the embryonic limb bud *in vitro*;

ii) administration of neuromuscular blocking agents; and

iii) grafting the limbs to the chorio-allantoic membrane.

A few authors e.g. Yasuda (1973) and Rajan and Merker (1975) have also studied the effect of the human embryonic movement on the development of the skeletal system and synovial joint by culturing *in vitro* the human embryonic limb buds.

General effects of immobilization

The external features of the chick embryos, paralysed by injection with botulin toxin, were described by Murray and Drachman (1969). At 19 days the embryos were smaller and lighter in weight than the controls. They were approximately equal in size to untreated 10 day embryos, but otherwise had matured normally for their age, and had no bizarre malformations. Internally they showed severe fixation of many joints, and protrusion of the tip of the lower beak beyond the upper. The facial part of the skull was narrowed, while the cranial part was of normal width. All of the skeletal muscles of the body 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

occurred normally on the 19th day.

Sullivan, in his extensive studies (1966, 1967, 1971, 1973) on the development of the paralysed chick embryos, found that their rate of development was slightly retarded, and some of them showed deformations in the shape of the body or abnormal postures of the limbs. The neck was markedly bent and immobilized, while the trunk often had a compressed appearance associated with abnormal curvatures of the vertebral column. The beak was also protruded. He suggested that these features were probably caused by pressure arising from contact between the embryo and the actively contracting amniotic membrane.

Persson (1983) immobilized chick embryos using injection of decamethonium iodide into the air sac, as described by Hall (1975), and found that, in general, paralysed embryos differed from control embryos by a distorted position of the body, indicating retention of a more embryonic position. In some paralysed embryos, large amounts of clear fluid were found subcutaneously in the abdominal area. Apart from these observations, no obvious malformation of the trunk or limbs were seen externally in paralysed embryos. The tip of the lower beak protruded in front of the upper beak. The total body weights of paralysed embryos were significantly lower than those of controls. He also found that the developmental stages of the paralysed embryos, based on external characteristics according to Hamburger and Hamilton (1951), indicated a slight delay compared to control embryos.

In a recent study, Dogra (1987) paralysed the chick embryos using injections of the decamethonium iodide into the air sac as described by Hall (1975) and observed that over the area of subcutaneous oedema feather germ development was retarded; protrusion of the cerebral hemispheres occurred through the malformed skull bones; paralysed embryos were lighter than controls of a similar stage; and the rate of weight gain of paralysed embryos was slower than that in the controls.

Effect of immobilization on the development of the synovial joint cavity

(a) Avian synovial joints

Several authors such as Fell and Canti (1934), Hamburger and Waugh (1940), and Mitrovic (1982) observed the early stages of the cavity formation in the absence of the chick embryonic movement and suggested that intrinsic factors were essential for the initial cavity formation, but that movement was necessary for full differentiation and maintenance of the joint cavity. Other authors, such as Lelkes (1958), Drachman and Coulombre (1962), Drachman and Sokoloff (1966), Murray and Drachman (1969), Bradley (1970), Drachman et al (1976), Ruano-Gil et al. (1978, 1985), Persson (1983), did not observe the early stages of the cavity formation in the absence of the chick embryonic movement and suggested that movement was essential for the cavity formation.

Fell and Canti (1934) studied the development of the avian knee joint *in vitro* and found that, when a whole limb was explanted, a joint developed but eventually disappeared by secondary (cartilaginous) fusion. When presumptive knee joint tissue was explanted without adjacent shaft tissue, only a single piece of cartilage without a joint appeared. As a result of these findings, they concluded that joint formation was not produced (a) as a result of the presence of non-chondrogenic tissue at the site of the joint; (b) as a rigidly localized part of the limb mosaic; (c) as a result of muscular, nervous, or vascular influence; (d) by mass movement of cells at the joint region. But it appeared due to the mechanical result of differential growth, caused by the resistance of the undifferentiated tissue of the joint region and perichondrium to the expansion of the more rapidly growing chondrification centres, and movement might be an essential factor in its subsequent development.

Hamburger and Waugh (1940) studied the development of the joint cavity in the transplanted limbs of the chick embryos and found that joint formation in nerveless limbs might be almost 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. Two types of fusion, primary and secondary, were distinguished. The first type of fusion, which was

fibrous, was more frequent and might be found at the final step of cavity formation in the transplanted limbs, with segregation of adjoining elements. The second type of fusion was cartilaginous, and developed either by direct differentiation of the joint tissue into cartilage, or by a second process of atypical chondrification of fibrous tissue. In view of these findings, they concluded that movement could be instrumental only in bringing about the final step in joint formation.

In a recent study, Mitrovic (1982) tested the role of movement in the formation of the joint cavity by paralysing chick embryos by administration of decamethonium and by culturing chick limb buds on the chorio-allantoic membrane. He found that the first stages of development of the cavity were not inhibited, but they did not progress further. The joint space was rapidly invaded by blood vessels and loose connective tissue and finally disappeared. The two elements of the joint became fused together either by fibrous tissue or by cartilage. He suggested that intrinsic factors were important for differentiation of the early stages of the joint cavity and that movement was necessary for full differentiation and maintenance of the joint cavity.

On the other hand, Lelkes (1958) cultivated knee joints of 6-7 day old chick embryos, either in a watch glass, or in flasks containing a mixture of fowl blood-plasma and fowl embryonic extract as a medium. In each case the two limbs of the same embryo were explanted in the

same watch glass or flask, and one limb was subjected to passive movement, while the other served as a control. He concluded that in control explantations, where no movement was applied, the undifferentiated interarticular tissue generally became chondrified, leading to complete cartilaginous fusion across the joint. Movement prevented this fusion. An articular cavity was sometimes formed in the moved explants but not in the controls. Movement also exerted a formative effect on the shape and structure of the articular surfaces. He suggested that proper differentiation of a joint depended on its undergoing movement.

To study an experimental model of clubfoot and arthrogryposis multiplex congenita, Drachman and Coulombre (1962) administered d-tubocurarine intravenously to the chick embryos by a technique of continuous infusion, and found that the joints were ankylosed due to the contracture of the periarticular and intra-articular soft tissues and occasionally, due to the fibrous adhesions between the joint surfaces. They also noted that the toes were commonly clawed or hyper-extended; the ankle joints were often fixed in a semiflex position, although acute flexion or hyper-extension were sometimes encountered. They concluded that congenital ankylosis of joints, whether occurring spontaneously or produced experimentally, might have resulted from relatively brief immobilization of the embryo.

Drachman and Sokoloff (1966) studied the effect of the paralysis on the development of the joint in the chick embryos using three experimental methods; administration of decamethonium bromide, botulinus toxin, and spinal cord extirpation and found that joint cavities failed to develop in the paralysed limbs, although the preparatory changes in the cellular architecture of the joint regions proceeded otherwise normally. The joint interzones were occupied first by fibrovascular tissue, and later by compact fibrous or cartilaginous tissue which bounded the articular elements together. The articular surfaces were flattened and distorted. They concluded that muscle contractions were essential for primary joint cavity formation, and for sculpturing of the form of the articular surfaces, but were not necessary for morphogenesis of articulations prior to the stage of cavity formation.

Murray and Drachman (1969) studied the development of the joints of the head and neck in paralysed chick embryos and found that the joint cavities were completely absent and fusion occurred across the joint regions by fibrous connective tissue, cartilage or bone, depending on the composition of the articular elements appeared. They concluded that the normal development and maintenance of mobile joints were dependent on an active musculature.

Bradley (1970) cultured chick embryo limb buds on the chorio-allantoic membrane and found that the joint formation was sometimes almost perfect, but the fibrous and cartilaginous fusions occurred in most cases. She

suggested that the proper differentiation of a joint depended on its undergoing movement. This view was supported by Drachman et al. (1976) who had studied the effects of paralysis produced by a viral myopathy on joint development. They injected coxsackie virus A_2 intravenously into chick embryos on the 7th day of incubation and found that primary myopathy with paralysis produced arthrogryphotic joint deformities.

Ruano-Gil et al. (1978, 1985) studied the effect of the paralysis on the development of the articular system and found that the joint cavity did not develop in paralysed embryos and was replaced by an undifferentiated mass of mesenchymatous tissue. Para-articular structures such as capsule, ligaments and meniscus did not develop, but the articular surfaces developed normally. They concluded that extrinsic factors such as movement were essential to the cavity formation.

In a recent study, Persson (1983), tested the role of movement on the development of the synovial and sutural joints by inducing decamethonium iodide for long term paralysis in chick embryos as described by Hall (1975), and concluded that movement of neuromuscular origin played no essential role in the development of sutures but was an essential factor in the development of synovial joints. He also reported that cartilaginous and fibrous fusion commonly occurred at the normal site of articulations in paralysed embryos.

Llusa-Perez (1988) studied the relationship of joint formation and embryonic movement in chicks, and concluded that a close relationship could be demonstrated between the dates on which the first movement was recorded and the commencement of joint formation.

(b) Mammalian synovial joints

In 1975 Rajan and Merker studied the limb buds of human embryos in culture, and found that after 10 days the joint cavity of the digits appeared, in the absence of movement. There was evidence of acid mucopolysaccharide material in the joint cavity, and occasional pyknotic cells lined the cavity. They suggested that necrosis of cells and alteration in glycosaminoglycans might be responsible in part, if not wholly, in the formation of a joint, and that blood supply, nervous control and movement might not be essential for the formation and differentiation of the joint cavity.

Effect of the immobilization on the development of the intra-articular structures

Hamburger and Waugh (1940) described the formation of fibrous articular tissue between the articular cartilages proceeded normally in the transplant limbs which was in agreement with Fell and Canti (1934) who studied the development of the chick knee joint *in vitro*.

In contrast, Drachman and Sokoloff (1966) found that intra-articular structures did not develop in the paralysed limbs. They concluded that movement was essential during embryonic development for differentiation of the intra-articular structures. This view was confirmed by Persson (1983), Ruano-Gil *et al*. (1978).

Effect of the immobilization on the development of the skeleton

(a) Avian skeletons

Murray and Huxley (1925) studied the self differentiation in chick limb buds grafted on to the chorio-allantoic membrane on the fourth day of incubation and found that, at 4 days of incubation, the limb-bud was a mosaic structure and the femur and patellar rudiments could undergo self differentiation, both anatomical and histological.

Murray and Selby (1930) used the same technique to study the effect of the intrinsic and extrinsic factors in the primary development of the skeleton, and concluded that the general shape of bones was determined by intrinsic

factors and that extrinsic factors, such as movement, produced minor sculpturing.

Niven (1933) studied the development of the avian patella in vivo and in vitro and found that, in cultures of the whole patellar mesenchyme, cartilage developed in the explants in all cases, and resembled that seen in the normal development, but the articular surfaces did not appear even after 7 weeks' cultivation. In cultures of parts of patellar mesenchyme, cartilage appeared, but the size of the cartilage mass was smaller and the form did not resemble that which developed in cultures of the undivided patellar mesenchyme. In cultures of the knee joint region, including the end of the long bones, from 7 day and 9 day old 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 chick skeleton in wings and legs transplanted into the coelomic cavity, or to the umbilical cord and found that, in the absence of innervation, morphogenesis was normal in a high percentage of cases. Hypodactyly and hypophalangy were the most common abnormalities in transplants and were occasionally combined with the absence of the fibula or ulna. They also found that chondrification and the first phases of ossification proceeded normally in the absence of innervation, but skeletal growth of transplants was reduced by about 20 percent, which they thought might be due either

to an inadequate establishment of vascularization of the transplants or to deficiencies in the innervation. The girdles were also more poorly developed than were the free limbs. In particular, the dorsal girdle elements, the scapula, and the ilium, were disproportionately small.

Drachman and Sokoloff (1966) produced paralysis of embryonic movement of chick embryos between 6 and 8 days of incubation by administration of decamethonium bromide, botulinus toxin and spinal cord extirpation and found that the bones of the lower limb were of normal maturity in the treated embryos, as judged by the degree of ossification, vascularization, and marrow invasion, although they were shorter than in controls. They also found that the plantar tarsal sesamoid was absent in 4 of the 11 day embryos, while the other 2 specimens showed a projection of cartilage attached to the tibia. A small, but 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 cartilages but were not necessary for differentiation of the patellar sesamoid cartilage and bones.

Murray and Drachman (1969) studied the development of the skull and cervical vertebrae in paralysed chick embryos and reported several distortions of cartilaginous and bony structures such as protrusion of the mandible; the retro-articular process of Meckel's cartilage extended straight

backwards in the paralysed embryos, rather than being angled upwards, as in the normal. They concluded that these distortions were due to (a) failure of skeletal muscles to exert their normal moulding influences on these structures 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 (H.H.) on the chorio-allantoic membrane and concluded that chondrification and the first phase of ossification proceeded normally in the absence of innervation.

Sullivan (1971, 1973, 1974, 1975) treated chick embryos with different paralysing drugs and studied the skeleton in cleared specimens stained with alizarin red-S. He observed bony abnormalities such as fusion and asymmetries of cervical vertebrae, scoliosis, and deformed clavicles and scapulas. Owing to jamming of the pelvis against the posterior part of the thorax, some of the ribs were pushed close together in some embryos, while the scapulae adopted exaggerated curvatures, becoming C-or L-shaped as a consequence of lack of space to elongate normally. The vertebral fusions were attributed to lack of movement at the developing articulations. The other effects on the skeleton appeared to be caused by compression resulting from the abnormal body postures.

(b) Mammalian skeletons

Yasuda (1973) cultured human limb buds in nutrient agar medium for four to eighteen days, and found that differentiation of mesenchyme into cartilages or bone primordia and other supporting tissues took place, in a manner similar to that seen in vivo. He noted some developmental retardation, less in the lower limbs than in the upper.

Effect of immobilization on the development of the muscular system

Muscle degeneration in the absence of movement in the embryonic chick has been reported by various authors.

Hunt (1932) studied muscle differentiation in chick limb buds cultured on the chorio-allantoic membrane and concluded that the limb muscles were capable of initial independent differentiation, but they did not invariably maintain this independence. At a certain period, about the 10th day of incubation, some external factors such as nerve supply seemed necessary for continuation of normal development, and prevented the fatty degeneration of the muscle. He also noted that tendons and other fibrous formations occurred in the grafts to some extent, but frequently did not have a typical relation to the muscle and bone.

The process of muscle degeneration was described in detail by Eastlick (1943), Eastlick and Wortham (1947), who studied the muscle differentiation in chick limb buds

cultured on the chorio-allantoic membrane and observed that the muscle underwent an initial differentiation in the nerveless grafts. Cross-striated fibrils appeared usually by the 10th day, but they were not maintained in the nerveless grafts, and the embryonic muscle tissue was destroyed by sarcolysis and phagocytosis. As the embryonic muscle broke down and was removed, a loose embryonic-like connective tissue remained. The capillary network, which nourished the muscle, appeared to persist within the connective tissue. Numerous sprouts grew out from these vessels and a ramified vascular plexus developed in the nerveless graft.

The mesenchymal cells which lay in close proximity to the vessels multiplied actively by mitosis. This complex of vessels and cells formed primitive fat organs. In view of these findings, they suggested that innervation was necessary for (a) the multiplication or increase of myofibrils, (b) the continued differentiation of the fibrils and the organization of the fibres into typical muscle bundles, and (c), the maintenance of muscle fibres in a healthy condition.

Sullivan (1963, 1966, 1967) studied the abnormalities of the muscular anatomy in the shoulder region of the paralysed chick embryos and observed that there was considerable atrophy of the muscle fibres and a number of abnormalities of the muscular anatomy in the paralysed embryos. In normal development, the chick wing musculature

was first visible in the wing bud as opposed to dorsal and ventral condensations of mesenchyme, the premuscle masses. M. supracoracoideus and M. coracobrachialis anterior separated from each other by cleavage in the supracoracoid division of the ventral mass. In paralysed embryos this cleavage seemed not to occur. Another common feature in the paralysed embryos was a deflection of fibres of M.triceps brachii pars scapularis so that instead of lying parallel with the humerus they diverged from it proximally and tended to become aligned parallel to M. latissimus dorsi. These results indicated that functional activity was important for the formation and maintenance of the muscle fibres. He did not describe the histological appearance of the paralysed muscle.

Drachman (1964) studied the atrophy of skeletal muscle in chick embryos treated with botulinum toxin and found the following features on the 12th day of incubation; reduction in muscle bulk, in transverse and longitudinal sections; reduction in the diameter of individual fibres, increase in number of sarcolemmal nuclei, with rounding of their contours, and clumping of groups of nuclei; interstitial fatty infiltration, and large intramuscular deposits of fat; phagocytosis of degenerated muscle fibres; increased endomysial fibrous tissue, with small spindle-shaped cells distributed interstitially in the more severely affected regions.

Drachman and Sokoloff (1966) observed that, in the paralysed embryos, most of the muscle mass was replaced by

adipose tissue. The remaining fibres showed atrophy and degeneration, and were devoid of striation. Sarcolemmal nuclei were aligned in chains, or clumped in some areas. Histiocytes were present in the regions of more advanced muscle degeneration.

The effect of movement on the development of the muscle was also noted by Murray and Drachman (1969) in their study of paralysed chick embryos. They found, that the muscles were extremely atrophic and loose in texture with fatty infiltration, although all of the muscles were identifiable and could be traced from their normal origins to their normal insertions. Tendons and ligaments closely associated with muscle, were more tenuous than in normal embryos, or were absent.

Histologically, the most striking finding was a large reduction in muscle bulk, with the greatest part of the muscle replaced by adipose tissue. The remaining muscle fibres were atrophic and degenerating. In these fibres, there was an apparent increase in the number of sarcolemmal nuclei, with alignment, clumping and pyknosis of nuclei. Some of the muscle fibres retained their striations while undergoing atrophy; others showed marked degeneration, characterized by eosinophilia, swelling, and floccular changes, with phagocytosis by histiocytes.

Bradley (1970) studied the process of muscle degeneration in chick limb buds cultured on the chorio-allantoic membrane and found that there was a gradual

accumulation of vacuoles containing fat within the muscle cells, which coalesced to form a single large vacuole. The cells became spherical and their nuclei were displaced to one side. Further fat accumulation distorted the nuclei, which then degenerated either in situ, or, if the cell membrane ruptured, on liberation. Eventually the pyknotic nuclei were removed by macrophages. She concluded that the limb muscles, although capable of initial independent differentiation, required a nerve supply to continue their normal development.

Hall (1975) paralysed the chick embryos by a single injection of decamethonium iodide or D-tubocurarine into the air sac and found that the total muscle mass of the paralysed limb was very much reduced as compared with that in untreated embryos. The myofibrils of peroneus longus were separated by fibrous connective tissue and were much fewer in number than in control embryos. The fibres were thin, lacked striations and were not as well organized into discrete bundles as were those from control embryos. He concluded that paralysis was accompanied by severe reduction in muscle mass and by delayed differentiation of muscle.

Effect of immobilization on the development of tendons

Various authors, such as Hunt (1932), Murray and Drachman (1969), Bradley (1970), Beckham et al. (1977), Kieny and Cheval ier (1979), and Dogra (1987), have suggested that tendon differentiation was initiated and proceeded to a considerable extent in the absence of movement, but that final completion and maintenance of the tendon required the mechanical action normally provided by the contraction of the embryo's own muscles.

Hamburger and Waugh (1940) found that the absence of the innervation resulted in an atrophy and degeneration of the skeletal muscle. The tendinous attachment of such muscles were structurally deficient, and it was certain that no pull was ever exerted on these points of attachment.

Bradley (1970) cultured chick limb buds on the chorio-allantoic membrane and found that the tendons developed normally in the grafts, but they did not have a typical relation to the muscle and bone. The nuclei of tendon fibres were slightly disorganized but otherwise the structure was remarkably normal despite the lack of attachment. She concluded that the tendon was initially self-differentiating but showed a gradation of dependence on nerve supply for their continued differentiation.

Beckham et al. (1977) studied the development of a digital flexor tendon in paralysed chick embryos and found that the synovial cavity around the tendon,

fibrocartilaginous area and elastic vinculum failed to form, as a result of the paralysis of the digit. They concluded that movement was required to produce a functional tendon apparatus in the embryo; they also predicted that movement might be required for the tendon regeneration after injury.

Kieny and Chevalier (1979) studied autonomous tendon development in the embryonic chick wing. They destroyed somitic mesoderm by local irradiation, at wing somite level, in the 2 day-old chick embryos, to prevent the development of forearm muscles. They observed that despite the absence of both flexor and extensor muscles, differentiation of the tendons took place, and that differentiation occurred at the same time and in the same positions as in the control chicks. However, these tendons were transient structures, which disappeared within three days, by disintegration and cell death. They concluded that tendons started to develop autonomously from the muscle bulk, but for their maintenance and further development they required connection to a muscle belly.

Dogra (1987) studied the effect of paralysis on the normal development of the chick flexor tendon and observed that in the absence of movement, the formation of a synovial cavity around the flexor tendons did not occur, the elastic tissue component of the vincula did not form, and the cartilaginous area, which was found on the volar aspect of the deep tendon, failed to develop. He also observed that all three flexor tendons were greatly reduced

in size and did not separate from each other, the visceral and parietal cell layers did not form and many stellate fibroblasts occupied the potential synovial cavity.

V. AREAS OF DISAGREEMENT

Following this review of published work on the development of the synovial joint and its associated structures under the normal and immobilized conditions, it is apparent that there are still controversies in the findings.

Development of the synovial joint is thought to involve intrinsic factors such as cell death or "liquefaction" in the interzone, and extrinsic factors such as muscular movement.

In the case of intrinsic factors, such as cell death, it is not clear if this is genetically programmed. Moreover the disappearance of dead cells may be due to their own destruction or phagocytosis by macrophages, the origin of which is unclear.

The role of extrinsic factors such as muscular movement on the development of joint cavity, can be studied by inhibition of muscular activity, to provide answers to the following questions: i) does the initial stage of joint formation, the three-layered interzone, differentiate in the absence of movement?; ii) if cell death still occurs in the absence of movement, is this sufficient for the cavity formation? iii) If not, what is the fate of the

interzone?; is it replaced by fibrous connective tissue, cartilaginous tissue or both? iv) is there a single pattern in the development of such different joints as the knee, the femoro-patellar and the metatarsophalangeal joints?

v) do the intra-articular structures arise from "synovial mesenchyme" or from the interzone?

vi) does synovial mesenchyme arise from the interzone or from the general mesenchyme around the joint interzone?

vii) Is the origin of the patella independent of the quadriceps tendon or is it an intratendinous sesamoid?

viii) what is the role of movement in the development of the following:

a. articular cartilage
b. intra-articular structures
c. synovial mesenchyme
d. patella
e. skeletal muscle
f. tendon and ligament such as tibialis anterior and patellar ligament?

In order to answer the above questions, it was decided to adopt a procedure which involved the comparison of the development of the metatarsophalangeal and the knee joints, in normal subjects, with those of the immobilized.

The subject chosen for study was the chick embryo, the reasons being: (a) it has been used for most experimental studies; (b) embryos of known age are readily available; (c) embryonic movement can be directly observed throughout the experiment; (d) the metatarsophalangeal joints of the

chick are similar to those of the human; (e) it has been reported that the knee joint of the chick is generally similar to that of the human (O'Rahilly and Gardner, 1956; Abu-Hijleh, 1987), although there are two main differences:

i) In the chick, the fibula articulates with a groove on the lateral condyle, and the lateral meniscus shares in part of this articulation, ii) the tibialis anterior tendon is intra-articular and arises from the lateral femoral condyle.

VI. PREVIOUS METHODS USED FOR IMMOBILISATION

In previous studies at least three ways have been used to immobilize the embryonic chick (isolated limbs or whole embryo).

A) The injection of neuromuscular blocking agent *in ovo*

B) Organ culture of the limb *in vitro*

C) Grafting the limbs to the chorio-allantoic membrane.

Both organ culture and chorio-allantoic membrane grafting have the following disadvantages that they alter the normal environment for development, they cannot be studied stage by stage for a long period, and they involve the risk of a traumatic effect.

Injection of a neuromuscular blocking agent *in ovo* is therefore a preferable method of inducing total muscular paralysis.

Since this method was introduced by Drachman and

Coulombre (1962), it has been used in three ways as follows:

1. Infusion of the drug into the chorio-allantoic blood vessels (Drachman and Coulombre, 1962[b]; Drachman and Sokoloff, 1966).
2. Periodically applying the drug directly on to the chorio-allantoic membrane (Murray and Smiles, 1965; Sullivan, 1965, 1974; Mitrovic, 1982).
3. Single injection of the drug into the air sac (Hall, 1975; Beckham et al., 1977; Persson, 1983; Dogra, 1987), all of whom claimed that a single injection of the drug into the air sac provided a simple technique for achieving long-term total paralysis.

VII. DRUG ADMINISTRATION

In the present study, to suppress the skeletomuscular contractions, decamethonium bromide was administered. This neuromuscular blocking agent is thought to depolarize postsynaptic membranes, 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).

As it was reported that the joint formation and movement in the knee and metatarsophalangeal joints occur in the 7-10 day old chick embryos (O'Rahilly and Gardner, 1956; Abu-Hijleh, 1987; Mitrovic, 1977; Kuo, 1932(1), 1932(2), 1938; Lusa-Perez et al., 1988; Beckham et al., 1977; Dogra, 1987), it was decided to prevent the effect of

any muscular movement on the knee and the metatarsophalangeal joints and their associated structures by paralysing the chick embryos from the beginning of the 6th day of incubation (stage 28) onwards.

CHAPTER TWO

MATERIALS AND METHODS

Fertile Ross brown eggs were obtained from Ross Poultry, Aberdeenshire, and incubated from day 0 in a "forced draught" type incubator (Westernette) which provided hourly rotation of eggs and maintained a humid atmosphere at 37-38^{o}C.

At day 3 of incubation, the eggs were transferred to the Western static incubator at 38^{o}C, and the eggs were allowed to settle for a period of minutes before being windowed. This allowed the embryos to float to the top. The eggs were then 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 embryo was marked by a 2 x 1 cm rectangle pencilled on the shell. The air sac was also marked. The egg was then placed into a crokboard which allowed the egg to be kept in the same horizontal position as it had been in the static incubator.

Using a fine grinding wheel, fitted to a dental drill, a small hole was made in the shell overlying the air sac, and the shell was cut along the margins of the rectangle taking care to avoid damage to the underlying membranes. The shell was then swabbed with a 90% Hibitane-alcohol solution to remove the sawdust 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 air sac were sealed with sellotape, and the eggs replaced in the static incubator. Embryonic movements were subsequently looked for under the binocular microscope twice daily, each egg being viewed for at least 3-4 minutes.

At the beginning of the 6th day of incubation, before any appearance of movement in the knee joint and the lower limb digits, the embryos were divided into 2 groups (182 experimental and 88 control).

A solution of decamethonium bromide, a post-synaptic blocking agent, (1 mg dissolved in 0.5 ml normal saline for each embryo) and dropped directly on to the chorio-allantoic membrane of the experimental groups using a disposable 1 ml syringe fitted with a sterile 26 gauge needle.

To maintain paralysis with a reasonable survival, each experimental embryo was again exposed at days 8 and 10 of incubation to 0.5 mg decamethonium bromide in 0.25 ml normal saline.

Control embryos were similarly treated with normal saline. To determine if immobilization was complete in the experimental group, embryos were again examined under the binocular dissecting microscope from the first administration of the drug for 3-4 minutes twice a day and compared with the control group. Any experimental embryos

which showing any sign of movement or which were found dead, were discarded.

Experimental and control embryos were collected at intervals from 7 to 17 days of incubation. This was done by opening each egg into a petri dish; a final record of embryonic movement was made again as the embryos were removed. If any sign of movement was seen within the experimental group, the embryos were discarded.

Embryos were then sacrificed by rupturing of the chorio-allantoic membrane. 27 paralysed and 17 control embryos were stained with Alcian blue and Alizarin red and the rest of the paralysed and control embryos were fixed either in Bouin's solution or in 5% glutaraldehyde.

Embryos aged 10 to 17 days of incubation from both control and paralysed groups, before fixation, were laid on a clean tissue, and oedema fluid, which was present subcutaneously in the paralysed embryos, was removed by making a small incision in the overlying skin. The embryos were then blotted to dry, and were weighed on an Electronic Reading Balance (LJBBOR ED.200).

The development of the 3rd metatarso-phalangeal and the knee joints in both control and paralysed chick embryos were investigated in this study using four methods: Alcian blue/Alizarin red transparencies; paraffin wax histology; semi-thin histology and transmission electron microscopy.

a) Alcian-blue/Alizarin Red transparencies

27 paralysed and 17 control embryos were sacrificed and staged according to developmental stages of Hamburger and Hamilton (1951).

The embryos were skinned and stained with Alcian blue and Alizarin red, using the technique of Kimmel and Trammel (1981). They were then stored in pure glycerine with crystal of thymol (for details of these procedures, see Appendix I).

b) Paraffin wax histology:

25 paralysed and 20 normal control 3rd metatarsophalangeal joints and 30 paralysed and 30 normal control knee joints were studied by serial wax histology with the following procedures:

1. Fixation: embryos were fixed in Bouin's fixative for 7 days.
2. They were then placed in 70% ethanol for 1-2 hours.
3. Using a dissecting microscope, they were staged according to the developmental stages of Hamburger and Hamilton (1951).
4. Right lower limbs were removed. In younger specimens, the entire limb was processed, while in older ones, the knee joints were isolated by cutting through the thigh and the leg. The 3rd metatarsophalangeal joints were also isolated by cutting through metatarsal bones and distal portions of the third digits. Those specimens over 14 days old were decalcified using E.D.T.A.

5. The specimens were then dehydrated through an ascending series of ethanols, cleared in amyl-acetate, using an automatic tissue processor.

6. The knee joints were embedded in paraffin wax, lateral side downwards, while the 3rd metatarsophalangeal joints were embedded in paraffin wax either on the lateral side or medial side downwards.

7. Serial sagittal sections were cut on a Jung microtome at a thickness of 5 μm, stretched in a water bath and mounted on albuminised glass slides, dried for 24 hrs, in an oven at 37^{o}C.

8. Slides were dewaxed in xylene, hydrated, and then stained with Meyer's Haematoxylin and eosin.

9. Slides were dehydrated, cleared in xylene and mounted in Histomount. [For details of this procedure, see Appendix II].

c) Semi-thin histology and transmission electron microscopy

The 3rd metatarsophalangeal and the knee joints of 11 paralysed and 10 normal controls were studied by the following procedures:

1. Fixation: Embryos were fixed by immersion in cold 5% glutaraldehyde in Millonig's buffer (pH 7.4) for 48 hours.

2. They were then placed in the same buffer for a further 24 hours and staged according to the developmental stages of Hamburger and Hamilton (1951).

3. The 3rd metatarsophalangeal joints and the knee joints of the right limbs were isolated as before and were post-

fixed in osmium tetroxide buffered with phosphate (pH 7.4) for 1 hour. The specimens over 14 days old were decalcified using E.D.T.A. before post-fixation.

4. They were then washed in Millonig's buffer and dehydrated through a graded series of acetone.

5. The knee joints were embedded in Spurr's resin, lateral side downwards while the 3rd metatarsophalangeal joints were embedded in Spurr's resin, either lateral side or medial side downwards.

6. Both the knee joints and the 3rd metatarsophalangeal joints were then cut in the sagittal plane on a Reichert-Jung Autocut (Mod. 1140) ultra-microtome at a thickness of 1.2 μm, using 6 mm wide glass knives of the Latta-Hartman type.

7. Sections were mounted on glass slides and left to dry and were stained by Azur blue II. [For details of these procedures, see Appendix III].

In order to study the detailed cytological events of the interzone in normal control and paralysed joints during the developmental stages, the desired area was determined from semi-thin sections, and the block was trimmed with a razor blade and sections were cut at 50 nanometers, using a Reichert-Jung ultra-cut microtome and a diamond knife.

Sections were then mounted on to uncoated 200 mesh copper grids and stained with uranyl acetate and lead citrate (Reynolds, 1963) and then examined in a Jeol TEM 100S. [For details of the staining procedure, see Appendix

IV].

d) Cell Counts

Counting of "dark" cells was begun in a preliminary way in the joint space of the 3rd metatarsophalangeal joint of both control and paralysed embryos at developmental stages 34-38.

"Dark" cell counts were made on every 3rd section of a wax series to rule out the possibility of counting the same cell more than once, as even the largest dark cells did not spread over more than 3 sections.

Sections were projected on to drawing paper by a slide projector (LEITZ PRADO with slide projection head) and the outline of the joint space, between the chondrogenic layers of the interzone, traced in pencil.

Before and after drawing of the joint space, a slide micrometer was used to determine the magnification of the system, and the mean value was taken as the magnification of the system.

Lines AB and CD were then drawn tangential to the most prominent points of the adjacent cartilage models (Fig. 1).

The actual areas of the joint spaces were determined by dividing their areas, measured with KONTRON MOP-AMO2 (Manual Image Analysis) by the square of the magnification of the system.

Using a research microscope equipped with a graticule in the eye piece, "dark" cell counts were made using an X40 objective lens. "Dark" cells were distinguished from

mesenchymal cells and nucleated red blood cells by their small size with darkly stained nuclei and cytoplasm, whereas nucleated red blood cells were always visible with a clear cytoplasm around the darkly staining spiral shape nucleus.

The result was expressed as the number of "dark" cells per millimeter square of the joint space, by dividing the sum of the "dark" cells counted in the joint spaces by the sum of the areas of the joint spaces.

The numbers of "dark" cells in normal controls and paralysed were compared statistically.

The mean value of the joint spaces of the normal controls and paralysed was also determined by dividing the sum of the areas by the sum of the cross sectional planes and computed statistically.

CHAPTER THREE

RESULTS

I. Observations on movements in control and paralysed embryos

A binocular microscope was used to detect movement in the embryos, from 3 days of incubation onwards. At least three types of movement were observed in control embryos, before and after the administration of normal saline. These movements were observed to be unaffected by the administration of normal saline, which took place on the 6th, 8th and 10th days of incubation. The following movements were observed:

1) Contractions of the amnion produced a "swinging" movement of the embryo, beginning at the end of the 4th day. These movements were not continuous, but of a phasic nature and are known to be caused by the contractions of the smooth muscle of the amnion. This could be divided into the active and inactive phases. The duration of the active phase increased until the 10th day, after which it began to decrease.

2) Pulsation of the chorionic and vitelline vessels, in phase with the contractions of the heart.

3) Movements of the embryo itself, beginning in the head and then progressing caudally.

Lifting and bending movements of the head were observed on the 4th day, but at later stages these two movements transformed into the lateral turning of the head. Trunk movement appeared on the 5th day, after the head lifting and bending, but before any movements in the limbs. Trunk movements included flexion and extension of the

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

Movements of the upper and the lower limbs began by the 6th day, starting at the root of the limb buds and progressing distally.

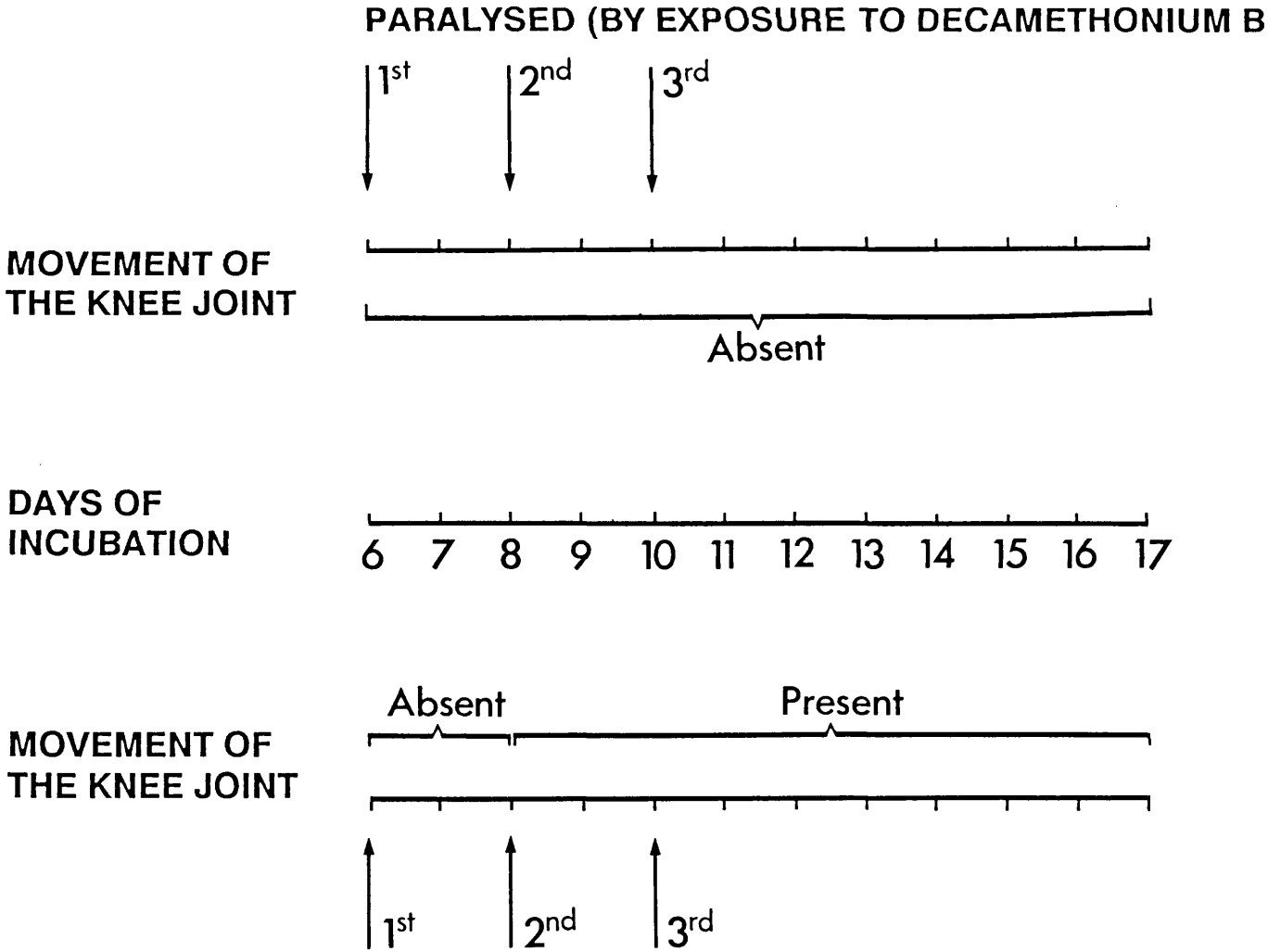
Flexion and extension of the knee and ankle joints began on the 8th day. On the 10th day, these movements extended to the toes which showed flexion, extension, abduction and adduction.

These embryonic movements were also phasic; phases of activity lasted for about 15 secs., and were followed by longer phases of inactivity, lasting between 25 and 30 secs., during which the embryo did not show any movement.

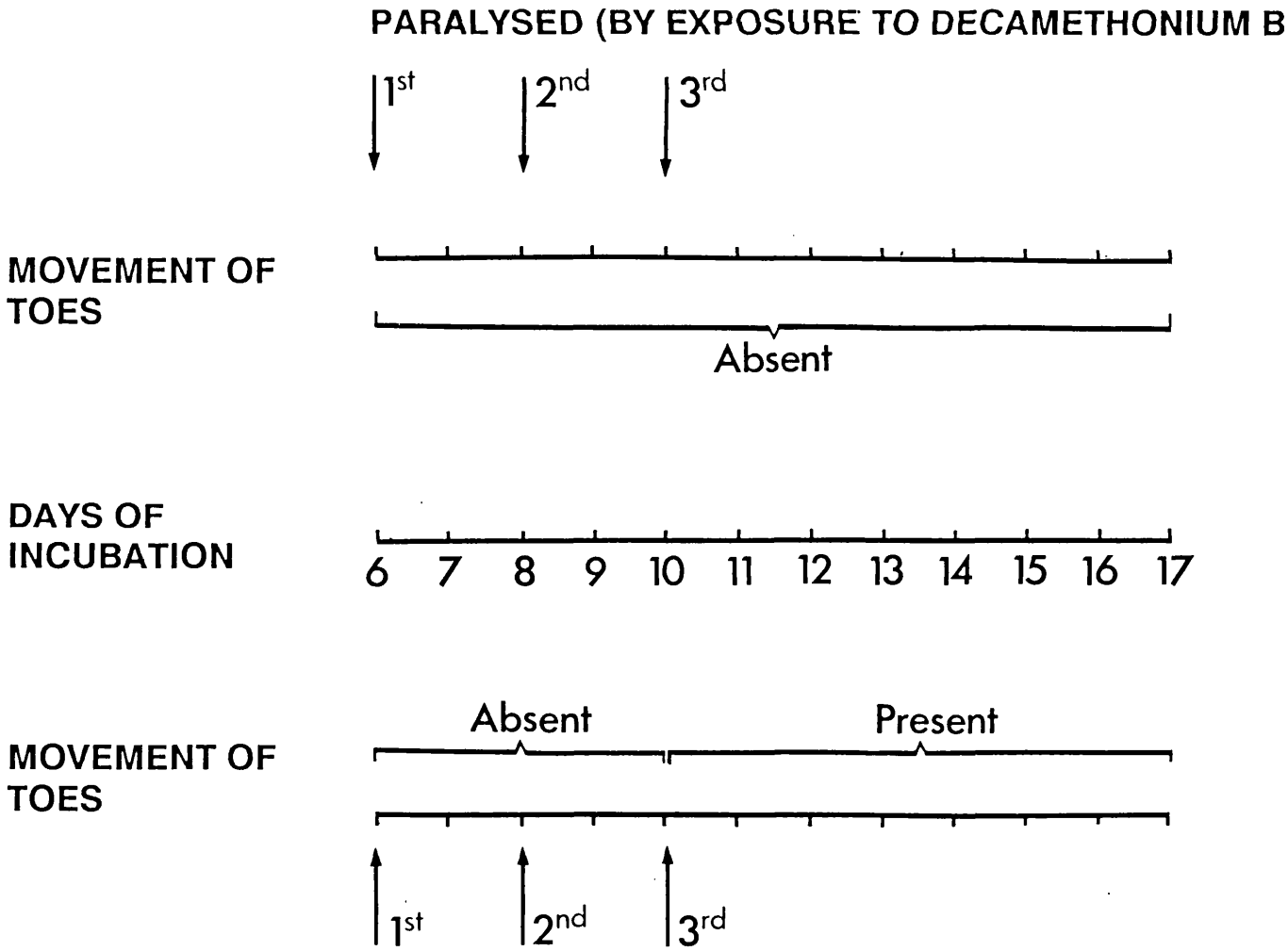
The duration of the activity phase gradually increased and that of the inactivity phase decreased, but the duration of the activity phase began to decrease at later stages. Although amniotic movement was independent of embryonic movement, movement of the limbs occasionally appeared to initiate amniotic movement.

In paralysed embryos, after the administration of decamethonium bromide, no movements were observed apart from those due to contractions of amnion and those of the chorionic vessels.

12 of a total of 182 embryos (7%) showed some movements after the first administration of decamethonium bromide, and they were therefore discarded. The timing of movements of the knee and the metatarsophalangeal joints in paralysed and control embryos are depicted in Text figs. 1 & 2.



Text. Fig. 1: shows timing of movement of the knee joint in paralysed and control embryos.



Text Fig. 2: shows timing of movement of the toes in paralysed and control embryos.

The totally paralysed embryos showed the following features:

a) Up to, and including, day 13, the developmental stages of paralysed embryos corresponded with those of controls. After 13 days the developmental stages of paralysed embryos lagged behind those appropriate to their chronological age.

b) oedema was observed beneath the skin of the abdominal region and around the thigh after day 10;

c) fewer feather germs developed in the oedematous region;

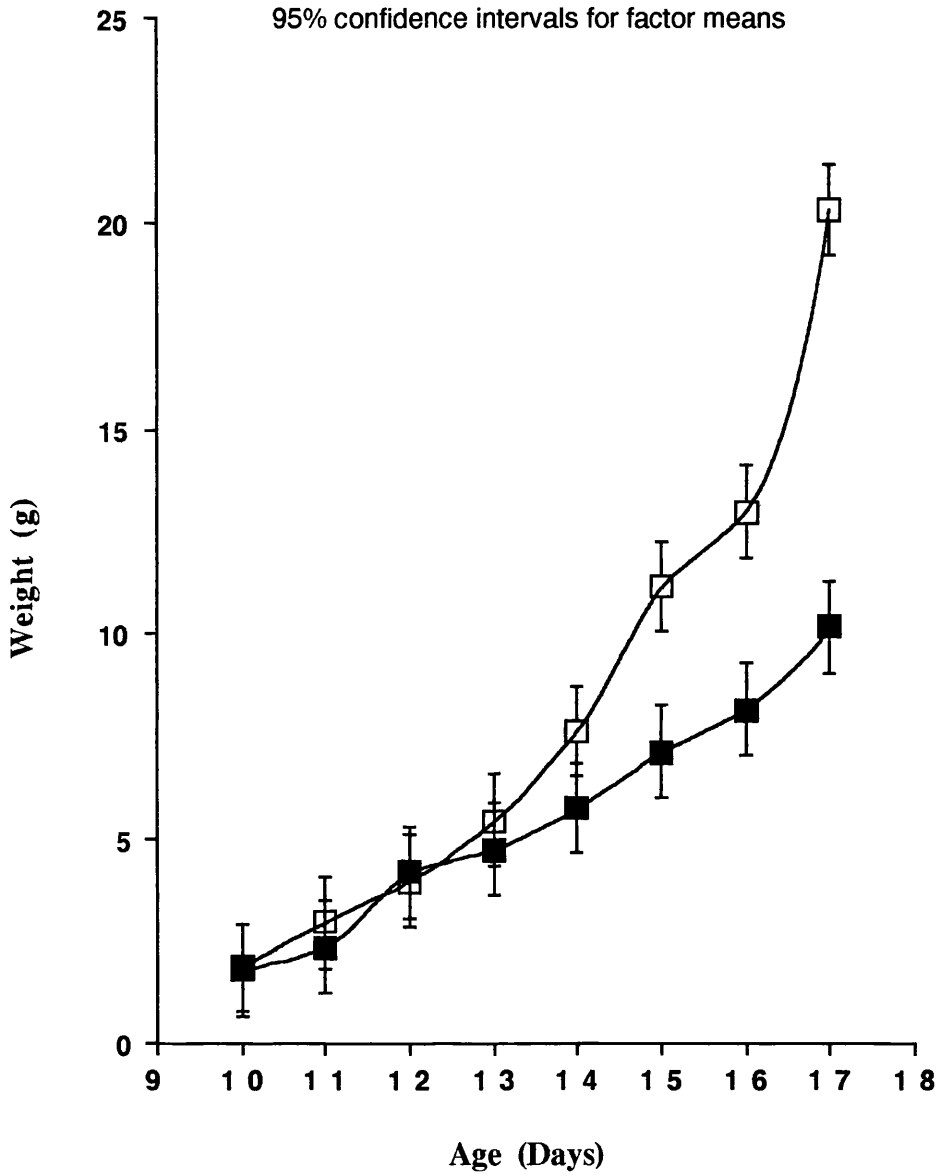
d) a bent and rigid neck was obvious from day 12 onwards, and was severe from day 15 onwards. The head was turned to the right. The rigidity of the neck was obvious when the paralysed embryos were suspended by their feet immediately after removal from the egg;

e) the vertebral column was S-shaped in some of the embryos.

f) the toes were in abnormal positions of either hyperflexion or hyperextension.

g) in later stages, paralysed embryos appeared shorter and wider; the upper beak was shorter than the lower, after 16 days of incubation.

In general the paralysed embryos were smaller than the controls. Their body weight was significantly less than that of controls from day 14 onwards. They were weighed <u>after</u> allowing the subcutaneous oedema fluid to escape. (Text. Fig. 3). (See detail in Appendix V).



Text Fig 3: Statistical comparison between the weight of the control (□) and paralysed (■) chicks.

Survival rate

The death rate was similar in the two groups: 9/88 (10%) of controls died during the experimental period, compared with 18/182 (10%) of the paralysed chicks.

II. General morphology of the lower limbs

The general morphology of the lower limbs was studied in the cleared whole-mounts of controls and paralysed embryos. Cartilage was stained blue with Alcian blue, and mineralized bone red with Alizarin red S. (Figs. 2-9).

In control and paralysed embryos at Stage 36 (10-11 days old), the femur, tibia, fibula, metatarsal and some of the proximal phalangeal cartilage elements showed a pale ring of perichondral ossification which was completely red at the next stage.

At Stage 38, control (13 days) and paralysed embryos (13-15 days) the appearances were similar to those at the previous stage.

In control embryos at Stage 39/40 (14-15 days) and paralysed embryos a mineralized periosteal collar was observed around the mid-diaphysis of the cartilage model of some phalanges which were more advanced in the paralysed embryos due to the older ages. The articular surfaces of the lower limb joints were reciprocally curved in controls, whereas they were flattened and showed fusion of the apposed cartilaginous epiphyses across the joint in paralysed embryos. (Figs. 2-9).

The patella and the plantar tarsal sesamoid cartilage were distinguishable in control embryos whereas, out of 27 paralysed embryos, only 2 specimens showed a patellar cartilage which was smaller than those in controls (Figs. 2-3) and the plantar tarsal sesamoid cartilage was not present at all (Figs. 6-7).

III. **Development of the knee joint**

Stage 30 controls (7 days)

The shafts of the femur, tibia and fibula were still cartilaginous but the chondrocytes were beginning to undergo hypertrophy, heralding the appearance of the primary centre of ossification.

A periosteal collar of bone was present around the mid-diaphysis of the shafts of the three cartilage models. The femoral and tibial condyles were prominent. The distal epiphysis of the femur and the proximal epiphyses of the tibia and fibula showed large cells with pale nuclei and eosinophilic cytoplasm, indicating the differentiation of precartilaginous cells at these sites. Towards the future articular surfaces of cartilage models these cells became flattened in a direction tangential to the surface, and gradually merged with the chondrogenous layers of the interzones, where the cells were more closely packed and elongated. The chondrogenous layers were continuous with the perichondrium at the periphery of the interzones.

The interzone showed mesenchymal condensations of the future menisci, which seemed to have developed in situ.

(Fig. 10). In the central area of the sectional profile of a meniscus, the cells had round to oval nuclei, while at its periphery they had flattened nuclei and were continuous with those of the chondrogenous layers of the blastema. Numbers of "dark cells" were seen between the lateral condyle of the femur and the lateral meniscus.

The posterior meniscofemoral and posterior meniscotibial ligaments, and the posterior and anterior cruciate ligaments were seen as condensations of mesenchyme which seemed to have developed in situ from the mesenchyme of the interzone (Fig. 11).

The posterior cruciate ligament arose between condensations of the posterior meniscotibial and posterior meniscofemoral ligaments and extended to the front of the intercondylar notch of the femur and the medial surface of the lateral femoral condyle. The anterior cruciate ligament arose from the front of the intercondylar area of the tibia under cover of the transverse tibiofibular ligament and passed upwards and backwards, lateral to the posterior cruciate ligament, to be attached to the back of the femur above the sharp medial ridge of the lateral femoral condyle.

The tibialis anterior tendon, which arises, in the chick, from the lateral condyle of the femur and descends through the knee joint, was clearly differentiated and could be traced through the loose, vascular tissue deep to the quadriceps tendon (Fig. 12). The tendon of the ambiens muscle was seen immediately in front of the origin of the

tibialis anterior tendon; it ran downwards and traversed the ligamentum patellae, from the medial to lateral.

The vascular mesenchymal tissue deep to the quadriceps tendon was more densely cellular and less vascular medially, in the site of future development of the patella.

Blood vessels were seen in the intercondylar fossa and at the periphery of the interzone.

Stage 30 paralysed (7 days)

The appearance of the cartilage models and of the interzones was, in general, similar to that in Stage 30 controls, except that the chondrogenous layers of the interzone between the femur and fibula had not developed, and the interzone here was a dense cellular and avascular interzone, with cells uniformly distributed, without any special orientation. The menisci (Fig. 13) and the cruciate ligaments (Fig. 14) were identifiable as orientated condensations within the mesenchyme of the interzone, but the tendons of tibialis anterior and ambiens muscles had not differentiated.

A few dark cells were observed between the lateral condyle of the femur and the lateral meniscus.

Occasional mitoses were seen throughout the interzone and at the precartilaginous portions of the cartilage models.

Stage 31 control (7 days)

The appearances were generally similar to those found in Stage 30 controls. The chondrogenic layers of the

interzone were still formed by closely packed cells, elongated tangentially to the surfaces of the adjacent cartilages. The interzone was still homogeneous, especially that between the femur and fibula. "Dark cells" were observed as before between the lateral condyle of the femur and the lateral meniscus, and, at some new sites: between the lateral condyle of the tibia and the lateral meniscus, and, occasionally, between the medial condyle of the femur and the medial meniscus and the medial condyle of the tibia and the medial meniscus.

Stage 31 paralysed (7 days)

The appearances were generally similar to those found in Stage 31 controls. The interzones were homogeneous and relatively more compact in comparison to controls. A small number of dark cells were distributed within the interzone, in the same areas as controls. The early development of the tibialis anterior tendon was observed in this stage, but it was not as advanced as in controls (Fig. 15).

Stage 32 controls (8 days)

The first appearance of the joint cavity was seen at the anterior aspect between the lateral condyle of the femur and the lateral meniscus (Fig. 16). The lateral meniscus had started to separate from the articular surface of the femur at the site where cavitation had begun. Elsewhere the interzone was still homogeneous, especially that between the femur and fibula. Occasional "dark cells" were found scattered along the future articular surfaces of the femur and among the intra-articular ligaments. The

cruciate ligaments were more differentiated than in the previous stage (Fig. 17).

The precartilaginous condensation of the patella had differentiated from the mesenchymal tissue in front of the femur, and several of its cells were in mitotic division. On the anterior surface of the patellar condensation, there was a dense cellular condensation of the future quadriceps tendon and patellar ligament; posteriorly the patellar condensation was separated from the femur by loose vascular mesenchyme (Fig. 18).

The blood vessels were still closely related to the peripheral parts of the interzone.

Stage 32 paralysed (8 days)

The state of ossification of the cartilage models of the femur, tibia, and the fibula was similar to that in Stage 32 controls. The interzones were still homogeneous and similar to that in Stage 31 paralysed chicks.

"Dark cells" were seen within the interzone, especially between the lateral condyle of the femur and the lateral meniscus (Fig. 19) and in front of and among the cruciate ligaments (Figs. 20 and 21), but there was no evidence of cavity formation.

The precartilagenous condensation of the patella was not differentiated from the mesenchymal tissue in front of the femur, but the tendon of the ambiens muscle was observed within the patellar ligament (Fig. 22).

Blood vessels were present in the peripheral parts of

the interzone.

Stage 33 control (8½ days)

Ossification of the mid-diaphysis of the femur, tibia, and the fibula had progressed. The chondrogeneous layers of the interzones were formed by the layers of densely packed cells, orientated tangentially to the surface. In the middle part of the interzone between the femur and the fibula the mesenchyme had become looser, so that a three-layered interzone was first distinguishable at this site (Fig. 23). Elsewhere the interzones were still homogeneous.

"Dark cells" were found scattered throughout the interzone, but especially between the lateral condyle of the femur and the fibula, among the intra-articular structures and deep to the origin of the tibialis anterior tendon from the lateral condyle of the femur. The joint cavity between the lateral condyle of the femur and the lateral meniscus was well developed and the first signs of cavitation were beginning to appear on the posterior aspect of the joint, between the medial condyle of the femur and the medial meniscus and the medial condyle of the tibia and the medial meniscus (Fig. 24).

Blood vessels were still present in the peripheral parts of the interzone, and in the posterior aspect of the femoral intercondylar notch they had begun to penetrate the superficial layer of its perichondrium.

The patellar condensation was still precartilaginous and separated from the femur by the loose vascular tissue.

Stage 33 paralysed (8½ days)

The state of ossification of the mid-diaphyses of the femur, tibia and the fibula was similar to that of controls of Stage 33. A typical three-layered interzone had not yet differentiated between the lateral condyle of the femur and the fibula (Fig. 25). The interzone between the medial condyles of femur and tibia showed many "dark cells" lying in a layer between two layers of mesenchymal cells of the chondrogenous zones of femur and tibia (Fig. 26).

Elsewhere, few "dark cells" were found throughout the homogeneous interzone and there were no signs of cavity formation.

The patellar condensation had still not differentiated. Blood vessels were present in the peripheral parts of the interzone, and in the posterior aspect of the femoral intercondylar notch they had begun to penetrate the superficial layer of its perichondrium as in controls at the same stage.

Stage 34 controls (9 days)

The periosteal collars of the femur, tibia and fibula were thicker, through the formation of additional trabeculae of woven bone in between which lay vascular mesenchyme.

The intercondylar notch of the femur contained prominent blood vessels posteriorly. Blood vessels were also seen in the peripheral part of the interzone and adjacent to the bony attachments of the cruciate ligaments.

The superficial layers of the chondrogenous layers were tangential to the surface. The interzone between the lateral condyle of the femur and the fibula was similar to that at Stage 33 controls, and showed a loose intermediate layer with "dark cells" (Fig. 27). Dark cells were also detected along the borders of the menisci, of the cruciate ligaments and along the articular surfaces and the posterior aspect of the origin of the tibialis anterior tendon (Fig. 28).

Cavitation between the lateral condyle of the femur and the lateral meniscus, between the medial condyle of the femur and the medial meniscus and between the medial condyle of the tibia and the medial meniscus showed further development. It was also occurring between the lateral condyle of the tibia and the lateral meniscus, in front of the cruciate ligaments, around the tendon of the ambiens muscle, and on the posterior aspect of the origin of the tibialis anterior tendon (Fig. 29). It was not present around the intra-articular ligaments which were still embedded in loose mesenchymal tissue. All of these cavities contained remnants of the loose tissue and were lined by "dark cells".

The patellar condensation was better developed, but in the absence of a perichondrium, its limits were not clearly defined. A thin layer of loose, vascular mesenchymal tissue was still situated between the patellar condensation and the femur.

Stage 34 paralysed (9 days)

The stage of ossification of the femur, tibia and the fibula was similar to that in Stage 34 controls. The intercondylar notch of the femur and the peripheral tissue of the interzone were also as vascular as in controls.

The interzone between the lateral condyle of the femur and the fibula had still not differentiated into a three-layered interzone. "Dark cells" were present and some of the cells within the interzone were beginning to differentiate into chondroblasts (Fig. 30). "Dark cells" were also found elsewhere throughout the interzone, except around the cruciate ligaments and the tibialis anterior tendon (Fig. 31). However, there was no sign of cavitation within the interzone or around the tibialis anterior tendon.

The cruciate ligaments appeared thinner than in controls of the same stage and they were poorly demarcated from the cells of the chondrogeneous layers of the interzone.

The medial meniscus had begun to disintegrate and the mesenchymal cells within the interzone at this site showed further differentiation to cartilage cells.

The patellar condensation had still not differentiated.

Stage 35 control (10 days)

In most respects the knee joint was now a miniature replica of the adult joint. Blood vessels appeared to have penetrated the cartilage of the intercondylar notch of the

femur posteriorly, to form cartilage canals. The chondrogenous layers of the interzone showed further reduction in their thickness to 2 or 3 superficial layers of cells, which maintained their tangential orientation; deep to them the cells were arranged more randomly.

The intermediate layer of the interzone between the femur and the fibula showed further loosening associated topographically with "dark cells". Towards the medial side of the joint, a cavity had appeared between the lateral meniscus and the lateral condyle of the femur above and the fibula below. Cavitation had extended further between the condyles of the femur and the tibia and both menisci (Figs. 32 and 33), around the tendon of the ambiens muscle and posterior to the tibialis anterior tendon. For the first time, cavities were also observed among the intra-articular ligaments, but there was no sign of cavitation on the posterior aspects of these ligaments, which were still associated with vascular mesenchymal tissue (Fig. 34). All of these cavities contained remnants of loose tissue and were bordered by "dark cells" (Fig. 33).

The patella showed early chondrification and had a perichondrium. Its anterior, superior, and posterior boundaries were densely cellular and distinct, whereas inferiorly it became indistinguishable from the infra-patellar tissue.

One specimen showed the appearance of the femoro-patellar joint cavity which was continuous above with the

loose vascular tissue of the supra-patellar area, and inferiorly with the main joint, and contained strands of tissue (Fig. 34).

Stage 35 paralysed (10 days)

In general, the appearance of the femur, tibia and the fibula was similar to that in Stage 35 controls.

The interzone between the lateral condyle of the femur and the fibula was still similar to that in Stage 34 paralysed chicks. Elsewhere the chondrogenous layers of the interzone were formed by 2 to 3 layers of the cells, which maintained their tangential orientation. Deep to them the cells had no regular orientation.

"Dark cells" were scattered throughout the interzone, except around the cruciate ligaments and the tibialis anterior tendon, but cavitation had not occurred in any part of the joint.

The cruciate ligaments were still distinguishable through the linear orientation of their cells, but there were no demarcation lines between them and the cells in the chondrogenous layers of the interzone.

The first sign of the fusion was detected between the medial condyle of the femur and the medial condyle of the tibia (Fig. 35). The anterior part of the medial meniscus had failed to develop and the interzone had differentiated to cartilaginous tissue; posteriorly, the meniscus and "dark cells" were conspicuous throughout the interzone (Fig. 36).

The tendon of the ambiens muscle was not clearly

defined within the patellar ligament.

The patellar condensation was just recognisable at this stage, but only in one specimen. It was smaller than in controls and there was no sign of the femoro-patellar joint cavity (Fig. 37).

Stage 36 control (10-11 days)

The centres of the shafts of the femur and tibia showed vascular invasion, whereas the shaft of the fibula still presented merely its periosteal bone collar, and additional trabeculae had not formed. The penetration of cartilage canals had progressed further in the intercondylar notch of the femur, and was also observed on the posterior aspect of the medial condyle of the tibia.

The articular surfaces of the bones showed three zones of cells; a deep zone of cells arranged in columns perpendicular to the surface' an intermediate zone of randomly arranged cells and a superficial layer of cells tangentially arranged.

"Dark cells" were found along the border of intra-articular structures especially around the cruciate ligaments (Fig. 38) and tibialis anterior tendon (Fig. 39).

The joint cavity was now complete in all parts of the joint, including the femoro-fibular (Fig. 40) and femoro-patellar joints (Fig. 41). Delicate tissue strands were seen crossing the knee and the femoro-patellar joint cavity, particularly near the centre of these joints.

The tendons of the tibialis anterior and ambiens

muscles were also surrounded by loose, sheath-like tissue, in which cavities had appeared.

All the intra-articular structures were identifiable: the menisci, the transverse ligament, the anterior and posterior meniscotibial ligaments, the posterior meniscofemoral ligament, and the cruciate ligaments were all identified (Figs. 42 and 38).

The gastrocnemius muscle appeared to take the place of the capsule posteriorly. The loose infra-patellar tissue, loose tissue posterior to the cruciate ligaments, the intra-capsular tissue in the peripheral parts of the joint, and the tissue surrounding the supra-patellar recess region, were all very vascularised and represented primitive synovial tissue or synovial mesenchyme.

Stage 36 paralysed (10-11 days)

The general appearance of the femur, tibia, and the fibula was similar to that at Stage 36 controls, but there was more progress in the extension of the cartilage canal within the intercondylar notch of the femur.

The interzone between the lateral condyle of the femur and the fibula was now formed by closely packed, parallel arrays of elongated cells which were continuous at the periphery with the perichondrium of the cartilage models. There were occasional blood vessels within the interzone (Fig. 43).

The distribution of "dark cells" was similar to that at Stage 35 paralysed and there were still no signs of cavitation within the interzone.

Some intra-articular structures, such as the medial meniscus and the cruciate ligaments had failed to develop completely or had disintegrated within the interzone (Fig. 44). The lateral meniscus was still identifiable, its borders outlined by "dark cells" (Fig. 45).

The tibialis anterior tendon was present but was thinner and more sinuous than in controls. Its borders were not lined by "dark cells" and no synovial cavity had developed around it. (Fig. 46). As at the previous stage the anterior aspect of the interzone between the medial condyle of the femur and the tibia showed cartilaginous fusion. The synovial mesenchyme was highly vascularized and the blood vessels occasionally penetrated into the interzone.

Stage 37 control (12 days)

The osseous trabeculae of the shaft of the femur and tibia showed a radiating pattern, while the fibula had merely its original periosteal collar.

Cartilage canals were observed in the medial and the lateral condyle of the femur and the medial condyle of the tibia.

The articular surfaces of femur, tibia and fibula appeared more condensed than at the previous stage, due to the compaction of the cartilage cells and the appearance of the collagen fibres between them.

"Dark cells" were no longer seen in the interzone. The joint cavity had become one continuous extensive space,

and showed a synovial lining with macrophages in some areas (Fig. 47). The projected synovial folds were also present in some areas of the joint cavity. The intra-articular structures were now fully developed (Fig. 48) and the central part of the menisci showed chondrocytes in lacunae. The patella was better defined and chondrification was advanced. The femoro-patellar cavity was still crossed by tissue strands, and showed a slight extension superiorly toward the supra-patellar recess.

Stage 37 paralysed (12 days)

The appearance of the osseous trabeculae of the shaft of the femur, tibia and the fibula was similar to those in Stage 37 controls. Numerous cartilage canals were observed at the distal end of the femur and proximal ends of the tibia and the fibula, and they occasionally extended towards the interzone.

"Dark cells" had disappeared within the interzone, while there was no evidence of cavity formation.

The interzone between the lateral condyle of the femur and the fibula showed flattened elongated fibroblast cells with numerous collagen fibres. Blood vessels and nucleated red blood cells were occasionally observed within the interzone, and some of the cells also seemed to be differentiating to cartilage cells (Fig. 49). Elsewhere the interzone also had progressively narrowed and showed fibrous fusion across the joint (Fig. 50) except the anterior aspect of the interzone between the medial condyle of the femur and the medial condyle of the tibia, which

showed cartilaginous fusion. The patellar condensation was not present in any of four specimens. The synovial mesenchyme was looser and more highly vascularized than in the controls, and showed early development of preadipocytes c.f. (Fig. 51 (normal), Fig. 52 (paralysed)). The bulk of the muscles around the knee joint was less than in controls, and most of the muscles were replaced by loose connective and adipose tissue. The remaining muscle fibres were atrophied and disorganized; sarcolemmal nuclei were increased in number and aligned in chains, or clumped in some areas so endomysial fibrous tissue was also increased. c.f. (Fig. 53 (normal), Figs. 54, 55 (paralysed)).

Stage 38 control (13 days)

There were several changes as compared with the previous stage:

1. Blood vessels were more numerous in the synovial mesenchyme and deep to the synovial lining cells, but there was no evidence of preadipocytes within the synovial mesenchyme;
2. the articular cartilage surfaces were reciprocally curved and showed more fine collagenous fibres;
3. all boundaries of the patella were well defined by the formation of the perichondrium;
4. the femoro-patellar cavity now extended superiorly to form the supra-patellar recess, but was still crossed by tissue strands;

5. cartilage canals were more numerous in the distal epiphysis of the femur and the proximal epiphysis of the tibia.

Stage 38 paralysed (13-15 days)

There were several changes as compared with Stage 37, paralysed:

1. Most of the muscle mass was replaced by connective and adipose tissue, and numerous macrophages and white blood cells were observed in the regions of the muscle degeneration (Fig. 56).
2. Blood vessels were more numerous in the synovial mesenchyme and adipose tissue was accumulating (Fig. 57);
3. Numerous white blood cells were also seen in the synovial mesenchyme (Fig. 58);
4. Numerous cartilage canals were present in the distal end of the femur and the proximal end of the tibia;
5. The articular surfaces were flattened and showed fibrous fusion across the joint, except at the site of the medial condyle of the femur and the tibia, which showed cartilaginous fusions. Two specimens showed almost complete cartilaginous fusion across the joint (Figs. 59, 60, 61).
6. The tibialis anterior tendon had disintegrated in some of the specimens (Fig. 62).

Stage 39 control (13-14 days)

The bony collar of the shafts of the femur and the tibia extended approximately to the level of the epiphysis. The fibular shaft showed invasion of blood vessels in its centre. Numerous cartilage canals were present in the distal epiphysis of the femur and the proximal epiphysis of the tibia and the fibula.

The menisci and the intra-articular ligaments showed more collagenous fibres in their structure.

The joint cavity showed more synovial villi and folds (Fig. 64). The femoro-patellar cavity and the supra-patellar recess were well formed, but still crossed by tissue strands. Preadipocytes were now seen in the infra-patellar tissue and in the subsynovial tissue at the posterior part of the joint (Fig. 63).

Stage 39 paralysed (16-17 days)

Cartilage canals had extended further in the distal epiphysis of the femur and the proximal epiphysis of the tibia and fibula. The bony collar of the shafts of the femur and the tibia extended approximately to the level of the beginning of the epiphysis and bone marrow was well developed in the diaphyses.

Most of the specimens showed cartilaginous fusion of the whole knee joint, even between the proximal end of the tibia and fibula. The perichondria of two cartilaginous epiphyses were continuous with one another at the site of the fusion (Fig. 65). Blood vessels were occasionally

observed at the site of the fusion. The tibialis anterior tendon was absent in some of the specimens. One out of four specimens at this stage showed a small cartilaginous patella, and the site of the femoro-patellar joint was replaced by the adipose tissue (Figs. 66, 67).

The synovial mesenchyme showed further accumulation of adipose tissue and white blood cells.

The patellar ligament was thin and disorganized and was invaded by blood vessels (Figs. 65, 67). The muscle mass of the lower limb had almost completely degenerated and been replaced by connective and adipose tissue.

IV. Development of the 3rd metatarsophalangeal joint

Stage 34 control (9 days)

The shaft of the metatarsal and of the proximal phalanx of the 3rd digit were cartilaginous and the chondrocytes were beginning to undergo hypertrophy. The perichondrium around the mid-diaphysis of the shafts of the two cartilage models showed periosteal ossification.

The distal end of the metatarsal and the proximal end of the proximal phalanx showed large cells with pale nuclei and eosinophilic cytoplasm, indicating the differentiation of precartilage at these sites. Towards the future articular surfaces of the cartilage models, these cells became elongated in a direction tangential to the surface, and gradually merged with the chondrogenic layers of the interzone. The chondrogenic layers were formed by elongated cells and were continuous with the perichondrium

at the periphery of the interzone. There was occasional mitotic cell division throughout the interzone and in the chondrogenic layers.

Dark cells were present between the chondrogenic layers but still a three-layered interzone was not recognizable (Fig. 68). Some of the "dark cells" seemed to have been engulfed by macrophages (Fig. 70).

Condensation of the joint capsule was seen at the periphery of the interzone.

Stage 34 paralysed (9 days)

The appearance of the cartilage models, and the interzone was similar to that found at Stage 34 in controls. "Dark cells" were seen throughout the interzone (Fig. 69).

Stage 35 control (10 days)

The general appearance was similar to that at Stage 34, but the condyles of the cartilage model of the 3rd metatarsal were more prominent. The tissue between the chondrogenic layers showed loosening and there were "dark cells" present, so that a three-layered interzone was now recognizable (Fig. 71).

Blood vessels were seen at the periphery of the interzone.

Stage 35 paralysed (10 days)

The appearance was similar to that of Stage 35 controls.

Stage 36 control (10-11 days)

Ossification of the metatarsal blastema was as advanced as that of the proximal phalangeal blastema of the 3rd digit and showed a thickening of the centrol bony cylinder with the formation of additional trabeculae, in between which were placed blood vessels. The chondrogenic layers of the interzone formed by two layers of cells, which were flattened in a plane tangential to the cartilage models.

The first sign of cavitation appeared within the loosening intermediate layer, at the periphery of the interzone (Fig. 72). "Dark cells" were not conspicuous. Blood vessels were more prominent at the periphery of the interzone.

Stage 36 paralysed (10-11 days)

The general appearance was similar to that of controls, but the intermediate layer, showed many "dark cells", but no cavity formation. As in the controls, blood vessels were present in the peripheral parts of the interzone (Fig. 73). A few specimens showed a small cavity at the periphery of the interzone which was invaded by the blood vessels (Fig. 74).

Stage 37 control (12 days)

The two cartilage models were similar to those at the previous stage. The chondrogenic layers were still formed by 2-3 superficial layers of flattened cells, deep to which the cells were more randomly orientated. The joint cavity had continued to develop but contained persisting tissue

strands (Fig. 75). "Dark cells" were seen in small numbers, lying along the free border of the articular cartilage.

The loose mesenchymal tissue at the periphery of the interzone had become more vascular, a sign of the differentiation of synovial mesenchyme from the general mesenchyme (Fig. 78). In the centre of the joint, there was still loose mesenchymal tissue with some blood vessels (Fig. 77).

Stage 37 paralysed (12 days)

There were now clear differences from the appearance in controls of Stage 37. The interzone was narrowed, in some placed to the point of disappearance, so that the apposed cartilaginous surfaces were in contact and were flattened, the boundary between them marked by a conspicuous line of "dark cells" (Fig. 79). The cells in the superficial layer of the chondrogenic zones were not tangentially flattened, as in controls but appeared as "plump" active chondroblasts, producing extracellular matrix. Where the interzone was wider, the intermediate layer showed some loosening of the tissue, but no obvious cavitation. Blood vessels and some extravasated free red blood cells were present in the peripheral part of the interzone, and also occasionally appeared within the interzone (Fig. 76).

Stage 38 control (13 days)

The centre of the shaft of the metatarsal bone showed vascular invasion whereas the shaft of the proximal phalanx presented only the periosteal bone collar. Blood vessels appeared to have penetrated the metatarsal cartilage in the dorsal and the plantar portions, to form cartilage canals. The articular surfaces were similar to those at the previous stage and showed a discontinuous lining of occasional "dark cells". The joint cavity showed further development, but some areas still showed tissue strands (Figs. 80,82).

The macrophages were occasionally found within the joint cavity.

Stage 38 paralysed (13-15 days)

The metatarsal and the proximal phalanx were similar to those of controls and the state of development of cartilage canals in the dorsal and the plantar surfaces of the metatarsal. The interzone remained very narrow and showed moderate numbers of "dark cells" (Fig. 81). There was no joint cavity, except that a few specimens showed small cavities at the periphery of interzone lining by the "dark cells" but had been invaded by the blood cells and the blood vessels (Fig. 83).

Stage 39 control (13-14 days)

The articular cartilage was formed superficially by two or three layers of the tangential and the flattened cells, deep to which the cells were still more randomly orientated.

The joint cavity was fully developed apart from occasional persisting strands of loose mesenchyme (Fig. 84).

Stage 39 paralysed (16-17 days)

There was now virtually complete cartilaginous fusion between metatarsal and proximal phalanx, although it was still possible to trace the line of the former interzone. "Dark cells" had disappeared in most specimens (e.g. Fig. 85), but one specimen still showed few "dark cells" at the site of joint fusion (Fig. 86). The synovial mesenchyme was invaded by numerous white blood cells (Fig. 85).

V. Histogenesis of the dead cells within the joint interzone

One of the main aims of this study was to assess the role and significance - if any - of "dark cells" in the development of the joint cavity. A previous study in the Department (Abu-Hijleh, 1987) had suggested that the "dark cells" appeared to be undergoing apoptotic cell death; that they appeared focally within the developing chick knee joint; that each focus of apoptosis was followed by a similarly focal development of part of the joint cavity. These observations were regarded as circumstantial evidence that programmed death of cells in the interzone was an initial cause of cavitation. The present study was designed to test this hypothesis by comparing the distribution and numbers of "dark cells" in two joints, one, the knee joint, large and complex, the other, the metatarsophalangeal smaller and simpler, in normal and paralysed chicks.

Detailed quantitative study was confined to the M-P joint, for the following reasons:

a) It is a simple condyloid joint whereas the knee joint in the chick consisted of the joints between three elements, the femur, tibia and fibula.

b) The knee joint is complicated by the presence of intra-articular structures, the cruciate ligaments and the menisci.

The numbers of "apoptotic" cells were determined per

millimeter square of the interzones of the 3rd metatarsophalangeal joint in both control and paralysed embryos and compared statistically. Text Fig. 4 (See detail in Appendix VI).

At Stage 34, the first "dead cells" were seen in the peripheral part of the interzones of both controls and paralysed embryos indicating that "dead cells" appeared independently of joint movement, a finding consistent with the view that cell death was due to intrinsic factors.

At Stage 35, there were still no significant changes in number and distribution of the "dead cells" in both control and paralysed chicks.

In control Stage 36, dead cells were reduced in number, as compared with the previous stage, but not significantly.

Joint cavity first appeared at the periphery of the interzone.

However, in paralysed chicks at Stage 36, the numbers of dead cells were increased significantly either in comparison to previous stages of paralysed or to control embryos, but joint cavity did not develop. Two specimens showed small cavities at the peripheral aspect of the interzone but were invaded by the blood vessels (Fig. 74).

At Stage 37 control, the number of dead cells showed a reduction from previous stage but not significantly. Cavity was well developed.

In the paralyzed same stage, dead cells showed no

significant increase in number compared to the previous stage of paralysed chicks, but still were significantly different in comparison with controls.

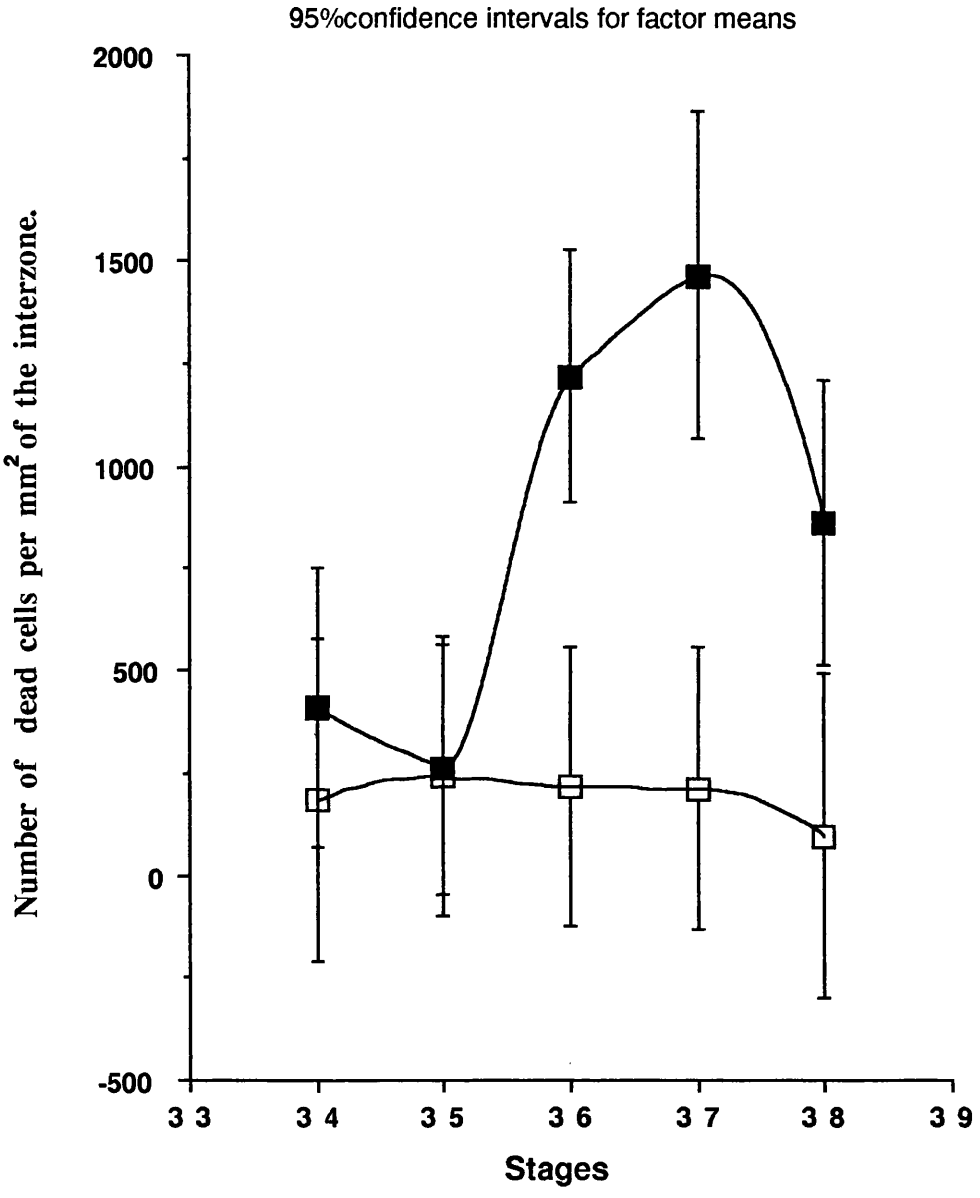
At Stage 38 controls, numbers of dead cells showed no significant changes in comparison to the previous stage.

In paralysed chicks at the same stage, dead cells showed no significant decrease in number compared to the previous stage, but were still significantly different in comparison with controls.

A few specimens showed some slight cavitation in the peripheral part of the interzone but they contained blood vessels and some extravasated red blood cells (Fig. 83).

At Stage 39, the controls showed no sign of dead cells and cavity formation was almost complete.

In paralysed chicks at this stage, the two cartilage models showed complete cartilaginous fusion. A few dead cells were present in only one specimen, scattered along the line of fusion (Fig. 86).

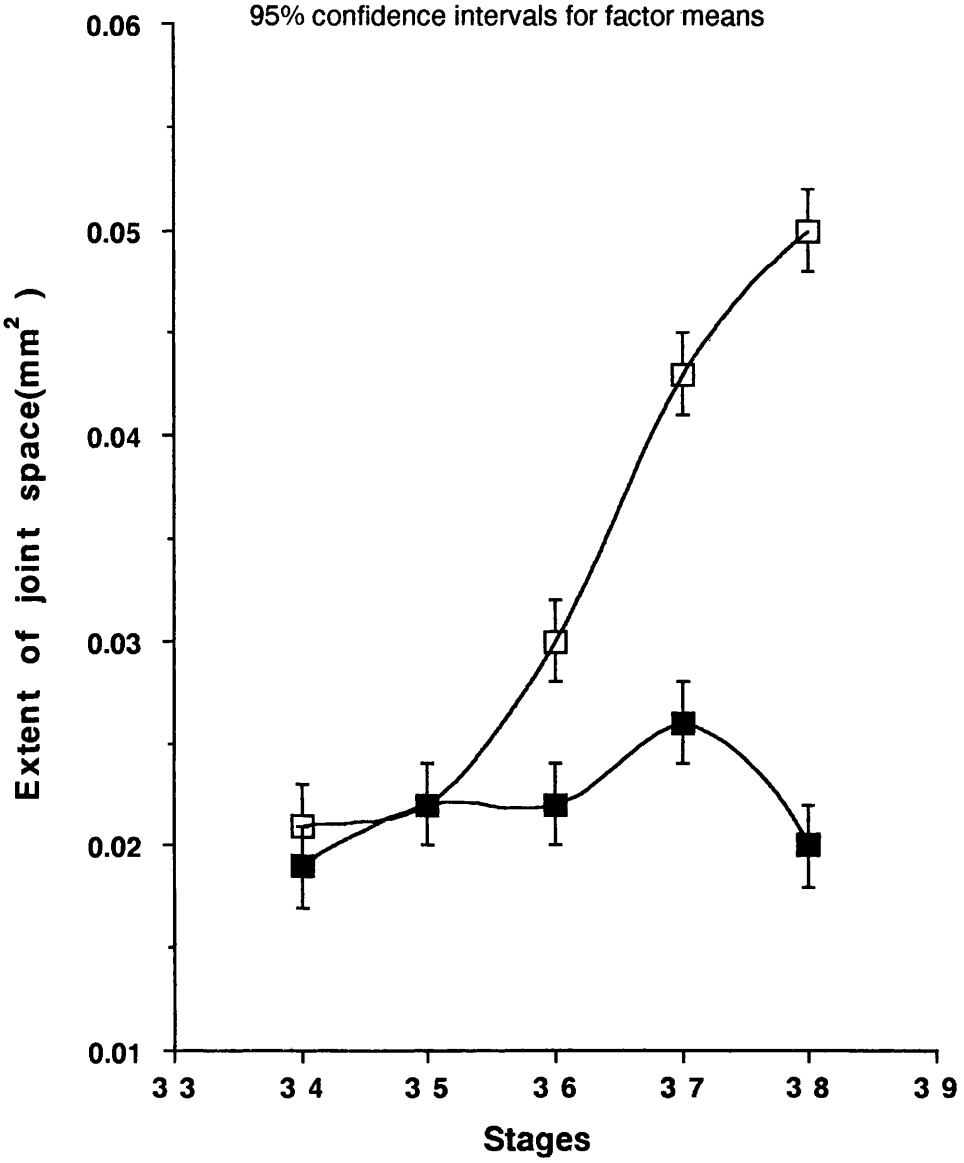


Text Fig 4: Statistical comparison between the number of dead cells in the interzone of the 3rd M-P joint in control (□) and Paralysed (■) chicks.

VI. **Extent of the joint space**

The mean value of the joint space (intermediate layer of interzone) of the 3rd metatarsophalangeal joint of controls and paralysed chicks were obtained in millimetre square and compared statistically. Text Fig. 5 (see detail in Appendix VII). Results showing significant reduction in the joint space of the paralysed chick from Stage 36 onwards which led to total fusion across the joint space at Stage 39.

The reduction of the joint space in the paralysed joint was coincident with the appearance of the first sign of joint cavity in control.



Text Fig 5: Statistical comparison between the joint space of control (□) and paralysed (■) 3rd M-P joint.

VII. Results of transmission electron microscopic studies

In view of the extensive changes seen by light microscopy in the joint interzone, the muscles and the synovial mesenchyme of paralysed embryos, it was decided to make a detailed study of these changes by the use of TEM.

1. Joint interzone

Two of the most important changes that took place in the interzone in paralysed chicks were cartilaginous and fibrous fusions. The 3rd metatarsophalangeal joint exhibited only the cartilaginous fusion, while the knee joint was affected by both types.

The events leading to cartilaginous fusion in the 3rd metatarsophalangeal joint were as follows:

a) Cartilaginous fusion

The 3rd metatarsophalangeal joint interzones were studied in control and paralysed embryos of H.H. Stages 34-38 and appearances were compared in the two groups in the same region at each stage.

In control embryos of Stages 34 and 35, the opposed surfaces of the cartilaginous epiphyses formed two chondrogenic layers, separated by an intermediate layer of looser mesenchyme. The mesenchymal cells of this loose intermediate layer showed certain characteristic features.

The nuclei were relatively large and palely stained. The nuclear matrix contained fibrillar and granular elements, and a very small amount of marginal chromatin. Each nucleus also contained one to three prominent electron dense nucleoli. The cytoplasm was relatively scanty and

contained a large number of free ribosomes, frequently arranged in rosettes, with a few membranes of endoplasmic reticulum. The mitochondria were numerous, with their matrix being mostly electron translucent. The Golgi apparatus was not prominent but lysosomes and lipid inclusions were frequently seen.

The intercellular spaces were almost translucent, but contained scarce aperiodic microfibrillar material and cell processes, which were frequently interconnected (Fig. 87). In other areas of the interzone, there were already signs of degenerative changes in some mesenchymal cells at the centre of the intermediate layer (Fig. 88).

Some showed extensive ballooning of the outer nuclear membrane. Their cytoplasm was also shrunken and darker staining (Fig. 88).

The appearances of the interzone of Stages 34-35 of paralysed chicks were indistinguishable from those found in control embryos.

In control embryos of Stages 36-38, the formation of cavities at the periphery of the joint was observed. These were initially small and separate, but later they coalesced to form a single cavity. The formation of initial cavities was not due to massive degenerative processes although signs of cell degeneration and macrophages were occasionally observed.

These cavities contained small amounts of microfibrillar material, cell profiles and elongated cells

with thin cytoplasm (Figs. 89a,b).

In the same Stages of the paralysed chicks, many cells of the loose intermediate layer showed various degrees of degeneration, but this did not seem to be associated with the same "loosening" of the mesenchyme which in controls was leading to formation of the joint cavity (Fig. 90). It is noteworthy that although in both control and paralysed chicks, degenerating cells were mainly confined to the central area of the intermediate zone, within that area there were also cells which appeared perfectly normal. The intercellular spaces contained sectional profiles of cytoplasmic processes of both normal and degenerating cells, and microfibrillar material.

Lipid droplets and remnants of nuclear membranes were occasionally seen in the intercellular spaces together with condensed nuclear fragments.

A particularly interesting finding was that some of the cells within the interzone were considerably enlarged, with large, pale ovoid nuclei, very prominent nucleoli and more compact cytoplasm.

In the light of subsequent events, these cells were interpreted as young chondroblasts (Fig. 91).

Developing blood vessels were also seen within the interzone. They were lined by a single continuous layer of endothelial cells, which lacked a basement membrane. As is usual in young developing capillaries, the endothelium was quite thick. It showed occasional fenestrae. (Fig. 92). In some paralysed chicks, there were small foci of

cavitation, with flattened darkly stained apparently degenerating cells arranged in a discontinuous layer along the margin of the space. The space contained a few extravasated red blood cells (Fig. 93).

Observation of Stage 39 controls revealed that the joint cavity was fully developed and electron translucent. The chondrogenic layers of the articular cartilage were densely cellular with flattened cells in the most superficial layer.

By contrast in paralysed chicks at Stage 39, the interzone had been transformed into cartilage, leading to fusion of the cartilaginous epiphyses of metatarsal and phalanx.

b) Fibrous fusion

Fibrous fusion took place only at the paralysed knee joint, typically between the lateral condyle of the femur and the tibia and fibula. The sequence of events leading to fibrous fusion has been followed in a series of Stages.

At Stage 36 of the paralysed chicks, the central area of the interzone contained many elongated spindle-shaped cells, orientated along the joint line. Many of these cells showed signs of degenerative change: very dark shrunken nuclei, ballooning of the outer layer of the nuclear membrane. These degenerating cells were interspersed with similarly spindle-shaped cells with large pale nuclei, prominent nucleoli. Intercellular space contained sectional profiles of cellular processes, debris

of degenerated cells and some microfibrillar material (Fig. 94).

At Stage 37 of the paralysed chicks, the interzone contained typical active fibroblasts, indicating fibrous fusion across the joint (Fig. 95). The cells are typical young active fibroblasts, with cigar-shaped, pale nuclei, with prominent nucleoli, and abundant cytoplasm with a large complement of rough endoplasmic reticulum. The bundles of collagen fibrils between them provide clear evidence of their activity.

Figs. 96a,b,c show a later stage in the process of fibrous fusion. All the cells are characteristic of actively synthetic fibroblasts, with small darkly stained nuclei, with prominent nucleoli and with the cytoplasm which shows abundant rough endoplasmic reticulum, filled with finely granular pre-secretory products. Some of the cells show very well the continuity between the distended sacs of rough endoplasmic reticulum and the space between inner and outer nuclear membranes. The intercellular space contains fine collagen fibres.

c) Cell death within the joint interzone

We can now summarize the various events in cellular degeneration within the interzone: an early stage of degeneration, cells showed increased density of the nucleus, and aggregation of chromatin material beneath the nuclear membrane ("margination"). The nuclear outline became very irregular as compared with the smooth contour

of normal nuclei. There was separation of the two layers of the nuclear membrane.

The cytoplasm also appeared more dense due to compaction of its organelles especially the free ribosomes. Mitochondria, the endoplasmic reticulum and the Golgi apparatus were swollen, leading to vacuolation of parts of the cytoplasm.

At more advanced stages of the degenerative process, the nucleus became shrunken, and irregular in shape, and so densely stained that details of their internal structure were obscured.

Nuclei at this advanced stage were surrounded by cytoplasm which showed deterioration of its organelles. Often only a thin rim of cytoplasm remained surrounding the nucleus (Fig. 97). Finally dead cells were found either dispersed in the interzone of both control and paralysed joints or phagocytosed by adjacent viable mesenchymal cells.

d) Phagocytosis of dead cells

In both control and paralysed joint interzones, dead cells were found either dispersed in the intercellular spaces or engulfed by adjacent viable mesenchymal cells, which later differentiated to a typical macrophage.

In some cases, apoptotic bodies phagocytosed by the adjacent mesenchymal cells, which were not a typical macrophage. (Figs. 98, 99).

In some cases, the ingesting cells can be considered as typical macrophages which contained many phagocytic

vacuoles. Red blood cells and the dead cells were distinguishable within those vacuoles and showed signs of digestion (Fig. 103).

In other cases, the macrophages also contained many phagocytic vacuoles but the dead cells were mostly at a later stage of digestion (Figs. 101, 102, 103).

2. **Muscle degeneration**

Muscles of the lower limbs began to degenerate about Stage 37 (12 days old) in the paralysed chick embryos.

At first, muscle fibres lost their regular longitudinal organisation and then began to be replaced either by loose connective or by adipose tissue. Some muscle fibres showed fatty degeneration and when compared with normal muscle fibres showed the following characteristics c.f. (Fig. 104 (normal), Fig. 105 (paralysed)).

The sarcolemmal nuclei were increased in number and were centrally located. They contained large and prominent nucleoli. Numerous lipid droplets were detected within their cytoplasm. Myofibrils were disrupted and the mitochondria and the Golgi organs were not clearly recognisable. The preadipocytes, macrophages, white blood cells which were mostly heterophil were present among the degenerative muscle fibres. Some of the macrophages showed phagocytic activity, and contained dense phagocytic material with lipid droplets. The fibroblast cells which synthesised collagen fibres were also present.

3. **Synovial mesenchyme of the paralysed joint**

The synovial mesenchyme of the paralysed knee and the 3rd metatarsophalangeal joints became looser and more highly vascularized than that of controls. At the later stages of paralysis it was invaded by the numerous heterophils, which showed the following ultrastructural features using TEM (cf. with description by Maxwell and Trejo (1970)) (Figs. 106, 107 and 108).

The heterophils were round with small pseudopodia and showed sectional profiles of up to 4 lobes of their nuclei. The heterochromatin was particularly dense towards the periphery of the nuclear lobes. The cytoplasm contained small, round or oval shape mitochondria distributed among dense heterophil granules. A few dense mature lysosomes were present, but occasionally it was almost impossible to differentiate between the smaller type of the heterophil granules and the lysosomes. The Golgi apparatus and rough or smooth endoplasmic reticulum were not recognized.

In the case of the paralysed knee joint, the adipose cells were also developed at the site of the infra-patellar fat pad, earlier than those of controls.

These adipose cells were large and variable in shape: some were elongated or rounded but many were polyhedral in sectional profile, and contained lipid which ranged from many small lipid droplets occupying a relatively small part of the total cytoplasmic area to several large lipid droplets, caused bulges in the cell surface (Figs. 109a,b,c).

At no stage did the lipid appear to coalesce complete to form the signet-ring cell characteristic of mammalian white fat. This difference from the mammalian pattern is well known and is thought to be associated with the presence of fine filamentous meshwork at the lipid droplet cytoplasm junction (Fig. 110).

The cell nucleus was first centrally located but later, due to the accumulation of the lipid droplets, lay at the margin of the cells, and it tended to take on a scalloped appearance, due to the pressure from the large lipid droplets.

At a later stage of the paralysis, even the endothelial lining of the blood vessels within the synovial mesenchyme contained lipid droplets and the boundary of the individual adipose cells, as a result of more compaction, were not fully recognizable from each other (Fig. 111).

CHAPTER FOUR

DISCUSSION

PREAMBLE

Despite extensive investigation of the development of synovial joints, reviewed in the Introduction, there remain areas of doubt and uncertainty. Some of these may be due to species (or even strain) differences; some to differences in different joints; some to postulations based on the study of normal material only, which do not allow assessment of the effects of movement, which requires the use of paralysed embryos; some to inadequate quantitation or microscopic technique.

The present study has attempted to resolve some of these areas of doubt and uncertainty by examining, in carefully staged embryos, the development of two joints of widely different structural complexity, the knee joint and the metatarso-phalangeal joint. The sequence and timing of major developmental events in these joints was compared in normal chicks with those in chicks in which <u>all</u> active movement had been prevented by the use of decamethonium bromide, from a time <u>before</u> that at which movement of the hind limbs normally starts, to a time when joint development is normally completed.

Similar studies of the normal development of the knee joint, in staged chick embryos, had previously been reported by O'Rahilly and Gardner (1956) and, in this Department, by Abu Hijleh (1987). It was felt to be necessary to repeat the study of normal development of the knee for two main reasons:

i. although there was broad general agreement on the chronology of development in the two studies, Abu Hijleh (1987) described extensive cell death in the interzone, and attributed to it considerable morphogenetic importance. O'Rahilly and Gardner (1956), on the other hand, had found signs of cell death in fewer than 2% of all their embryos and had therefore discounted its significance.

ii. it was decided to prepare a new series of control embryos, rather than to use the baseline provided by Abu Hijleh's (1987) results, in order to make a direct comparison between normal and paralysed chicks produced by incubation of eggs from the same batch, under identical conditions.

There has also been a previous study of the development of the metatarso-phalangeal joints of chicks (Mitrovic, 1977). It was repeated, not only to allow comparison between a simple and a complex joint, but to provide comparative quantitative data on the distribution and amount of cell death in normal and paralysed chicks. As Abu Hijleh (1987) had already found, this quantitation was very difficult in the knee joint, because of the presence of intra-articular structures which increased its complexity. It was hoped that the metatarso-phalangeal joint would provide a simpler model and this hope proved to be justified.

In addition to its main topic, this thesis has also recorded some observations on incidental effects of

paralysis: degenerative changes in paralysed skeletal muscle; the cytology of adipose cells which appeared in increased numbers in the synovial mesenchyme and at the site of degenerating muscles; general effects on the embryos.

Normal development of synovial joints

a) Knee joint

The youngest chick embryos examined in the present study were at Stage 30. For the sake of completeness, the appearances at earlier stages are referred to briefly, based on the study by Abu Hijleh (1987). He found that, in the normal knee joint, at Stage 27, chondrification had begun in the shafts of the three blastemata and the cells of the distal end of the femoral blastema and the proximal ends of the tibial and fibular blastemata gradually merged into the interzone region which appeared as an area of continuity between the three blastemata. The interzone was densely cellular, and avascular, and did not chondrify at any stage. This was in disagreement with Whillis (1940), O'Rahilly and Gardner (1956) and Andersen (1961, 1962a,b, 1963, 1964) who all suggested that there was a continuity between the cartilaginous elements due to the chondrification of the interzone.

By Stage 30, when the present study began, periosteal ossification had begun around the mid-diaphysis of the shafts of the femur, tibia and fibula and the chondrocytes in the shafts of the three blastemata had begun to

hypertrophy. The cells of the distal end of the femoral blastema and the proximal end of the tibia and fibular blastemata differentiated into precartilaginous cells which became elongated tangentiallly to the future articular surface and gradually merged with the chondrogenic layers of the interzone, where the cells were more closely packed and elongated. At the site of the future menisci, the cells of the interzone became rounded up and were readily distinguishable from the elongated cells of the chondrogenic layers. It is concluded that the menisci developed directly in situ, from the mesenchyme of the interzone. Similarly, the other intra-articular structures, such as cruciate ligaments, transverse tibiofibular ligament, posterior meniscotibial and the posterior meniscofemoral ligaments were also found to differentiate in situ from the interzone, at Stage 30. This was in agreement with those who studied the development of the intra-articular structures in birds, e.g. Hepburn (1889) and Abu-Hijleh (1987) and was also in agreement with those who studied it in mammals e.g. Hepburn (1889), Bardeen (1905), McDermott (1943), Andersen (1961), Schenck (1965), Rooket (1979) and Mitrovic (1978). It disagreed with Haines (1947) (human), Gray and Gardner (1950) (human), O'Rahilly and Gardner (1956) (birds) who studied the development of the intra-articular structures and claimed that they arose in two stages: in the first stage, the interzone was invaded by cells of the

peripherally placed future synovial mesenchyme, which in the second stage, gave rise to intra-articular structure.

Also by Stage 30, the tibialis anterior tendon, which arises in the chick from the lateral condyle of the femur and descends through the knee joint, was clearly differentiated from the loose vascular mesenchymal tissue deep to the quadriceps tendon. O'Rahilly and Gardner (1956) reported the beginning of the differentiation of the tibialis anterior tendon at Stage 27, while Abu-Hijleh did not identify it until Stage 29.

The condensation of the fibrous capsule, reinforced anteriorly by the quadriceps tendon and patellar ligament, differentiated from the general mesenchyme at the periphery of the joint interzone and was continuous with the perichondrium of the adjacent cartilage elements. It appeared before the formation of a joint cavity and of a typical three-layered interzone, which confirms Gardner and O'Rahilly (1959) and Schenck (1965), who also described the condensation of the fibrous capsule as appearing before cavity formation and in some cases, before the appearance of a three-layered interzone.

At Stage 32, the first sign of cavity formation appeared in the anterior part of the periphery of the interzone, between the lateral condyle of the femur and the lateral meniscus. This was in agreement with Mitrovic (1977, 1978) (chick and rat) and Abu-Hijleh (1987) (chick) but in disagreement with Andersen and Bro-Rasmussen (1961) (human), Wassilev (1972) (rat), Rooket (1979) (human) all

of whom found the first appearance of cavity formation to be within the central part of the interzone. These differing views may be due to differences in the species of animal used. It was also in disagreement with Haines (1947) (human) and Gray and Gardner (1950) (human), who claimed that the joint cavity first appeared more peripherally, but within the future synovial mesenchyme.

Of particular interest is the relation between the stage at which cavitation was first seen in normal chicks and the stage of onset of movement. The results of the present study are compared with those of 3 previous studies in the chick.

O'Rahilly and Gardner (1956), who studied only the knee joint, did not find cavitation until Stage 34 (9 days of incubation), although they detected movement of the joint much earlier, at Stage 30 (7 days of incubation).

Abu-Hijleh (1987) found early signs of cavitation in the knee joint at Stage 31 (7 days), but did not record the timing of first movement.

Llusa-Perez et al. (1988) found that the onset of movement and cavitation coincided, at Stage 35 (9 days).

In the present study, it was also found that movement and cavitation coincided but earlier, at Stage 32 (8 days).

The disagreements might be due to one or more of three factors: (i) variations in different strains of chicks used, (ii) the misinterpretation of artificial splitting within the interzone as cavitation, or (iii) to

misinterpretation of the possible early onset of embryonic movement, through the difficulty of telling whether onset of small movements were intrinsic to the knee, or a passive expression of movements at the hip, or of movements produced by contractions of the amnion. Because of these disagreements, it is clearly difficult to establish a relationship between movement and cavitation by studying only normal embryos. It is clearly essential to study paralysed embryos as well.

At Stage 33, the joint cavity between the lateral condyle of the femur and the lateral meniscus was well developed and very small cavities were beginning to appear in the posterior aspect of the interzone, between the medial meniscus and medial condyle of the tibia and femur. Also at this stage, a three-layered interzone (two chondrogenic layers and a loose intermediate layer) appeared between the lateral condyle of the femur and fibula, coinciding with, or following, cavity formation. This was in agreement with Abu Hijleh (1987) but disagreed with O'Rahilly and Gardner (1956) who did not find a typical three-layered interzone in the chick knee joint at any stage.

At Stage 34, joint cavities had also appeared in the posterior part of the interzone between the lateral condyle of femur and fibula, on the anterior and posterior aspect between the lateral condyle of tibia and the lateral meniscus, in front of the cruciate ligaments, around the tendon of the ambiens muscle, and on the posterior aspect

of the tibialis anterior tendon. All these findings agreed with those of Abu-Hijleh (1987), except on the timing of the cavity formation between the lateral condyle of the tibia and the lateral meniscus, which he found earlier, at Stage 33.

At Stage 35 cavities appeared in relation to the cruciate ligaments and, by Stage 36, cavitation was almost complete in all parts of the knee joint and all of the intra-articular structures were completely defined within the interzone.

That part of the mesenchymal tissue on the periphery of the interzone which was separated from the general mesenchyme of the limb by the fibrous capsule now became gradually vascularized and differentiated to form the synovial mesenchyme. This early synovial mesenchyme lined the joint cavity, except the articular surfaces of the long bones and the menisci, a finding which was in agreement with Haines (1947), Gray and Gardner (1950), Gardner and Gray (1953), O'Rahilly (1957), Mashuga (1964), Schenck (1965), Gardner and O'Rahilly (1968), Mitrovic (1978) and Abu-Hijleh (1987). It was in disagreement with Andersen (1961, 1962, 1963, 1964) and Wassilev (1972), who claimed that the synovial mesenchyme was laid down as a part of the interzone and was derived, like the latter, from the original skeletal blastema. At later stages the synovial mesenchyme included numerous macrophages in agreement with Andersen (1961, 1962, 1963, 1964), who also reported the

presence of mast cells and white blood cells which were not seen in the control specimens in the present study.

At Stages 39-40 (14 days), mesenchymal cells at the site of the future infra-patellar fat pad, which was now vascularized, began to accumulate fat droplets and differentiate into preadipocyte cells. This was at a stage earlier than that observed by O'Rahilly and Gardner (1956), who described this event as starting at Stage 44 (18 days). This difference might be due to strain differences in the chicks used, but not to the method of staining, because they used metachromatic staining as applied in the course of the present work.

b) Femoro-patellar joint

The patellar condensation was found to be separated from the femur at the outset by loose vascular mesenchymal tissue, in agreement with the findings of Haines (1947) (human), Gray and Gardner (1950) (human), Doskocil (1985) (human), O'Rahilly and Gardner (1956) (chick) and Abu-Hijleh (1987) (chick), but in disagreement with Walmsley (1940) (human), Andersen (1961) (human), Schenck (1965) (rabbit), all of whom found, between the patella and femur, a dense interzone, which passed through a typical three-layered stage. The femoro-patellar joint cavity began to develop at the time when the patella became cartilaginous, at Stage 35. This was in agreement with O'Rahilly and Gardner (1956) and Abu-Hijleh (1987), except on the timing of cavity formation, which they found later at Stage 36. Cavity formation in the femoro-patellar joint appeared

after the main knee joint cavity was almost completely established.

c) The 3rd metatarsophalangeal joint

At the beginning of the 5th day of incubation (Stages 26 and 27), the chick metatarsophalangeal joint was represented by a closely packed, homogeneous, avascular cellular tissue (Mitrovic, 1977 and Graig et al., 1987). At Stage 34 (9 days), the earliest examined in the present study, the mid-diaphysis of the metatarsal and proximal phalangeal cartilage models of the 3rd digits showed hypertrophic cells, and the onset of ossification in the surrounding periosteum. The cells in the distal end of the metatarsal and the proximal end of the proximal phalangeal cartilage models differentiated into precartilaginous cells which became flattened, tangentially to the surface and gradually merged into a typical 3-layered interzone at Stage 35. This result was in agreement with Mitrovic (1977), who also found a three-layered interzone, before cavity formation, but was in disagreement with Henrikson and Cohen (1965) and Graig et al. (1987), who did not find a three-layered interzone until the time of cavity formation, and also in disagreement with Gardner and O'Rahilly (1959) who found a three-layered interzone after cavity formation in the joints of the human foot.

At Stage 35, fibrillar material, similar to that described by Henrikson and Cohen (1965), Mitrovic (1977) and Graig et al. (1987), was detected within the

intermediate layer of the interzone. While Henrikson and Cohen (1985) and Mitrovic (1987) did not explain its significance, Graig et al. (1987) considered that a decrease in the amount of this material might provide an area of weakness, which might allow the production of cavitation through movement.

Although embryonic movement of the toes of the chick embryo has been reported as early as the 8th day by Kuo (1932a, 1932b, 1938, 1939), all other authors give a later time: on the 10th day by Beckham et al. (1977), and "after 10 days" by Dogra (1987). In the present study, movement of the toes was first seen at Stage 35 (10th day of incubation) preceding the onset of cavitation in the 3rd metatarsophalangeal joint, at Stage 36. This was in agreement with those authors (Graig et al., 1987; Mitrovic, 1977; Henrikson and Cohen, 1965) who found the joint cavity in the digits either at Stage 35 (10 days) or later.

These early cavities appeared in the periphery of the joint interzone, as in the knee joint; they soon extended towards the central part of the interzone and contained a small amount of microfibrillar material, and profiles of elongated cells with scanty cytoplasm as previously described by Henrikson and Cohen (1965).

Invasion of the interzone by blood vessels, or their development in situ within the interzone (Henrikson and Cohen, 1965; Mitrovic, 1977), were also observed in the present investigation at Stage 37. The presence of these blood vessels had been suggested as of possible

significance in cavity formation by Henrikson and Cohen (1965), but this is unlikely for three reasons: a) the blood vessels appeared after the first signs of cavity formation; b) they were not observed in the area in which cavitation first appeared; and, most significantly, c), they were also present in the paralysed joint, in which the cavity did not appear.

However, the present study indicated that these blood vessels did take part in the formation of the synovial lining with which they were later displaced to the periphery of the joint. At Stage 39, the articular surfaces became reciprocally curved and the joint cavity had completely developed.

Effect of immobilization on the development of the synovial joints

a) Knee joint

In the present study, paralysis of skeletal muscles from the 6th day of incubation (Stage 28) was found to produce the following effects on the development of the knee joint:

1) At Stage 30, the cells of the mid-diaphysis of the femur, tibia and fibula had begun to hypertrophy, and periosteal ossification developed around the mid-diaphysis of the three blastemata in a fashion entirely similar to

events in control embryos. This showed that the timing of onset of periosteal ossification was independent of movement, and fully confirmed the findings of Murray and Selby (1930), Hamburger and Waugh (1940), Drachman and Sokoloff (1966), Bradley (1970) and Yasuda (1973).

2) The cells of the proximal tibial and fibular epiphyses and of the distal femoral epiphysis differentiated into precartilaginous cells which became elongated in a direction tangential to the surface and gradually merged with the chondrogenic layers of the interzone, where the cells were more closely packed and elongated, in a fashion similar to that in controls. Between the lateral condyle of femur and fibula the chondrogenic layers did not form and the cells were uniformly distributed without any special orientation, unlike the controls.

3) The intra-articular structures, such as the menisci, cruciate ligaments, transverse tibio-fibular ligaments, posterior menisco-tibial and the posterior menisco-femoral ligaments first differentiated from the mesenchyme of the interzone at Stage 30, at the same time, and in the same manner, as in control embryos.

4) The tibialis anterior tendon began to differentiate from the loose vascular mesenchymal tissue deep to the quadriceps tendon, at Stage 31, later than in control embryos. The tendon of the ambiens muscle appeared within the patellar ligament at Stage 32 also later than that in ontrols.

5) The joint cavity, which normally first appeared at Stage 32, between the lateral condyle of the femur and the lateral meniscus, did not appear at all in the absence of movement. This finding is in agreement with those of Lelkes (1958), Drachman and Coulombre (1962), Drachman and Sokoloff (1966), Murray and Drachman (1969), Drachman et al. (1976), Ruano-Gil et al. (1978) and Persson (1983) none of whom observed cavitation in the absence of movement. It disagrees with the findings of Fell and Canti (1934), and of Hamburger and Waugh (1940) who observed early cavity formation in cultured chick limb buds and suggested that intrinsic factors were solely responsible for initial cavitation, but that embryonic movement was necessary for full differentiation and maintenance of the joint cavity.

At Stage 33, the interzone between the lateral condyle of the femur and the fibula had not differentiated into a typical three-layered interzone, unlike the control, and the cells, instead of becoming more loosely arranged, seemed to remain as active mesenchyme, and differentiated either into fibroblasts or precartilaginous cells.

6) At Stage 34, the tendon of the ambiens muscle and the tendon of the tibialis anterior muscle lost their normal arrangement and began to degenerate. The associated synovial cavity, which normally appeared at Stage 34 - Stage 36, completely failed to develop around the tendons. At Stage 35, the tendon of the ambiens muscle disintegrated completely within the patellar ligament and the tibialis

anterior tendon became "wavy" and began to disappear in some specimens from Stage 38. It is concluded that the initial differentiation of the tendons of the tibialis anterior and the ambiens muscle was independent of movement, but that muscular activity is required for their maintenance and the formation of their associated synovial cavities. This confirmed the conclusion of Bradley (1970), Beckham et al. (1977), Kieny and Chevallier (1979) and Dogra (1987), who studied the development of various tendons in the chick in the absence of the embryonic movement.

7) At Stage 35, the medial meniscus completely disintegrated within the interzone and the first sign appeared of cartilaginous fusion across the joint cavity, between the medial condyle of the femur and the medial condyle of the tibia. The rest of the intra-articular structures disintegrated completely during Stages 36 and 37, and were no longer recognizable within the interzone. This result was in agreement with Drachman and Sokoloff (1966), Persson (1983), Ruono-Gil et al. (1978) all of whom reported the absence of the intra-articular structures in the paralysed chick embryo, although none of them mentioned whether the primordia of the intra-articular structures formed. From the present results it is concluded that intra-articular structures develop autonomously from the mesenchyme of the interzone, but that their maintenance and further development is dependent upon movement.

8) Articular cartilage did not develop, and the interzone

became progressively narrower as compared with that of controls, from Stage 37 onwards. The articular surfaces became flattened and distorted compared to those of controls, which were reciprocally curved. Mesenchymal cells within the interzone now differentiated into fibroblasts, which were responsible for complete fibrous fusion across the joint. An early development of blood vessels was also detected within the interzone.

At later stages of paralysis, most of the specimens showed cartilaginous fusion. This can be interpreted in two ways: that either some mesenchymal cells remained inactive and later differentiated into cartilage cells or that fibroblasts differentiated into cartilage cells. A similar result was also reported by Hamburger and Waugh (1940), but other authors, such as Mitrovic (1982), Drachman and Coulombre (1962), Drachman and Sokoloff (1966), Murray and Drachman (1969), Bradley (1970), Drachman et al. (1976), Persson (1983), although they reported fibrous and cartilaginous fusion in the absence of movement in the chick embryo, did not clarify the fate of the fibrous fusion. Ruano-Gil et al. (1978, 1985) reported normal articular surfaces and the replacement of the interzone by undifferentiated mesenchymal cells in the joints of paralysed chicks, findings quite different from those observed in the present study.

In the paralysed knee joint, synovial mesenchyme differentiated from the mesenchymal tissue at the

peripheral part of the joint interzone, in association with invasion by blood vessels, in a manner similar to that in controls at Stage 36, but the synovial lining, synovial folds and villi did not differentiate, because of the absence of the joint cavity. This finding confirmed those of Mashuga (1964), who found the differentiation of the synovial membrane and villi in the joints of the mammalian embryos at the time of onset of embryonic movement and postulated that differentiation of synovial membrane and villi from the synovial mesenchyme depended upon the appearance of the movement in the joint. Continuing his work on the synovial membrane after birth, he reached the conclusion that the intensity of vascularization of the synovial membrane increased in line with an increase of dynamic loading on the joint, and, on the contrary, diminished in response to the conditions of partial or full immobilization of the joint. This finding was in disagreement with that in the present study, which showed that in paralysed embryos, blood vessels were more numerous within the synovial mesenchyme than that in controls. This divergence of view may be because Mashuga (1964) did not carry his experiments under the paralysed condition. However, the reason why blood vessels were more abundant in the synovial mesenchyme of paralysed embryos is still not entirely clear. It may be that the venous drainage was poor, due to the loss of muscle tone, and caused blood stagnation within the capillaries, thereby initiating further blood vessel formation.

9) In paralysed chicks, the infra-patellar adipose tissue began to differentiate from the mesenchymal cells around the blood vessels within the synovial mesenchyme at Stage 37, earlier than in controls, and started to accumulate fat very rapidly. Similar results were reported in denervated animals (Barnett, 1966). The reason why adipose tissue appeared earlier, and accumulated fat more rapidly, in paralysed embryos, is not clear and needs further investigation.

In addition to these changes, at the later stages of paralysis, further changes were observed: (i) the blood vessels within the synovial mesenchyme and within the joint interzone became more permeable, which could have been due to the release of histamine (decamethonium releases detectable amounts of histamine when administered in very high doses (Koelle, 1975)). (ii) Numerous white blood cells, mostly heterophils, invaded the surrounding tissue. (iii) On either side of fibrous or cartilaginous fusion across the joint, the perichondria of two cartilaginous epiphyses were continuous with one another. (iv) The patellar ligament and the fibrous capsule lost their regular arrangement and began to disintegrate, and the synovial mesenchyme became continuous with the general periarticular tissue, which was now mostly loose connective and adipose tissue, due to muscle degeneration.

In view of these findings, it can be concluded that the synovial mesenchyme can initially differentiate from

the mesenchymal tissue which was separated from the general mesenchyme by fibrous capsule, but that its further development depends upon the embryonic movement.

b) Femoro-patellar joint

In the majority of paralysed specimens, the patella did not differentiate within the mesenchyme in front of the femur, but a few specimens showed development of a small patella, without the appearance of a femoro-patellar joint cavity. Vascular mesenchymal tissue between the patella and the femur differentiated into the adipose tissue or invaded by blood cells at a later stage of paralysis.

c) The 3rd metatarsophalangeal joint

1. In paralysed embryos, periosteal ossification began to develop around the mid-diaphysis of the metatarsal and phalangeal cartilage models of the 3rd digits at Stage 34, as in controls. This result is similar to that already described above for the knee joint and reinforces the idea of those authors (Murray and Selby, 1930; Hamburger and Waugh, 1940; Drachman and Sokoloff, 1966; Bradley, 1970; Yasuda, 1973) who believed that the timing of onset of periosteal ossification was independent of movement.

2. As we have seen, a three-layered interzone did not differentiate at any stage in the paralysed knee joint, but in the metatarsophalangeal joint it differentiated at Stage 35, at the same time as in controls.

3. In most cases, a joint cavity did not develop. However, in a few specimens, at Stages 36-38, a small

cavity appeared at the periphery of the joint. These cavities were very soon invaded by blood vessels.

It cannot be concluded that joint cavitation, when present, had developed in the complete absence of movement, because the possibility exists that passive motion of the joints, produced by contraction of the smooth muscle of the amnion, (which was not, of course, affected by decamethonium), accounted for these findings. Whatever the stimulus for their formation, these incipient cavities give evidence that there are subsidiary mechanisms, in addition to skeletal muscular contraction, which aid in the formation of joint cavities.

4. Blood vessels developed within the interzone and, like developing capillaries elsewhere, they sometimes lacked basement membranes and were fenestrated.

5. The mesenchymal cells of the interzone began to differentiate into cartilage cells from Stage 37, and the interzone became progressively narrower. The articular surfaces became flattened and distorted as compared with those of controls, from which it can be concluded that embryonic movement is necessary to perfect the form of apposed bone ends, as suggested by Murray and Selby (1930), Hamburger and Waugh (1940) and Drachman and Sokoloff (1966).

6. Finally, the two bony elements were united by cartilaginous fusion at a late stage of paralysis. No fibrous fusion was observed in this joint, such as was seen in the knee joint.

Phenomenon of cell death within the synovial joint interzone

Cell death is a phenomenon which has been recognized within the synovial joint interzone by Fell and Canti (1934), Saunders et al. (1962), Dawd and Hinchliffe (1971), Wassilev (1972), Hinchliffe and Thorogood (1974), Rajan and Merker (1975), Rooket (1979) and described as necrosis by Mitrovic (1977, 1978, 1982) and, most recently, regarded as programmed cell death or apoptosis by Abu-Hijleh (1987).

In the present study degenerating cells were also observed within the joint interzone and showed similar cytological appearances within paralysed and control joints.

They were recognizable, even in wax sections, at medium power of magnification, by their small size and their darkly stained nuclei and cytoplasm. In semithin plastic sections, their nuclei showed condensation of chromatin and their cytoplasm appeared to contain clear vacuoles. With TEM it was evident that many of them were in various stages of degeneration. The earliest sign of degeneration was increased density of the nucleus and the aggregation of chromatin material beneath the nuclear membrane. Then the nuclear outline became very irregular as compared with that of normal nuclei. This irregularity of the nuclear outline was associated with separation of the two layers of the nuclear membrane, the outer layer ballooning out into the cytoplasm and forming what had

appeared to be cytoplasmic vacuoles in semithin sections. In more advanced stages of degeneration the cytoplasm appeared more dense due to compaction of its organelles, especially the free ribosomes. The mitochondria, endoplasmic reticulum and Golgi apparatus were swollen. At a more advanced stage of degeneration, the nucleus became shrunken, the nuclear chromatin became more concentrated beneath the nuclear membrane, the two layers of the nuclear membrane separated further and gave the appearance of electronlucent cytoplasmic vacuoles. Nuclei at this advanced stage were surrounded by only a thin layer of cytoplasm which showed deterioration of its organelles.

Finally these degenerative cells either disintegrated in the interzone in both control and paralysed chicks, or were ingested and digested by adjacent viable mesenchymal cells, which later differentiated to typical macrophages.

The cytological changes in these degenerative cells were similar to those described as occurring in embryonic development by many workers, including Glucksmann (1951), Behnke (1963), Weber (1964), Farbman (1968), Dawd and Hinchliffe (1971), O'Connor and Wyttenbach (1974), El-Shershaby and Hinchliffe (1974), Ojeda and Hurle (1975), Mitrovic (1977, 1978), Abu-Hijleh (1987).

The changes differed from those associated with necrotic cell death, as described by Wyllie et al. (1980, 1985), in the following respects:

a) degeneration of cells in the interzone did not affect all the cells at the same time; they were scattered

between apparently healthy mesenchymal cells;

b) there were no accompanying signs of inflammation, such as increased vascularity, vascular stasis, exudate of leucocytes;

c) they were phagocytosed by adjacent viable mesenchymal cells.

It is concluded that these degenerative changes in cells in the joint interzone bore a closer resemblance to those described elsewhere as programmed cell death or apoptotic cell death rather than to those associated with necrotic cell death.

Time and sites of appearance of cell death in the interzone of normal joints

In the present investigation, cell death preceded cavity formation in the interzone in both the knee and the 3rd metatarsophalangeal joints of control embryos, continued during the process of cavitation and declined and ceased when cavitation was complete.

In the knee joint, degenerating cells first appeared between the lateral condyle of the femur and the lateral meniscus at Stage 30 and then extended elsewhere in the interzone during Stages 34 and 35; by Stage 36 the numbers began to decline and they had disappeared completely by Stage 37. They were not observed at any stage between the patella and femur, confirming the previous finding of Abu-

Hijleh (1987). In the metatarsophalangeal joint a "first wave" of cell death had been observed at Stages 30-31 by Mitrovic (1977) but this was not studied in the present investigation. The "second wave" of cell death found at Stage 34 by Mitrovic (1977) was fully confirmed in the present study.

In the M-P joint, dead cells were confined to the periphery of the interzone. Although they were less conspicuous in the 3rd metatarsophalangeal joint than in the control knee joint, their numbers bore a similar relation to cavity formation; they first appeared before cavity formation, continued to be present during cavitation, and disappeared by Stage 39, when cavity formation was complete.

Time and sites of appearance of cell death in the interzone of paralysed joints

To my knowledge, no one, other than Mitrovic (1982), has mentioned the presence of dead cells within the joint interzone of paralysed embryos. Although he reported the presence he did not present any data regarding the timing and sequence of their appearance, or their numbers as compared with those of control embryos.

In the present study, statistical comparison between the number of the dead cells in the interzone of the 3rd metatarsophalangeal joint in control and paralysed chicks, showed that signs of cell death appeared initially in the paralysed interzone in numbers and distribution similar to

those in controls. However, a new and surprising finding was made, that the numbers of degenerating cells increased greatly at about the time when the first sign of cavity formation had been detected in control groups, and in the absence of cavitation in the paralysed joint. The reason for this increase in the numbers of degenerating cells in the metatarsophalangeal joint of paralysed embryos is not clear, but there are some possibilities:

a) decamethonium could itself be the cause of more degeneration of cells; or

b) that degeneration did indeed affect a greater number of cells in paralysed embryos; or

c) that phagocytic activity in paralysed chicks was less than in controls; or

d) that in the absence of movement, cell degeneration was a slower process and did not progress to complete disintegration in paralysed embryos, so that degenerating cells accumulated.

There is not sufficient evidence at present to say which of these is more likely: although the well-established mode of action of decamethonium makes (a) very unlikely. Possibly (c) could be tested by counting macrophages (more specifically stained e.g. for acid phosphatase) in normal and paralysed joints.

In the paralysed knee joint, degenerating cells were first seen, at Stage 30, as in control embryos, between the lateral condyle of the femur and the lateral meniscus.

After Stage 32 they were seen everywhere in the interzone except among the cruciate ligaments and along the margins of the tibialis anterior tendon, at which sites of dead cells were not detected at any stage.

Finally these cells disappeared within the interzone of paralysed embryos in the same stage of controls. Therefore it can be concluded that the times of their appearance, distribution and disappearance appear to be independent of movement.

Significance of cell death in relation to cavity formation

Fell and Canti (1934) found an area of degeneration in the region of the prospective knee joint of the chick embryo and called this area the opaque patch, because of its opacity to transmitted light in the living embryo. However, they did attribute significance to the opaque patch in development of the knee joint. Saunders et al. (1962) found a similar area at the elbow region of chick embryo and thought that it played an important role in the differentiation of the elbow joint.

Dawd and Hinchliffe (1971) and Hinchliffe and Thorogood (1974) suggested that cell death might play a role in the separation of the mesenchymal condensations for radius and ulna. Wassilev (1972) found that cavitation proceeded with degenerative changes of mesenchymal cells in the central part of the interzone. Mitrovic (1977, 1978) found a "first wave" of cell degeneration in an early stage

of the joint interzone and thought that this early cell necrosis might i) prevent chondrification of the interzone by getting rid of cells with chondroblastic potentialities; and ii) provide for loosening of the medial part of the interzone, leading to differentiation of a three-layered interzone. He saw a "second wave" of cell degeneration in the peripheral parts of the developing articular cavity, and suggested that cell death might play a role in the development of the joint cavity.

Abu-Hijleh (1987) found that what he regarded as "programmed cell death" preceded cavity formation in the chick knee joint and he also presented evidence that it was responsible for sculpturing of the articular surfaces and the intra-articular structures.

The present results, in the normal knee and the 3rd metatarsophalangeal joint, showed that cell death precedes cavitation, continues during cavitation, is present along the intra-articular structures and articular surfaces, and ceases once cavitation is complete. But from the results in paralysed embryos, it is clear that cell death is not the only factor necessary for cavity formation. Indeed, in the metatarsophalangeal joint, although degenerating cells were much more numerous in paralysed chicks as compared with controls, the cavities were either very small or were not present at all. Neither can it be said that cell death is solely responsible for sculpturing of the articular surfaces and the intra-articular structures. This sculpturing was defective in paralysed chicks, despite the

presence of dead cells in the interzone, in numbers similar to, or even much greater than those in normal joints.

Furthermore, it cannot be said that cell death prevents the interzone becoming cartilaginous, because cartilaginous fusion occurred in the interzones of paralysed joints, despite the presence of cell death.

Therefore the full significance of cell death within the joint interzone and its relation to cavity formation has still to be discovered. At present it seems likely that cell death is responsible for loosening of the mesenchymal tissue and therefore for the appearance of foci of cavitation within the interzone; wherever it was not present, in paralysed chicks as for example along the cruciate ligaments after Stage 32 and around the tibialis anterior tendon, the interzone never became looser and cavitation did not occur.

As the tissue of the interzone became looser, and small foci of cavitation appeared through death of cells, it seems that movement of the joint by muscular activity was necessary for the completion of the process of cavitation, for its continued maintenance and for the prevention of fibrous or cartilaginous fusion.

In a sentence, it is concluded that cell degeneration in the interzone is a necessary, but not a sufficient cause of cavity formation, for which movement is also required.

While the experimental evidence supports this conclusion as far as the knee joint and the metatarso-

phalangeal joint are concerned, it must be said that the conclusion is not supported by events in the formation of the femoro-patellar joint. The present study has confirmed the findings of a previous Ph.D. study in the Department (Abu-Hijleh, 1987) that cavitatation occurs in the normal femoro-patellar joint without any evidence of cell death. This means, of course, that the statement that cell death is a necessary cause of cavity formation is not of universal application. It may indeed be invalid, but there is the possibility that the femoro-patellar joint is "the exception which proves the rule". In support of this idea it can be said that development of the femoro-patellar joint showed two other features which distinguished it from the remainder of the knee joint:

1. from the outset, the patellar cartilage was separated from the femur by loose vascular mesenchyme, rather than by the densely cellular avascular interzone seen between femur, tibia and fibula;

2. when cavity formation began in the femoro-patellar joint, it occurred very much later than elsewhere, and by extension from the rest of the cavity of the knee joint.

Comparative sequential development of chick knee and metatarsophalangeal joints.

Sequential development of chick knee joint and metatarsophalangeal joint compared to previous investigators and summarized as follows.

(a) Chick knee joint:

Structures	O'Rahilly & Gardner (1956) Stage	Abu-Hijleh (1987) Stage	In present study
Homogeneous interzone	26	27	Not studied
Skeletal chondrification	27	27	
Quadriceps femoris muscle	27	27	
Tibialis anterior tendon	27	29	30
Ambiens tendon	29	29	30
Three-layered interzone	?	32-33	33
Patellar condensation	29-30	30 (not localised)	32
Diaphyseal ossification	28-29	30	30
Menisci	30	30	30
Cruciate ligaments	30	28	30
Embryonic movement	30	not studied	32
Foci of apoptosis localized between			
L.C.F. and L.M.		30-37	30-36
L.C.F. and fibula	"pyknotic cells"	31-37	33-36
L.C.T. & L.M.	found in only	31-36	31-36
M.C.F. and M.M.	2/125 embryos	31-36	31-36
M.C.T. and M.M.		31-36	31-36
Cavitation:			
L.C.F and L.M.		31 anterior	32 anterior
L.C.F. and fibula	femoro-meniscal	34 post	34 post
L.C.T. and L.M.	at stage 34	33 post	34 ant.&post
M.C.F. and M.M.		32 post	33 post.
M.C.F. and M.M.		33 post	33 post.
Patellar peri-chondrium	36	35	35
Chondrification in patella	36	36	35
Synovial tissue	36	36	36
Synovial folds and villi	37	37	37
Cartilage canals	35 femur 36 tibia	35 femur 36 tibia	35 femur 36 tibia
Infra-patellar fatpad	44	?	39-40

Embryos of all investigations have been staged according to Hamburger and Hamilton (1951).

L.C.F. = Lateral condyle of femur.

L.C.T. = Lateral condyle of tibia.

L.M. = lateral meniscus.

M.C.F. = Medial condyle of femur.

M.C.T. = Medial condyle of tibia.

M.M. = Medial meniscus.

(b) Chick metatarsophalangeal joint:

Structures	Mitrovic (1977) Stage	Graig et al. (1987) Stage	In the present study
Homogeneous interzone	26-27	26-27	Not studied
Three-layered interzone	34	35-37	35
Degenerative cell death	30-31 "first wave" ?		Not studied
	34 "second wave"		34
Cavitation	35-37	35-37	36

All staging according to Hamburger and Hamilton (1951).

Differences in the development of various normal joints

The major differences between the joints studied in the present investigation of control embryos are summarized as follows:

a) Commencement of a three-layered interzone

- In the knee joint it first appeared after the onset of cavity formation;
- In the femoropatellar joint, it did not appear at all;
- In the 3rd metatarsophalangeal joint, it appeared before the onset of cavity formation.

b) Cell death in the interzone

- In the knee joint it preceded cavitation, continued during cavitation, and ceased once cavitation is complete;
- In the femoropatellar joint, it was not seen at any stage;
- In the 3rd metatarsophalangeal joint it preceded cavitation, continued during cavitation, and ceased once cavitation was complete, but it was less conspicuous than in the normal knee joint.

c) Vascularity within the interzone

- In the knee joint it did not occur at any stage;
- In the femoropatellar joint, the patella was separated from the lower end of the femur by loose vascular mesenchymal tissue from the outset, and until cavity formation began;
- In the 3rd metatarsophalangeal joint it appeared after the commencement of cavity formation;

d) Relation of onset of cavity formation to the movement

- In the knee joint, the first sign of cavity formation was coincident with the appearance of movement;
- In the femoropatellar and the 3rd metatarsophalangeal joint, the first sign of cavity formation was preceded by the appearance of movement.

Development of the patella

In the present study, the primordium of the patella was found to develop behind the quadriceps tendon and patellar ligament. The quadriceps tendon was found to be inserted into the superior margin of the patella, and the patellar ligament began from the anterior aspect of the patella and descended towards the tibia. This result showed that the patella was blastemal in origin rather than an intra-tendinous sesamoid, a finding in agreement with those of Niven (1933), Brooke (1937), O'Rahilly and Gardner (1956), Andersen (1961), Schenck (1965), Doskocil (1985) and Abu-Hijleh (1987), but in disagreement with those who considered the patella as an intra-tendinous sesamoid bone e.g. Bardeen (1905), Walmsley (1940) and Haines (1947). When the patellar condensation appeared, the lower end of the femur had already chondrified and the two were separated by loose vascular mesenchymal tissue from the outset. This result fully confirms those of Haines (1947), Gray and Gardner (1950), O'Rahilly and Gardner (1956) and Abu-Hijleh (1987) but disagrees with that of Doskocil

(1985), who claimed that the anlage of the patella was associated at the very beginning of its development with the blastema of the lower end of the femur and separated from it at the early prechondral blastema stage by loose mesenchymal tissue.

Timing of onset of development of the patella

O'Rahilly and Gardner (1956) and Abu-Hijleh (1987) claimed that the mesenchymal condensation for the patella could be recognized at about the time of initial ossification of the femur (Stage 30) and prior to the onset of knee joint cavitation. The finding of the present study was that the patellar condensation began at Stage 32 within the mesenchyme in front of the femur, at the time of the first cavitation in the knee joint and that it began to chondrify and develop a perichondrium at Stage 35. Its anterior, superior and posterior boundaries were densely cellular and distinct, whereas inferiorly it became indistinguishable from the infra-patellar tissue. These findings confirmed those of Abu-Hijleh (1987), except in the time of appearance of the patellar chondrification, which he found appeared at Stage 36, later than our finding here. Niven (1933) found isolated patellar condensation from the surrounding tissue at the beginning of the 11th day (Stage 36); chondrification between the 11th and 12th days (Stage 37), and the anlage was surrounded by a perichondrium at the end of 12 days (Stage 38), which was considerably later than our finding.

Effect of immobilization on the development of the patella

Niven (1933) found that the patella did not differentiate in cultures of the whole knee joint. However, he considered this failure to develop due to inadequate nutrition, resulting from the large size of the explants, while in our experiment the knee joint was not isolated, and its nutrition was not obviously disturbed. Drachman and Sokoloff (1966), found that the patella developed in 5 out of 6 paralysed chicks within the quadriceps tendon, although it was smaller than normal. They concluded that embryonic movement was not necessary for differentiation of the patella.

The results of the present investigation are somewhat equivocal: 2 out of 27 cleared paralysed specimens showed the presence of the patella, which was smaller than that in controls. Histological paraffin wax sections also showed occasional differentiation of the patellar cartilage from the mesenchymal tissue behind the quadriceps tendon similar to those of controls, but smaller in size. In view of these new findings, it can be concluded that although embryonic movement is not essential for the initial differentiation of the patellar cartilage, it does encourage its further growth.

Cytology of the adipose cells

In the present investigation, the adipose cells either around the muscle degeneration or within the synovial mesenchyme of the normal and paralysed knee joints

showed similar cytological features. The cytoplasm contained numerous lipid droplets of various sizes which later coalesced and formed several large lipid globules, which caused bulges at the cell surface.

An interesting incidental finding was that, at any of the stages which were studied here, the large lipid globules did not coalesce completely to form the monolocular fat cell, so familiar in mammalian white fat presumably due to the presence of fine filamentous meshwork at the lipid cytoplasmic interfaces which were reported as membranous structures in mammals (Williamson, 1964; Rhodin, 1974).

These results were in agreement with Luckenbill and Cohen (1966) who studied the subsynovial adipose tissue in the ankle joint of the chick after hatching. Although these adipose cells remained multilocular, they were clearly different from the multilocular cells of mammalian brown fat, which are characterised by numerous mitochondria.

Effect of immobilization on skeletal muscle fibres

In the paralysed embryos, the bulk of the muscles of the lower limbs began to reduce from Stage 37 onwards compared to those of controls and they were eventually largely replaced by loose connective and adipose tissue. The muscle fibres were atrophied and lost their regular organisation; their nuclei were increased in number and

aligned in chains, or clumped in some areas, and were centrally located in the fibres; some muscle fibres showed accumulations of lipid droplets; myofibrils were disrupted and the cytoplasmic organelles lost their electron density; endomysial fibrous tissue increased and degenerating muscle fibres were phagocytosed by numerous macrophages and heterophils. These results were similar to those in studies of degenerating muscle fibres in cultured and paralysed limb buds of chick embryos; e.g. Hunt (1932), Eastlick (1943), Eastlick and Wortham (1947), Sullivan (1963, 1966, 1967), Drachman (1964), Drachman and Sokoloff (1966), Bradley (1970), Hall (1975). All of these authors concluded that embryonic movement was important for the maintenance of the muscle fibres.

General effect of paralysis

In the present study, the general effects of skeletal muscle paralysis were similar to those described by Murray and Drachman (1969), Sullivan (1966, 1967, 1971, 1973), Persson (1983) and Dogra (1987). The embryos were smaller and lighter in weight than controls, and their developmental status, based on external characteristics according to Hamilton & Hamburger (1951), were retarded, particularly after 13 days of incubation. The neck was bent to the right and the vertebral column became S-shaped. The lower beak was shorter than the upper beak in a majority of paralysed embryos at later stages. The digits became either hyperflexed or hyperextended. These

deformities, as described by Sullivan (1966, 1967, 1971, 1973), were probably caused by pressure from contact between the embryo and the actively contracting amniotic membrane. Dogra (1987) also reported protrusion of the cerebral hemispheres through the malformed skull bones; this was not observed in the present study.

After the 3rd administration of decamethonium bromide, the subcutaneous tissue of paralysed embryos became severely oedematous and feather germ development was retarded over the area of oedema. This oedema may have been due to one or both of the following possibilities:

a) deficiency of venous return due to skeletal muscle degeneration;

b) increased permeability of blood vessels, because of over-dosage of decamethonium bromide; over-dosage of decamethonium bromide is known to cause release of histamine (Koelle, 1975), which itself is the main cause of increased permeability of blood vessels.

CONCLUSIONS

In the Introduction (pp.90-91) a number of specific questions were asked. Answers to most of these have already been given at various places in Results and Discussion, but it is thought appropriate to answer each of them in turn at this point:

Q.i. In the absence of movement, does the initial stage of joint formation, a three-layered interzone, differentiate?

In the paralysed knee joint, a typical three-layered interzone is less obvious, mainly because the intermediate layer, between the two chondrogenic zones, does not undergo the same loosening as it does in the normal joint.

In the paralysed metatarso-phalangeal joint, on the other hand, a typical three-layered interzone is formed.

Q.ii. In the absence of movement, does cell death still occur and, if so, is this sufficient for cavity formation?

In both the paralysed knee and metatarso-phalangeal joints, cell death still occurs; indeed it has been shown to be increased in amount in the paralysed M-P joint.

Despite this, cavity formation is very limited and, particularly in the knee joint, is totally absent

in most embryos. Cell death is therefore not a sufficient cause of cavity formation, which also requires joint movement. (In the femoro-patellar joint, cell death is not seen in either normal or paralysed limbs, and it is therefore neither sufficient nor necessary for cavity formation).

Q.iii. In the absence of movement, what is the fate of the interzone?

In the paralysed knee and M-P joints, the interzone becomes narrower, and its surviving cells differentiate into cartilage cells (in the M-P joint) and, in the knee joint, to cartilage cells or fibroblasts. In each joint therefore, fusion occurs, either cartilaginous or fibrous.

Q.iv. Is there a single pattern of development in knee, femoro-patellar and M-P joints?

Although there is a common general pattern of development, there are clear differences in detail between the three joints.

a. In the femoro-patellar joint, the femur and patella are separated from the outset by loose vascular mesenchyme, in which cavitation occurs by extension from the cavity of the rest of the knee joint, without evidence of cell death.

b. In the knee joint, a three-layered interzone appears after the onset of cavitation, and it is vascular only at the periphery.

c. In the M-P joint, a three-layered interzone, precedes cavity formation. It is initially avascular, becomes vascularised by invasion and by in situ development of vessels, which, as cavitation progresses, are displaced to the periphery, where they persist in the synovial mesenchyme.

Q.v. Do intra-articular structures of the knee joint arise in situ from the interzone, or from general mesenchyme which secondarily invades the interzone from the peripherally placed synovial mesenchyme?

The evidence indicates in situ differentiation.

Q.vi. Does the synovial mesenchyme arise from the interzone or from the general mesenchyme around the joint interzone?

It arises from the general mesenchyme, which is cut off from the periarticular mesenchyme by the development of the capsule.

Q.vii. Is the origin of the patella independent of quadriceps tendon or is it an intratendinous sesamoid?

It develops independently of the quadriceps tendon, appearing posteriorly to it, rather than within it.

Q.viii. What is the role of movement in the development of each of the following?

a. articular cartilage: typical articular cartilage did not develop in the absence of movement.

b. intra-articular structures and joint capsules: in the paralysed knee joint, the primordia of all the

intra-articular structures appeared initially, but they failed to complete their development and eventually disappeared or disintegrated. Joint capsules also appeared but atrophied later.

c. synovial mesenchyme: initial development in paralysed knee and M-P. joints was similar to that in normal joints, but later it showed greater vascularity and, in the knee joint, the earlier appearance of the infra-patellar fat pad.

d. patella: this failed to develop in most embryos; when it did appear it was very much smaller than normal.

e. skeletal muscle: paralysed muscle underwent typical disuse atrophy and was replaced by adipose tissue and fibrous connective tissue.

f. tendons: The tendons of tibialis anterior and ambiens muscles initially developed but subsequently atrophied, and disintegrated and even disappeared. Their synovial sheaths did not develop at all.

APPENDICES

Appendix I: Clearing of cartilage and bone with Alcian blue and Alizarin red (Kimmel and Trammel, 1981).

Staining Solution:

Alcian blue, 0.14% in 70% ethanol	2 ml
Alizarin red, 0.12% in 95% ethanol	4 ml
Glacial acetic acid	2 ml
70% ethanol	50 ml

1. The paralysed and control embryos were skinned, rinsed in running water and stained with the above staining solution for 48 hours, changing solution with fresh stain after 24 hours.
2. Decant staining solution and replace with acetone for 3-4 days.
3. Transfer to 90% ethanol for 12-24 hours.
4. Then treat with 1% KCl from 6 hours to 24 hours. The paralysed embryos being fragile, were treated with 0.5% KCl. This made control of maceration and differentiation of tissue easier to regulate. Early stages of normal embryos were also treated similarly.
5. They were then cleared in increasing concentrations of glycerine 20%, 40%, etc. in 1% KCl. (Fragile ones were treated with 0.5% KCl).
6. Store in pure glycerine with crystal of thymol.

Appendix II: Processing for wax histology

Bouin's fixative

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

Staining with Myer's haematoxylin and eosin

1.	Xylene 1-2 hrs 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.	Meyer'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

The sections were then mounted in Histomount.

Appendix III: Processing for Semi-thin Histology

Buffered glutaraldehyde fixative (Sabatini et al. 1963).

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°C and used cold.

Phosphate buffered osmium tetroxide fixative (Millonig, 1961).

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°C.

Millonig's buffer (Millonig, 1961)

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 hours 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 oven 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 minutes

The sections were then mounted in Histomount.

Appendix IV: Processing for Transmission Electron Microscopy

A double staining method was employed, using uranyl acetate and lead citrate (Echlin, 1964).

Uranyl acetate:- a saturated solution of uranyl acetate in 40% ethanol was used.

Lead Citrate method (Reynolds, 1963):-

1. Lead citrate is prepared by mixing 1.33 gms lead nitrate and 1.76 gms sodium citrate and 30 mls of distilled water in a 50 mls volumetric flask.
2. The suspension was shaken vigorously for 30 minutes to ensure complete conversion to lead citrate.
3. 8.0 mls of N sodium hydroxide were added and the suspension mixed and made up to 50 mls with distilled water.

Staining method:-

Using a clean pipette, drops of uranyl acetate solution were placed on a clean piece of dental wax in a Petri dish. The grids, with the mounted sections on the dull side of the grid, were placed, dull side downwards, on to the stain for 1 minute. The grids were then quickly washed in water and allowed to dry in air.

Similarly drops of lead citrate solution were placed on to a piece of dental wax and sections stained for 1 minute and washed quickly in water. In order to avoid contamination of sections while staining with lead citrate, pellets of sodium hydroxide were placed in the Petri dish and the staining process was carried out under cover of

another Petri dish to prevent the formation of lead carbonate by reaction of lead citrate with atmospheric CO_2 on surface of sections.

Stained sections were viewed in the transmission electron microscope.

Appendix V: Table of means for the weight of embryos by days and treatment c=control and p=paralysed.

age(days)	n	mean	Stnd. Error (internal)	Stnd. Error (pooled s)	95% Confidence for mean	
10 c	5	1.85	.07	.56	.72	2.98
10 p	5	1.79	.04	.56	.66	2.92
11 c	5	2.97	.24	.56	1.84	4.10
11 p	5	2.35	.07	.56	1.22	3.47
12 c	5	3.95	.25	.56	2.82	5.08
12 p	5	4.17	.31	.56	3.04	5.30
13 c	5	5.43	.47	.56	4.30	6.56
13 p	5	4.74	.14	.56	3.61	5.87
14 c	5	7.63	.81	.56	6.50	8.75
14 p	5	5.74	.35	.56	4.61	6.86
15 c	5	11.17	.93	.56	10.04	12.29
15 p	5	7.12	.33	.56	5.99	8.24
16 c	5	13.00	.78	.56	11.87	14.12
16 p	5	8.17	.35	.56	7.04	9.30
17 c	5	20.35	1.39	.56	19.22	21.47
17 p	5	10.18	.37	.56	9.05	11.30
Total	80	6.91	.14	.14	6.63	7.19

Analysis of Variance for weight of embryos by days and treatment.

Source	Sum of Squares	d.f.	Mean square	F	p
MAIN EFFECTS	1589.30	8	198.66	124.62	.001
Days	1436.92	7	205.27	128.77	.001
Treatment	152.37	1	152.37	95.58	.001
INTERACTIONS	216.59	7	30.94	19.41	.001
Day & treatment	216.59	7	30.95	19.41	.001
RESIDUAL	102.02	64	1.59		
TOTAL (CORR.)	1907.92	79			

0 missing values have been excluded.

Appendix VI: Table of means for number of dead cells in the inter zone of the third metatarsophalangeal joint by stage and treatment. c=control and p=paralysed.

stage	n	mean	Stnd. Error (internal)	Stnd. Error (pooled s)	95% Confidence for mean	
34 c	3	184.33	85.61	193.37	-211.87	580.54
34 p	4	409.50	132.74	167.47	66.36	752.63
35 c	4	240.75	54.43	167.47	-102.38	583.88
35 p	5	261.40	35.01	149.79	-45.50	568.30
36 c	4	216.00	28.96	167.47	-127.13	559.13
36 p	5	1219.80	231.79	149.79	912.89	1526.70
37 c	3	112.00	13.05	193.37	-284.21	508.21
37 p	3	1466.66	497.40	193.37	1070.45	1862.87
38 c	3	96.00	10.26	193.37	-300.21	492.21
38 p	4	863.50	145.75	167.47	520.36	1206.63
Total	38	523.73	54.33	54.33	412.41	635.06

Analysis of Variance for number of dead cells in the inter zone of the third metatarsophalangeal joint by stage and treatment.

Source	Sum of Squares	d.f.	Mean square	F	p
MAIN EFFECTS	5814579.1	5	1162915.8	10.366	.001
stage	2109907.9	4	527477.0	4.702	.005
treatment	3853972.3	1	3853972.3	34.353	.001
INTERACTIONS	2235526.2	4	558881.55	4.982	.003
stage &treatment	2235526.2	4	558881.55	4.982	.003
RESIDUAL	3141224.1	28	112186.57		
TOTAL (CORR.)	11191329	37			

0 missing values have been excluded.

Appendix VII: Table of means for the joint space of the third metatarsophalangeal joint by stage and treatment. c=control and p= paralysed.

stage	n	mean	Stnd. Error (internal)	Stnd. Error (pooled s)	95 % Confidence for mean	
34 c	3	.02	.0015	.001	.01	.02
34 p	4	.01	.0010	.001	.01	.02
35 c	4	.02	.0016	.001	.02	.02
35 p	5	.02	.0010	.001	.01	.02
36 c	4	.03	.0012	.001	.02	.03
36 p	5	.02	.0008	.001	.02	.02
37 c	3	.04	.0026	.001	.04	.04
37 p	3	.02	.0005	.001	.02	.02
38 c	3	.05	.0008	.001	.04	.05
38 p	4	.02	.0004	.001	.01	.02
Total	38	.02	.0003	.0003	.02	.02

Analysis of Variance for the joint space of the third metatarsophalangeal joint by stage and treatment.

Source	Sum of Squares	d.f.	Mean square	F	p
MAIN EFFECTS	.002	5	.0004	74.170	.001
Stage	.001	4	.0002	45.813	.001
Treatment	.001	1	.0010	178.387	.001
INTERACTIONS	.001	4	2.81689E-004	46.772	.001
Stage &treatment	.001	4	2.81689E-004	46.772	.001
RESIDUAL	1.68633E-004	28	6.02262E-006		
TOTAL (CORR.)	.0035289	37			

0 missing values have been excluded.

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Effect of Paralysis of Skeletal Muscles on the Development of Synovial Joints in the Chick Embryo

By

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Volume II : Figures

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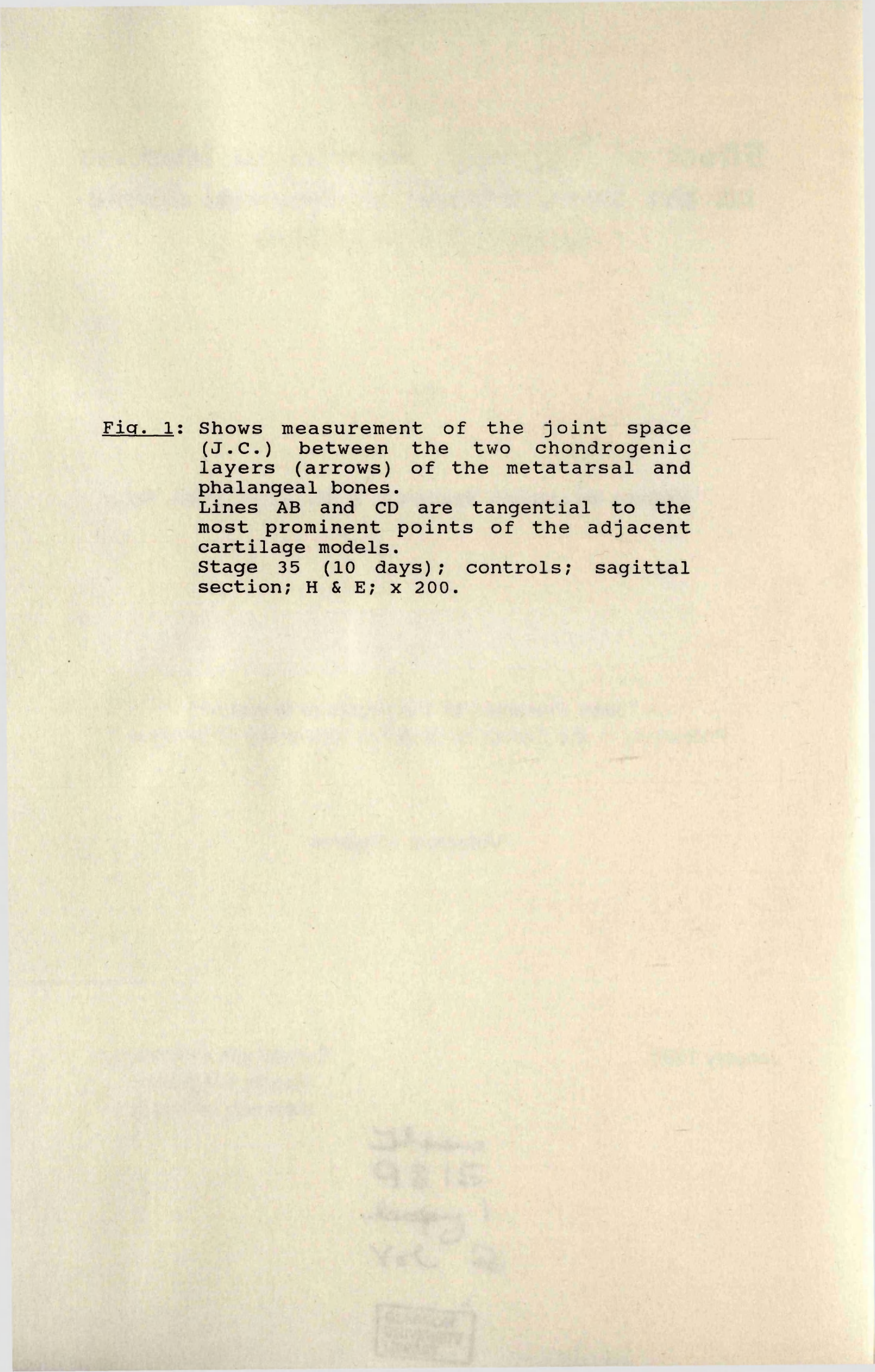


Fig. 1: Shows measurement of the joint space (J.C.) between the two chondrogenic layers (arrows) of the metatarsal and phalangeal bones.
Lines AB and CD are tangential to the most prominent points of the adjacent cartilage models.
Stage 35 (10 days); controls; sagittal section; H & E; x 200.

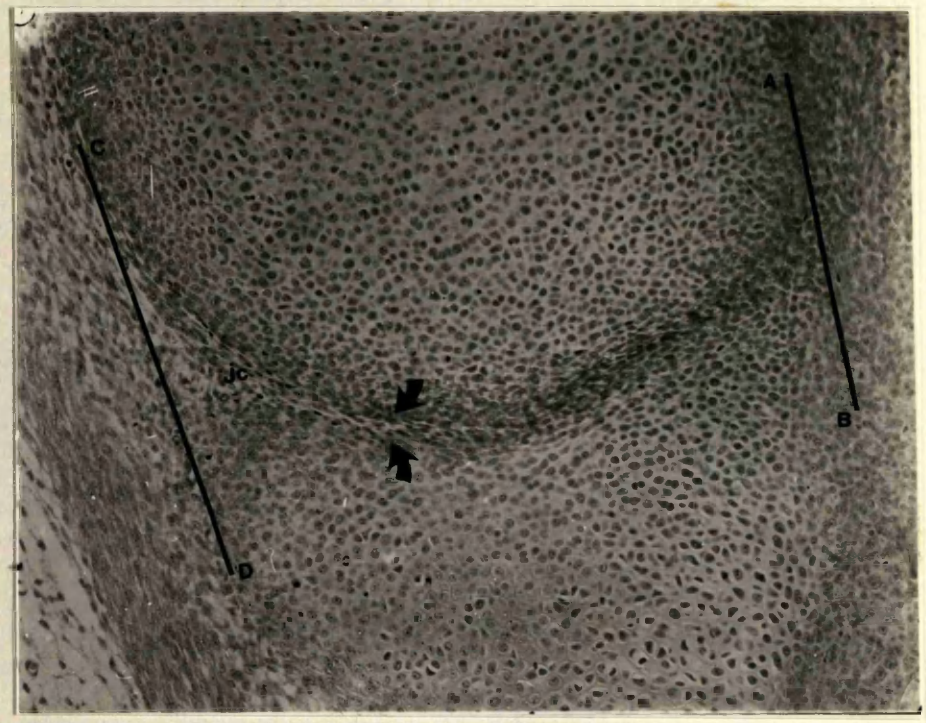
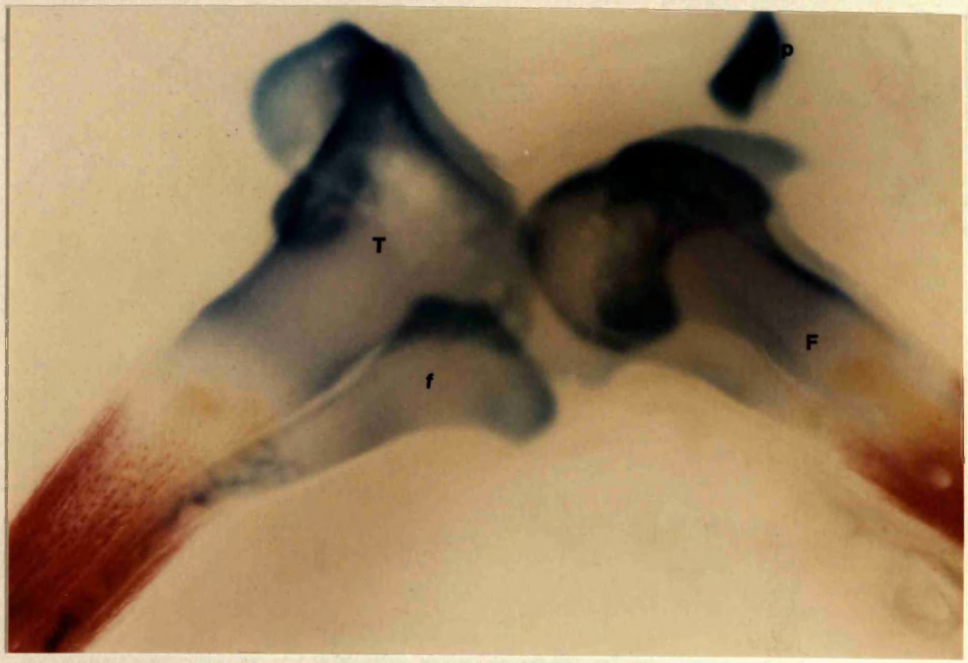

A
B
C
D
JC

Fig. 2: Normal chick knee joint, lateral view. Articular surfaces of the femur (F), tibia (T) and the fibula (f) are reciprocally curved. Patella (P) is still cartilaginous.
Stage 39 (14 days); control; Alcian blue, alizarin red. x 23.

Fig. 3: Paralysed chick; knee joint, lateral view. Articular surfaces of the femur (F), tibia (T) and the fibula (f) are flattened. Patella (P) is present, but very small compared with control (Fig. 2).
Stage 39 (16 days); paralysed; Alcian blue and alizarin red; x 23.


P
T
F
f

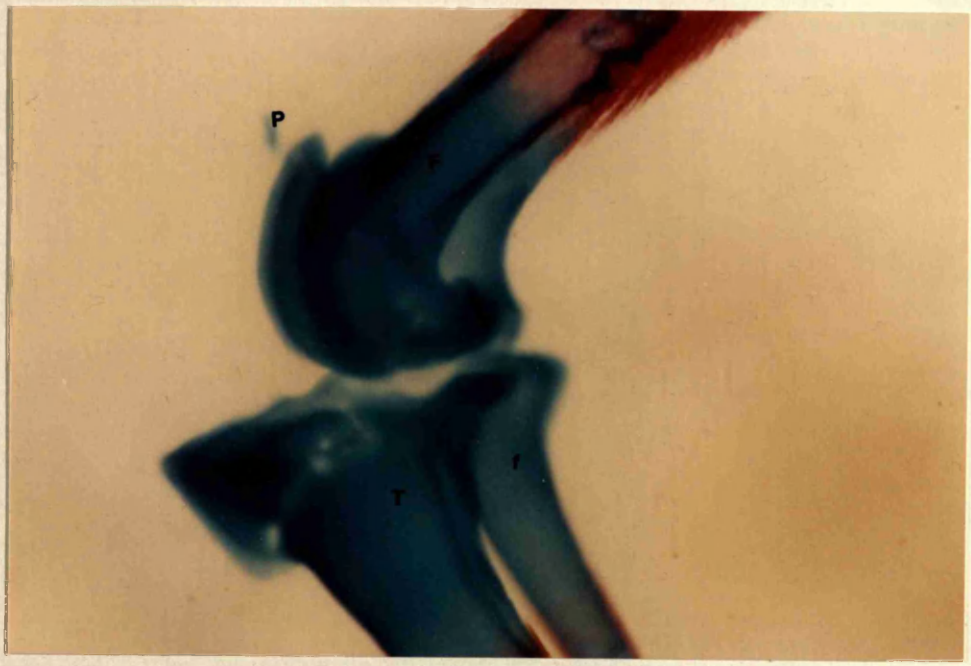
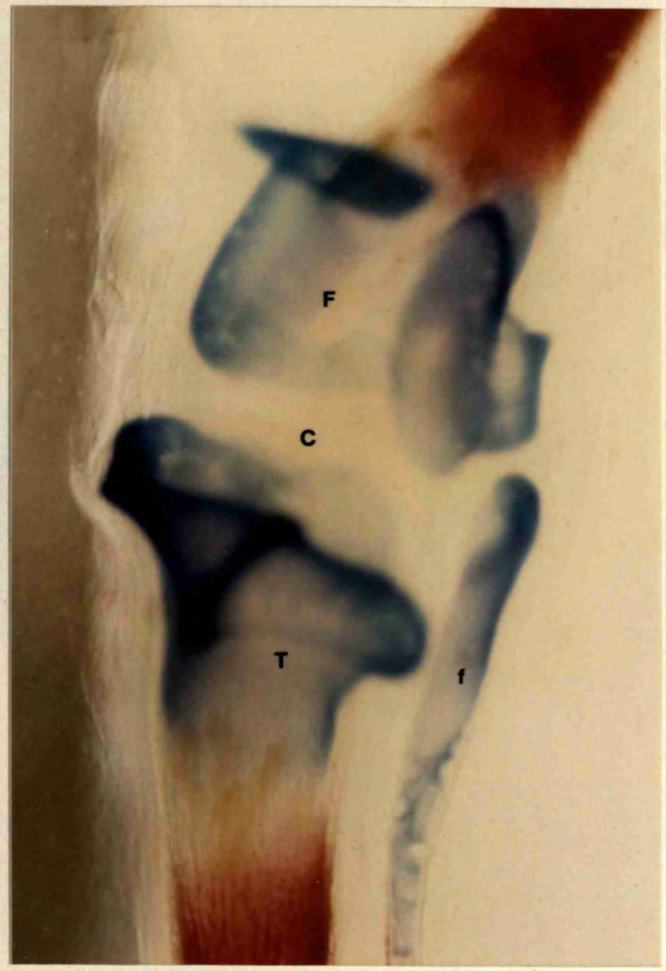

P
T
f

Fig. 4: Normal chick; knee joint, anterior view of specimen illustrated in Fig. 2. The joint cavity (C) is well developed between the femur (F), tibia (T) and the fibula (f).
Stage 39 (14 days); control; Alcian blue and alizarin red, x 23.

Fig. 5: Paralysed chick; knee joint, anterior view of specimen illustrated in Fig. 3. The joint space (J.C.) between femur and tibia is very much narrower than that in control (Fig. 4). There is complete cartilaginous fusion between the lateral condyle of the femur and the fibula.
Stage 39 (16 days); paralysed; Alcian blue and alizarin red. x 23.


F
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f

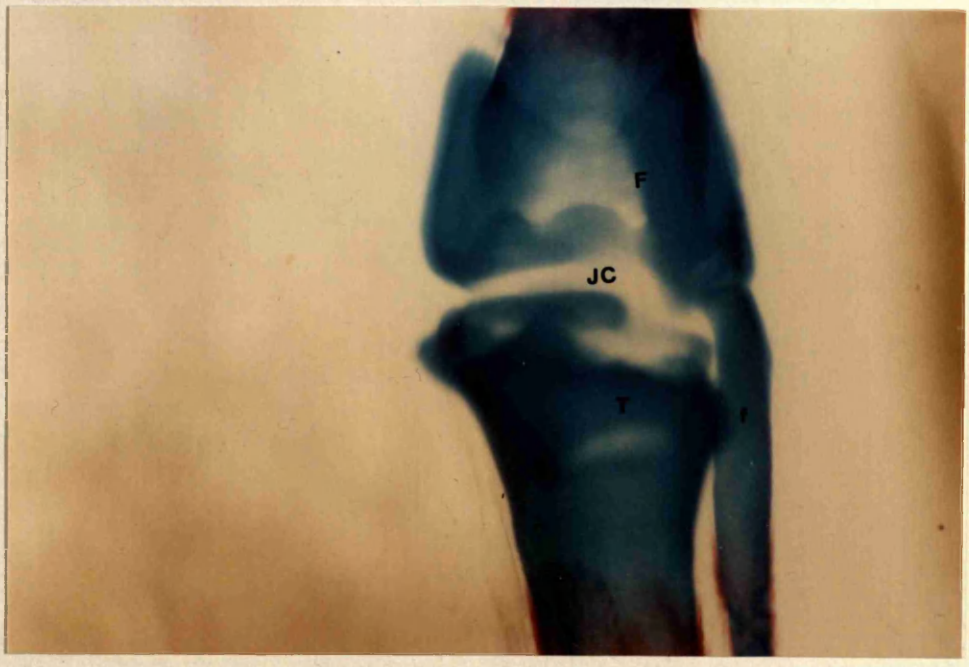

F
JC
T
f

Fig 6: Normal chick; ankle joint. The articular surfaces are reciprocally curved. The plantar tarsal sesamoid cartilage (P) is present on the posterior aspect of the joint.
Stage 40 (15 days); control; Alcian blue and alizarin red; x 23.

Fig. 7: Paralysed chick; ankle joint. The articular surfaces are flattened and show fusion across the joint. The plantar tarsal sesamoid cartilage is not present on the posterior aspect of the joint.
Stage 40 (17 days); paralysed; Alcian blue and alizarin red; x 23.

Fig. 8: Normal chick: skeleton of foot. Joint cavity is well developed (arrows) and articular surfaces reciprocally curved. Periosteal collar of bone present in metatarsals. Signs of endochondral ossification in diaphysis of some phalanges.
Stage 39 (14 days); control; Alcian blue and alizarin red; x 11.5.

Fig. 9: Paralysed chick: skeleton of foot.
Joint cavities not developed; articular surfaces flattened and show fusion across the joint (arrows). Periosteal collar of bone present around the mid-diaphysis of the metatarsals and some phalanges. Note bone development is more advanced than in control of same stage, which is however 2 days younger.
Stage 39 (16 days); paralysed; Alcian blue and alizarin red; x 11.5.

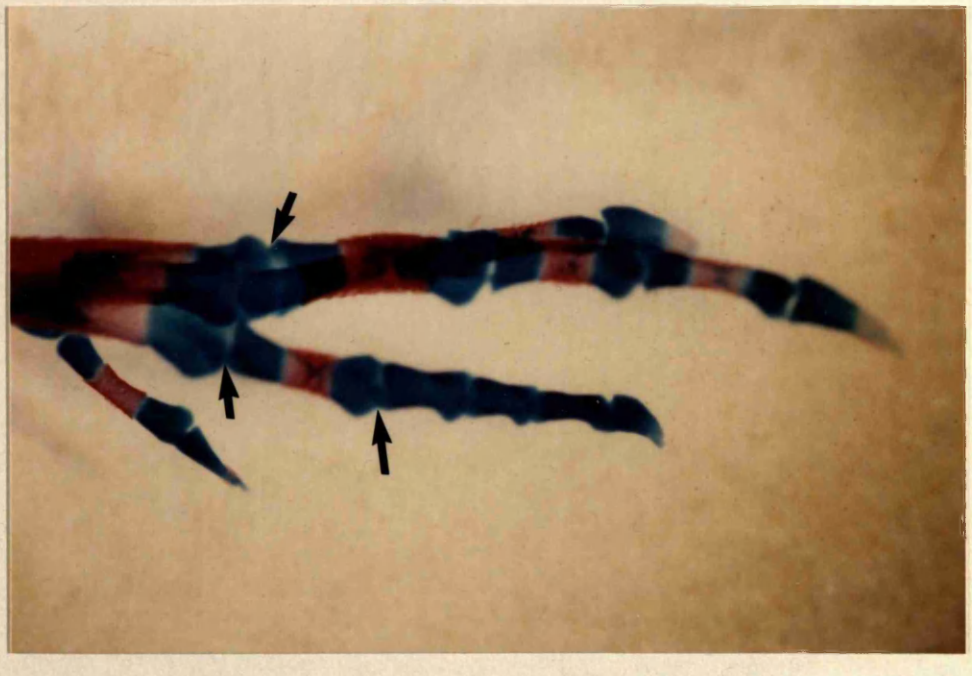
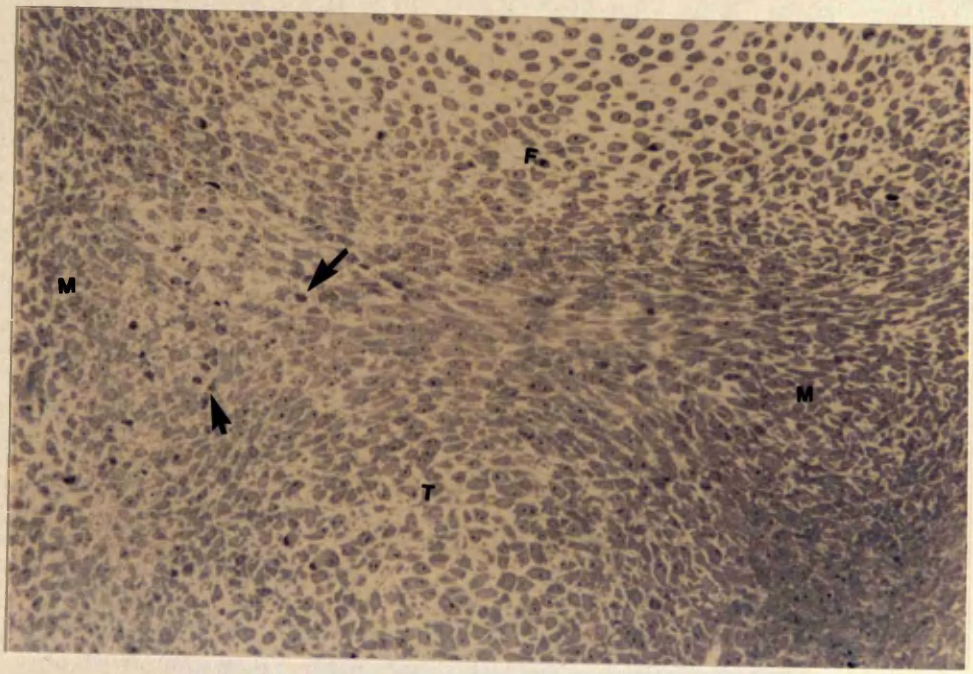


Fig. 10: Normal chick, knee joint; articulation between the lateral condyle of the femur (F) and the tibia (T) represented by early cartilage models. The triangular sectional profile lateral meniscus (M) is beginning to differentiate from the interzone; its borders are lined by small darkly stained cells ("dark cells") (arrows). Stage 30 (7 days); control; sagittal section; plastic; Azur blue II; x 276.

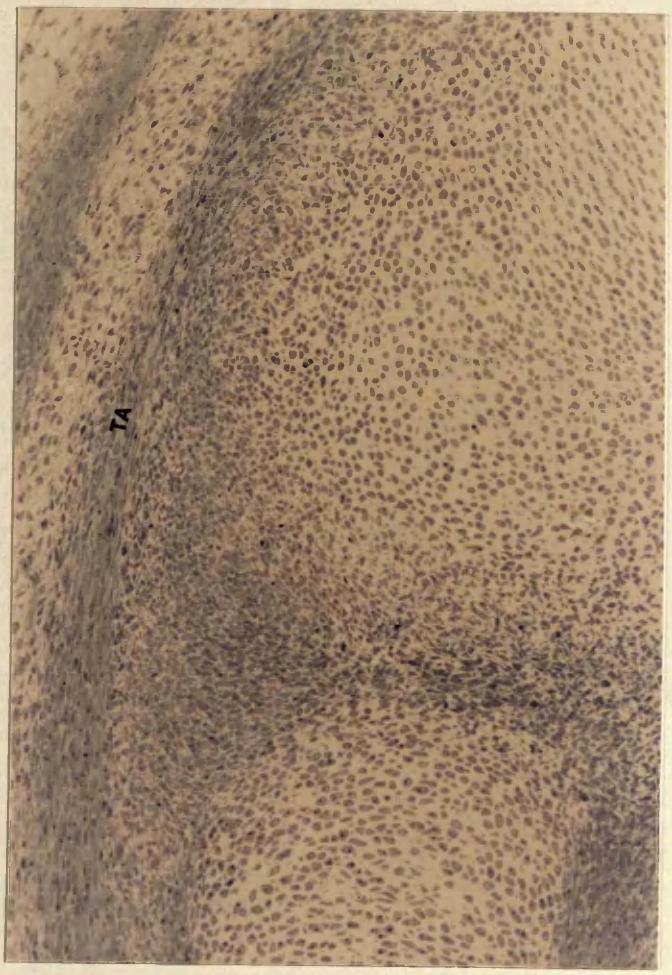
Fig. 11: Normal chick, knee joint. A cellular condensation extending from the posterior aspect of the lateral condyle of the femur (F) towards the tibia (T), represents the precursor of the anterior cruciate ligament (AC). Stage 30 (7 days); control; sagittal section; plastic; Azur blue II; x 187.


F
M
M
T


F
A
P
AC
T

Fig. 12: Normal chick; developing tendon (TA) of tibialis anterior muscle is present as a band of condensed mesenchyme, with longitudinally orientated nuclei.
Stage 30 (7 days) control; sagittal section; plastic; Azur blue II; x 144.

Fig. 13: Paralysed chick; Articulation between the lateral condyle of the femur (F) and the tibia (T). The lateral meniscus (M) is represented by a triangular profile of condensed mesenchyme, developing from the interzone and lined by a few dark cells (arrows).
Stage 30 (7 days); paralysed; sagittal section; plastic, Azur blue II; x 276.


TA

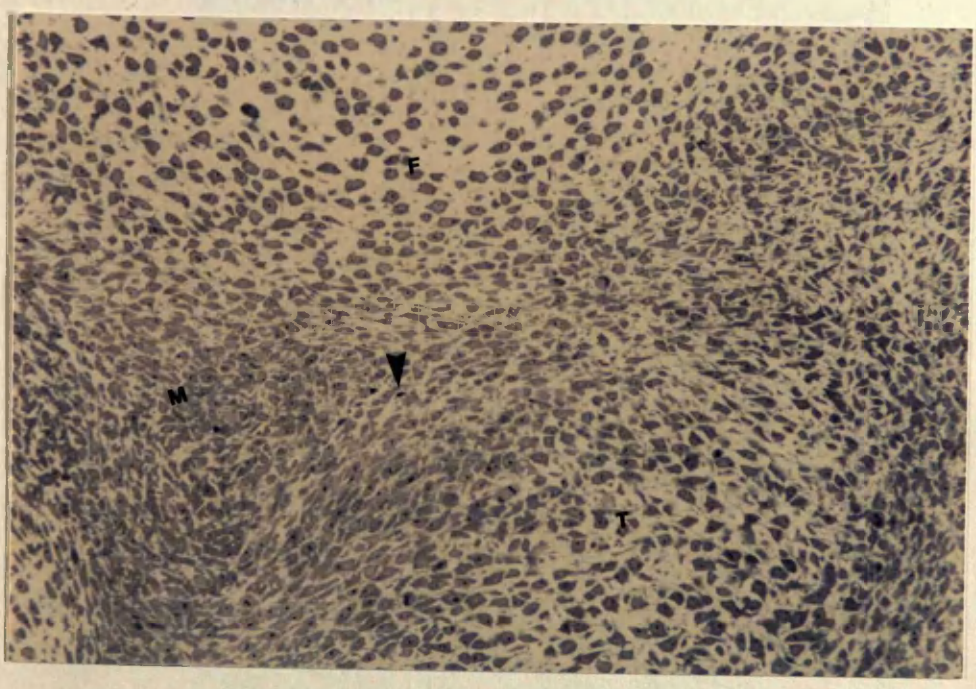

F
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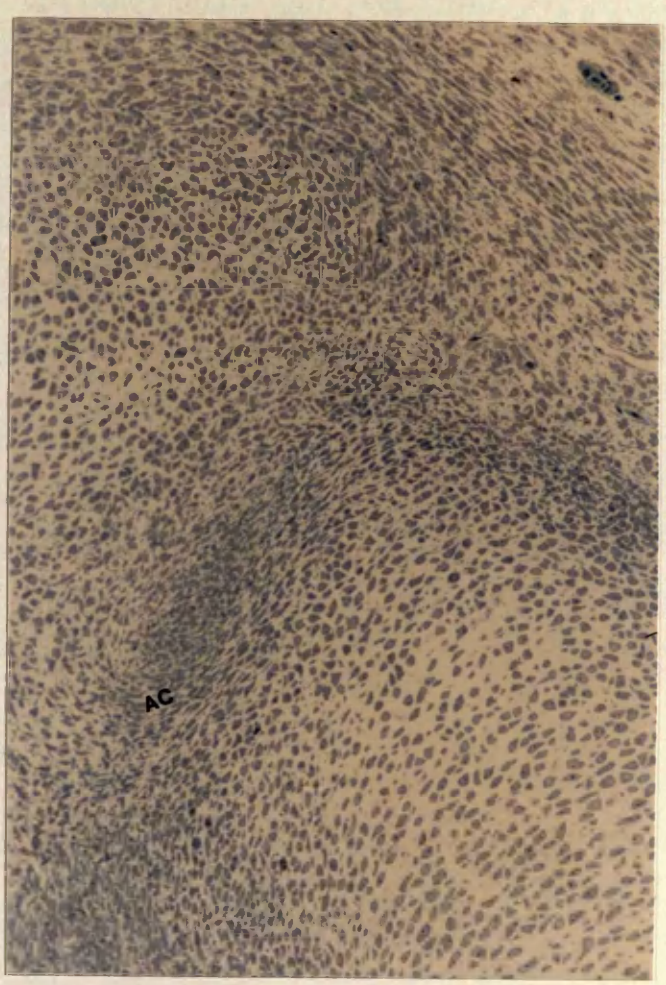
Fig. 14: Paralysed chick; The primordium of the anterior cruciate ligament (AC) is present, as a mesenchymal condensation within the interzone.
Stage 30 (7 days); paralysed; sagittal section; plastic; Azur blue II; x 187.

Fig. 15: Paralysed chick; shows developing tendon (TA) of tibialis anterior muscle. It is represented by a band of condensed mesenchyme, in which the cells are much less regularly orientated than in the normal chick (cf. Fig. 12).
Stage 31 (7 days); paralysed; sagittal section; plastic; Azur blue II; x 175.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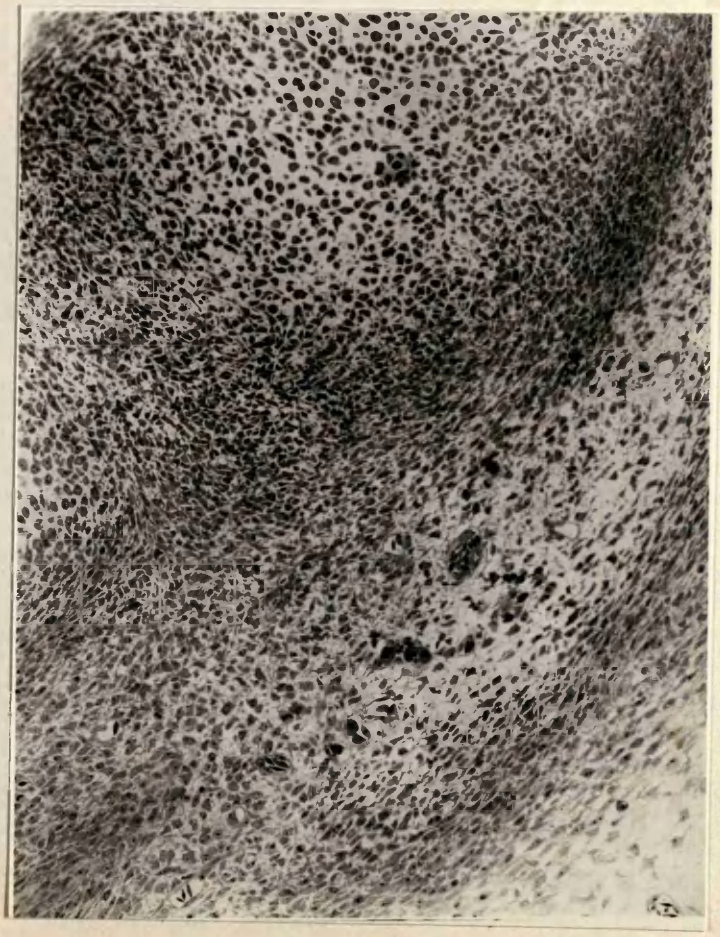
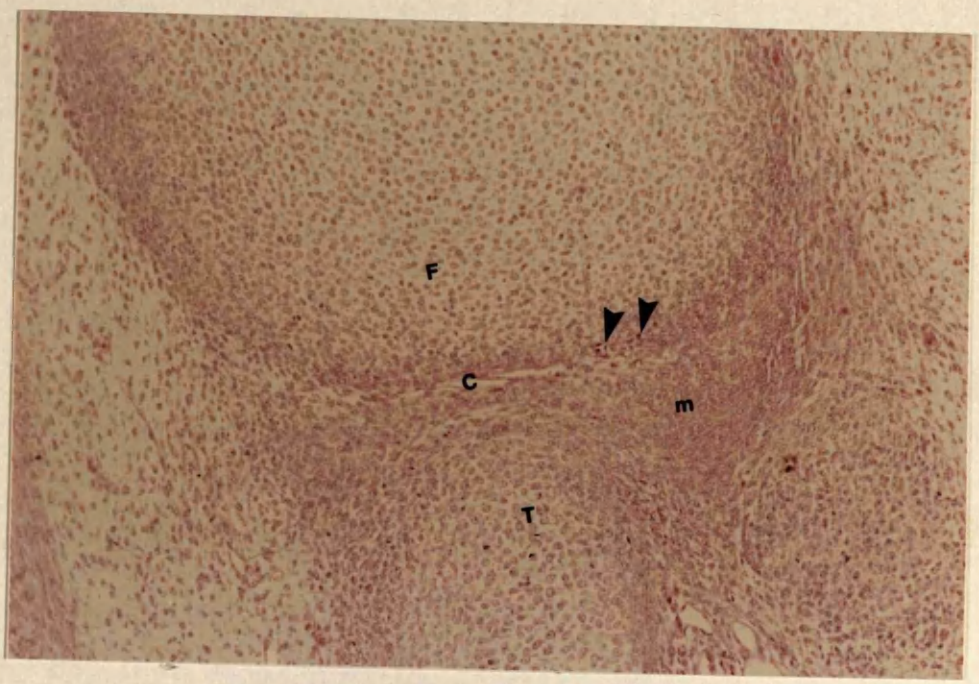


Fig. 16: Normal chick; knee joint: shows articulation between the lateral condyle of the femur (F), lateral meniscus (m) and lateral condyle of tibia (T). The joint cavities (C) have developed between the femur and lateral meniscus. Their margins are outlined by scattered "dark cells" (arrows).
Stage 32 (8 days); control; sagittal section; wax; H & E; x 158.

Fig. 17: Normal chick; knee joint: shows further development of the anterior cruciate ligament (A.C.).
Stage 32 (8 days); control; sagittal section; plastic; Azur blue II; x 240.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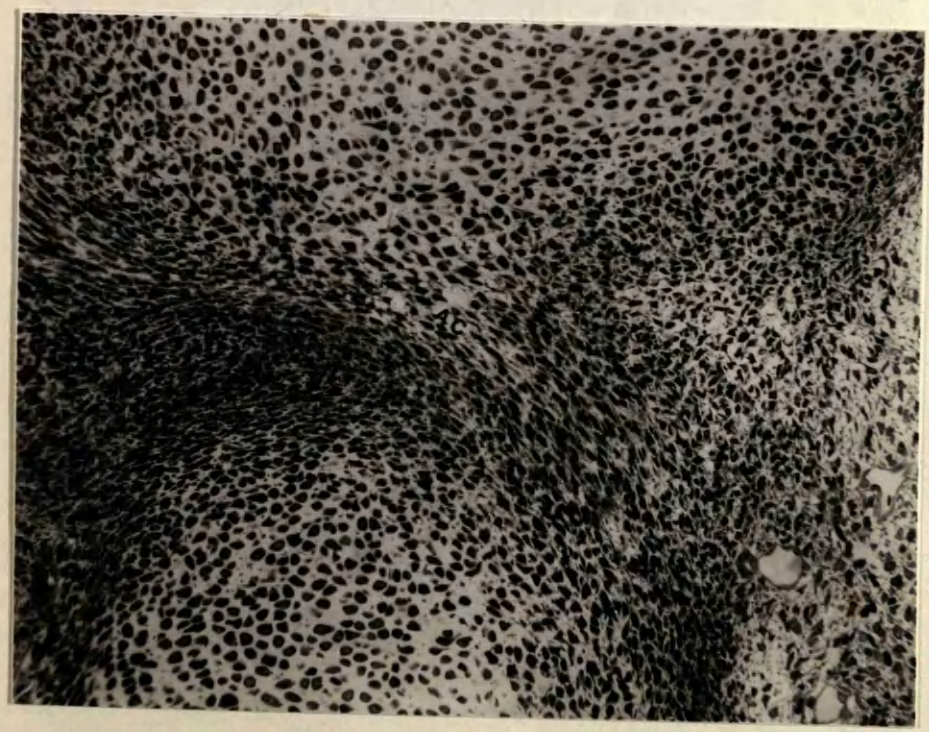

Fig. 18: Normal chick; knee joint; shows the articulation between the lateral condyle of the femur (F) and the tibia (T). The patellar condensation (P) is present anterior to the femur. The ambiens muscle (a.m.) is present within the patellar ligament.
Stage 32 (8 days); control; sagittal section; plastic; Azur blue II; x 65.

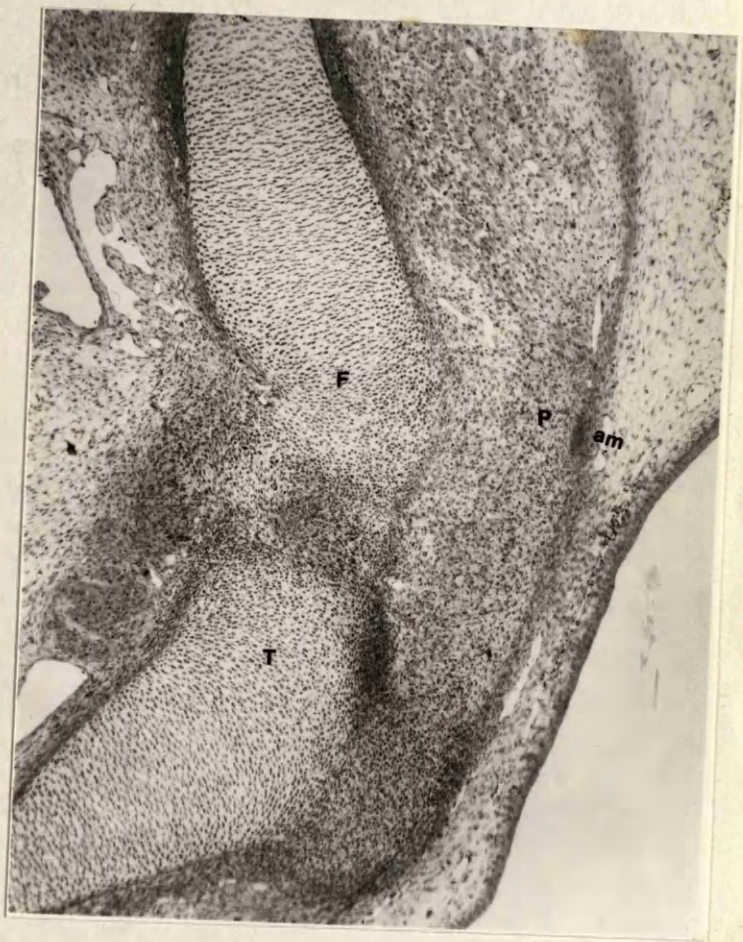
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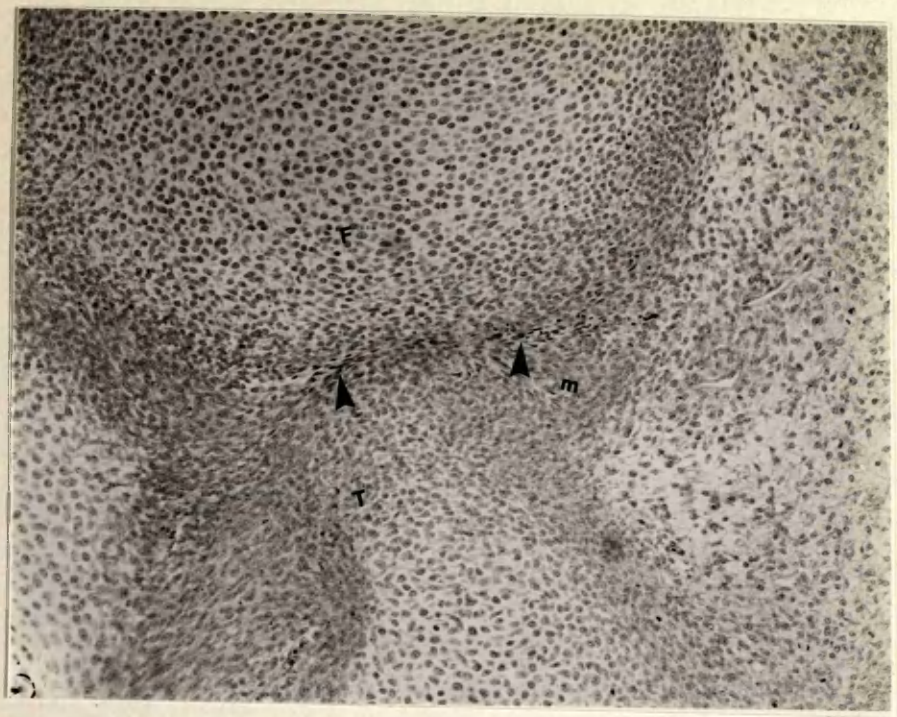
Fig. 19: Paralysed chick; knee joint; shows articulation between the lateral condyle of the femur (F), lateral meniscus (m) and lateral condyle of tibia (T). The joint cavities have not developed yet, but "dark cells" (arrows) are present in the interzone between the femur and the lateral meniscus.
Stage 32 (8 days); paralysed; sagittal section; wax; H & E; x 137.5.

Fig. 20: Paralysed chick; knee joint: shows cellular condensations, the precursors of the anterior cruciate (AC) and the posterior cruciate ligaments (PC). "Dark cells" (arrows) are scattered in front of, and between, the ligaments.
Stage 32 (8 days); paralysed; sagittal section; plastic; Azur blue II; x 240.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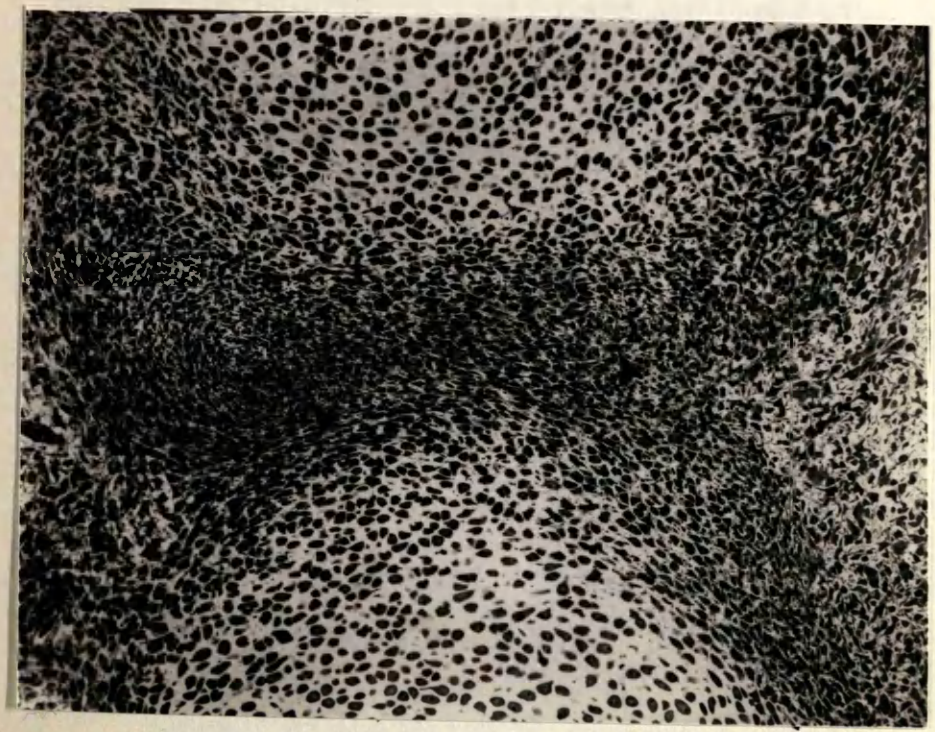
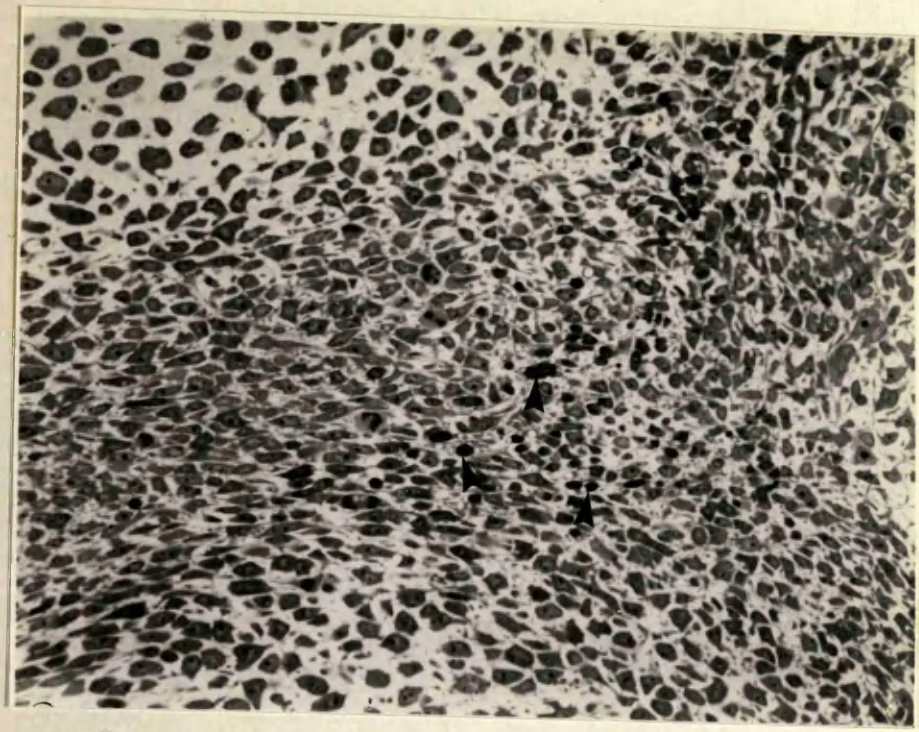


Fig. 21: Paralysed chick; knee joint: higher magnification of Fig. 20, to show numerous "dark cells" in the region anterior to the cruciate ligaments.
Stage 32 (8 days); paralysed; sagittal section; plastic; Azur blue II; x 500.

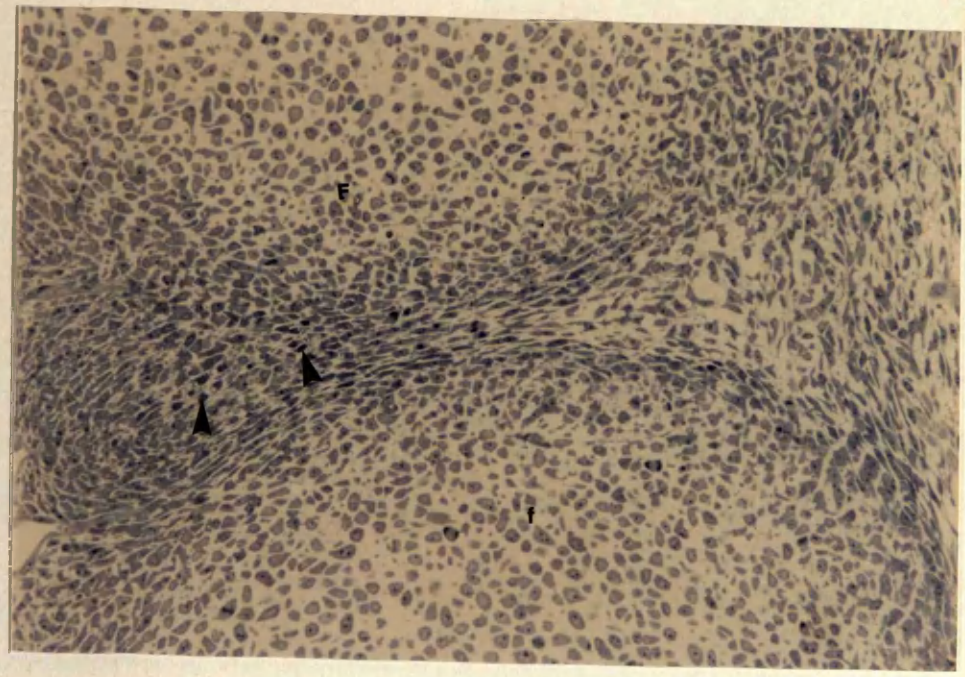
Fig. 22: Paralysed chick; knee joint: shows the articulation between the lateral condyle of the femur (F) and the tibia (T). The patellar condensation is not developed but an area of condensed mesenchyme represents an early stage in development of the ambiens muscle (a.m.), within the patellar ligament.
Stage 32 (8 days); paralysed; sagittal section; plastic; Azur blue II; x 65.




F
T

Fig. 23: Normal chick; knee joint: shows the articulation between the lateral condyle of the femur (F) and the fibula (f). A three-layered interzone is becoming evident: a layer of loose cellular mesenchyme, sandwiched between two more condensed layers (the chondrogenic layers) which cover the adjacent epiphyseal surfaces. Scattered "dark cells" (arrows) are present within the loose layer and along the margins of the chondrogenic zones.
Stage 33 (8½ days); control; sagittal section; plastic; Azur blue II; x 276.

Fig. 24: Normal chick; knee joint: shows articulation between the medial condyle of the femur (F), medial meniscus (m) and medial condyle of tibia (T). A few "dark cells" (arrows) are present within the interzone. Small cavities (C) are forming between the articular surfaces and the medial meniscus. Blood vessels (bv) are present at the periphery of the joint.
Stage 33 (8½ days); control; sagittal section; wax, H & E; x 276.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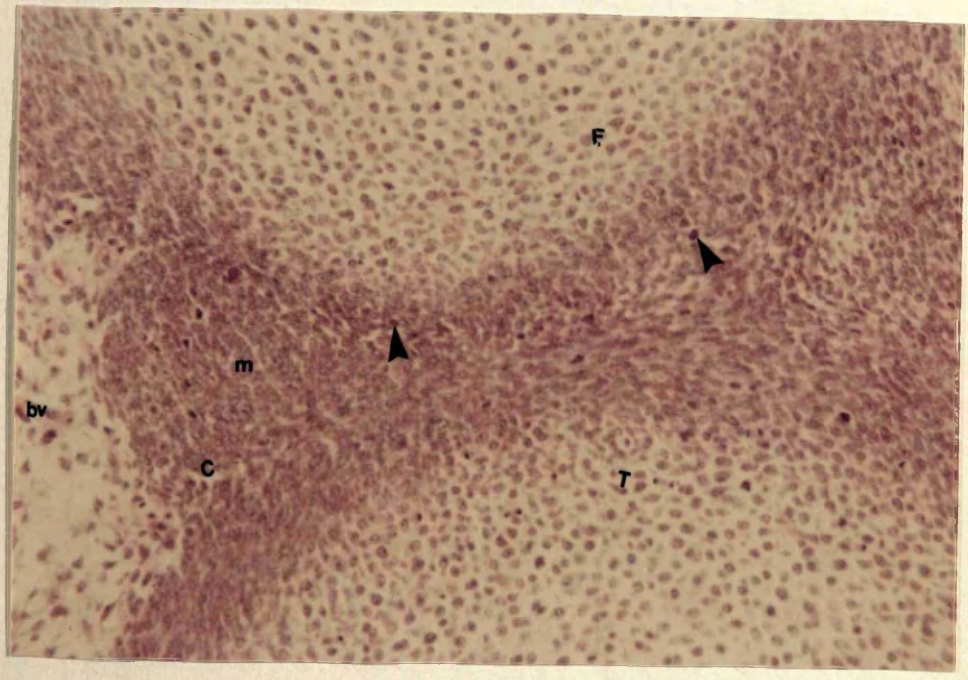
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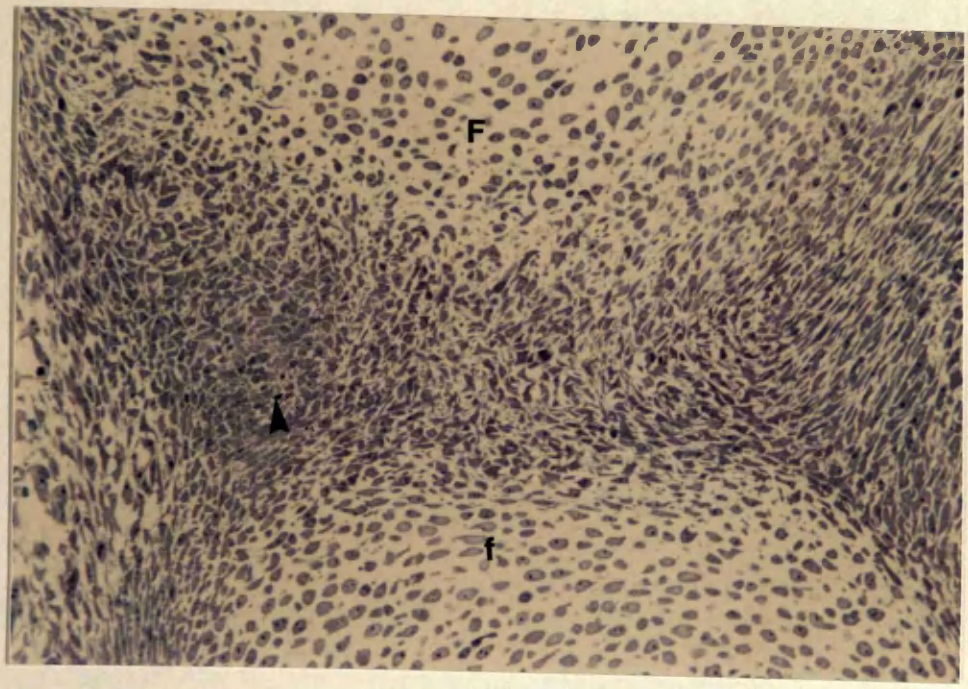
Fig. 25: Paralysed chick; knee joint: shows articulation between the lateral condyle of the femur (F) and the fibula (f). The interzone consists of young active mesenchymal cells without any specific orientation. A three-layered interzone has not formed. A few "dark cells" (arrows) are present within the interzone.
Stage 33 (8½ days) paralysed; sagittal section; plastic; Azur blue II; x 276.

Fig. 26: Paralysed chick; knee joint: shows articulation, between the medial condyle of the femur (F), medial meniscus (m) and medial condyle of tibia (T). Large numbers of "dark cells" (dc) are present within the interzone, which also contains some young chondroblasts (arrows). Blood vessels (bv) are present at the periphery of the joint.
Stage 33 (8½ days); paralysed; sagittal section; wax; H & E; x 240.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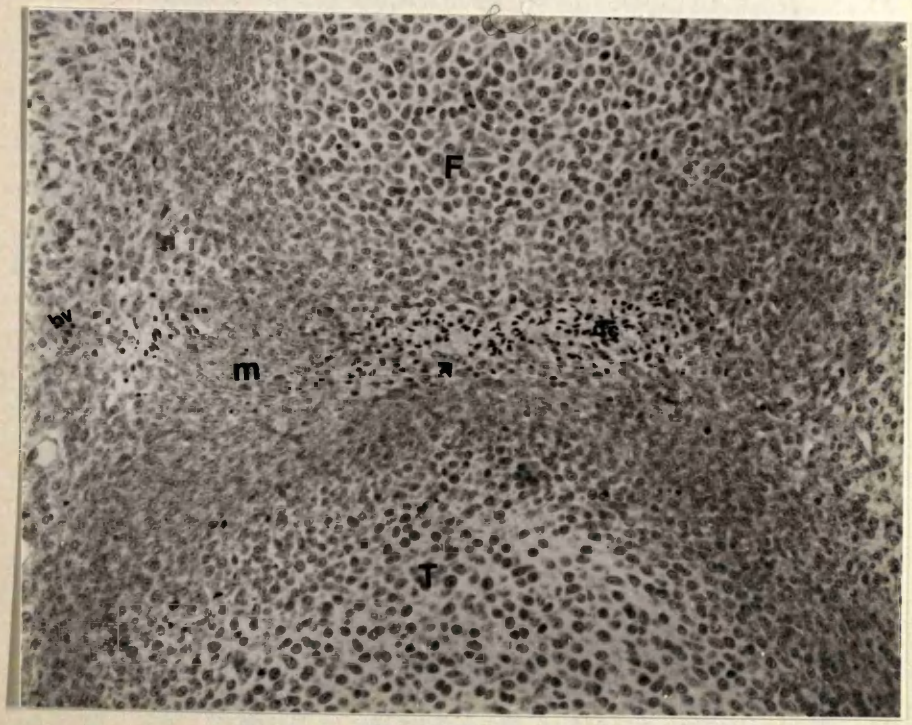
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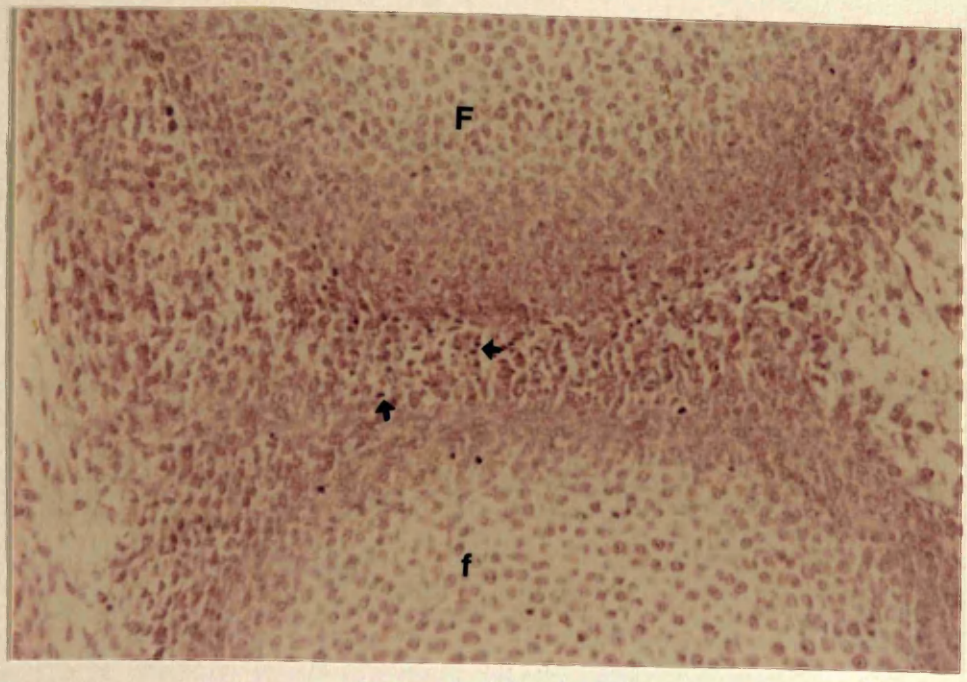
Fig. 27: Normal chick; knee joint: shows articulation between the lateral condyle of the femur (F) and the fibula (f). Each epiphysis is covered by a well defined chondrogenic zone. The two zones are separated by a looser mesenchyme in which there are scattered numerous "dark cells" (arrows).
Stage 34 (9 days); control; sagittal section; wax; H & E; x 230.

Fig. 28: Normal chick; knee joints: shows a linear group of numerous "dark cells" (dc) under the attachment of the tibialis anterior tendon (T.A.) to the lateral condyle of the femur (F).
Stage 34 (9 days); control; sagittal section; wax; H & E; x 253.


F
f

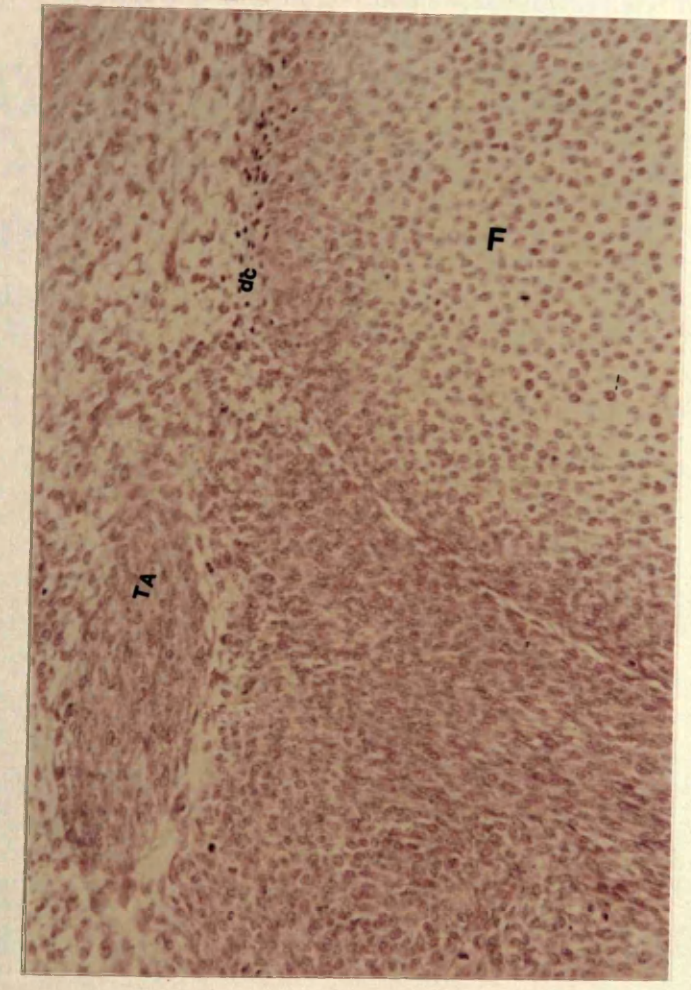
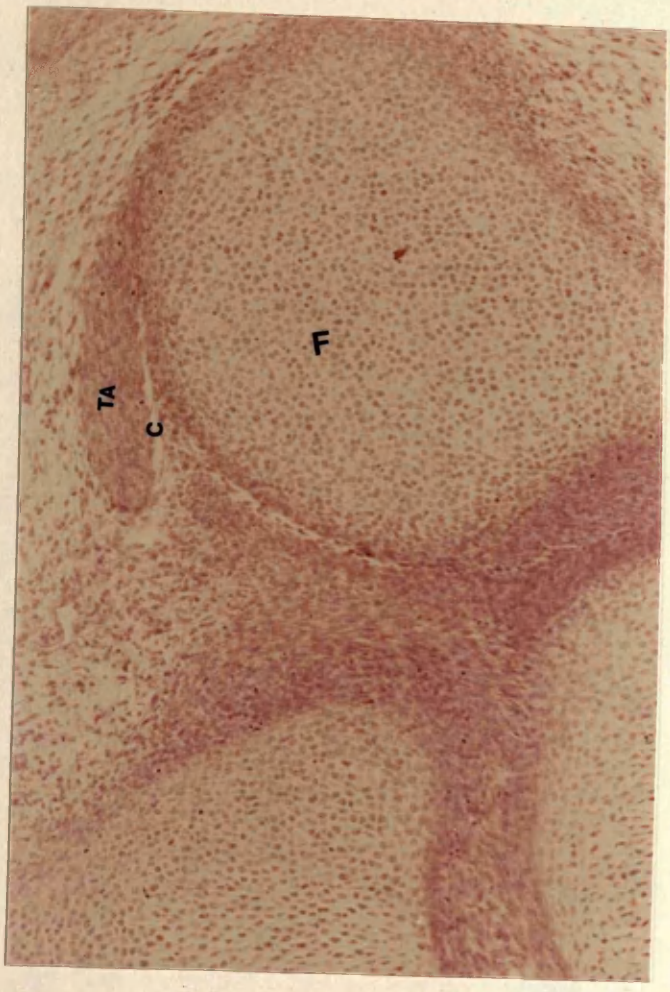

F
dc
TA

Fig. 29: Normal chick; knee joint: shows cavity formation (C) on the posterior aspect of the origin of the tibialis anterior tendon (T.A.) from the lateral condyle of the femur (F).
Stage 34 (9 days); control; sagittal section; wax; H & E; x 144.

Fig. 30: Paralysed chick; knee joint: shows articulation between the lateral condyle of the femur (F) and the fibula (f). Numerous "dark cells" (arrows) are present within the posterior aspect of the interzone, but still a three-layered interzone has not formed. Some cells within the interzone appear to be differentiating into chondroblasts (C). Mitotic cells (m) are also seen within the interzone.
Stage 34 (9 days); paralysed; sagittal section; plastic; Azur blue II; x 253.


F
TA
C

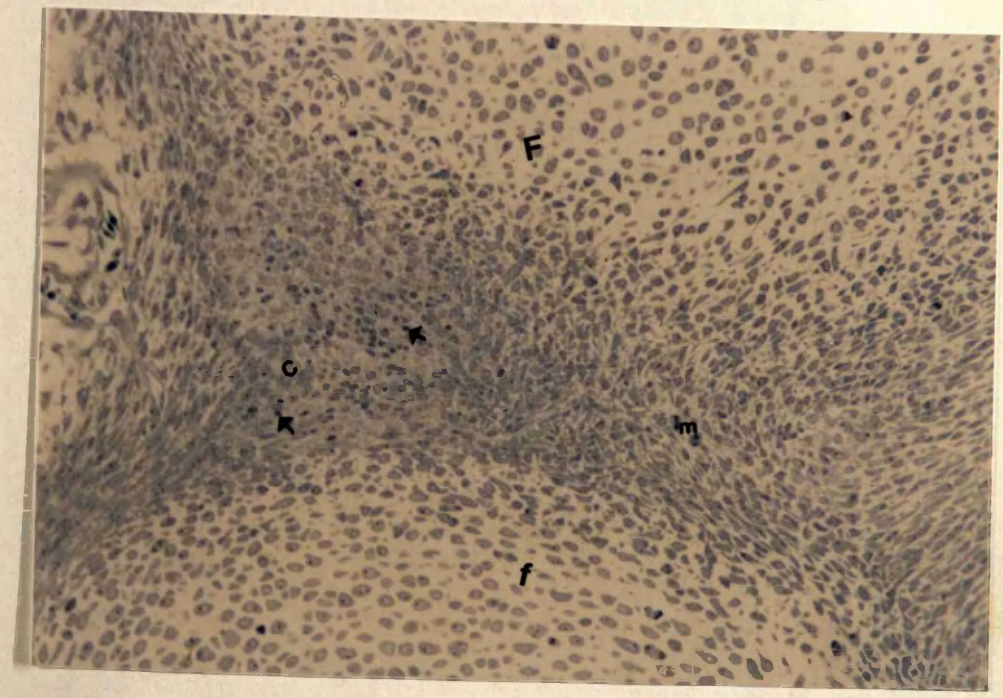

F
C
Im
f

Fig. 31: Paralysed chick; knee joint: shows the origin of the tibialis anterior tendon (T.A.) from the lateral condyle of the femur (F). There are no "dark cells" along its borders and there is no formation of a synovial cavity.
Stage 34 (9 days); paralysed; sagittal section; wax; H & E; x 144.

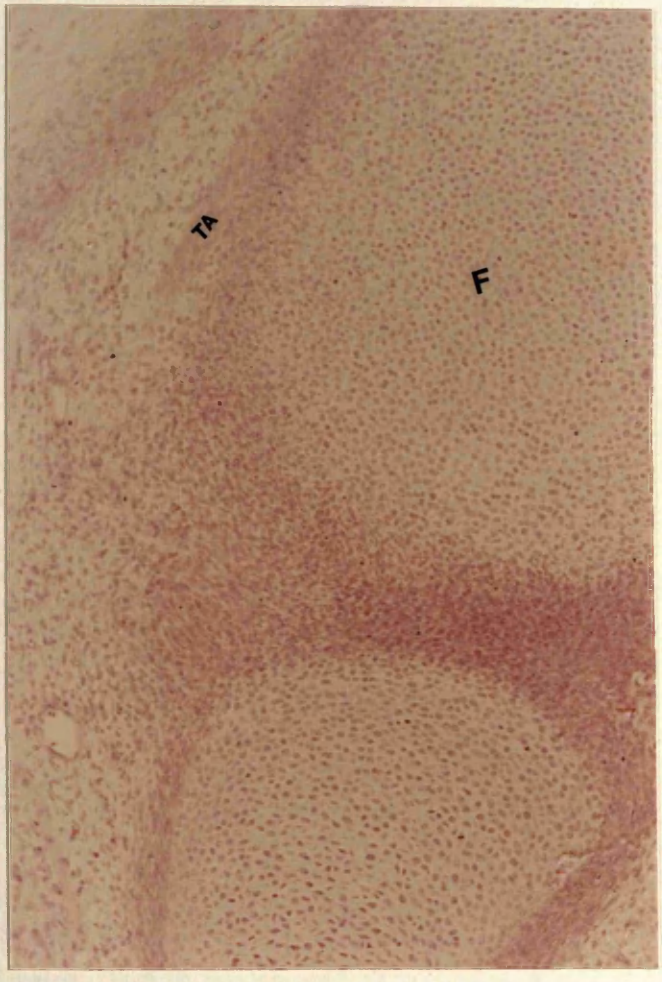
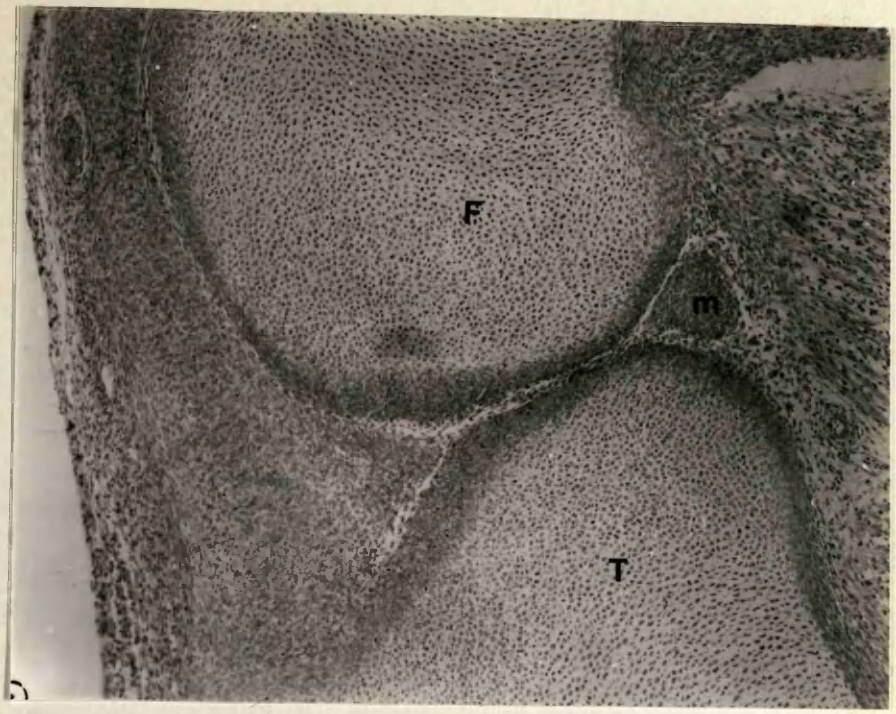
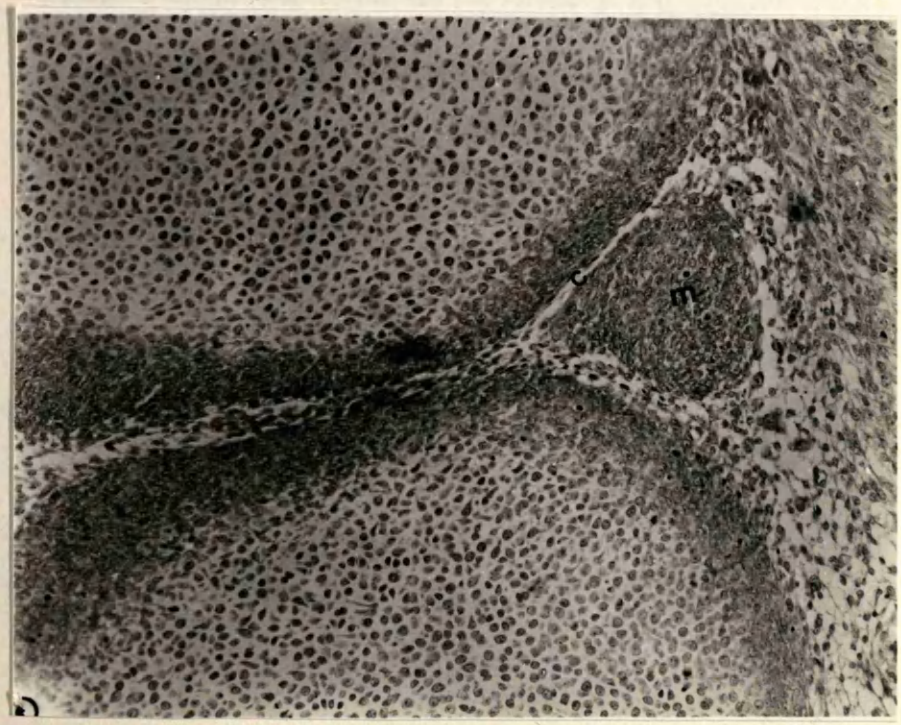

TA
F

Fig. 32: Normal chick; knee joint; shows the articulation between the medial condyle of the femur (F), medial meniscus (m) and medial condyle of tibia. Further loosening of the intermediate part of the interzone clearly anticipates the appearance of the joint cavity. Formation of an actual cavity is confined to the upper and lower margins of the meniscus.
Stage 35 (10 days); control; sagittal section; wax; H & E; x 75.

Fig. 33: High power of Fig. 32. Cavities (C) are well developed along the margin of the medial meniscus (m). Scattered "dark cells" are aligned along the chondrogenic zones of femur and tibia.
Stage 35 (10 days); control; sagittal section; wax; H & E; x 240.


F
m
T


c
m

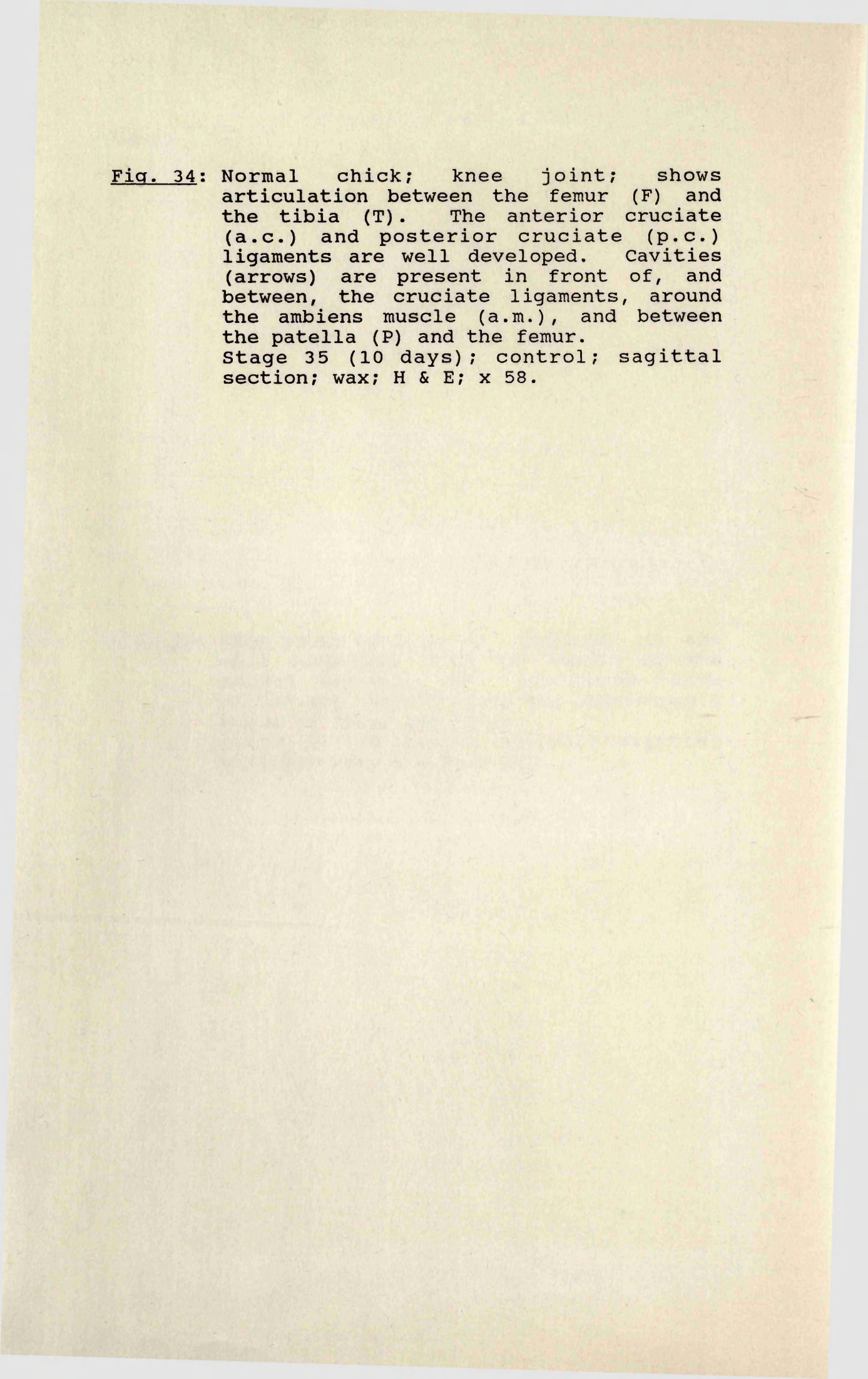


Fig. 34: Normal chick; knee joint; shows articulation between the femur (F) and the tibia (T). The anterior cruciate (a.c.) and posterior cruciate (p.c.) ligaments are well developed. Cavities (arrows) are present in front of, and between, the cruciate ligaments, around the ambiens muscle (a.m.), and between the patella (P) and the femur.
Stage 35 (10 days); control; sagittal section; wax; H & E; x 58.

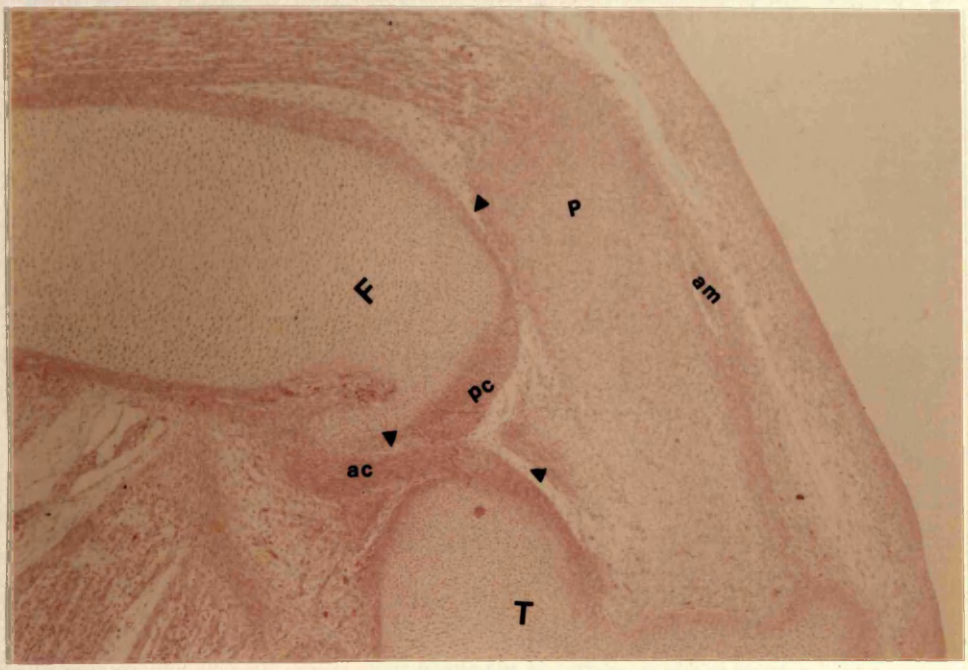
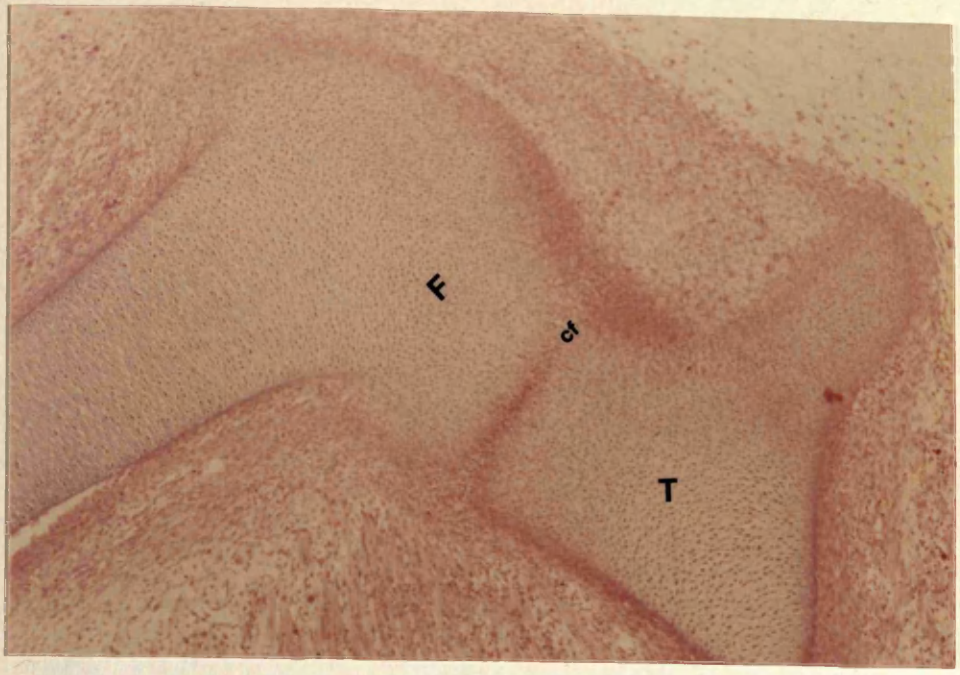
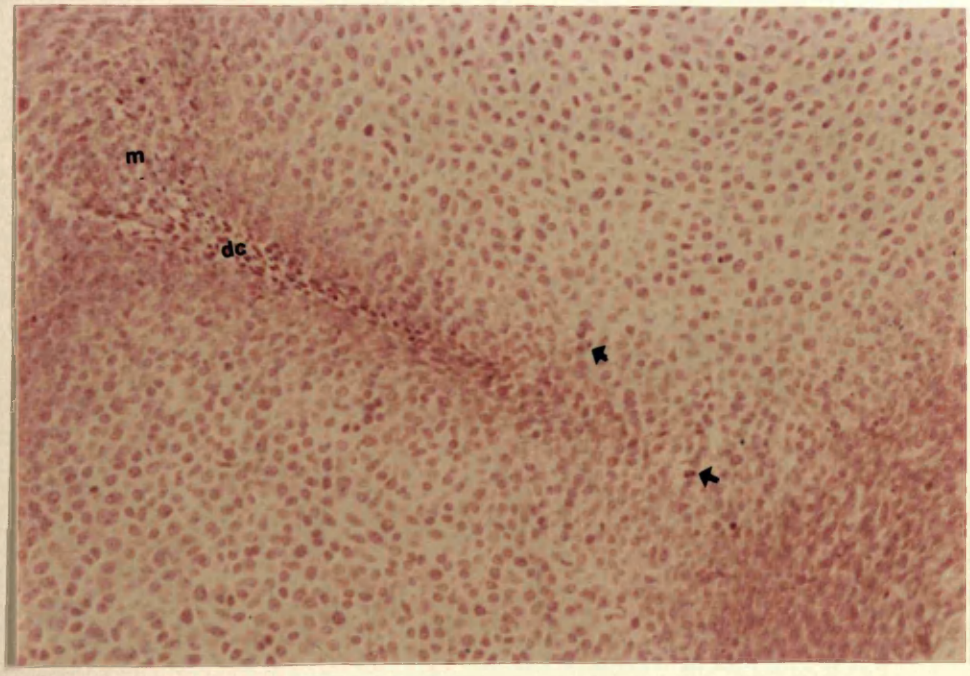

p
am
pc
ac
T

Fig. 35: Paralysed chick; knee joint; shows cartilaginous fusion (c.f.) anteriorly between the medial condyle of the femur (F) and medial condyle of the tibia (T). The medial meniscus has disintegrated.
Stage 35 (10 days); paralysed; sagittal section; wax; H & E; x 80.

Fig. 36: High power of Fig. 35. Mitotic figures (arrows) are present at the site of the fusion. Numerous "dark cells" (dc) are present within the interzone posteriorly. Posterior aspect of the medial meniscus (m) is still present.
Stage 35 (10 days); paralysed; sagittal section; wax; H & E; x 276.


F
cf
T


m
dc

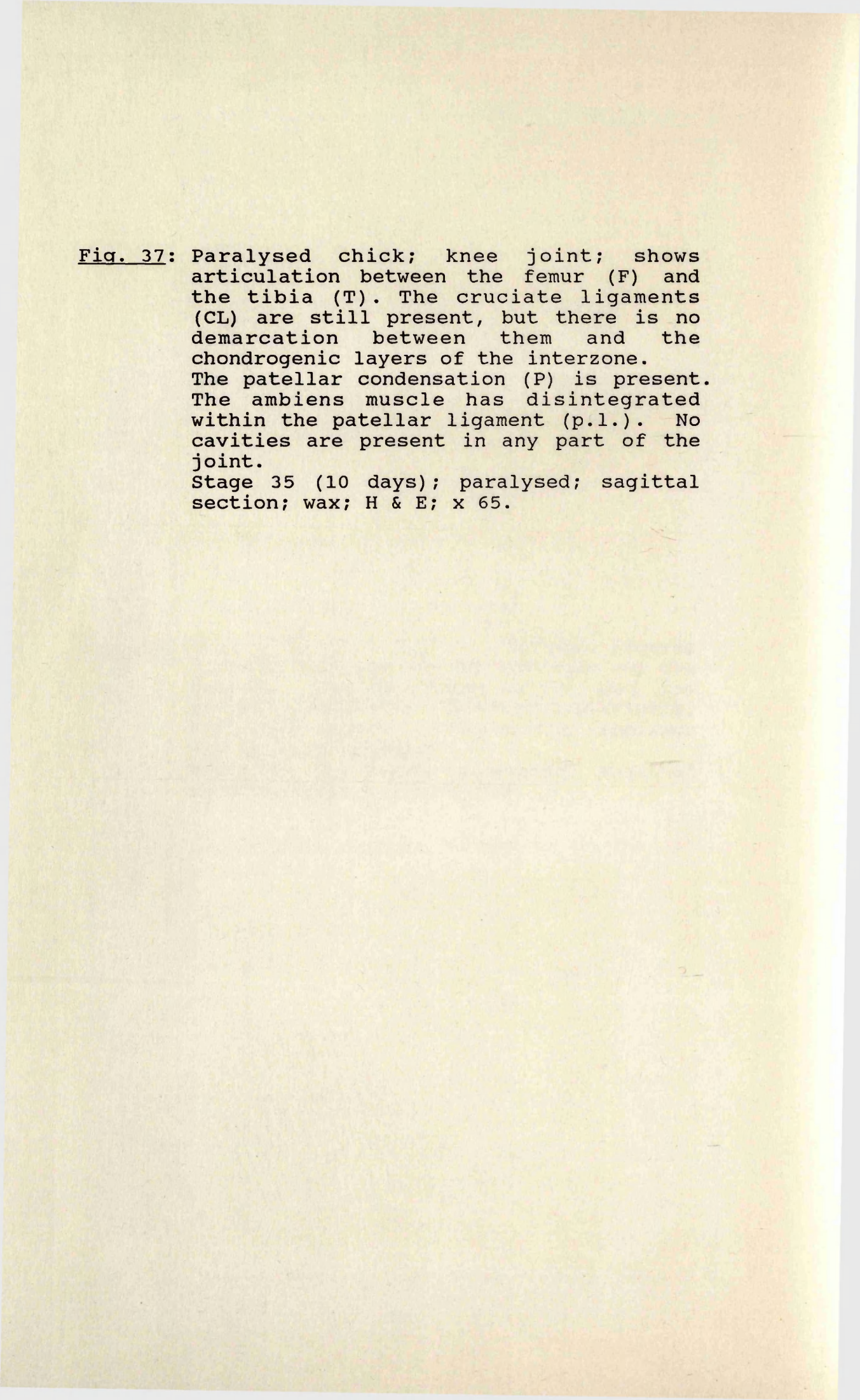


Fig. 37: Paralysed chick; knee joint; shows articulation between the femur (F) and the tibia (T). The cruciate ligaments (CL) are still present, but there is no demarcation between them and the chondrogenic layers of the interzone. The patellar condensation (P) is present. The ambiens muscle has disintegrated within the patellar ligament (p.l.). No cavities are present in any part of the joint.
Stage 35 (10 days); paralysed; sagittal section; wax; H & E; x 65.

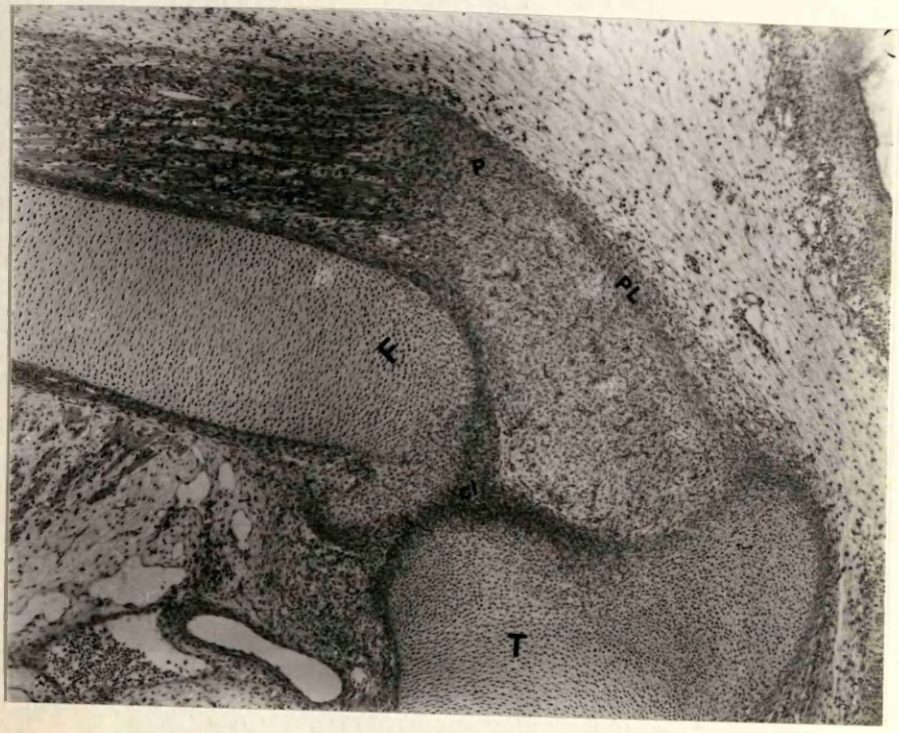
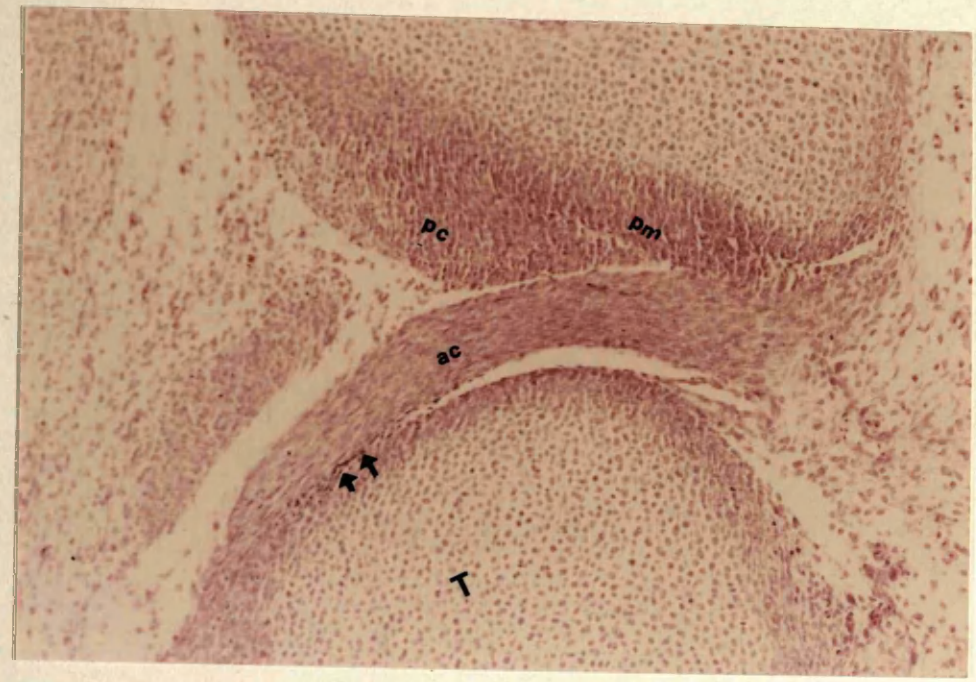

P
PL
L
T

Fig. 38: Normal chick; knee joint; shows advanced cavitation between the intra-articular ligaments. The posterior cruciate (p.c.) and anterior cruciate (a.c.) ligaments and posterior meniscofemoral (p.m.) are well formed. Numerous "dark cells" (arrows) are localized between the anterior cruciate ligament and the lateral condyle of the tibia (T).
Stage 36 (10 days); control; sagittal section; wax; H & E; x 144.

Fig. 39: Normal chick; knee joint; shows numerous "dark cells" (arrows) arranged along the margins of the tibialis anterior tendon (T.A.), on the posterior aspect of which is a well developed cavity.
Stage 36 (10 days); control; sagittal section; wax; H & E; x 144.


pc
pm
ac
T

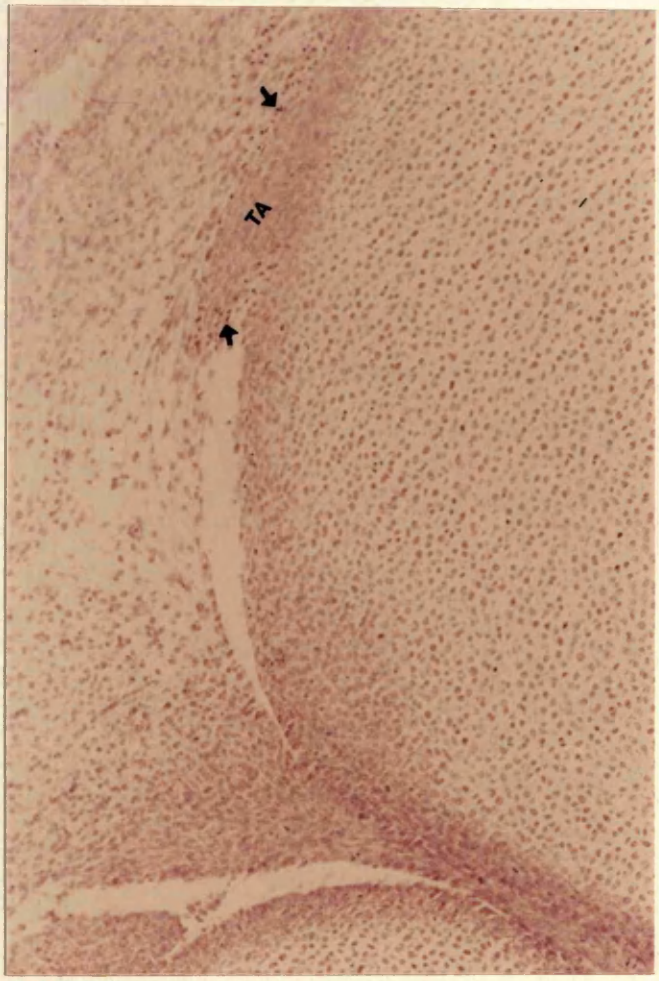
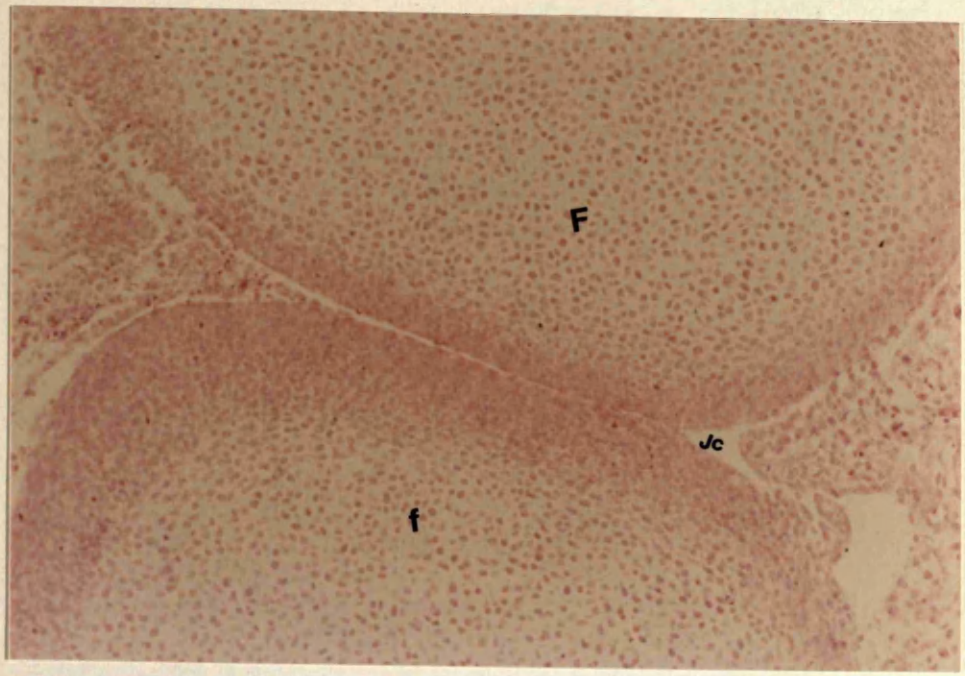

TA

Fig. 40: Normal chick; knee joint; shows articulation between the lateral condyle of the femur (F) and the fibula (f). The joint cavity (J.C.) is fully developed.
Stage 36 (11 days); control; sagittal section; wax; H & E; x 172.

Fig. 41: Normal chick; knee joint; a cavity (C) is forming between the femur (f) and the patella (P), but it still contains many loose connective tissue strands.
Stage 36 (10 days); control; sagittal section; wax; H & E; x 240.


F
Jc
f

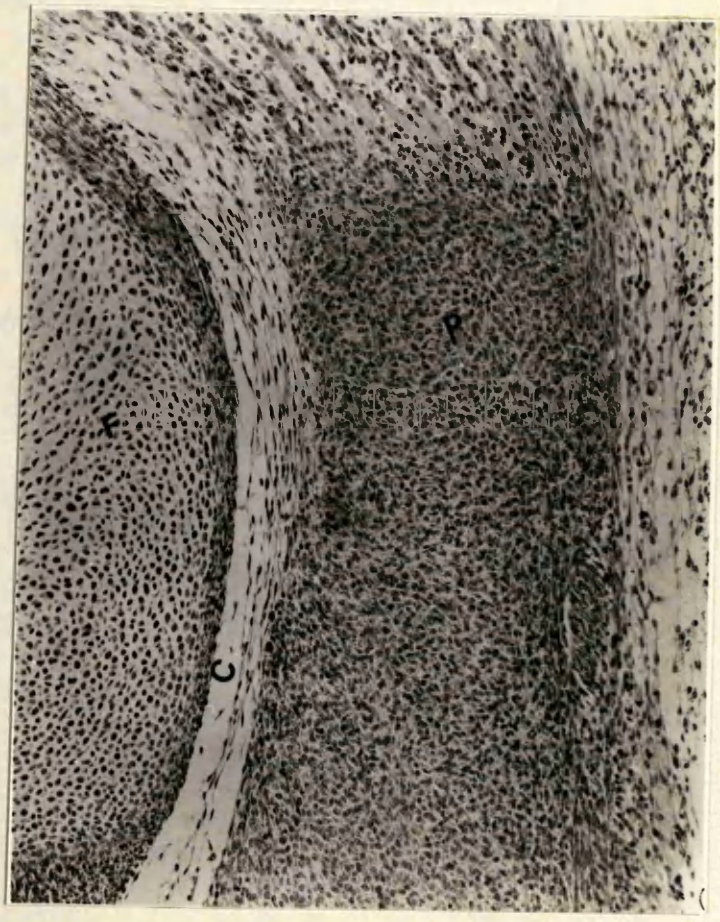
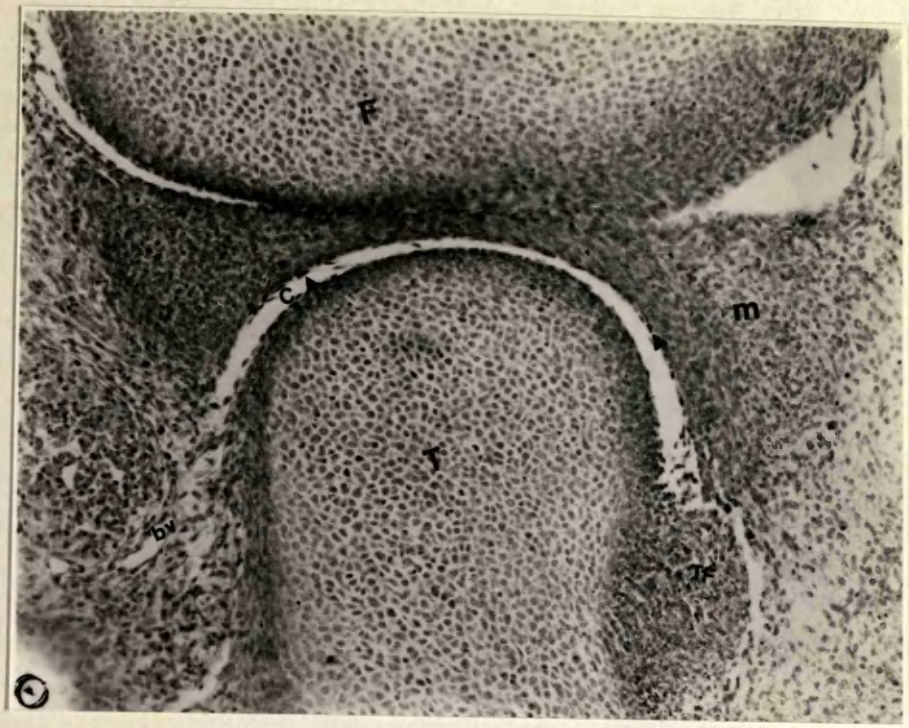

P
F
C

Fig. 42: Normal chick; knee joint; shows articulation between the lateral condyle of the femur (F), lateral meniscus (m) and lateral condyle of tibia (T). Joint cavity (C) is now well developed along the margin of the lateral meniscus. Its boundaries are lined by "dark cells" (arrows). Blood vessels (bv) are present at the periphery of the lateral meniscus. Transverse tibio-fibular ligament (T.F.) can be distinguished from the lateral meniscus.
Stage 36 (11 days); control; sagittal section; wax; H & E; x 187.5.

Fig. 43: Paralysed chick; knee joint; shows articulation between the lateral condyle of the femur (F) and the fibula (f). The interzone has become narrower, and consists of flattened cells continuous with the prechondrium of the adjacent cartilage models. Blood vessels (bv) are present within the interzone.
Stage 36 (11 days); paralysed; sagittal section; plastic; Azur blue II; x 187.5.


F
m
T
bv
Tf

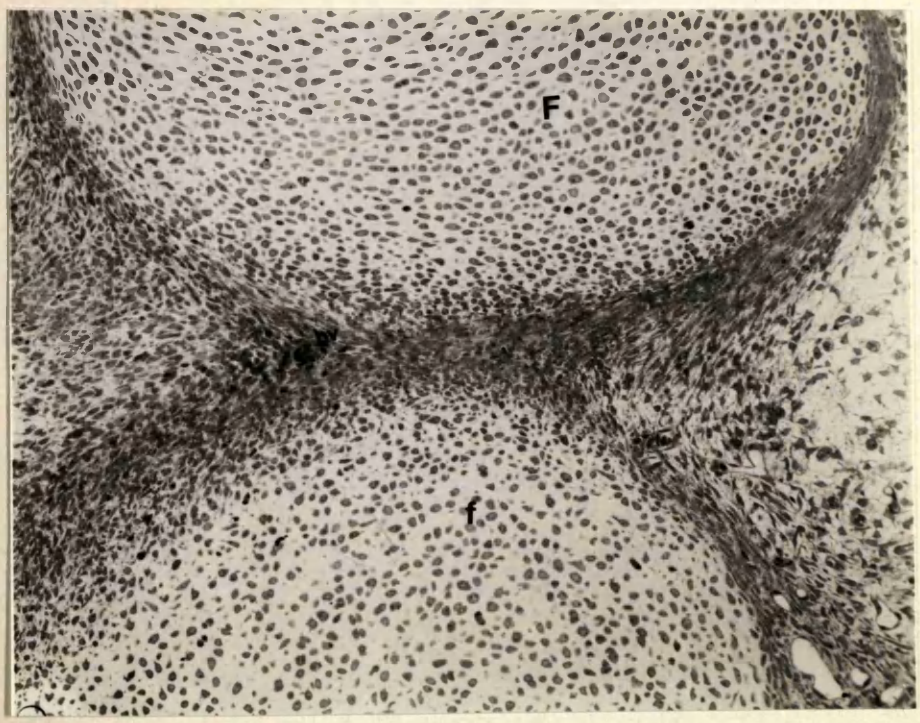
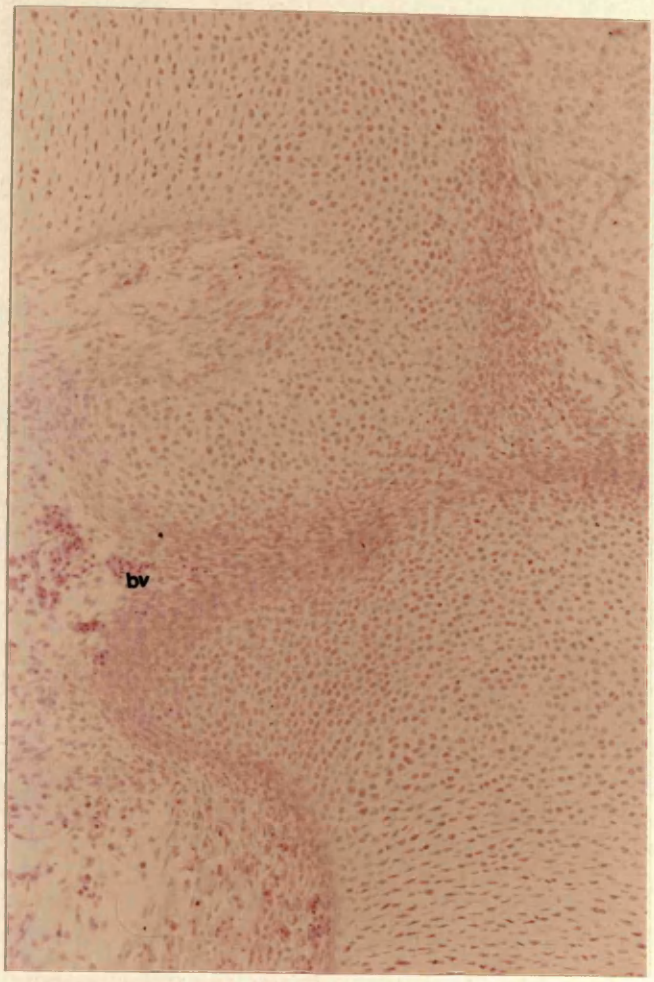

F
f

Fig. 44: Paralysed chick; knee joint; The intra-articular ligaments are no longer recognizable within the interzone. Blood vessels (bv) are present at the peripheral part of the interzone.
Stage 36 (11 days): paralysed; sagittal section; wax; H & E; x 144.

Fig. 45: Paralysed chick; knee joint; shows articulation between the lateral condyle of the femur (F), lateral meniscus (m) and lateral condyle of tibia (T). A cavity has not developed, but the upper margin of the lateral meniscus is lined by a large number of "dark cells" (arrows). The transverse tibiofibular ligament cannot be distinguished from the lateral meniscus.
Stage 36 (11 days); paralysed; sagittal section; wax; H & E; x 276.


bv

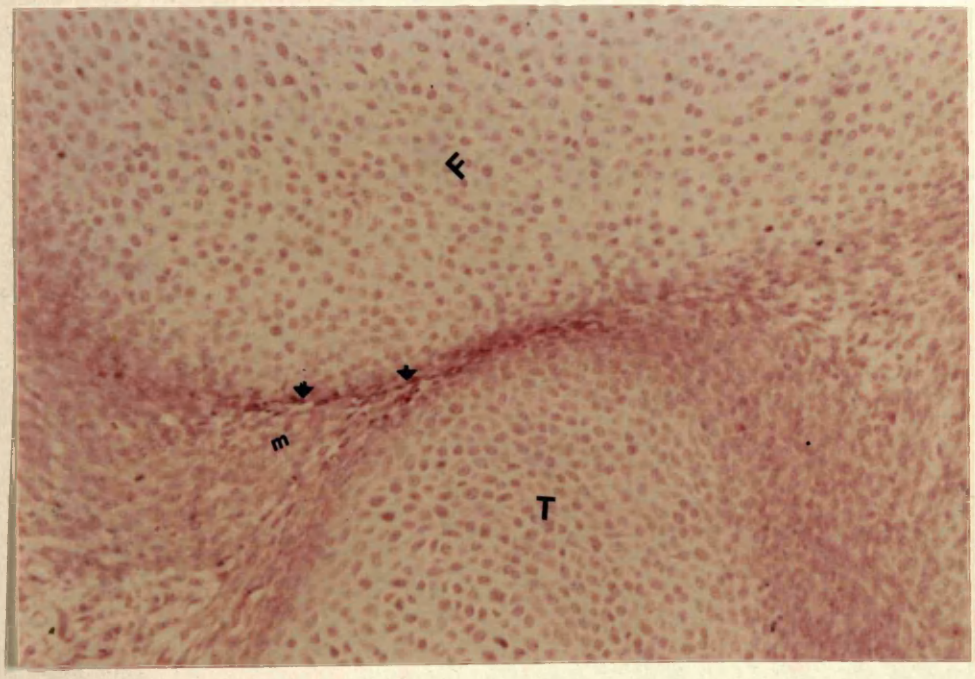

F
m
T

Fig. 46: Paralysed chick; knee joint; shows no sign of "dark cells" or of cavity formation around the origin of the tibialis anterior tendon (T.A.). The synovial mesenchyme is highly vascularized (compare with that in Fig. 39).
Stage 36 (11 days); paralysed; sagittal section; wax; H & E; x 158.

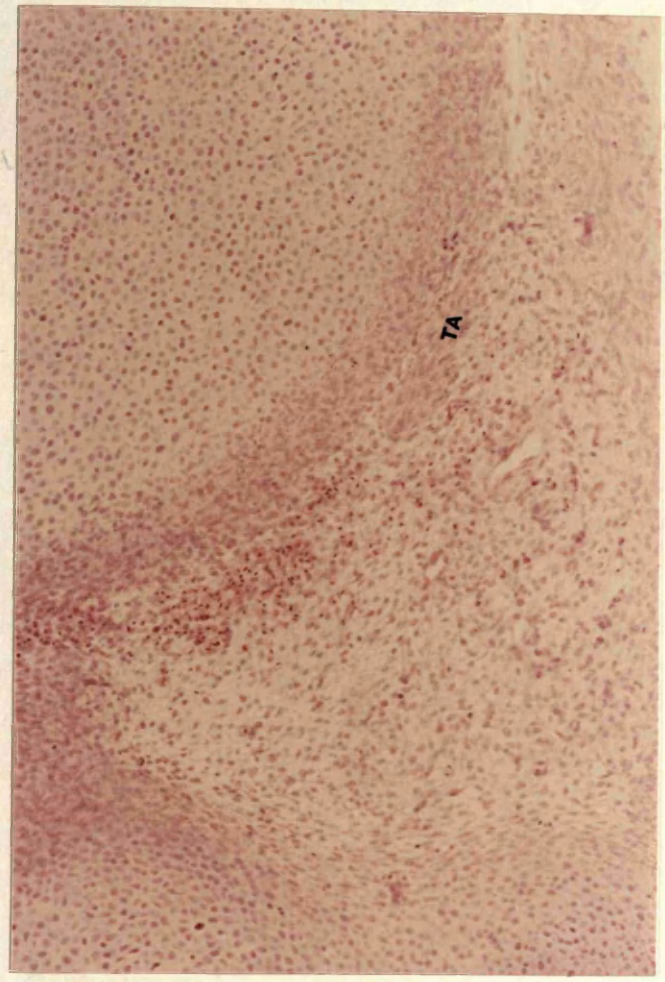
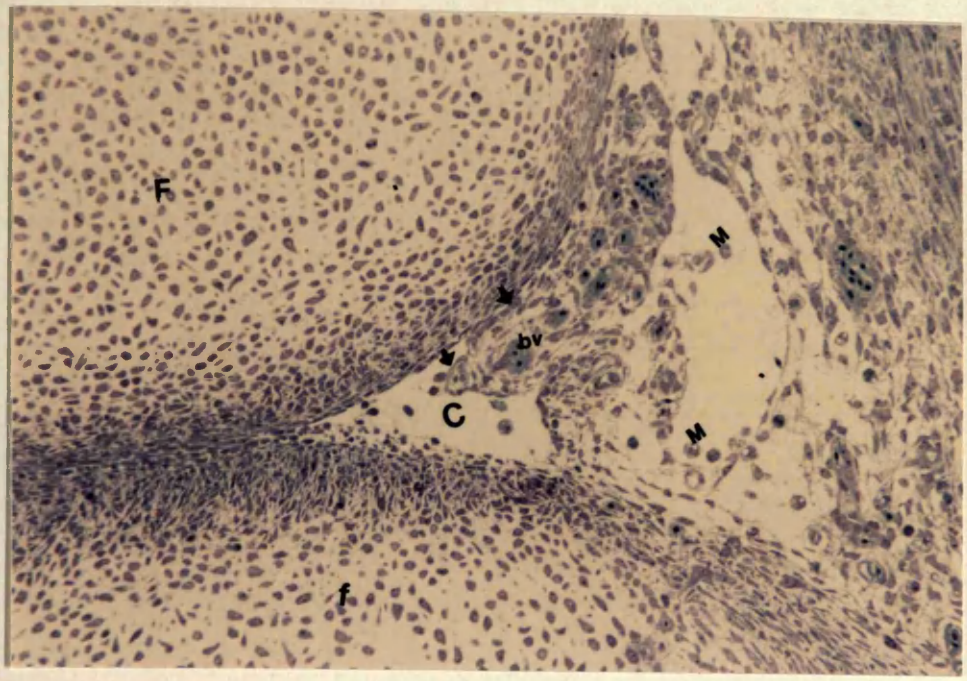

TA

Fig. 47: Normal chick; knee joint; shows articulation between the lateral condyle of the femur (F) and the fibula (f). The joint cavity (C) is well developed. It is lined by a synovial membrane (arrow), from which a synovial fold ("villus") projects into the cavity. In the fold, numerous blood vessels (bv) lie immediately beneath the synovial lining cells. Numerous macrophages (M) are present both within the joint cavity and the synovial fold.
Stage 37 (12 days); control; sagittal section; plastic; Azur blue II; x 230.

Fig. 48: Normal chick; knee joint; shows well developed lateral meniscus (m), transverse tibiofibular ligament (T.F.), and tibialis anterior tendon (T.A.); around the latter is a well defined synovial cavity.
Stage 37 (12 days); control; sagittal section; wax; H & E; x 65.


F
M
bv
C
M
f

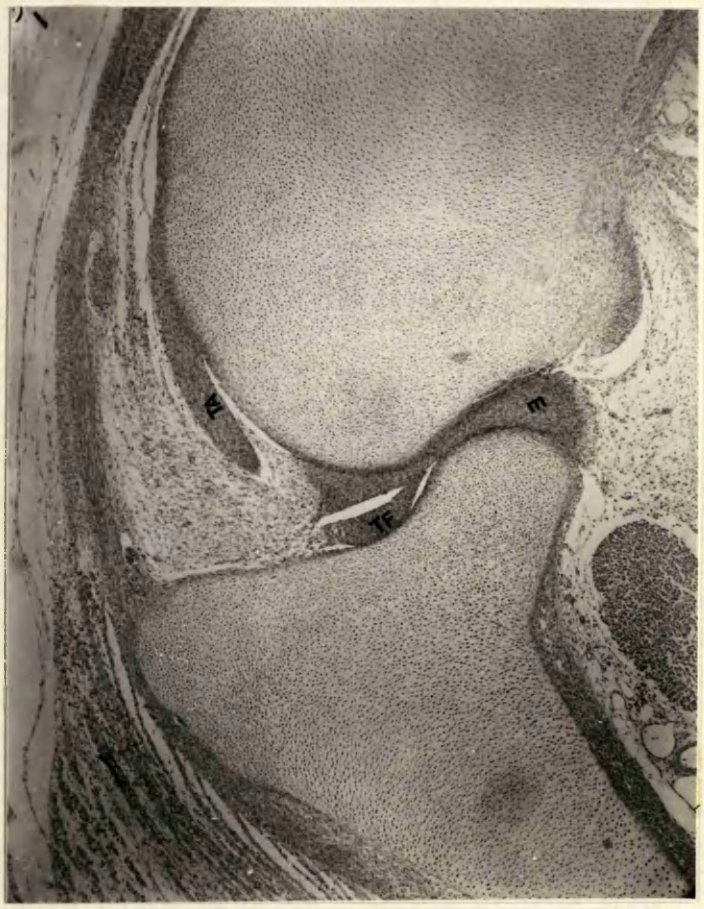
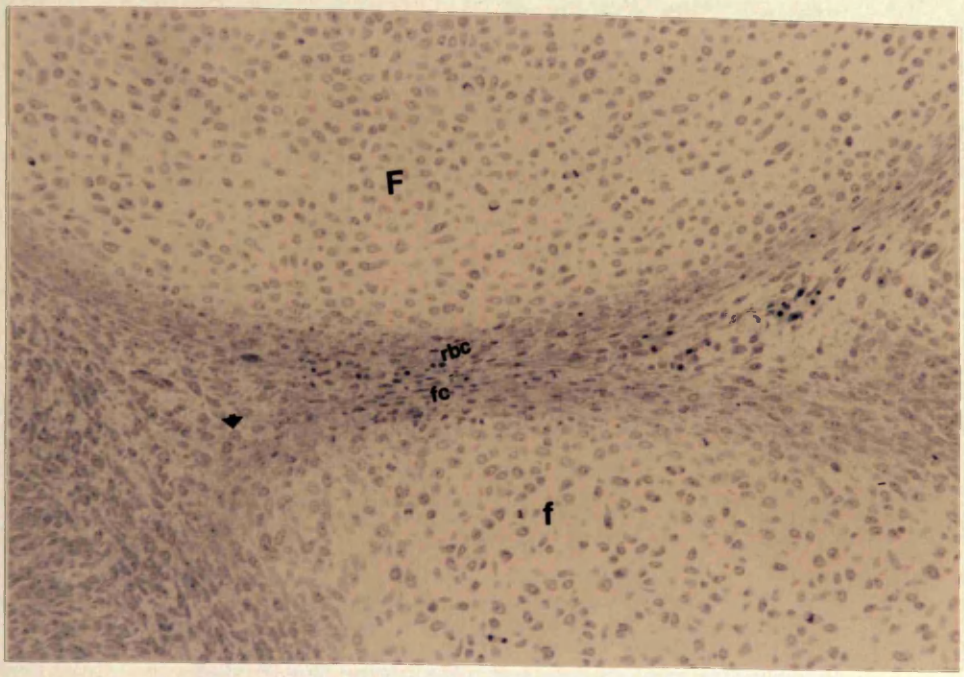

m
TF

Fig. 49: Paralysed chick; knee joint; shows early fibrous fusion between the lateral condyle of the femur (F) and the fibula (f). Blood vessels and red blood cells (rbc) are present at the site of incipient fusion. Some of the cells in the interzone appear to be differentiating to cartilage cells (arrows), the rest are fibroblasts with elongated nuclei (fc).
Stage 37 (12 days); paralysed; sagittal section; plastic; Azur blue II; x 230.

Fig. 50: Paralysed chick; knee joint; shows fibrous fusion between the lateral condyle of the femur (F), fibula (f) and lateral condyle of tibia (T). The lateral meniscus and transverse tibiofibular ligament have disintegrated completely.
Stage 37 (12 days); paralysed; sagittal section; plastic; Azur blue II; x 65.


F
rbc
fc
f

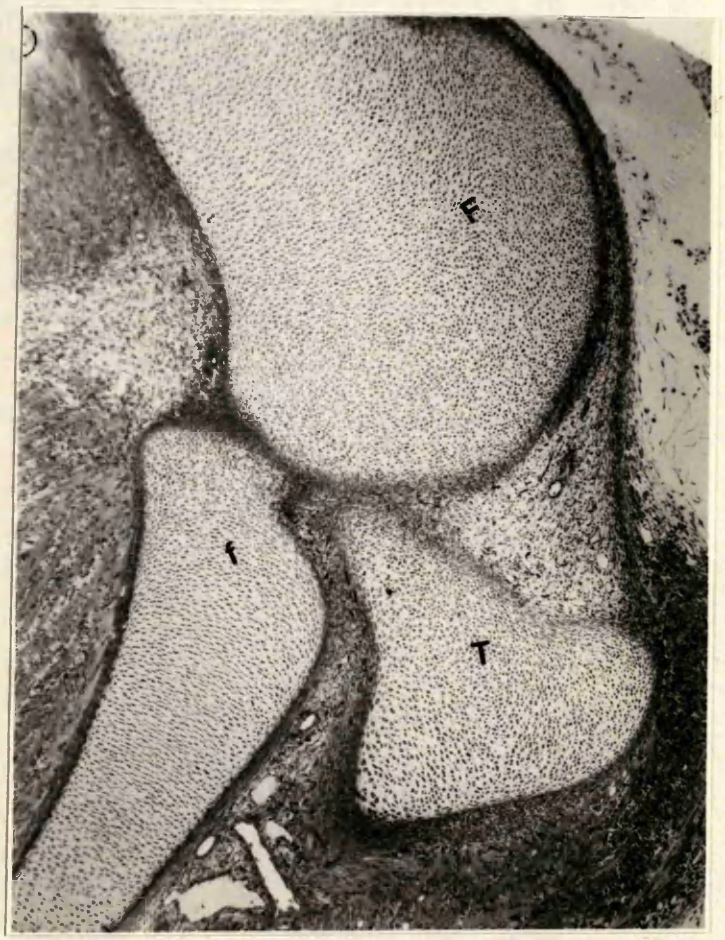
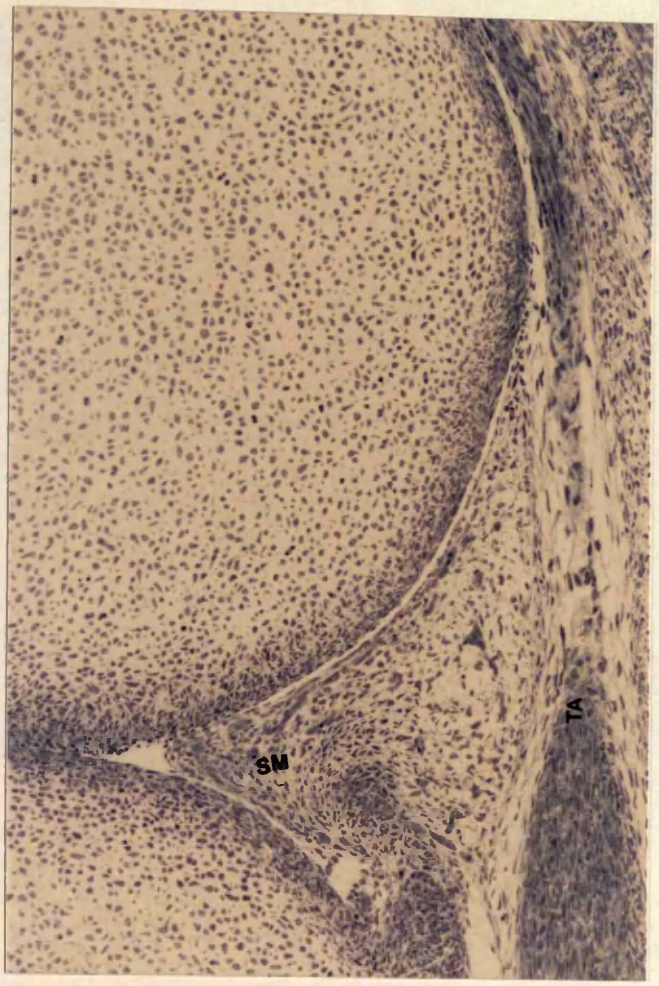

F
f
T

Fig. 51: Normal chick; knee joint; shows the synovial mesenchyme of the knee joint (SM), at the site of the tibialis anterior tendon (T.A.). There is no evidence of preadipocytes.
Stage 37 (12 days); control; sagittal section; plastic; Azur blue II; x 144.

Fig. 52: Paralysed chick; knee joint; shows the synovial mesenchyme of the knee joint (SM), at the site of the tibialis anterior tendon (T.A.). The mesenchyme is looser than that shown in Fig. 51 and shows a few preadipocytes (arrows).
Stage 37 (12 days); paralysed; sagittal section; plastic; Azur blue II; x 248.


SM
TA


SM
TA

Fig. 53: Normal chick; skeletal muscle; shows part of the gastrocnemius muscle. The muscle fibres (m.f.) are at the immature (myotube) stage of development, with many nuclei arranged in a line in the centre of the fibre. Between the fibres are blood vessels and connective tissue cells, including macrophages.
Stage 37 (12 days); control; sagittal section; plastic; Azur blue II; x 200.

Fig. 54: Paralysed chick, skeletal muscle; shows part of the paralysed gastrocnemius muscle. The muscle fibres (mf) have lost their regular longitudinal organisation.
Stage 37 (12 days); paralysed; sagittal section; plastic; Azur blue II; x 144.


mf

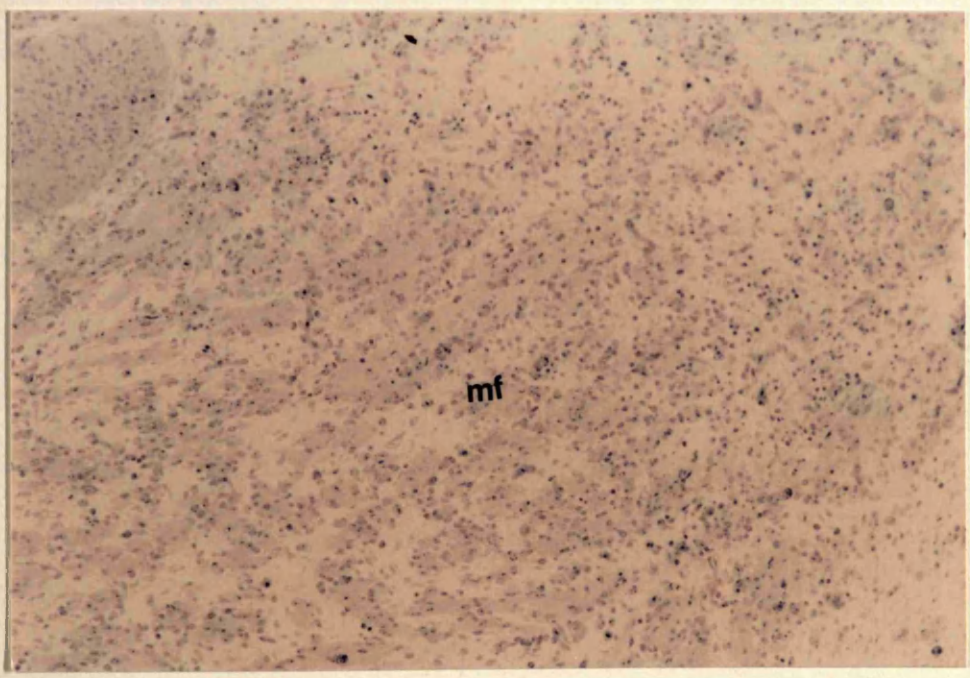
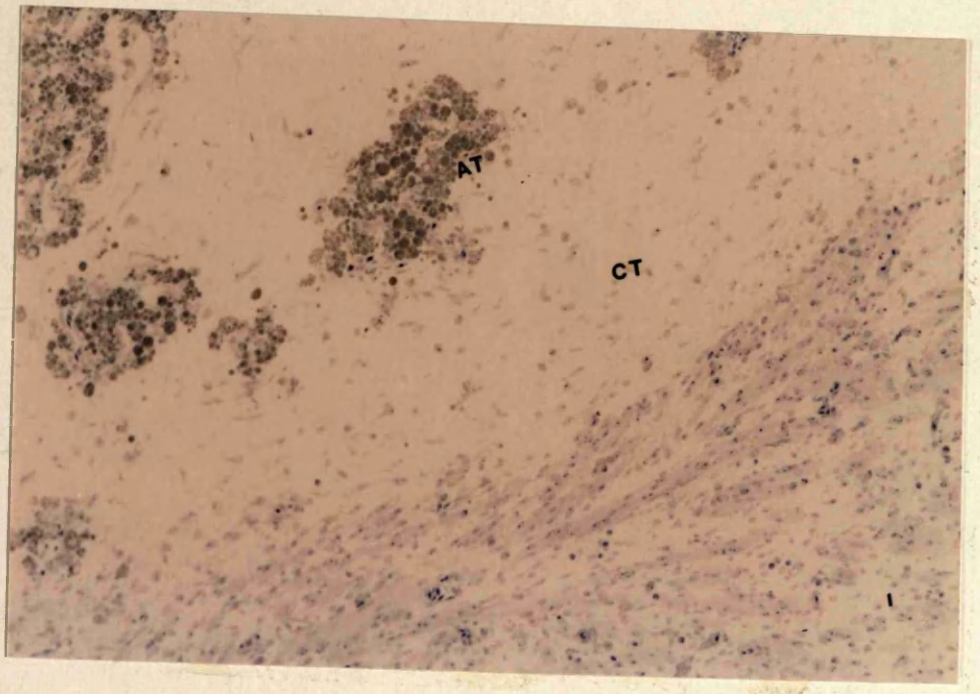

mf

Fig. 55: Paralysed chick; skeletal muscle; shows the paralysed gastrocnemius muscle fibres (mf) which are being replaced by loose connective tissue (CT) and adipose tissue (AT).
Stage 37 (12 days); paralysed; sagittal section; plastic; Azur blue II; x 144.

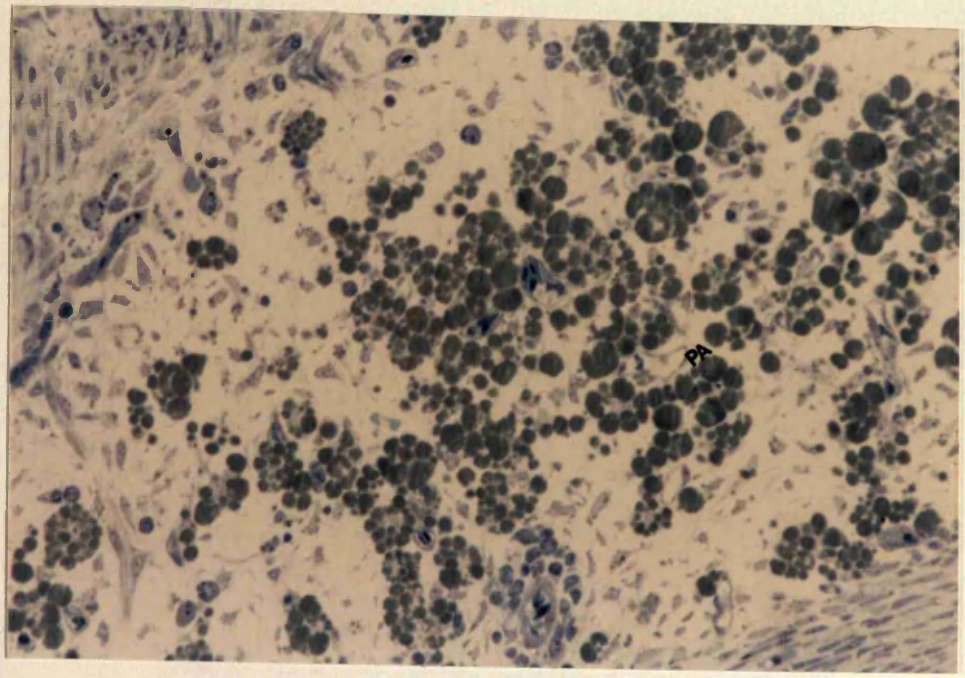
Fig. 56: Paralysed chick; skeletal muscle; shows part of the paralysed tibialis anterior muscle. Sarcolemmal nuclei are increased and some are centrally located. Numerous blood vessels (bv), macrophages (M), white blood cells (w.b.c.), preadipocytes (arrows) and fibroblast cells are present among the degenerative muscle fibres.
Stage 38 (15 days); paralysed; sagittal section; plastic; Azur blue II; x 240.


AT
CT
I


BV

Fig. 57: Paralysed chick; knee joint; shows the synovial mesenchyme with large numbers of preadipocytes (P.A.) and abundant blood vessels.
Stage 38 (13 days); paralysed; sagittal section; plastic; Azur blue II; x 290.

Fig. 58: Paralysed chick; knee joint; shows the synovial mesenchyme with large numbers of invading white blood cells (w.b.c).
Stage 38 (13 days); paralysed; sagittal section; plastic; Azur blue II; x 230.


PA

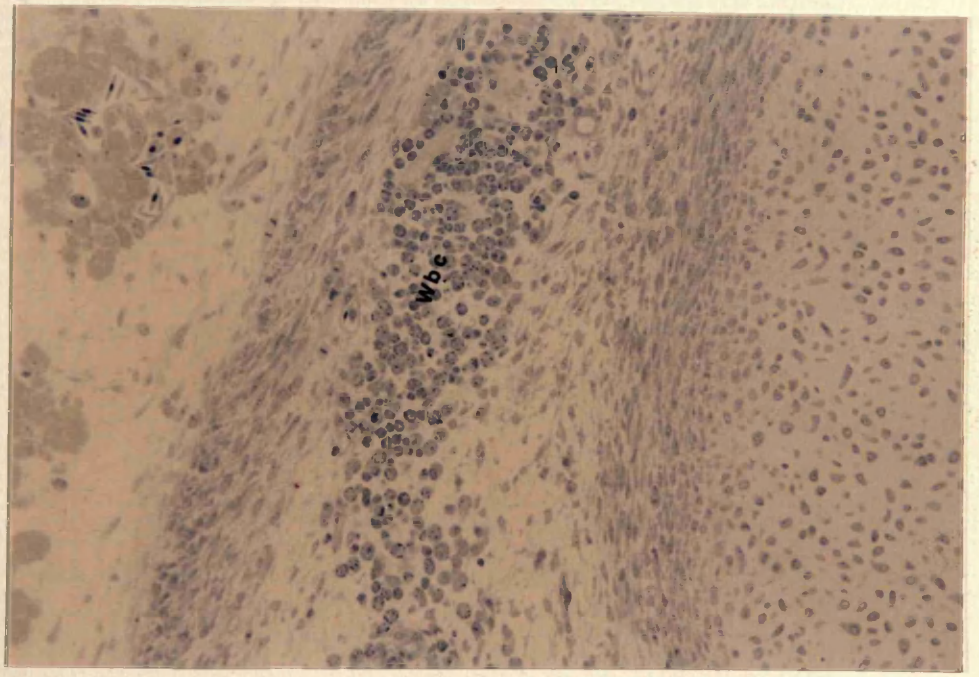
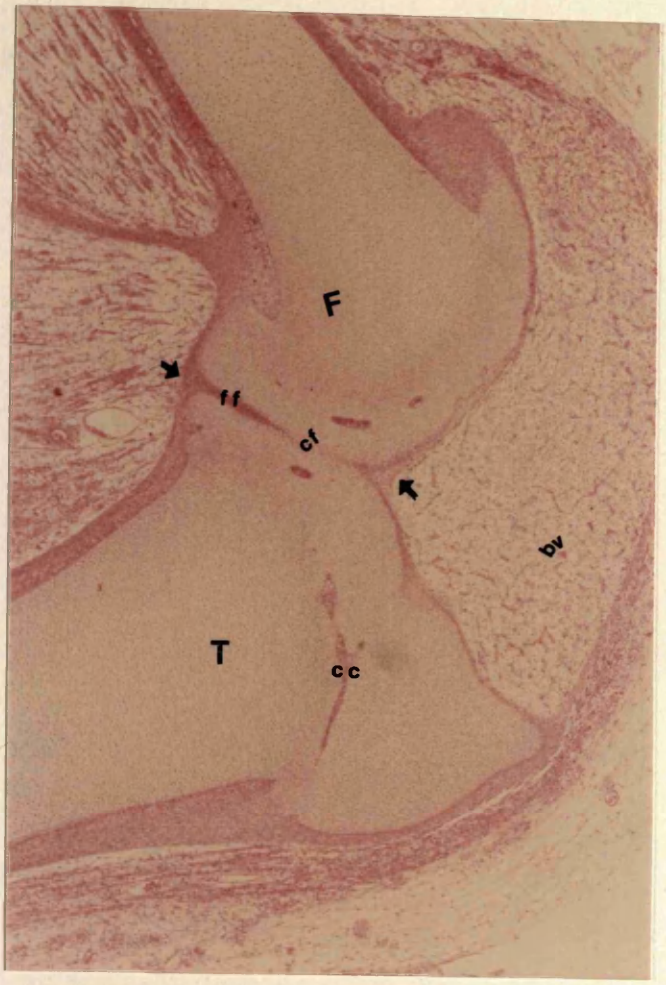
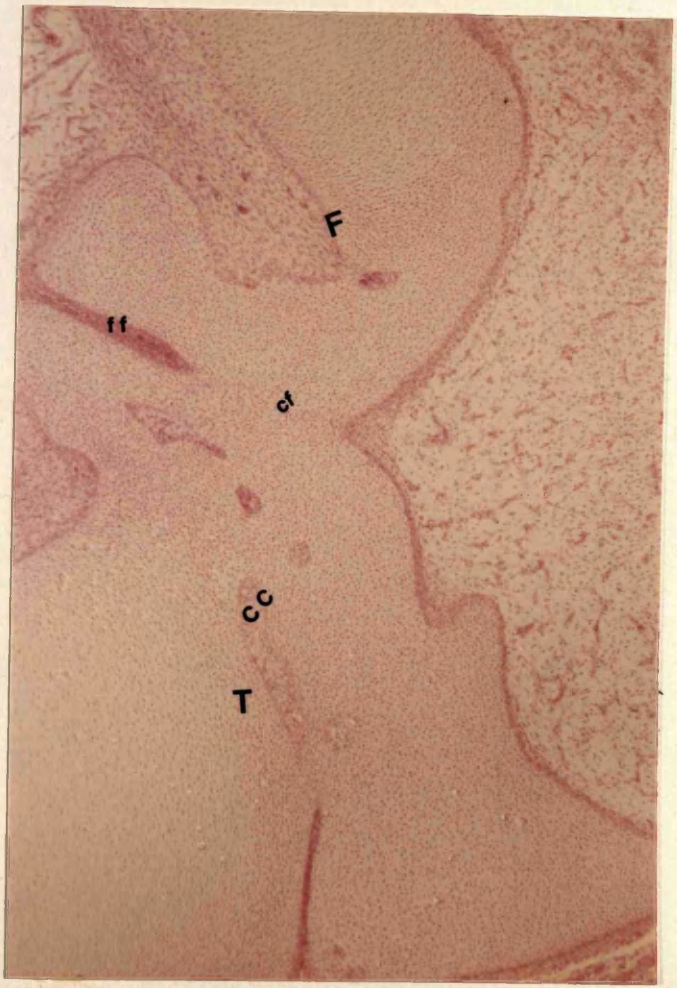

Wbc

Fig. 59: Paralysed chick; knee joint; shows cartilaginous fusion (C.F.) on the anterior aspect and fibrous fusion (f.f.) on the posterior aspect of the joint between the lateral condyle of the femur (F) and lateral condyle of tibia (T). The patella has not developed. Numerous blood vessels (b.v.) are present within the synovial mesenchyme. Cartilage canals (CC) are present in the condyles of the femur and tibia. The perichondria (arrows) of two cartilaginous epiphyses are continuous with one another.
Stage 38 (15 days); paralysed; sagittal section; wax; H & E; x 58.

Fig. 60: Paralysed chick; knee joint; shows cartilaginous fusion (cf) on the anterior aspect and fibrous fusion (ff) on the posterior aspect of the joint between the femur (F) and the tibia (T) at the site of the intra-articular ligaments. Numerous cartilage canals (CC) are present in the proximal epiphysis of the tibia and the distal epiphysis of the femur.
Stage 38 (15 days); paralysed; sagittal section; wax; H & E; x 92.


F
ff
cf
bv
T
cc


F
ff
cf
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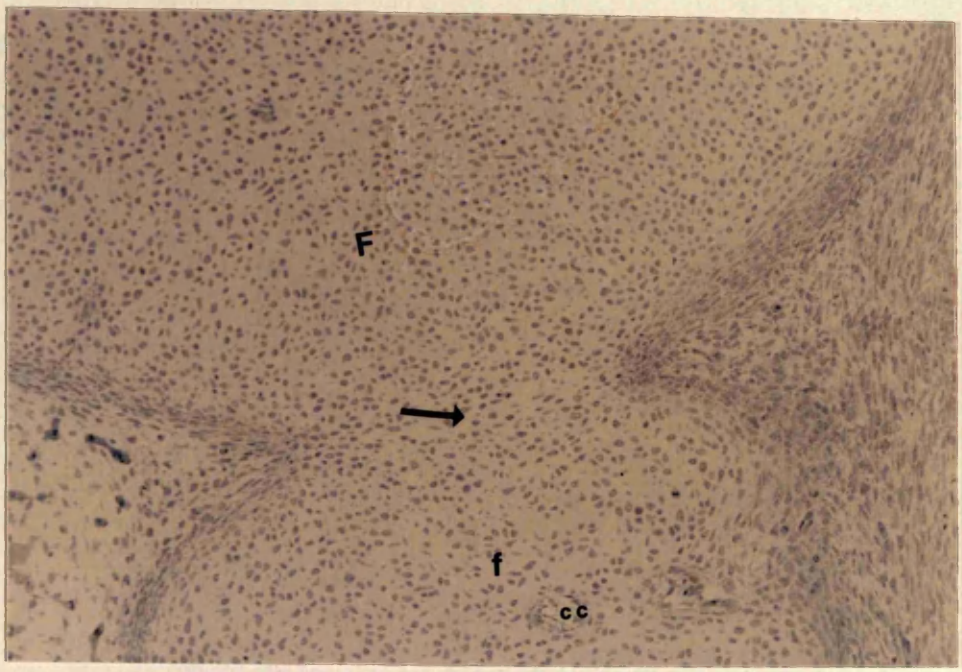
T

Fig. 61: Paralysed chick; knee joint; shows cartilaginous fusion (arrow) between the lateral condyle of the femur (F) and the fibula (f). Note (i) continuity of the perichondria of the two cartilaginous epiphyses and (ii) presence of a cartilage canal (CC) within the fibula. Stage 38 (15 days); paralysed; sagittal section; plastic Azur blue II; x 144.

Fig. 62: Paralysed chick; knee joint; shows fusion between lateral condyle of femur (F) and fibula (f) and between proximal end of tibia and fibula.
Note: . The absence of tibialis anterior tendon
. blood vessels (bv) at the site of fusion
. abundant blood vessels (arrows) within the synovial mesenchyme.
Stage 38 (15 days); paralysed; sagittal section; wax; H & E; x 65.


F
f
cc

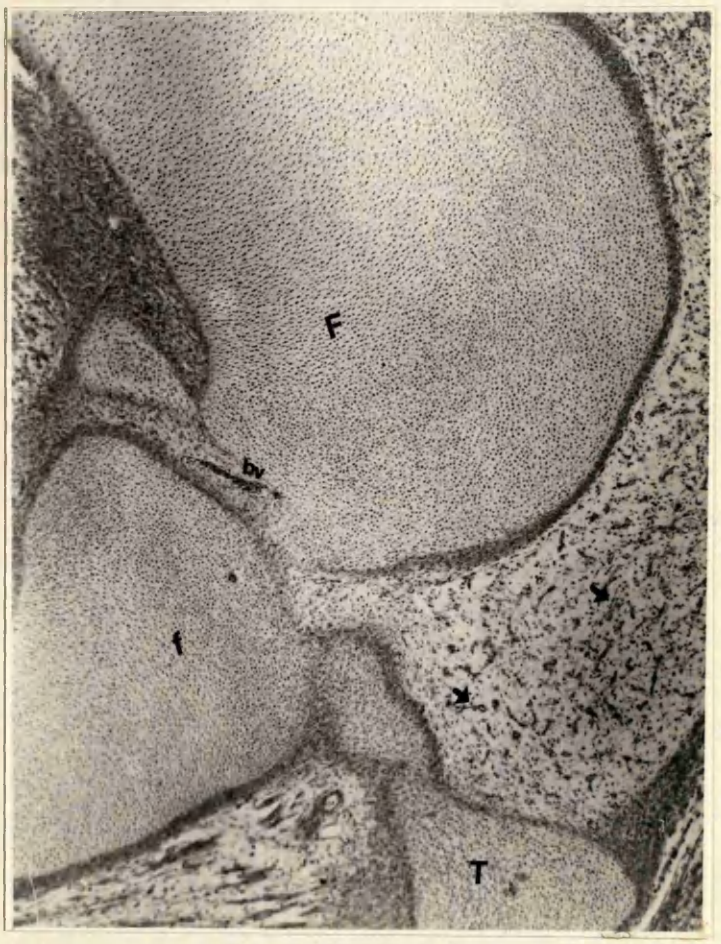
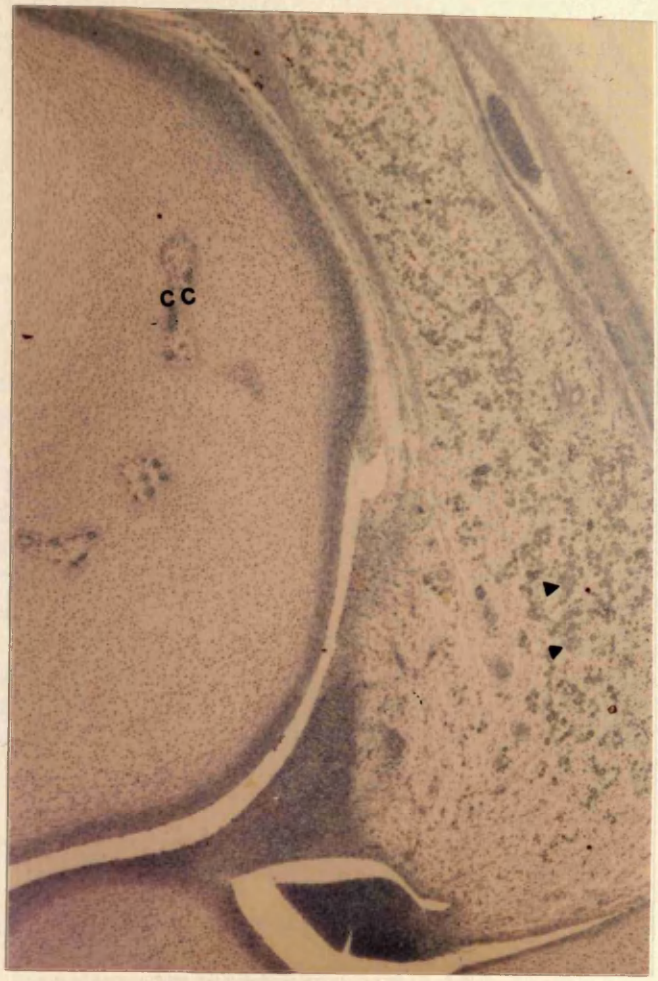

F
bv
f
T

Fig. 63: Normal chick; knee joint; shows the appearance of the preadipocytes (arrows) at the site of the future infrapatellar fat pad within the synovial mesenchyme of the knee joint. Note cartilage canals (CC) within the lateral condyle of the femur.
Stage 39 (14 days); sagittal section; plastic; Azur blue II; x 144.

Fig. 64: High power of part of Fig. 63. A small synovial fold (sf) projects into the joint cavity.
Stage 39 (14 days); control; sagittal section; plastic; Azur blue II; x 230.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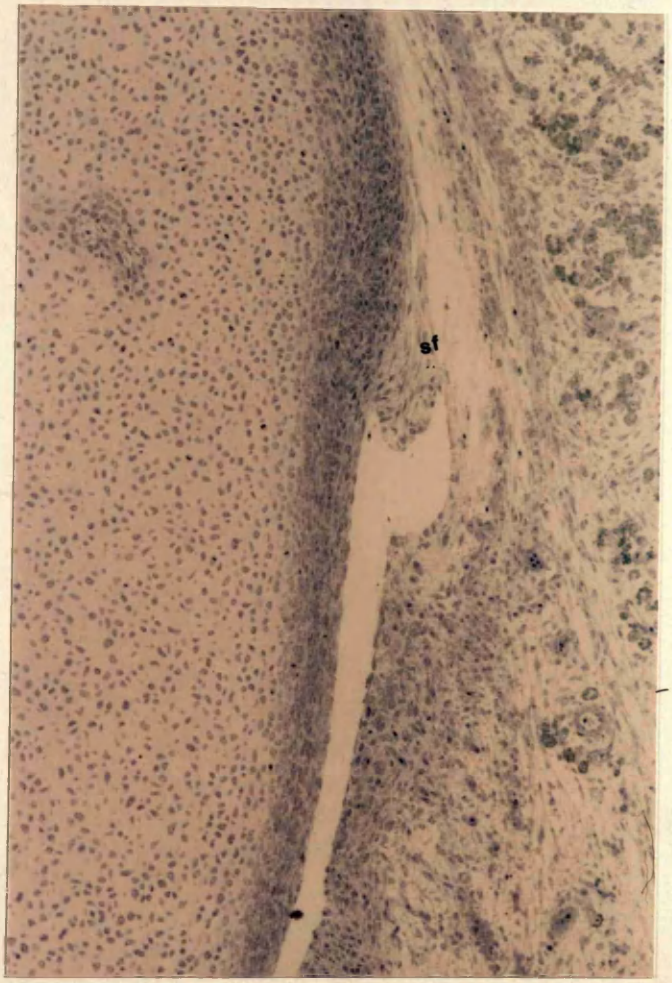

sf

Fig. 65: Paralysed chick; knee joint; shows the patellar ligament (PL) has lost its regular arrangement and has been invaded by blood vessels (bv). Numerous preadipocytes (PA) and white blood cells (wbc) are present within the synovial mesenchyme. The perichondria (arrow) of two cartilaginous epiphyses are continuous with one another.
Stage 39 (17 days); paralysed; sagittal section; plastic; Azur blue II; x 144.

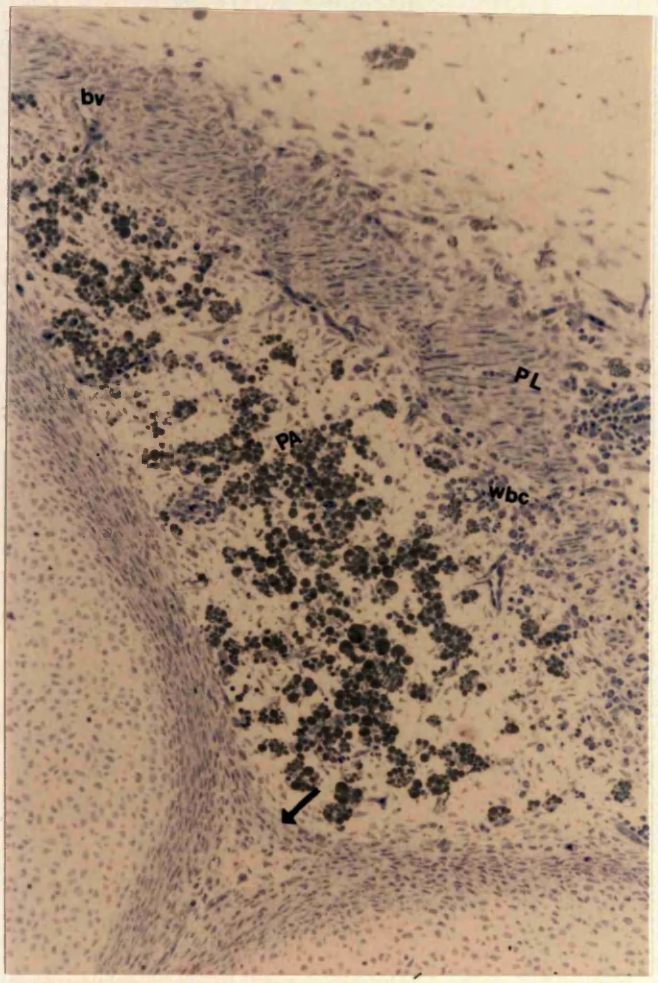
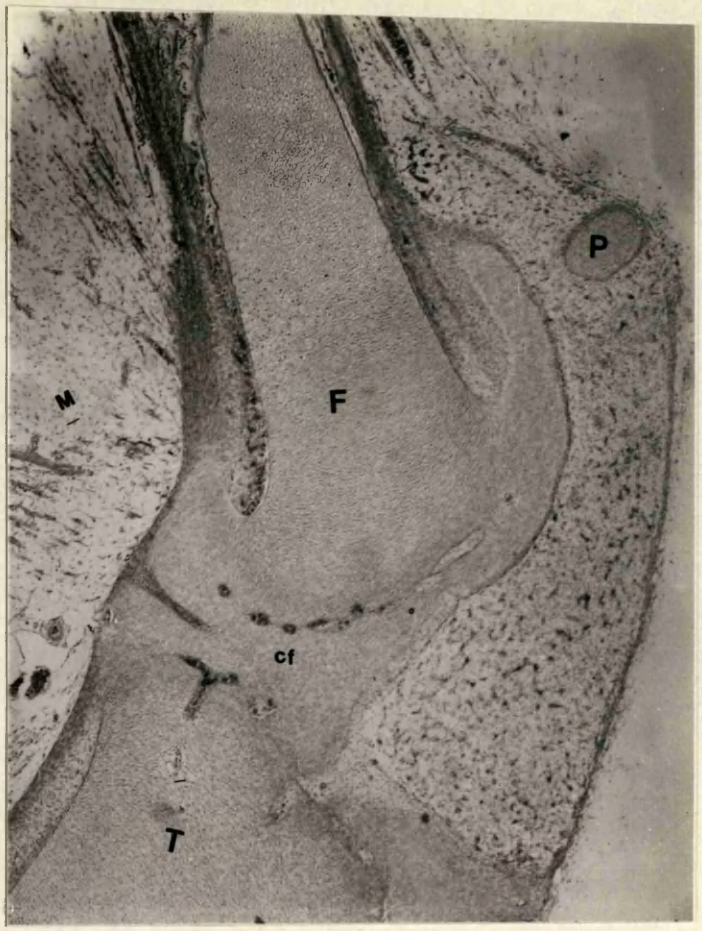

bv
PL
PA
wbc

Fig. 66: Paralysed chick; knee joint; shows cartilaginous fusion (c.f.) between the femur (F) and tibia (T). A small patella (P) is present in front of the femur. The muscles (M) around the knee joint have degenerated almost completely.
Stage 39 (17 days); paralysed; sagittal section; wax, H & E; x 65.

Fig. 67: High power of part of Fig. 66. The femoro-patellar joint cavity has not developed. Its place is taken by very vascular adipose tissue. The patellar ligament (PL) is very thin, and disorganized.
Stage 39 (17 days); paralysed; sagittal section; wax; H & E; x 200.


P
F
M
cf
T

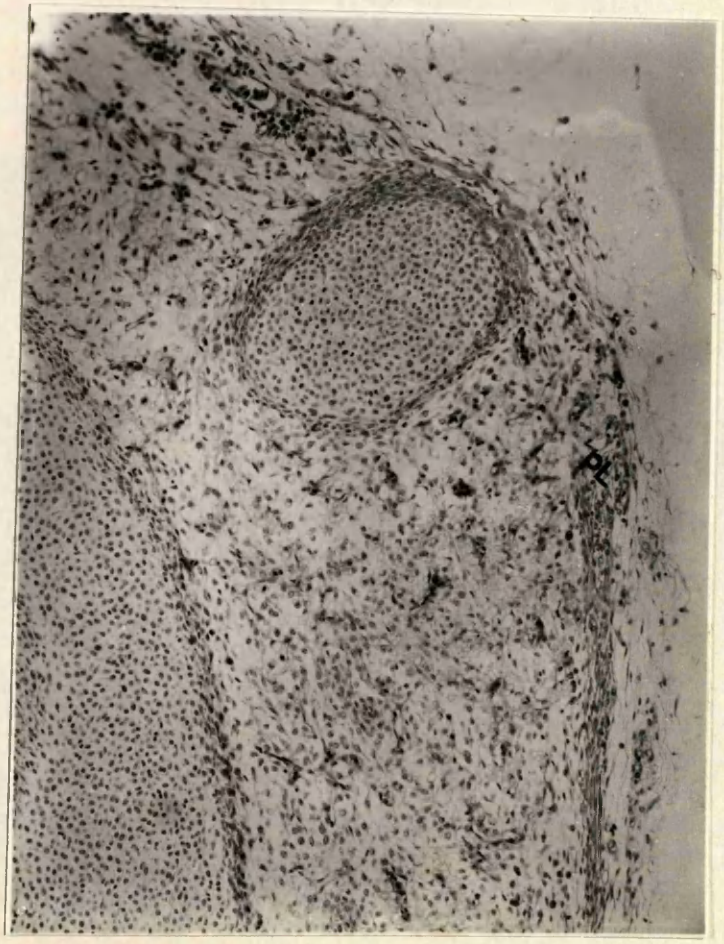
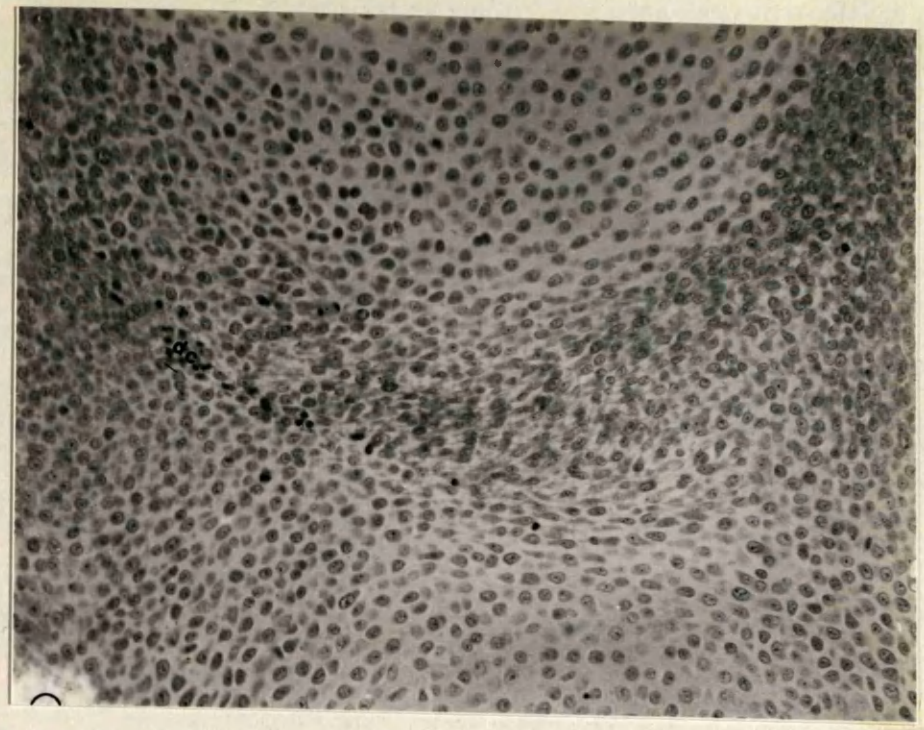

OL

Fig. 68: Normal chick; 3 M.-P. joint; shows the chondrogenic layers are mostly defined and there is none if any loose intermediate zone. "Dark cells" (dc) are present within the interzone.
Stage 34 (9 days); control; sagittal section; wax; H & E; x 320.

Fig. 69: Paralysed chick; 3 M.-P. joint; shows the appearance of interzone similar to that of Fig. 68. "Dark cells" (dc) are present within the loose intermediate layer.
Stage 34 (9 day); paralysed; sagittal section; wax; H & E; x 320.



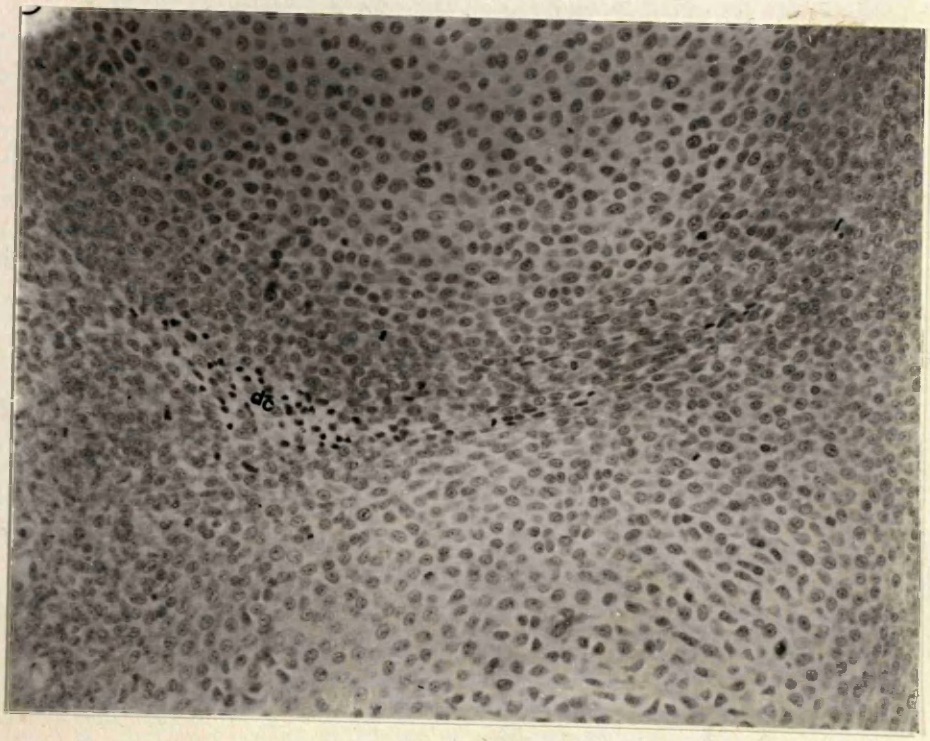
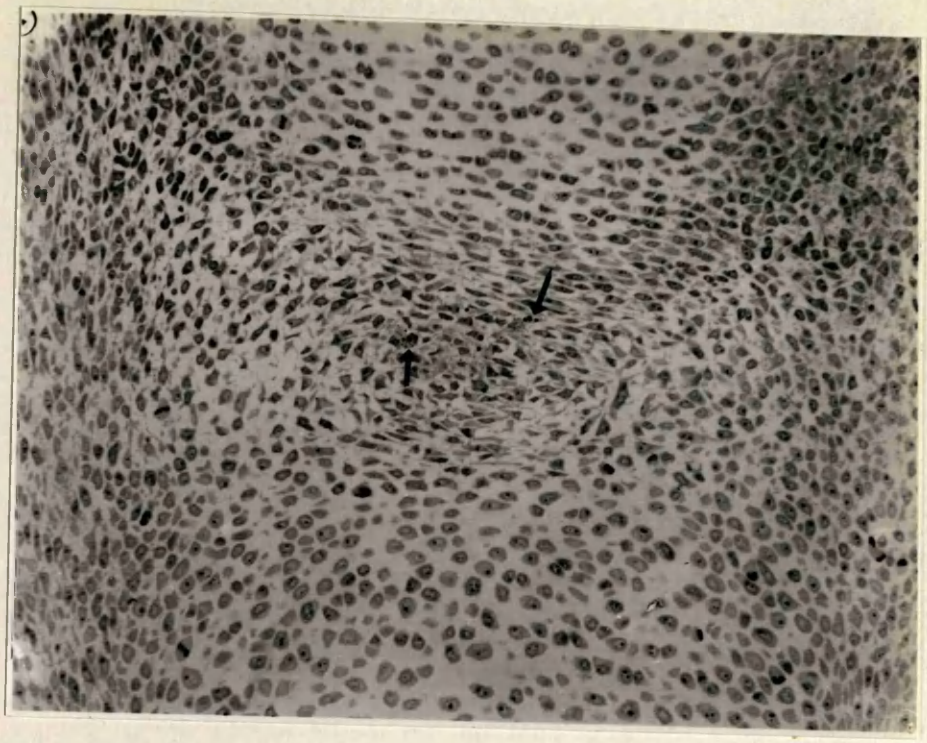


Fig. 70: Normal chick; 3 M.-P. joint; shows "dark cells" within the interzone to have been engulfed by macrophages (arrows).
Stage 34 (9 day); control; sagittal section, plastic; Azur blue II; x 320.

Fig. 71: Normal chick; 3rd M.-P. joint; shows further loosening of the intermediate layer of the interzone, which contains a number of "dark cells". The two chondrogenic layers are better defined than at Stage 34, so that the interzone now appears 3-layered.
Stage 35 (10 days); control; sagittal section; H & E; x 200.



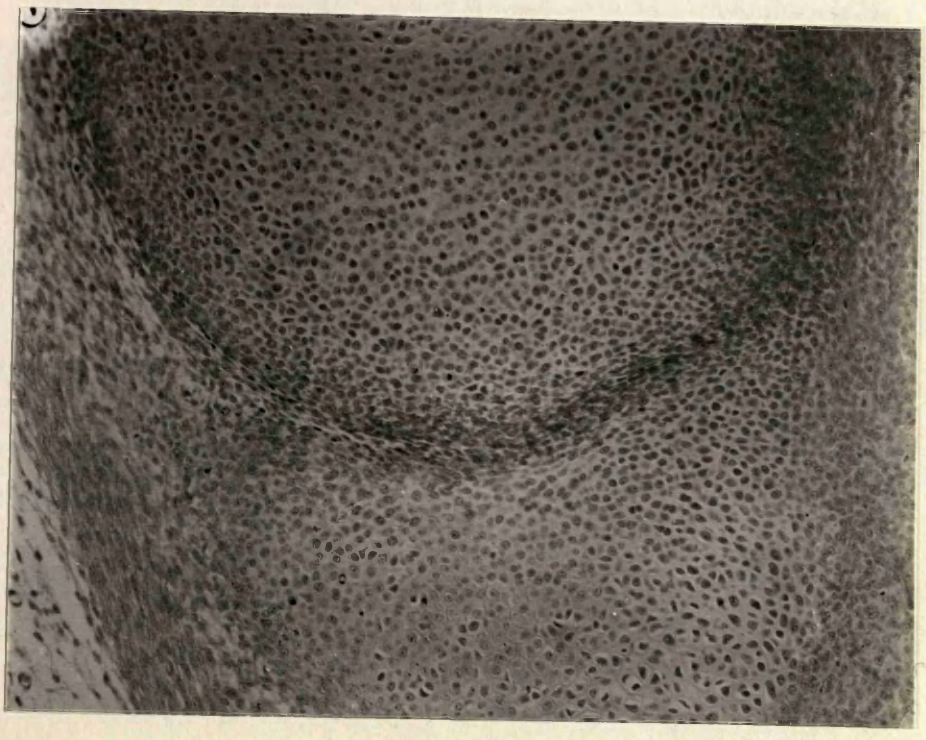
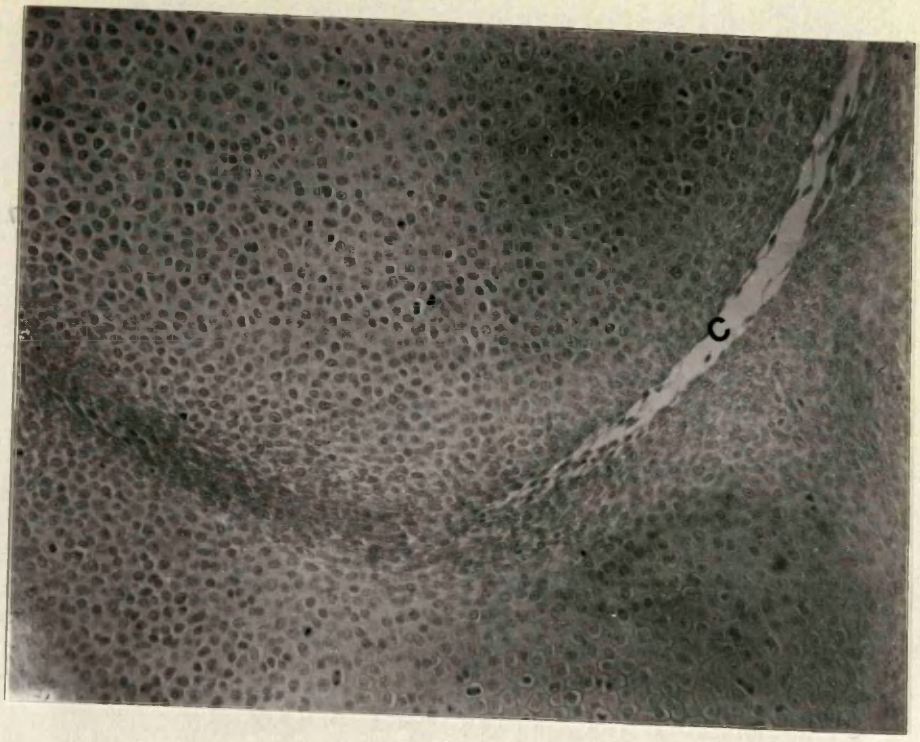


Fig. 72: Normal chick; 3rd M.-P. joint; shows first appearance of joint cavity (C) within the intermediate layer of the interzone.
Stage 36 (11 days); control; sagittal section; wax; H & E; x 252.

Fig. 73: Paralysed chick; 3rd M.-P. joint; shows many "dark cells" (dc) within the interzone. There is no sign of a joint cavity. Blood vessels (bv) are present in the peripheral part of the interzone.
Stage 36 (11 days); paralysed; sagittal section; plastic; Azur blue II; x 252.


C

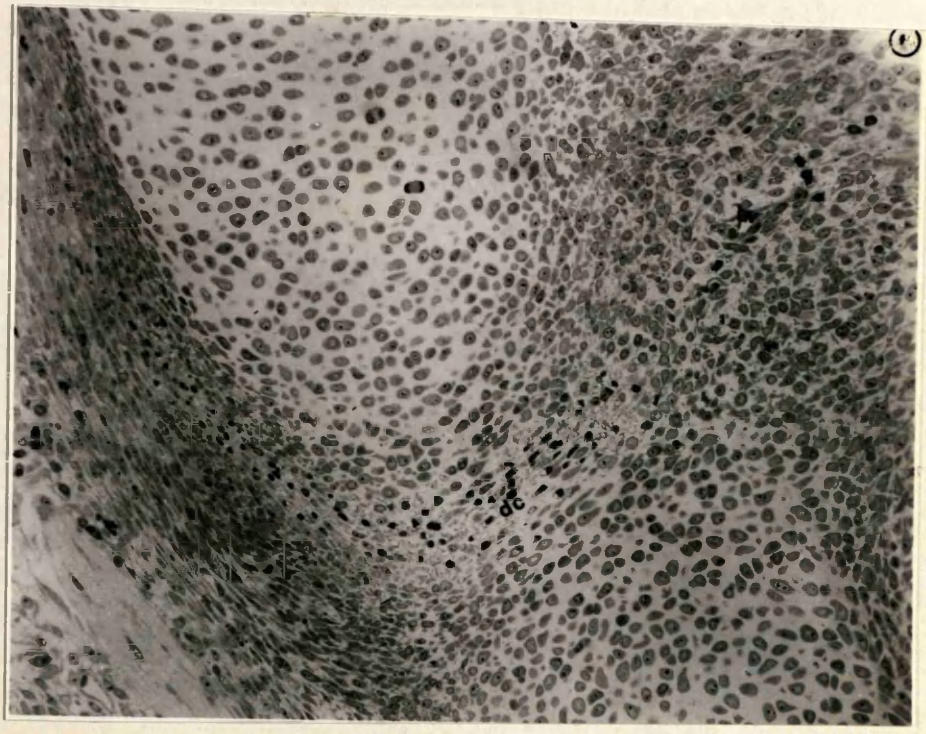

dc

Fig. 74: Paralysed chick; 3rd M.-P. joint; shows the development of a small cavity (C) within the interzone. It has been invaded by blood vessels (bv). A large number of "dark cells" (dc) are also present within the interzone.
Stage 36 (11 days); paralysed; sagittal sections; wax; H & E; x 240.

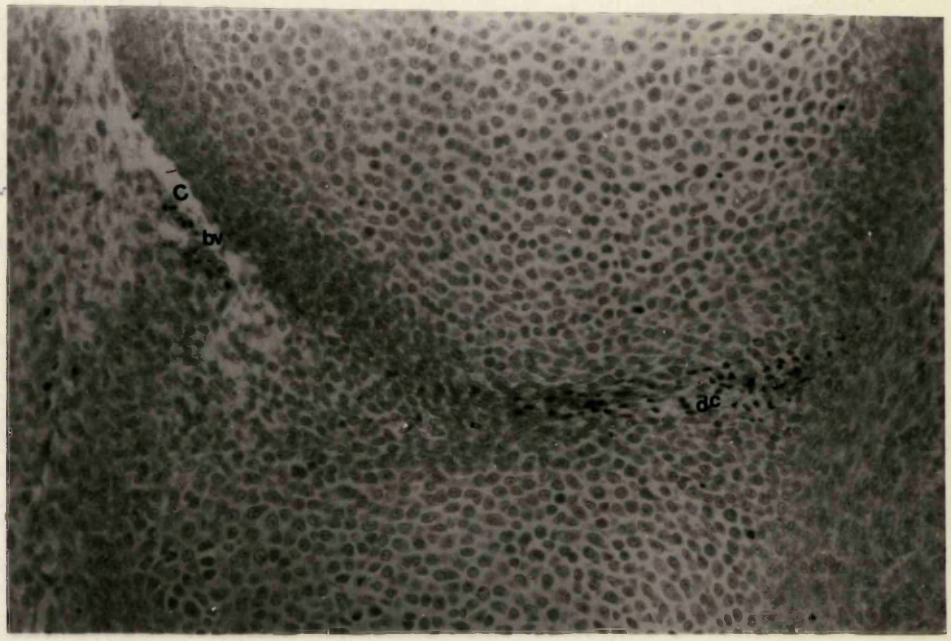
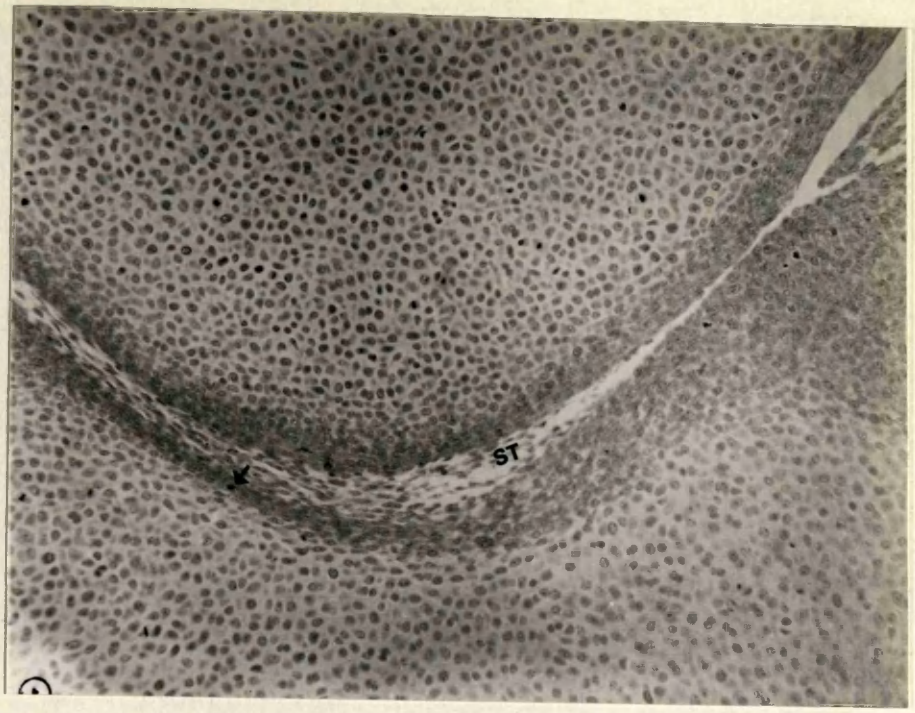

C
bv
dc

Fig. 75: Normal chick; 3rd M.-P. joint; shows the peripheral part of the joint. The joint cavity is well developed in places but elsewhere the future cavity is still occupied by loose connective tissue strands (ST). The articular surfaces are reciprocally curved and show a few dark cells (arrow) along their margins.
Stage 37 (12 days); control; sagittal section; wax; H & E; x 240.

Fig. 76: Paralysed chick; 3rd M.-P. joint; shows the peripheral part of the joint, the joint cavity is not developed and the articular surfaces are flattened. Many "dark cells" (arrowhead) and free red blood cells are present within the interzone. Some interzonal cells are differentiating into precartilage cells (P.C.). Blood vessels (bv) are present at the periphery of the interzone.
Stage 37 (12 days); paralysed; sagittal section; wax; H & E; x 240.


ST

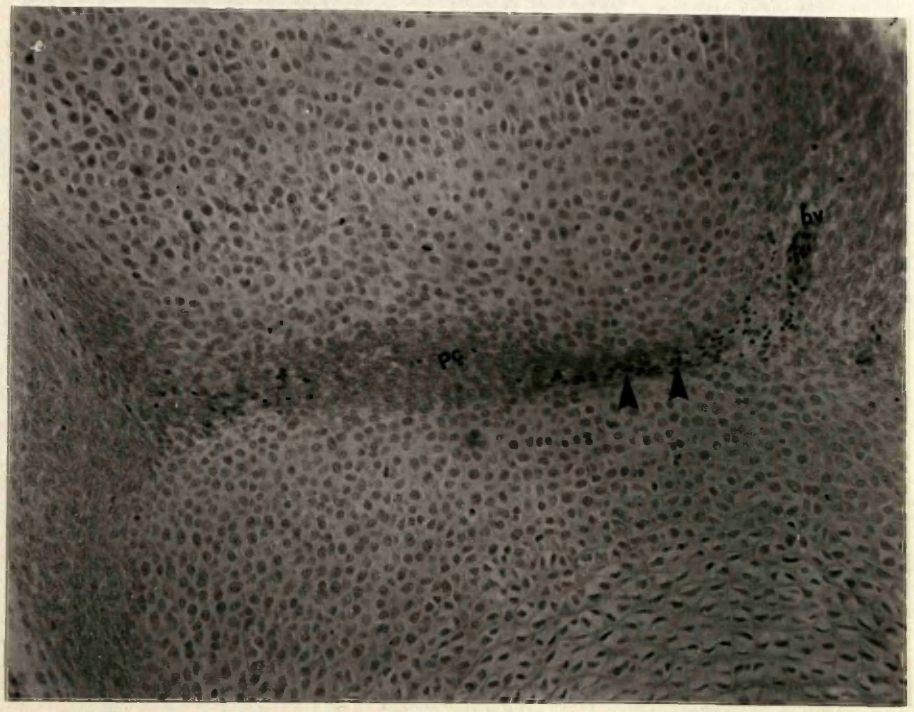
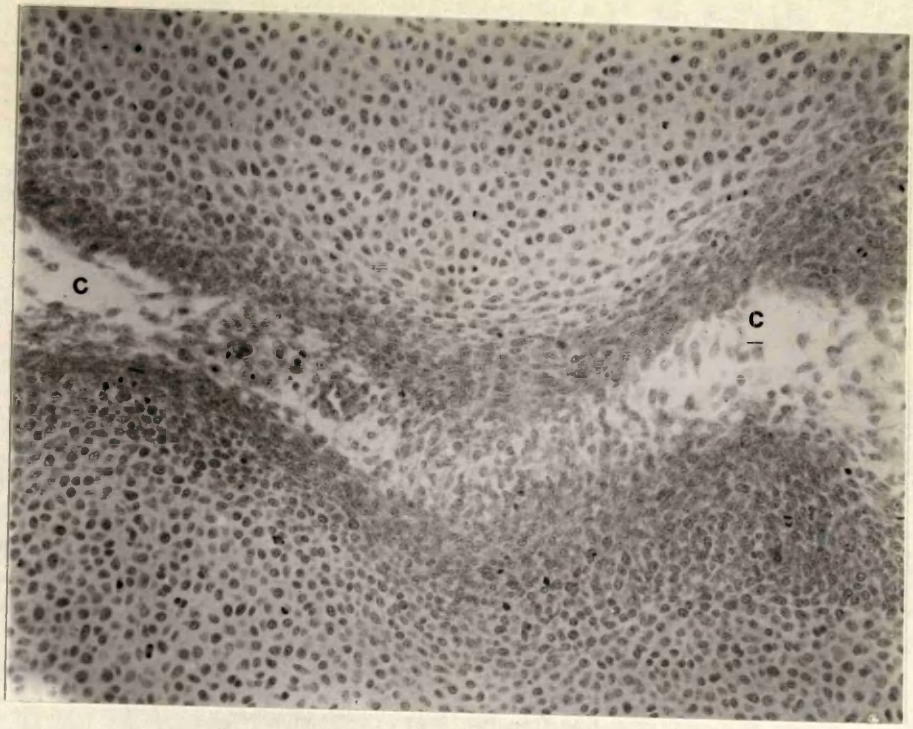

PC
bv

Fig. 77: Normal chick; 3rd M.-P. joint; shows the middle part of the joint. A typical three-layered interzone; the intermediate layer is now very loose and shows early cavity formation (C). It also contains some blood vessels.
Stage 37 (12 days); control; sagittal section; wax; H & E; x 240.

Fig. 78: Normal chick; 3rd M.-P. joint; shows the peripheral part of the joint. The joint cavity is well developed (right of photo); to the left, the future cavity is still occupied by highly vascular loose mesenchyme.
Stage 37 (12 days); control; sagittal section; wax; H & E; x 150.


C
C

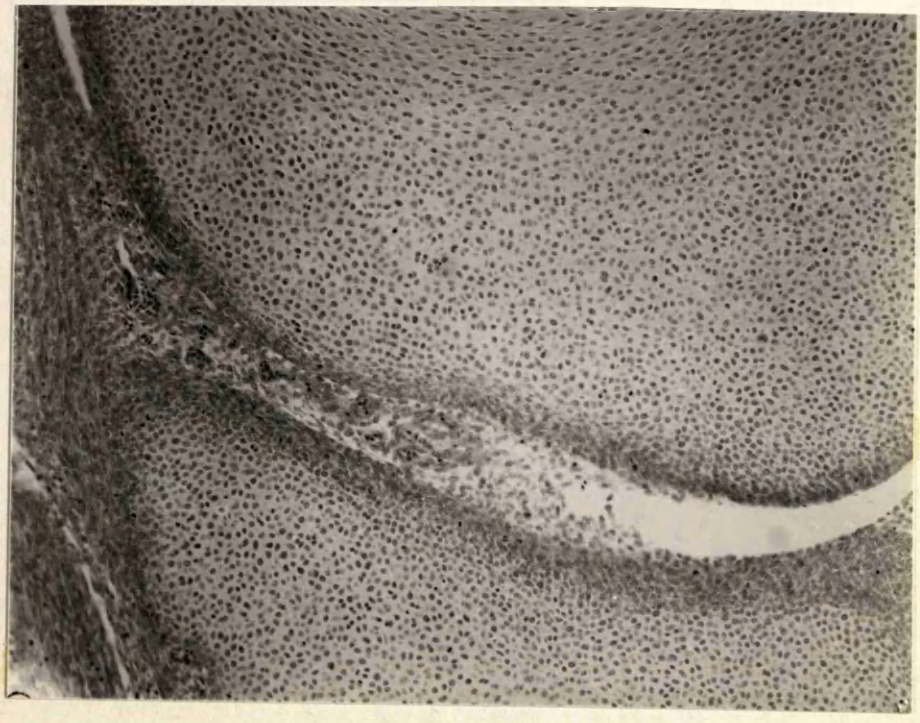


Fig. 79: Paralysed chick; 3rd M.-P. joint; Small cavities (C) are developed at the peripheral part of the interzone. There are some invading blood vessels (bv). Numerous dark cells (arrow) are present within the interzone.
Stage 37 (12 days); paralysed; sagittal section; wax; H & E; x 240.

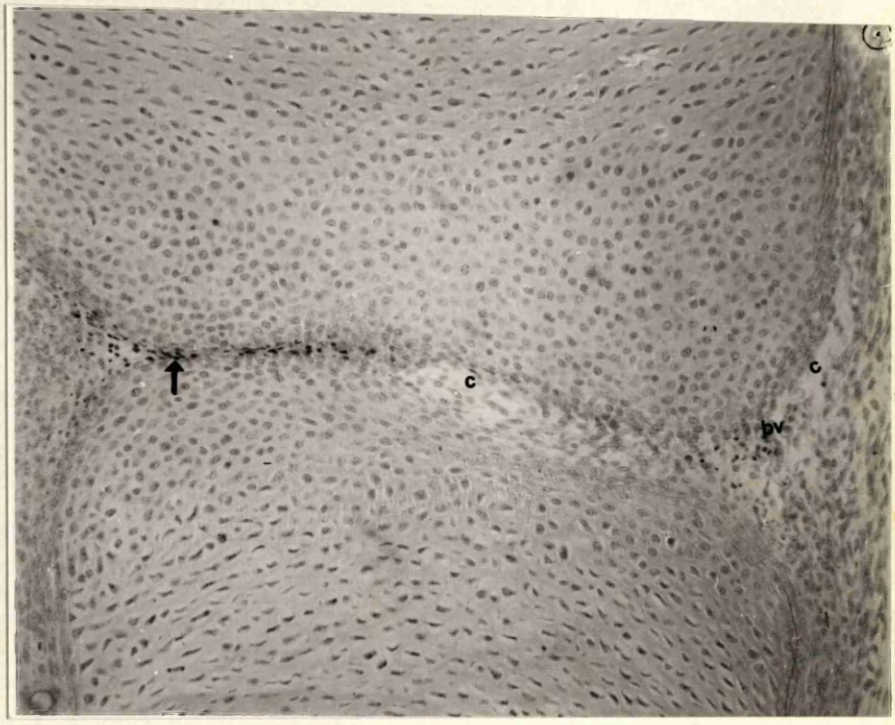
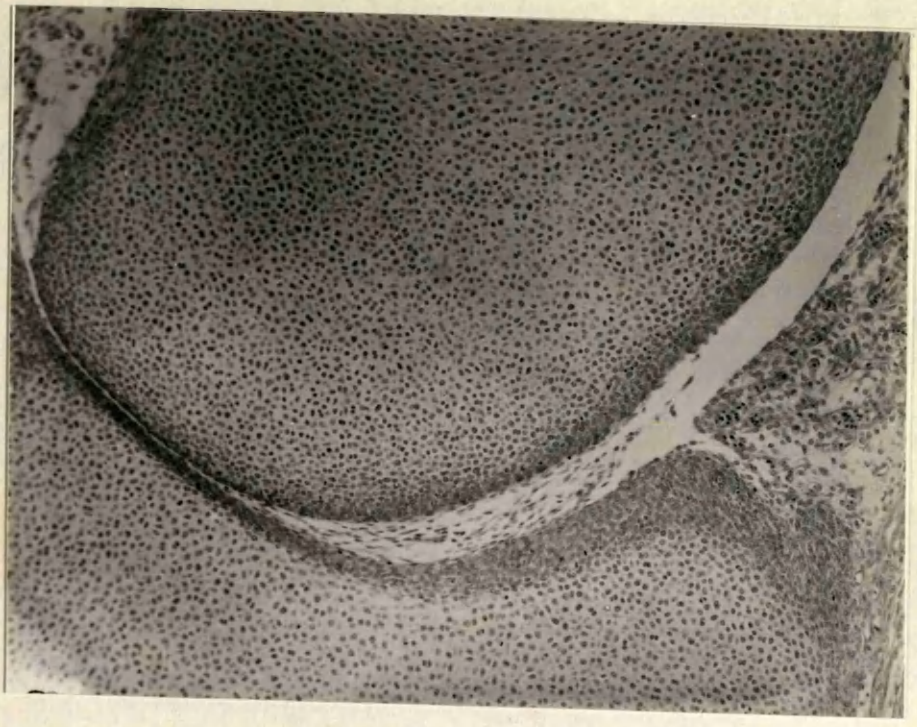

c
c
pv

Fig. 80: Normal chick; 3rd M.-P. joint; The section passes through the peripheral part of the joint. Anteriorly, the joint cavity is well developed; posteriorly it is represented by loose mesenchyme of the intermediate layer of the interzone. Here "dark cells" are present, aligned along the reciprocally curved articular surfaces.
Stage 38 (13 days); control; sagittal section; wax; H & E; x 150.

Fig. 81: Paralysed chick; 3rd M.-P. joint; The joint cavity is not developed, the articular surfaces are flattened, the interzone is very narrow and in some areas the cells have differentiated to cartilage cells (CC)."Dark cells" (d.c.) are also present within the interzone.
Stage 38 (13 days); paralysed; sagittal section; wax; H & E; x 252.



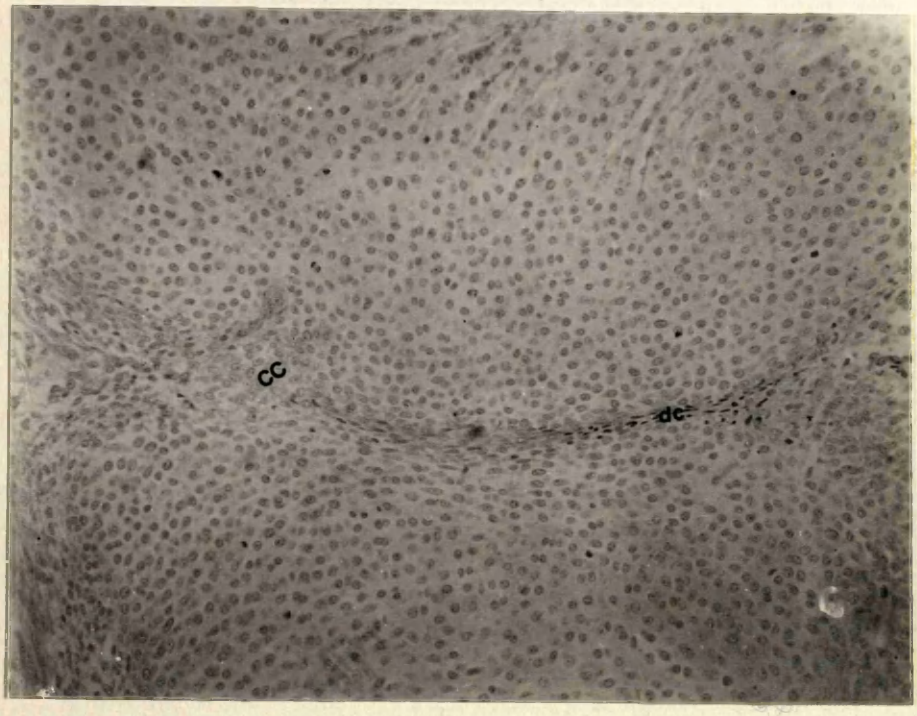
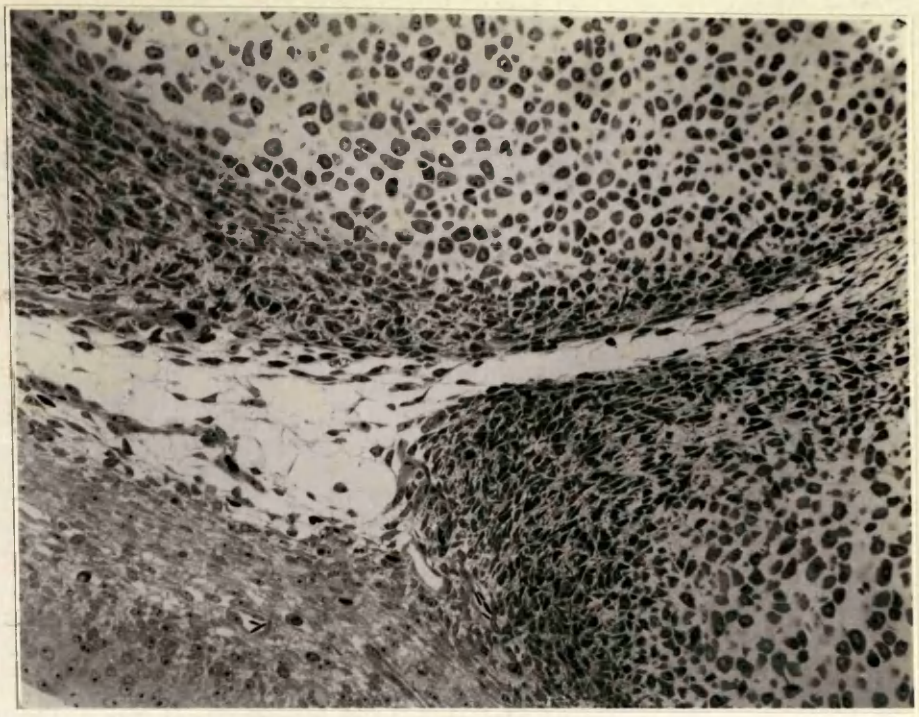

cc
dc

Fig. 82: Normal chick; 3rd M.-P. joint; In this area, cavitation is still proceeding and strands of loose connective tissue are still present.
Stage 38 (13 days); control; sagittal section; plastic; Azur blue II; x 280.

Fig. 83: Paralysed chick; 3rd M.-P. joint; A very small cavity is present, largely occupied by blood vessels (bv) and extravasated red blood cells (rbc). A few "dark cells" can be seen.
Stage 38 (14 days); paralysed; sagittal section; plastic; Azur blue II; x 240.



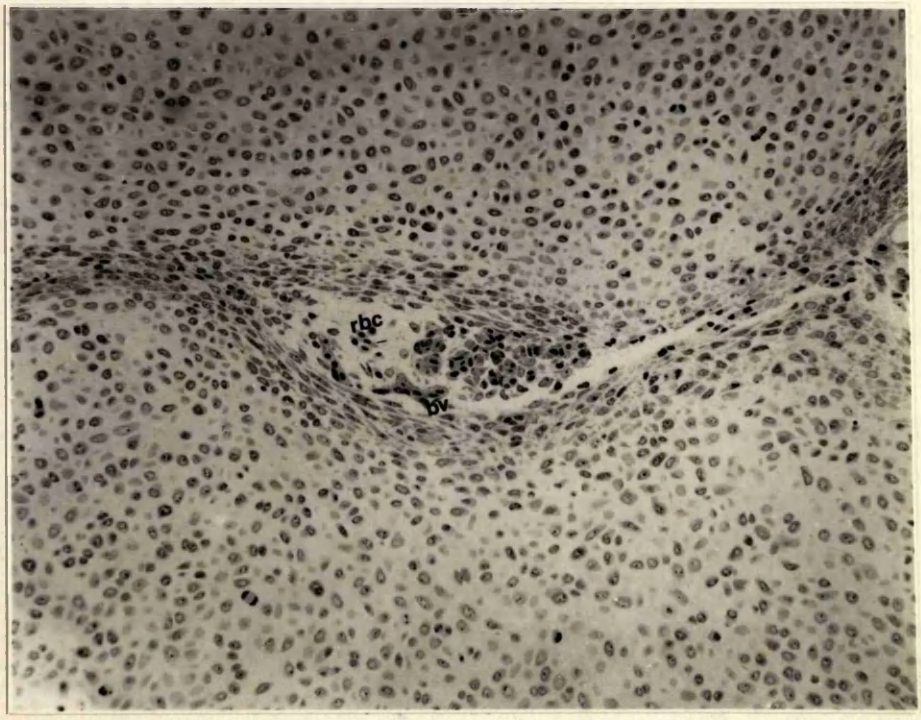
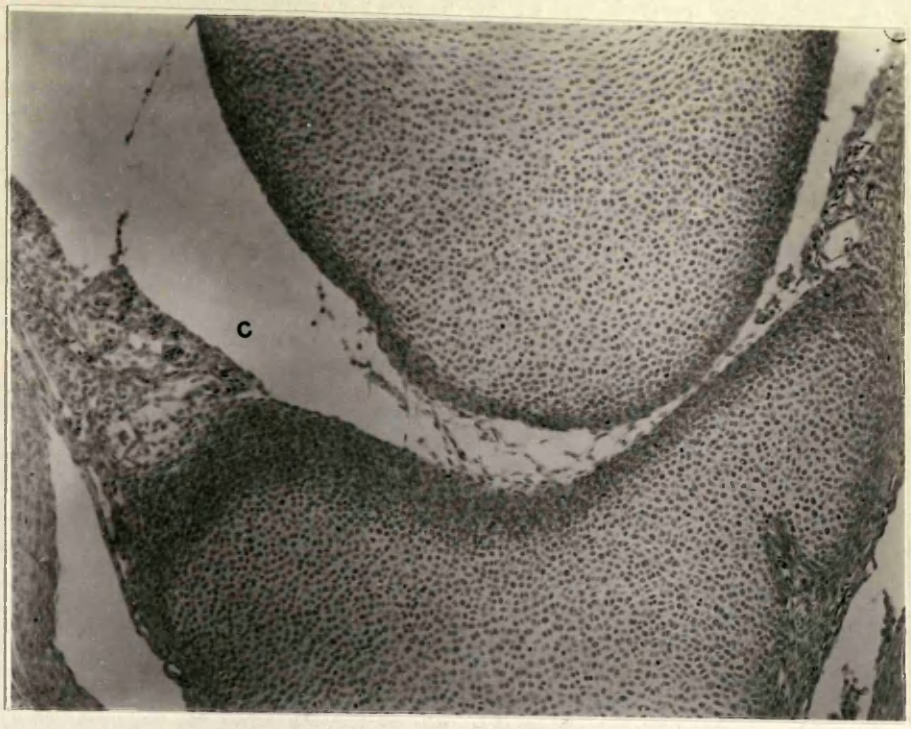

rbc
bv

Fig. 84: Normal chick; 3rd M.-P. joint; The joint cavity (C) is almost complete, but it still contains a few connective tissue strands.
Stage 39 (14 days); control; sagittal section; wax; H & E; x 150.

Fig. 85: Paralysed chick; 3rd M.-P. joint; shows cartilaginous fusion (CF) across the joint. Numerous white blood cells (w.b.c) are present within the synovial mesenchyme at the anterior margin of the joint.
Stage 39 (17 days); paralysed; sagittal section; wax; H & E; x 150.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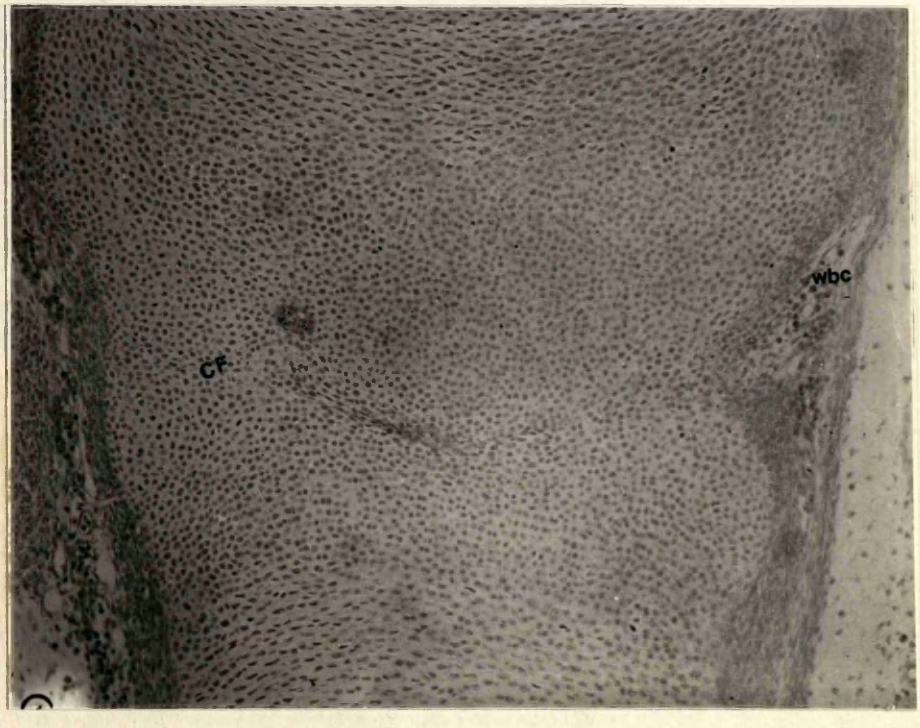

wbc
CF

Fig. 86: Paralysed chick; 3rd M.-P. joint; shows cartilaginous (CF) fusion across the joint. A few "dark cells" (d.c) are present along the line of fusion in the posterior part of the joint. Numerous cartilage canals (CC) are present within the metatarsal cartilage model.
Stage 39 (15 days); paralysed; sagittal section; wax; H & E; x 280.

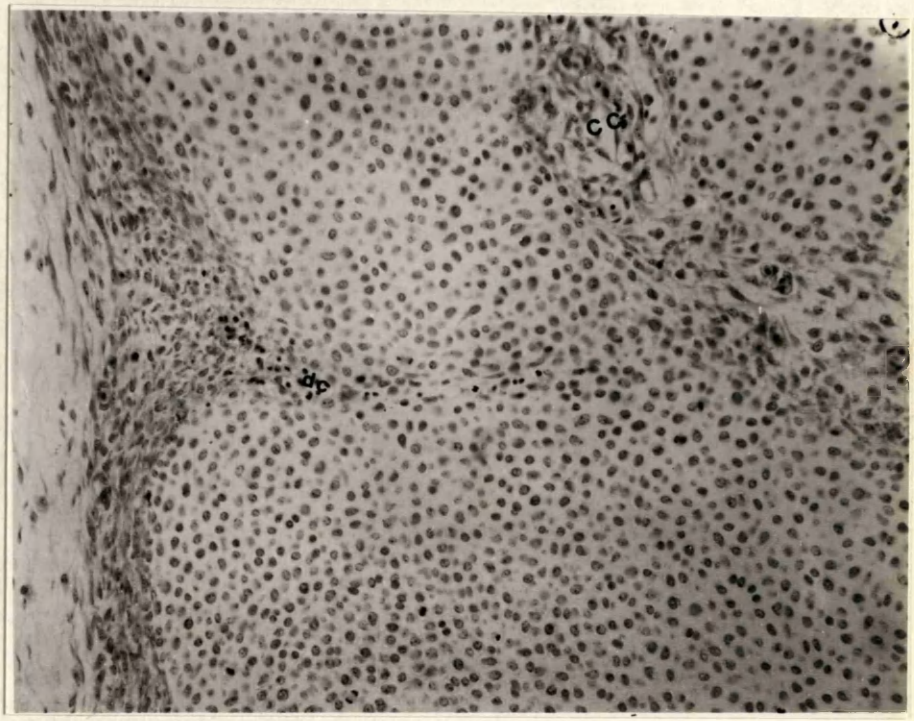

CC
AP

Fig. 87: Normal chick; 3rd M.-P. joint; a montage of EM's taken from the interzone. The interzone contains well spaced, mainly healthy, pleomorphic mesenchymal cells.
Note:
1) Most cells have large, rather pale nuclei, with a thin line of marginal chromatin, and one to three nucleoli. These are regarded as normal, healthy, mesenchymal cells.
2) A few mesenchymal cells have darker more irregularly shaped nuclei, with some aggregation of chromatin, and smaller amounts of cytoplasm.
3) frequent contacts between the cells
4) electron-lucent intercellular space containing sectional profiles of cell processes and microfibrillar material.
Stage 35 (control); EM x 3300.

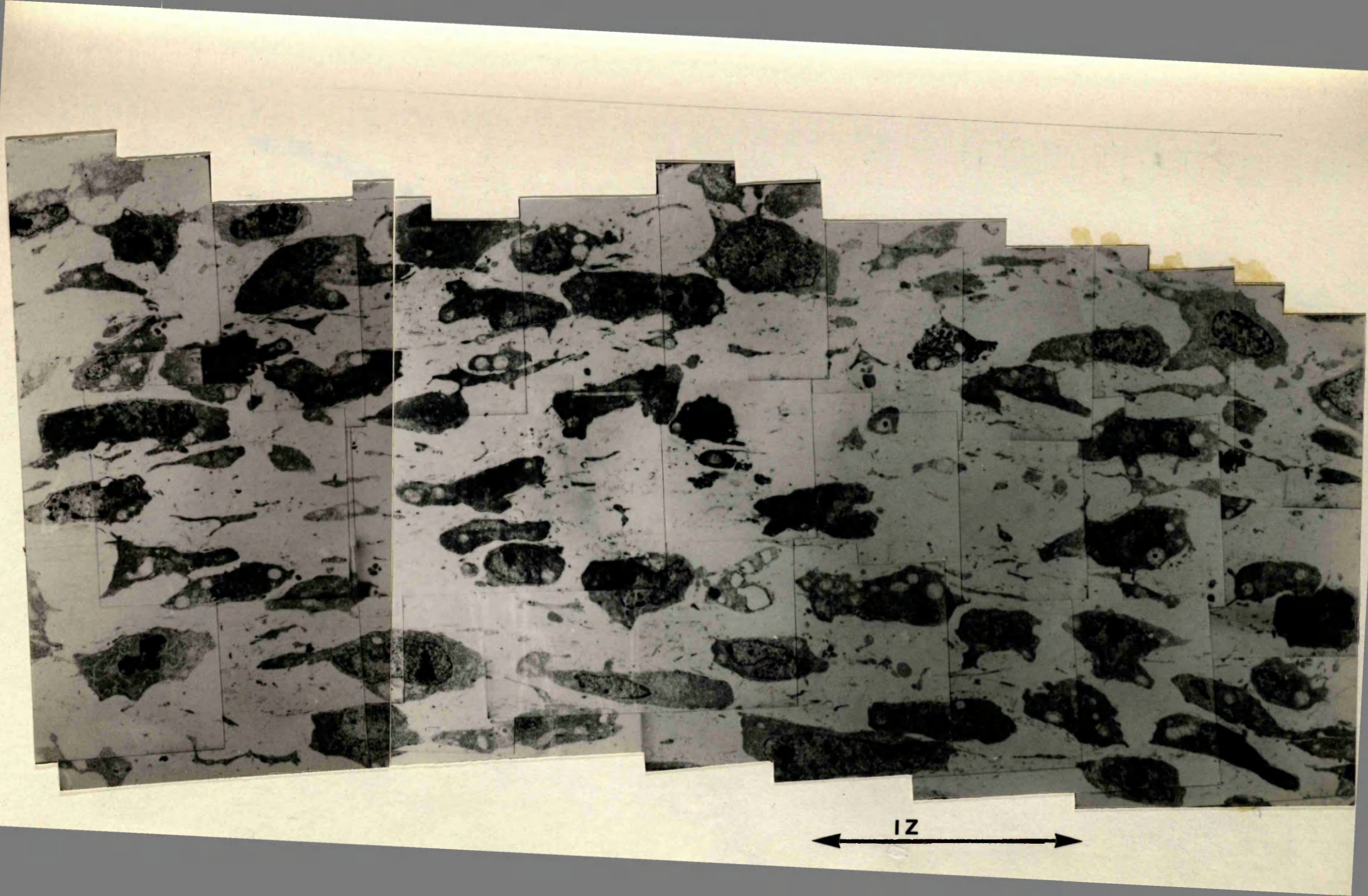

12

Fig. 88: Normal chick; 3rd M.-P. joint; a montage of EM's taken from the interzone. Most of the cells are normal mesenchymal cells (arrowheads). Scattered amongst them are a small number of cells which are obviously not normal. (arrows). Their nuclei are much darker and somewhat shrunken. In places the outer layer of the nuclear membrane is widely separated from the inner, forming large vacuoles extending into the cytoplasm, which is darker and less abundant than in the normal cells.
Stage 35 (control); EM x 4850.

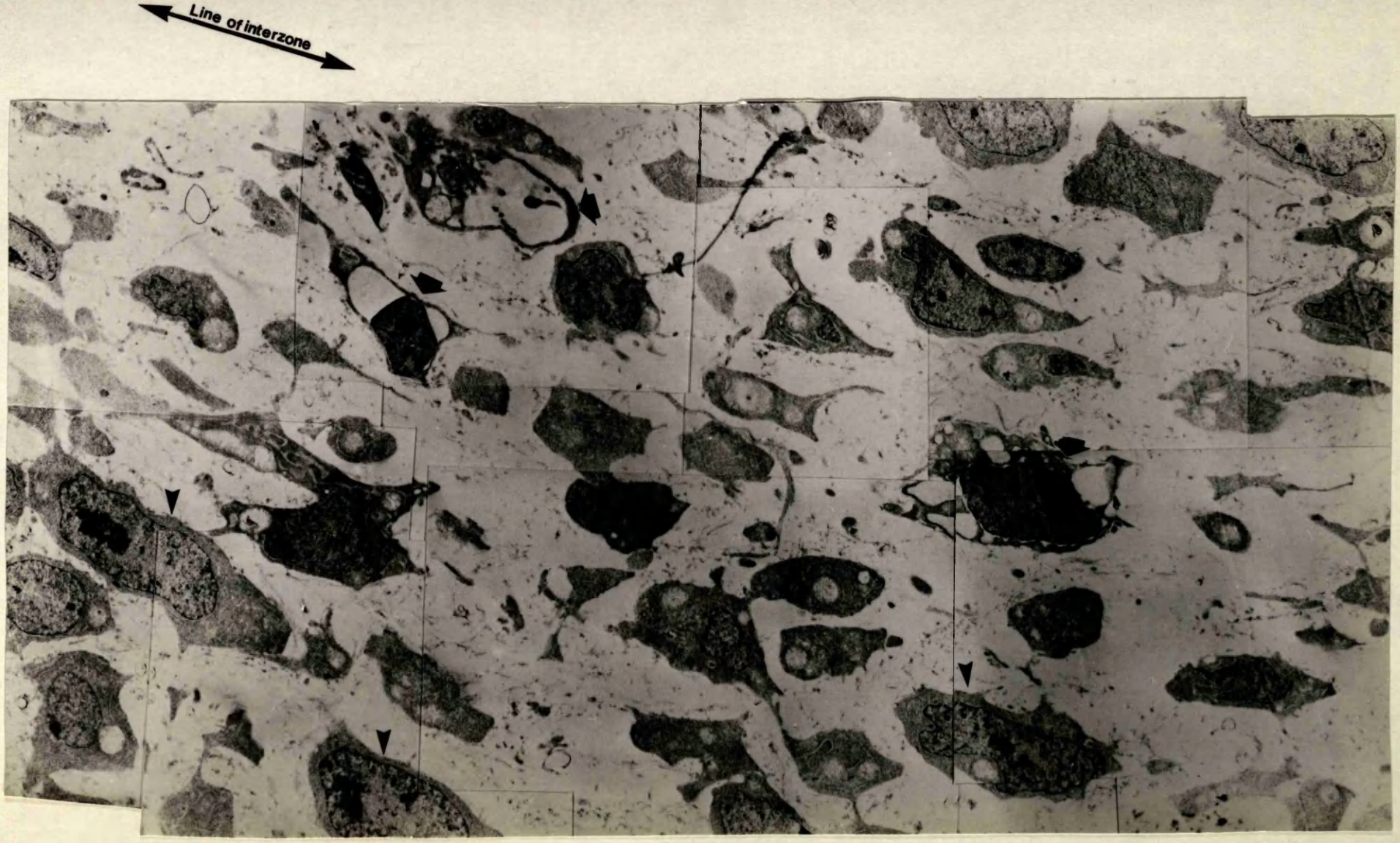
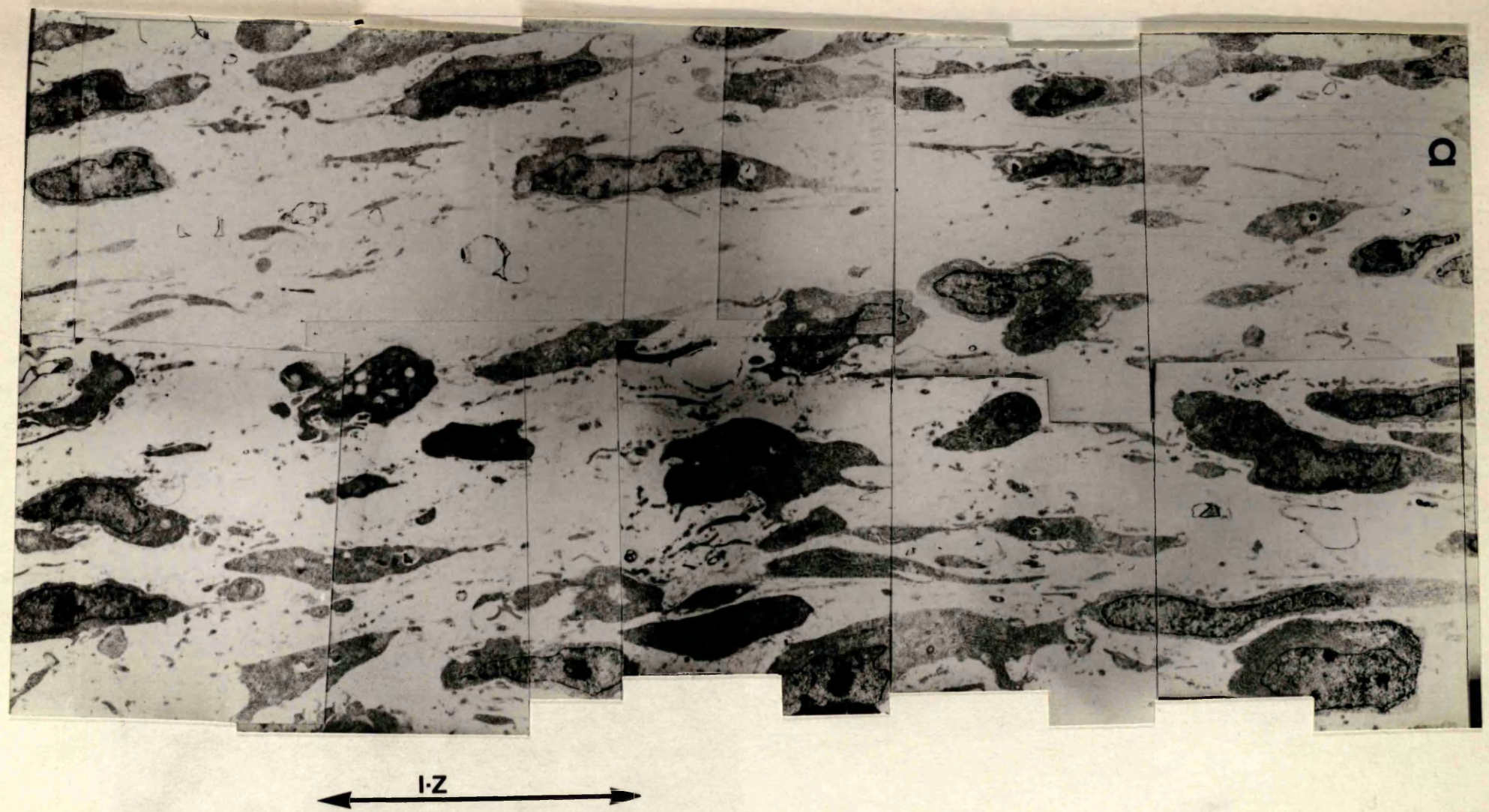

Line of interzone

Fig. 89a,b.: Normal chick; 3rd M.-P. joint; a montage of EM's of the earliest stages in loosening of tissue of the intermediate zone leading to formation of small cavities. Several of the cells, lying in a band extending from top left to bottom right of the montage, show early degeneration. The nucleus and cytoplasm are darker and more compact. Some show marked aggregation of nuclear chromatin. Intercellular space contains a small amount of microfibrillar material and cell sectional profiles.
Stage 37 (Control); 3rd M.-P. joint; x 4668.


a
Z-I

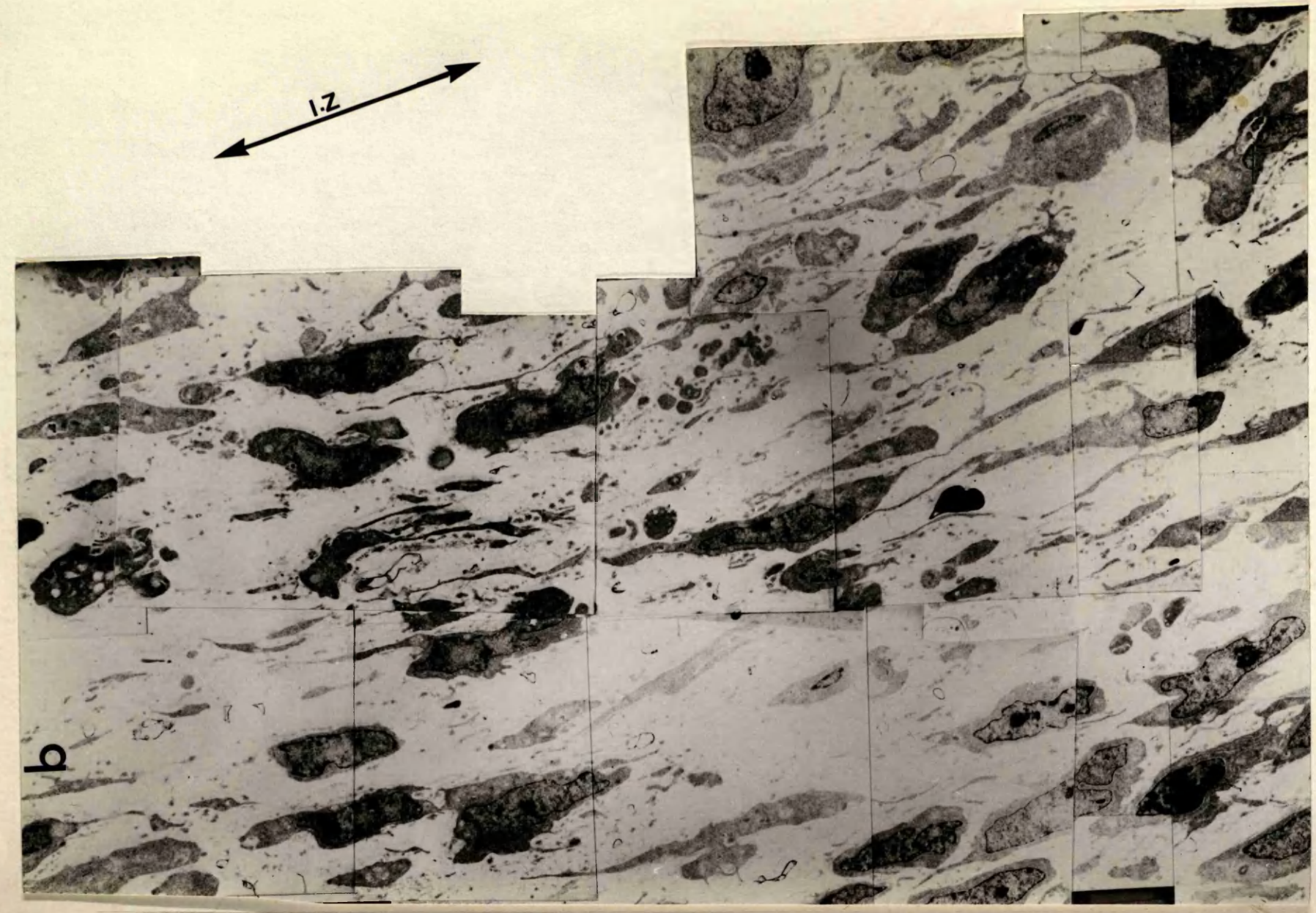

I.Z
b

Fig. 90: Paralysed chick; 3rd M.-P. joint; a montage of EM's of the interzone, the plane of which runs horizontally across the page.
Note:
1. In the lower one quarter of the montage the normal mesenchymal cells have large, pale, ovoid nuclei, of smooth outline and containing one or more nucleoli, and abundant cytoplasm, with irregular processes making contact with those of neighbouring cells.
2. In the upper three quarters of the montage, many (but not all) of the cells show early degenerative changes: the nuclei are smaller darker and more irregular in outline; in some there is wide separation of the inner and outer nuclear membranes, with the formation of large vacuoles extending into the cytoplasm.
Stage 36 (Paralysed); 3rd M.-P. joint.
EM x 2625.

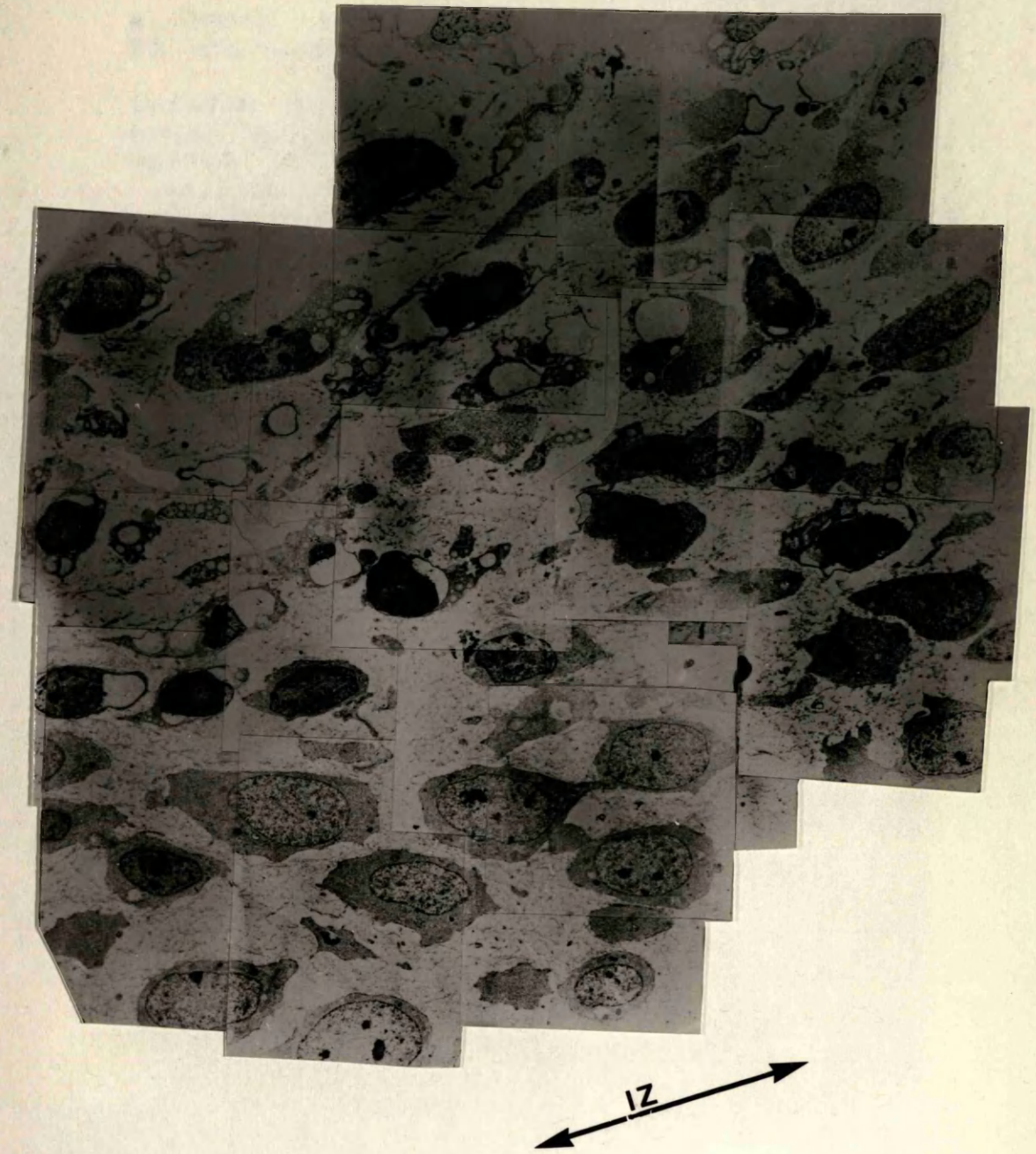

12

Fig. 91: Paralysed chick; 3rd M.-P. joint; a montage of EM's of the interzone of the joint.
. degenerative changes in several mesenchymal cells; with very large intracellular vacuoles and darkly stained nuclei, of irregular outline.
. several mesenchymal cells show signs suggestive of differentiation into cartilage cells (C) (See Text).
. intercellular space contains cellular fragments and microfibrillar materials.
Stage 37 Paralysed; 3rd M.-P. joint; EM, x 3079.

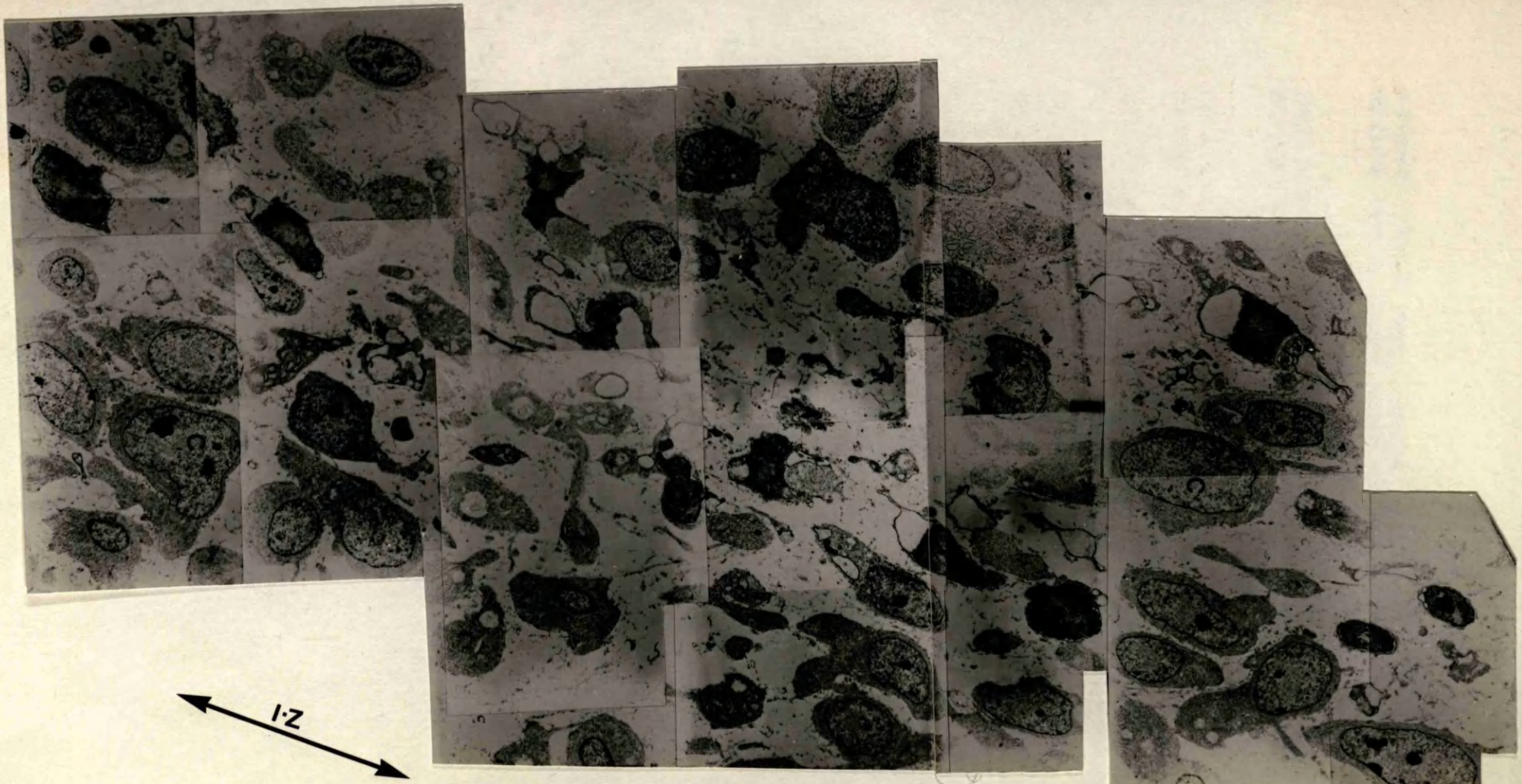

1·2

Fig. 92: Paralysed chick; 3rd M.-P. joint; shows a developing blood vessel within the interzone.
Note:
1. The lining of thick endothelial cells, characteristic of young developing capillaries.
2. variety of adjacent cells:
i. to the left, a sectional profile of a "dark cell", with darkly stained nucleus and scanty cytoplasm.
ii. to the right, part of a healthy "active" mesenchymal cell.
Stage 37; paralysed; EM x 5949.

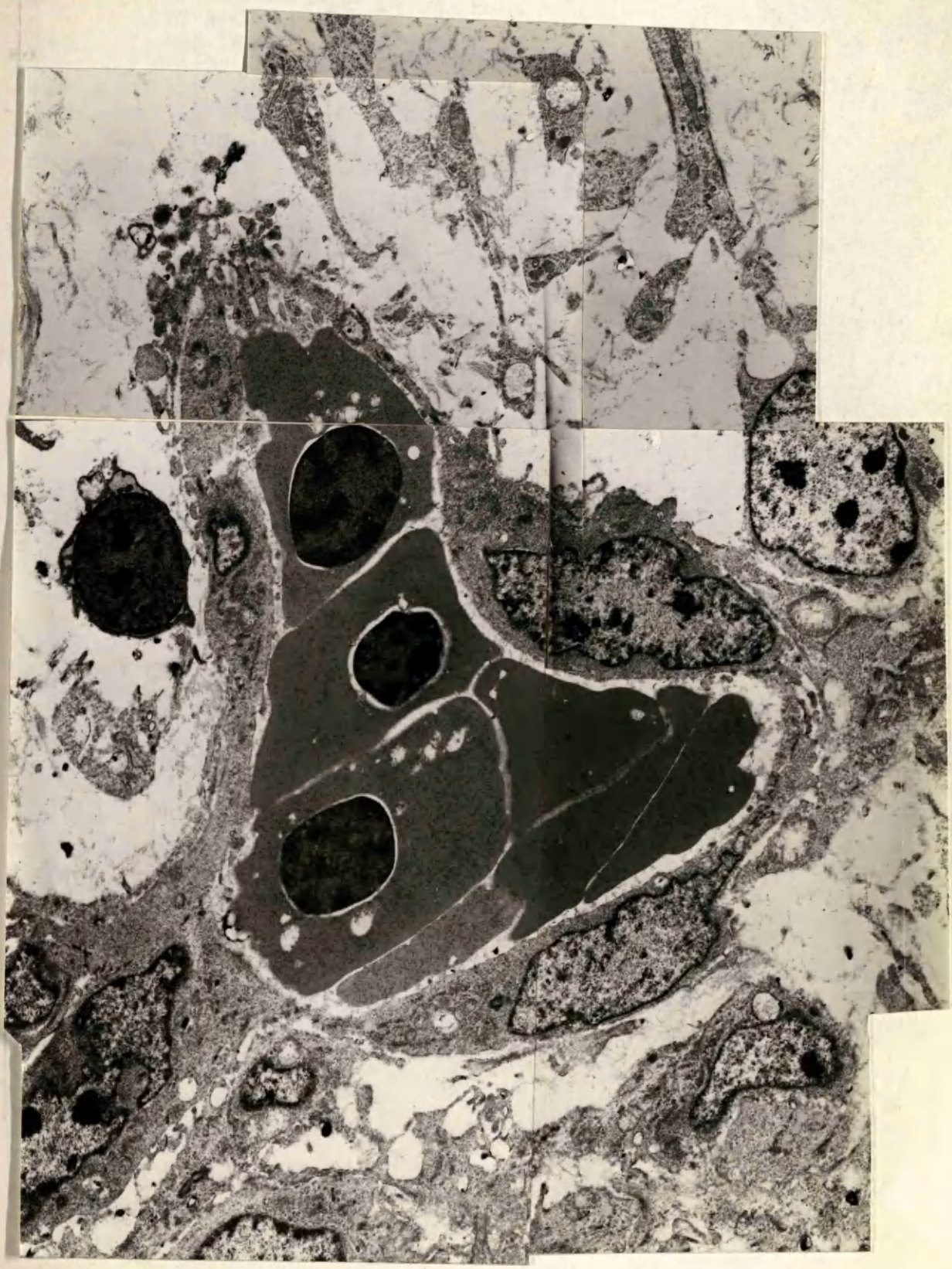


Fig. 93: Paralysed chick; 3rd M.-P. joint; a montage of EM's of the interzone of the joint. Shows a small electron lucent cavity lying along the plane of the interzone along the margins of the cavity are scattered flattened "dark cells" shown by EM to be degenerating. A few extravasated RBCs are also seen (arrows).
Stage 38; paralysed chick; 3rd M.-P. joint; EM x 2940.

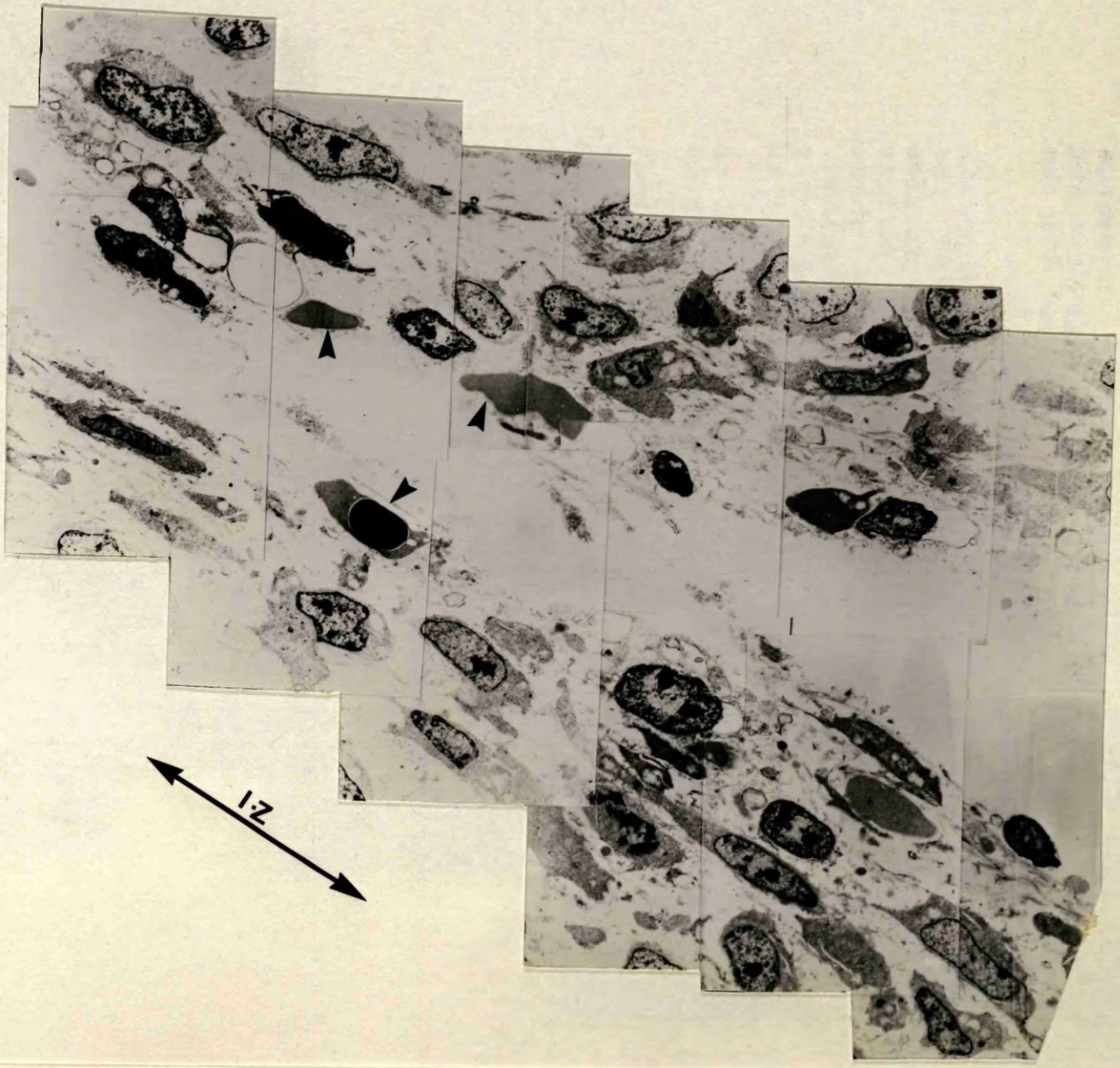

IZ

Fig. 94: Paralysed chick; knee joint; a montage of EM's of the interzone between the lateral condyle of the femur and the tibia.
Shows:
i. spindle-shaped cells (arrows), elongated along the line of the interzone. Some of them show massive separation of inner and outer nuclear membranes forming vacuoles which occupy virtually all the cytoplasm. The nuclei are very compact and darkly stained. The cells are interpreted as dying or dead.
ii. lying immediately adjacent to degenerating cells are others which appear entirely normal (arrowheads).
iii. between the cells are sectional profiles of cellular processes, debris of degenerated cells and some microfibrillar material.
Stage 36; paralysed; EM x 2999.

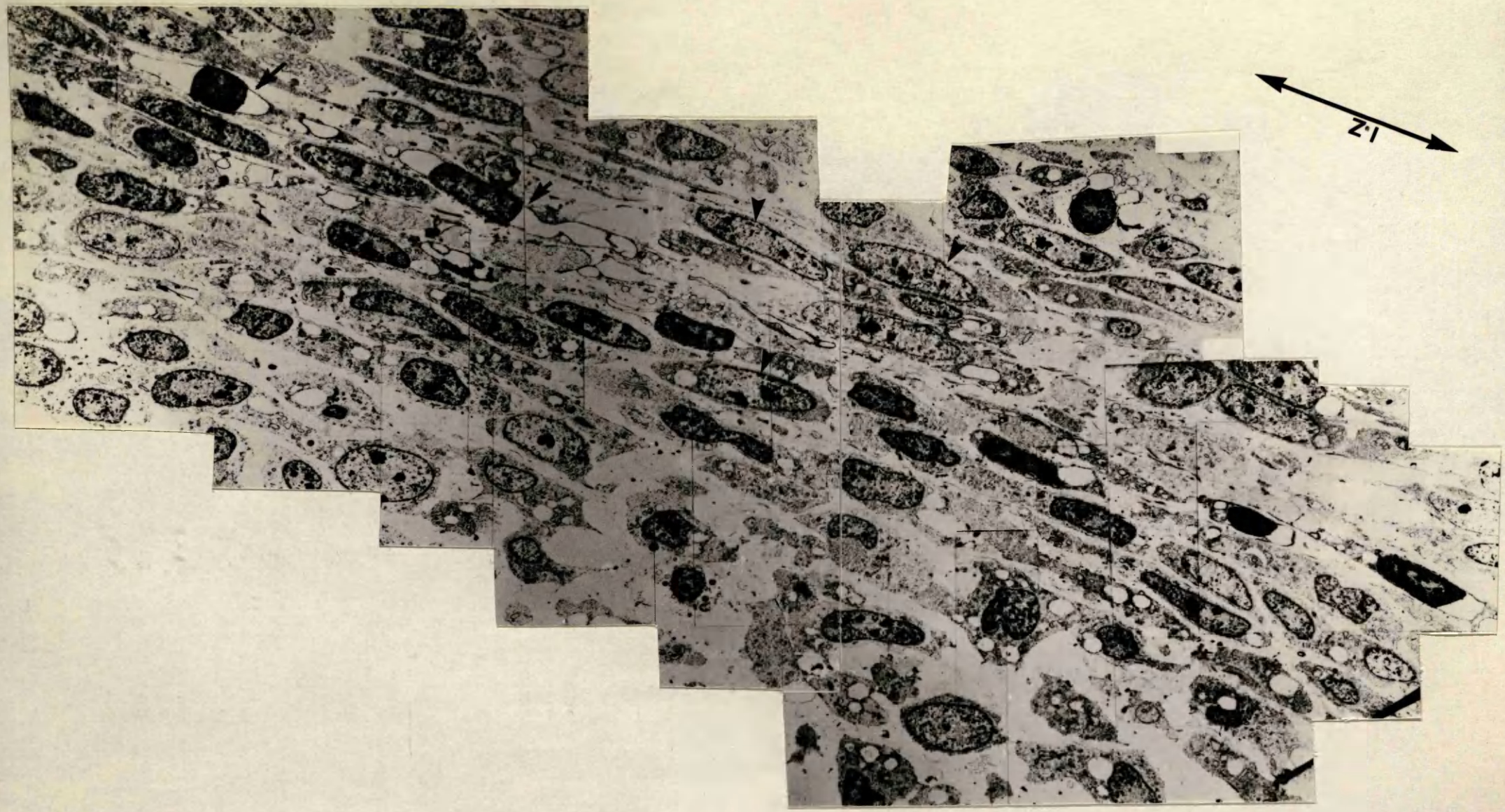
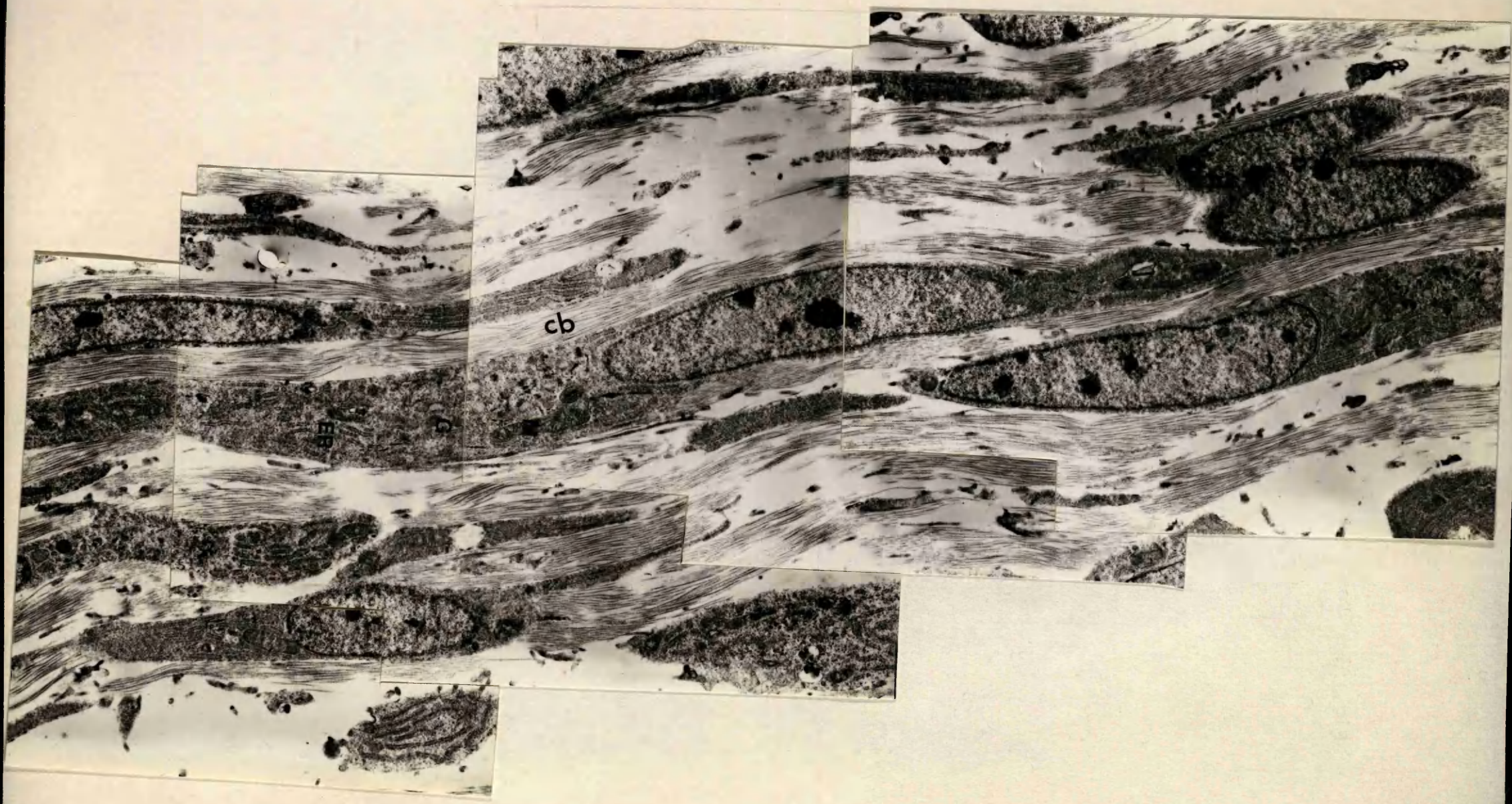

Z·1

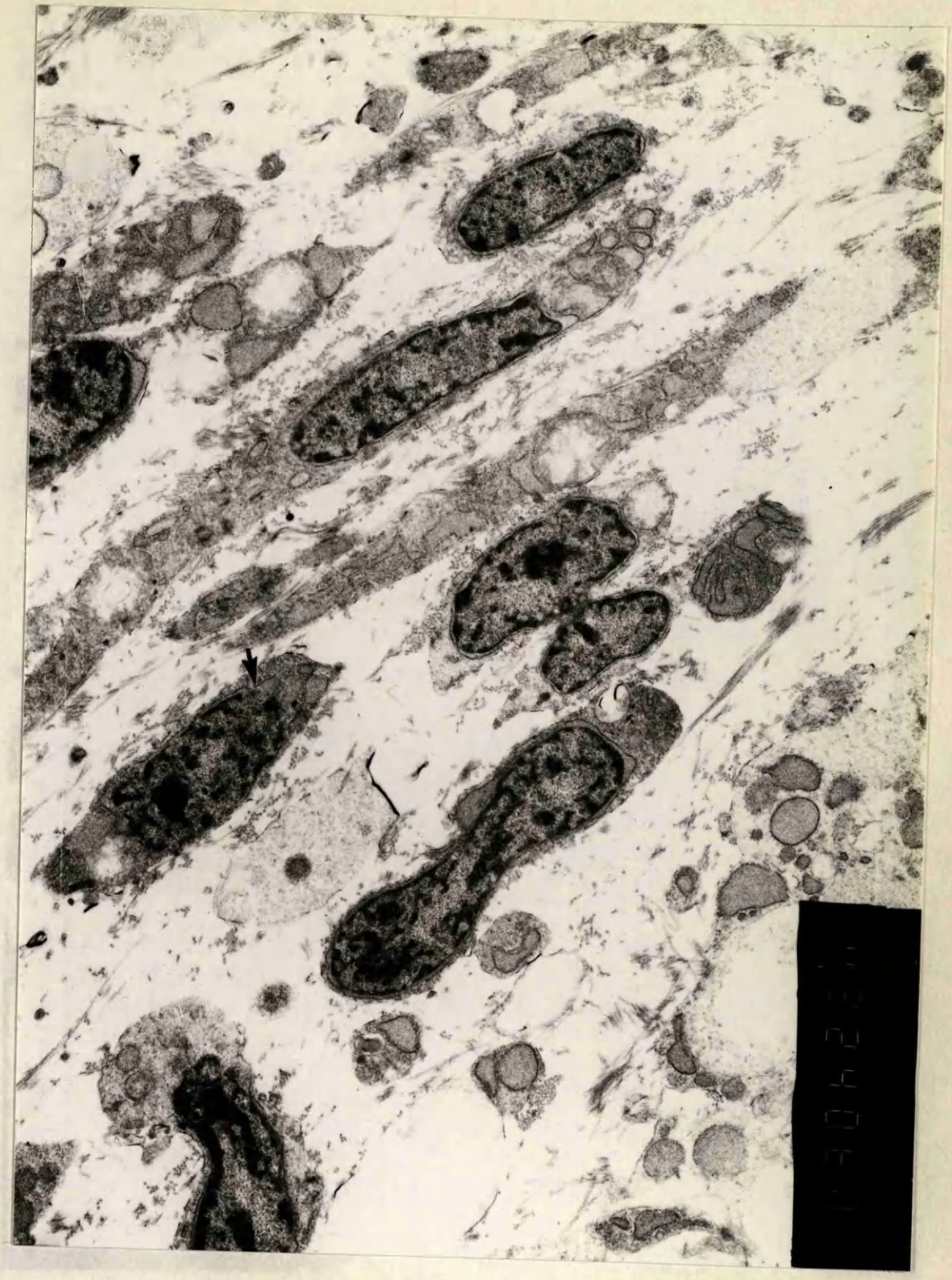
Fig. 95: Paralysed chick; knee joint; shows typical active fibroblasts at the site of fibrous fusion within the interzone of the paralysed joint.
Note:
. The fibroblasts contain elongated nuclei with one to three nucleoli;
. The elongated cytoplasm contains rough endoplasmic reticulum (ER), Golgi organs (G), slender shaped mitochondria (M);
. The intercellular space is largely filled by collagen bundles (cb).
Stage 37; paralysed chick; EM x 3400.


cb
ER

Figs. 96a,b,c: Paralysed chick; knee joint; shows appearances in a region of fibrous fusion. All the cells shown are characteristics of actively synthetic fibroblasts: the nuclei contain prominent nucleoli; the cytoplasm shows abundant rough endoplasmic reticulum, filled with finely granular pre-secretory products. Some cells show very well the continuity between the distended sacs of RER and the space between inner and outer nuclear membranes (abc arrows). The intercellular space contains fine collagen fibres.
Stage 38; paralysed chick; EM x a.7900, b. x 10000, c. x 10000.

a

b



C

Fig. 97: Paralysed chick; knee joint; most of the cells are normal. Cell (A) is at an early stage of degeneration and shows aggregation of the nuclear chromatin and separation of nuclear membrane. Cell (B) is at a later stage of degeneration and shows rounded up nucleus with large vacuoles (V) and deterioration of the cell organelles.
Stage 36; paralysed chick; EM x 6950.

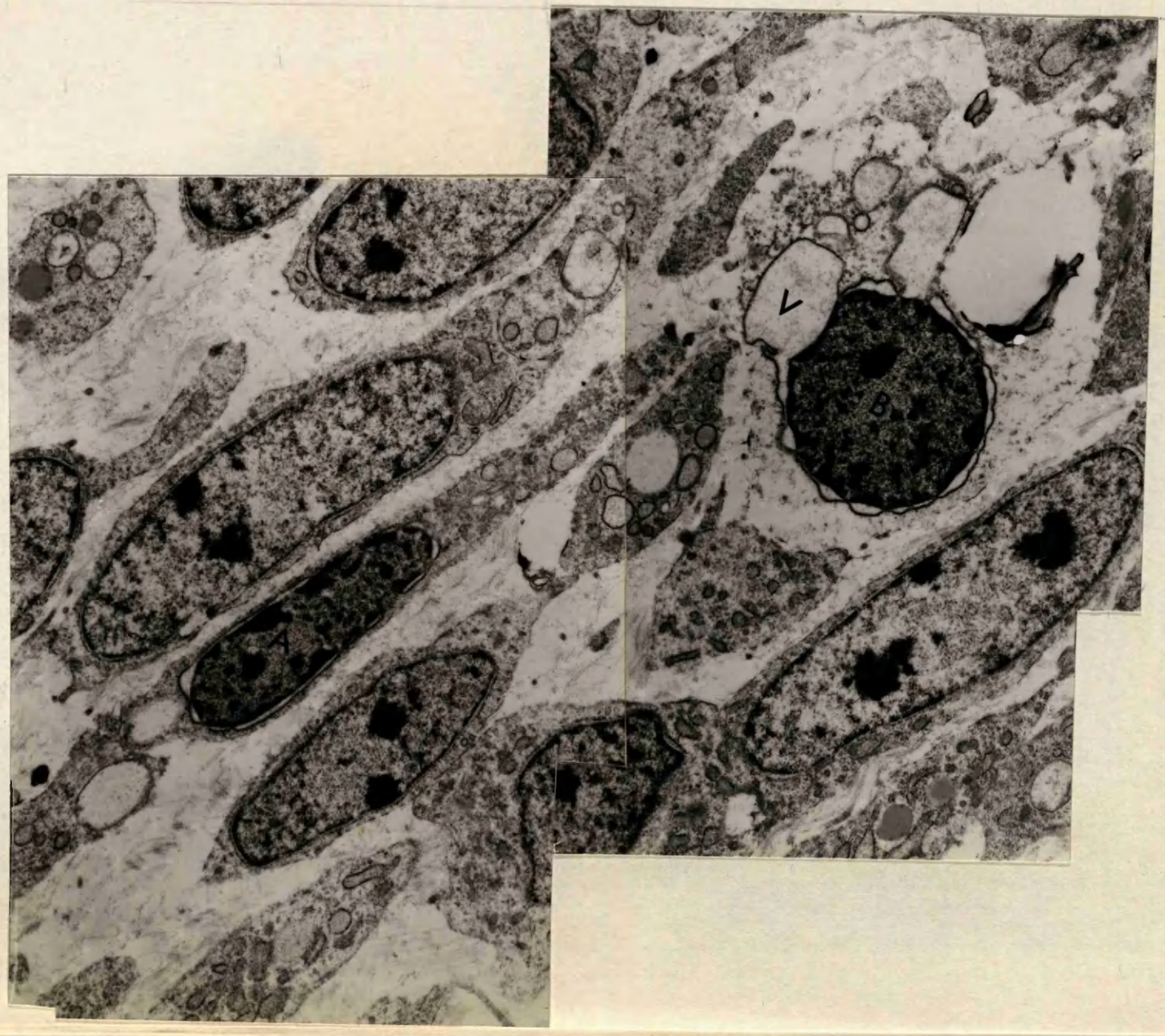
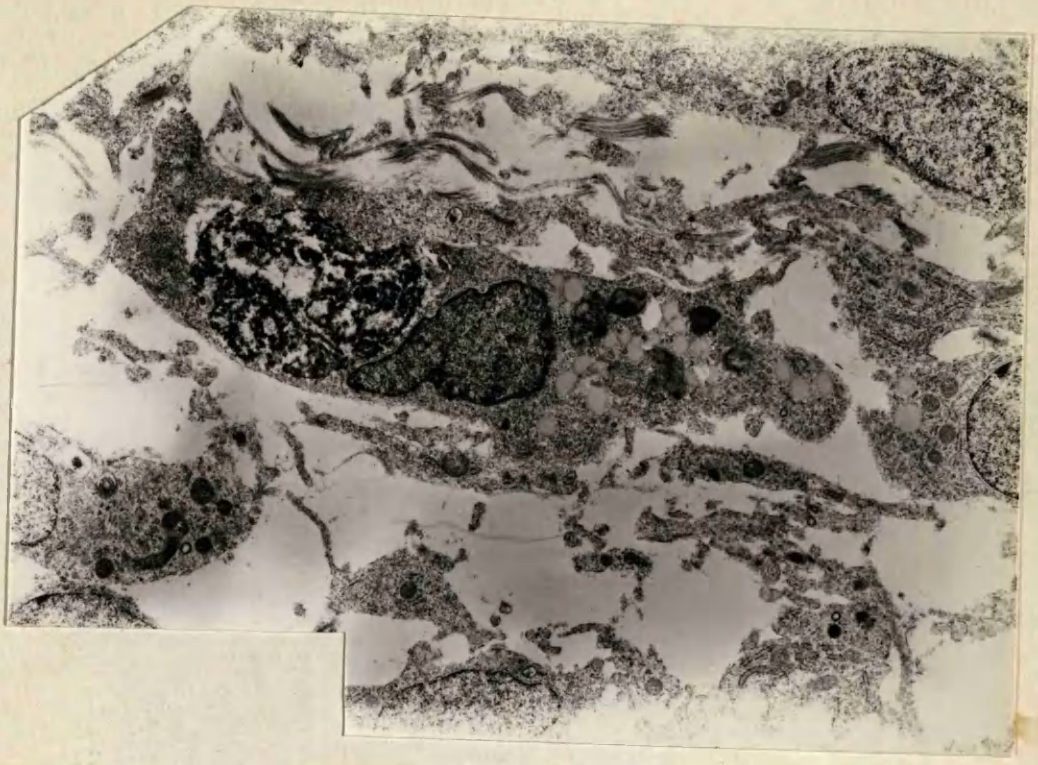

A
B

Fig. 98: Paralysed chick; knee joint; shows apoptotic body phagocytosed by a cell which appears to be a modified mesenchymal cell. The apoptotic body shows coarse banded granules.
Stage 36; paralysed chick; EM x 7832.

Fig. 99: Control chick; 3rd M.-P. joint. Shows two phagocytosed apoptotic bodies.
Stage 37; control chick; EM x 8300.



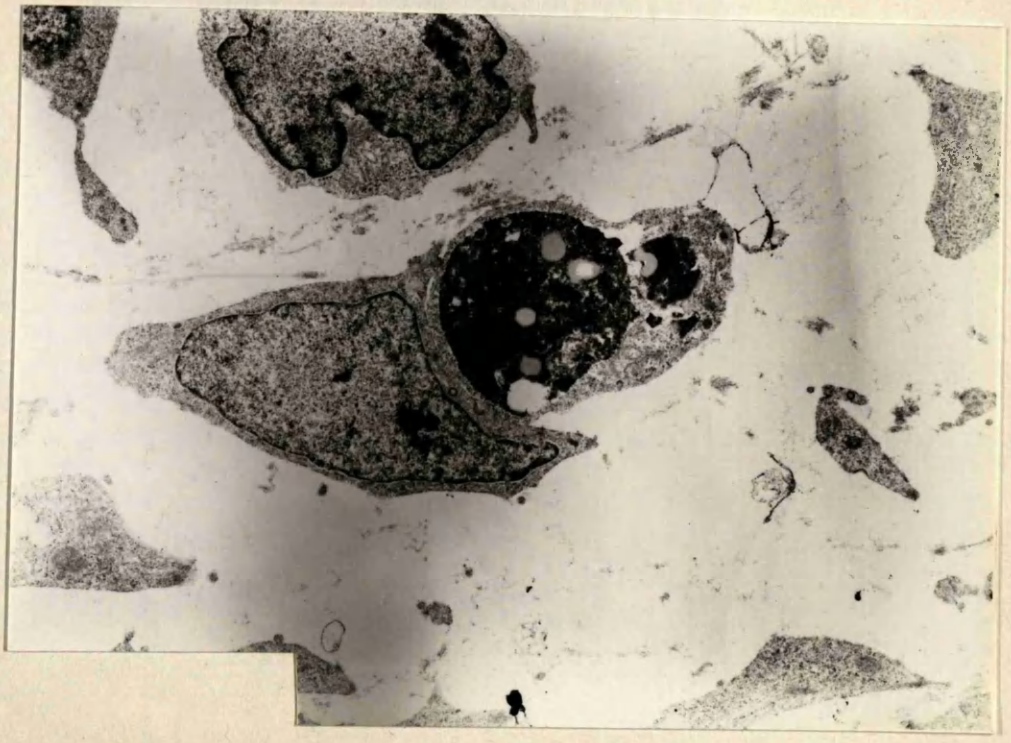


Fig. 100: Paralysed chick; 3rd M.-P. joint. Shows a typical macrophage in the interzone. It has phagocytosed an apoptotic body and fragments of R.B.C. Stage 38; paralysed chick; EM x 6600.

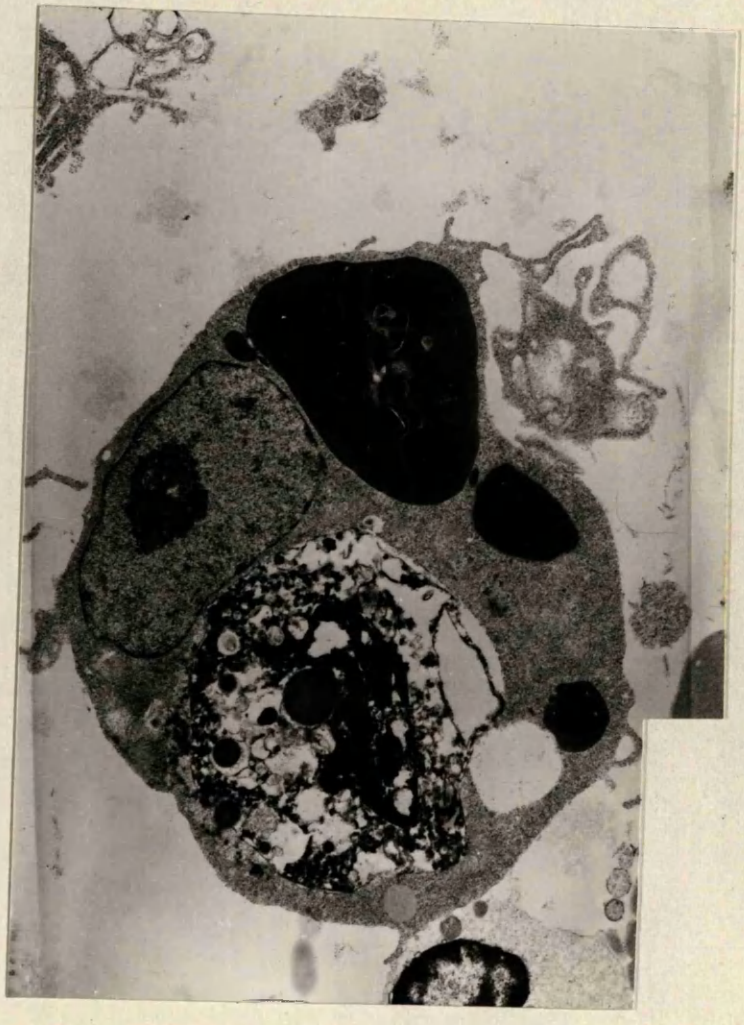


Fig. 101: Normal chick; 3rd M.-P. joint; shows two typical macrophages within the interzone.
Stage 37; control chick; EM x 8099.

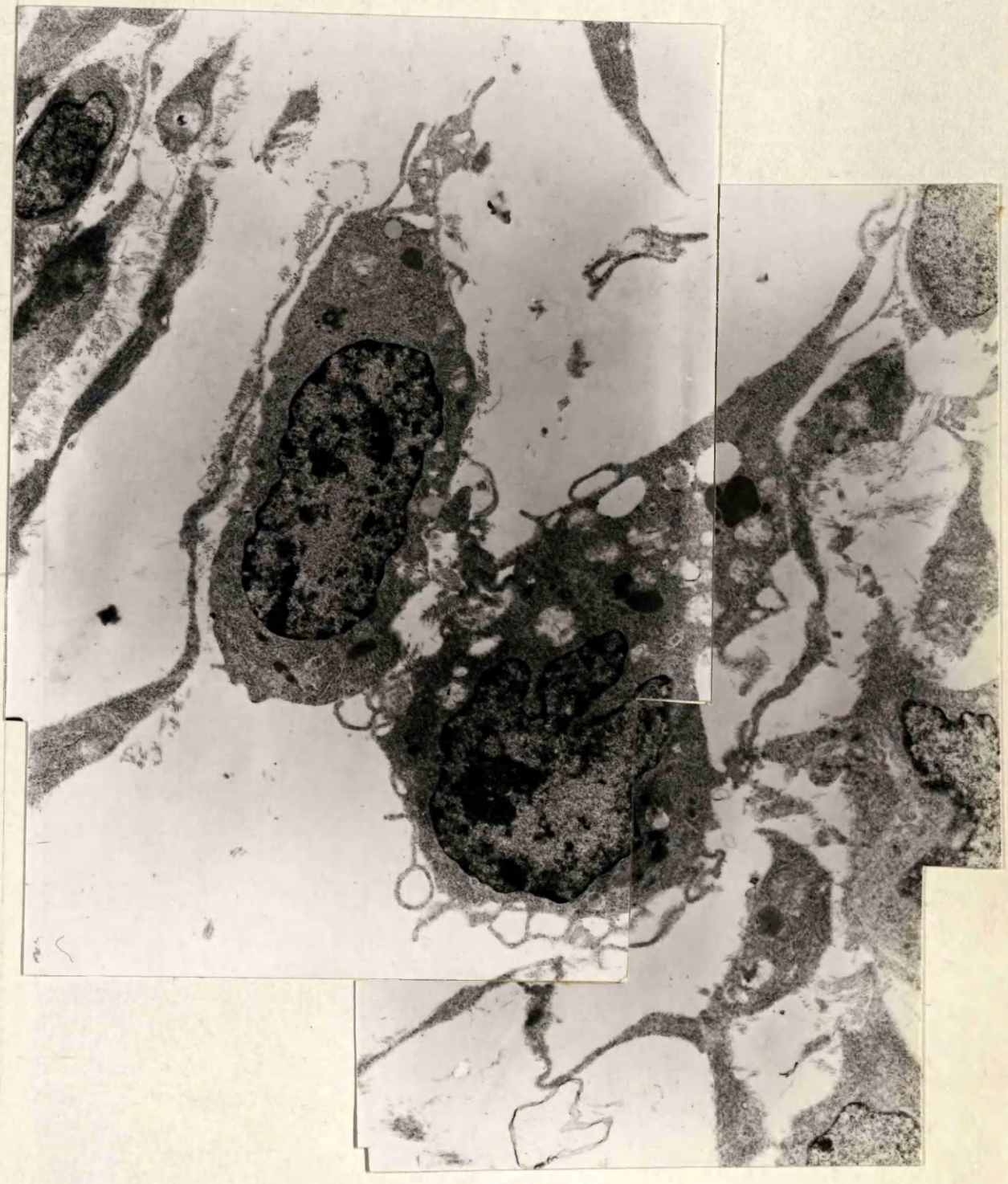


Fig. 102: Paralysed chick; M.-P. joint; cell debris, ingested and digested by typical macrophages within the interzone of the joint.
Stage 37, paralysed chick; EM x 7932.

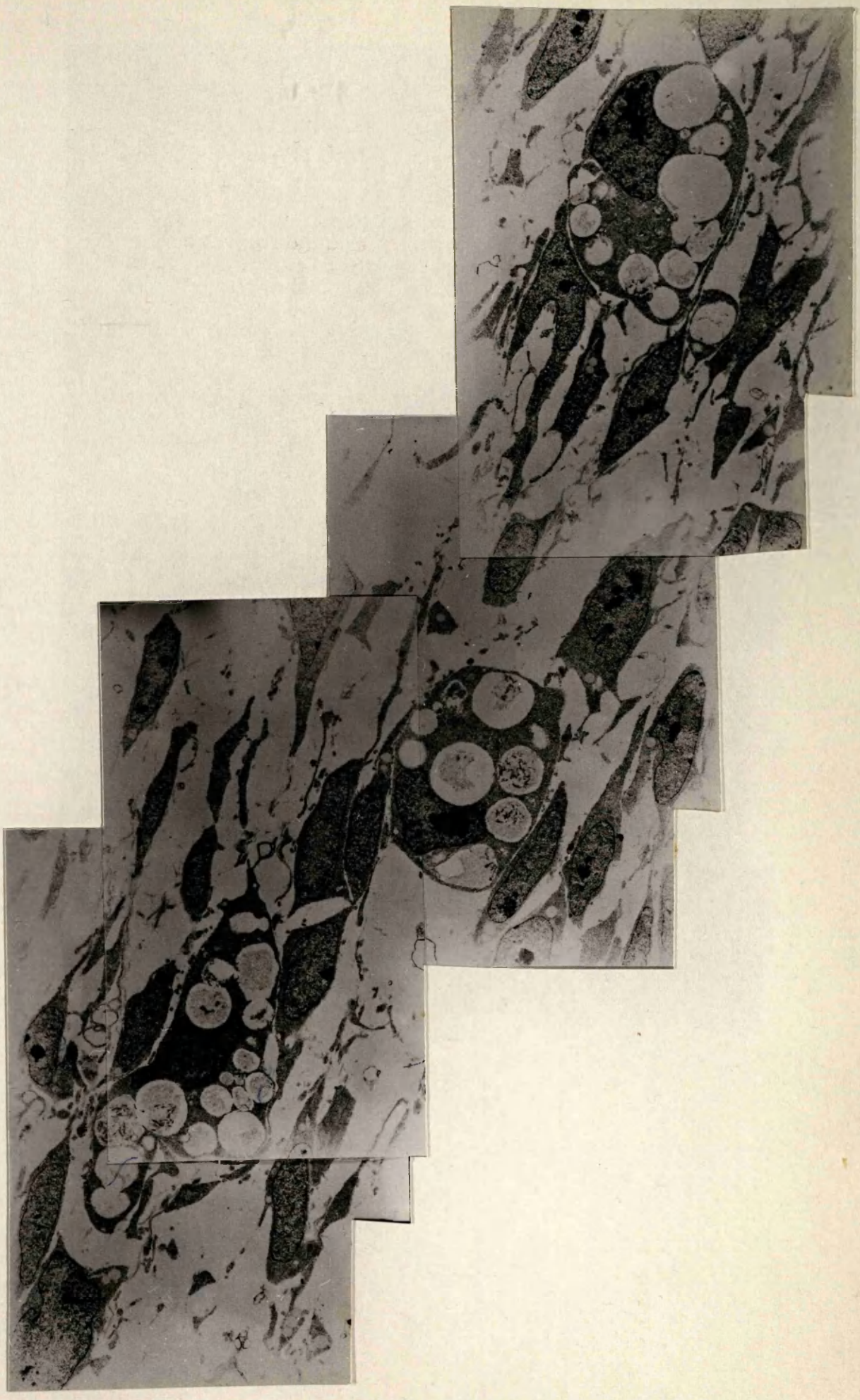


Fig. 103: Normal chick; knee joint; a montage of EM's of the interzone between the lateral condyle of femur and the fibula. At the right hand side of the montage, the cells of the chondrogenic zone of the femur are elongated tangentially to the articular surface compactly arranged and all healthy. The remainder of the montage is of the intermediate layer. The cells are widely scattered. Some appear normal (arrows), others show varying degrees of degeneration (arrowheads). The intercellular space contains extravasated RBC's, fragments of degenerated cells, sectional profiles of cell processes, and macrophages containing phagocytosed cell debris. Stage 35; control; EM x 3120.

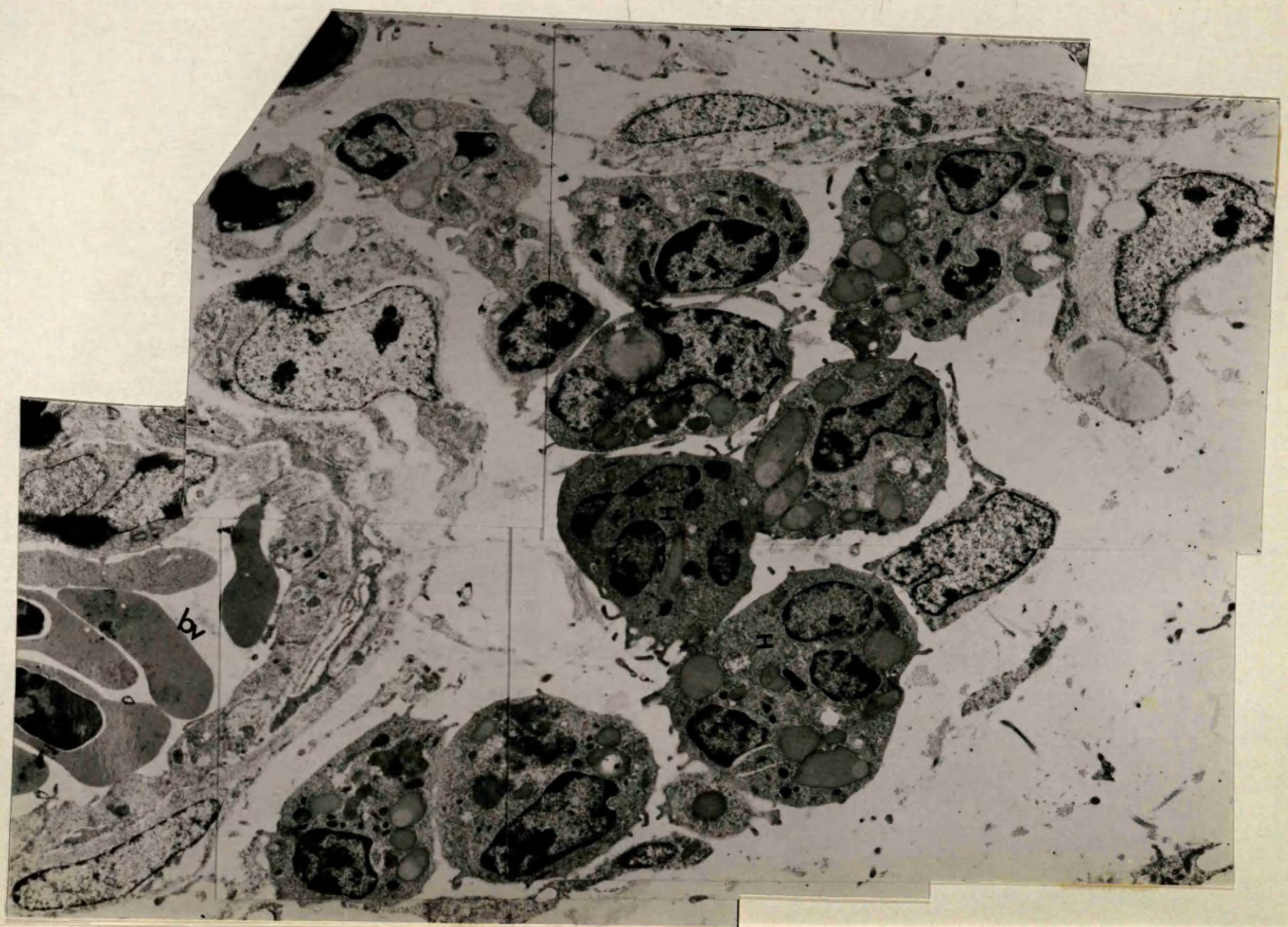
MAC

Fig. 104: Normal chick; skeletal muscle; shows normal muscle fibres.

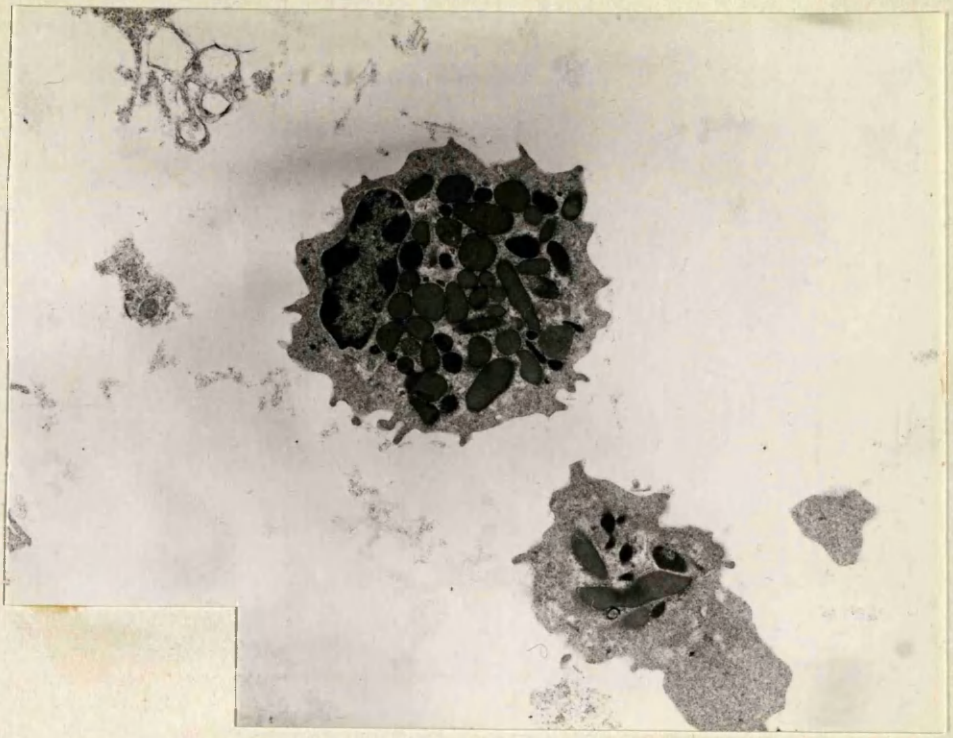
Note:
i. Centrally placed nuclei, typical of myotube stage of development.
ii. myofibrils developing within abundant sarcoplasm.
Stage 38; normal chick; EM x 3360.

Fig. 105: Paralysed chick; skeletal muscle. The muscle fibres are swollen, with extensive degenerative changes in the cytoplasm. Only scattered fragments of myofibrils are to be seen. The sarcoplasm, palely stained, and without recognisable organelles, contains many lipid droplets. One of the fibres shows a close-packed cluster of nuclei. Between the degenerating muscle fibres is one macrophage, which contains many lipid droplets and phagocytosed cell debris, presumably remnants of degenerated muscle fibres.
Stage 38; paralysed chick; E.M., x 3150.

Fig. 106: Paralysed chick, knee joint; shows numerous heterophils (H) around the blood vessels (bv) within the synovial mesenchyme of the joint.
Stage 38; paralysed chick, E.M., x 5250.


bv
H
H

Figs. 107 & 108: Paralysed chick; 3rd M.-P. joint; show heterophils within the synovial mesenchyme.
Stage 38; paralysed chick; E.M., x 6720.



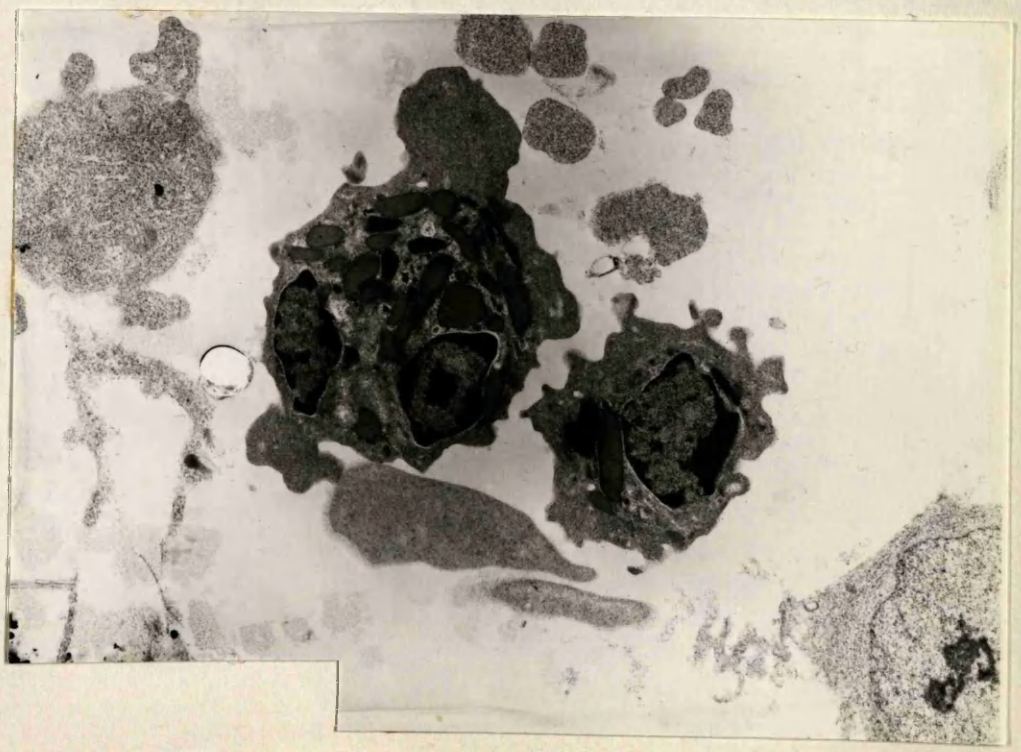
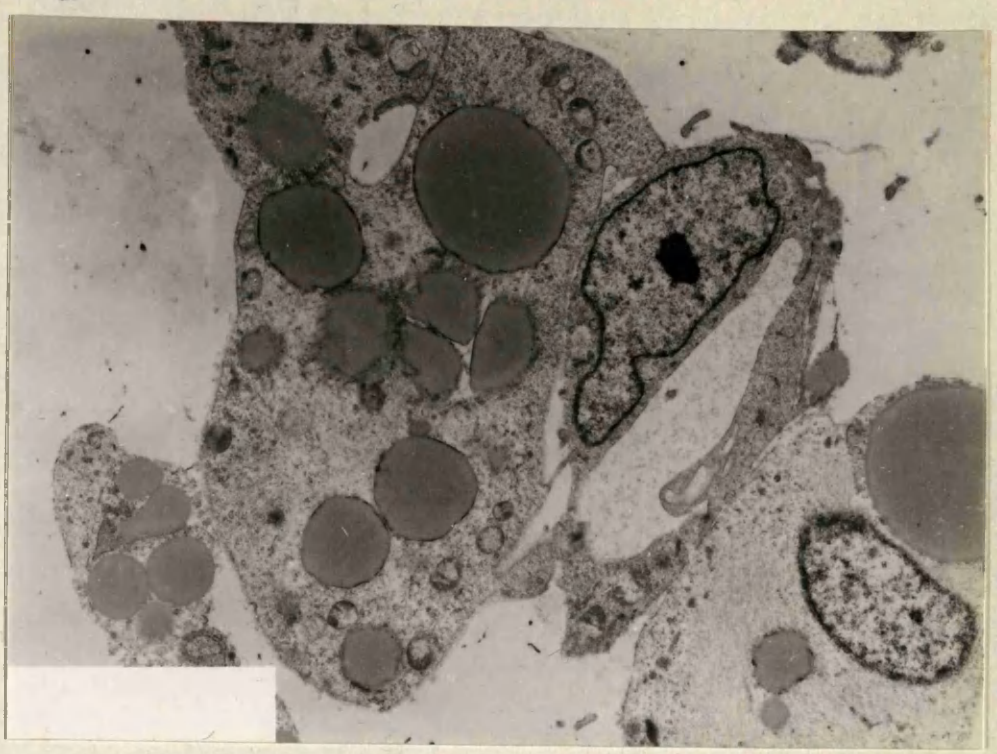


Fig. 109a,b,c: Paralysed chick; knee joint; shows various examples of preadipocytes, which contain a number of lipid droplets of varying size. Mitochondria are few in number.

E.M., a. x 8238; b. x 110; c. x 7049.

a



b

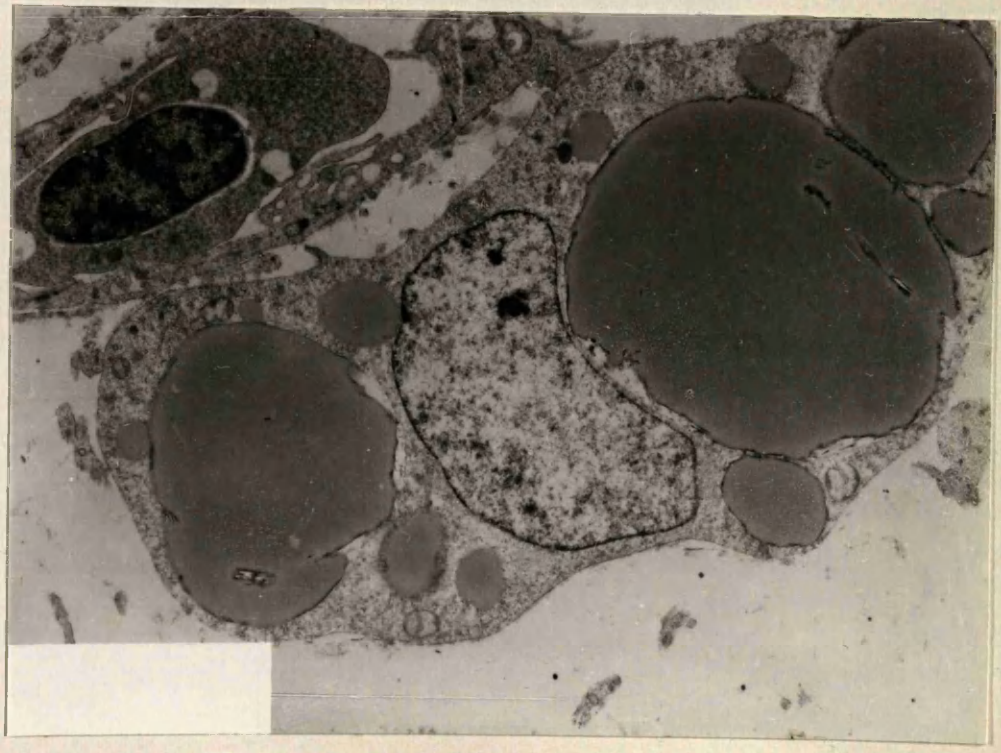
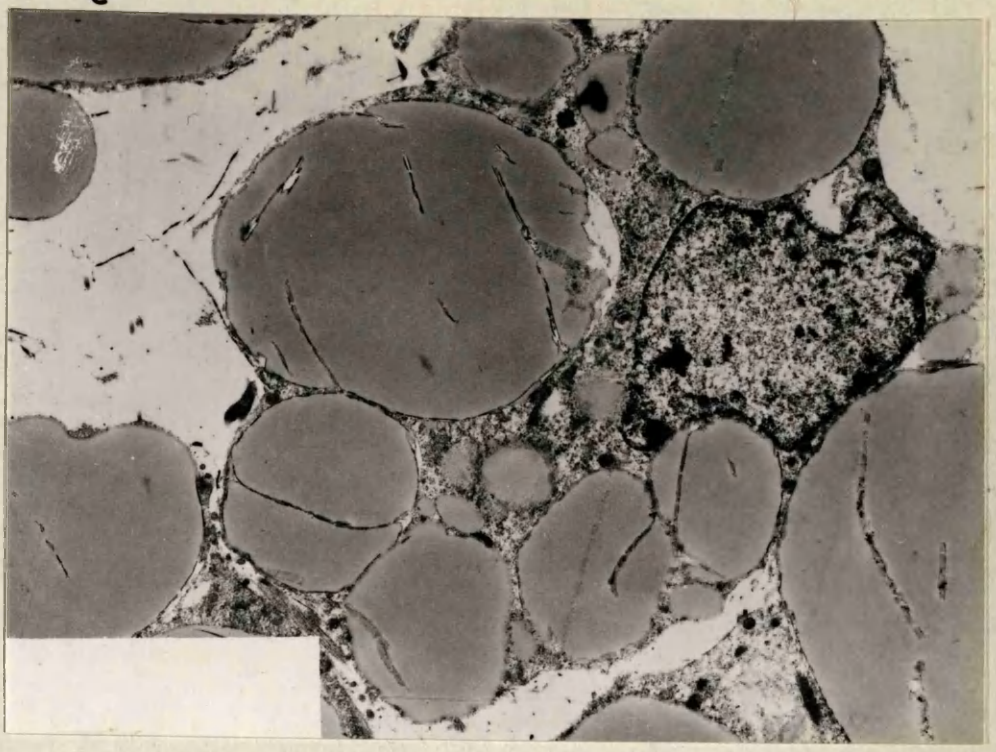


Fig. 109c:

Fig. 110: shows the relationship between lipid droplets (L) and cytoplasmic filaments. Each lipid droplet is associated with the parallel bundles of fine cytoplasmic filaments. Mitochondria (M) are located between lipid droplets.

E.M., x 14000.

C



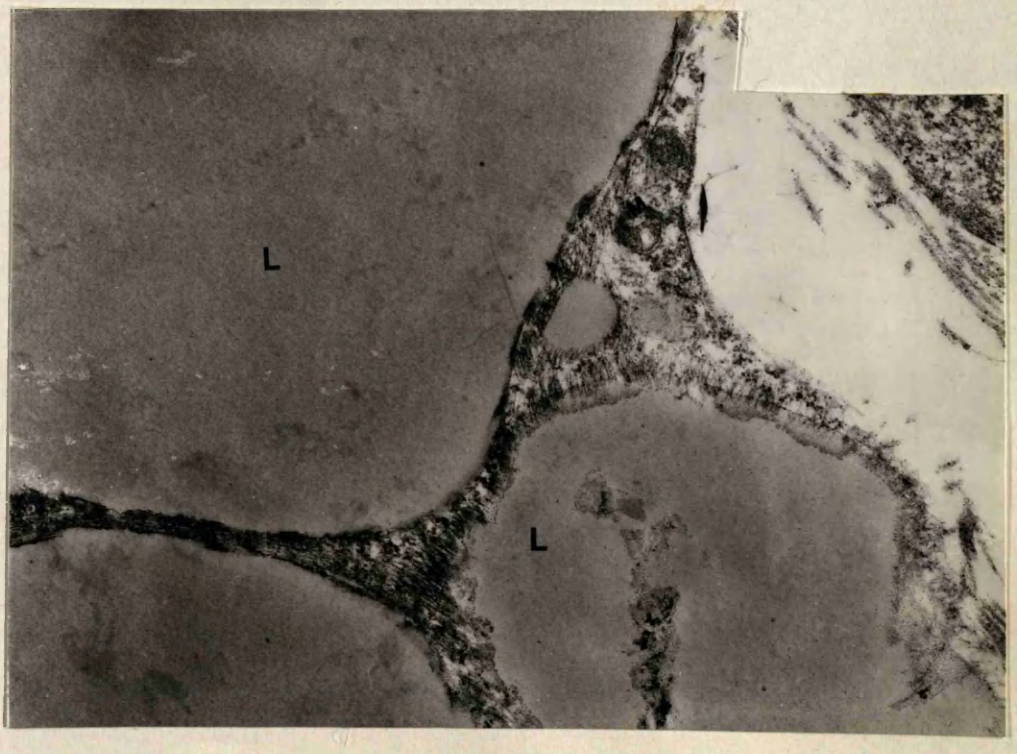
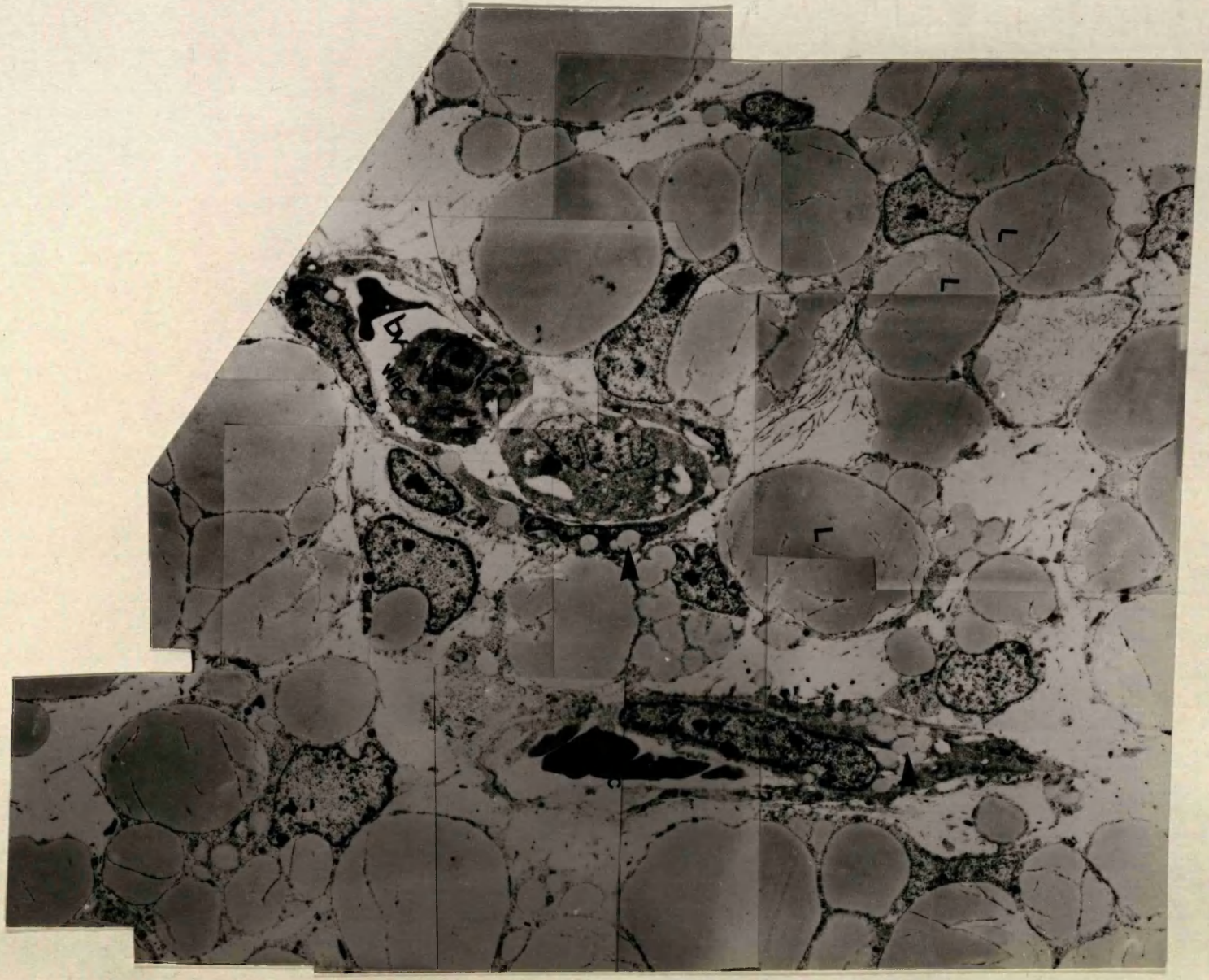


Fig. 111: Paralysed chick; knee joint; a montage of EM; shows accumulation of the preadipocytes within the synovial mesenchyme.

Note:
. the cytoplasm of preadipocytes contain several large and small lipid droplets (L).
. the nucleus of the preadipocytes convoluted by the large lipid droplet.
. blood vessels (bv) among the preadipocyte cells, formed by a single continuous endothelial cell and containing white blood cells (w.b.c.) and red blood cells (r.b.c.). Several lipid droplets (arrows) are observed within the cytoplasm of the endothelial cells.
Stage 39; paralysed chick; E.M., x 3360.


L
L
L
BM
WBC