STUDIES ON THE DEVELOPMENT OF THE AVIAN KNEE JOINT, WITH SPECIAL REFERENCE TO THE ROLE OF APOPTOTIC CELL DEATH

By

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In the name of Allah, the Beneficent, the Merciful.

Certainly We created man of an extract of clay, then We made him a small life germ in a firm resting place. Then We made the life germ a clot, then We made the clot a lump of flesh, then We made (in) the lump of flesh bones, then We clothed the bones with flesh, then We caused it to grow into another creation, so blessed be, Allah, the best of the creators. Then after that you will surely die, then surely on the day of resurrection you shall be raised.

The Believers, Holy Qoran, Chapter 23, verses 11-16.

DEDICATED

TO MY FAMILY

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SECTION 1 - INTRODUCTION

INTRODUCTION

I. General

It is almost 100 years since the publication of the early classic studies of the development of synovial joints such as those of Hepburn (1889), Parsons (1900), Lewis (1902) and Bardeen (1905). Since then, the subject has been repeatedly restudied, using more refined techniques, including histochemistry and electron microscopy, and the experimental analysis of causative factors.

Although there is still much that remains controversial, the general sequence of events is now established, and this will be described in broad outline, as seen in a "typical" synovial joint in a limb, before the main papers are reviewed in detail.

1. The mesenchymal condensation, or scleroblastema, appears in the central axis of the limb bud, outlining the future skeleton and joints. This condensation follows a proximo-distal sequence.

2. Chondrification occurs in the condensation, proximal and distal to the future joint, so that the two articulating bones are now recognizable as cartilage models. The mesenchyme which surrounds their shafts forms a perichondrium, which provides for appositional growth in girth.

3. The ends of the two cartilage models are united by a densely cellular and avascular interzone. This develops into a three-layered structure: a chondrogenic layer

covering each articular surface and a looser intermediate layer. The chondrogenic layer covering the future epiphysis is continuous with the perichondrium of the future diaphysis, and serves the same function, namely providing for appositional growth of the cartilage model. Condensed mesenchyme at the periphery of the interzone provides for the formation of the capsule, deep to which is the looser synovial mesenchyme. Within the looser intermediate zone of the interzone there will develop the joint cavity and intra-articular structures, such as menisci and cruciate ligaments.

II. Some areas of disagreement

Despite the general agreement, outlined above, there are many remaining questions to be answered:-

1. When the articulating bone rudiments chondrify, what factors determine the different pathway followed by the interzone mesenchyme? Does the interzone itself ever become cartilaginous?

2. Does the formation of the joint cavity (by cavitation or "clefting") depend on extrinsic factors, such as muscular movement, or on intrinsic factors, such as cell death or "liquefaction" in the interzone, or are both sets of factors required if the joint cavity is to appear and to persist? If cell death is involved, is it genetically programmed? What is the origin and role of macrophages in cavitation?

3. Does cavitation start centrally or peripherally?

4. Do intra-articular structures arise from the interzone or from "synovial mesenchyme?"

5. Is the developmental sequence of events in synovial joint development closely correlated with developmental stages of the embryo, as claimed for the chick knee joint by O'Rahilly and Gardner (1956)?

6. In general do synovial joints, of the most commonly studied species such as man, laboratory rodents and the chick follow the same developmental pattern, or are there important variations between species?

7. Within a single species, do all synovial joints develop in a similar fashion, or are there important individual variations e.g. does the femoro-patellar joint follow the standard pattern? What effect does the presence of intra-articular structures, such as menisci, or intra-articular tendons, have on joint development?

III. Review of published work

This is divided into two sections:

a) mammalian synovial joints and b) avian synovial joints, for two reasons:-

i) because the present study is concerned with the chick knee joint, and because the chick has been the animal of choice for most experimental studies, and ii) because it is important, for a human anatomist, to relate the developmental pattern in the chick to that in mammals and particularly in man.

I. THE FORMATION OF THE INTERZONE

A. MAMMALIAN SYNOVIAL JOINTS

Bardeen (1905) studied the development of the human skeleton, and reported that, in general, the development of the skeleton of the limb passed through three stages, blastemal, chondrogenous, and osseogenous. In the knee joint, the peripheral blastemal tissue at the joint became transformed into a capsular ligament. The blastemal tissue in which the cartilaginous precursors of femur and tibia developed, became condensed at their articulating ends, while in the region of the knee joint, the tissue became less dense and finally disappeared, leaving a joint cavity.

Keith (1933) studied the development and differentiation of human limb buds and claimed that the part of the original blastema which was left between the cartilaginous precursors of the femur, tibia and fibula, formed the first basis of a joint, and he named it the "interchondral disc". The cells in the peripheral part of the blastema condensed and formed the perichondrium, a membrane surrounding the growing cartilages. The perichondrium was continued from segment to segment over the interchondral disc and became the basis of the capsular ligament. He also claimed that, at first, the ends of the cartilages which projected into the joint cavity were also covered by an extension of the perichondrium. The peripheral cells of the interchondral disc lined the capsule and formed the synovial membrane. He also claimed

that there were three interchondral discs in the knee joint; an internal tibio-femoral, an external tibio-femoral and a fibulo-femoral. When the fibula became excluded from the knee joint, the fibulo-femoral disc, from which fibres of the popliteus took origin, was included in the tendon of that muscle.

Whillis (1940) studied the development of the interphalangeal joints of the human embryo, and he found that, at stage 30 mm, the two chondrifying elements were separated by relatively undifferentiated mesenchymal cells which constituted the "joint disc". The cells of the disc were arranged parallel to the surfaces of the cartilage elements. In the region of the future articular surfaces the cells were flattened, very closely packed and arranged with their long axis in the joint line. At the extreme periphery of the disc the mesenchymal cells were arranged longitudinally and formed the perichondrium. The mesenchymal tissue which remained between the joint disc and the perichondrial condensation became much looser than the central part of the disc. In succeeding stages, the peripheral mesenchymal tissue became very loose and cavities appeared within it, while the joint disc became thinner in its central part so that, in an embryo of 72 mm, it was represented by a single layer of flattened cells. At 125 mm, the joint disc between the cartilages had entirely disappeared and the two elements were united across the joint line by a primitive type of cartilage, or "precartilage". He also claimed that the dissolution of

continuity between the cartilage elements was accomplished by liquefaction of the matrix of the primitive cartilage uniting the two elements. The cause of the liquefaction was not explained.

Haines (1947) studied human embryos, and found that the joints first appeared as interzones, formed from the remains of the skeletal blastema between the cartilages. He described each interzone as passing through a three-layered stage, with two chondrogenous layers and an intermediate loose layer. The fibrous capsules formed as condensations in the extrablastemal tissue near the joints, which 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. In a subsequent study of the early development of the femoro-tibial and tibiofibular joints of the human embryo, Haines (1953) found that the interzones for the medial and lateral condylar regions are separate from the time of their first appearance, and are not formed by the subdivision of one originally single interzone as mentioned by Gray and Gardner (1950). He also found that, before chondrification, the fibular and femoral regions of the skeletal blastema were continuous with one another but, soon after chondrification, the fibula was found to be unattached to either the femur or the tibia. The fibula never articulated with the femur at any stage, but eventually formed an interzone with the tibia.

Gray and Gardner (1950) studied the development of the human knee joint, and found that, in a 6-week embryo, the cartilaginous precursors of the femur and the tibia were separated from each other by an avascular and undifferentiated blastemal zone. At $6\frac{1}{2}$ weeks, due to continuing chondrification, the cartilage elements lay closer to each other and the interzone became denser in appearance. At $7\frac{1}{2}$ weeks, the interzone differentiated into a three-layered structure. The middle looser layer closely resembled the mesenchyme which was located adjacent to the joint and with which it appeared to be continuous. The outer layers of the interzone arranged along the distal ends of the cartilage elements were continuous with the perichondria. They also claimed that there was no apparent cartilaginous continuity between the femur and tibia, such as had been described by Bardeen (1905) and by Whillis (1940), nor did the fibula enter into the formation of the knee joint, as Keith (1933) had claimed.

O'Rahilly (1951), who studied the developing human knee joint, found that, in embryos of 15 mm, the tibial condensation was directly in line with that for the femur, and the fibula, which was lateral to the femoro-tibial axis, appeared to reach the same level as the upper surface of the tibia. At 16 mm, the fibula lost its eccentric position and was situated under the femur, and the tissue between the femur and fibula was arranged as a three-layered interzone. At 18 mm, the femoral condyles overhung the tibia and fibula on each side, and the upper

end of the fibula reached, or even exceeded, the same level of the tibia but appeared bent backwards. At 25 mm, the fibula was separated from the femur by the lateral condyle of the tibia, and a three layered interzone was found between the femur and tibia, but the tibia and fibula were not yet in contact with each other. At 28 mm, a three-layered superior tibio-fibular interzone was present.

Gardner and Gray (1953) studied the human shoulder and acromioclavicular joints. In the shoulder joint, a three-layered interzone was noted for the first time at stage 20 mm, and at stage 22 mm, the capillaries penetrated the tissue immediately adjacent to the interzone and converted it into a "synovial mesenchyme". In the acromio-clavicular joint, on the other hand, they did not find a three-layered interzone in any of the specimens studied. They found that the acromio-clavicular joint was represented as a wide zone at 12 mm, and remained as a broad zone in specimens between 22 and 45 mm. A cavity in this joint was first noted at 49 mm, and it was unilateral and located much closer to the acromion than to the clavicle.

O'Rahilly (1957) studied various human synovial joints, and described the formation of the interzone. At about $5\frac{1}{2}$ weeks, the interzone was composed from three layers: a loose middle layer, and two chondrogenic layers, each of which was continuous with the perichondrium of the adjacent cartilage element. The extrablastemal mesenchyme was invaded by blood vessels and became vascularised and

continuous with the loose middle layer of the interzone - that portion of the vascular mesenchyme adjacent to the blastemal interzone, called "synovial mesenchyme". In a subsequent study of the human knee joint, O'Rahilly (1968) described the formation of the interzone. He found that the blastema between the femur and tibia became a homogenous interzone at (Carnegie) stage 19 (16-21 mm), and recognized it as a three-layered structure at stage 21 (22-24 mm). At stage 22 (23-25 mm) all the constituent structures of the joint were recognizable although cavitation did not occur until stage 23 (27-31 mm).

Gardner, Gray and O'Rahilly (1959) studied the skeleton and joints of the human foot, and found the interzone to develop in a manner similar to that described by previous workers. They claimed that cavitation began in a few joints during the embryonic period, but that formation of three-layered interzones, followed by cavitation, began in most joints during the early fetal period. Capsules and ligaments were present as cellular condensations before cavities appeared and, in some, before the appearance of a three layered interzone.

The findings of more recent workers support previous results on the early formation of the interzones. Andersen (1961, 1962a,b, 1963, 1964), in his histochemical studies on the histogenesis of the knee, elbow, shoulder and acromio-clavicular joints in human foetuses, described the formation of interzones in detail. The cartilaginous centres of the individual skeletal structures were laid

down in the condensed mesenchymal cord, or "skeletal blastema", and underwent appositional growth in all directions. Part of the original blastema was left between them, and this condensed mesenchyme was called the articular interzone. The cells of this interzone had round or oval nuclei and a very sparse, basophilic cytoplasm. At the outset, they were not arranged according to any definite plan, but, owing to the growth of the adjacent cartilaginous primordia, the cells of the interzone gradually became oriented with their long axes transverse to the long axes of the skeletal primordia. Then a striking change took place in the interzone. Its cells began to secrete a highly metachromatic and alcian-blue stained intercellular substance, forcing the cells apart, especially in the central layer. Now the interzone was distinctly three-layered, consisting of two chondrogenous layers serving as appositional growth layers for the epiphyses adjacent to the interzone, and continuing at the periphery of the joints into the ordinary perichondria of the cartilage (Anderson, 1962). Between the two chondrogenous layers, there was an intermediate looser, more highly metachromatic and alcian blue-stained layer made up of long spindle-shaped cells. This layer was continuous towards the peripheral marginal layers of the interzone with a similar tissue which was clearly separated, by the deep staining of its intercellular substance, from the surrounding vascular general

mesenchyme. This separating zone was at the site where the joint capsule was formed by the peripheral areas of the skeletal blastema, originally as a cellular strand continuous with the perichondria of the cartilages concerned (Anderson,1961). The intercellular substance of the interzone consisted of chondroitin sulphate A or C (possibly both), and not hyaluronic acid. Cavity formation started centrally after the intermediate layer of the interzone became incorporated on each side with the two chondrogenous layers, which in turn were incorporated in the epiphyseal cartilages. Later on the epiphyses faced each other in the central parts of the joint, by smooth, hyaline cartilaginous surfaces, separated only by a narrow central joint cavity. He also found that the tissue of the interzone which had been completely avascular, was invaded by vessels from the surrounding general mesenchyme so that the peripheral areas of the interzone were converted from a dense cellular tissue to a looser vascular structure called "synovial mesenchyme".

Anderson and Bro-Rasmussen (1961) reached similar conclusions in their study of the hand and foot of human fetuses, but they considered the synovial mesenchyme as apart from the surrounding general mesenchyme, projecting wedge-shaped into the peripheral parts of the joint. The joint cavity first appeared in the central area of the joint, then spread peripherally, where the synovial mesenchyme projected into the joint.

Schneck (1965) studied the knee and ankle joints of

the rabbit and reported that, in their development, the joints went through the typical mesenchymal, pre-cartilaginous and cartilaginous blastemal stages, with the formation of a characteristic interzone at the site of the developing joint. A capsular condensation was observed prior to the development of the three typical layers of the interzone.

O'Rahilly and Gardner (1978) in their review of the embryology of movable joints, reported that a number of synovial joints differed from the general process in their development. Some, especially some of the small joints, showed considerable delay between differentiation and cavitation. Some of these, such as the acromio-clavicular, might never display the usual homogeneous and three-layered interzones. Others such as the sternochondral joints, showed cartilaginous continuity across the interzone in early stages, and might or might not subsequently develop cavities. Some joints, such as the temporo-mandibular, developed where a continuous blastema never existed.

Mitrovic (1978) studied the development of diarthrodial joints in the rat, and described the events in the development of the interzone. He found an early wave of cell necrosis occurring immediately after differentiation of the interzone, and thought that this cell death 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 interzone and for the differentiation of a three-layered interzone. 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 joint; this invasion did not extend into the interzone between the cartilages at any stage of development.

B. AVIAN SYNOVIAL JOINTS

Hepburn (1889) in his study of the development of diarthrodial joints in birds and mammals, noted the close similarity in the morphogenesis of the early knee joint in mammalian and avian embryos.

Fell and Canti (1934) in their classic study of the development *in vitro* of the avian knee joint, described its normal early histological development. Cartilage matrix was first seen in a limb bud 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. Very shortly after this stage, in a 3 mm limb bud, the first sign of the future knee-joint appeared. 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, they found that cartilage matrix of a very young and immature type 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 a 6-day limb bud, they found that the cartilage was well developed throughout the epiphyseal region, except in the interzone mesenchyme, 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 on disappeared.

O'Rahilly and Gardner (1956) in their comprehensive study of the development of the knee-joint of the chick and its correlation with the staging of embryonic development, also described the mode of formation of the interzone. By stage 26 the early cartilaginous blastema was segmented into three portions, thereby indicating the site of the future knee joint as an interzone or intercartilaginous blastemal disc. The interzone was a dense cellular region which, on its first appearance, was uniform or homogeneous in structure. At stage 27, beginning chondrification in the future bone elements accentuated the homogeneous femoro-tibial and femoro-fibular interzones of the knee, and in some instances the primordia of the future bones seemed to be continuous, owing to an appearance of

chondrification extending partly across the interzones. It is important to note that they found that a typical three-layered interzone was quite difficult to recognize in the knee of the chick embryo. Gardner and O'Rahilly (1962) in their study of the development of the chick elbow joint, found that, at stage 29, a three-layered interzone was established, and the central portions of the humero-radial and humero-ulnar part of the interzone were looser in texture, and presented blood vessels in their peripheral parts. At stage 37, condensations for the capsule, and for the medial and lateral ligaments were noted, and loose, vascular tissue was observed deep to these condensations. This was believed to be early synovial tissue.

Henrikson and Cohen (1965) studied the chick interphalangeal joint, and they found that occasionally the interzone showed a three-layered structure, two compact cell layers forming the chondrogenous layers for the adjacent cartilage elements, and an intermediate layer lying between them. They also found that a band of metachromatic substance was occasionally present in the joint interzone. This area was shown by the electron microscope to be structurally distinct from the metachromatic phalangeal cartilage. They thought it likely that this metachromasia was a distinct entity, and not an extension of the articular surface. They claimed that the differentiating joint interzone included blood vessels which extended from the synovial mesenchyme, which was present in the peripheral parts of the joint.

Mitrovic (1977) studied the chick meta-tarsophalangeal joint. The events are similar to those which he described in the rat embryo (1978) (see p. 12).

Several authors have considered the presence of vessels within the interzone during development. Henrikson and Cohen (1965) in the chick interphalangeal joint, found blood vessels within the differentiating joint 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) who also studied the chick interphalangeal joint, frequently found some blood vessels and free erythrocytes in the axial part of the joint. These findings in the chick contrast with those of several studies on mammalian joints e.g. in the rat, Mitrovic (1978) reported that blood vessels were confined exclusively to the periphery of the joint, in the synovial mesenchyme, a finding in agreement with those of Haines (1947), Gardner (1950), O'Rahilly (1957) and Anderson and Bro-Rasmussen (1961) among others.

II. THE FATE OF THE INTERZONE

A. FORMATION OF THE INTRA-ARTICULAR STRUCTURES

(a) Mammalian synovial joint

Two questions have been frequently raised: do intra-articular structures arise from the interzone or from the "synovial mesenchyme" and does the synovial mesenchyme form from the interzone or is it really a separate entity?

Some workers have claimed that the "synovial mesenchyme" is formed by the invasion of blood vessels into the peripheral parts of the interzone, which are thereby converted into a vascular and looser tissue: (e.g. Anderson 1961, 1962, 1963 and 1964). Others have claimed that the synovial mesenchyme is formed from the general mesenchyme at the peripheral parts of the joint e.g. Haines (1947), Gray and Gardner (1950), Gardner & Gray (1953), O'Rahilly (1957), Schenck (1965), Gardner & O'Rahilly (1968) and Mitrovic (1978).

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

Bardeen (1905) studied human skeletons, and found that, in 20 mm embryos, the cruciate ligaments and the menisci were differentiated directly from the blastema. The peripheral blastemal tissue of the joints became

transformed into a capsular ligament, strengthened in front by the tendon of the quadriceps.

Keith (1933) regarded the menisci as remnants of the interchondral discs, projecting into the gap between the articular surfaces. According to this author, the backward extension of the femoral condyles toward the popliteal space isolated a posterior part of the capsule, which came to lie within the joint and formed the cruciate ligaments.

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

Andersen (1961) studied the human knee, and found that at 23 mm, the cruciate ligaments and the menisci were developed *in situ*, as a cellular condensation in the interzone. Schenck (1965) studied the rabbit knee, and he also observed that the cruciate ligaments and the menisci developed *in situ* from the interzone.

Mitrovic (1978) studied rat embryos, and reported that the peri-articular ligaments, capsular, and synovial tissue differentiated by a distinct and usually retarded segregation of the cells of the mesenchyme that surrounded the interzone, but that the menisci and intra-articular ligaments differentiated from the cells that constituted the interzone.

On the other hand, Haines (1947) thought that the fibrous capsules which developed as condensations in the

extrablastemal tissue near the joints, cut off a part of the general mesenchyme to form the synovial mesenchyme, and that it was this which gave rise to the more central parts of the synovial cavities, the synovial and sub-synovial tissues and all intracapsular structures including ligaments, tendons, and fibro-cartilages. Gray and Gardner (1950) studied the human knee, and claimed that at $7\frac{1}{2}$ weeks, before a fibrous capsule is formed, and due to increase in joint size, adjacent mesenchyme became intra-articular in position. At 8 weeks of development, the menisci and cruciate ligaments arose from the characteristically vascular synovial mesenchyme.

b. Avian synovial joints

O'Rahilly and Gardner (1956) studied the chick knee, and found that the synovial tissue, cruciate ligaments, and the menisci, arose from the synovial mesenchyme which became incorporated in the joint from the vascular extrablastemal tissue. They also said that the lack of a clearly defined capsule in the development of the knee joint of the chick lends support to the contention that capsule formation is not necessary to the inclusion of synovial mesenchyme within the joint.

B. CAVITY FORMATION

The question has been frequently raised, does the formation of the joint cavity depend on either extrinsic factors, such as muscular movement, or intrinsic factors,

such as cell death or "liquefaction' in the interzone, or are both factors required if the joint cavity is to appear and to persist? Also does cavitation start centrally or peripherally? Many authors have suggested that mechanical factors are important in joint development basing their studies on experimental analysis, while others basing their studies on normal histological material, have attached greater importance to intrinsic, genetic, factors.

1. FACTORS LEADING TO CAVITY FORMATION AS SEEN IN NORMAL HISTOLOGICAL MATERIAL:-

A) CAVITY FORMATION DUE TO CELL DEATH

(a) Mammalian synovial joints

O'Rahilly & Gardner (1978), in their study of the embryology of movable joints, quoted Wassilev's (1972) finding that, in the development of the knee joint of the rat, cavitation proceeded with degenerative changes of mesenchymal cells in the central part of the interzone, but gave no detail of the mechanism of degeneration.

Rajan and Merker (1975), in a brief abstract, reported on joint formation of the human embryo digits in culture. They found that there was evidence of material in the joint cavity, which was acid mucopolysaccharide in nature, with occasional cells showing pyknotic nuclei lining the cavity. Under the electron microscope there was single cell necrosis with evidence of loss of glycosaminoglycans. They did not give any detail for the evidence in their findings.

Mitrovic (1978) in his study of the development of the diarthrodial joints in the rat embryo, noted two waves of cell degeneration. The first, which occurred was after differentiation of the joint tissue he thought might serve one (or both) of two purposes, i) to prevent chondrification of the interzone by removing cells with chondroblastic potentialities; and ii) to provide for loosening of the interzone and the differentiation of a three-layered interzone. A second wave was noted immediately preceding and accompanying the joint cavitation. These cells showed pyknotic nuclei, poorly stained cytoplasm, and "fuzzy" limits. He also reported that the later stages of cell death, such as cell disintegration and macrophagocytosis, were not seen during the second wave, and he suggested that these cells either disappeared or changed their staining pattern and constituted a surface cell layer of the articular cartilage. He claimed that these dead cells might have played some role in the cavitation process, and he considered them as a type of physiological cell death comparable to that found by others in the interdigital areas.

TenCate, Freeman and Dickinson (1977) in their study of the development of the sutural joints in rat embryo, found that cell death occurred in the central part of the suture associated with newly differentiated osteoblasts, engaged in phagocytosis of the cellular debris. They

claimed that these cells were implicated in the development of the suture and considered it as programmed cell death, but they did not give any detail of the cytological changes in dying cells.

b. Avian synovial joint

O'Rahilly and Gardner (1956), in their study of the development of chick knee joint, did not give any explanation about the mechanism of cavity formation, which started as a femoro-meniscal cavity, at stage 33. They found pyknotic cells in the joint region in only 2 embryos out of 122 embryos at stage 34, and did not comment on their nature or significance.

Mitrovic (1977), in his study of the development of the metatarso-phalangeal joint of the chick embryo, found two waves of cell degeneration: an early wave, which was noted after differentiation of the joint tissue, he thought might provide for loosening of the interzone and the differentiation of a three-layered interzone. A second wave which was noted immediately preceding and accompanying the joint cavitation, he thought might be implicated in some way in the cavitation process. These cells under the light microscope showed pyknosis, basophilia and vacuolization. Under the electron microscope, these dead cells showed strong basophilia, electron opacity and enlarged endoplasmic reticulum. He suggested that these dead cells either rapidly disintegrated and were phagocytosed by the surrounding cells, or cells remained

after the joint cavity was fully differentiated as a discontinuous cover for the articular surfaces. Also in addition to cell degeneration, he found the presence of an organic component, presumably mucopolysaccharide, in the primitive synovial fluid, which was thought might be implicated in cavitation at the sites of its accumulation.

Mitrovic (1982) in a subsequent study of the development of the articular cavity in paralysed and cultured limb buds of chick embryo, found dead cells in the joint region and thought that these were involved in the cavity initiation.

B. CAVITY FORMATION DUE TO "LIQUEFACTION'

(a) Mammalian synovial joints

Several workers have suggested that "liquefaction" of cells was responsible for cavity formation, (e.g. Retterer, 1902; Keith, 1933; Walmsley, 1940; Whillis, 1946; McDermot, 1943; Haines, 1947; and Schneck, 1965).

Retterer (1902) (cited in a review by Haines, 1947) found that the intercellular network was composed of scattered strands containing irregularly disposed nuclei. Some of the cells were eventually destroyed, but most became attached to one or other of the walls of the cavity, and persisted as its lining. This view was supported by Haines (1947), who studied the human synovial joints and found that the synovial surface was ragged, and that 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. He also reported that when the articular surfaces were fully chondrified, the remains of the liquefied tissue of the interzone or synovial mesenchyme came to form a thin fibrillar layer overlying the cartilage, containing flattened cells some of them pyknotic or reduced to debris. Eventually these flattened cells disappeared.

Keith (1933) studied the development of various human synovial joints and found that, during the 3rd month, the skeletal blastema between the chondrified bases of the bones, by a process of "vacuolation within and between the cells", opened out into a cavity and formed the joint cavity.

Willis (1940) studied the development of human and rat synovial joints and found that the two elements in the joint were united for a time by primitive cartilage. The dissolution of continuity in the later stages of joint formation was accomplished by "liquefaction" of the matrix of the primitive cartilage uniting the two bones. The cause of liquefaction and its nature was not explained. He 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) found in human embryos that, at the age of 60 days, the interchondral disc (interzone) had begun to show early and small degrees of decreased density

and irregular loss of cellular substance anteriorly and posteriorly. These changes appeared to be in the nature of a rapid and complete dissolution of the cells in the area, without finding cells in different stages of disintegration. This loss of substance was the first sign of the formation of the cavity of the joint.

(b) Avian synovial joints

Henrikson and Cohen (1965), in their study of the developing chick interphalangeal joint, found that the joint cavity was first seen either between or lateral to the articular surfaces, and emphasised that there was no sign of liquefaction or cell degeneration preceding cavity formation. They found blood vessels within the differentiated joint interzone and they suggested that their presence may be necessary for the initiation of cavity formation.

2. FACTORS LEADING TO CAVITY FORMATION AS SEEN IN EXPERIMENTAL STUDIES

Several workers have studied the effect of extrinsic factors, such as muscular movement, on the development of the synovial joints using one (or both) of two experimental methods:

i) culturing the embryonic limb bud *in vitro*; and ii) by paralysing the embryo.

a. Avian synovial joints

Experiments performed by Murray and Selby (1930), and those subsequently made by Fell and Canti (1934) and by Hamburger and Waugh (1940) using chick embryo limb buds, cultured in vitro, all showed that the joint mesenchyme (interzone) underwent normal development but that separation of skeletal anlagen does not occur. In the absence of movement the joint tissue underwent chondrification, progressively disappearing through fusion with the adjacent cartilaginous skeletal elements.

This result was supported by Bradley (1970), who cultured chick embryo limb buds on the chick chorioallantoic membranes. He found that, morphologically, limb development was retarded by at least 1 day, although feather development was normal for the graft age. He concluded that the skeleton of the chick limb may be regarded as capable of self-differentiation, since chondrification and the first phase of ossification proceeded normally in the absence of innervation. He also found that joint formation was sometimes almost perfect but the presence of fusions in the majority of cases supported the suggestion that proper maintenance of the differentiation of a joint depended on its undergoing movement.

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 watchglass or flask, and one limb was subjected to passive movement, while the other served as control. He concluded that, in the control explanted limbs, where no movement was externally applied, cartilaginous fusion across the joint of the skeletal parts was generally found. Movement prevented this fusion. An articular cavity was sometimes formed in the moved explants but not in the controls. He concluded that movement exerted a formative effect on the shape and structure of the articular surfaces, and on the histotypical proliferation of cartilage tissue.

This hypothesis was supported by Drachman and Sokoloff (1966), who produced paralysis of embryonic movement of the chick embryo through administration of decamethonium bromide, botulinus toxin, or by spinal medulla section. They observed that an articular cavity did not form and that the foot sesamoids were absent. Their results underlined the importance of skeletal muscle contraction in both the formation of articular cavities and in determination of the form of articular surfaces. This view was supported by Sullivan (1966), Murray and Drachman (1969), Drachman et al (1976), Rauno-Gil et al (1978) and more recently by Persson (1983) who produced paralysis of the embryonic movement of the chick embryo through administration of decamethonium iodide. He found that all embryos whose movements were paralysed, showed regression of the articular cavity and its replacement by

mesenchymatous tissue; this regression also affected all para-articular structures such as the capsule, ligaments and menisci. He concluded that the skeletal muscle contractions were necessary to maintain the articular cavity and para-articular formations.

However, other workers reported the presence of small cavities at the periphery of joints of paralysed embryos, suggesting that some degree of cavitation had occurred even under such conditions of paralysis.

Murray and Drachman (1969) while studying the development of head and neck joints in paralysed chick embryos, found that, in a few cases, joint cavities underwent complete development. This was supported by Mitrovic (1982) who produced paralysis of chick embryos by administration of decamethonium bromide, and then cultured the limb buds on the chorioallantoic membrane. In general, he found that in embryos paralysed prior to differentiation of the joint cavity, the first stages of development of the cavity were not inhibited, but that the initial cavities did not progress further, and were rapidly invaded by blood vessels and loose connective tissue, and finally disappeared. Fusions across the joint developed after a few days by a process of fibrosis and chondrification. He suggested that intrinsic factors were important for differentiation of the early stages of joint cavitation, and that movement appeared to be the necessary condition for full differentiation and maintenance of the

joint cavity.

b. Mammalian synovial joint

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

Rajan and Merker (1975) observed the development of normal cavities in cultured human embryonic digits. After 10 days in culture, the joint cavity appeared and this was in the absence of movement. They also found that the joint cavity contained some acid mucopolysaccharide material, with occasional cells having pyknotic nuclei, and thought that this cell death might be implicated in cavity formation. They concluded that, as the organ cultures are devoid of blood supply, nervous control and movement, these factors were not essential to the formation and differentiation of the joint cavity.

3. WHERE DOES CAVITATION BEGIN?

A. Mammalian synovial joints

Several workers found that cavitation was seen first in the peripheral region of the joint e.g. Haines (1947); Gardner and Gray (1950, 1953); Gray and Gardner (1950) and Schneck (1965).

Haines (1947), in various human synovial joints, found that, at 29 mm, just before the joint cavities appeared, the three layered interzone became sharply distorted. In the intermediate layer the cells became flattened and at the same time the matrix of this tissue prepared for "liquefaction". At 30 mm, the tissue matrix of the peripheral regions of the interzone broke down and small cavities were formed. In general, he concluded that the cavities of the larger joints appeared at, or soon after, the onset of periosteal ossification in the long bones, and that, in the smaller joints, the cavities appeared in later stages than the larger joints.

Gray and Gardner (1950) who studied the development of the human knee joint, found that in one of three fetuses of 8 weeks, 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. They also reported that the first cavities were irregular in outline, and that they frequently contained strands of tissue and scattered cells. They concluded that cavitation started in the synovial mesenchyme before it appeared in the interzone. They did not explain how the cavity was formed.

Gardner and Gray (1953) who studied the development of the human shoulder and acromioclavicular joints, found that, in the shoulder joint, cavities were first seen at 25 mm, located in both the anterior and posterior parts of the

joint. They also reported that cavitation was preceded by the loosening of the middle layer of the interzone in its peripheral part. In the case of acromioclavicular joint, they found that a joint cavity first appeared at 49 mm, and it was unilateral and located much closer to the acromion than to the clavicle. They also reported that the acromioclavicular joint differed significantly from the typical pattern exemplified by the shoulder joint, in its rate and manner of development. A three-layered interzone was not seen at any stage.

Schneck (1965) who studied the development of the rabbit knee joint, also found that cavitation first appeared in the peripheral parts of the knee joint, through a loosening of the tissue, both above and below the menisci, while the central parts of the joint, where the femur and tibia were in tight contact, showed no cavitation.

Other workers however, have found that cavitation started centrally in the interzone (e.g. McDermott (1943), Andersen (1961, 1962, 1963, 1964) and Andersen and Bro-Rasmussen (1961)).

Andersen (1961) found that in the human knee joint at 34 mm, the intermediate layer of the interzone consisted of only one layer of cells centrally, while peripherally, between the menisci and the femur, as well as between the menisci and the tibia, it consisted of 2-3 layers. At 35-41 mm, the intermediate layer of the interzone disappeared

centrally and a cavity was first seen in the central area of the joint, and then spread towards the periphery. Cavity formation was preceded by the formation of large quantities of chondroitin sulphate in the three layers of the interzone and in the loose infrapatellar tissue. He found no sign of cell degeneration or of "liquefaction" in the interzone. In subsequent studies of the development of the human elbow joint (1962) and shoulder and acromioclavicular joints (1963) he again found that cavitation began in the central part of each of these joints. In the case of the elbow joint, a small cavity was observed centrally in the humero-radial joint at 28 mm, thereafter extending to the radio-ulnar and humero-ulnar joints. In the case of the shoulder joint, a small central cavity appeared at 28 mm, and the cavity subsequently extended to the peripheral parts of the joint. In the acromioclavicular joint, a small cavity was first seen between the articular disc and the acromion at 45 mm, while the cavity between the disc and the clavicle did not appear until 68 mm. He also reported that in the acromioclavicular joint a distinct three-layered interzone could not be seen, the articular disc being formed as a fibrous, vascular strand extending through the joint and dividing it into two parts. In all the joints which he studied, he found that cavity formation was preceded by the accumulation of large amounts of acid mucopolysaccharides, and that there was no sign of cell degeneration or liquefaction appearing in the joints. The same conclusion

was reached by Anderson and Bro-Rasmussen (1961) who studied the development of the hand and foot joints in human embryos.

Others such as Mitrovic (1978) reported a compromise view. In the rat embryo, he found that the first cavity was seen as a small and narrow cleft. In single joints, such as the shoulder or interphalangeal joints, the cavities were first seen in the periphery of the mesenchyme, while in the more complex joints such as hip, elbow, knee and tarsal joints, they were seen simultaneously in the periphery and in the centre of the joint, as two or even three separate clefts. He also found that, at any particular stage of development, the larger joints were more advanced than the smaller ones. He claimed that cavity formation was preceded by cell degeneration in the interzones and he thought that cell death might be implicated in the cavity formation.

B. Avian synovial joints

O'Rahilly and Gardner (1956) who studied the development of the chick knee joint, found the first cavity in the femoro-meniscal region at stage 33, but gave no explanation of the mechanism of formation.

Gardner and O'Rahilly (1962) found that in the chick elbow joint the cavity was first seen in the humeroradial region at stage 35, but again offered no explanation of the mechanism of cavity formation.

Henrikson and Cohen (1965) found, in the chick interphalangeal joint, that the first cavity was seen in the interzone as a narrow cleft either between or lateral to the phalanges at stages 37-41. This early cleft contained small amounts of collagen, thin cytoplasmic processes which extended from the lining cells and free cells without any connection with the walls of the cavity. They also found blood vessels in the intermediate layer of the interzone and thought that they might play a role in cavity formation. Like Andersen (1961, 1962, 1964) and Andersen and Bro-Rasmussen (1961), they found no sign of cell degeneration or liquefaction preceding cavity formation.

THE PHENOMENON OF CELL DEATH

I. GENERAL

It is now well established that cells may die "by accident or by design", (Wyllie *et al*, 1980, 1985). When they die "by accident" e.g. as a consequence of environmental trauma or disease, the process is called *necrosis*. When a cell dies "by design" as the result of events occurring within itself, apparently as part of a genetically determined programme, the process is known variously as *physiological* or *programmed cell death* or as *apoptosis*.

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, 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.

A. *Necrosis*

Wyllie *et al* (1980, 1985) in their comprehensive review of cell death, described the morphology and mechanism of cell necrosis. The early changes which occur in cell 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 flocculent and sometimes also granular 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, but with swelling of the nucleus and rupture of its membrane, the marginated chromatin masses may become evident as small discrete masses. 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 remainder is eventually phagocytosed and digested by specialized phagocytic cells.

Necrotic cells have leaky membranes 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.

These changes can be produced by several conditions, such as severe hypoxia and ischemia. Kerr (1971) who studied the effect of hypoxia on the rat liver, found that ligation and complete obstruction of the hepatic artery produced hepatic necrosis. Other agents, such as raised temperature, have been shown to cause necrosis of lymphocytes (Shrek et al, 1980). McDowell (1972) found that administration of sodium fluoro-acetate in the rat caused necrosis of the proximal convoluted tubule of the kidney.

B. Apoptosis

The phenomenon of programmed cell death has long been a subject of speculation among biologists, who found it difficult to believe that this process was a normal occurrence in living organs, and particularly in connection with morphogenesis.

Since dead cells often assume the appearance of dense granules, which stain intensely with nuclear dyes and often lie within the cytoplasm of a phagocyte, they were variously interpreted by earlier workers either as invading leukocytes living in symbiosis with host cells, or as intracellular deposits, or as "concentrated protoplasm", or as "mitotic metabolites" (i.e. granules formed as cell breakdown products during mitosis) (see review by Glucksmann, 1955). Some early

workers recognized cell death in various regions of vertebrate embryos, although it was not always appreciated that the degenerating cells were usually within macrophages. Glucksmann (1951) has reviewed the many examples of morphogenesis in which cell death is involved. He reported that cell deaths occur regularly at certain developmental stages of all vertebrate embryos. They cannot be considered as artifacts, due to fixation or other conditions of handling, since they occur in the best fixed specimens and can also be detected in the living embryo. He suggested a classification of normal cell death as follows:

i) morphogenetic degenerations are related to the shaping of organs, e.g. during the invagination of the optic cup, the formation of the crystalline lens, the olfactory pit, the neural tube, etc.

ii) histogenetic degenerations are related to the differentiation of tissues and organs, e.g. sex differentiation of the individual involves the partial degeneration of the Mullerian or of the Wolffian ducts.

iii) phylogenetic degenerations are of two types: those which occur in vestigial organs and those involved in the regression of larval organs. The difference between them is merely the stage which a particular structure has achieved before it regresses. The best examples for the first group is the degeneration of the paraphysis in higher vertebrates. Examples in the second group are the degenerations in the pronephros and mesonephros in higher vertebrates, of ganglion cells in the branchial region, and of the conjunctival

papilla.

He 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 occurrence of apoptosis during normal development, many workers found that apoptosis can be produced by an external minor stimuli.

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 (1980) also found that exposure to temperatures between 37 °C and 43 °C produced apoptotic degeneration of lymphocytes, while higher temperatures produced necrotic degeneration.

Recently Wyllie *et al* (1980, 1985) described the morphology, mechanism, and causes of apoptosis, in their comprehensive review about cell death.

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

Table 1 : Differences between Necrosis and Apoptosis.

	Necrosis	Apoptosis
- Causes	Environmental trauma, disease, toxins	In normal development - physiological and homeostatically regulated. Can also be triggered by relatively minor external stimuli.
- Mechanisms	Irreversible failure of membrane selectivity, uncontrolled entry of calcium, influx of water, activation of phospholipase and secondary lysosomal degradation.	Initiate through new gene expression. Nuclear chromatin is digested by an endogenous nuclease, and the cell surface undergoes biochemical modification which permits recognition by phagocytes.
- Histological appearance	Usually affects tracts of contiguous cells. Eosinophilic "ghosting" of entire cells.	Characteristically affects scattered individual 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	Marginate 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 cytoplasmic degradation advanced.	Disperses to shower granules while cytoplasm structurally intact.
Nuclear membrane	Retains pore structures until cytoplasmic degradation advanced; eventually destroyed with other organelles.	Pores retained adjacent to euchromatin, but lost next condensed chromatin. Eventually becomes discontinuous, so that dense chromatin masses lie among cytoplasmic organelles.
Cytoplasm	Swelling of all compartments followed by rupture of membranes and destruction of organelles. Mitochondrial matrix densities characteristic.	Endoplasmic reticulum may dilate focally. Structurally intact mitochondria and other organelles compacted together; protuberances from cell surface separate to form apoptotic bodies.

II. CELL DEATH IN EMBRYONIC DEVELOPMENT

Cell death occurs 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 tube, and in the course of differentiation of the retina and the lens (Glucksmann, 1965); these are all cases where evagination or invagination take 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. Hamburger and Montalcini (1949) have studied cell death during the differentiation of the cervical and thoracic spinal ganglia in chick embryos. This process is at its peak at 5 and 6 days of incubation and is accompanied by macrophages. No such degeneration occurs in those ganglia which innervate the limbs. The same conclusion was reached by O'Connor and Wyttenbach (1974) who studied cell death in the visceromotor neurons of the cervical region of the chick embryo's spinal cord.

Dead cells have been found in the branchial arches of human, 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.

Garcia-Porrero and Ojeda (1978) 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.

Pexieder (1975) also found physiological cell death in the bulbar cushions of the normal chick embryonic heart. These dead cells were characterized by the presence of numerous prenecrotic cells with cytosegresomes. Later on many macrophages appeared.

Because cell death in the developing knee joint is the principal theme of this thesis, the general phenomenon of cell death in limb morphogenesis is dealt with separately and in detail in the following section.

III. CELL DEATH IN LIMB MORPHOGENESIS

At an early stage of development the limb buds consist of two components, an inner core of mesenchymal tissue, covered by a thin layer of ectoderm. At the end of the bud, the ectodermal layer consists of a crest-like thickening of pseudo-stratified epithelium (the apical ectodermal ridge, AER).

The importance of cell death in limb development has been recognized in several studies: in the chick embryo [Saunders, Gasseling and Saunders (1962); Saunders (1966); Saunders and Fallon (1966); Dawd andHinchliffe (1971); and Hinchliffe, 1974; in the rat embryo [Ballard & Holt, 1968], and in the human embryo [Kelley (1970) and Hinchliffe (1982)].

Cell death occurs in the following areas:

i) in the apical ectodermal ridge - AER.

ii) in the limb mesenchyme:-

a) Interdigital areas.

b) The anterior and posterior necrotic zones (ANZ and PNZ)

c) Along the central axis of the mesoderm.

i) Cell death in the AER

Cell death has been observed in the chick AER, either by vital staining (Hinchliffe and Ede, 1967) or by histological or electron microscope studies (Saunders, 1948; Zwilling, 1961' Jurand, 1965). Zwilling (1966) claimed that cell death was found in the regressing AER, but in fact dead cells seem to be found during the period of its maximum activity. Cell death also occurs in the AER of other species. Dead cells have been found in the mouse AER [Chang (1939); Forsthoefel (1959); Jurand (1965) and Milaire (1962, 1967a)]. Milaire (1967a) found that dead cells were more numerous in the AER of the syndactylous mutant than in the normal. Chang and Hwei-Yan (1964) reported the presence of dense bodies, which were probably dead cells, in the AER of the mouse. The authors considered these dense bodies, which were positively stained for acid phosphatase, to be lysosomes or cytosomes but, as we shall see later, it is more probable that they were digestive vacuoles containing dead cells phagocytosed by neighbouring ectodermal cells. Jurand (1965) reported similar events in the chick AER. Dead cells were also found in the AER in the rat and the mole (Milaire, 1962). 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 mammalian apical ridge.

ii) cell death in the limb mesenchyme

Cell death has also been found in the superficial mesenchymal tissues, such as the interdigital areas found in many species and in the posterior and anterior areas between the limb bud and the body wall of the chick embryo (posterior necrotic zone - PNZ, anterior necrotic zone - ANZ).

a) Interdigital areas

Cell death has been observed in the interdigital areas of chick limbs at about 7.5 days (Saunders et al 1957; Saunders, Gasseling and Saunders, 1962; Saunders and Fallon, 1966; and Zwilling, 1960). Massive areas of cell death occurred along the anterior border of digit II, between digits II and III, at the posterior edge of the phalanx of digit IV, and later between digits III and IV. By contrast in the hind limb buds of the duck which develops webbed feet, there is very little cell death in the area between the digits.

Zwilling (1960) suggested that the AER played an active role in determining mesenchymal cell death, either by withholding some vital metabolite or by providing some inhibitory substance in the localized regions between the digits. In the developing limbs of other species, the interdigital zones show abundant cell death prior to and

during the emergence of the digits, e.g. in the mouse (Chang, 1939; Forsthoefel, 1959; Millaire, 1962, 1967a, Menkes, Deleanu and Ilies, 1965; Saunders, 1966 and Ballard and Holt, 1968), in the rat and mole (Milaire, 1962), and in man (Menkes et al, 1965 and Kelley, 1970). In all cases, the dead cells seem to be engulfed and digested by phagocytes.

Milaire (1962) has suggested that cell death in these interdigital regions is due to the regression of the overlying AER. Milaire (1965, 1967a) has also noted the absence of cell death in the interdigital areas of oligosyndactylous and syndactylous mutant mice. Cell death is thought to play an active role in carving the contours of the limb and separating the digits, through the removal of the mesenchyme lying between them. The failure of interdigital cell death to occur results in a degree of soft-tissue syndactyly, and in those species, such as the duck, with webbed feet, there is reduction or absence of interdigital cell death. Further evidence for this last point comes from the observations of Deleanu (1965), and of Saunders and Fallon (1966) who found that absence of cell death is correlated with the presence of webbing. A massive area of cell death occurs in the area between digits which are free of webbing (digits I and II), while between digits connected by webbing only shallow zones of necrosis are found.

Some support for this suggestion comes from the observations of Menkes and Deleanu (1964), Deleanu (1965), and Saunders and Fallon (1966). They injected chick embryos

with Janus green. Syndactylism was induced beginning with a tota dose of 3-3.5 ug; it appeared in 88% of treated embryos by using a dose of 4-5 ug and in 100% following administration of 5-6 ug. Survivors showed a high frequency of soft-tissue syndactyly in the hind limb with thin webs remaining between digits, and an examination by using vital dyes at suitable stages showed the absence of macrophages interdigitally. The failure of digital separation was clearly observed. According to the authors, this was due to a toxic effect on the interdigital mesenchyme, "inhibiting the macrophage reaction and disturbing ecto-mesodermal relations". It has to be said that since this suggested mechanism of action was itself speculative, it is uncertain what weight to attach to the experiment.

The interaction of mesenchyme and ectoderm was illustrated by Zwilling's (1960) experimental combination of duck mesoderm and chick ectoderm; there was little interdigital necrosis and duck-like webbed feet developed, while the reverse combination of chick mesoderm and duck ectoderm, resulted in some suppression of interdigital necrosis. Both Zwilling (1960) and Saunders and Fallon (1966) concluded that duck ectoderm inhibits the necroses of the chick interdigital mesoderm and that treatment of chick embryos with Janus green produces a rather similar effect, thus producing a webbed foot.

b) The anterior and posterior necrotic zones

Other areas of superficial mesenchymal necrosis in chick

embryos have been found, by vital staining, to be located in the anterior and posterior zones, in the area of attachment of the limb-buds to the body wall.

Saunders et al (1962) originally suggested that these areas play an active role in the shaping of the upper arm and forearm in the wing. 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.

Another suggestion about the ANZ and PNZ was that they may play a role in the mesodermal-ectodermal inductive interactions claimed to be responsible for limb morphogenesis (Zwilling, 1961). Zwilling claimed that the AER had an inductive effect on the underlying mesenchyme causing it to proliferate, but that the AER was still dependent on a maintenance factor (AEMF) from the mesoderm. Gasseling and Saunders (1964) suggested that the PNZ was not a source of AEMF on the basis of their experimental grafting of PNZ under the AER, which then flattened. This suggestion was supported by Hinchliffe and Ede (1967), who observed that in the normal chick hind limb bud at stage 24, the AER was a series of irregular waves over the PNZ, and could be seen to be breaking down, subsequently disappearing at stage 26.[3] Hinchliffe and Ede (1967) also suggested that the talpid mutant provided evidence for the idea that PNZ is not a

source of AEMF. In the talpid^{3} mutant, ANZ and PNZ are 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^{3} mesoderm concerned in AEMF production or distribution as a result of the failure of cell death to occur in the area of mesoderm which underlies the termination of the AER anteriorly and posteriorly in normal embryos.

It has also been suggested that the fore limb PNZ may have some responsibility for the pattern of limb symmetry (Gasseling and Saunders, 1964). This suggestion was based on experiments in which the PNZ was grafted preaxially in the wing-bud rim, resulting in the appearance, immediately posterior to the graft, of a supernumerary wing tip of left-hand asymmetry, mirror imaging the normal right-hand. Hinchliffe (1974, 1982) in reviews of the patterns of cell death in chick and vertebrates limb morphogenesis, concluded that the study of mutants, in which the pattern of cell death in limb development is affected, makes it clear that foci of cell death are under genetic control and that they can be either extended or restricted, thus causing major alterations in limb form.

c) Cell death along the central axis of the mesoderm

Fell and Canti (1934) described an area of degeneration in the region of the prospective knee joint of the chick embryo. They briefly mentioned a similar zone in the elbow joint subsequently described in more detail by Saunders et al

(1962) and Dawd & Hinchliffe (1971). These areas are known as the opague patches because of their opacity to transmitted light in the living embryo. Fell and Canti (1934) described the morphology of the opaque patch and its appearance in relation to the mesenchymal condensation in the chick embryo hind limb bud. They reported that the significance of the opaque patch in knee joint development was not known.

Saunders et al (1962) claimed that the corresponding opaque patch in the fore limb is of importance in the differentiation of the elbow joint, but gave no critical evidence to support this view. Also Dawd and Hinchliffe (1971) suggested that the opaque patch may play a role in the initial separation of mesenchymal condensations for radius and ulna, and in suppression of chondrogenesis in the mesenchyme between the two bones.

Thorogood (1971) and Hinchliffe and Thorogood (1974) examined the situation in the talpid limb bud, which lacks the opaque patch of cell death, and in which, in subsequent development, the radius and ulna are fused. Although this area of degeneration has not been described in limb development of most other tetrapods, it seems to be present in the mouse and the rat. Milaire (1967b) demonstrated an area of necrosis at the end of the 11th day of development in the central mesoderm. At 12 days, he stated that important degenerating areas may be demonstrated in the central mesoderm of the zeugopodium between the presumptive tibia and fibula.

Also Mitrovic (1977, 1978, 1982) described two waves of dead cells in the central axis of limb buds of rat and chick.

IV. CYTOLOGY OF CELL DEATH

Several workers have studied the morphology of cell death in vertebrate embryos by light, transmission and scanning 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 hyperchromatosis of the nuclear membrane. Both the nucleus and the cytoplasm shrank by 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 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 by using both light and electron microscope have been published by Bellairs (1961), Franchi and Mandl (1962, 1964), Weber, (1964), Jurand (1965), Ballard (1965), Saunders and Fallon (1966), Dawd and Hinchliffe (1971), Hammer and Mottet (1971), Mitrovic (1977, 1978), and, more recently, by Wyllie et al (1981, 1984). Bellairs (1961) studying cell death and phagocytosis in the early blastoderm of the chick by electron microscopy, found similar nuclear changes and noticed several changes in the cytoplasm which generally seemed denser than that of normal cells. The mitochondria seemed more electron-opaque than those in normal cells. The cell membrane of these degenerating cells was found to be intact, despite the degenerative changes which had taken place inside the cell. The dead cell appeared to be surrounded by a single membrane (i.e. its own membrane) before becoming engulfed, but when it was in the process of being phagocytosed, it appeared to be surrounded by two membranes. One was the invaginated cell membrane of the host, while the other was the cell membrane of the degenerating cell.

Franchi and Mandl (1962) studied germ cells of foetal and neonatal female rats, using electron microscopy, and found several stages of cell death. Cells presumed to be at mitotic prophase at an early stage of degeneration showed condensation of the nuclear material and contained in the cytoplasm large rounded or irregularly shaped vesicles surrounded by a double membrane. Cells presumed to be in a

more advanced stage of degeneration showed swollen vesicles of endoplasmic reticulum, disrupted mitochondria and granulated cytoplasm. The first sign of phagocytosis was indicated by partial or total engulfing of a degenerating germ cell by a neighbouring somatic cell. The somatic cell seemed to change to a phagocyte and the nucleus frequently became irregular in shape. The cytoplasm of the phagocytic cell contrasted sharply with the abnormal cytoplasm of the degenerating germ cell; the two were frequently continuous due to the fragmentation of the cell membrane. The engulfed germ cell was found to be diminished in size until only a small mass of electron dense nuclear material persisted, which appeared to be the final stage of digestion.

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 striation, 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.

Jurand (1965) studying the apical ectodermal ridge of chick and mouse limb buds, claimed that the first signs of cell death occurred in the cytoplasm and not the nucleus. These changes consisted of the successive development of lysosomes, Golgi groups, and then cytolysosomes, all of which were positive for acid phosphatase. The cytolysomes contained mitochondria and endoplasmic reticulum, which

suggested that the cells were undergoing autolysis.

Ballard (1965) studied the course of cell death and digestion in the mesenchymal interdigital tissue of the rat foetus, using both light and electron microscopy and suggested the following sequence of events:-

1. Cells died and initially remained spaced out in the tissue as in life and then shrank by the loss of fluid which collected in vacuoles. These seemed either to empty their contents into the intercellular space or to be extruded whole into it.

2. The dead cells were then engulfed by similar, but viable, neighbouring mesenchymal cells, that resembled the other non-phagocytic mesenchymal both in their acid hydrolase activity and in their morphology and ultrastructure.

3. These phagocytic cells differentiated and became typical macrophages. This process was reflected in the altered appearance of their nuclei which became denser and more indented, and in an increase in cytoplasm and cytoplasmic organelles (mitochondria and endoplasmic reticulum), and in increased acid hydrolase activity. Additional dead cells might be ingested at any stage in this process.

4. 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. The same suggestions were reported by Dawd and Hinchliffe (1971) in their study of the opaque patch

in the central mesenchyme of the developing chick limb.

Saunders and Fallon (1966) studied, by EM, the cytological details of the posterior necrotic zones (PNZs) of limb buds of chick embryos. They identified dead cells, at first isolated, and later being engulfed by macrophages. Macrophages were found containing fragmentary remains of dead cells, which appeared to be in various stages of digestion.

Hammer and Mottet (1971) also observed degenerating cells in the interdigital areas of the developing chick limb, that appeared most often in the stage of hyperchromatosis of the nuclear membrane. Dead cells, located within macrophages, were quite evident under light microscopy. 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, basophilia and vacuolization, under the light microscope. On the 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.

Recently, Wyllie et al (1981, 1984) in their study of the nuclear changes in apoptosis in murine lymph cell lines and rat thymocytes, described the morphology of chromatin in apoptosis. The cell nucleus normally possessed a large nucleolus and contained little peripheral heterochromatin. The earliest recognized change was dispersion of the nucleolus into a shower of osmiophilic fragments, and the

appearance of aggregates of granular, condensed chromatin immediately subjacent to the nuclear membrane. Sometimes these aggregates appeared to fill blister-like protrusions of the nuclear membrane. Nuclear pores were absent from the nuclear membrane overlying the condensed chromatin. Adjacent to the crescentic peripheral masses of condensed chromatin, but apparently distinct from them, roughly spherical masses of finely granular osmiophilic material were frequently observed. The authors were uncertain about the nature of this material, but they thought it might have derived from the nucleolus. Cells at what were presumably later stages showed deeply convoluted nuclear profiles. Eventually the nucleus was represented by several discrete masses of condensed chromatin, initially membrane bounded, but later with incomplete or absent nuclear membranes. Cells at this stage frequently showed degenerative changes in the cytoplasmic organelles such as mitochondrial swelling and rupture of the cristae, but the earlier nuclear changes tended to occur in cells with compacted but structurally intact cytoplasmic organelles.

These studies on the cytology of cell death suggest that there is a common pattern of degenerative changes 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.

Several workers studied dead cells by scanning electron microscopy. Hurle and Hinchliffe (1978) presented a general survey of the PNZ under the SEM. Within the area, healthy mesenchymal cells, rounded dead cells and cell fragments and large macrophages could be easily identified. Healthy mesenchymal cells had a typical stellate appearance, with numerous cell processes. In the PNZ they formed a loose mesh-work within which were located the different types of degenerative cells. Cells in the initial stages of degeneration tended to lose their stellate profile, became rounded and showed constrictions which suggested that they were in initial stages of fragmentation. Cells in a more advanced stage of degeneration were clearly recognized as fragmenting cells and appeared to consist of four or five or more rounded bodies detaching from each other. In addition to these changes in cell surface structure, the surface was frequently pitted with small holes. Macrophages were clearly differentiated from mesenchymal cells by their round shape, lack of cell processes and large size; they had ovoid protrusions, "probably representing recently ingested dead cells". Similar results showing budding and fragmenting cells have been obtained by Pexieder (1975), Garcia-Porrero and Ojeda (1979) and Kaplan *et al* (1975).

V. GENETIC CONTROL OF CELL DEATH

It is clear from the examples cited that cell death is a normal part of many morphogenetic processes, and that it may be regarded in some organs as an end point of cellular differentiation. One expects, therefore, to find evidence that cell death is under genetic control, and this is provided by a group of mutant genes which affect cell death, usually increasing normally occurring cell death, but more rarely suppressing it.

Zwilling (1949) studied the wingless mutant in chick embryos, and found that the number of dead cells was greater than normal seen in the apical ectodermal ridge (AER). This augmented cell death led to degeneration of the AER during the third day of embryonic development, and therefore to failure of wing development, since the AER plays an important role in induction of limb development. A group of mutations in the mouse cause shortening or complete disappearance of the tail, and sometimes of part of the posterior body. These mutations are more severe in the case of the homozygote than in the heterozygote. In all of the above mutations the gene acts either by directly causing necrosis in the notochord, and simultaneous, or consequent, necrosis of the somites and neural tube (reviewed by Gruneberg, 1963).

In the case of another mutation of the fowl, cell death is suppressed rather than increased. In the polydactylous talpid^3 mutation, the PNZ and ANZ, which are present in the superficial mesenchyme of normal limb buds of chick embryos

at stages 21 to 29 (3.5 - 6 days), are absent in the case of talpid^3 embryos at this time of development. In addition the "opaque patch" of necrosis of the deeper mesenchyme of the limb (Fell and Canti, 1934) is either suppressed or very much smaller in talpid^3 embryos (Hinchcliffe, 1967).

VI. MECHANISMS OF CELL DEATH: THE ROLES OF LYSOSOMES AND OF MACROPHAGES

Embryologists have frequently attempted to account for cell death, but the question remains unresolved.

Glucksmann (1951) suggested that certain stimuli for the cell proliferation and for the differentiation of organs were active for limited periods only, and that, when these stimuli cease, cells fail to divide and complete their specialisation, and thus age and subsequently die. There is, however, no critical evidence for this theory.

More recently, the discovery of lysosomes has led to new approaches to the problem of the cause of cell death. It has been suggested that lysosomes may release, intracellularly, enzymes capable of killing and digesting the normal viable cells.

Lysosomes were first discovered in rat liver by de Duve, using differential centrifugation to determine the distribution of the enzymes involved in the metabolism of carbohydrates. The first of the enzymes to attract attention

was acid phosphatase. At first it was believed that the particles containing acid phosphatase were mitochondria, but later it was discovered that they formed a distinct group of cell particles different from both mitochondria and microsomes. By the aid of electron microscopy, de Duve et al (1955) identified the dense particles and gave the name of "lysosomes".

Lysosomes are defined as having the following characteristics:-

1 - diameter 0.4 - 0.5 μm.

2 - contain hydrolases with an acid pH optimum.

3 - possess enzyme latency, related to the limiting lipoprotein membrane, which retains the enzymes and resists the penetration of small molecules from outside.

4 - function as the intracellular digestive system of the cell.

Rat liver for a long time was the most common material used for study, both qualitative and quantitative, of the hydrolytic enzymes. Rat liver lysosomes are now known to contain at least twelve acid hydrolases, which together are capable of digesting all the major cell constituents.

The question which has been frequently raised, is whether all these enzymes are present together in all lysosomes, as claimed by de Duve (1963a), or whether there exist several kinds of lysosomes, differing qualitatively in their enzymic equipment.

An important characteristic of lysosomes is the single

lipoprotein membrane; enzymes can be released from lysosomes both *in vitro* and *in vivo*, by treating them with certain agents which appear to act by decreasing the stability of the lysosomal membrane. Amongst the factors having this effect are repeated freezing, detergents (Wattiaux *et al*, 1963); UV-irradiation, streptolysins, some steroids (de Duve, 1963 and Weissman *et al*, 1964) and excess vitamin A *in vitro* or *in vivo* (Dingle, 1961; Fell and Dingle, 1963; Weissman *et al* 1964).

By contrast, some other substances have been found to increase the stability of the lipoprotein membrane of lysosomes, thus slowing down the rate of release of enzymes. Such substances include chloroquinone, cortisone, cortisol (Weissman *et al*, 1964), cholesterol (de Duve *et al*, 1962).

Lysosomes and cell death

The role of acid hydrolases released from lysosomes, in embryonic cellular degeneration, is controversial. It has been suggested that if the lysosomal enzymes are released into the cytoplasm of the cell *in vivo* they will cause damage or even the death of the cell. This is the so-called "suicide bag" hypothesis (de Duve, 1959; 1963, and de Duve and Beaufay, 1959).

Zwilling (1964) supported this view. Jurand (1964) also implied that the acid phosphatase activity was closely associated with the cellular degeneration in the apical ectodermal ridge cells of the chick embryo.

However, more recent work has not supported this view. For example, Ballard and Holt (1968) studying the dead cells

within the interdigital zones of the foetal rat foot, found no increase in acid phosphatase activity of the degenerating cells prior to the onset of phagocytosis. Dawd and Hinchliffe (1971) reported that isolated dead cells, and dead cells recently ingested by macrophages, contained no more acid phosphatase activity, either discrete or diffuse, than either neighbouring living mesenchymal cells, or mesenchymal cells which had ingested 1-3 dead cells. Increased acid phosphatase activity was found, however, within the macrophages, where activity was localized within the digestive vacuoles containing the dead cells, and within the Golgi apparatus and Golgi vesicles.

Hammar and Mottet (1971) also found no lysosomal activity in the interdigital cells prior to phagocytosis of the dead cells. Once phagocytosis occurred, however, intracellular digestion appeared very active.

Pannese et al (1976), in their study of the lysosomes in normal and degenerating neuroblasts of the chick embryo spinal ganglia, reported that a diffuse distribution of acid phosphatase activity was only found in a limited number of cases during the terminal stage of the process. Their results indicated that lysosomes do not play a primary role in the degenerative process.

Most recently, Umansky (1982), in his study of the genetic programme of cell death, said it had been shown for many cases that degradation of the genetic material occurred in morphologically intact cells and was not the consequence

of activation of hydrolytic enzymes in dying cells.

Role of phagocytes in cell death

Whatever initiates cell death, the digestion of necrotic tissue is apparently completed by lysosomal enzymes, by one of two methods. The first is an autophagic process, in which cell components are digested by the enzymes of the same cell within autophagic vacuoles, which shut off the hydrolases from the rest of the cell and from neighbouring cells. The second method is by a phagocytic process in which the degenerating cell or cells are ingested by a phagocyte within which the digestion of dead cells is carried out by the numerous lysosomes of the phagocyte. The dead cells, which are in digestive vacuoles with a high hydrolytic enzyme activity, are found at different stages of digestion.

Ballard (1965), Ballard and Holt (1968), and Dawd and Hinchliffe (1971), using both light and electron microscopy, identified isolated dead cells which showed the same number of lysosomes and the same distribution of enzyme activity as normal viable mesenchymal cells. Then the isolated dead cell was engulfed within the pseudopodia-like projections of a neighbouring viable mesenchymal cell. At this stage the engulfing mesenchymal cell did not show any changes, but soon after ingesting the dead cell, the living mesenchymal cell became enlarged, its nucleus and cytoplasm more intensively stained and its nucleus sometimes distorted by the ingested dead cells. An increase in the number of lysosomes and the association of hydrolytic enzymes with the ingested dead cell or cells was observed at this stage within the mesenchymal

cell, which was now regarded as showing all the morphological features of a macrophage.

According to the differences in hydrolytic enzyme activity and distribution and the stages of digestion, Ballard (1968) studied cell death and digestion in foetal rat foot, and he classified the phagocytes into three stages:

Stage 1: phagocyte with least enzyme activity containing large pyknotic nuclei showing little sign of digestion.

Stage 2: phagocyte showing intermediate enzyme activity and containing mixed types of dead cells, some in early , and others in later stages of digestion.

Stage 3: phagocyte with most enzyme activity containing dead cells showing the most pronounced signs of digestion.

The digestion of dead cells by macrophages which have differentiated directly in situ from mesenchymal cells, has also been described by Hammar and Mottet (1971). Hinchliffe and Ede (1973) and Mitrovic (1977; 1978) all working on various aspects of limb development.

This phenomenon, the phagocytosis of dying cells by neighbouring cells, which are not macrophages in the strict sense, has also been described in other situations:-

(i) Schluter (1973) found engulfment of dead cells, in the closing neural tube, by neighbouring neuroepithelial cells.

(ii) Garcia-Porrero et al (1979) described phagocytosis of dead ectodermal epithelial cells by their neighbours, in the process of invagination which leads to lens vesicle formation.

(iii) Hurle et al (1977) found that, in the developing heart, apoptotic myocardial cells are ingested by their healthy myocardial cell neighbours.

(iv) in involution of hormone dependent epithelia e.g. breast and prostate, dying cells are ingested by their normal neighbours.

Saunders (1966) and Saunders and Fallon (1966) reported that dead cells of the PNZ undergo digestion in macrophages, but they provided no evidence to show whether these macrophages had differentiated in situ, from mesenchymal cells, or had migrated into the tissue.

VII. SUMMARY

What general conclusions emerge from this survey of cell death?

1) That physiological or programmed cell death (apoptosis) can be distinguished from necrosis (caused by external environmental change).

2) That apoptosis is widespread in embryonic development, and plays an essential part in the shaping of organs and in the removal of redundant, transient, structures.

3) That apoptosis also occurs postnatally, contributing to the continued remodelling of structure during growth, and to processes of tissue atrophy (e.g. of muscle after denervation) and of involution (e.g. of endocrine dependent epithelia.

4) That in apoptosis, the first changes to be seen are in the nucleus, followed by changes in cytoplasmic organelles. Cell death is <u>not</u> initiated by endogenous lysosomal activity (the "suicide bag hypothesis").

5) Dead and dying cells are ingested by phagocytes, which are often normal neighbouring cells, and not conventional macrophages. Digestion of cell remnants is lysosomal.

6) Apoptosis is preceded by evidence of nuclear (genetic) activity, and its immediate cause seems to be genetically determined, although external triggers have been identified in some cases, and may yet be found in others.

IV. DEVELOPMENT OF THE PATELLA AND THE FEMOROPATELLAR JOINT

There is disagreement in some of the published papers about both these topics.

1) The development of the patella

The main question is this:

"Is the patellar primordium independent of the quadriceps tendon, or is it an intra-tendinous sesamoid?"

A. MAMMALIAN SYNOVIAL JOINTS

Some authors, e.g. Andersen (1961), Schneck (1965), and Doskocil (1985), claimed that the patellar condensation was formed independently to the quadriceps tendon, and that a secondary invasion of the tendon took place later. Other workers have considered that the patella develops initially within the tendon, e.g. Bardeen (1905), Walmsley (1940) and Haines (1947).

Gray and Gardner (1950) studied the human knee joint and found that the patellar condensation appeared in front of the femur at 7.5 weeks and that it was separated from the femur, by a loose mesenchymal tissue, from the outset. The patellar condensation became precartilaginous at 8.5 weeks and cartilaginous at 10 weeks. They did not give any explanation about the relation between the patella and the quadriceps tendon.

Andersen (1961) also studied the human knee joint, and found that the patellar primordium originated in the blastema behind the quadriceps tendon with a sharp distinction in nuclear morphology between the patellar cells and the cells

of the quadriceps tendon, which were elongated with long spindle-shaped nuclei. He also found that the patellar primordium was separated from the femur by a typical three-layered interzone. He suggested that secondary invasion of the quadriceps tendon took place later.

Schneck (1965) reached the same conclusion in the rabbit. He found that the developing patella consisted at first of cells of the prechondral variety, which exhibited no definite evidence of lacunae, and that the dense cellular condensation of the quadriceps tendon and patellar ligament lay on its anterior surface. Posteriorly the patella was separated from the femur by a typical layered interzone.

More recently, Doskocil (1985), in his study of the formation of the femoropatellar part of the human knee joint, 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, from which it separated at the early prochondral blastema stage. The anlage of the quadriceps tendon formed in front of the anlage of the patella. The tendon of the rectus femoris lay in front of the patella, the tendons of the vastus medialis and lateralis inserted in connective tissue discs on either side of the patella, the vastus intermedius inserted in the upper edge of the patella. In view of these findings, he claimed that the patella is not a sesamoid bone.

In contrast to all these findings, several workers (e.g. Bardeen, 1905; Walmsley, 1940; Haines, 1947) have considered

that the patella develops in the quadriceps tendon.

Bardeen (1905) found that the human patella first formed in the quadriceps tendon, in embryos of 17 mm. Walmsley (1940) studied the development of the human patella, and found that a precartilaginous patella was present in the deeper part of the quadriceps tendon at the level of the lower end of the femur, at 20 mm stage. The quadriceps tendon was separated from the femur by a layer of loose tissue which was continuous below with loose mesenchymal tissue. An articular disc was secondarily formed, at the 35-40 mm stage, between the patella and the femoral condyles by the fusion of the patellar and femoral perichondria.

Haines (1947) also found that the patella was formed in the quadriceps tendon, and separated from the femur by a broad layer of loose tissue.

Patterson (1945) mentioned in his abstract that the mammalian digital sesamoids develop within the articular capsule and their association with the tendon is a secondary one. Gardner, Gray and O'Rahilly (1959) reached a similar conclusion about the origin of the digital sesamoids in human embryos.

B. AVIAN SYNOVIAL JOINTS

Niven (1933) studied the development of the avian patella *in vivo* and *in vitro*, and found that the patella was formed by a 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 day,

and it was surrounded by a perichondrium at the end of the 12th day. In cultures of the whole patellar mesenchyme, the cartilage appeared in the explants in all cases, and it resembled that seen in the normal development. 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. Articular surfaces did not appear in the cultures even after 7 weeks cultivation.

O'Rahilly and Gardner (1956) found that the anlage of quadriceps tendon first appeared in the chick at stage 28, while the patellar anlage appeared later, at stages 29-30, as a densely cellular condensation deep to the expanded quadriceps insertion. They also found that, from its first appearance, the patella was separated from the femur by loose vascular tissue. At stage 36, the patella showed early cartilage and had a perichondrium. They suggested that the patella is a peri-articular rather than an intratendinous sesamoid.

2) The development of the femoropatellar cavity

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; and Gray and Gardner, 1950).

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

Andersen, 1961; Schneck, 1965).

Haines (1947) found that development of the femoro-patellar joint differed from that of more typical joints in that no dense interzone was formed at any stage, so that the loose tissue which separated the patella from femur persisted as such from an early stage of development. The same conclusion was reached by Gray and Gardner (1950), who found that the patella was separated from the femur by loose mesenchymal tissue from the outset until cavity formation began, and the joint did not form in blastemal tissue nor did a typical interzone arise. The cavity was first seen in the 10 weeks embryo, extending for a short distance proximal to the patella, and at 14 weeks the suprapatellar recess reached the level of the transition of the femoral perichondrium to periosteum.

Doskocil (1985) also found that the patella was separated from the femur by loose connective tissue, and that expansion of the extracellular spaces and destruction of the cells between the patella and femur gave rise to spaces which were the anlage of the future joint cavity of the femoropatellar articulation. The femoropatellar cavity was the first cavity to develop in the knee joint, and from the outset it formed a single entity with the suprapatellar bursae.

Walmsley (1940) recognized a typical articular disc (interzone), which formed secondarily between the patellar and femoral perichondria. The patello-femoral synovial cavity developed in the articular disc by "liquefaction" in a

manner which he regarded as typical of the diarthroses. Haines (1947) disagreed with Walmsley and regarded such fusion of the two cartilages as an artifact.

Andersen (1961) found that the femoro-patellar cavity formed in the blastemal interzone between the femur and patella and passed through a three-layered stage. He found that cavity formation was preceded by the formation of large quantities of chondroitin sulphates A and C in the three layers of the interzone. Schneck (1965) also found that the patella was separated from the femur by a typical layered interzone, and that the cavity was formed by loosening of the interzone cells.

B. AVIAN SYNOVIAL JOINTS

Niven (1933) found that in the early stages of development. the anterior and posterior surfaces of the patellar rudiment were convex but during the 16th day of incubation two concavities developed on the posterior surface for articulation with the condyles of the femur. He did not explain how the cavity was formed.

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

V. THE CHICK AND HUMAN KNEE JOINTS COMPARED

The chick knee joint shows two main differences from the human knee joint:

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.

Table (2), adapted from Gardner and O'Rahilly (1968) and O'Rahilly and Gardner (1956), summarises the sequence of events in development of the human knee joint, using the criteria for staging devised by Streeter (1951) and compares the sequence with that for the chick, according to criteria for staging devised by Hamburger and Hamilton (1951).

In interpreting this Table, it must be stressed that it is the sequence of developmental events which is being compared: the stage numbers have no absolute value for interspecific comparisons, since they are based on the external features and are particular to each species. Attention is directed to the following points, marked by asterisks on the Table:-

* Embryonic movements, in both species, begin after the main outlines of the joint have been sketched out, but before the first appearance of cavitation.

** Gardner and O'Rahilly (1956) did not find a typical three-layered interzone in the developing chick knee joint.

*** In the chick, chondrification of the patella begins much

later, relative to other developmental events, than it does in man; it appears at H.H. stage 36, later than the time of periosteal ossification in the femur, tibia and fibula, and after cavitation has begun.

Table 2 . Comparative sequential development of human knee joint and that of chick embryo.

(The data relating to Gallus domesticus are from O'Rahilly and Gardner, 1956).

(The data relating to human are from Gardner and O'Rahilly, 1968).

Feature	Homo stage	Gallus stage
Lower limb bud	13	17
Ectodermal thickening and ridge	13-18	18-30
Skeletal chondrification	18	27
Femorofibular proximity	18-19	27
Quadriceps insertion	18-19	27
Tibialis anterior in knee	-	27
Ambiens in knee	-	29
Homogeneous interzone	19-20	26
Patellar condensation	19-20	29-30
Intracapsular ligaments, cruciate and menisci	19-20	30
Embryonic movements	20	early 30s*
Three layered interzone	21	? **
Chondrification in patella	21-22	36 ***
Diaphyseal bone collars	22-23	28-29
Cavitation	23	34 +

Homo Stage, according to Streeter (1951).

Gallus Stage, according to Hamburger and Hamilton (1951).

SECTION 2 - MATERIALS AND METHODS

MATERIALS AND METHODS

Chick embryos were obtained by incubating fertile eggs from a commercial source (Ross Poultry, Aberdeen) in a "forced draught" type incubator (Westernette) which provided hourly rotation of the eggs and maintained a humid atmosphere at 38°C.

Embryos were staged, according to Hamburger and Hamilton (1951). In this study, a total of 117 embryos was used, arranged from stage 27 to stage 40.

Both lower limbs were removed. In the younger specimens, the entire limb was processed, while in the older ones, the knee joints were isolated by cutting through the thigh at a level between the upper 2/3 and lower 1/3 of the femur, and through the leg between the upper 1/3 and lower 2/3 of the tibia and fibula.

In most cases one knee joint from each embryo was studied, but in some, both were examined.

The following methods were used:-

I. PARAFFIN WAX HISTOLOGY

A total of 68 knee joints was studied by serial wax histology. Procedures:- A. H & E, PAS, and selective stain for mitotic figures.

1. Fixation:- embryos were fixed directly in Bouin's fixative, (for H & E and PAS stains), or modified Bouin's fixative (for mitotic figure stain), for 24-48 hours.
2. They were then placed in 70% ethanol for 1-2 hours.

3. Staged, using a dissecting microscope.

4. Both 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.

5. The limbs, or the knee joints were dehydrated through an ascending series of ethanols, then cleared in amyl-acetate, using an automatic tissue processor and embedded in paraffin wax, lateral side downwards.

6. Serial sagittal sections were cut on a Jung microtome at a thickness of 5-7 μm, stretched in a water bath and mounted on albuminised glass slides (serially for H & E, interrupted series for PAS stain and for mitotic figures), dried for 24 hrs, in an oven at 37 ^{o}C.

7.Slides were dewaxed in xylene, hydrated, and then stained with one of the following stains:-

a - Haematoxylin and eosin:- a total of 42 knee joints was stained.

b - PAS, using H & E as a counterstain:- a total of 10 knee joints was stained.

c - A selective stain for mitotic figures (Frazer, 1982):- a total of 5 knee joints was stained.

8. Slides were dehydrated, cleared in xylene and mounted in Histomount. [For details of the procedures, see Appendix 1].

B. Acid phosphatase:- a total of 11 knee joints was stained.

1. Embryos were fixed in formal/Ca fixative for 24-48 hours,

and staged.

2. Both knee joints were isolated, and decalcified in 10% buffered EDTA at 0 °C, changed each day for 2-4 days.

3. Dehydration, clearing and embedding:

50% acetone	2 hrs.
3 changes of absolute acetone	2 hrs. each.
acetone/wax	2 hrs.
1st wax	2 hrs.
2nd wax	3-4 hrs.

and embedded in wax, lateral side downwards.

4. Serial sagittal sections were cut on a Jung microtome at a thickness of 5-7 μm. An interrupted series was stretched in a water bath, mounted on albuminised glass slides, dried for 24 hrs, in an oven at 37 °C, dewaxed in xylene, hydrated through decreasing concentrations of acetone to water and stained by the Azo-dye method (Barka and Anderson, 1962). [Details of the procedure are given in Appendix 1].

II. SEMI-THIN HISTOLOGY

A total of 26 knee joints was examined.

Procedure:-

1. Embryos were fixed by immersion in cold 5% glutaraldehyde in Millonig's buffer (pH 7.4) for 24-48 hours.

2. They were then placed in the same buffer for a further 24-48 hours, and staged under a dissecting microscope.

3. Both lower limbs were removed, the knee joints isolated, and post fixed in 1% osmium tetroxide for 1.5-2 hours.

4. The knee joint specimens were dehydrated through a graded series of acetone. Acetone was used rather than the more usual ethanol series because it had been shown to give better results in previous projects involving chick limbs.

5. Embedded in Spurr's resin, lateral side downwards.

6. Serial sections were then cut on a Reichert-Jung Autocut (Mod. 1140) ultramicrotome at a thickness of 1.2-1.5 um, using 6 mm wide glass knives of the Latta-Hartman type. Most of the knee joints were cut in the sagittal plane.

7. Sections were mounted on glass slides and left to dry. They were then placed in a solution of saturated sodium hydroxide in absolute ethanol for 15-20 minutes.

8. Staining:-

Sections from each specimen were stained by one or other of the following:

a) Haematoxylin and Eosin.

b) Periodic acid Schiff (PAS):-

As a control for the success of the staining procedure,

a piece of rat liver was processed in identical fashion to the knee joint.

c) Azur blue II stain.

Some specimens were stained by Azur blue II only. [Details of the staining procedures are shown in Appendix II].

III. TRANSMISSION ELECTRON MICROSCOPY

A total of 21 knee joints at various stages (27-40) was examined.

This method was used principally to study the detailed cytological events which occurred during cell death in the interzone.

Procedure:-

1. Embryos were fixed by immersion in cold 5% glutaraldehyde in Millonig's buffer (pH 7.4) for 24-48 hours.
2. They were then placed in the same buffer for a further 24 hours, and staged.
3. Both lower limbs were removed, and the knee joints isolated.
4. The knee joint specimens were post-fixed in osmium tetroxide buffered with phosphate (pH 7.4) for 1.5-2 hours, dehydrated through a graded series of acetone, and embedded in Spurr's resin, lateral side downwards.
5. After the desired area for study had been determined from semi-thin sections, the block was trimmed with a razor blade and sections were cut at 60-80 nanometers, using a Reichert-Jung Ultra-cut microtome and a diamond knife.
6. Sections were mounted on to uncoated 200 mesh copper grids and stained with uranyl acetate and lead citrate (Reynolds, 1963). Sections were then examined by TEM on a Jeol TEM 100S.

[For details of the staining procedure, see Appendix III].

IV. SCANNING ELECTRON MICROSCOPY

23 knee joint specimens at various stages (30-40) of development were studied.

Procedure:-

1. Embryos were fixed intact in 5% glutaraldehyde in Millonig's buffer (pH 7.4) for 24-48 hours.

2. They were then placed in the same buffer for a further 24-48 hours and staged.

3. Both lower limbs were removed, the knee joints isolated, dehydrated in a graded series of ethanols and embedded in paraffin wax, lateral side downwards.

4. The blocks were cut on a Jung microtome at a thickness of 5-7 μm. At intervals, a section was mounted on to a glass slide and examined unstained under the light microscope. The process was repeated until the required area was reached, when the section was mounted, left to dry, then dewaxed in xylene and stained with H & E.

5. The remainder of the block was then placed in an oven at 60 °C to melt the surrounding wax. The specimens were then placed in xylene for 2-3 days, in a 37 °C oven. The specimen was transferred to fresh xylene frequently to ensure complete removal of wax.

6. Each specimen was then transferred to a clean bottle of absolute ethanol for 4 hours and critical point dried, using ethanol as a transition fluid. Each specimen was placed in a membrane basket to minimise damage.

7. Dried specimens were examined under a dissecting

microscope to determine the cut surface, then each specimen was mounted on to an aluminium stub, using double-sided Sellotape or conductive-silver paint, with the cut surface uppermost.

8. The specimen was then sputter-coated with gold, using a Polaron sputter-coater, and the specimen was then examined using a Jeol T300 scanning microscope.

[For further details see Appendix IV].

SECTION 3 - RESULTS

RESULTS

As a preliminary to an account of the embryonic development, a description of the main features of the internal structure of the joint, based on a number of personal dissections of late fetuses, is included.

As shown in Figure 1, there are two major differences between the chick and human knee joint:

1) The head of the fibula articulates with a groove on the lateral condyle of femur and the lateral meniscus shares, in part, in the articulation between them.

2) The tibialis anterior tendon, which arises from the anterior surface of the lateral condyle of femur, is an intra-articular structure.

The developmental events in the normal chick embryo knee joint have been studied in a total of 115 embryos, of stages 27-40.

I. SURVEY OF DEVELOPMENT OF VARIOUS CONSTITUENTS OF THE KNEE JOINT.

In this section, description is deliberately restricted to an account of the general morphogenesis of the joint. As will emerge later, a major factor contributing to the internal shaping of the cavity of the joint and of intra-articular structures is the phenomenon of cell death. The role of cell death in the histogenesis of the joint is dealt with separately in Section II.

Stage 27 (5 - 5½ days)

At this stage, the distal end of the femoral blastema was expanded indicating the future form of the femoral condyles (Fig. 2). The femoral blastema was bounded by a layer of closely packed cells, somewhat elongated in a direction tangential to the blastemal surface, and with basophilic nuclei. The cells in the enlarged distal end of the blastema showed relatively darkly basophilic nuclei, contained scant cytoplasm and showed a fair amount of non-staining matrix between the cells (Fig. 3). By contrast, the cells in the shaft portion of the blastema showed early chondrogenesis: the cells had larger pale staining nuclei with somewhat larger amounts of eosinophilic cytoplasm and were more closely packed. The tibia and fibula showed many layers of deeply basophilic cells around their shafts, and, within the shafts, large cells with pale nuclei and eosinophilic cytoplasm. The cells of the distal end of the femoral blastema and the proximal ends of the tibial and fibular blastemata merged insensibly into the interzone region which appeared as an area of continuity between the three blastemata (Fig. 2). The dense marginal layer of cells (future perichondrium) around the tibial and fibular blastema was continuous across the periphery of the interzone with that around the femur. At this stage, therefore the site of the future knee joint was seen as a dense cellular, avascular interzone, whose cells were uniformly distributed, without any special orientation. There was occasional mitotic activity present throughout the interzone and at the

articular ends of the pre-cartilaginous models. The mitotic figures showed no specific localization. There were also a few darkly stained cells scattered in the interzone, located in the area bounded by the femoral, tibial and fibular blastema. Most of these "dark" stained cells seemed to have been engulfed by macrophages (Fig. 3). There was no indication of the differentiation of future intra-articular structures, such as the menisci or cruciate ligaments. There were early signs of the differentiation of the major muscle groups, and the quadriceps femoris condensation was recognized in front of the femoral blastema, and separated from it by loose and vascular mesenchymal tissue (Fig. 2). A number of blood vessels extended between the tibial and fibular shafts.

Stage 28 ($5\frac{1}{2}$ - 6 days)

The shafts of the femur, tibia and fibula were cartilaginous. Femoral and tibial condyles were recognizable, but the tibial tubercle was not prominent. The distal end of the femur, and the proximal ends of the tibia and fibula showed large cells with pale nuclei and a moderate amount of eosinophilic cytoplasm, indicating the differentiation of precartilage at these sites. There was no sharp demarcation between the cartilaginous and precartilaginous parts of the three skeletal blastemas. Most of the cells of the interzone were orientated transversely to the long axis of the limb, especially along the future articular surfaces of the cartilage models, forming the

chondrogenous layers, which were continuous with the differentiating perichondrium at the periphery of the interzone (Fig. 4). The perichondrium was clearly visible around the shafts; it stained densely with Azur blue II, and the chondroblastic and fibroblastic layers were distinguishable (Fig. 5). Occasional mitotic activity was noticed throughout the interzone, especially in its chondrogenous layers, and at the distal end of femur and proximal ends of tibia and fibula. In a few sections, occasional "dark" cells were seen scattered in the interzone (Fig. 6). A condensation of cells extending from the anterior part of the lateral femoral condyle to the tibia probably represented the primordium of one of the cruciate ligaments (Fig. 4). At each end of this ligament, its elongated cells passed into continuity with those of the chondrogenous layer of the interzone. The cells of this cruciate ligament primordium showed mitotic activity, and seemed to have developed *in situ*, from the mesenchyme of the interzone. No definite condensations indicating the menisci or the tibialis anterior tendon were seen. There was a condensation of closely packed elongated cells, extending between the quadriceps femoris tendon and the tibia, which indicated the position of the future patellar ligament (Fig. 5).

The mesenchymal tissue deep to the quadriceps femoris muscle was loose and vascular, and no definite patellar anlage was seen at this stage. Small blood vessels were present near the peripheral parts of the interzone (Fig. 4).

Stage 29 (6 - 6½ days)

Appearances were, in general, similar to those at stage 28, with slight progress in development. The form of the femoral and tibial condyles was well established, but the tibial tubercle was not prominent. The shafts of the femur, tibia and fibula were cartilaginous and the chondrocytes were beginning to undergo hypertrophy, prior to endochondral ossification. The distal end of the femur and the proximal ends of the tibia and fibula were still precartilaginous. Towards their free surface, their cells became increasingly elongated in a direction tangential to the surface, and gradually merged with the chondrogenous layer of the interzone, where the cells were still more closely packed and elongated. There was no sharp dividing line between the precartilaginous end of the cartilage model and the chondrogenous layer of the interzone. Occasional mitotic activity was noticed, and the mitotic figures were scattered throughout the interzone, chondrogenic layers and the articular surfaces of the cartilage models. No dark stained cells were seen in the interzone. The interzone was devoid of any stainable material with PAS stain, while in the cartilaginous elements of the femur, tibia and fibula, the intercellular material was faintly stained by PAS. A marginal condensation of cells continuous above and below with the femoral and fibular perichondria, indicated the posterior part of the fibrous capsule of the knee joint (Fig. 7), while the anterior part was formed by the patellar

ligament. This capsule separated the mesenchymal tissue at the periphery of the interzone from the surrounding mesenchyme. The femoral inter-condylar fossa showed discrete condensations of cells which clearly indicated the location and direction of the cruciate ligaments. There were no definite condensations to indicate the menisci or the tibialis anterior tendon. The tissue deep to the quadriceps femoris muscle and tendon was still vascular and loose, and no definite patellar anlage was seen. Blood vessels were seen in the surrounding mesenchyme, adjacent to the periphery of the interzone (Fig. 8).

Stage 30 (6½ days)

The tibial tubercle was prominent and its cells were arranged in elliptical rows at its junction with the shaft. The femoral blastema was still not fully chondrified. Cartilage had differentiated leaving a narrow transitional zone which intervened between cartilage itself and the chondrogenous layer of the interzone. In this transitional zone the cells became increasingly flattened in a direction tangential to the surface, and became more condensed. This transition zone gradually merged with the chondrogenous layer of the interzone, where the cells were even more closely packed and flattened. The chondrogenous zone of each of the cartilage models showed occasional mitoses which were also seen scattered throughout the interzone. The interzone showed no material stainable with PAS. In the perichondrium around the mid-portions of the shafts of the femur, tibia and fibula, the first signs of periosteal ossification were seen.

The interzone showed a condensation for the lateral meniscus (Fig. 9). In the central area of the meniscus, the cells had round to oval nuclei, while at its periphery they had flattened nuclei and were continuous with those of the chondrogenic layers of the blastemata. The meniscus seemed to have developed *in situ* from the mesenchyme of the interzone. Condensations were seen, for the first time, forming the primordia of the posterior menisco-femoral and posterior menisco-tibial ligaments. A bifurcated condensation was seen, one limb representing the posterior menisco-femoral ligament, the other the posterior menisco-tibial ligament. The posterior cruciate ligament crossed medially to the angle of divergence between these two limbs, on its way to attachment to the posterior intercondylar area of the tibia (Fig. 10). Another condensation of cells extending from the anterior surface of the lateral condyle of femur downward and between the tibia and fibula, represented the primordium of the tibialis anterior tendon (Fig. 11). At its attachment to the lateral condyle of femur, its elongated cells passed into continuity with those of the chondrogenous layer of the condyle. The tendon of the ambiens muscle was also seen immediately in front of the site of origin of the tibialis anterior tendon from the lateral condyle of femur. The tendon of ambiens muscle crossed the front of the patellar condensation, and, running downwards, traversed the ligamentum patellae, from medial to lateral. The mesenchymal tissue deep to the quadriceps tendon was still vascular and

loose, apart from the site of the patellar primordium, where it was densely cellular and less vascular medially (Fig. 12). The intercondylar fossa was well vascularized and some of these primitive vessels were associated with the anterior cruciate ligament near its attachment to the lateral femoral condyle (Fig. 13). Blood vessels were seen posteriorly, closely related to the interzone. A very few blood cells were seen in one section only, within the interzone itself (Fig. 13). Careful study, focussing up and down, showed that these were not in blood vessels, but had probably been extravasated from adjacent blood vessels.

Stage 31 (7 days)

The articular ends of the femur, tibia and fibula were similar to those found at stage 30, and 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 femur and fibula. The collar of periosteal bone was well established around the middle of the shafts of the femur, tibia and fibula (Fig. 14). Part of the lateral meniscus appeared between the lateral condyle of femur and fibula. Occasional mitotic figures were seen scattered throughout the menisci, cruciate ligaments, and the chondrogenic layers of the interzone. A condensation of cells extending between the head of fibula and the tibia, represented the interosseous tibio-fibular ligament (Fig. 15). At the ends of this tibio-fibular ligament, its elongated cells passed into continuity with those of the

chondrogenous layer of the interzone. One specimen showed what might be interpreted as the first sign of a small cavity between the lateral condyle of the femur and the lateral meniscus. It is difficult to be absolutely sure that this was indeed the first sign of cavitation and not an artifact. However the edges of this "cavity" showed several "dark" cells, which at later stages were regularly found within, and adjacent to, areas of cavitation (Fig. 16). No PAS stainable material was seen in the interzone, while the cartilaginous elements of the femur, tibia and fibula showed faint staining of the intercellular material.

The patellar condensation had become more dense and was differentiating to precartilage. Some mitoses were seen in the condensation. On the anterior surface of the patellar condensation there was a dense cellular condensation, forerunner of the quadriceps tendon and patellar ligament. Posteriorly the patellar primordium was separated from the femur by loose vascular mesenchyme which, like the femoro-tibio-fibular interzone, showed no material stainable with PAS.

As at the previous stage, blood vessels were seen in the intercondylar fossa and at the periphery of the interzone (Figs. 16, 17).

Stage 32 (7 days)

At this stage, the central part of the diaphyses of the femur and tibia showed further subperiosteal bone formation, while the fibula still showed only a thin collar of

periosteal bone.

It was at this stage that the first unequivocal evidence was seen of the appearance of small cavities in the interzone. Localized cavitation was seen between the lateral condyle of the femur and the lateral meniscus (Fig. 18) and between the medial condyle of femur and the medial meniscus (Fig. 19). Elsewhere the interzone was still homogeneous, especially that between femur and fibula. Associated with both cavities were small numbers of "dark" cells; neither cavity showed any PAS positive material. One specimen showed loosening of the interzonal tissue between the lateral condyle of femur and the fibula (Fig. 20). Intra-articular structures showed some progress in their development: the closely packed cells of the cruciate ligaments were elongated in the long axis of the ligaments. The meniscus had started to separate from the articular surface of the femur at the site where cavitation had begun. The patellar condensation was still separated from the femur by loose and vascular tissue, and blood vessels were still closely related to the peripheral parts of the interzone. One specimen showed prominent blood vessels localised in the inter-condylar notch of the femur posteriorly, and penetrating the superficial layer of the perichondrium (Fig. 19). Some blood vessels were seen on the superficial posterior surface of the medial meniscus (Fig. 19).

Stage 33 ($7\frac{1}{2}$ - 8 days)

The femur, tibia and fibula showed further thickening of

the periosteal collar. The articular surfaces of the bones were covered by a layer of densely packed cells, tangentially arranged. The interzone tissue between the lateral condyle of femur and fibula showed further loosening, so that a typical three-layered interzone became distinguishable (Fig. 21). Localized cavity formation was seen at a number of sites (see e.g. Fig. 22). All the visible cavities were lined by "dark" cells and none contained any PAS stainable material.

Fine collagenous fibres, stained dark blue with Azur II and pinkish with PAS, were detected in the tendon of tibialis anterior and in the cruciate ligaments. The patellar condensation was still precartilaginous and was separated from the femur by very loose and vascular tissue. Blood vessels were still confined to the peripheral parts of the interzone, penetrating the periphery of the menisci and the superficial layers of the cruciate ligaments near to their bony attachments. Prominent blood vessels in the posterior aspect of the femoral intercondylar notch penetrated the superficial layer of its perichondrium.

Stage 34 (8 days)

Appearances were, in general, similar to those at stage 33, but there was further progress in the development of the joint cavity. The superficial zone of cells on the femoral, tibial and fibular articular surfaces maintained their tangential orientation. The mesenchymal interzone localized between the lateral condyle of femur and fibula had become very loose and one specimen showed a very small cavity in

this situation (Fig. 23). Most of the specimens showed cavity formation in various parts of the femoro-meniscal and tibio-meniscal regions (the sites of cavitation are summarized in Table 3). All the cavities were lined by "dark" cells, and contained remnants of loose tissue (Fig. 24). No PAS stainable material had accumulated inside the cavity. (Fig. 25). Due to loosening of the mesenchymal interzone tissue and cavity formation, the menisci were now clearly defined as wedge-shaped sectional profiles. The mesenchymal tissue surrounding the cruciate ligaments and the tibialis anterior tendon had become very loose, and in one specimen a cavity had appeared along the posterior surface of the tibialis anterior tendon. This cavity was bordered by a layer of "dark" cells (Fig. 26).

The patella was more defined but was still neither surrounded by a perichondrium nor fully chondrified. The tissue between the patella and femur was very loose and vascular, but there was still no definite cavity. The site of the future supra-patellar recess was indicated by loose vascular mesenchyme. The cruciate ligaments showed a capillary plexus in their superficial layers, adjacent to their bony attachments, and the menisci also showed a capillary plexus in their peripheral parts (Fig. 23). The blood vessels in the intercondylar notch of the femur had penetrated most of the thickness of the perichondrium.

Stage 35 (8 - 9 days)

At this stage, the knee joint looked in most respect

like a miniature replica of the adult joint. The chondrogenous zone was further reduced in thickness to 2 or 3 layers of cells, which maintained their tangential orientation. Deep to them, the cells were arranged more randomly. In one specimen the cells deep to the chondrogenous zone of the femoral condyles were arranged in columns perpendicular to the surface (Fig. 27).

Almost all embryos showed cavities at all articular sites except at the femoro-patellar region. Small cavities were present between the lateral meniscus and the lateral condyle of femur above, and the fibula below (Fig. 28) between the anterior cruciate ligament and the lateral tibial condyle (Fig. 30) and along the posterior surface of the tibialis anterior tendon (Fig. 31). All these cavities were bordered by lines of dark cells. When traced in serial sections, the lateral meniscus was seen to form a complete disc, while the medial meniscus was a C-shaped structure. Tangentially to the posterior, superior, and anterior surfaces of the patella was a cellular condensation indicating the primordium of its perichondrium (Fig. 32). The patella was still separated from the femur by a very loose and vascular strip of tissue, continuous above with similar tissue occupying the site of the future supra-patellar recess (Figs. 32, 33). Blood vessels in the posterior aspect of the inter-condylar notch of the femur had now penetrated the condylar cartilage and lay within cartilage canals (Figs. 30, 32). Small blood vessels penetrated the perichondrium of the posterior aspect of the

tibial condyle.

Stage 36 (10 days)

At this stage, cavitation was completed at all aspects of the knee joint, including the femoro-patellar joint. 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. The small cavities which had appeared in various sites in the joint had extended, and coalesced to form a single more or less continuous cavity. However the cavity between the lateral condyle of femur and fibula, although well formed (Fig. 34), appeared in most specimens to be still separate from the general cavity. Most parts of the knee joint cavity were lined by "dark" cells, and contained tissue remnants. Cavitation was also well advanced between the intra-articular ligaments which were therefore much more clearly defined, especially the menisco-tibial and menisco-femoral ligaments (Fig. 35). The menisci showed chondrogenic cells in their central parts, and fine collagenous fibres appeared in their superficial layers. The gastrocnemius muscle appeared to take the place of the capsule posteriorly (Fig. 36). 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 characterized by the presence of numerous blood vessels, indicating the primitive

synovial tissue or "synovial mesenchyme". An early synovial lining was detected in some places as shown in Figure 37. The synovial lining was formed from cells of variable shape, most of them were flattened, but others oval or rounded in shape. A very small amount of metachromatic material appeared in sections stained by Azur blue II in the peripheral part of the joint closely related to the synovial lining. This material is interpreted as precipitated synovial fluid. The patella showed chondrification centrally; its anterior, superior and posterior boundaries were well defined by perichondrium while its inferior border was still not sharply demarcated from the infra-patellar tissue (Fig. 36). An important new development was the appearance of the femoro-patellar joint cavity, continuous above with loose and vascular tissue of the supra-patellar area, and inferiorly with the main joint cavity, but separated from it by tissue strands (Fig. 36). The cartilage canals in the distal end of the femur had become more prominent and penetrated further into the condylar cartilage than at the previous stage. Blood vessels penetrated the posterior aspect of the medial condyle of the tibia (Figs. 35, 36).

Stage 37 - (11 days)

At this stage, the joint cavity was almost a continuous space. The articular surfaces were covered by two layers of flattened cells, arranged tangentially to the surface, while the deeper cells were arranged in columns.

The articular surfaces appeared more condensed than at the previous stage, due to compaction of the cells and the appearance of the collagen fibres between them. The shafts of the femur and tibia showed radiating bony trabeculae, while the fibula still showed a periosteal collar bone. One specimen showed the cavity between the lateral condyle of femur, fibula and lateral meniscus, still lined by dark stained cells. The central part of the menisci showed chondrocytes in lacunae (Fig. 38). The joint cavity showed a synovial lining in certain areas, and a capillary network lay immediately beneath the synovial lining cells. Some areas showed synovial folds projecting into the cavity. The patella was more defined, and chondrification was advanced. The femoro-patellar cavity, still crossed by tissue strands, showed a slight extension superiorly toward the supra-patellar connective tissue which continued to loosen (Fig. 39). The distal end of the femur showed more further advance of cartilage canals which extend from the posterior aspect of the inter-condylar fossa, but still did not reach the articular cartilage zone. Blood vessels penetrated the medial condyle of the tibia from various directions.

Stage 38 (12 days)

The main changes were:

i. there was an increased vascularity of the synovium, with

many vessels lying immediately deep to the synovial lining cells (Fig. 40).

ii. the femoro-patellar cavity now extended superiorly to form the suprapatellar recess.

iii. cartilage canals were more numerous in the distal end of the femur (Fig. 41) and the proximal end of the tibia.

Stage 39 (13 days)

Appearances were similar to those at stage 38. The articular cartilage surfaces showed more fine collagenous fibres. The joint cavity was well formed, and the lining of synovial tissue showed more synovial villi (Fig. 43). One specimen showed obliteration of the cavity in some aspects due to contact between the articular surfaces (Fig. 42). This contact is post-cavitation, most probably due to static posture of the limb of the embryo at that time.

Stage 40 (14 days)

At this stage, the knee joint looked like the adult one (Fig. 44). The articular cartilages became more dense, with more fine collagenous fibres between the cell columns, and the superficial area of the articular surfaces was formed by a very flattened cell layer (Fig. 46). The bony collar of the shafts of the femur and tibia extended approximately to the level of the epiphysis. The fibular shaft showed bony trabeculae in its centre. The menisci showed more collagenous fibres and were now fibro-cartilaginous structures, but still very cellular. The intra-articular ligaments appeared as dense fibrous structures. The joint cavity showed more synovial villi and folds. The femoro-

patellar cavity and supra-patellar recess were well formed and continuous with each other, but still partly separated from the main cavity by tissue strands. The patella was well formed, and penetrated by blood vessels from its anterior and superior surfaces (Fig. 45).

II. CELL DEATH IN THE HISTOGENESIS OF THE INTERZONE

Particular attention was paid to the presence in the interzone of cells which were initially identified simply as "dark cells". In serial wax sections, cut at 5 um and stained routinely with H & E, these "dark cells" were small, with darkly stained nucleus and cytoplasm. As the study proceeded, using semithin plastic embedded sections and TEM, it became evident that the "dark cells" were, in fact, undergoing apoptotic cell death. Once this became clear, it was decided to determine:-

1. the distribution of dark cells within the interzone
2. their relative numbers at each major site of distribution,

and to relate these two parameters to 3. the stage of embryonic development and 4. the stage of appearance of the various components of the joint cavity and of intra-articular structures.

The use of three techniques - serial wax sections, semithin sections and TEM - was essential to the completion of this study. The true nature of the "dark cells" could only be determined by TEM; the details of their distribution and relative numbers could only be determined in serial wax sections. The results from serial wax and semithin sections are presented first.

The distribution and frequency of cell death

The distribution of dead cells, their relative numbers at each main site of the knee joint, their relation with the stage of embryonic development, and the time of appearance of the various components of the joint cavity, are summarized in Table 3.

Explanation of the Table:-

Dark cells were seen in scattered foci in the interzone. It was important to have an assessment of the following:-

1. The developmental stage at which the various foci first appeared in each region of the joint.
2. The stage at which "dark" cells were most numerous in each joint.
3. The stage at which "dark" cells were no longer seen at each focus in each joint.
4. The relationship (if any) between the time and site of cavity formation and time and site of appearance of the maximal number of "dark" cells.

Assessment of site and approximate density of "dark" cells was done as follows:-

Each joint was systematically studied in serial sections. When a focus of "dark" cells was found, it was followed in successive serial sections and the number of "dark" cells was estimated in a sample of the sections. The mean number counted in the sections of a particular focus were expressed on the following scale:

+ very occasional dark cells 1-2 cells/focus

++ small numbers of dark cells 3-10 cells/focus

+++ moderate/large numbers of dark cells 11-30 cells/focus

++++ very large numbers of dark cells > 30 cells/focus

Scoring of dark cells present at each developmental stage:-

i) In each embryo (numbered 1,2,3, etc. at each stage), foci of dark cells found anteriorly and posteriorly in the joint were assessed separately. The total "score" of dark cells at each developmental stage was obtained by adding all the +'s in all the embryos studied at that stage.

ii) This total "score" obviously had no absolute value, since the number of embryos at each developmental stage varied. The total score was therefore expressed as a proportion of the maximum possible score which could have been achieved at each stage calculated by:

number of joints x [(4 +'s (anterior) + (4 +'s (posterior)]

Key to abbreviations

L.c.f.	lateral condyle of femur
fib.	fibula
L.c.tib.	lateral condyle of tibia
M.c.f.	Medial condyle of femur
M.c.tib.	Medial condyle of tibia
L.m.	Lateral meniscus
M.m.	Medial meniscus
ant.	anterior
post.	posterior
C	cavity
(a and b)	both knees belonged to the same embryo

Table 3. Distribution and relative numbers of "dark" cells at each stage and time and site of cavity formation.

Stage	L.c.f. & fibula		L.c.f. & L.m.		L.c.tib. & L.m.		M.C.f. & M.M.		M.C.tib.&M	
30	ant.	post.	ant.	post.	ant.	post.	ant.	post.	ant.	pos
1	–	–	++	+	–	–	–	–	–	–
2	–	–	++	++	–	–	–	–	–	–
3	–	–	–	–	–	–	–	–	–	–
4	–	–	–	–	–	–	–	–	–	–
score / poss.max.	0/32		7/32		0/32		0/32		0/32	
Stage 31										
1	++	+	++	++	++	++	+	+	+	++
2	–	++	++C	++	–	+	–	++	–	–
3	++	+	++	+	+	+	++	+	+	+
4	–	–	++	+	–	–	–	+	–	+
5	–	+	–	+	–	++	–	–	–	–
score / poss.max.	9/40		14/40		9/40		8/40		6/40	
Stage 32										
1	++	+	+++C	++	++	++	++	++	++	++
2	+	++	++	+	+	++	+++	++C	+	++
3	+	+++	++	++	+	++	–	++	+	+
score / poss.max.	10/24		12/24		10/24		11/24		9/24	

Table 3 (Contd.)

Stage	L.c.f. & fibula		L.c.f. & L.m.		L.c.tib. & L.m.		M.C.f. & M.M.		M.C.tib.&M.m	
33	ant.	post.	ant.	post.	ant.	post.	ant.	post.	ant.	post.
1	++	+++	+++C	+++	+++	++	++	++	+++	+++C
2a	+	+++	++	+++C	++	++	-	++C	-	+
2b	+	+++	-	+++	+++	+++	++	++	++	+
4	++	-	+++	+++	++	++C	++	++	++	+
5	++	++++	-	+++	-	++	++	++	-	+
score	21		23		21		18		18	
poss.max.	40		40		40		40		40	

Stage 34										
1a	++	+++C	+++C	++	+++	+++	++C	++C	++	++
1b	+++	+++	+++C	++C	+++	+++C	++	++	-	++
3	++++	++++	++C	+++	-	++	++C	++C	++	+++C
4	+++	+++	+++C	++	+++	+++	-	++	++	++
5	+++	+++	++	+++	-	++	++	++	-	+++C
6	++	+++	+++C	++C	+++	+++C	++	++	-	++
7	+++	+++C	++C	+++	-	++	-	++	++	++
score	42		37		30		24		22	
poss.max.	56		56		56		56		56	

Stage 35										
1a	+++C	+++C	+++C	++C	+++C	+++C	+++C	+++C	+++C	+++C
1b	+++C	+++C	++C	++C	-C	++C	-C	++C	+++C	+++C
3	+++C	++C	++C	++C	+++C	+++C	+++C	+++C	+++C	+++C
4	++++	++++	+++	++	-	++	-	++	++	++
5	+++C	+++C	+++C	++C	+++C	+++C	+++C	+++C	++C	++C
6	++	+++	+++C	++C	+++C	+++C	+++C	+++C	+++C	+++C
7	+++C	++C	++C	++C	+++C	+++C	-C	+++C	++C	++C
8	++++C	++++C	+++C	++C	-C	++C	-C	++C	+++C	+++C
score	49		38		36		32		44	
poss.max.	64		64		64		64		64	

Table 3 (Contd.)

Stage	L.c.f. & fib.		L.c.f. & L.m.		L.c.tib. & L.m.		M.C.f. & M.M.		M.C.tib. & M	
36	ant.	post.	ant.	post.	ant.	post.	ant.	post.	ant.	pos
1	+++C	+++C	++C	+++C	−C	++C	−C	−C	−C	++C
2	++C	++C	++C	++C	++C	++C	−C	−C	−C	++C
3	+++C	+++C	+++C	+++C	−C	++C	−C	++C	++C	++C
4	+++C	+++C	++C	+++C	−C	++C	−C	−C	−C	++C
5	++C	++C	+++C	+++C	++C	++C	−C	−C	−C	++C
6a	+++C	+++C	+++C	+++C	−C	++C	−C	−C	−C	++C
6b	+++C	+++C	++C	+++C	−C	++C	−C	++C	++C	++C
8	++C	++C	+++C	+++C	−C	++C	−C	++C	++C	++C
score / poss.max.	42/64		43/64		20/64		6/64		22/64	
Stage 37										
1	+++C	−C	+++C	−C	−C	−C	−C	−C	−C	−C
2	−C	−C	−C	−C	−C	−C	−C	−C	−C	−C
3	−C	−C	−C	−C	−C	−C	−C	−C	−C	−C
score / poss.max.	3/24		3/24		0/24		0/24		0/24	

Note:- The use of the symbols +, ++, +++, and ++++ give only a very general indication of the extent of cell death.

On the basis of the detailed results shown in Table 3, certain general statements can be made:

- Apoptotic cell death was first seen, to a limited extent, at stage 30 ($6\frac{1}{2}$ days), between the lateral condyle of femur and lateral meniscus (Fig. 9).
- The number of apoptotic cells increased steadily, to reach maxima, in each site, at stages 34/35 (8-9 days), declining rapidly thereafter to negligible levels at stage 37 (11 days), at which apoptotic cell death was seen, to a limited extent only between the lateral condyle of femur, lateral meniscus and fibula.
- The largest numbers of apoptotic cells were seen between the lateral condyle of femur and fibula (Figs. 47-50), and between the lateral condyle of femur and lateral meniscus (Fig. 60).
- The smallest numbers of apoptotic cells were seen between the medial condyle of femur and medial meniscus.
- The numbers of apoptotic cells varied not only in the knee joints of different embryos of the same stage, but also in the two knees of the same embryo.
- The signs of joint cavity formation were seen, at one or two sites only and in a very small number of embryos, at stages 31, 32 and 33 (7 -8 days).
- At stage 34 (8 days), a cavity between the lateral condyle of femur and lateral meniscus, was present in six out of seven embryos. This was the first site at

which cavitation was generally present at this stage.

- At stage 35 (9 days), almost all embryos showed cavities at all sites of the knee joint except the femoro-patell region.
- At stage 36 (10 days) cavitation was completed, by coalescence of the cavities at various sites.

There follows a more detailed examination of the possible relationship between apoptotic cell death and joint cavity formation.

The signs of cell death always preceded signs of joint cavity formation, at each of the main sites analysed in Table 3, (but not at the femoro-patellar joint, see later). Cell death continued to be seen, accompanying the process of cavitation, up to stage 37 (11 days), when the joint cavity was completely formed. Cell death was seen earliest between the lateral condyle of femur and lateral meniscus at stage 30 (6 days), which was also the site at which cavitation was first generally established at stage 34 (8 days).

The first large concentrations of cell death were localized between the lateral condyle of the femur and the fibula, at stages 31-35, and cavitation in this region was seen, in only a very small number of embryos, at stage 34 (8 days), and was generally established at stage 35 (9 days). A possible explanation of this long interval is that the region between the lateral condyle of femur and fibula was filled by mesenchymal tissue which had to be completely eliminated to form a joint cavity, and the lateral meniscus only shared in part of the articulation. By contrast, other regions of the

joint were occupied by both menisci and cruciate ligaments, and many fewer mesenchymal cells lay between the articular surfaces and the intra-articular structures. This may explain why cell death was smaller in amount, and generally absent at early stages, between the medial condyles of femur and tibia and the medial meniscus.

Besides the 5 sites analysed in the Table, foci of cell death were also seen in association with developing intra-articular structures, such as the tibialis anterior tendon and the cruciate ligaments.

At stage 30 (6 ½ days), a limited amount of apoptotic cell death was first seen in relation to the condensation for the cruciate ligaments in the middle of the interzone (Fig. 11).

At stage 31 (7 days), in one specimen only, limited signs of apoptotic cell death were seen, localized in the area between the posterior cruciate ligaments and the posterior menisco-femoral and menisco-tibial ligaments (Fig. 10).

At stage 32 (7½ days), signs of cell death were first seen, in relation to the surface of tibialis anterior tendon, to a limited extent (Fig. 53). Two specimens showed a small amount of cell death in relation to the surface of the cruciate ligaments, especially at their attachment to the condyles of the femur and tibia.

At stage 33 (7½ - 8 days), signs of cell death were seen, in most of the embryos, in relation to the surface of the

cruciate ligaments at various sites, and in relation to the surface of the tibialis anterior tendon, especially along its posterior surface, and at the site of its attachment to lateral condyle of femur (Fig. 54).

At stage 34 (8 days), signs of cavitation were seen in relation to the surface of the tibialis anterior tendon (Fig. 26). The tissue surrounding the cruciate ligaments generally became very loose at this stage.

At stage 35 (9 days) signs of cavitation were present, in relation to the surface of the tibialis anterior tendon (Fig. 31), and the surface of the cruciate ligaments (Fig. 30), in most of the embryos.

At stage 36 (10 days), cavitation in relation to the surface of cruciate ligaments was generally established. Signs of cell death still appeared in relation to the surface of the cruciate ligaments at various sites (Fig. 55). The intra-articular ligaments, were much more clearly defined (Fig. 35) at this stage. Cavitation in relation to the surface of the tibialis anterior tendon at this stage was seen in most of the embryos along the posterior surface of the tendon (Figs. 56, 57), but some showed cavitation along its anterior surface (Fig. 58). A moderate amount of cell death was still seen in relation to the tibialis anterior tendon in various sites (Figs. 56-58).

At stage 37 (11 days) signs of cell death were no longer seen in relation to the cruciate ligament, while they declined rapidly to negligible levels in relation to the

tibialis anterior tendon (Fig. 59). At this stage the intra-articular structures were well formed and looked like those of the adult; the tibialis anterior tendon was surrounded by a cavity like a sheath.

In complete contrast, no sign of cell death was found, at any stage, in the developing femoro-patellar joint. From the outset, the patella was separated from the femur by loose and vascular mesenchymal tissue, which became progressively looser until stage 36 (10 days), when a definite cavity first appeared between the patella and the femur.

The results described so far, were derived from study of routinely stained wax sections. Material stained by PAS, by a selective mitotic stain, and for acid phosphatase activity provided a limited amount of additional information.

1. Periodic acid Schiff. PAS

The results were largely negative. Before cavitation, the interzone showed no PAS positive material, although the intercellular material of femur, tibia and fibula were faintly positive. When cavities were established, there was no PAS positive material within them (Figs. 25, 63, 64).

2. Selective mitotic stain

This was used for two purposes:

i. in the early stages of the study, before the nature of "dark" cells became clear, it was helpful in distinguishing between "pyknotic" nuclei and those in mitosis (Figs. 65, 66).

ii. When the nature of "dark" cells was recognized, it became

important to know if cell death in the interzone was accompanied by cell division. In fact, mitoses were few in the interzone, and certainly insufficient to make good the loss of cells by apoptosis.

3. Acid phosphatase in cells of the interzone

The method used allows only a general assessment of the distribution of the enzyme, without intracellular detail.

Figures 67 and 68 show the interzone between lateral condyle of femur and the fibula at stage 34. Faint pink staining was seen in the chondrocytes of the cartilage models (Fig. 67); in the condensed mesenchyme of the two chondrogenic zones (Figs. 67 and 69) and in normal mesenchymal cells in the loose tissue of the interzone (Figs. 67 and 69).

"Dark" cells were recognized by their more darkly stained, compact nuclei as in routine H & E sections. Their scanty cytoplasm did not show a level of staining any greater than that of the adjacent normal mesenchymal cellls.

Only one or two cells in the interzone showed a strong staining reaction: these are interpreted as macrophages.

Similar strong staining was seen in cells which are morphologically recognizable as macrophages, in the synovial mesenchyme lining the joint cavity (Fig. 69) and within the joint cavity (Fig. 70) both in an embryo of stage 37.

These findings are based on study of material from a total of 11 knee joints. They indicate that "dark" cells do not show increased amounts of acid phosphatase activity, within the limits of the method used.

III. CYTOLOGICAL CHANGES IN CELL DEATH IN THE INTERZONE, STUDIED BY TEM.

Once the distribution, and the timing of the cell death in the interzone had been determined in serial wax and semithin sections, the detailed cytological events were studied by TEM.

The material studied was taken from embryos of Hamburger and Hamilton Stage 27, before any foci of cell death were detected as in either wax or semithin material, and from embryos of several intermediate stages, up to H-H Stage 40, when dead cells were no longer seen at all by light microscopy and the joint cavity was fully developed and similar to that of the adult knee.

The same region of the knee was selected for study at each stage, namely the interzone between the femur and the fibula. This was chosen for three reasons: (i) it was the site at which cell death was seen earliest by optical microscopy, (ii) it showed a high concentration of dead cells; and (iii) the knee joint was most conveniently embedded with its lateral side downwards; this therefore became the cutting face, and the laterally placed femoro-fibular articulation was the first part of the knee joint reached when semithin sections were sampled to select a suitable region for thin sectioning.

As already described in the wax and semithin material, cell death is seen during quite an extended period, beginning before cavitation is seen, continuing during and beyond

cavitation. The EM's which illustrate the following account have been chosen to show the sequence of events, from the normal mesenchymal interzonal cell, through the various degenerative changes, to disintegration and/or phagocytosis and digestion. Two general points should be noted:

i. the sequence of changes is not necessarily reflected in the stage number of the material illustrated; because degeneration is a continuing process, late cytological events may be seen at early H-H stages (and vice versa).

ii. the only region examined by TEM was the femoro-fibular interzone: however, from the material studied by optical microscopy, this is believed to be representative of the process in other areas of the joint.

A) NORMAL, VIABLE MESENCHYMAL INTERZONE CELLS

At early stages (27-30), the interzone consisted of loosely-packed, homogeneously dispersed mesenchymal cells, without special orientation. The cells were variable in shape: some were elongated or rounded but many were stellate in sectional profile, and they contacted each other either by cell to cell attachment or through their cytoplasmic processes.

The cell nucleus was large and usually had one or two, but sometimes three or four nucleoli, and the nucleo-cytoplasmic ratio was high as shown in Figure 71.

The intercellular spaces were large and electron translucent. Thin membraneous structures as well as electron dense granules were occasionally observed (Figs. 71, 75).

At later stages (31-36) the interzone consisted of more compact mesenchymal cells, which were arranged tangentially to the ends of the adjacent cartilage models. Most of the mesenchymal cells were elongated, especially in the centre of the interzone, but some were rounded, especially in the peripheral parts of the interzone, and intercellular connections between the mesenchymal cells were much less frequent.

The intercellular spaces were reduced and filled by sectional profiles of both normally stained cytoplasmic structures, and darkly stained cellular structures, which showed various degrees of degeneration (Figs. 72, 73).

At a higher magnification, viable mesenchymal cells showed the following characteristic features:-

1 - The nucleus.

The nucleus was relatively large and centrally placed, and of medium density. The nuclear envelope consisted of the usual outer and inner layers, enclosing the perinuclear cisterna, and the outer nuclear membrane was sometimes continuous with the cisternae of the endoplasmic reticulum (Fig. 74).

The nuclear matrix contained fibrillar and granular elements, and only a very small amount of marginal chromatin was seen. Each nucleus usually contained one or more eccentric nucleoli, each consisting of a large granular area, which was moderately electron dense, and a small fibrillar area, which was very electron dense (Fig. 74).

2 - The cytoplasm.

The cytoplasm was relatively scanty, and almost electron translucent. It contained numerous ribosomes, either singly (free ribosomes) or in groups of polysomes, or attached to the outer nuclear membrane, or to the profiles of the endoplasmic reticulum (rough endoplasmic reticulum). The endoplasmic reticulum consisted of smooth vesicles, but sometimes a few elongated and granular vesicles could also be seen (Fig. 74) and some were continuous with the nuclear cisternae. The contents of the endoplasmic reticulum were amorphous and electron translucent.

The mitochondria were numerous and appeared in sections, either as circular or elongated profiles, and their matrix was mostly electron translucent and contained a few dense granules.

The Golgi apparatus was not prominent, but was sometimes seen as a group of dilated lamellae, with electron translucent contents (Fig. 75).

Occasional lysosomes were seen. Some cells showed a centriole, in close proximity to the nucleus (Fig. 75). The cytoplasmic membrane was well-defined. There were many cytoplasmic processes which formed intercellular junctions with similar processes of neighbouring cells.

B) CELL DEATH

At low magnification the nuclei and cytoplasm of many of the mesenchymal interzones were darkly stained (Figs. 76-79). Sometimes, many cells showed various degrees of degeneration,

while others appeared normal, as did the cells of the chondrogenic zone (Figs. 76, 77).

The mesenchymal cells lost their intercellular cytoplasmic connections. The intercellular spaces were reduced and filled by numbers of sectional profiles of cytoplasmic structures of both normal and degenerating cells (Figs. 72, 73). Membranous structures, which were most probably remnants of nuclear membranes were occasionally seen in the intercellular spaces, together with darkly stained fine and coarse granules, which were most probably cytoplasmic remnants. Condensed nuclear fragments were also numerous in the intercellular spaces, and attached to the cytoplasmic membranes of the interzone cells.

At stages (38-40), dead cells were no longer seen; the joint cavity was fully developed and electron translucent in appearance. The chondrogenic zones of the articular cartilage were densely cellular, and the cells of their superficial layer were flattened.

At a higher magnification, the mesenchymal dead cell in the interzone showed the following characteristic features:-

1) The nucleus.

The major changes occurred first in the nucleus. In the early stages there was increased density of the nucleus, and aggregation of chromatin subjacent to the nuclear membrane (Figs. 80, 81).

The nuclear membrane became much folded as compared with the smooth contour of normal nuclear outline. Local

separations of the two layers of the nuclear membrane with dilatation of the perinuclear cisterna were seen. The nucleolus was more prominent and generally more darkly stained.

At later stages, the nuclei of dying cells showed many changes. The nucleus became rounded up, and the nuclear chromatin became more concentrated in patches beneath the nuclear membrane (Figs. 82-84). Nuclear pores subjacent to the marginal chromatic condensation disappeared, and the local dilatations between the inner and outer nuclear membranes increased and appeared as electron translucent vacuoles (Fig. 85). At the site of some nuclear pores, the inner and outer nuclear membranes were still attached to one another.

The nucleolus became large and homogeneously dense, and the nuclear matrix contained compact round masses of dense granules.

Some cells showed the chromatic material concentrated in one big homogeneous mass, strongly electron dense (Fig. 86). Sometimes the pyknotic nucleus was fragmented (Figs. 87, 93). Finally, some of the nuclear fragments were surrounded by cytoplasmic elements and formed spherical or ovoid bodies, the so-called apoptotic bodies (Figs. 88-90), either dispersed in the intercellular spaces, or engulfed by neighbouring mesenchymal cells.

2) The cytoplasm.

In the early stages of cell death, affected cells separated from their neighbours, and the cytoplasmic membrane

showed various degrees of indentation (Figs. 80, 81). The cytoplasm became condensed, due to compaction of the organelles, especially the free ribosomes. This condensation was associated with the appearance of translucent vacuoles of different sizes.

Most of the cytoplasmic organelles initially retained their integrity. Some mitochondria, endoplasmic reticulum, and parts of Golgi apparatus were swollen and formed cytoplasmic vacuoles.

At a more advanced stage, the cytoplasm became shrunken, and many organelles showed deterioration (Figs. 82-87). The cytoplasm frequently showed a characteristic aggregation of many cytoplasmic fine granules into parallel bands, which most probably represented a compaction of free ribosomes (Figs. 83, 85) and also coarse granules arranged in parallel bands (Fig. 91) most probably due to compaction of the rough endoplasmic reticulum. These granules were electron dense, and orientated in different directions in the same cell, These banded granules are similar to the banded granules found by Bellairs (1961) in dead cells of the chick blastoderm, and by Dawd & Hinchliffe (1971) in dead cells of the opaque patch of the chick limb. This banding has not been found in normal viable cells.

Finally, parts of the cytoplasm of dead cells separated from the rest of the cell into the intercellular spaces; some contained nuclear fragments in addition to condensed cytoplasm (Fig. 90), whereas others contained cytoplasmic

elements alone (Fig. 87).

3) Phagocytosis of dead cells.

The resulting apoptotic bodies were seen either isolated and dispersed in the intercellular spaces, or engulfed by neighbouring viable mesenchymal cells, which later differentiated to macrophages.

In some cases, the process of ingestion of the dead cell was observed (Fig. 93). The dead cell became surrounded by many cytoplasmic processes from a neighbouring, apparently viable, mesenchymal cell.

In other cases, mesenchymal cells were found which had ingested dead cells, but were otherwise identical in all respects to neighbouring normal, non-phagocytic mesenchymal cells (Figs. 94-96). The dead cells showed little sign of digestion, and the nucleus and cytoplasm could frequently be distinguished (Fig. 94) and others showed signs of digestion, and nucleus and cytoplasm could not be distinguished (Figs. 95, 96).

In other cases, the ingesting viable mesenchymal cell can be considered as a macrophage, and showed certain changes from an early stage (Figs. 97-100). The margin of the nucleus of the macrophage was more dense, and it tended to take on a convoluted appearance, due to the pressure from the ingested dead cells. The macrophage cytoplasm and contained many phagocytic vacuoles.

The dead cells showed signs of digestion inside the vacuoles; they presented a pitted appearance, and contained myelin figures (Figs. 97. 98).

Other phagocytes were large, and contained many digestive vacuoles, within which were dead cells mostly at a late stage of digestion. Some dead cells showed little sign of digestion, perhaps because they had been recently ingested (Figs. 102, 103).

IV. SCANNING ELECTRON MICROSCOPY

Once the distribution and the timing of cell death in the interzone had been determined in serial wax and semi-thin sections, and the detailed cytological events had been studied by TEM, an attempt was made to study the changes during cavitation in the interzone by SEM.

The material studied was taken from embryos of H-H stage 30, in which foci of cell death were first detected, through several intermediate stages up to H-H stage 40, when dead cells were no longer seen at all by light microscopy, and the joint cavity was fully developed and similar to that of the adult knee.

Various regions of the knee joint were selected for study at each stage, but mainly the interzone between the femur and the fibula. Figures 104 and 105 give a general low power view. Most of the cells are included in the sectional plane, and their sectional profiles show a wide range of shapes. Many are stellate, with an irregularly rounded or ovoid cell body, and two, three or four blunt, tapering cell processes. A few are more elongated and spindle shaped. The intercellular space is occupied by a three-dimensional network, (for detail, see Fig. 106), in the interpretation of which at least three possibilities have to be considered:

i. that the network consists of fine cytoplasmic extensions of the cells i.e. <u>filopodia</u>, indicating active outgrowth from the cells, involved in cell locomotion;

ii. that the network consists of <u>retraction fibres</u>, produced

as the mesenchyme becomes looser, by a drawing out of the cytoplasmic connections between cells; or

iii. that the network consists of young collagen fibres.

Of these three possibilities, the third can be dismissed by study of transmission EM's at the same stage, which show little if any evidence of collagen (see Figs. 72, 89).

The distinction between the first and second possibilities is more difficult to make. Revel and Solursh (1978), faced with a similar problem in the primary mesenchyme of chick and rat embryos, decided that "when encountering cells linked to each other by many taut thin processes it is ....tempting to interpret these as retraction fibers rather than filopodia". The intercellular space also shows structures larger than the network of "fibres". Some are obviously sectional profiles of larger cell processes (Fig. 106, PR). Others are not sectioned, but are seen as complete and roughly spherical structures (Fig. 106, DC): these are interpreted as apoptotic cells or apoptotic bodies.

When the joint cavity is well established, as in Figure 107, the articular surface of the lateral condyle of the femur is covered by layers of loosely overlapping flattened cells, forming the most superficial tangential layer of chondrocytes. Lying on this layer are scattered, roughly spherical cells with an irregular surface covered with blebs (Fig. 108). Similar cells are shown in Figure 109, where their surface is pitted, and Figure 110, where the surface is deeply constricted. All these cells are interpreted as

apoptotic cells for the following reasons:

1. Their external form is consistent with the appearances already described in TEM's of thin sections.

2. When sections were cut from the wax embedded tissue to expose the joint cavity for study by SEM, the last section cut was stained by H & E, to provide some familiar landmarks as a guide to the interpretation of the SEM appearances. The section showed "dark" cells lying upon the femoral articular surface as they are seen to do by SEM in Figure 107.

3. The surface appearances are similar to those described by others as typical of apoptotic cells found in other situations in development (e.g. Pexieder, 1975; Hurle and Hinchliffe, 1978; Garcia-Porrero and Ojeda, 1979; and Kaplan et al., 1975). The appearance of the articular surface at a later stage of cavitation is shown in Figures 111 and 112, when it is free of adherent apoptotic cells, and looks like a smoothly cobbled street. The low surface elevations are probably produced by underlying chondrocytes in the most superficial layer of the articular cartilage.

Similar appearances have been described in young children (see Ghadially, 1978).

SECTION 4 - DISCUSSION

DISCUSSION

In the general introduction, several areas of disagreement among previous workers were identified. These will now be discussed in turn, in the light of the results of the present study.

I. CELL DEATH IN THE INTERZONE OF DEVELOPING SYNOVIAL JOINT

The most important finding of the present study was the occurrence of scattered foci of cell death in the interzone. This was first seen at stage 30, increasing to reach maxima at stages 34/35, and subsequently declining to disappearing point by stage 37.

This finding raises several questions for discussion:

1) Is cell death a general phenomenon in the development of synovial joints?

Previous publications dealing with this question have been reviewed in detail in the Introduction. They fall into three groups:

a) those describing cell death as an early event in joint development;

b) those which specifically deny the occurrence of cell death at any stage in joint development, or describe it as only an occasional occurrence, lacking morphogenetic significance and,

c) those which describe results generally similar to those of the present study.

a) Cell death as an early event

i. the "opaque patch"

Fell and Canti (1934) described the position of the "opaque patch" in the lower limb of the chick embryo, and found that it appeared at stages 23 and 24, apparently lying in the prospective femoral blastema. By stage 25, it was reduced in amount, and at stage 26 it was T-shaped, with the top of the "T" across the joint region between femur, tibia and fibula. They reported that the significance of the opaque patch was not known.

Dawd and Hinchliffe (1971) studied the "opaque patch" in the forelimb of the chick embryo, and found that it appeared at stage 23, preceding the mesenchymal condensations, reached its maximum extent at stages 24 and 25, when it appeared as a progressively more distal zone of dead cells, lying between the radius and ulna which formed the two arms of the Y-shaped mesenchymal condensation. At stage 26, it was located in a small area between the proximal ends of radius and ulna. At stage 27 and 28 dead cells were reduced in number and were located in the triangular area bounded by the proximal ends of radius and ulna and by the distal end of the humerus. At stage 29 it had disappeared. They also found that the opaque patch was not present in the prospective humerus material, nor did it extend into the humero-ulnar joint. They suggested that the significance of the opaque patch was that it might play a role in the initial separation of radial and ulnar condensations, and in suppression of chondrogenesis in

the mesenchyme between radius and ulna.

ii. the "early wave"

Mitrovic (1977) found an "early wave" of cell degeneration, appearing at the 7th day (not staged, but probably about stage 31) in the interzone of the metatarsophalangeal joint of the chick embryo. This early cell necrosis progressively disappeared and could not be easily seen after the 9th embryonic day (Stage 35).

Mitrovic (1978) also found an early wave of cell degeneration appearing by the 12th-13th day, in the interzone of the diarthrodial joints of the rat embryo, immediately after differentiation of the interzone. This cell necrosis was clearly seen in the small digital joints of both limbs by the 15th-17th day, while in the larger joints such necrotic cells were more difficult to see. He did not give detailed accounts of any individual joint. In both studies of the chick and rat embryos, the significance of this early wave of cell degeneration was unclear, but he suggested that it might serve one (or both) of two purposes: 1) to prevent chondrification of the interzone by removing cells with chondroblastic potentiality; and 2) to provide for loosening of the interzone and the differentiation of a three-layered interzone.

b. Cell death denied

O'Rahilly and Gardner (1956) found that cavitation first appeared in the femoro-meniscal cavity at stage 33. In two specimens a small cavity was present between the medial condyle of the femur and the medial meniscus at stage 34. In

another embryo a small cavity was present between the lateral condyle of the femur and the lateral meniscus. At stage 34, in one embryo, they found a "striking number of pyknotic cells, flattened over the convexities of the articular surfaces of the femur, tibia and fibula, and above and below the medial meniscus". In another embryo at this stage, dark-stained cells were found scattered throughout the interzone. They did not make any suggestion about the significance of these dark stained cells in the joint, or any explanation for the mechanism of cavity formation.

Henrikson and Cohen (1965) found that the interzone of the chick interphalangeal joint became more compressed at stage 37. A definite joint cavity was first seen in the interzone at stages 37-41, as a narrow cleft either between or lateral to the embryonic phalanges. They stated explicitly that no degenerative phenomena were noticed in the joint cavity, but they found blood vessels within the differentiating joint interzone. They suggested that, if vessels do not play a direct role in cavity formation, "their presence may be necessary in some as yet undefined fashion for the initiation and sustenance of the articular space".

Andersen and Bro-Rasmussen (1961) found that cavitation in the hand and foot human joints started centrally, after the intermediate layer of the interzone had been incorporated in two chondrogenous layers which in turn were incorporated in the two joint surfaces. In the course of joint formation, fairly large quantities of chondroitin sulphate A and C form

in the three layers of the interzone, and cavity formation was not preceded by any sign of liquefaction or degeneration in the intermediate layer of the interzone. Andersen (1964) reached exactly the same conclusion in his study of the development of the human knee joint.

c. Cell death as a regular and significant event

Strayer (1943) found signs of degeneration in the hip joint of the human embryo. At 23 mm, he noticed an increase in the intercellular spaces, with evidence of degeneration in the area of cells lying between the head of the femur, ligamentum teres, and acetabulum. At 36-42 mm, cavities filled with fluid were apparently formed in the peripheral regions of the joint. Isolated cells suspended in the fluid showed signs of degeneration, by loss of their cellular outlines and the fading of their nuclear staining. Other nuclei were pyknotic in appearance. Besides these signs of degeneration, the cavities contained long fibrils which he suggested had resulted from cell degeneration. At the same time, he noticed long strips of well preserved cells stretched diagonally from the acetabulum to the head of the femur. The processes of the cells were so strongly attached to one another as apparently to stand considerable tension. Finally, he suggested that cavitation process was "at once, a degenerative and a mechanical process".

Rajan and Merker (1975) also found evidence of material which was acid mucopolysaccharide in nature, with occasional cells showing pyknotic nuclei, lining the human digital joint cavity, which appeared in the absence of movement. Under the

electron microscope, there was single cell necrosis with evidence of loss of glycosaminoglycans.

Mitrovic (1977) found a second wave of cell necrosis to appear in the course of joint cavitation of the metatarsophalangeal joints of the chick embryo. The cell necrosis of the second wave was morphologically distinct from that of the early wave in that the cells disappeared very rapidly and could not be easily identified. He noticed that the cavity was preceded by the appearance of flattened, elongated cells exhibiting strong basophilia, electron opacity and enlarged endoplasmic reticulum, along the margin of the cavity. When the joint cavity was fully formed, these basophilic cells were found to cover the articular surfaces in a discontinuous manner. On the basis of several morphological criteria (nucleocytoplasmic basophilia, electron opacity, enlarged endoplasmic reticulum and signs of organelle alteration) he suggested that these cells might be degenerative cells, whose death might account for loosening of the tissue and lead to cavity formation. He also suggested that these flattened basophilic cells might remain as "a distinct cell line which covered the articular cavity walls, preventing secondary fusions of the articular surfaces". He found an accumulation of intercellular material which might be analogous to the primitive synovial fluid. He suggested that accumulation of this material could account for the formation of a joint space by distending the intercellular spaces and later articular cavity walls, and

thus preventing them from secondary collapse and fusions. He also found some blood vessels and free erythrocytes in the axial part of the joint. He suggested that these blood vessels might somehow be associated either with cell degeneration or with the cavitation process.

Mitrovic (1978) found the most striking feature at the time of cavity formation of the diarthrodial joints of the rat embryo, was the appearance of flattened elongated cells which stained heavily with basic dyes. They were usually seen as two single lines of cells, bordering each side of the cavity. In a few digital joints, they were found in several layers. In the areas of cavity formation, the cells occasionally appeared to be degenerating. Fragmented, pyknotic nuclei, poor cytoplasmic staining and fuzzy limits were the most apparent characteristics. The elongated basophilic cells were not seen later in joint development and seemed either to disappear or to change their staining pattern. He found that the formed cavity did not contain any material stainable by PAS. He suggested that the role of these basophilic cells in cavity formation was achieved in two different ways: 1) by loosening of the interzone tissue due to cell degeneration, although he admitted that there were problems with this hypothesis since he could not see the later stages of cell degeneration, such as cell disintegration and macrophagocytosis, during cavitation of the axial part of the interzone. Instead, he observed degenerative changes in the peripheral, synovial part of the joint. Or 2) these cells were a special cell line,

differentiated by the time of cavitation, and taking an active part in the cavitation process, perhaps by "lowering intercellular adhesion". If this were so, cells remaining at the surface of articular cartilage could be considered different from the deeper chondrocytes. He thought that this hypothesis was consistent with the findings of others that in adult animals, superficial articular chondrocytes cannot produce sulphated proteoglycans.

In the present study, only a few dead cells were found at stage 27, located in the area bounded by the cartilage models of the femur, tibia and fibula; by stage 29 there were only one or two dead cells, in a very few sections, or none at all. These dead cells probably corresponded to those of the "early wave" described by Mitrovic (1977, 1978) or of the "opaque patch" described by Fell and Canti (1934), Dawd and Hinchliffe (1971) and others. We found continuity between the femoral, tibial, and fibular blastemas at stages 27-29, without any sign of chondrification in the interzone between them: The adjacent ends of the femoral, tibial and fibular blastemata, still unchondrified, merged insensibly into the interzone.

The significance of this "early" death of cells in the chick knee joint is a matter only for speculation. It may "remove cells with chondrogenic potential", as suggested for the opaque patch in the chick elbow by Dawd and Hinchliffe (1971), and for the "early wave" of cell death in joints of chick and rat by Mitrovic (1977 and 1978). However this is

only a speculation, without any support from observation or experiment. The other suggestion made by Mitrovic (1977, 1978), that the "early wave" of cell death leads to loosening of tissue at the centre of the interzone, so that it became three-layered, is not supported by the present study, in which three-layered interzones did not appear until much later, at stage 32, when most of the intra-articular structures had developed.

Mitrovic's (1977, 1978) interpretation of his "second wave" of cell death, which he found in both chick and rat embryo joints, is ambiguous. There were, he said, some morphological signs of cell degeneration, which might lead to loosening of the tissue and cavity formation. His other suggestion was that these basophilic cells were a special cell line, differentiated by time of cavitation, which covered the articular cavity walls, and prevented secondary fusions.

His doubts about the first suggestion, that the dark cells were degenerative, rested on his failure to find later stages in the process, including phagocytosis by macrophages. The present study has shown that these doubts were not justified: all stages of degeneration, up to complete disintegration, have been seen, together with clear evidence of phagocytosis by macrophages of local origin.

O'Rahilly and Gardner (1956) in their comprehensive account of the development of the chick knee joint, found a focus of pyknotic cells localized in the interzone, in only 2 specimens out of 125, at stage 34. They made no suggestion

about the significance of these cells, or any explanation of the mechanism of cavity formation. In view of the results of the present study, it seems strange that they did not notice foci of cell death in any of the other embryos of the large series which they studied. The reason is probably partly technical: the dark cells are not immediately obvious in routinely stained wax sections, although once they were seen and then deliberately looked for, they were obvious. "What one knows, one sees" (Goethe).

The failure of Henrikson and Cohen (1965) to find any sign of cell degeneration inside the developing joint is more difficult to understand, since they looked specifically for it. It may be that the mechanism of cavity formation is different in joints with large cavities - such as the knee - from that in the smaller plane joints of the carpus and tarsus. They found multiple blood vessels inside the interzone, and thought that these might be related to initiation of cavitation. The same suggestion was made by Mitrovic (1977), who found blood vessels and cells inside the interzone of the same joint. By contrast, in the present study no evidence was found of any blood vessels inside the interzone of the knee joint; blood vessels were confined to the peripheral parts of the interzone.

Andersen (1964) and Andersen and Bro-Rasmussen (1961) have also claimed that cavity formation was not preceded by any sign of liquefaction or degeneration in the intermediate layer of the interzone. Their failure to observe cell death

must have been an oversight, as with O'Rahilly and Gardner (1956), unless the mechanism of joint formation differs fundamentally in man (studied by Anderson and colleagues) from that in chick.

Searls (1965), in his work on the incorporation of radioactive sulphate in chondroitin sulphate during the differentiation of chick limb bud cartilage, first detected increased uptake in the chondrogenic areas at late stage 22, while by stage 24 a pronounced Y-shaped pattern was seen, corresponding to the pattern of mesenchymal condensation. Even so, the area between radius and ulna was only slightly less positive for acid mucopolysaccharides than the condensations themselves. This result was also reached by Hinchliffe and Ede (1967) who showed that the central core of the chick limb mesenchyme was more or less uniformly stained for acid mucopolysaccharides at stage 24, but that by stages 27 and 29, the area between radius and ulna was not stained. They suggested that the central mesenchymal core initially synthesised acid mucopolysaccharides uniformly, and that the Y pattern of synthesis emerged later, with suppression of synthesis between the arms of the Y. Dawd and Hinchliffe (1971) reached the same conclusion, and attributed the cessation of acid mucopolysaccharide synthesis between the radius and ulna to cell death of the opaque patch in this region. The present results neither support nor refute the presence of acid mucopolysaccharides in the joint cavities, since the PAS reaction used does not stain acid mucopolysaccharides.

It appears that the present study establishes the reality of cell death in the developing chick knee joint. This leads to a second question:

2) What is the significance of cell death?

To my knowledge, there is no previous comprehensive study of the amount and distribution of cell death in developing synovial joints or of its correlation with the development of the joint cavity and of intra-articular structures.

As summarized in Table 3 , signs of cell death always precede signs of cavitation at each of the sites analysed in the Table. The first signs of cavitation appeared anteriorly, between the lateral condyle of femur and the lateral meniscus, at stage 31. This was the site of the first appearance of cell death, at stage 30. It was also noticed that signs of cell death accompanied the signs of cavitation, up to stage 37 (11 days), when joint cavity formation was complete. Once cavitation was initiated, foci of cavity formation appeared at various new sites in the joint which eventually coalesced to form a continuous cavity. Signs of cell death were still seen after the initiation of cavitation at each site, presumably serving to clear away

remnants of mesenchymal tissue between the articular surfaces. The largest focus of cell death was found between the lateral condyle of the femur and the fibula: cavitation first appeared at this site at stage 34 (8 days), was generally established at stage 36 (10 days), and the signs of cell death continued until stage 37 (11 days). A possible reason for this long period of cell death is that the region between the lateral condyle of femur and fibula is occupied by a considerable amount of mesenchymal tissue, most of which is eliminated, apart from that which gives rise to part of the lateral meniscus; more time may therefore be needed to eliminate the tissue at this site. By contrast, other regions of the interzone are occupied mainly by menisci and intra-articular ligaments, with only a small amount of mesenchymal tissue lying between the articular surfaces and the intra-articular structures. Certainly the foci of cell death were generally smaller and shortest lived between the medial condyles of femur and tibia and medial meniscus.

In addition to the main sites, analysed in Table 3 signs of cell death were found to precede signs of cavitation around intra-articular structures such as the tibialis anterior tendon and the cruciate ligaments. Small foci of cell degeneration were first seen in relation to the cruciate ligaments, in a very small number of embryos at stages 30-32; most specimens showed signs of degeneration at stages 33 and 34, at various sites along the surface of the cruciate ligaments. The first signs of cavitation were seen in relation to the surface of the cruciate ligaments at stage

35, then cavitation extended rapidly around the ligaments, which became more clearly demarcated by stage 36.

The first signs of cavitation in relation to the surface of tibialis anterior tendon were noticed at stages 32-33, while first cavitation was noticed in a very small number of embryos at stage 34. Dead and dying cells were seen in relation to the surface of the tendon until stage 37, by which time the tendon was completely differentiated and surrounded by a cavity like a sheath. A possible explanation of this long interval (stage 32/33 - stage 37) may be that the tendon has a big surface, and is surrounded by a large amount of mesenchymal tissue, whose elimination takes time. By contrast, the cruciate ligaments are surrounded by a very small amount of mesenchymal tissue, which does not take long for its clearance.

These results, taken together, suggest that cell death in the interzone serve two purposes: i) the initiation of cavitation in the knee joint: degeneration of cells and removal of the debris by macrophages leads to loosening and cavity formation; and ii) the sculpturing of the articular surfaces and intra-articular structures such as the menisci, cruciate ligaments and tibialis anterior tendon.

The only site of cavitation which was <u>not</u> preceded by any sign of cell death in this study, was the femoro-patellar joint. The significance of this exception is discussed later.

3) What is the nature of cell death?

Interpretation of the cytology of cell death in the interzone, based on observation by electron microscopy, reveals a certain sequence of events.

Low power survey electron micrographs have shown many of the mesenchymal cells in various degrees of degeneration, interspersed with others which appeared normal, as did cells of the chondrogenic zone. There was also a reduction in the intercellular space and in the thickness of the interzone. This reduction is attributed to the orientation of the mesenchymal cells in the transverse plane, perpendicular to the long axis of the cartilage models.

At a higher magnification, the major changes were seen to occur in the nucleus. 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 interrupted separation of the two layers of the nuclear membrane. This local separation of the two layers of the nuclear membrane led to dilatation of the perinuclear cisternae which were continuous with the cisternae of the endoplasmic reticulum. Concomitant with these early nuclear changes, 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 of the apoptotic process, 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. Dense compact, round granules appeared inside the nuclear matrix. Sometimes, the chromatic material was concentrated in one large homogeneous and strongly electron dense mass. Nuclei at this advanced stage of dying were surrounded by cytoplasm which showed deterioration of its organelles. Often only a thin rim of cytoplasm remained surrounding the nucleus, presumably indicating the loss of cytoplasm into the intercellular space.

Finally, apoptotic bodies of different sizes and shapes, formed of nuclear fragments surrounded by cytoplasmic elements, were found either dispersed in the intercellular spaces, or phagocytosed by adjacent viable mesenchymal cells. Detached fragments of deteriorating cytoplasm, often containing banded granules were clearly seen in the intercellular spaces.

The banded granules found in the cytoplasm have also been reported by Bellairs (1961) and Dawd and Hinchliffe (1971). Bellairs (1961) suggested three possible ways in which they had formed:-

1) by combination of several granules of ribonucleoprotein

2) by the breakdown of other cytoplasmic constituents

3) by loss of water during degeneration resulting in the close packing of the granules.

The findings of the present study suggest that fine banded granules resulted from compaction of free ribosomes, and that packed groups of parallel membranes represented compaction of rough endoplasmic reticulum.

The process of cell degeneration occurring during development of a variety of organs, has been described by several workers under different terms, e.g. Haines (1947) described it as a liquefaction process; Strayer (1943); Rajan and Merker (1975) and Mitrovic (1977, 1978) described it as pyknosis; Kerr (1971) as shrinkage necrosis, and finally Kerr et al (1972) coined the new name, apoptosis. That the degenerative changes described above represent a process of apoptosis or genetically programmed cell death, is based on the following items of circumstantial evidence:

1) The changes characteristically affect some mesenchymal cells while others are normal.

2) The changes occur during normal development of the joint, without any apparent external precipitating factor.

3) The cytological changes described are similar to those described by others as typical of physiological cell death occurring during development of a variety of organs, as outlined in the Introduction.

4) What is the fate of the dead cells?

The degenerative changes described above take place during the apoptotic process, before ingestion by phagocytes. This finding suggests that the phagocytic process is not the

cause of apoptosis. This suggestion is in agreement with the interpretation of Ballard (1965) and Dawd and Hinchliffe (1971).

Under the electron microscope, some of the apoptotic cells were found partially surrounded by cytoplasmic processes of a mesenchymal cell, identical with its viable neighbours, and apparently acting as a phagocyte. Other cells contained within their cytoplasm one or two recently ingested apoptotic cells or bodies which showed little or no sign of digestion. These phagocytes represent the first stage of phagocytosis, described by Dawd and Hinchliffe (1971), who found that there was no sign of increased acid phosphatase activity within either the dead cells or the phagocyte at this stage.

Other phagocytes had 3-5 phagocytic vacuoles containing ingested apoptotic bodies, one or two showing signs of digestion such as smaller size and loss of stain intensity. The nucleus of the phagocyte took on a shape apparently imposed by pressure from the engulfed apoptotic cells. These phagocytes represent the 2nd stage of phagocytosis described by Dawd and Hinchliffe (1971), who found increased acid phosphatase activity in phagocytes at this stage.

Mature macrophages were large cells, containing several apoptotic bodies. Their nuclei showed increased electron density, and the quantity of macrophage cytoplasm was greatly increased compared with that of normal mesenchymal cells, and contained more mitochondria and an increased amount of

endoplasmic reticulum. These phagocytes represent the 3rd stage described by Dawd and Hinchliffe (1971).

If the EM findings are correlated with those on acid phosphatase activity as seen in wax sections, the following tentative suggestions emerge:

i) that when mesenchymal cells of the interzone begin to undergo apoptosis, there is no obvious increase in their acid phosphatase activity, as indicated by histochemical staining and by the number of lysosomes seen by E.M.

ii) that isolated dead cells and apoptotic bodies are phagocytosed by adjacent normal mesenchymal cells

iii) that some of these newly differentiated phagocytes may then assume the appearance of typical macrophages, which may contain groups of ingested dead cells. (Saunders et. al. 1962) interpreted such groups as clusters formed by the aggregation of dead cells, not within a macrophage).

This interpretation of the differentiation in situ of macrophages from mesenchymal cells is similar to that put forward by a number of workers, analysing cell death in the mesenchyme, in limb development: e.g. Ballard (1965); Ballard and Holt (1968); Dawd and Hinchliffe (1971); Hammer and Mottet (1971) and Hinchliffe and Ede (1973), all working on various aspects of limb development. It runs contrary to the currently fashionable concept of a "mononuclear phagocytic system", a widely distributed system of macrophages, all of which are said to be derived from blood borne precursors, the monocytes, of bone marrow origin. The evidence from the present study that macrophages differentiate in situ from

mesenchymal cells of the interzone may be summarized as follows:

i) in the early stages of phagocytosis, the cells involved did not differ morphologically from mesenchymal cells in the neighbourhood. Under EM they do not show features of either monocytes or macrophages.

ii) the interzone is avascular: blood vessels are confined to the more peripheral, presumptive synovial mesenchyme. No evidence was found of the migration of monocytes into the centre of the interzone and of their differentiation into macrophages.

iii) Acid phosphatase positive cells were sparse in the interzone at stages when it was clear, from the EM study, that phagocytosis of dead cells was under way.

5) What is the role of lysosomes in cell death?

The question of lysosomal participation in cell death has caused considerable controversy.

Material stained for acid phosphatase examined by light microscopy did not show greater activity in apoptotic cells than in their normal neighbours, a finding which contradicts the "suicide bag" theory of de Duve, that dead cells synthesise additional lysosomal enzymes which destroy the cell through release of hydrolytic enzymes into the cytoplasm (de Duve, 1959). This view confirms that of Mitrovic and of several other workers: [Ballard and Holt (1968); Dawd and Hinchliffe (1971); Hammar and Mottet (1971); Pannese et al (1976); and Umansky (1982)].

II. DEVELOPMENTAL SEQUENCE RELATED TO EXTERNAL STAGING

The present study reveals that there is in general a remarkable regularity of the developmental sequence in relation to external staging. Although slight variations in the time of onset may be expected, we found that, at any given stage, most of the specimens showed the same features. Chondrification of the femoral, tibial and fibular blastemata was established at stage 27, and confined to the shafts, while the distal end of the femur and proximal ends of the tibia and fibula, were chondrified at stage 30. The interzone appeared as a homogenous structure until stage 32, when, a three-layered interzone was distinguishable. The perichondrium around the femur, tibia and fibula was recognized at stage 28, when condensations for the quadriceps femoris muscle, and the cruciate ligaments were also noted. The tibialis anterior tendon formed at stage 29. The patellar condensation appeared at stage 30. At the same stage, condensations for menisci, ambiens tendon, and ossification around the centres of the shafts of femur, tibia, and fibular apeared. Cavitation was first generally established between the lateral condyle of femur and lateral meniscus at stage 34, which was also the site of the first appearance of cavitation, at stage 31. Cavitation was generally established at the various regions of the joint, except the femoro-patellar region at stage 35. At this stage, cartilage canals had appeared in the intercondylar notch of the femur posteriorly, and the primordium of the

patellar perichondrium appeared. The joint cavity was well developed between the articular structures, including the femoro-patellar region, by stage 36. At this stage, the patella became chondrified, an early synovial lining was detected in some places, and cartilage canals appeared in the medial condyle of the tibia. The articular cartilage started to show cell columns in its deep layer at this stage. The synovial folds and villi appeared at stage 37. The knee joint looked like that of the adult, and blood vessels started to penetrate the patellar perichondrium, at stage 40.

The present findings in general confirm those of O'Rahilly and Gardner (1956). The times given by them for the appearance of the tibialis anterior tendon, (Stage 27) and the ambiens tendon, and ossification around the middle parts of the shafts of femur, tibia and fibula (Stage 29) are earlier than those found here. Their findings of the condensation for the cruciate ligaments at stage 30, and the patellar perichondrium, at stage 36, are later than ours. These differences are all small and serve to emphasize the value of staging, and the close correlation between external features and internal structure.

The main differences between our results and those of O'Rahilly and Gardner (1956) are in the time and process of cavitation. They found that cavitation appeared in the interzone, in some specimens at stage 34, as menisco-femoral cavities, while in our results, cavitation was generally established between the lateral condyle of femur and lateral meniscus at stage 34, and the first appearance of cavitation

was noticed at stages 31/32, in the same site. They failed to recognize the darkly stained cells, except in two out of 125 specimens, and they attached no significance to these "dark" cells, nor did they offer any explanation of the process of cavitation in the joint. They also failed to recognize the three-layered interzone at any stage, while it is clearly established in our specimens at stages 32 and 33. These main differences can only represent errors in observation on their part: thus in their Figure 11, the three-layered interzone is clearly shown, while they claimed that it was quite difficult to recognize in the knee joint.

III. DEVELOPMENT OF THE PATELLA AND FEMOROPATELLAR JOINT

A) THE DEVELOPMENT OF THE PATELLA

a) Association with quadriceps tendon

O'Rahilly and Gardner (1956) found that the patellar anlage appeared deep to, and later than, the expanded quadriceps tendon. They therefore regarded the patella as a peri-articular rather than an intra-tendinous sesamoid. Anderson (1961) also found that the patellar primordium originated in the blastema behind the quadriceps tendon with a sharp morphological distinction between the cells of the two structures. Schneck (1965) also reached the same conclusion, that the quadriceps tendon and patellar ligament lay on the anterior surface of the patella. Doskocil (1985) found that the quadriceps femoris tendon was attached to the front and the edges of the patella, and he claimed that the

patella is not a sesamoid bone.

The present study fully confirms the conclusion of all the above workers, that the patella is not an intra-tendinous sesamoid bone. The quadriceps tendon was found to be inserted into the upper border of the patella, and the patellar ligament began from the anterior surface of the anlage of the patella and descended towards the tibia. These findings are in disagreement with those who regarded the patella as an intra-tendinous sesamoid bone e.g. Bardeen (1905), Walmsley (1940) and Haines (1947).

b) Time of appearance and chondrification

In this study, it was found that the patella appeared as a mesenchymal condensation, anteriorly to the femur at stage 30, which is the time of initial ossification of the long bones, and prior to the onset of joint cavitation. It became surrounded by a perichondrium at stage 35, and chondrified at stage 36. These findings confirm those of O'Rahilly and Gardner (1956) except in the time of appearance of the patellar perichondrium, which they found to appear at stage 36, which is later than our finding. Niven (1933) found that the patellar condensation was isolated from the surrounding tissue, and did not develop until the end of the 10th day (stage 36) or the beginning of the 11th day (stage 37). It chondrified between the 11th and 12th days (stage 38), and was surrounded by a perichondrium at the end of the 12th day. These times are considerably later than those found here.

Our findings are in disagreement with those 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 from which it did not become separated until the early prechondrial blastema stage. By contrast, we found that the patellar condensation appeared after chondrification of the femur.

c) Association with lower end of femur

The conclusion which was reached by e.g. Haines (1947), Gray and Gardner (1950), and O'Rahilly and Gardner (1956) that the patella was separated from the lower end of the femur by loose vascular mesenchymal tissue from the outset and until cavity formation began, and that the joint did not show a typical interzone, is completely confirmed by the present study. Doskocil (1985) found that the patella was separated from the lower end of the femur by loose tissue only after its anlage had separated from that of the femur. By contrast, Andersen (1961) and Schneck (1965) claimed that the patella was separated from the femur by a typical three-layered interzone.

B) THE DEVELOPMENT OF THE FEMORO-PATELLAR JOINT

From the present study, it was found that the cavity was formed by gradual loosening of the mesenchymal tissue between the femur and patella. No foci of dead cells were found preceding or accompanying cavity formation, between femur and patella unlike all the rest of the knee joint cavity.

This finding obviously rules out the view that cell

STUDIES OF THE LYMPHATICS OF THE LIVER AND THE UPTAKE OF INTERSTITIAL FLUID FROM THE SPACE OF DISSE

Vol. 2

FIGURES

By

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Fig. 1

An example from the Hunterian collection of lymphatics injected with mercury showing the superficial lymphatics of the liver forming a close arborescent plexus beneath the peritoneal covering of the organ. The larger trunks are seen running off into one of the ligaments on their way to the draining node.

Fig. 2

Elias's 3-dimensional scheme showing the way branches of the hepatic artery, the portal vein and the bile duct pierce the perilobular limiting plate on entering or leaving the lobule.

Note: the narrow space between each structure and the adjacent hepatocyte.

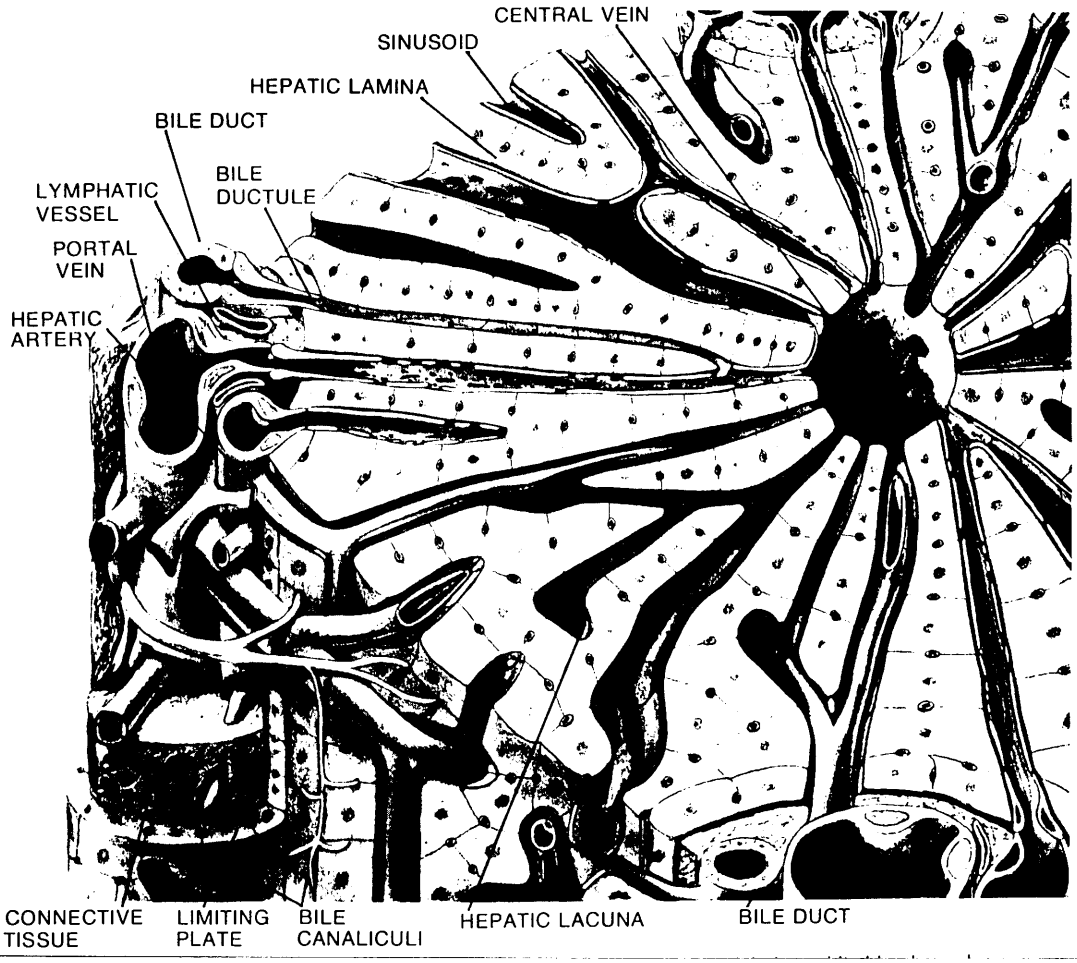

CENTRAL VEIN
SINUSOID
HEPATIC LAMINA
BILE DUCT
BILE DUCTULE
LYMPHATIC VESSEL
PORTAL VEIN
HEPATIC ARTERY
CONNECTIVE TISSUE
LIMITING PLATE
BILE CANALICULI
HEPATIC LACUNA
BILE DUCT

Fig. 3

Schematic drawing of blood-lymph barrier in the normal liver presented by Henrikson et al (1984) showing the way in which the portal vein (PV) and the hepatic artery (HA) pierce the limiting plate and entering a sinusoid (S). The flow along the blood-lymph barrier is shown in arrows. The flow at the point of entry of these vessels [described by Henrikson et al (1984) as less defined] is shown by dotted arrows.

Note: the proximity of the lymphatics (L) to the point of entry of the vessels (i.e. gaps) suggested by Henrikson et al.

AF - anchoring filaments
HV - hepatic vein
SP - sieve plates of the sinusoidal endothelium

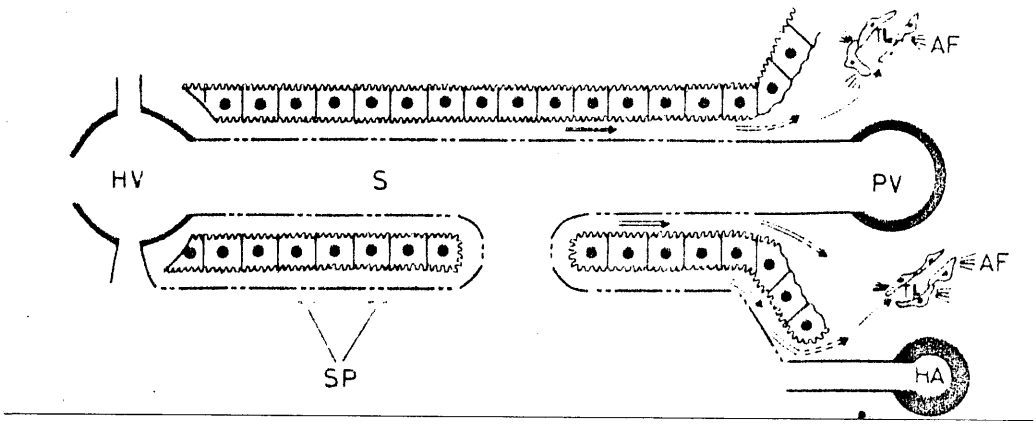

AF
HV
S
PV
AF
SP
HA

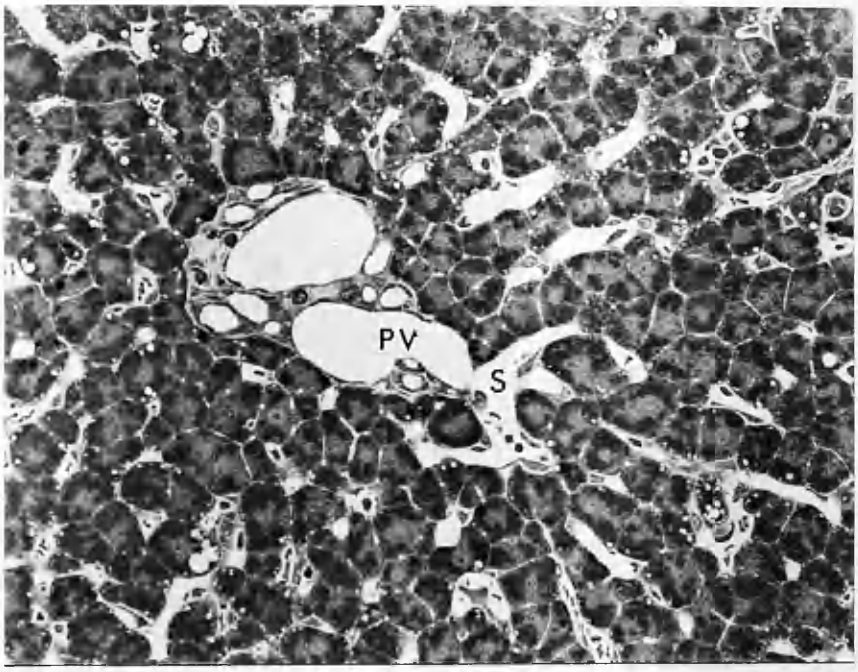
Fig. 4

A. As the portal vein branch (PV) enters the sinusoid (S) it is difficult to recognize a space between the wall of the vein and the hepatocytes of the limiting plate.
x 312

B. A T.E.M. of the same area which reveals that the space between the wall of the vein and the hepatocytes of the limiting plate is of the same order of size as Disse's space.

PV - portal vein
S - sinusoid

x 5880


PV
S

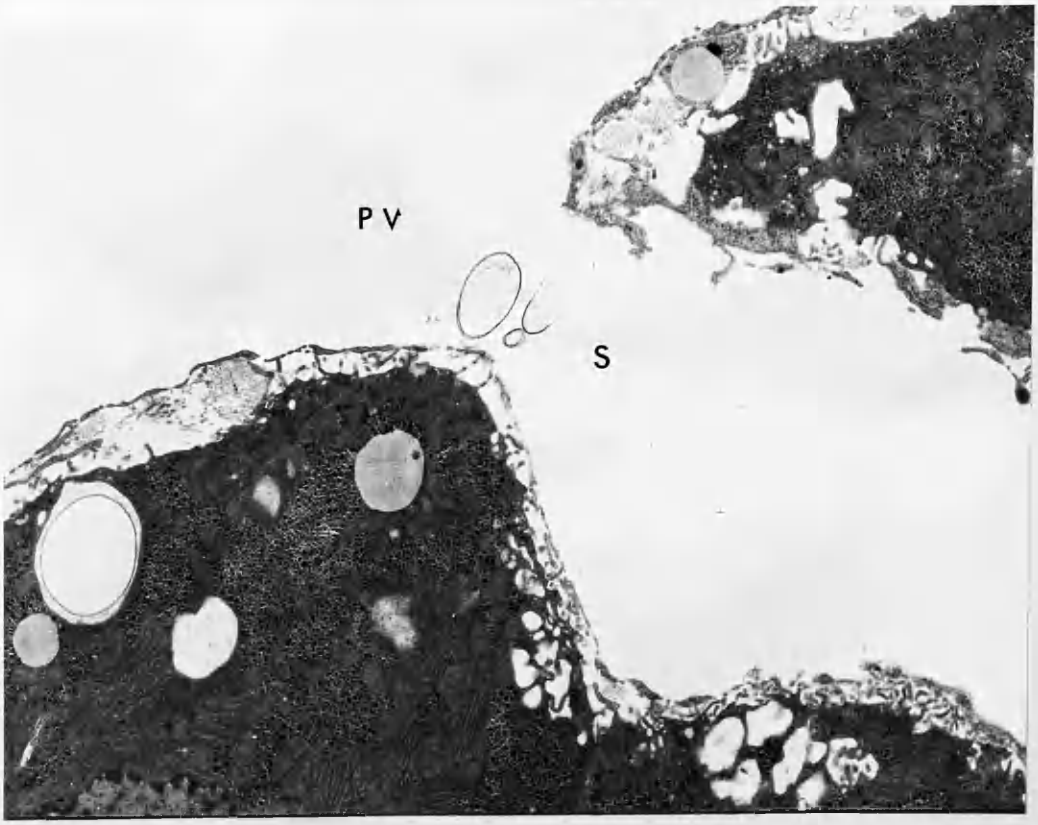

PV
S

Fig. 5

A gap (G) between 2 hepatocytes of perilobular limiting plate. It is 17 µm long, 2 µm wide, and extends between Disse's space (D) of a sinusoid (S) and portal tract (P) and space of Mall (M). It contains a fibroblastic process embracing a bundle of collagen.
x 7840


P
M
G
D
S

Fig. 6

A higher magnification of the contents of the previous gap in Fig. 5 showing the three main contents.

1. C - collagen
2. F.P. - fibroblastic process
3. M.V. - microvilli

x 28000

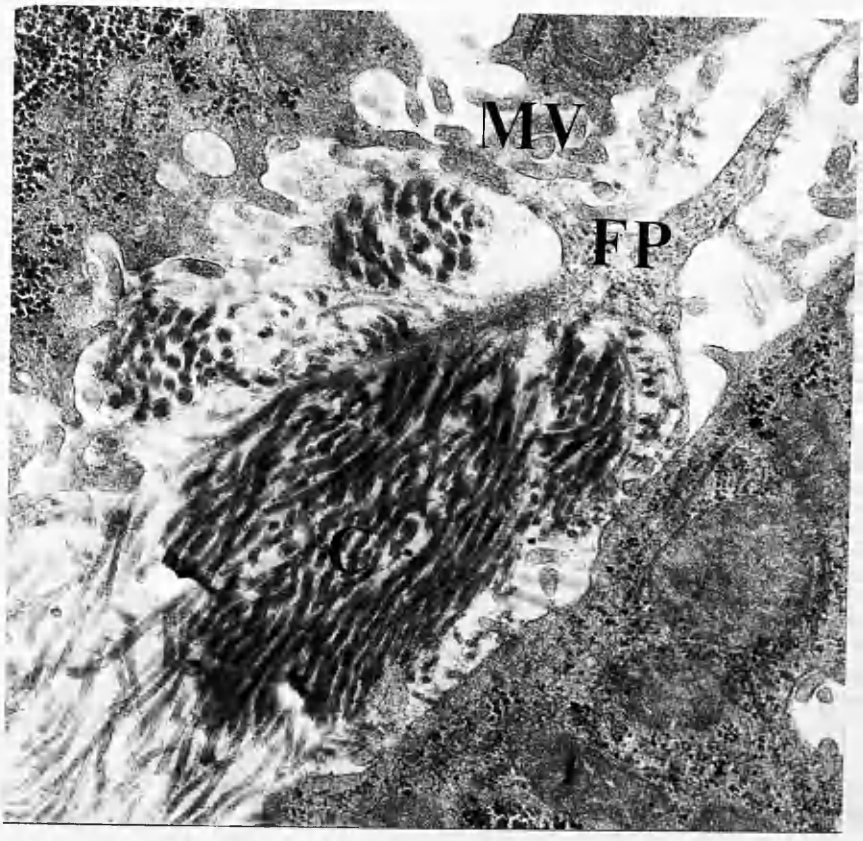

MV
FP

Fig. 7

A gap (G), 2 µm long, 4 µm wide between two hepatocytes of the limiting plate. It extends between Disse's space (D) of a sinusoid (S) and the space of Mall (M) and portal tract (P).
x 5880


P
M
G
D
S

Fig. 8

A gap (G), about 8 µm long and 1 µm wide, between two hepatocytes of a limiting plate.
x 7840

Fig. 9

A gap (G), about 5 μm long and 5 μm wide, between two hepatocytes of a limiting plate.

Note: A fibroblast (F) whose processes extend into the space of Disse (D).

x 7840

G
F
D

Fig. 10

A gap (G), about 3 µm long and 5 µm wide, between two hepatocytes of a limiting plate.

CM - chylomicrons

x 7840

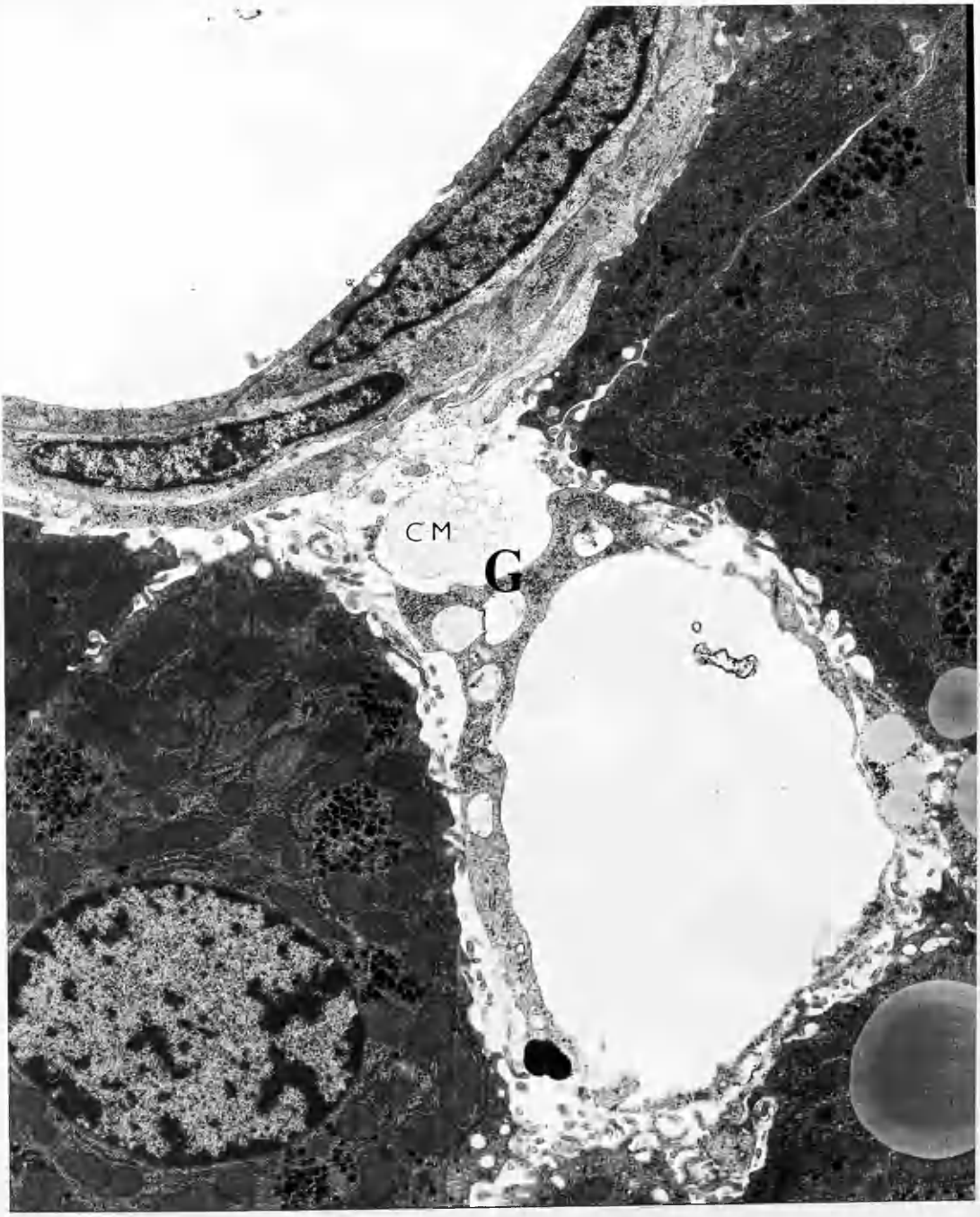

CM
G

Fig. 11

A gap (G), about 3 µm long and 2 µm wide, between two hepatocytes of a limiting plate.

Note: a bile ductule (BD) faces the gap.

x 7840

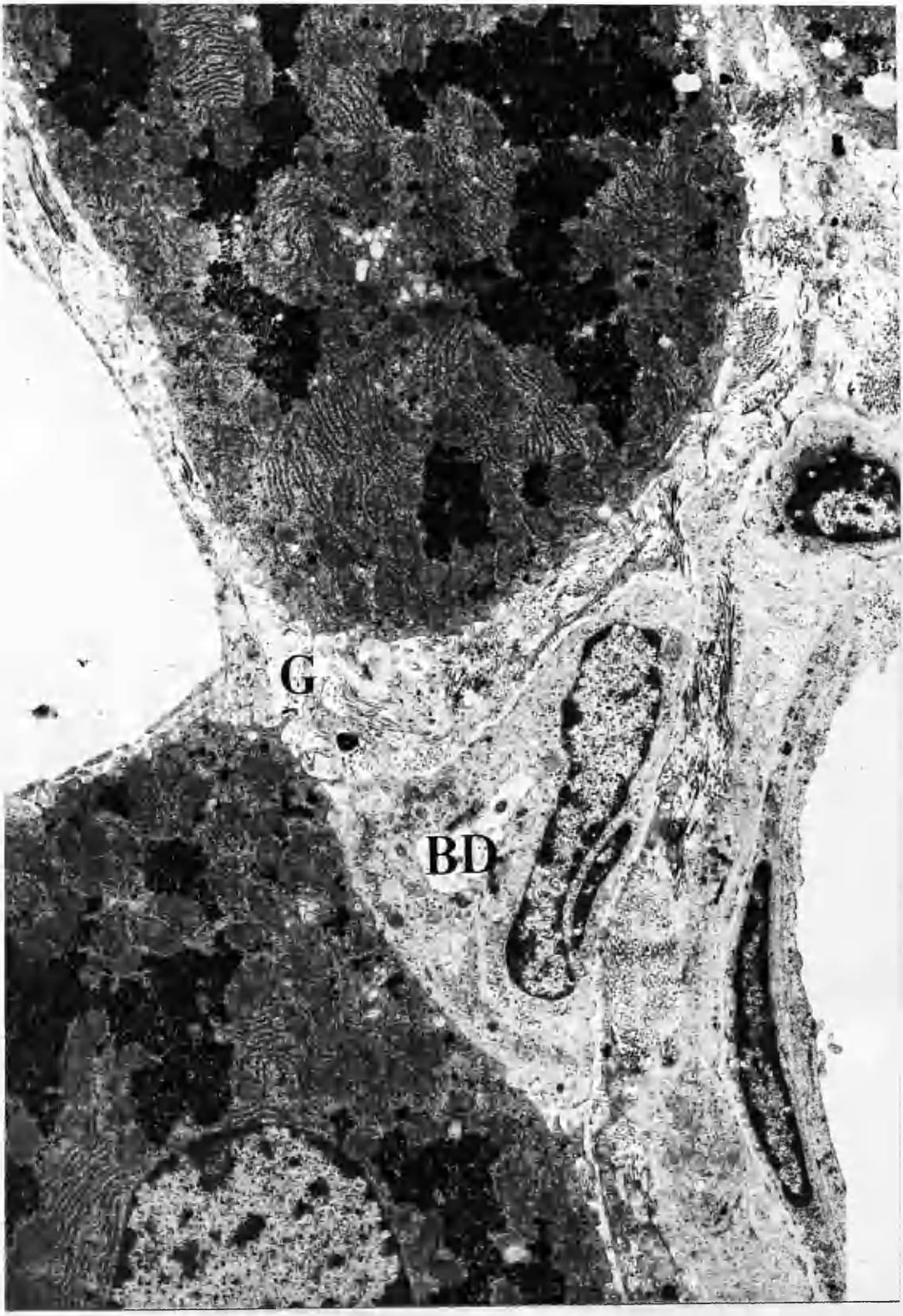

G
BD

Fig. 12

A gap (G), about 1.5 µm long and 3 µm wide, between two hepatocytes of a limiting plate.
x 9800

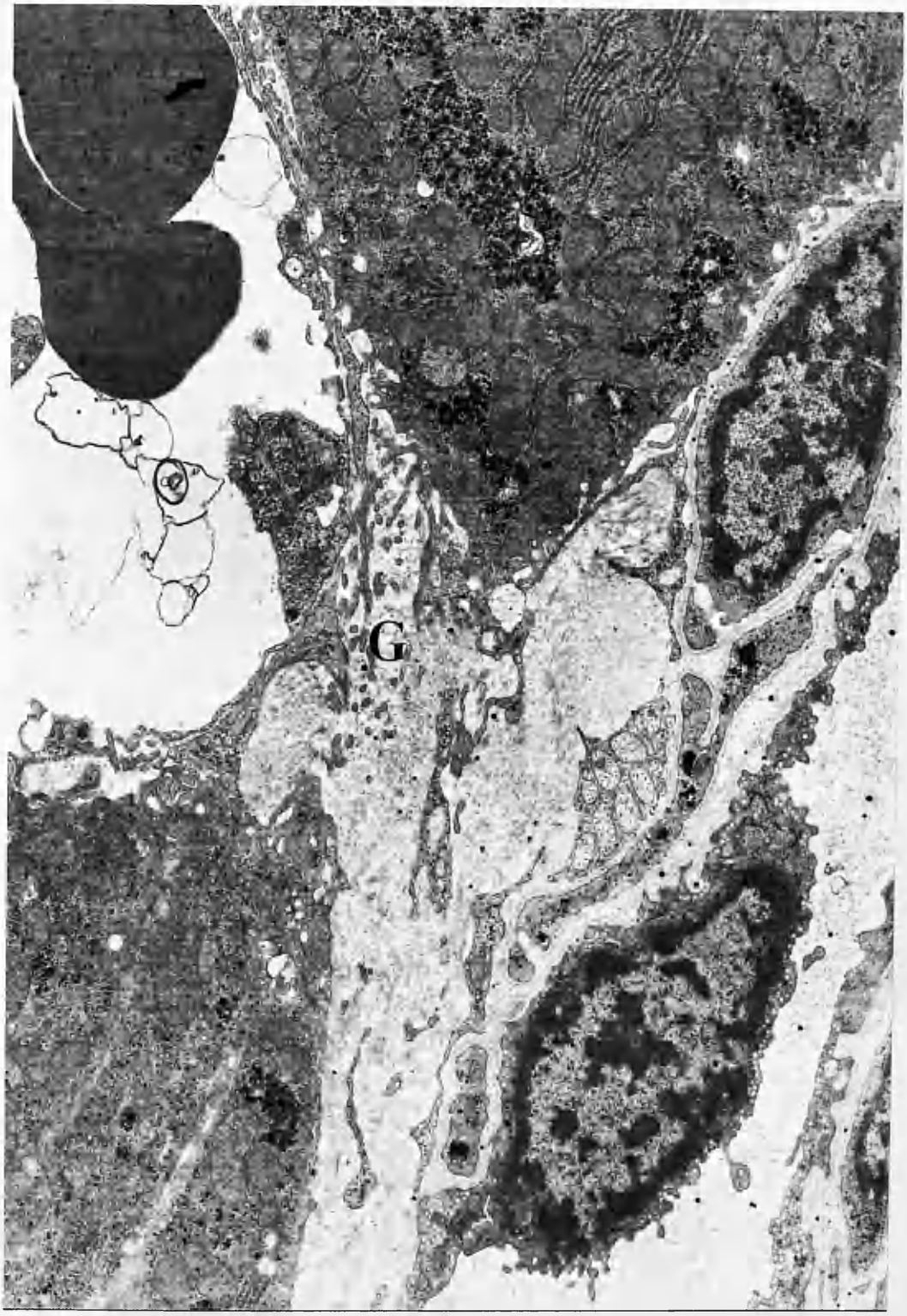

G

Fig. 13

A gap (G), about 3.5 μm wide, between two hepatocytes of a limiting plate.

Note: the sinusoidal endothelium (S.E.) nearly abuts on the portal tract interstitium (PT).

x 5880

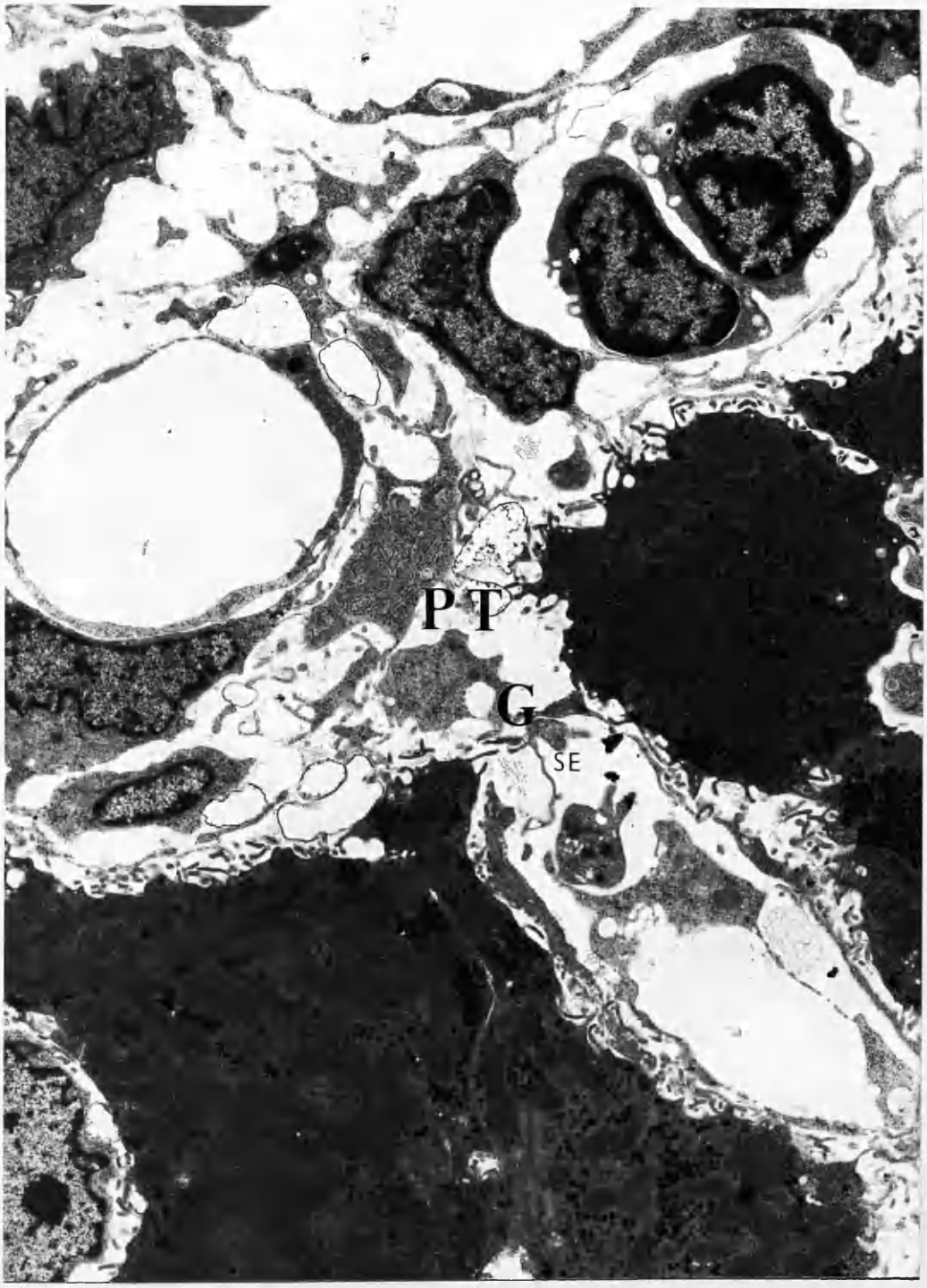

PT
G
SE

Fig. 14

A gap (G) about 3.5 μm wide between two hepatocytes of a limiting plate where the sinusoidal endothelium (SE) abuts directly on the portal tract interstitium (PT).
x 15680


PT
G
SE

Fig. 15

A gap (G) about 12 μm wide between two hepatocytes of a limiting plate where the sinusoidal endothelium (SE) abuts directly on the interstitium of the portal tract (PT).
x 5886

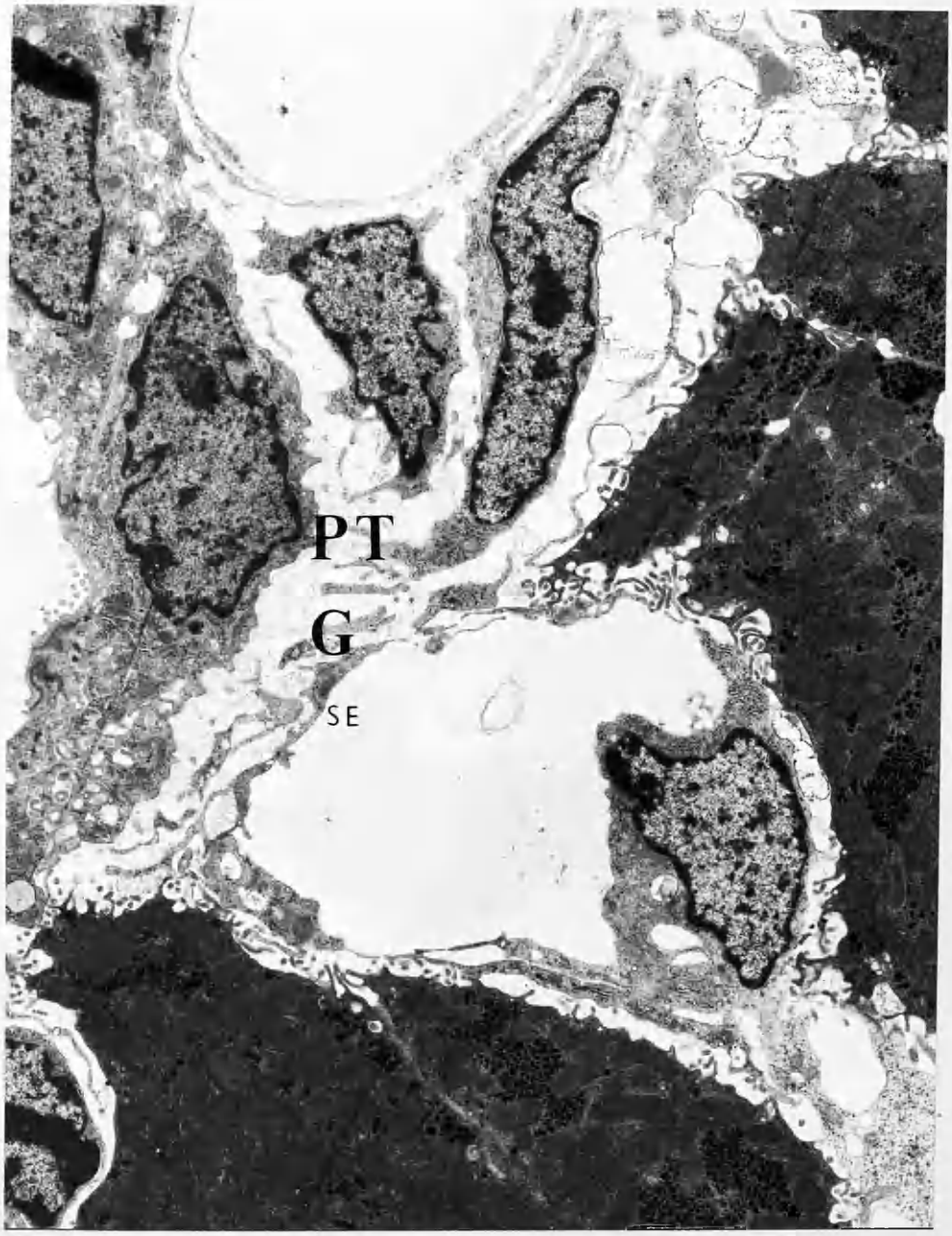
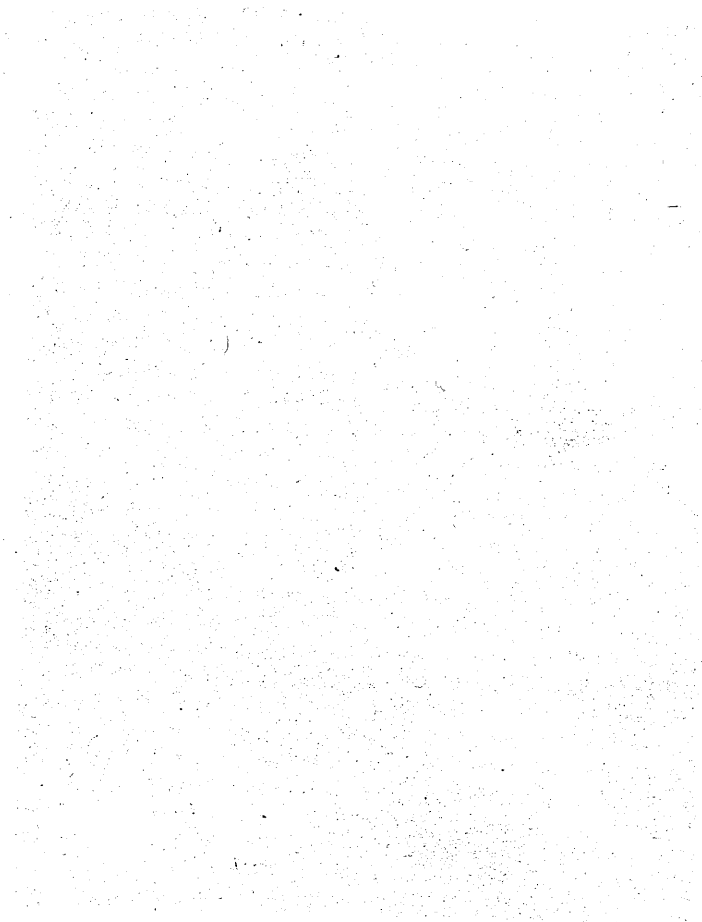

PT
G
SE

Fig. 16

The endothelium (E) of a longitudinal sectional profile of a sinusoid (S) abuts directly on the interstitium of the portal tract (PT) through a gap (G), which is about 32 µm wide, between two hepatocytes of a limiting plate.
x 3920




S E
G PT

Fig. 17

The gap (G) can be traced into continuity with the perisinusoidal spaces of Disse (D) associated with the two sinusoids S1 and S2 and their interconnections.
x 5880

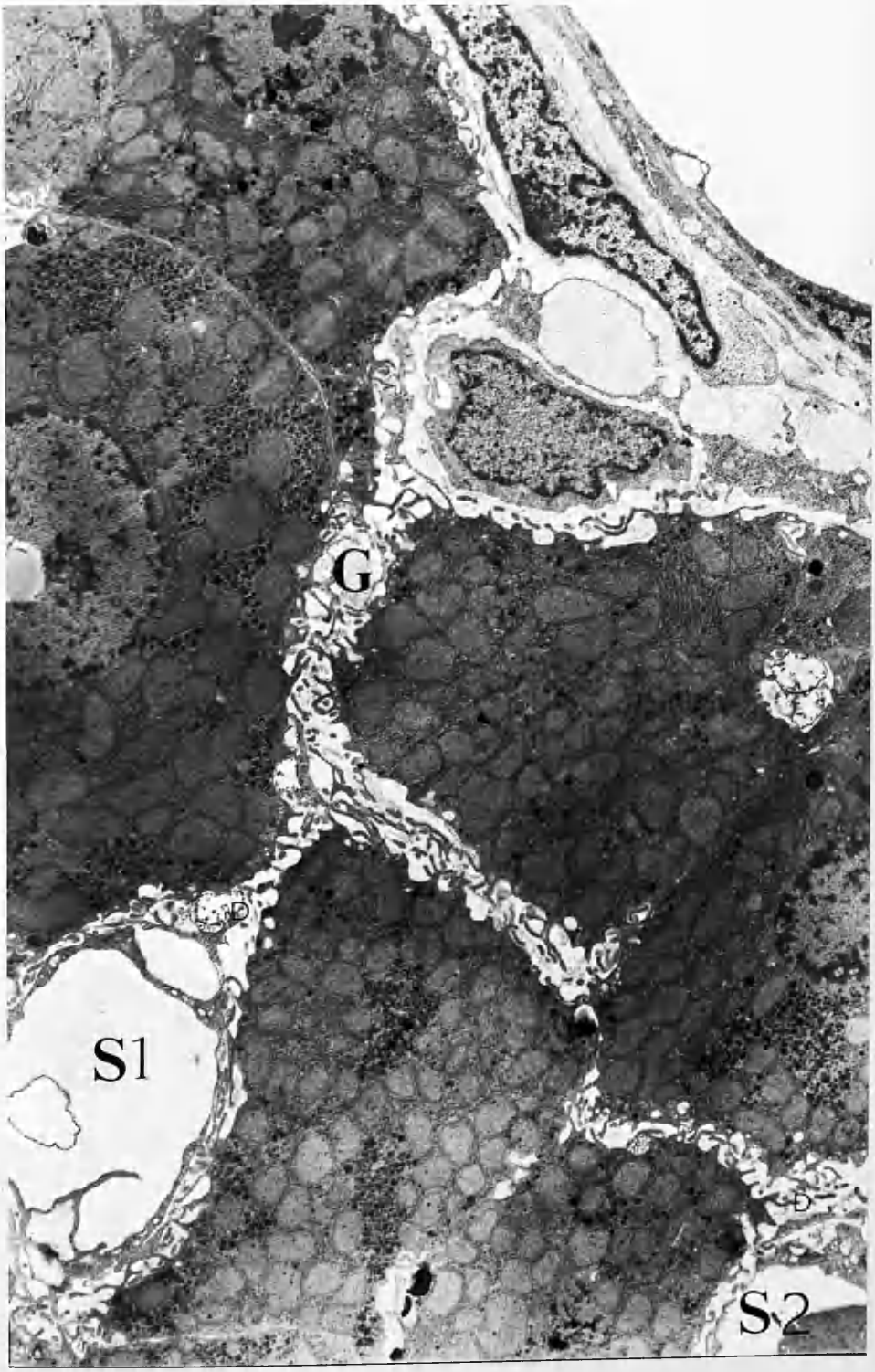

G
S1
S2

Fig. 18

This is one example of the ramifications of the space of Disse (D) between hepatocytes.

Note: the contrast has been deliberately increased to emphasize the intercellular expansions of Disse's space.

x 5880

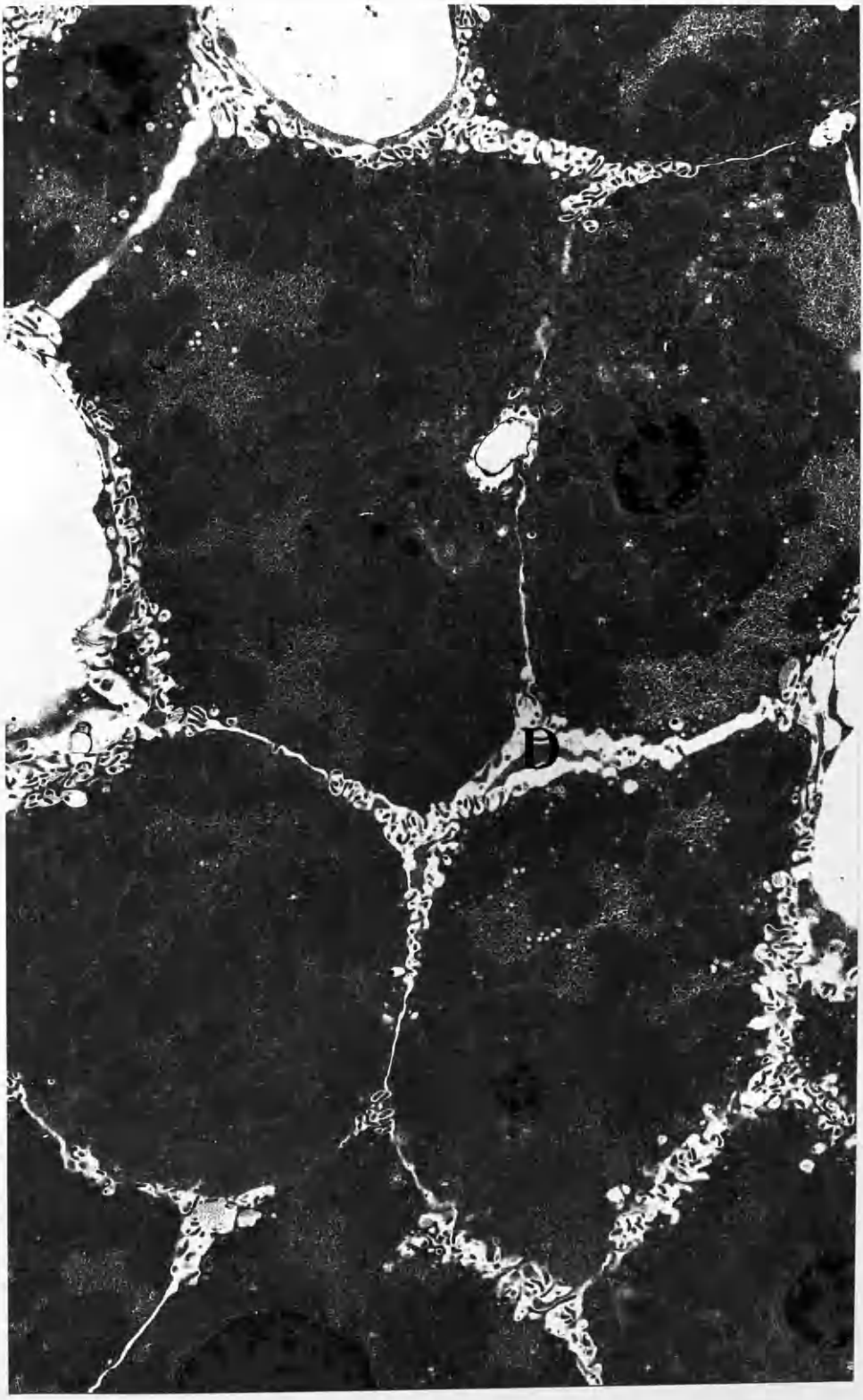

D

Fig. 19

This gap (G) between two hepatocytes of a limiting plate is mainly occupied by a bundle of collagen fibres which extend into spaces of Disse of several sinusoids.
x 3920

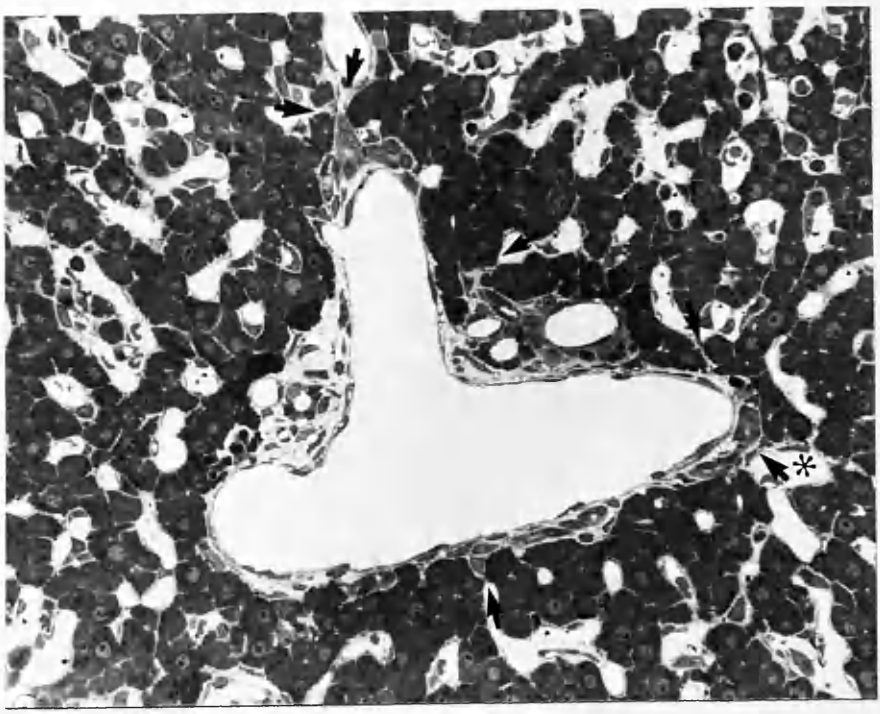
G

Fig. 20

In the sectional profile of this portal tract one can recognize 6 gaps (arrows) in the perilobular limiting plate, onle one of which (*) may possibly be associated with an entry of a portal vein branch.
x 360

Fig. 21

In the profile of this portal tract it is possible to recognize 7 gaps (arrows) in the perilobular limiting plate of which only one (*) can be related to an entry of a portal vein branch.
x 312



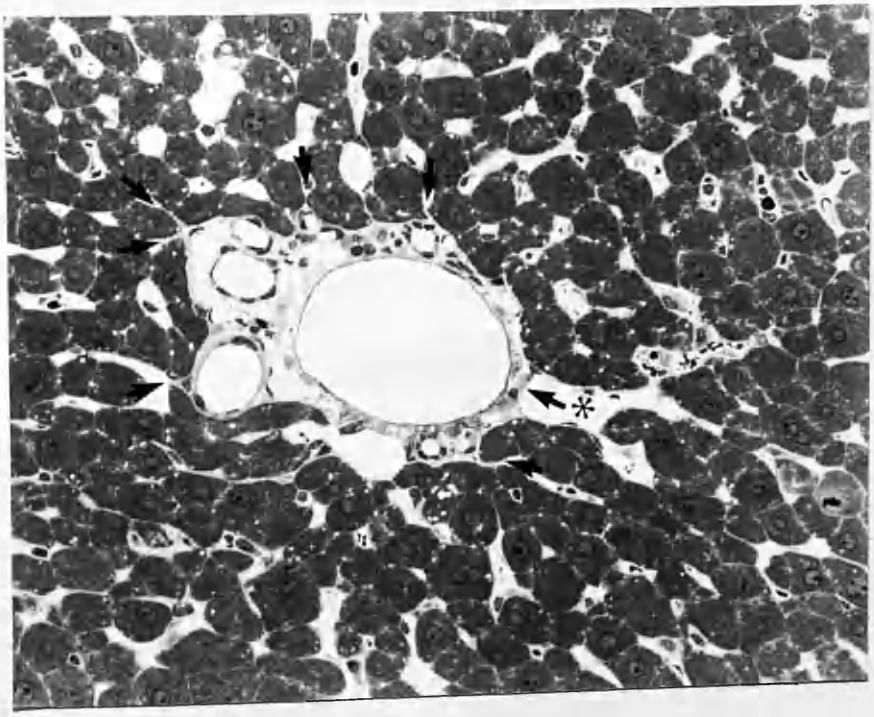


Fig. 22

There are 4 gaps (arrows) in the perilobular limiting plate of the sectional profile of this portal tract. x 312

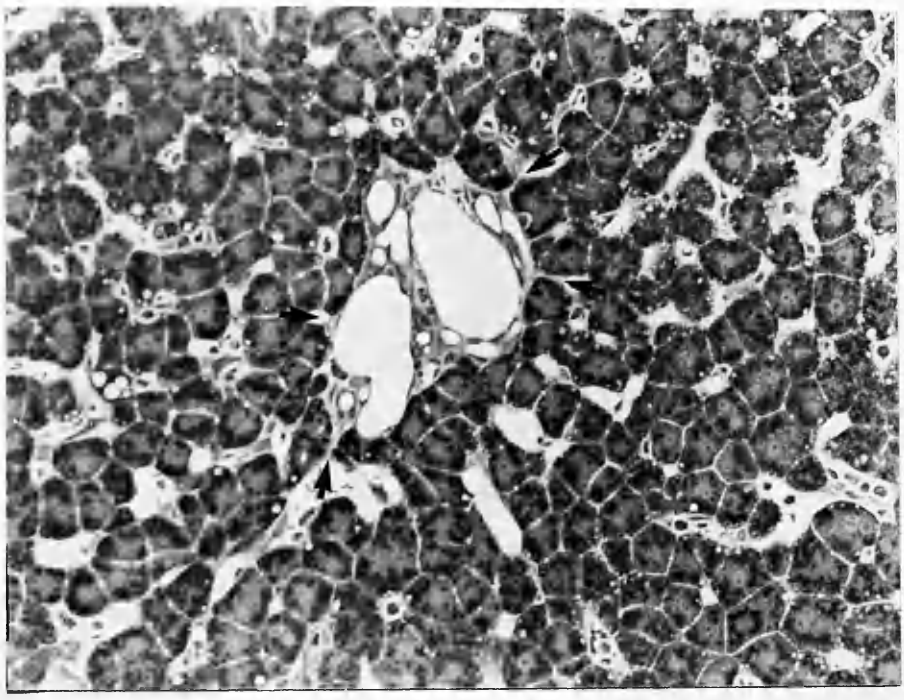


Fig. 23

It is possible to identify 2 gaps (G1, G2) in a TEM field at a relatively low power of 1400.
x 3920

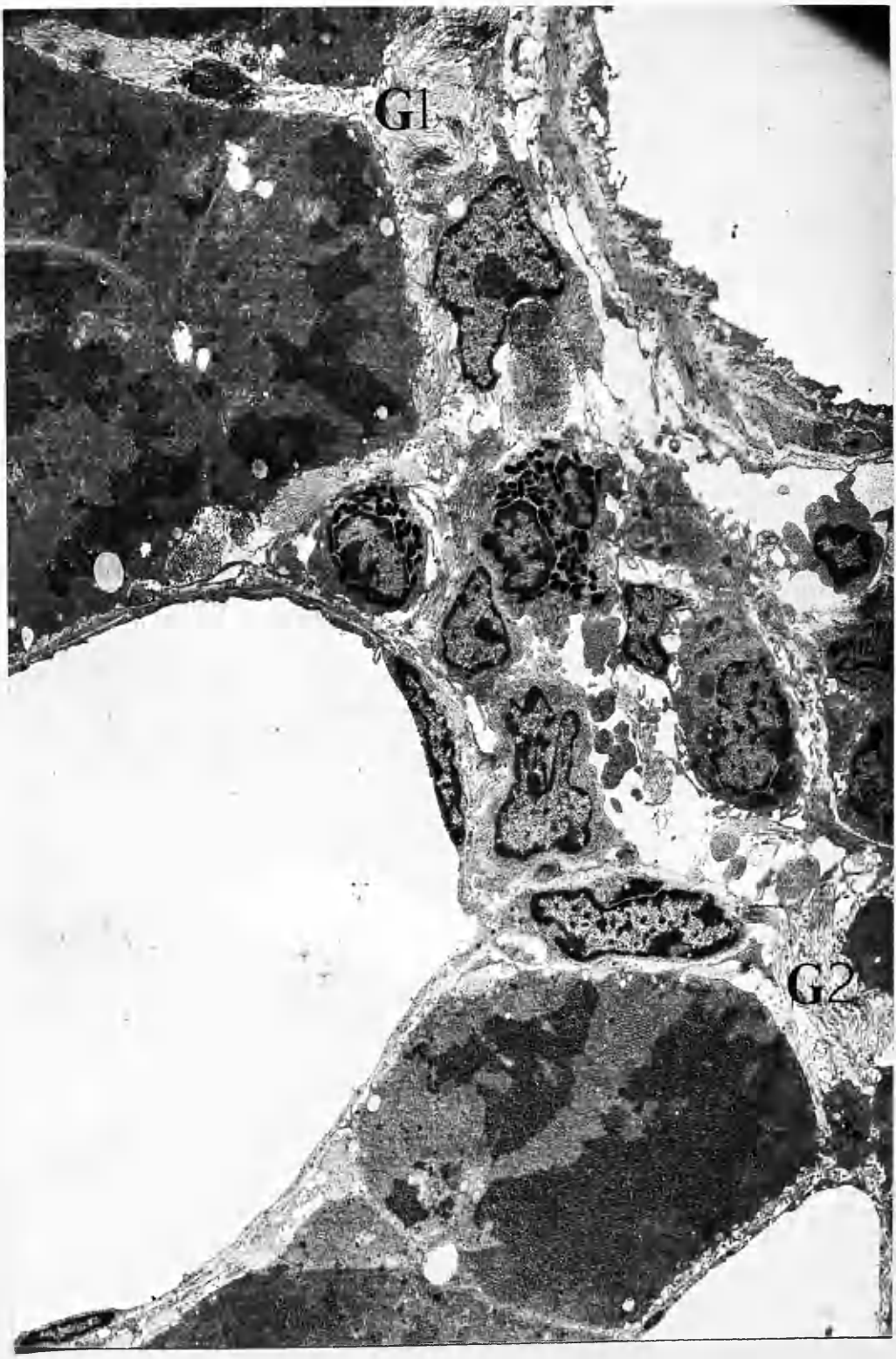

G1
G2

Fig. 24

Shows 3 adjacent gaps (G1, G2, G3) between 4 hepatocytes of a limiting plate.
x 5880

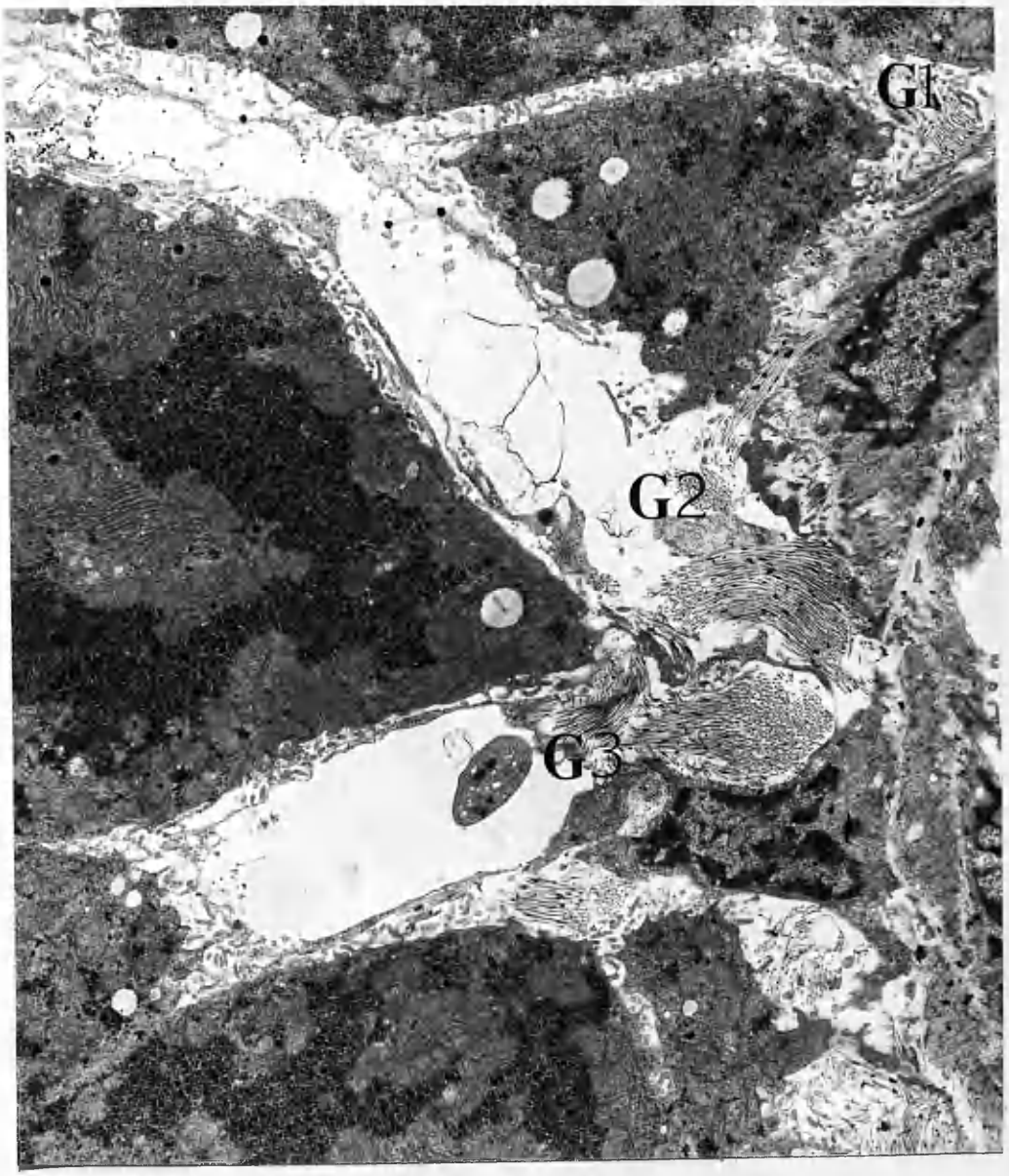
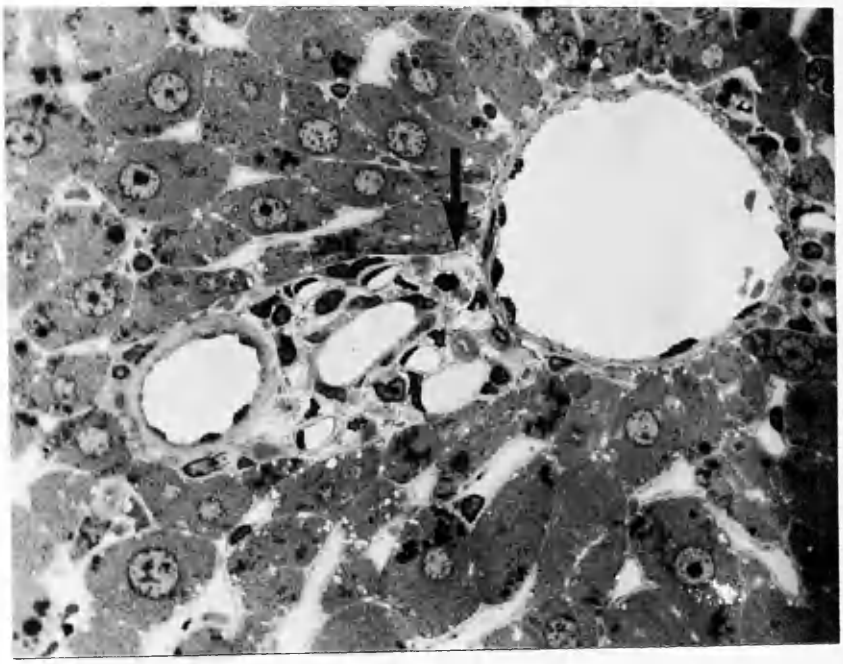

G1
G2
G3

Fig. 25

A. Photomicrograph of the smallest lymphatic profile (arrow) identified by optical microscopy.
x 320

B. An electronmicrograph of the adjacent section of the same area confirming that the vessel is in fact a lymphatic capillary (about 3 μm in diameter).
x 5880



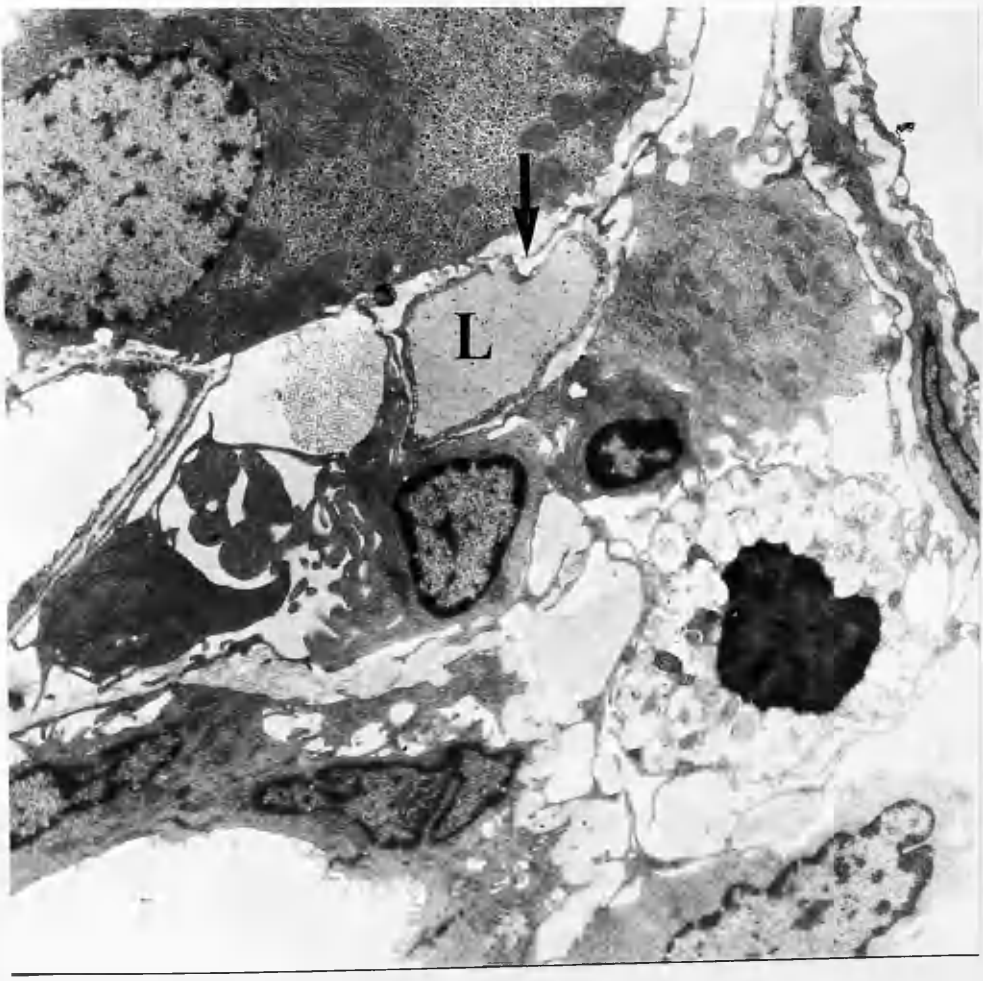

L

Fig. 26

Shows that in this portal tract profile, whose portal vein branch diameter is about 40 μm, lymphatics are absent.
x 520

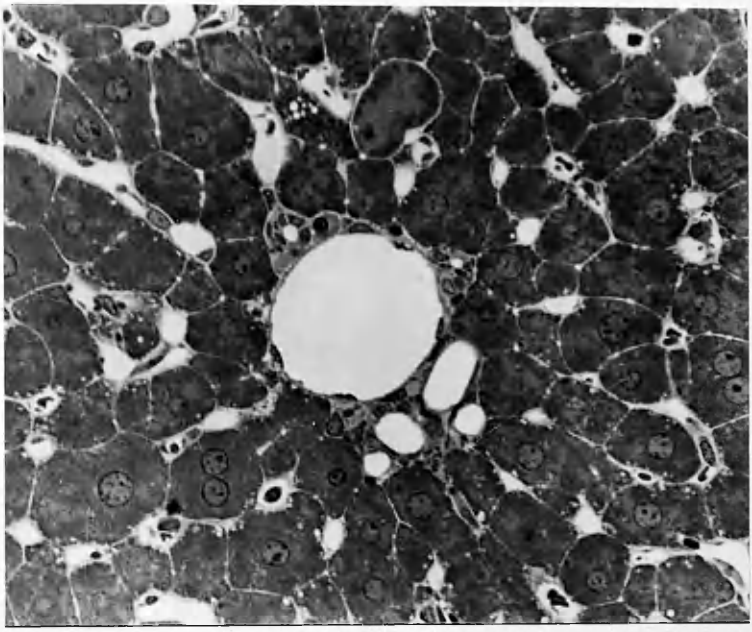
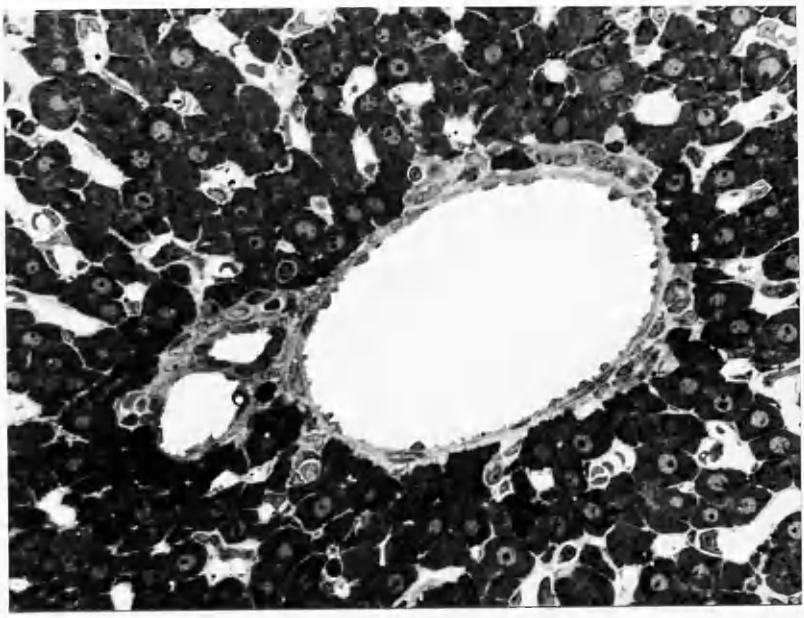


Fig. 27

A. Shows that there are no lymphatics in a portal tract sectional profile whose accompanying portal vein branch diameter is about 73 μm.
x 430

B. No lymphatic vessel could be seen even in this portal tract sectional profile, whose accompanying portal vein branch is about 125 μm in diameter.
x 430



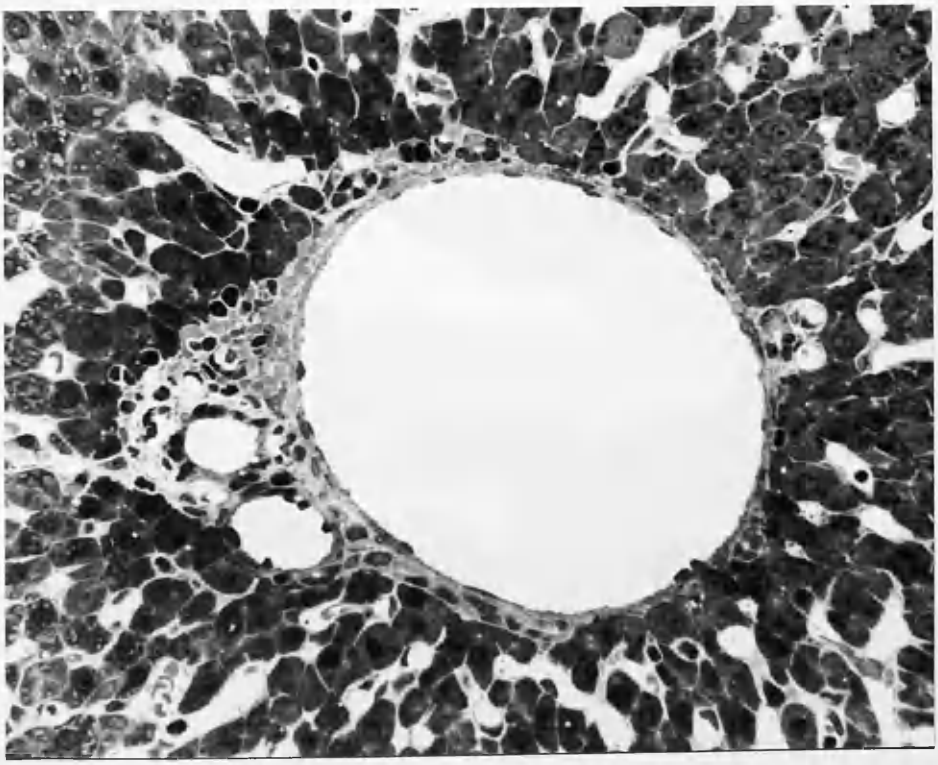


Fig. 28

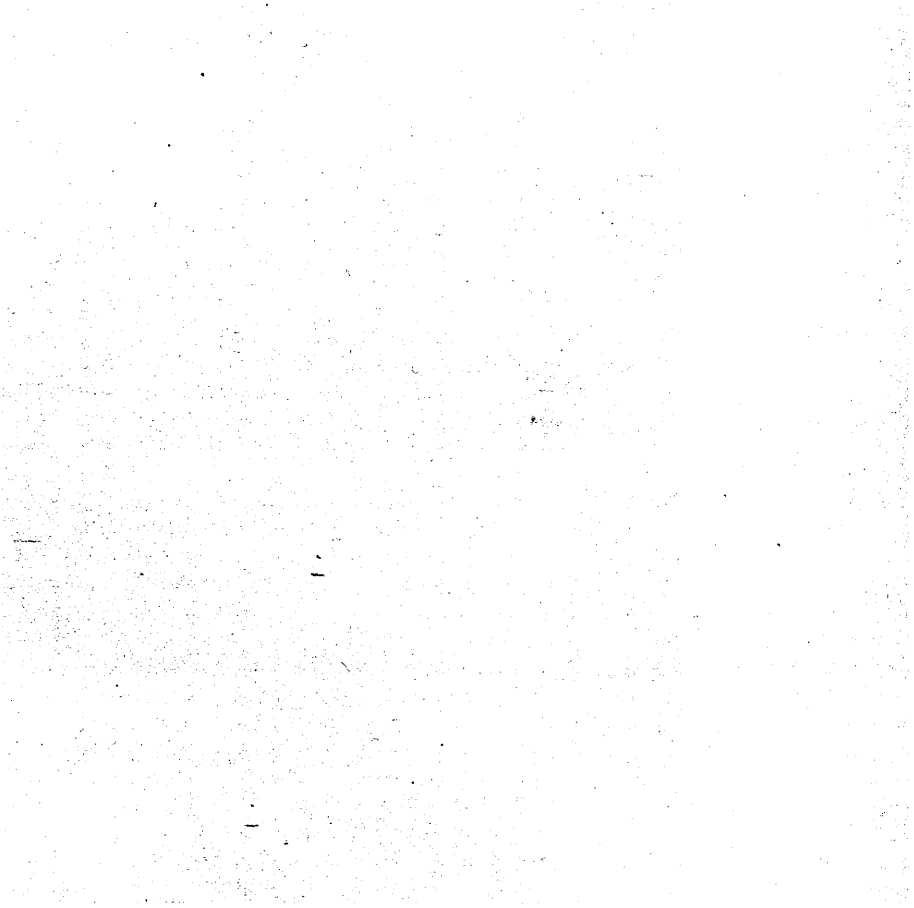
A SEM of part of a large portal tract.

Note: - the coarse, irregulary arranged collagen bundles (C).

- a typical scalloped lymphatic vessel (L) with a thin wall and proteinous contents.

BD - bile duct
C - capillary
V - vein

x 1030



C
D
V
L
C
01 L8!0 52 001

Fig. 29

A high magnification of a small area in the interstitium of a large portal tract.

Note: closely packed collagen bundles (C).
- a fibroblast (F)
- mast cell (M)
- bundle of nerve fibres (NF)
- a lymphatic vessel (L)
- a selnder fibroblastic process

x 15680

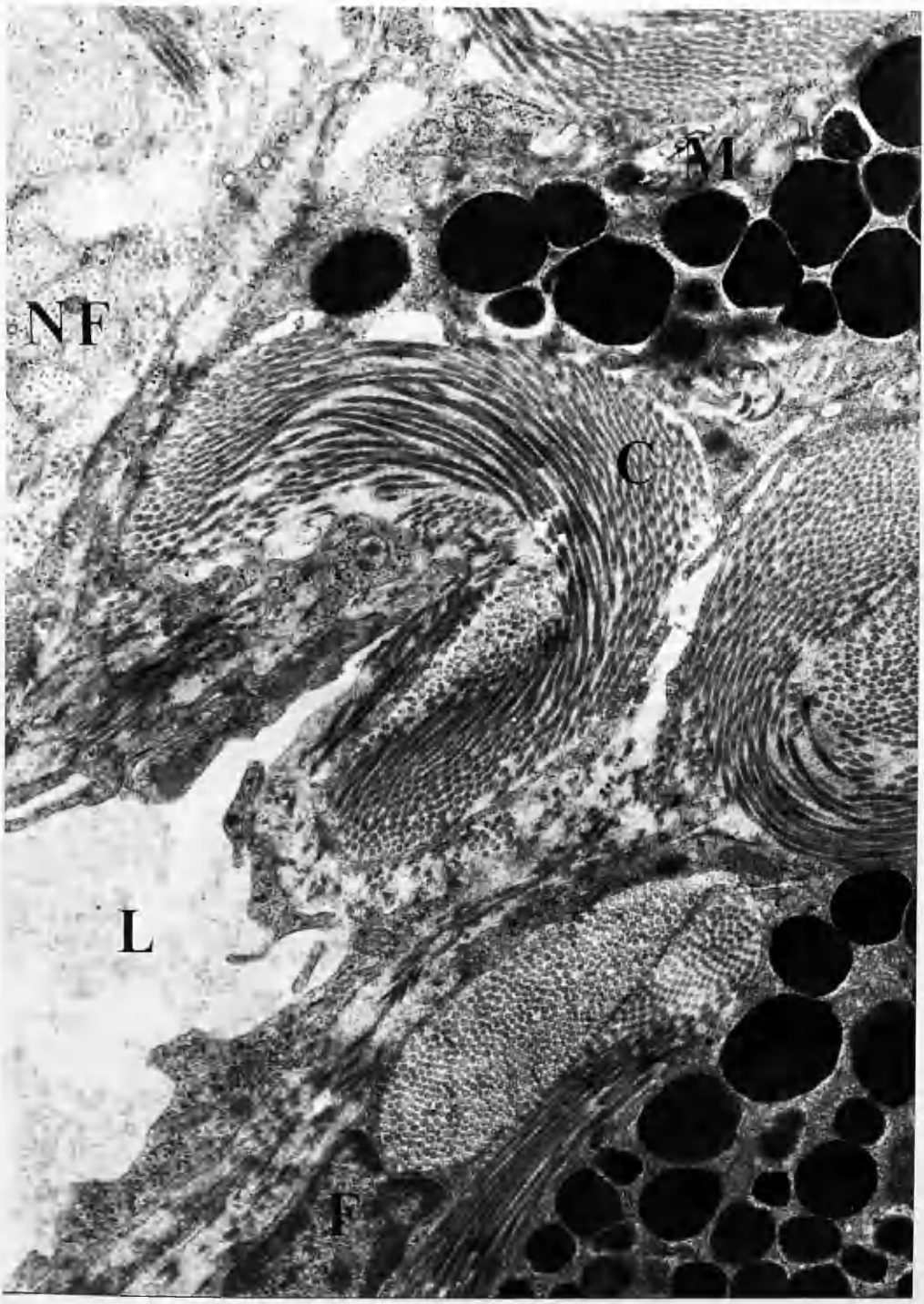

M
NF
C
L
F

Fig. 30

Showing an area in the interstitium of portal tract smaller than the previous one in Fig. 29.

Note:
- looser connective tissue
- compare the cytoplasmic processes of the fibroblast with the lymphatic endothelium of the lymph vessel (L)
- the intercellular junctions (J) between lymphatic endothelial cells.
- compare the density of the proteinous material in lymphatics with that in the loculi bounded by fibroblastic processes.
- the varying density of the collagen bundles.

x 15680

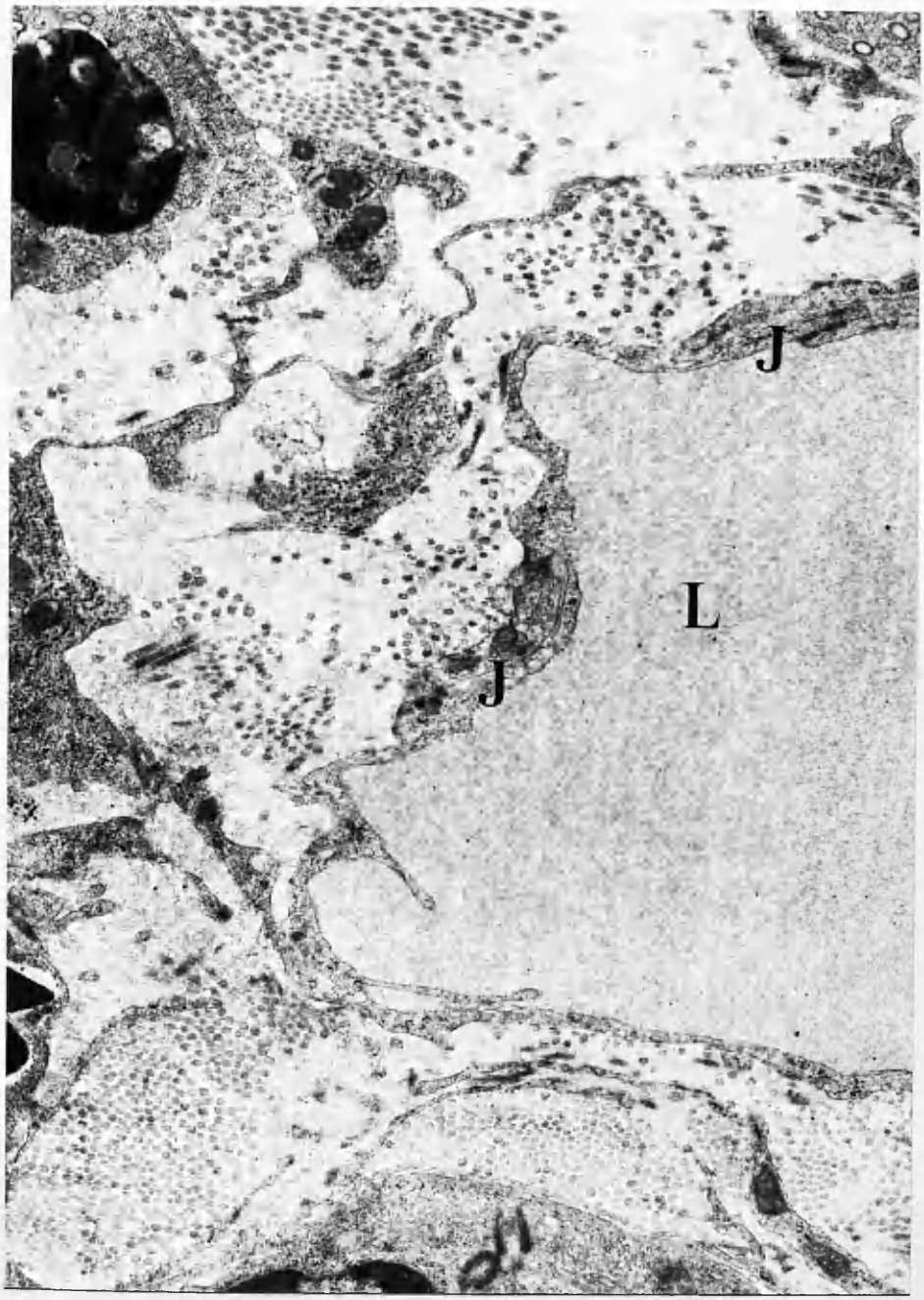

J
L
J

Fig. 31

Shows interstitial area of an even smaller and more peripheral portal tract.

Note: - much looser connective tissue.
- fibroblasts (F1, F2) sending out processes into the interstitium, thus creating loculi.

x 7840

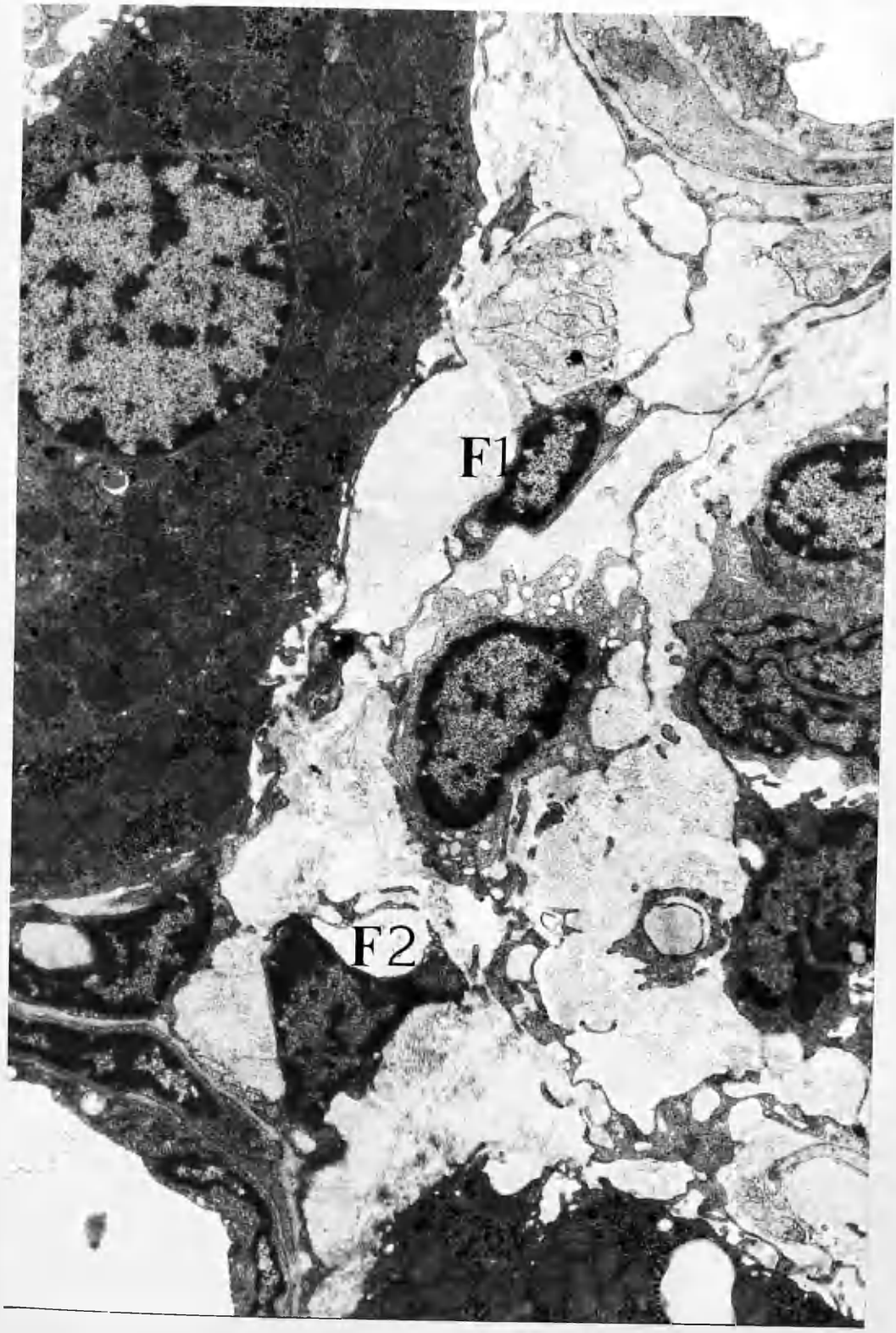
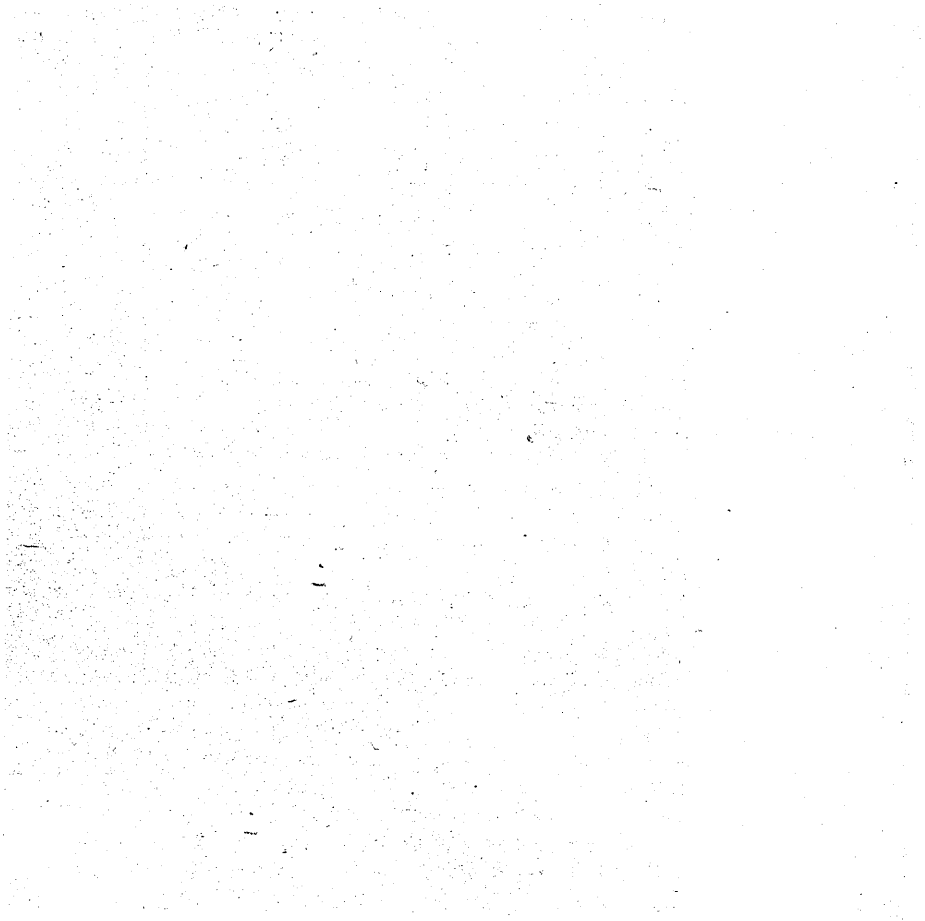

F1
F2

Fig. 32

Loculi (LOC) created by multiple processes of different fibroblasts with desmosome-like junctions (J).
x 19600



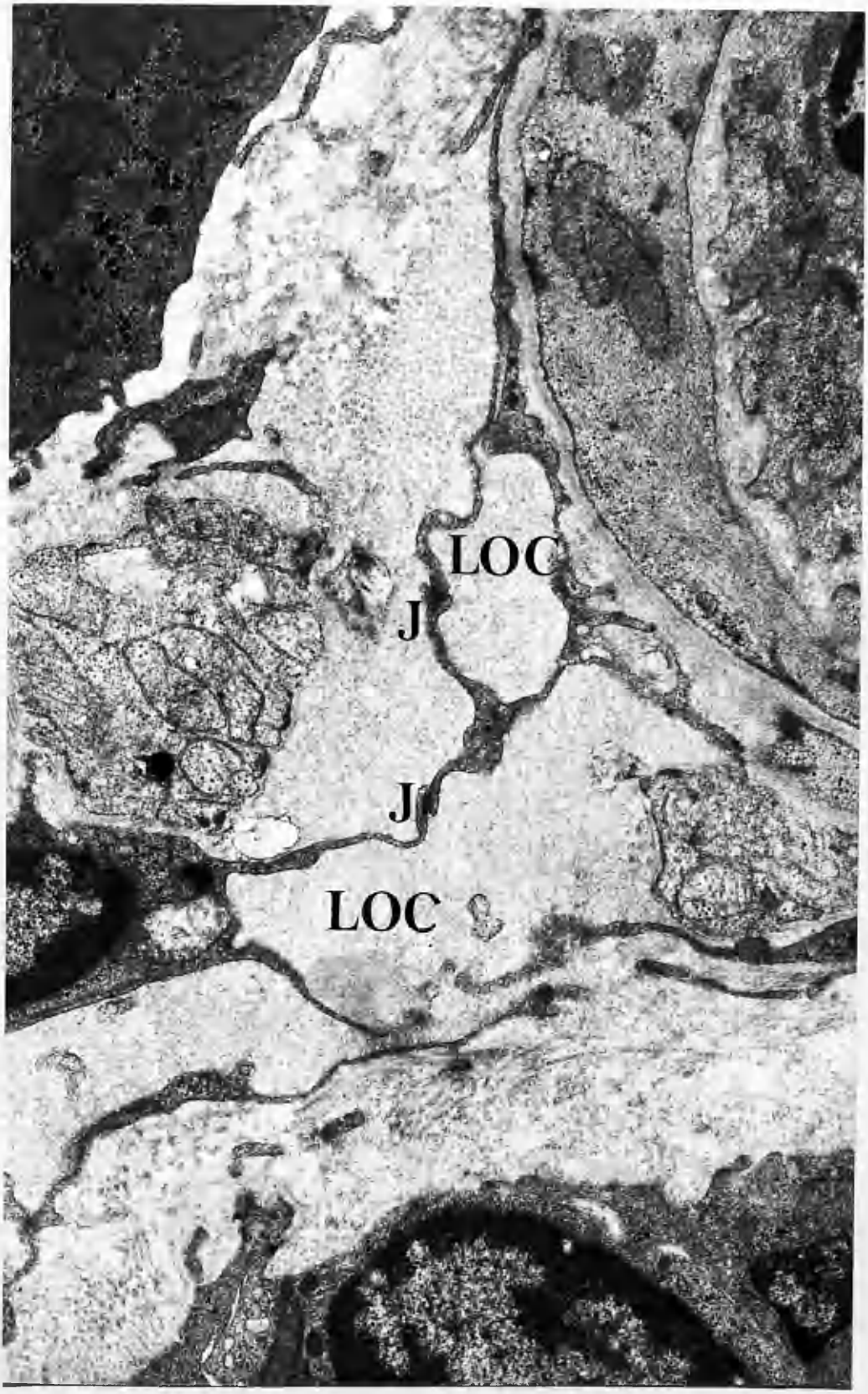
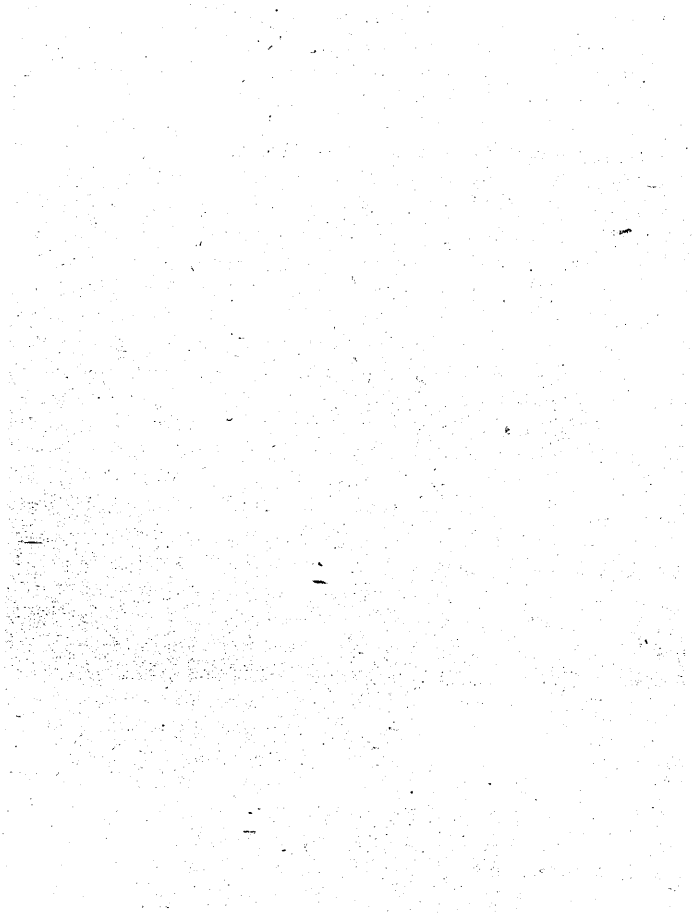

LOC
J
J
LOC

Fig. 33

Showing multiple fibroblastic processes (FP) enclosing a loculus that contains dense proteinous precipitate.

Note: - the area between the two lines AA', BB' might well be mistaken for endothelially lined lymphatics.
- collagen bundles CC' also make small part of the wall of the loculus.

x 15680



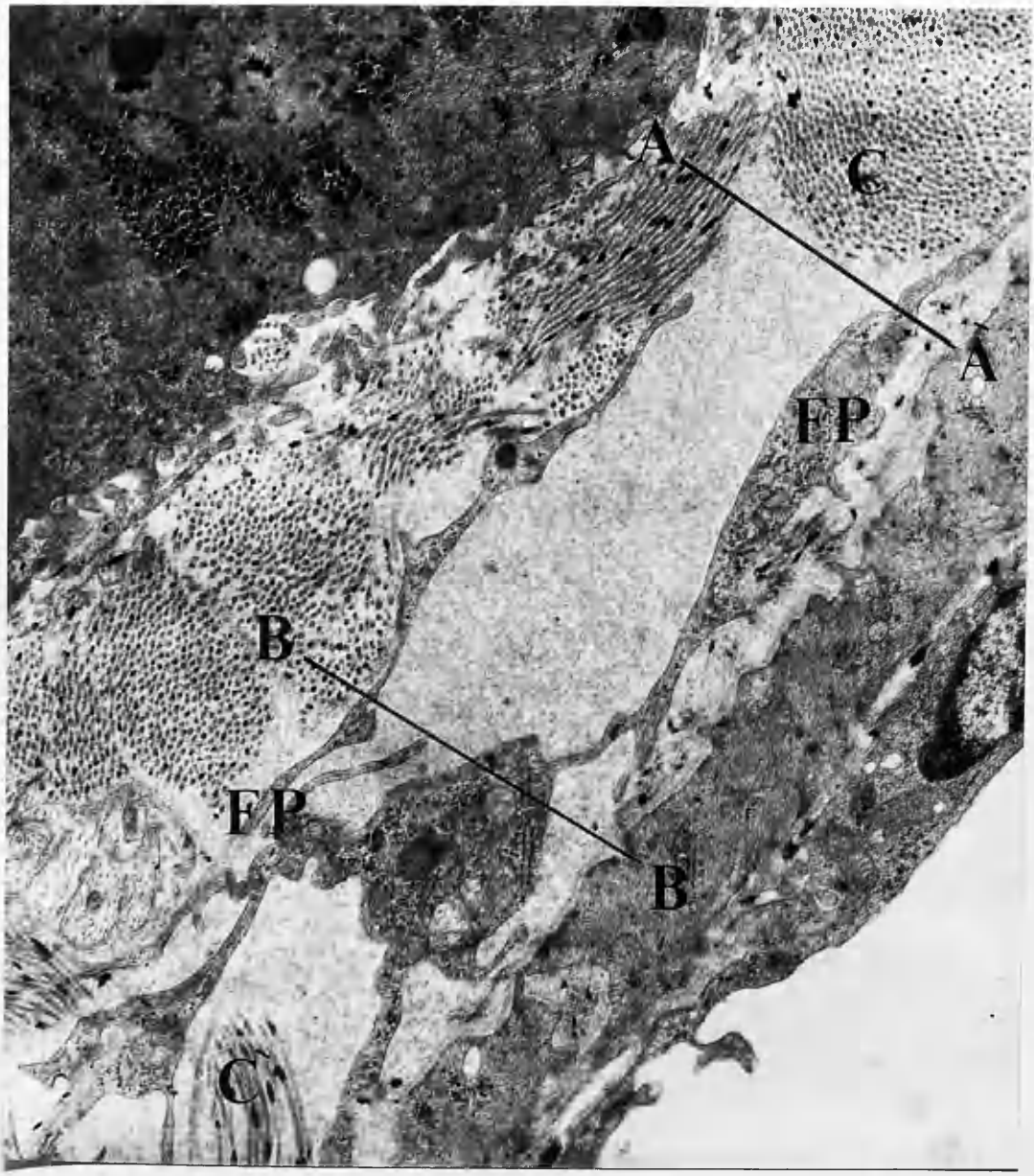
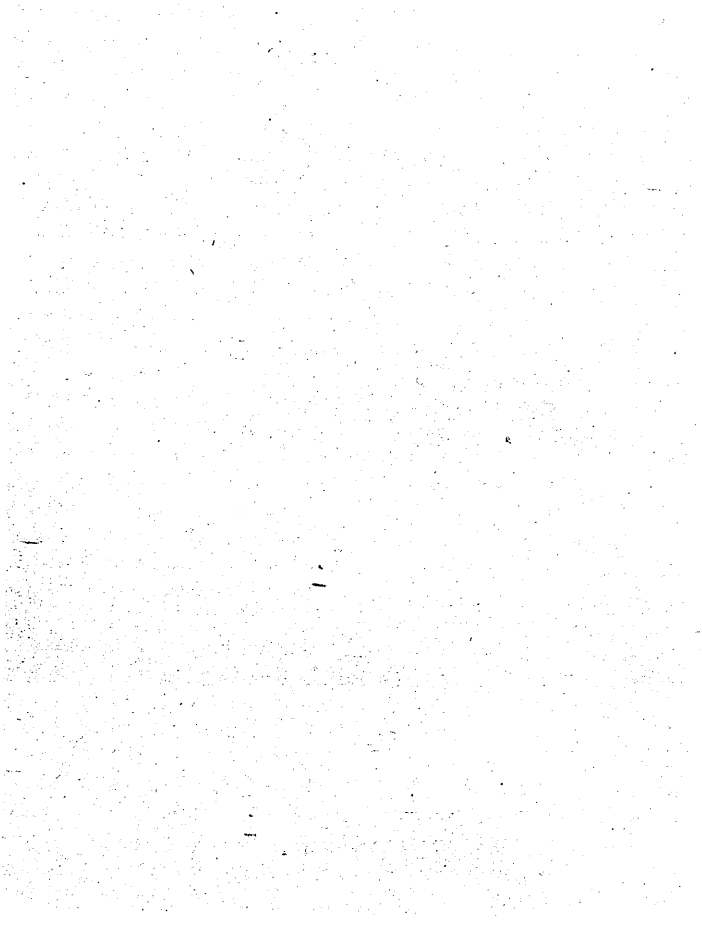

A
C
A
FP
B
FP
B
C

Fig. 34

Fibroblastic processes completely enclose a channel which might easily be mistaken for a lymphatic, although clearly it is not.

Note: - the nature and density of the contents
- the gaps (arrows) between fibroblastic processes that make the channel.

x 11760



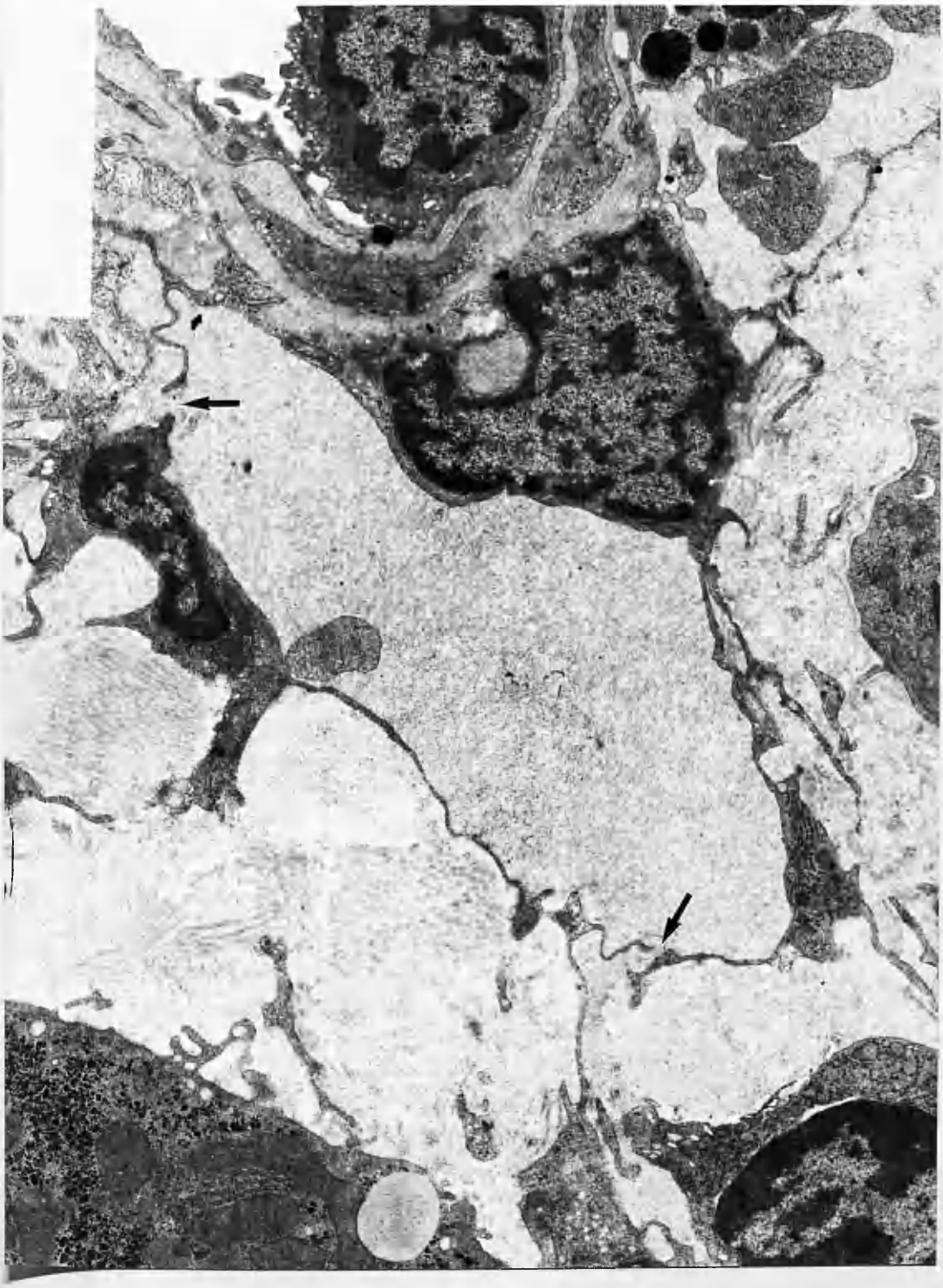


Fig. 35

A cross sectional profile of a fibroblastic channel (FC) which appeared to be a lymphatic by light microscopy. TEM revealed the fibroblastic nature of the lining, the collagen within the lumen (C) and the multiple openings.

- compare the contents with that of the neighbouring true lymphatics (L).

x 15680

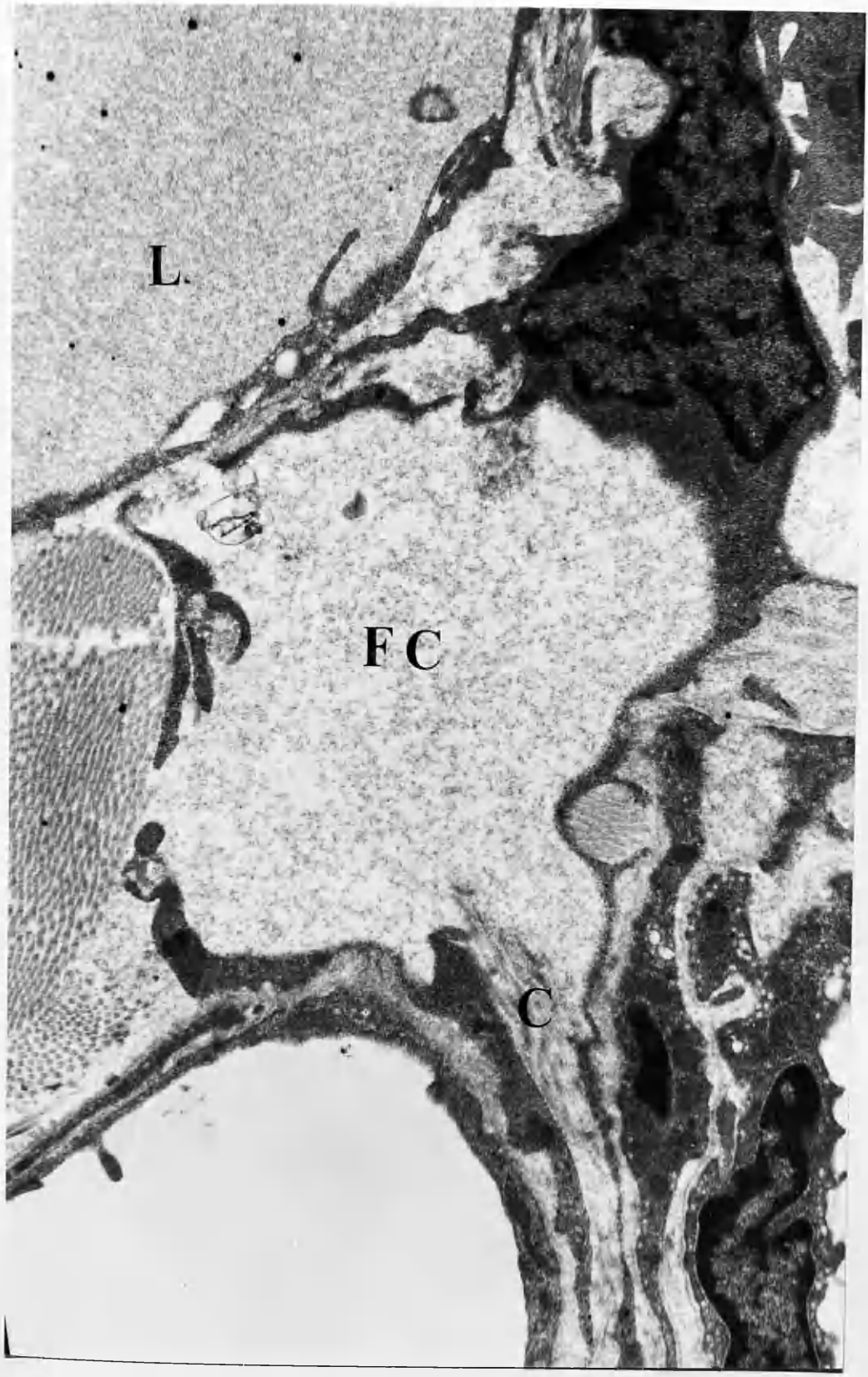

L.
F C
C

Fig. 36

Shows a longitudinal sectional profile of a fibroblastic channel which contains many cells (macrophages and neutrophils).

Note: the thin, interrupted and branching lining of fibroblastic processes.

x 7840

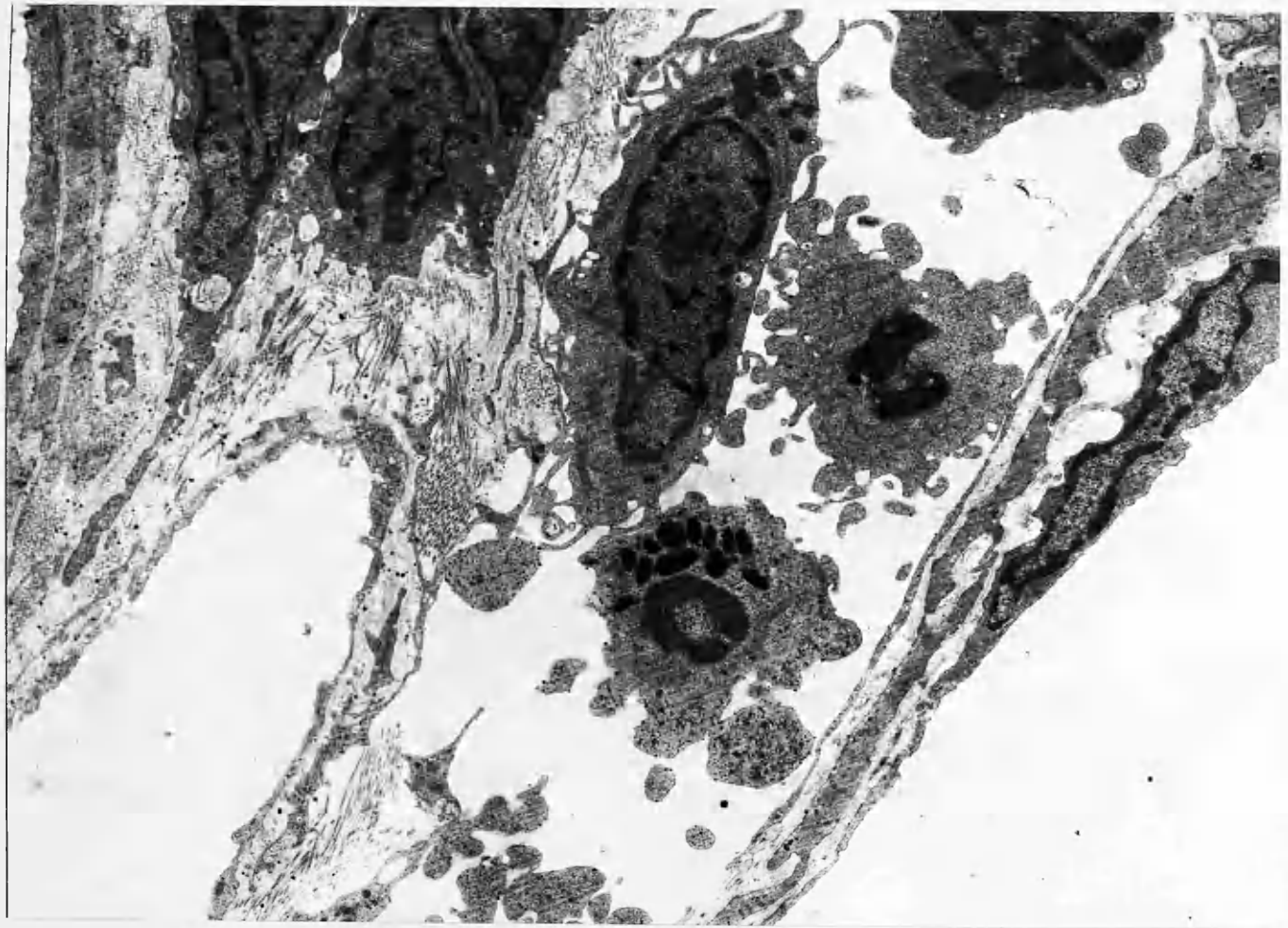
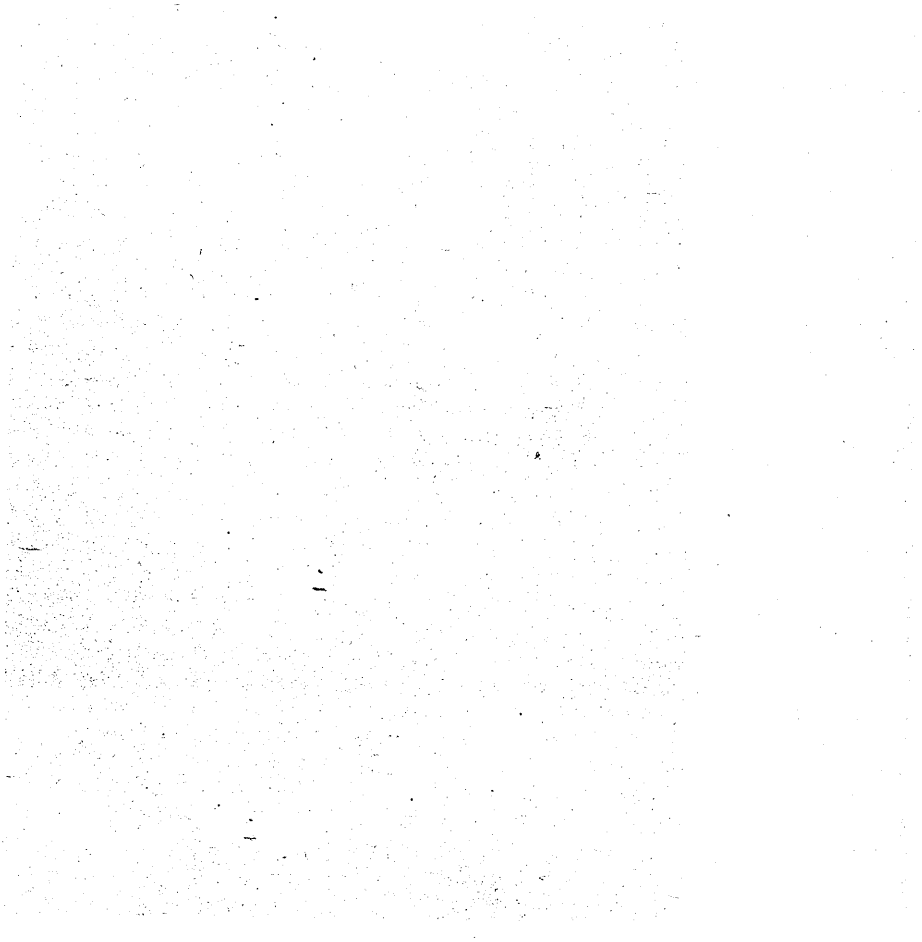


Fig. 37

Shows a longitudinal sectional profile of a fibroblastic channel.

Note:
- contents of precipitated protein and a mononuclear cell
- thin interrupted lining
- collagen (C) on the luminal and abluminal sides of the wall

x 15680



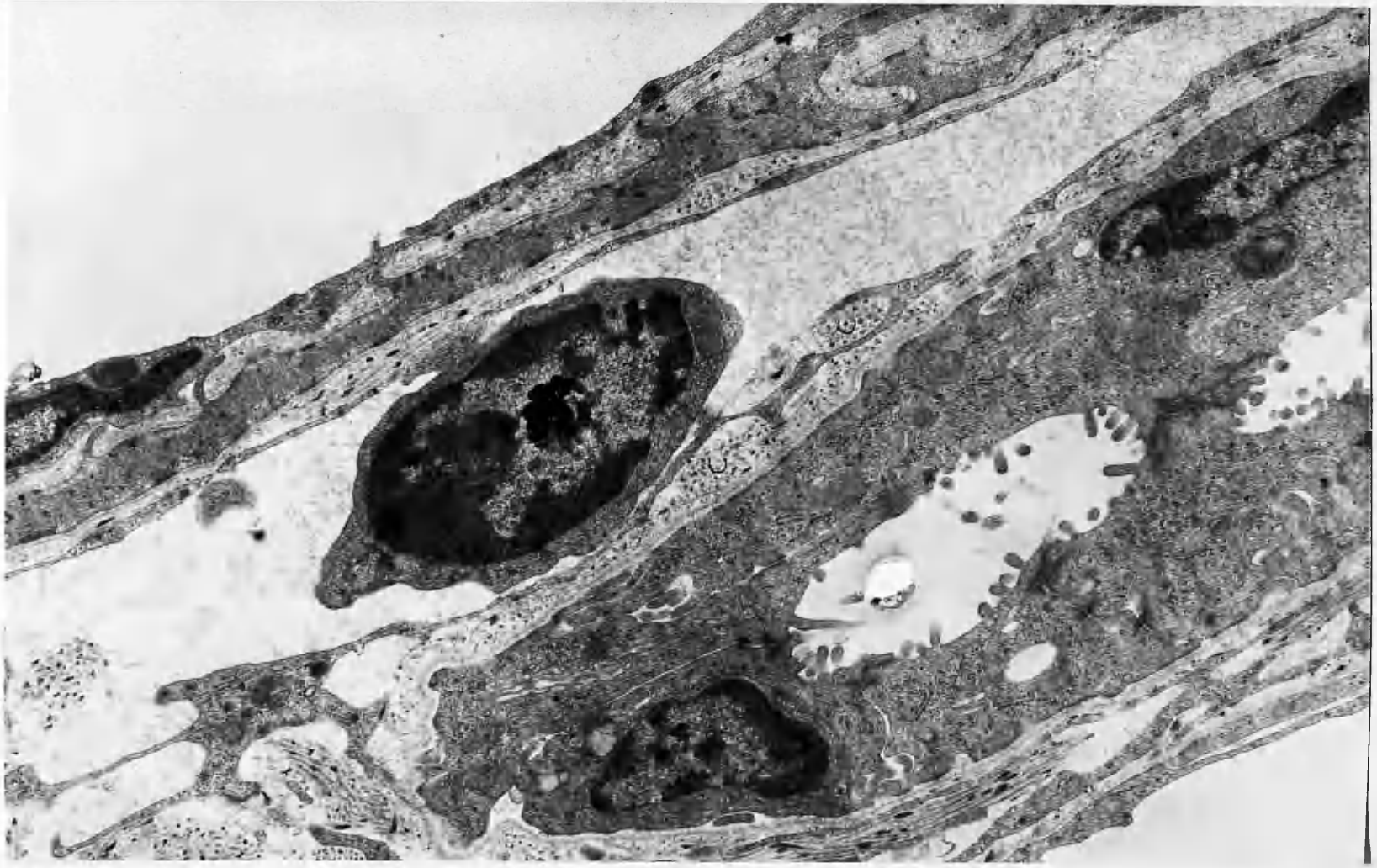
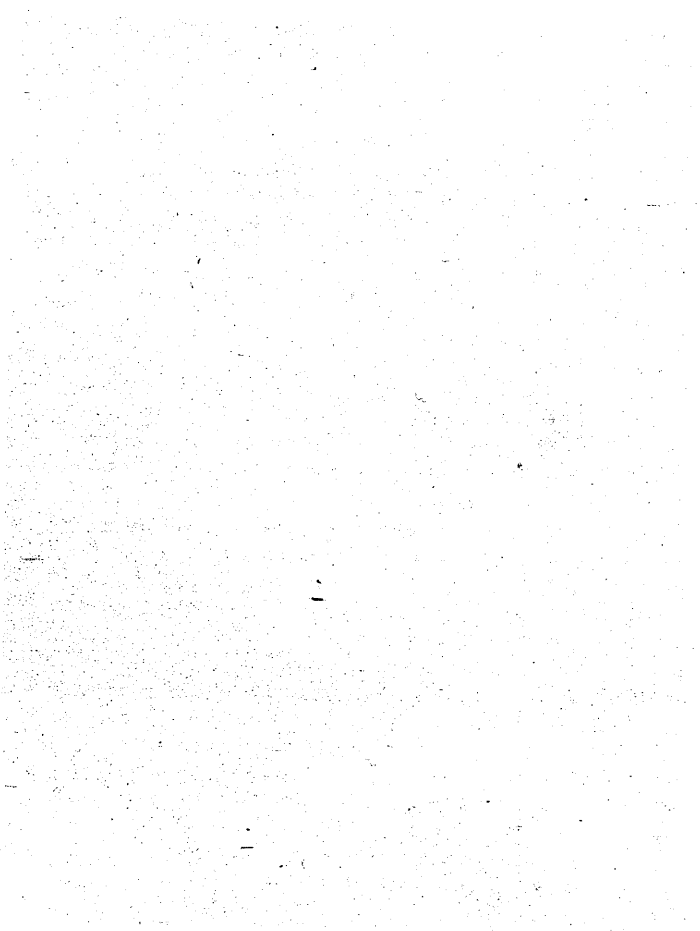


Fig. 38

Shows a cross sectional profile of a fibroblastic channel, almost completely enclosed by the processes of a single fibroblast. The gap left is completed by a bundle of collagen.
x 15680



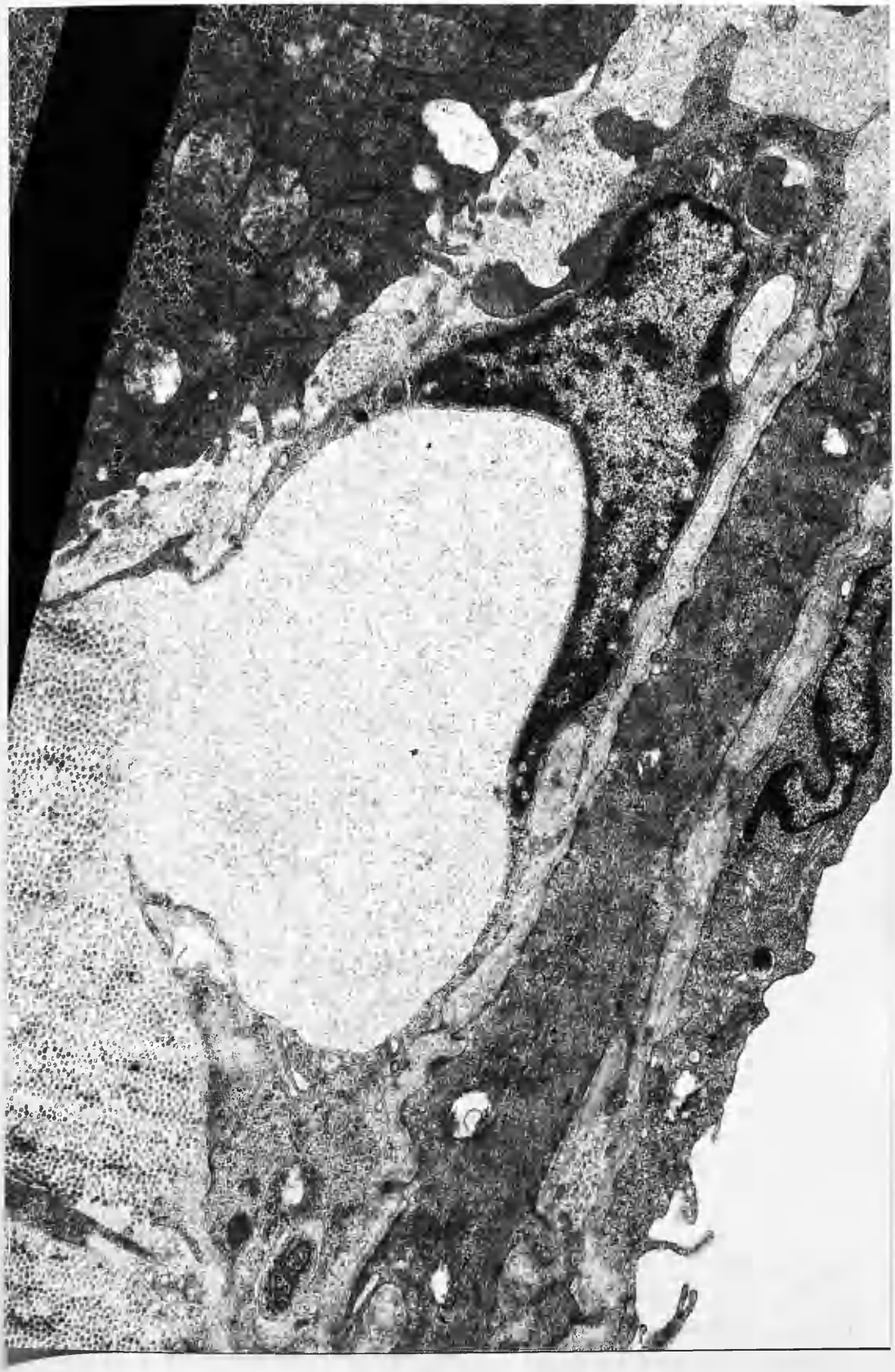


Fig. 39

Shows a sectional profile of a channel completely enclosed by fibroblastic processes.
x 7840

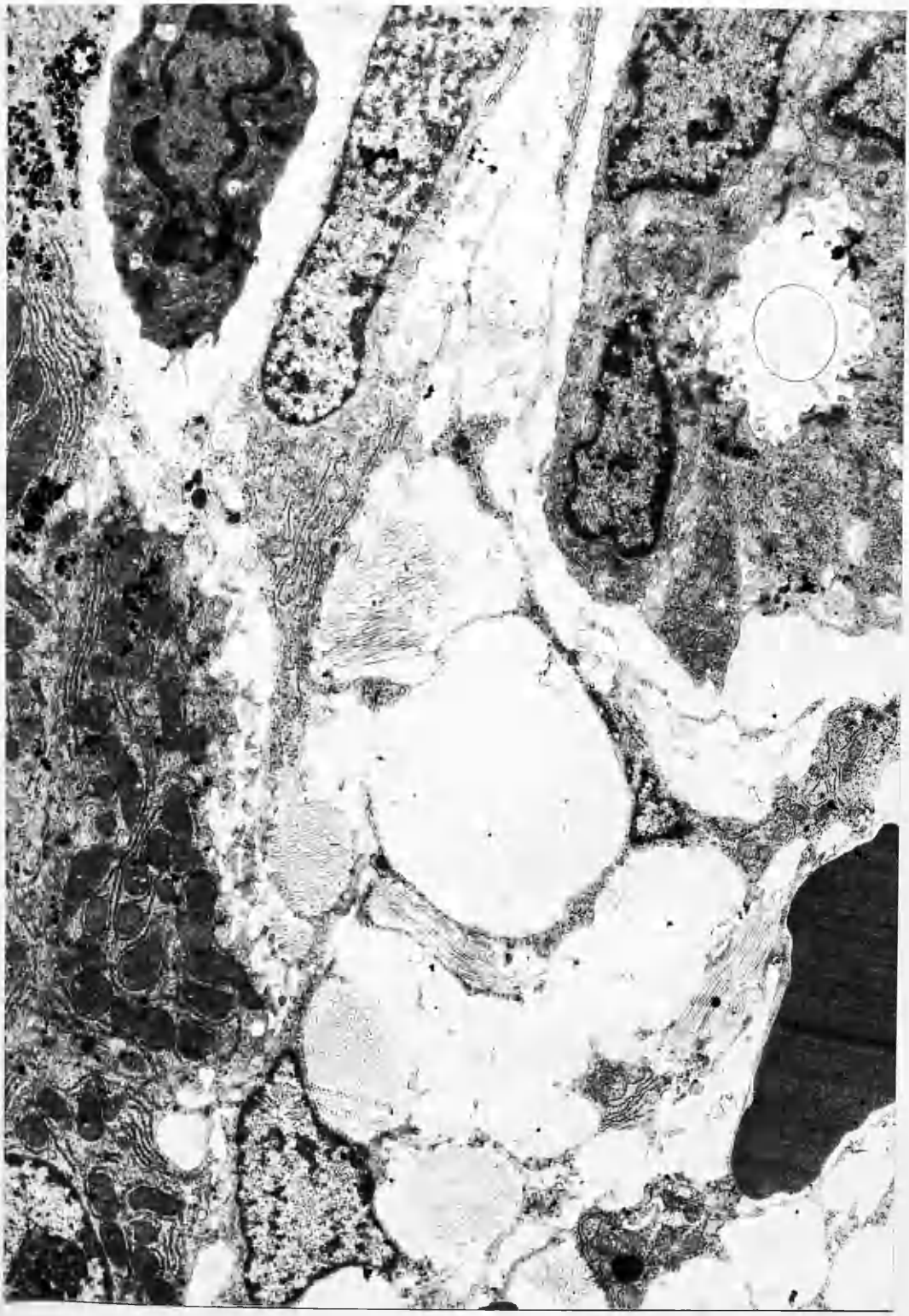


Fig. 40

Shows a fibroblastic channel (FC) adjacent to a blood capillary in a portal tract. Compare the size and proteinous content with that of the adjacent blood capillary.

Note: - the thin branching processes of fibroblastic cytoplasm which bound the channel
- the deficiency in the lining.

x 3920

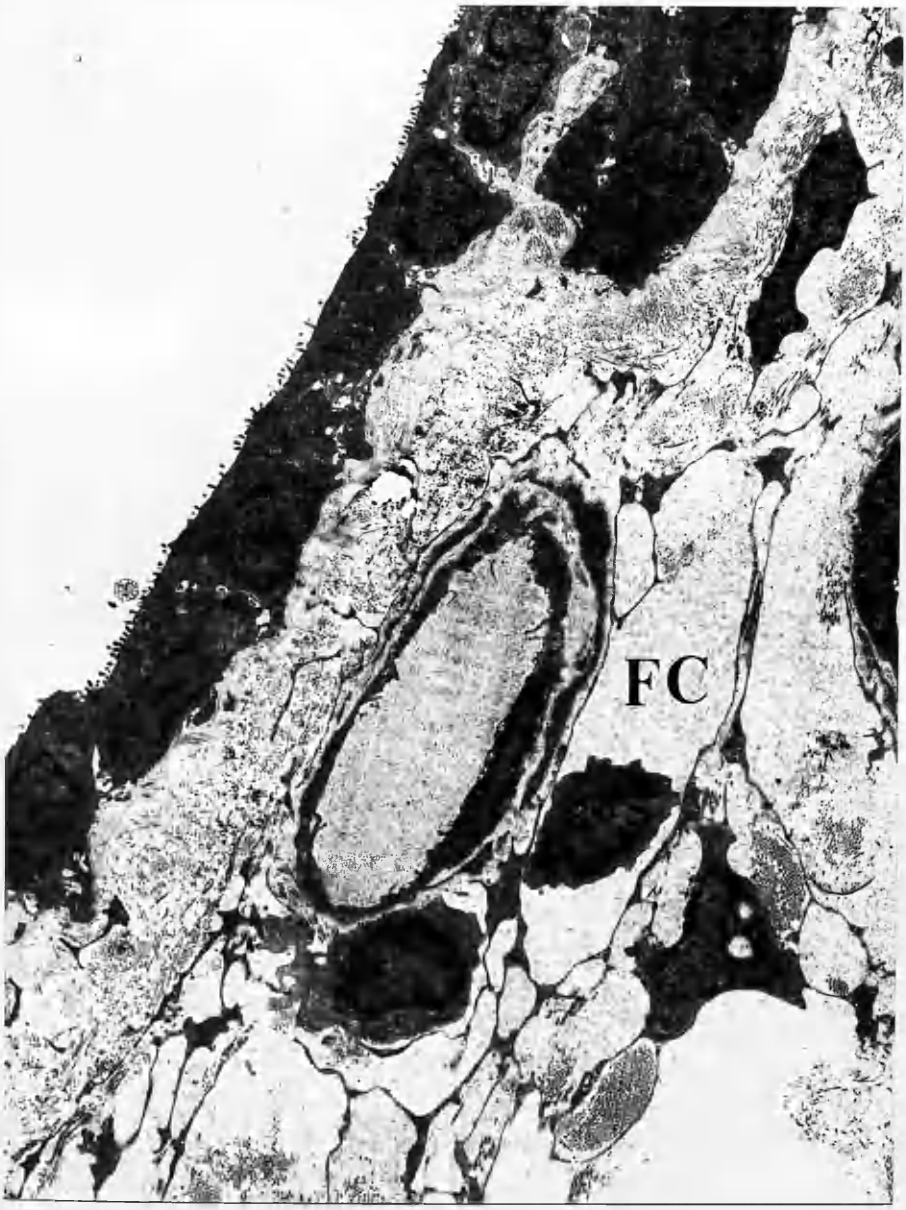

FC

Fig. 41

Shows the SEM appearance of fibroblastic channels which suggests that the wall is most likely made of sheets of fibroblastic processes (SH) with multiple gaps (G) and that the wall may be completed by collagen (C).
x 19000

C
SH
02 h81 0 52 1

Fig. 42

Shows a nearly complete fibroblastic channel (FC); the gap facing the hepatocyte of the limiting plate is completed by collagen (C).

Note: the relation of the gap in the fibroblastic channel to the space of Mall (M).

x 28000

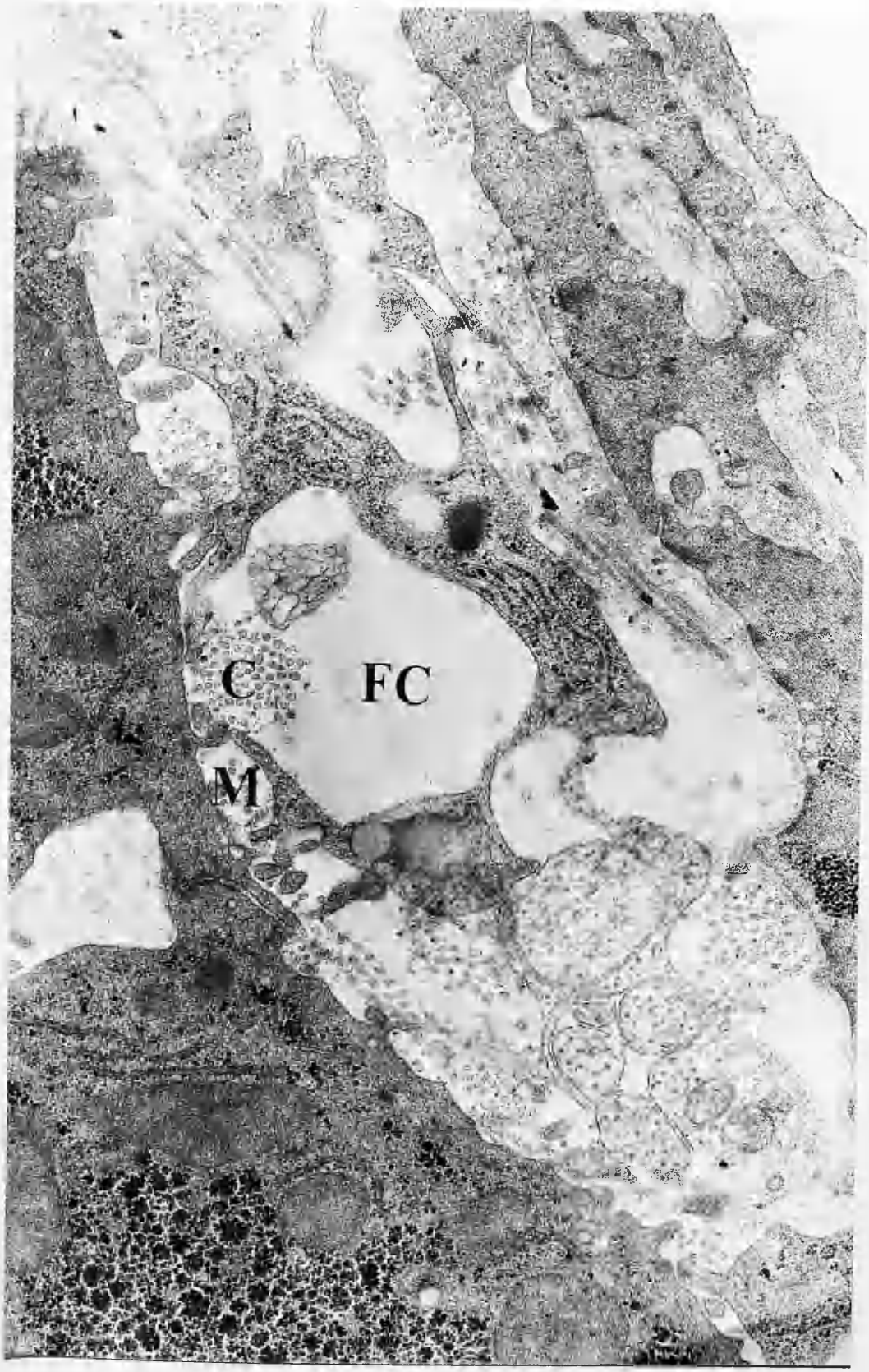

C
FC
M

Fig. 43

Illustrates the proximity of the fibroblastic channel (FC) to the space of Mall (M).
x 11760

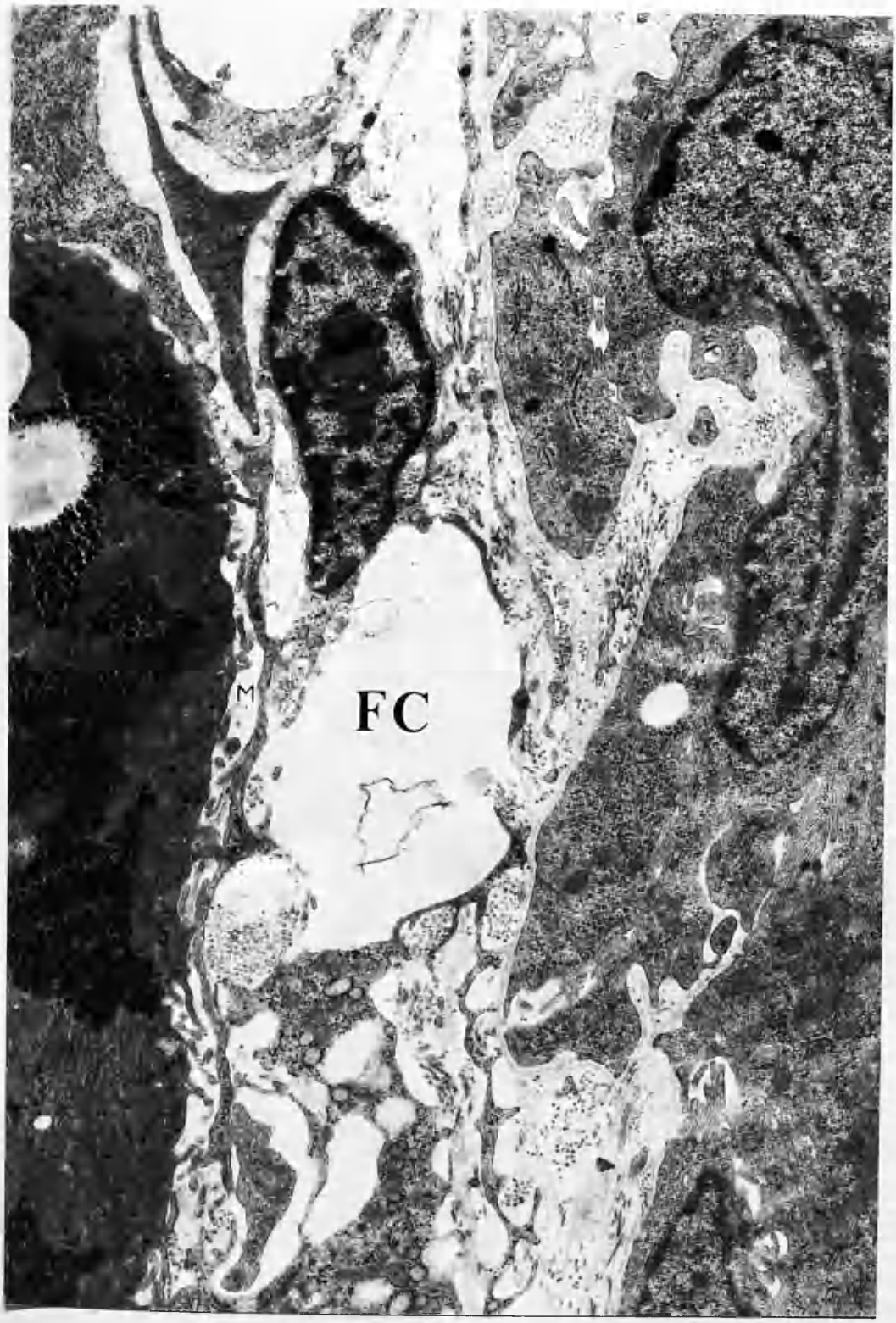

FC
M

Fig. 44

Demonstrates the proximity of the fibroblastic channel (FC) to the true lymphatic (L).

Note: the position of the gap (arrow) in the wall of the fibroblastic channel, which faces the true lymphatic.

x 5880

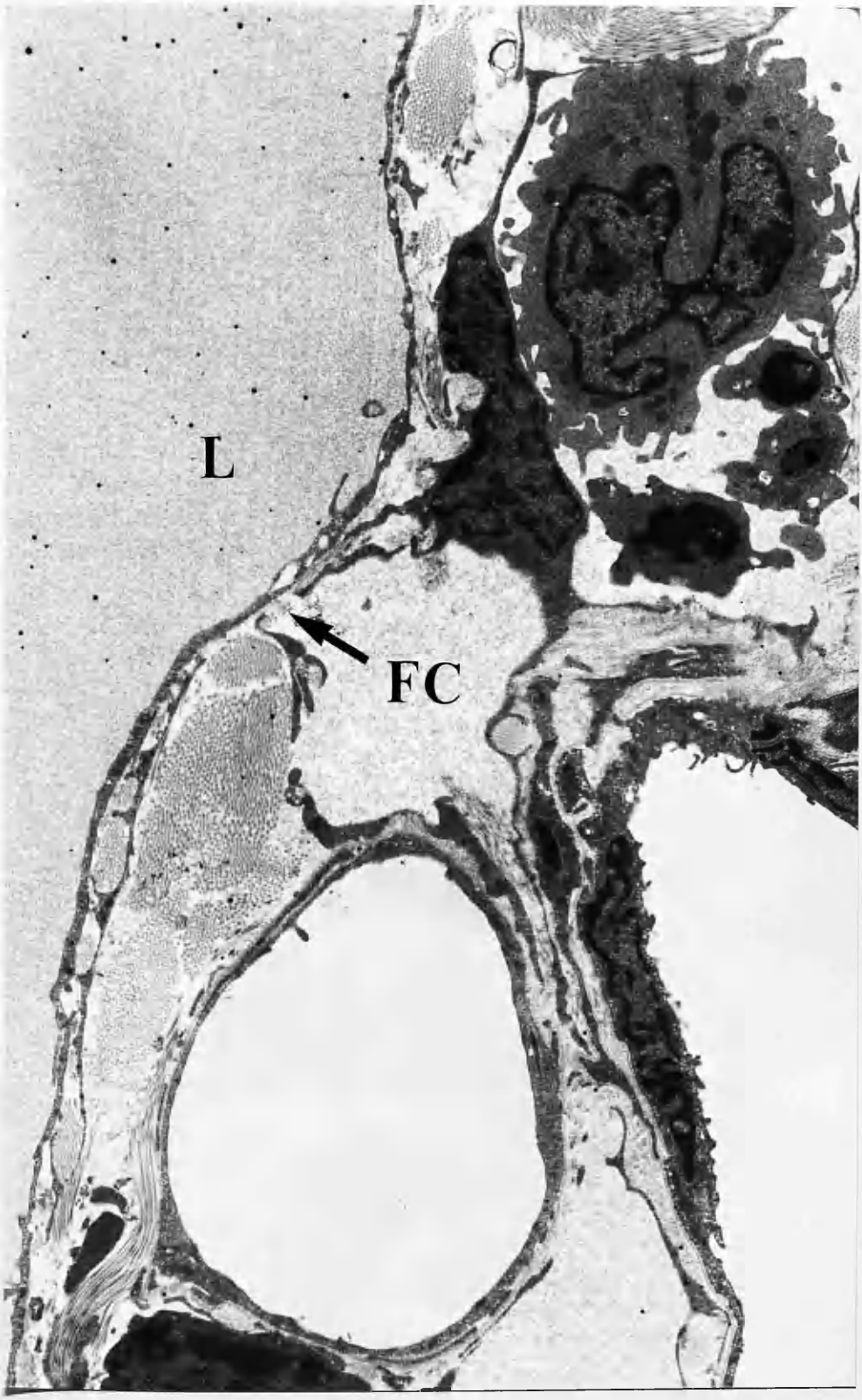

L
FC

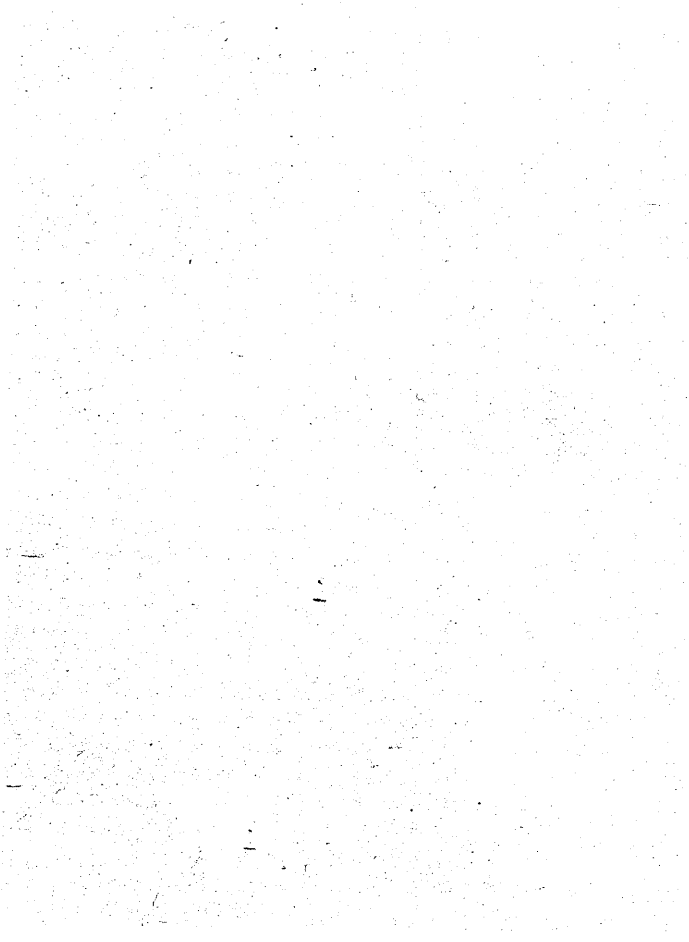
Fig. 45

Shows a bundle of collagen (C) in the space of Disse (D) bathed in proteinous material.
x 58800

C
D

Fig. 46

Shows two bundles of collagen (C) in the space of Disse, bathed in proteinous material.
x 19600



C
C

Fig. 47

Shows a collagen bundle, in a portal tract, bathed in proteinous material.
x 50400

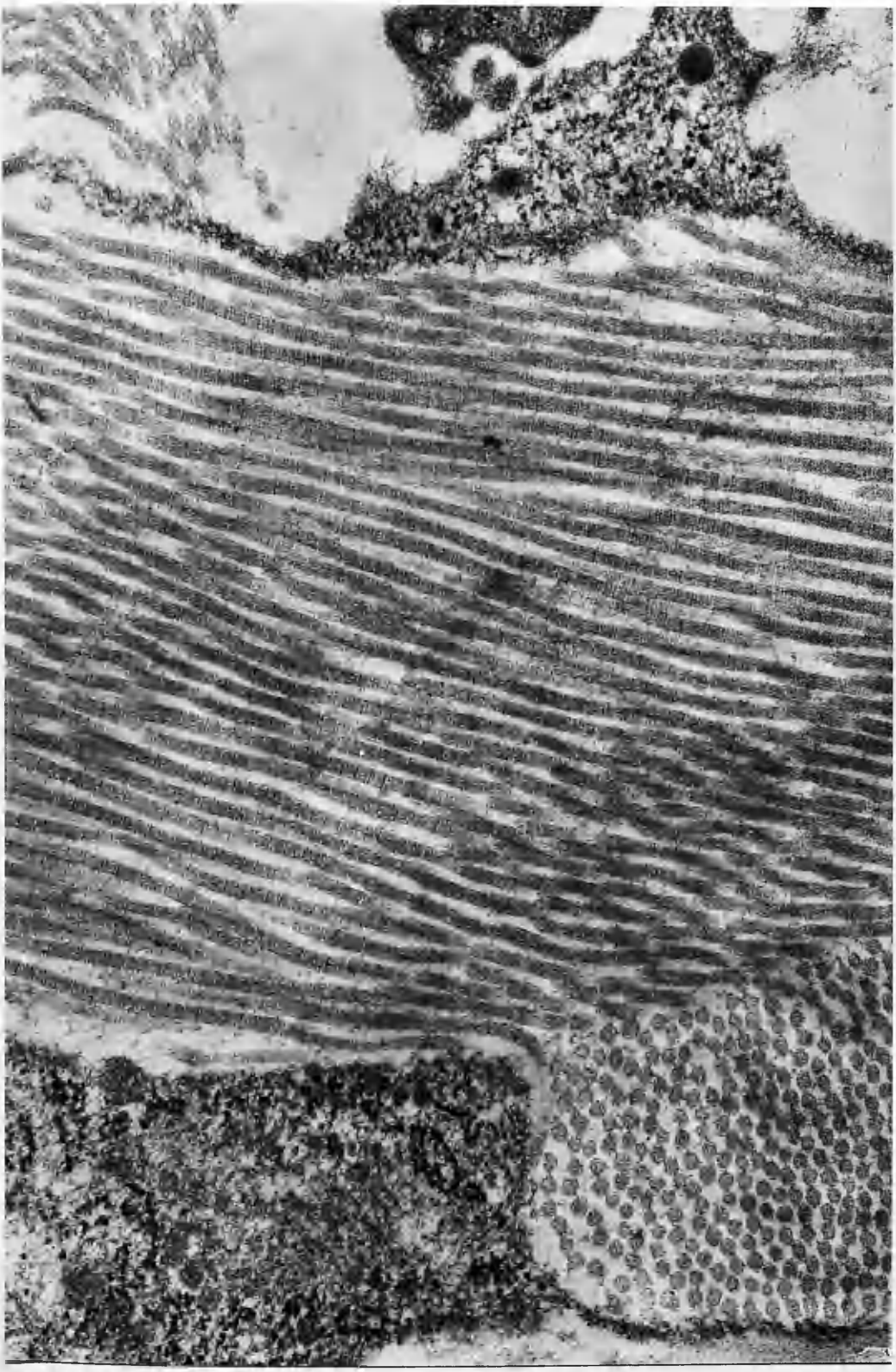


Fig. 48

Ferritin granules can be seen in the sinusoid (S) lumen and the space of Disse (D). These granules are scanty presumably because fixation was by perfusion. x 50400

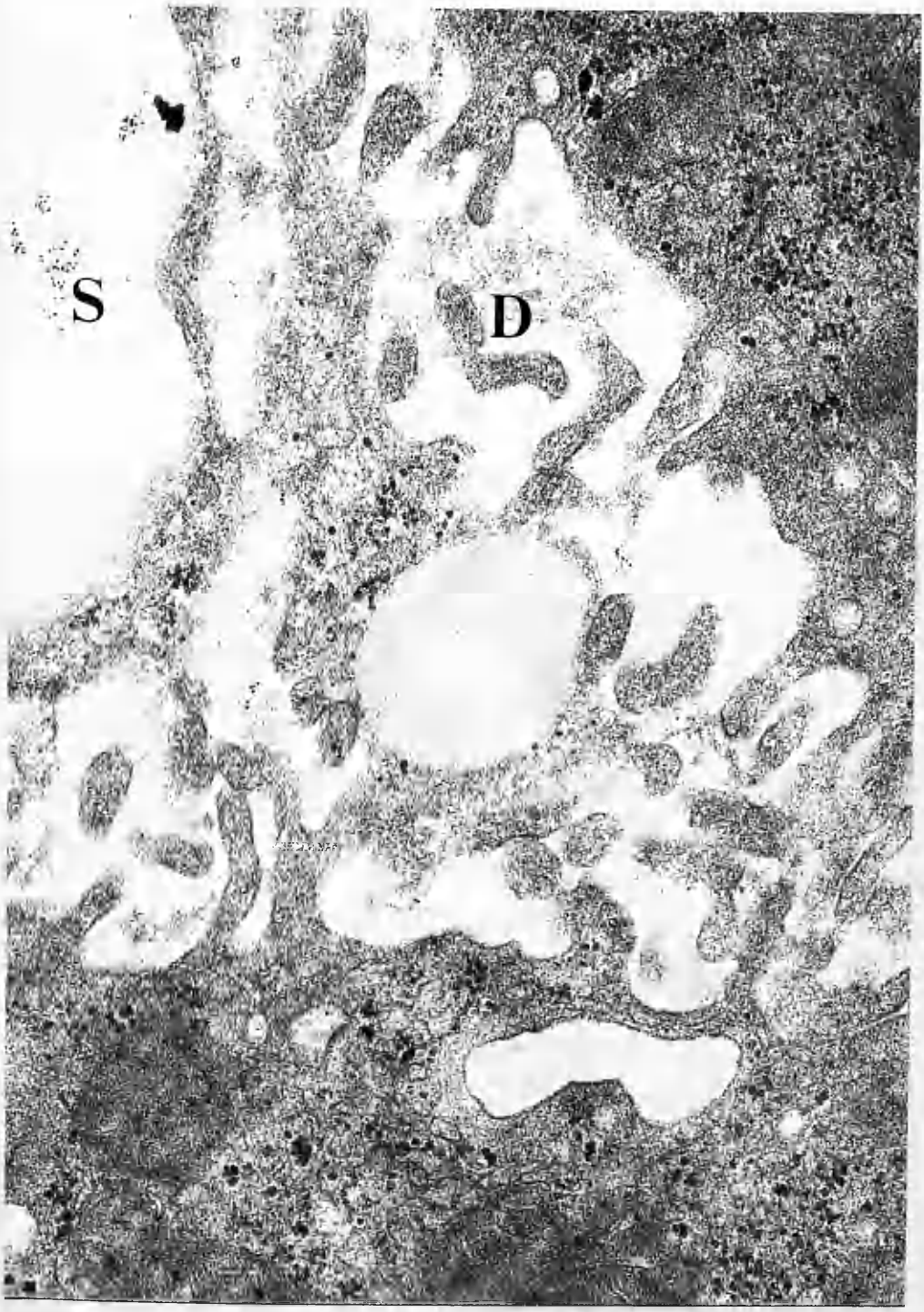
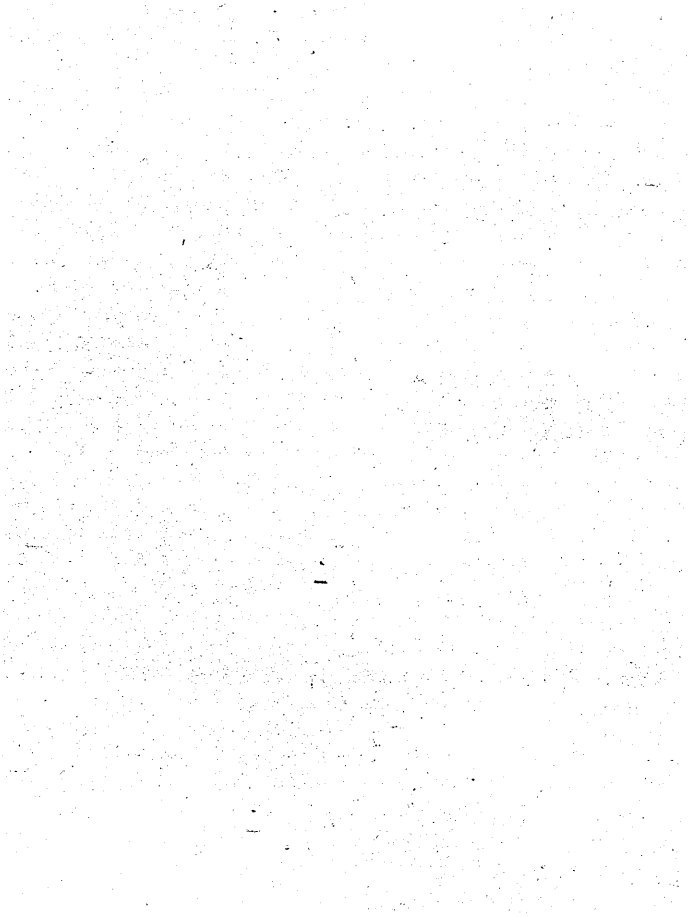

S
D

Fig. 49

Ferritin granules are seen in abundance in the sinusoidal lumen (S), in endothelial fenestrations (arrows) and in Disse's space (D). This specimen was taken from a decapitated animal and fixed by immersion.
x 39200



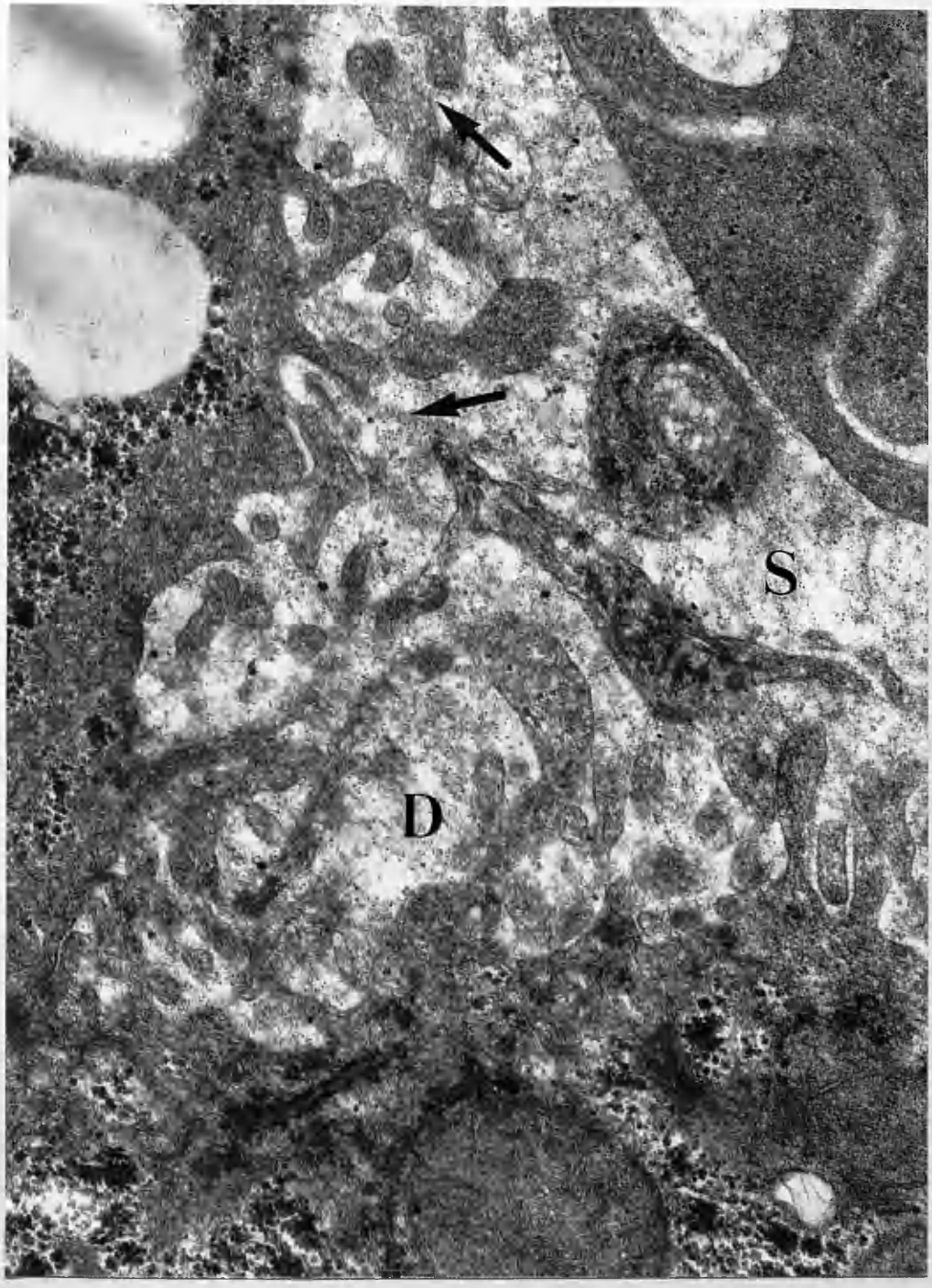
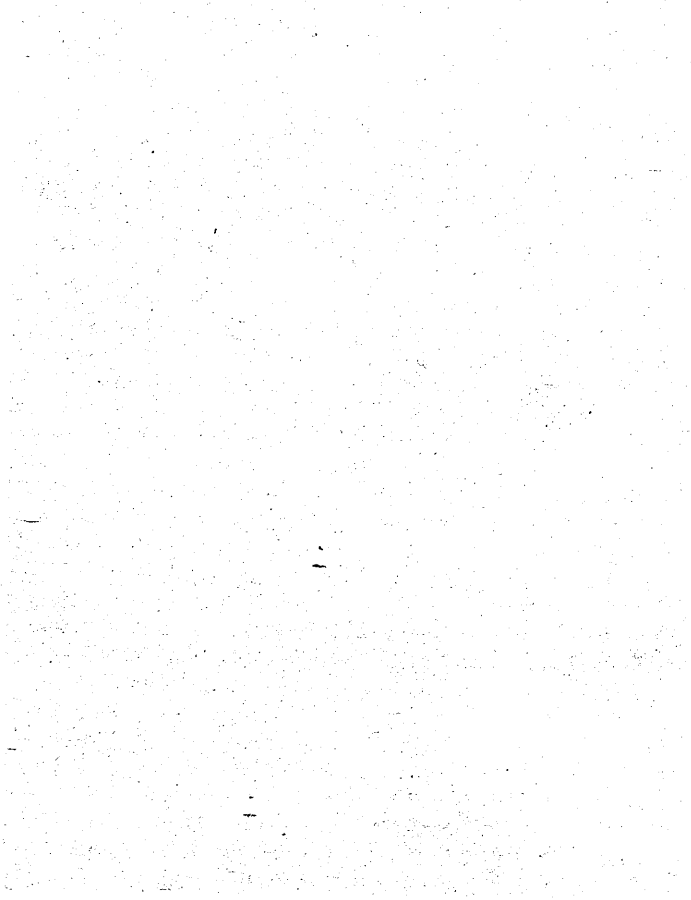

S
D

Fig. 50

A gap (G) in a limiting plate with ferritin granules between its various components.
x 50400



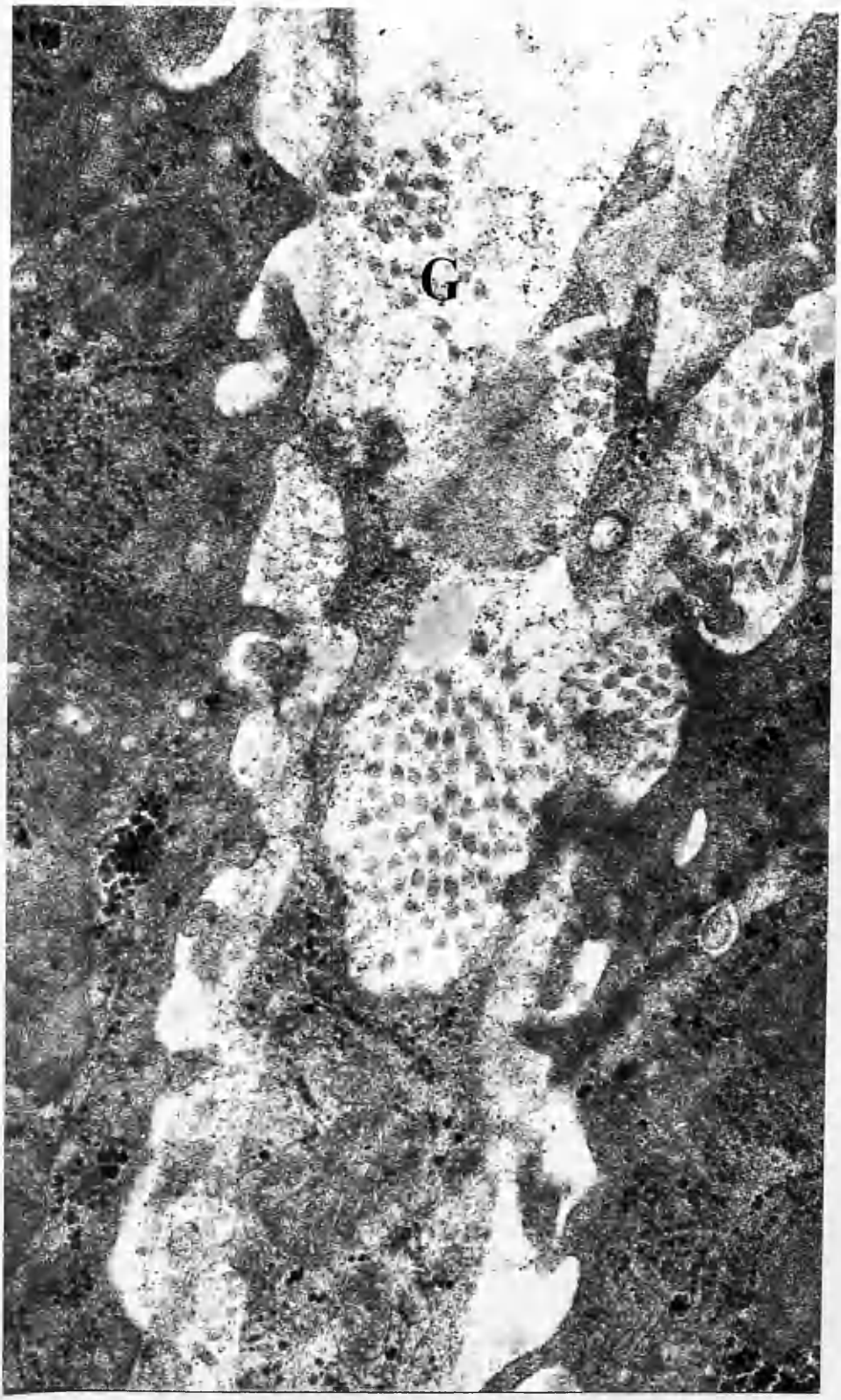

G

Fig. 51

Ferritin granules in the space of Mall (M).
x 15680

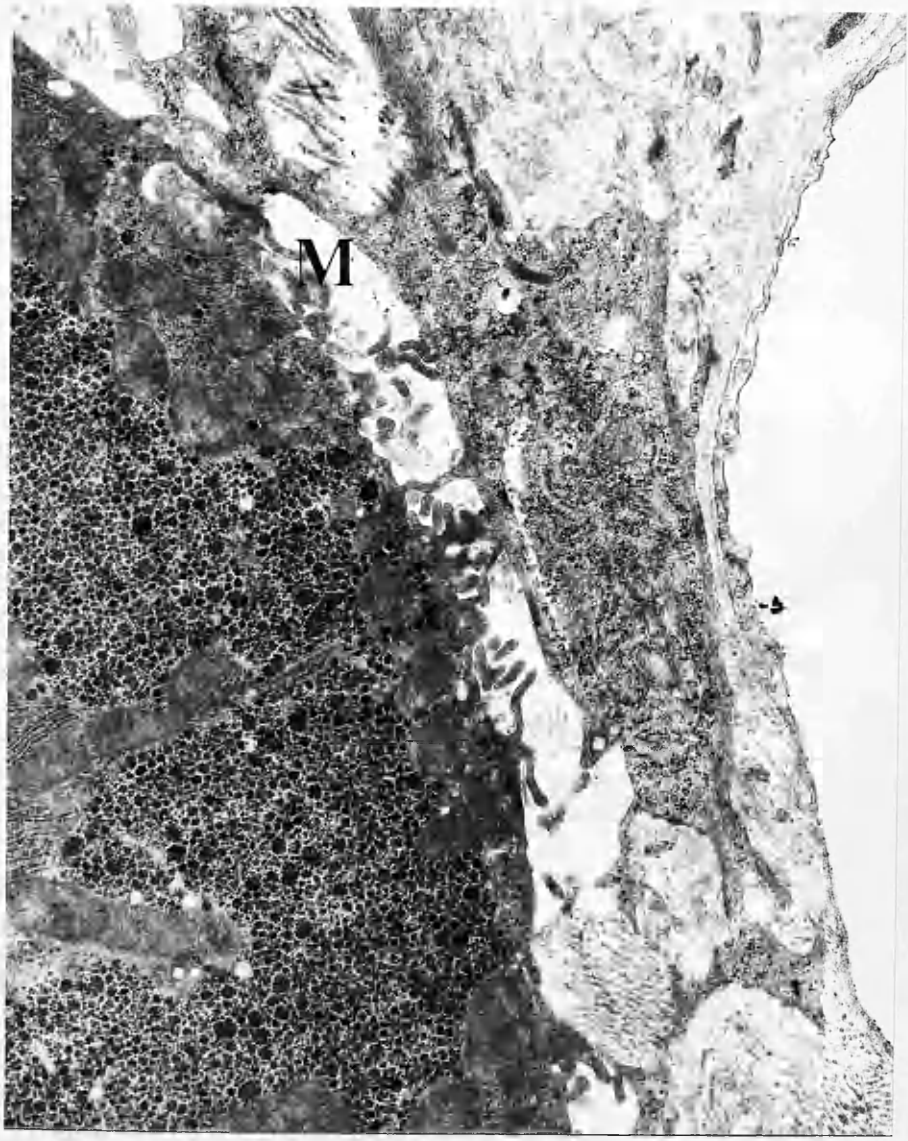

M

Fig. 52

Ferritin granules in the interstitium of a portal tract.

Note: (arrow) indicates site of early formation of a bristle coated vesicle taking up ferritin granules.

x 50400

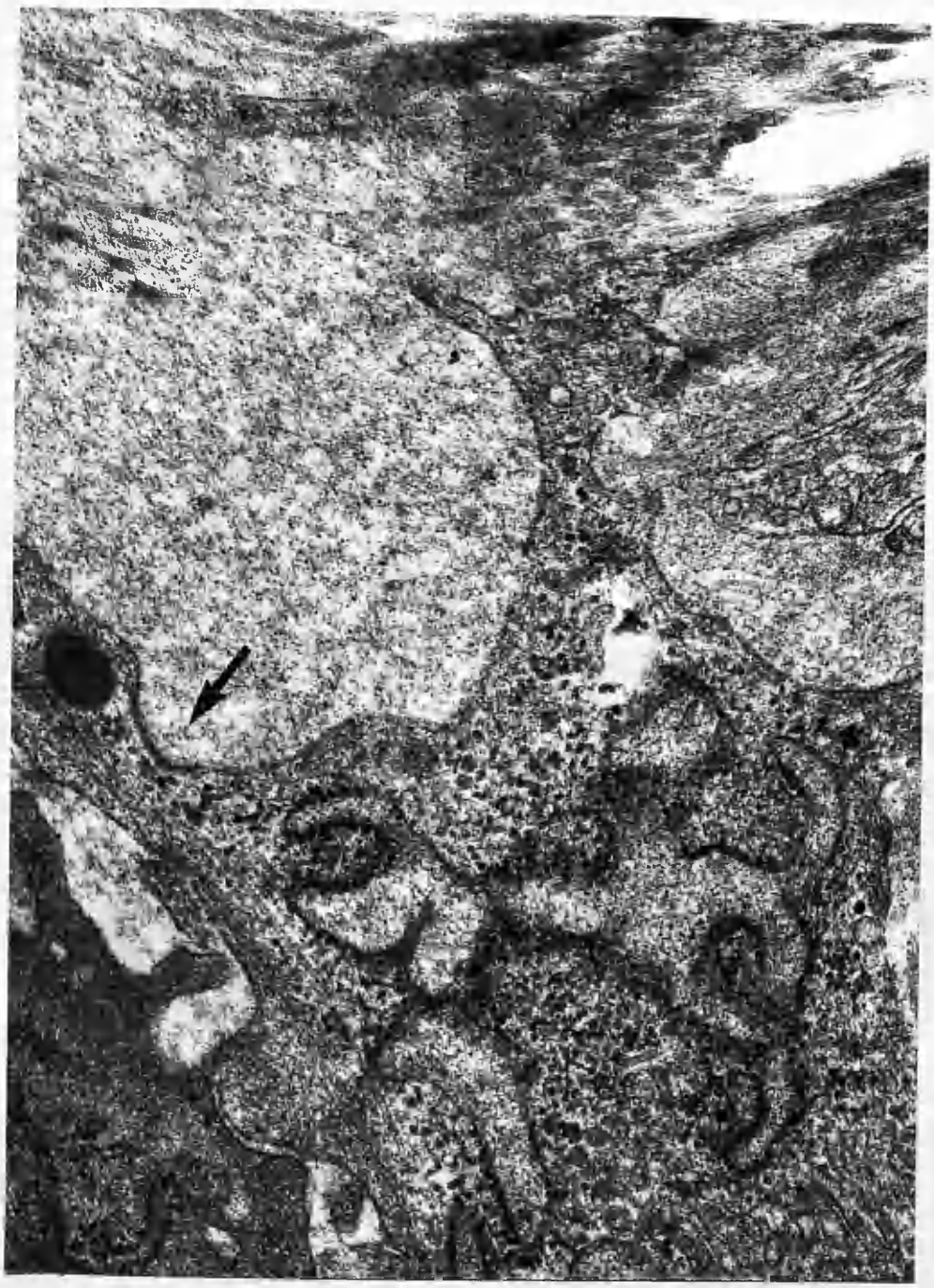
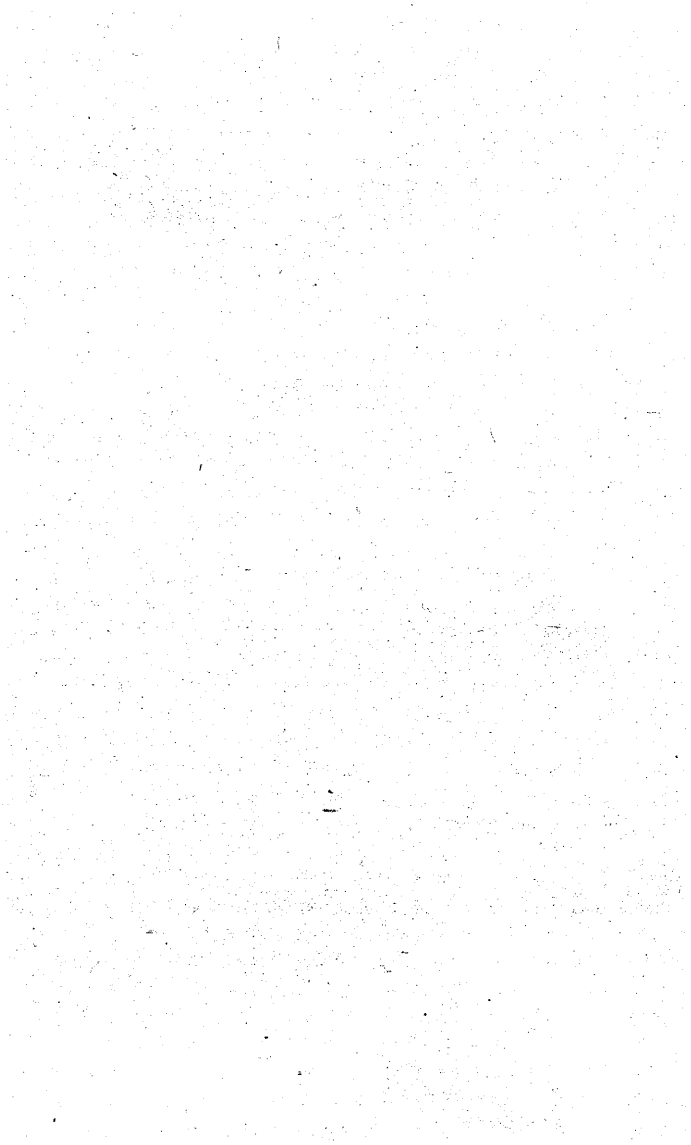


Fig. 53

Ferritin granules in a fibroblastic channel.
x 15680



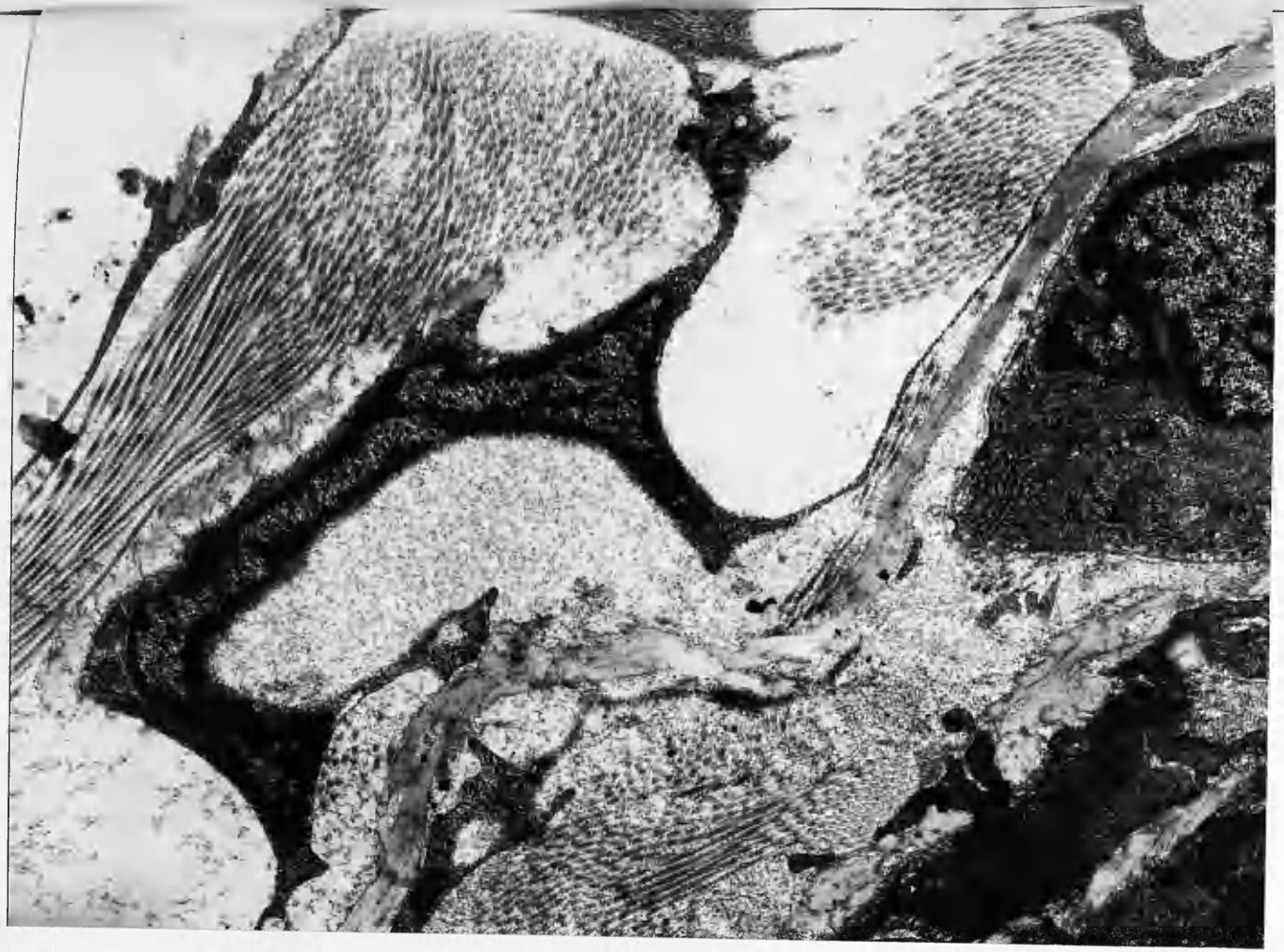


Fig. 54

Shows ferritin granules bathing a bundle of collagen fibres in the interstitium of a portal tract.

Note: compare with Fig. 47.

x 50400

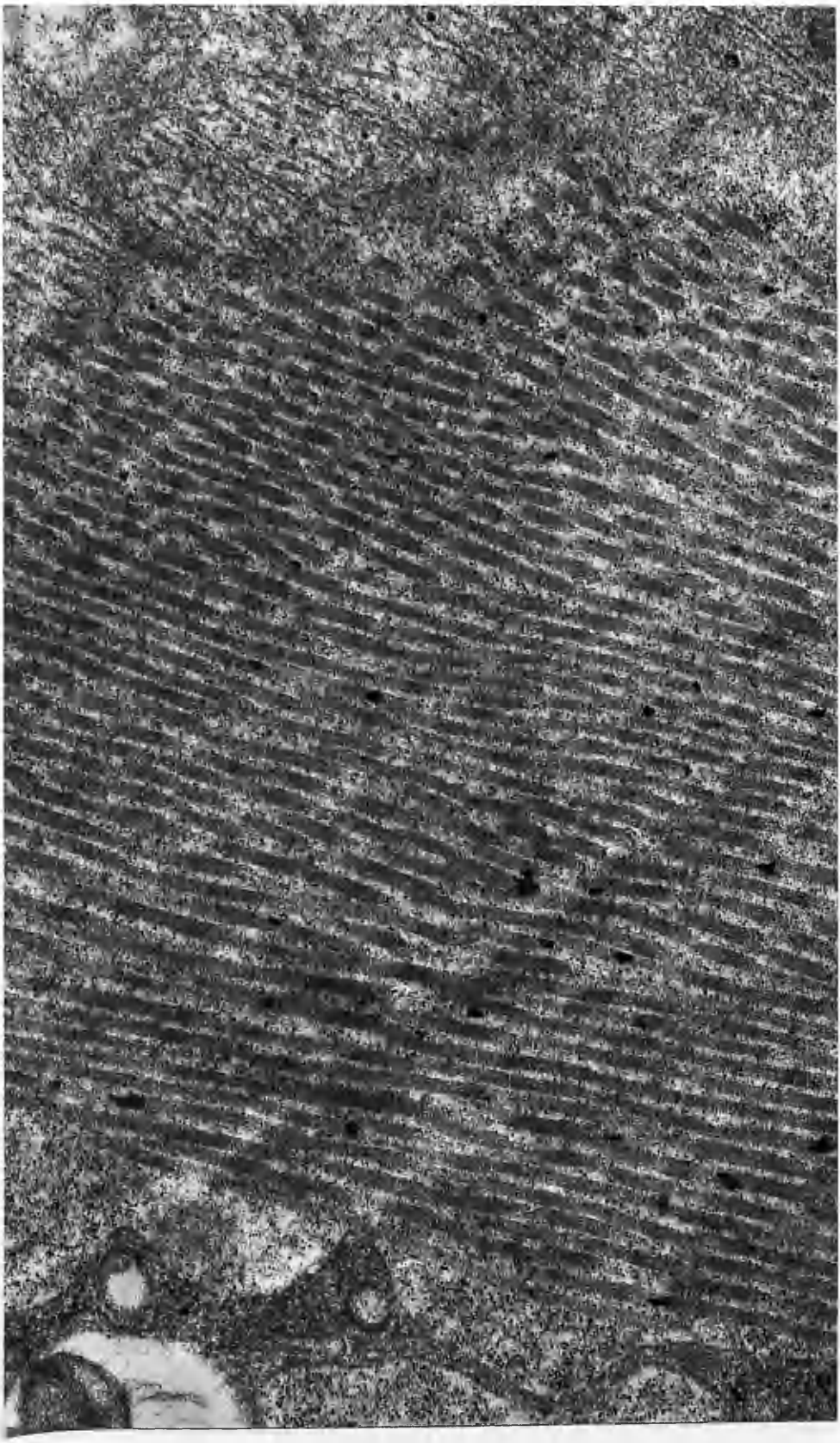


Fig. 55

Shows that lymphatics contained a higher concentration of ferritin granules than did other parts of the suggested pathway.
x 50400

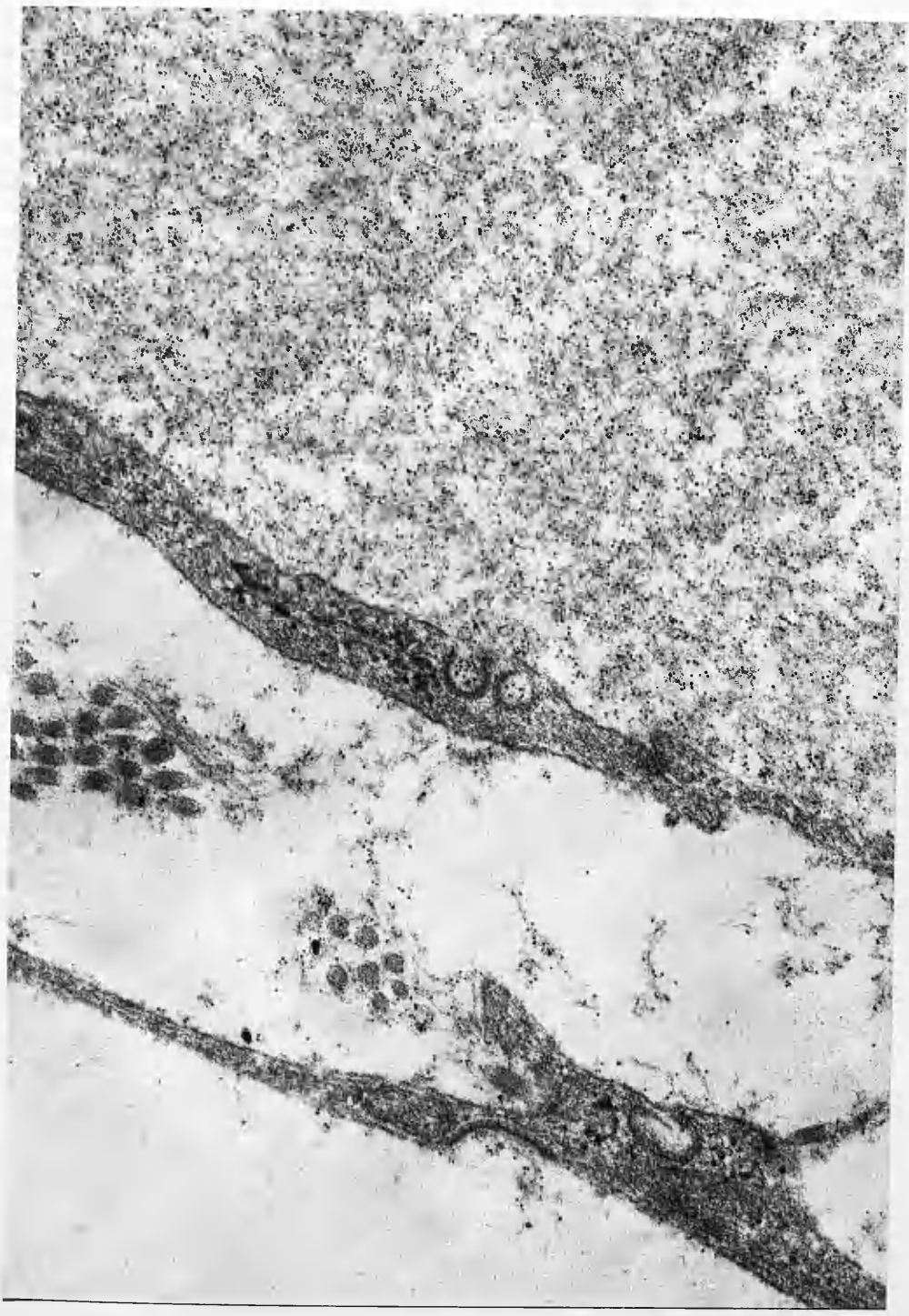
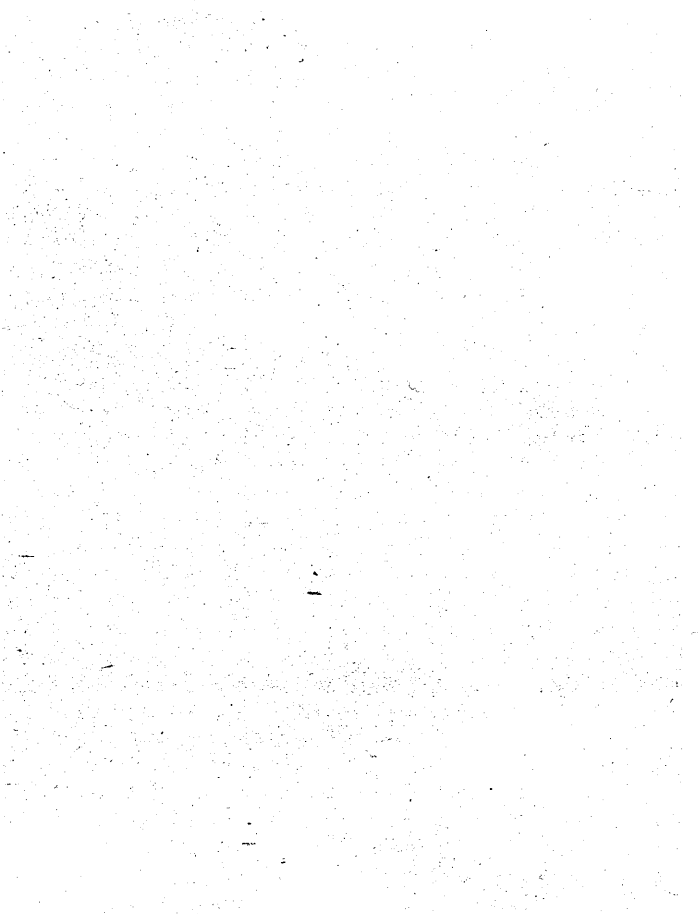


Fig. 56

A. Shows that ferritin granules were taken up by the sinusoidal endothelium (SE) in bristle coated vesicles (arrows).
x 11760



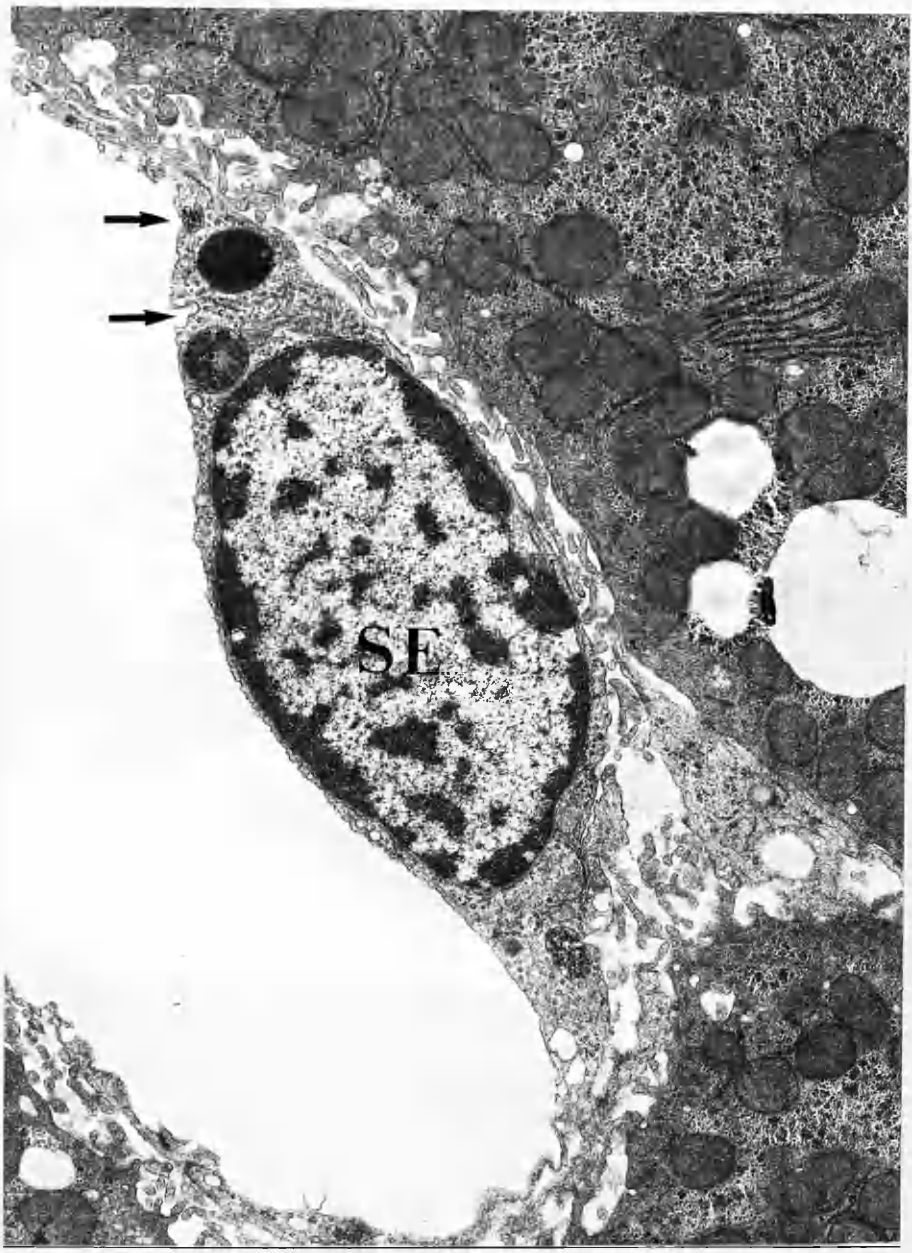

SE

Fig. 56

B. A higher power of the part of the sinusoidal endothelium taking up the ferritin granules in bristle coated vesicles (arrows).
x 39200

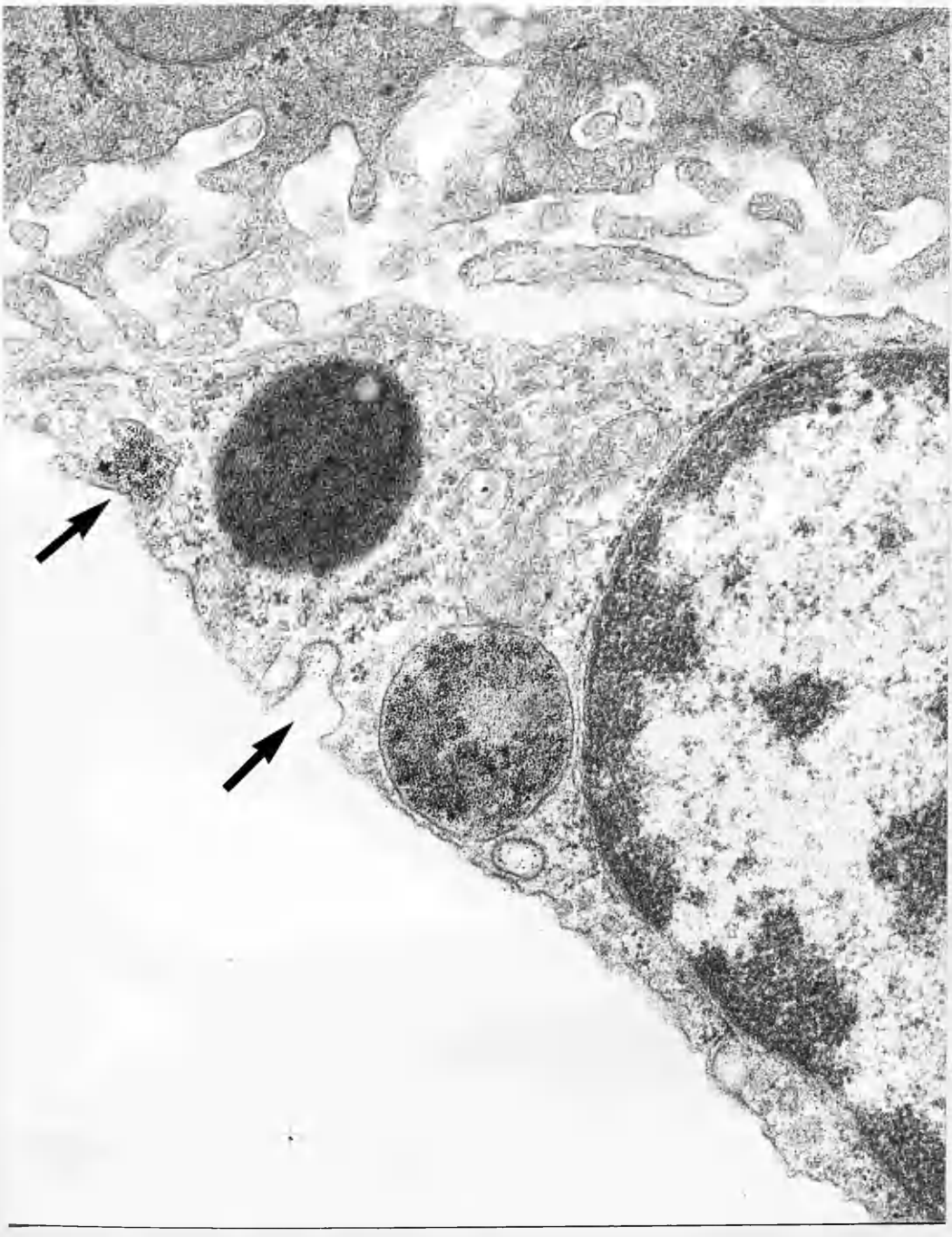
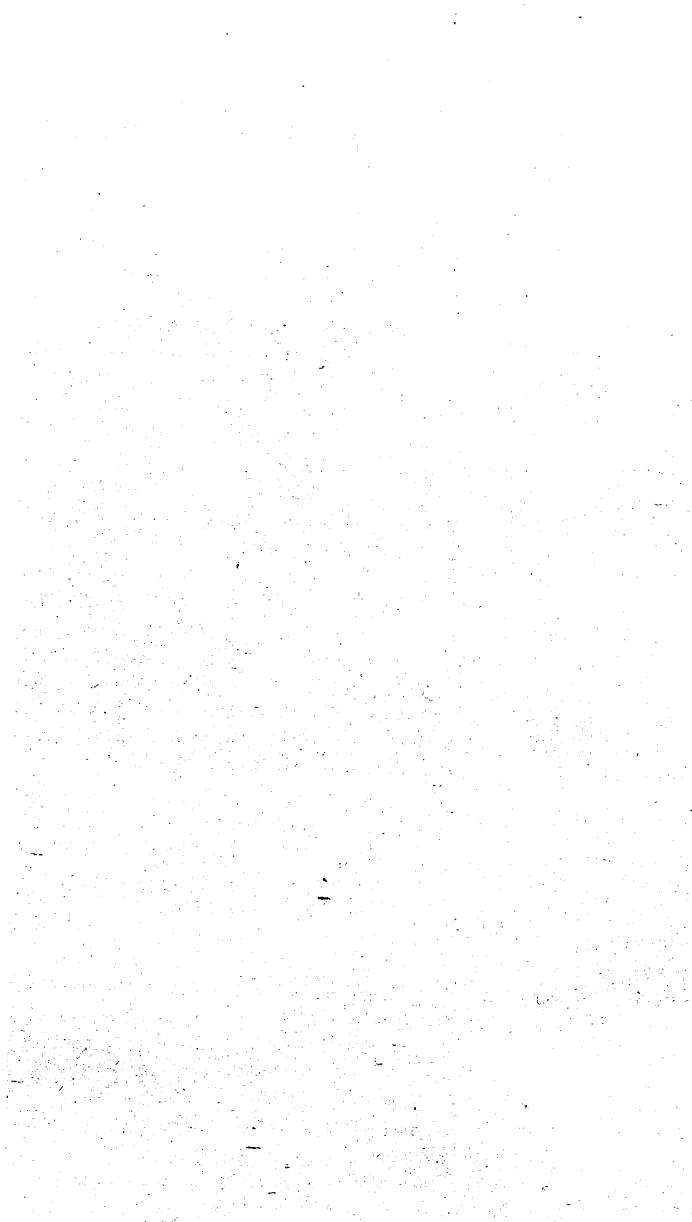


Fig. 57

Shows Pontamine Sky Blue particles in the sinusoidal lumen, fenestrations (arrows) and Disse's space. x 11760



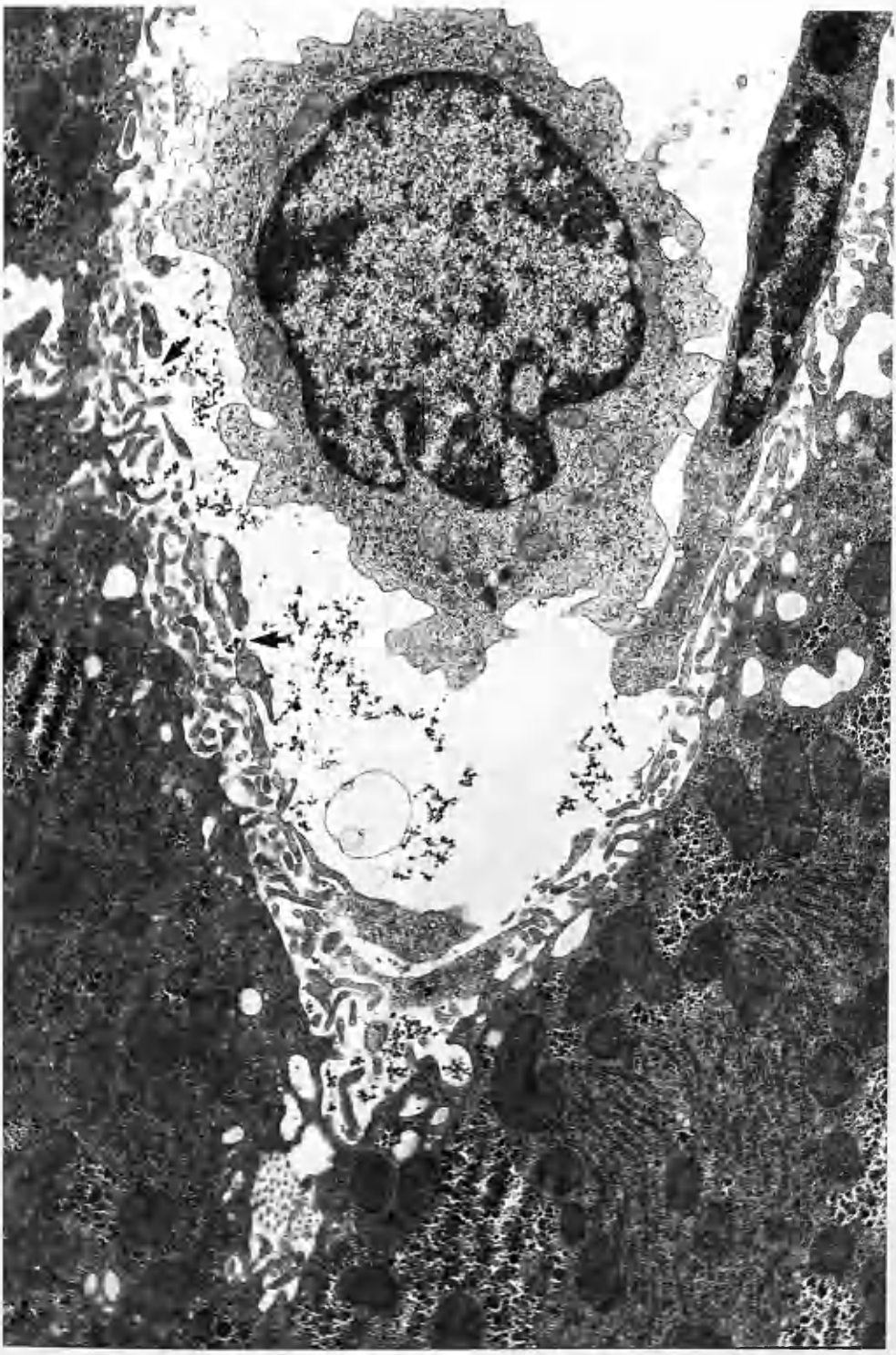


Fig. 58

Shows Pontamine Sky Blue particles in a gap (G) in the limiting plate.
x 11760

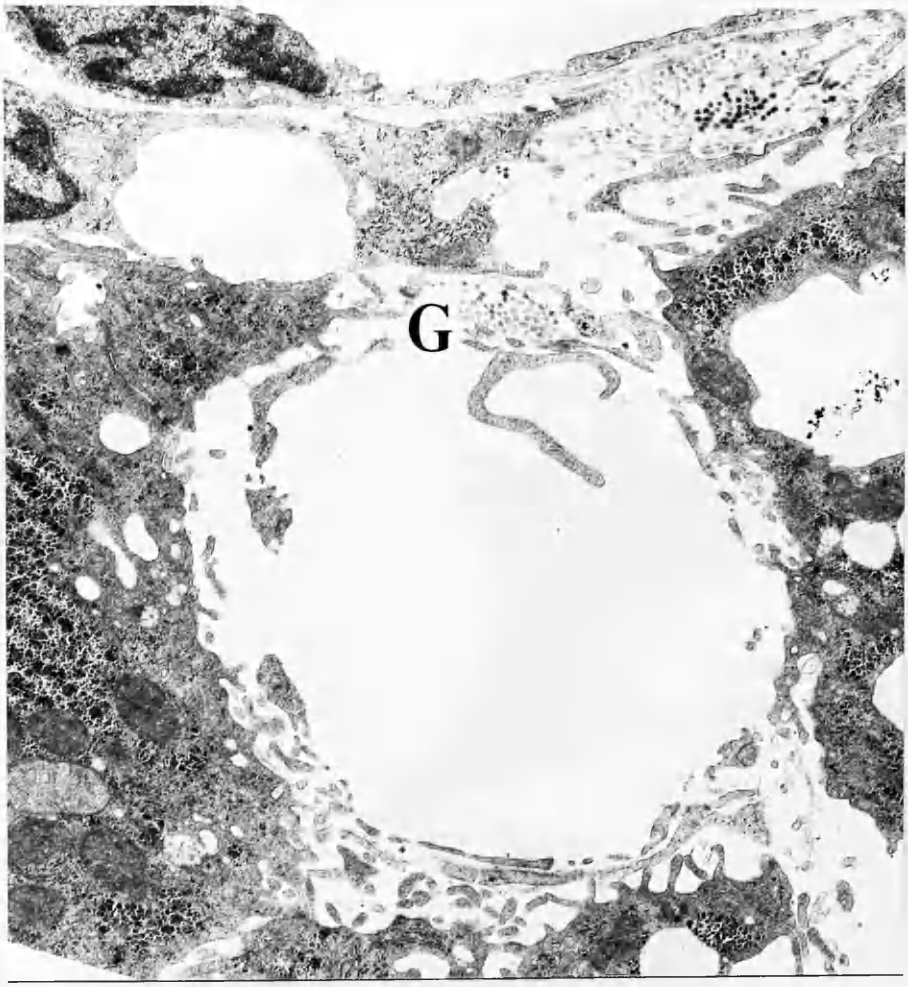

G

Fig. 59

Shows Pontamine Sky Blue particles in various parts of the interstitium of a portal tract.

Note: PSB particles in fibroblastic channels FC.

x 15680

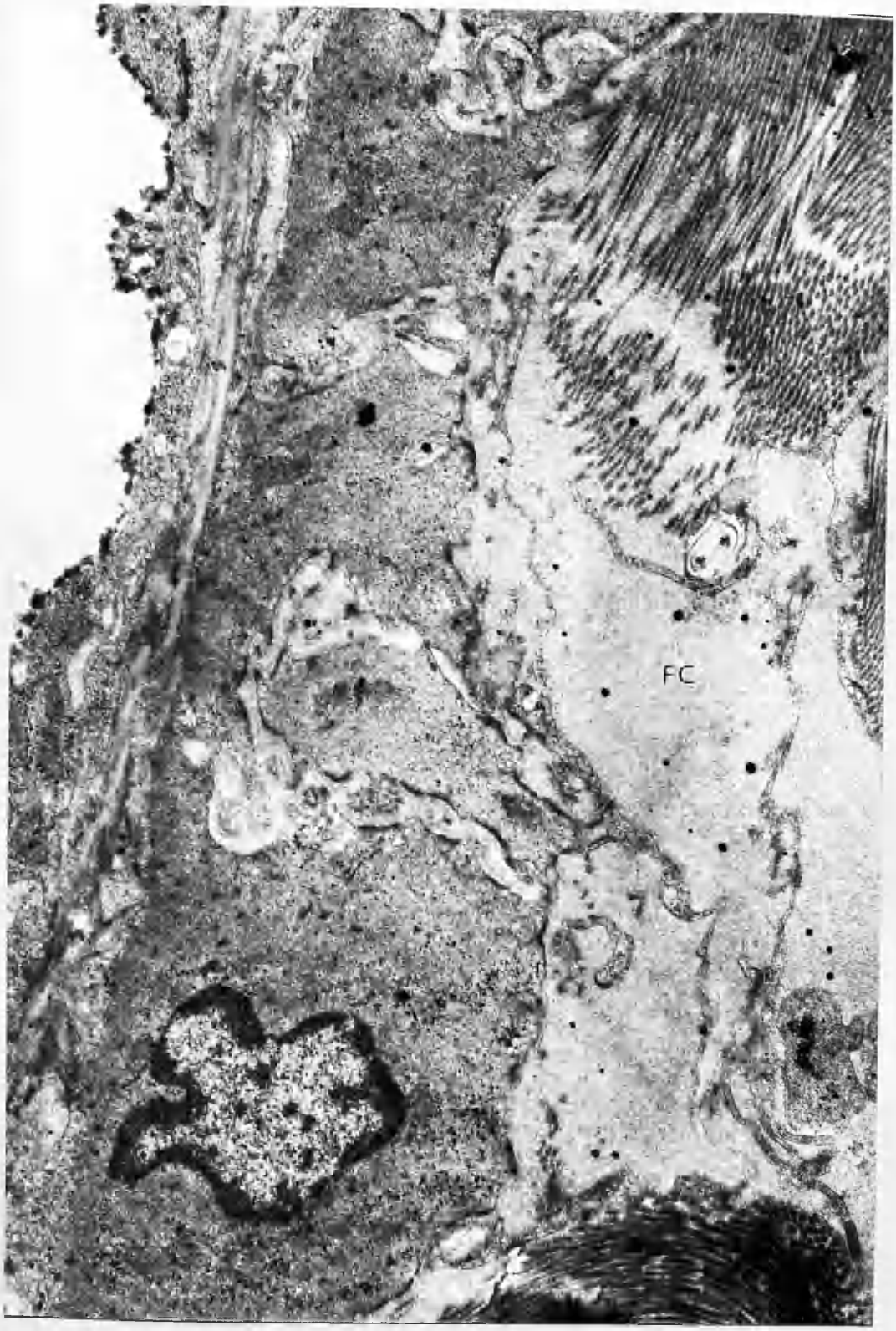

FC

Fig. 60

Shows Pontamine Sky Blue particles in the space of Mall (M), in portal tract interstitium, and in the lymphatic.

Note: the high concentration of the particles in the lymphatics.

x 9800

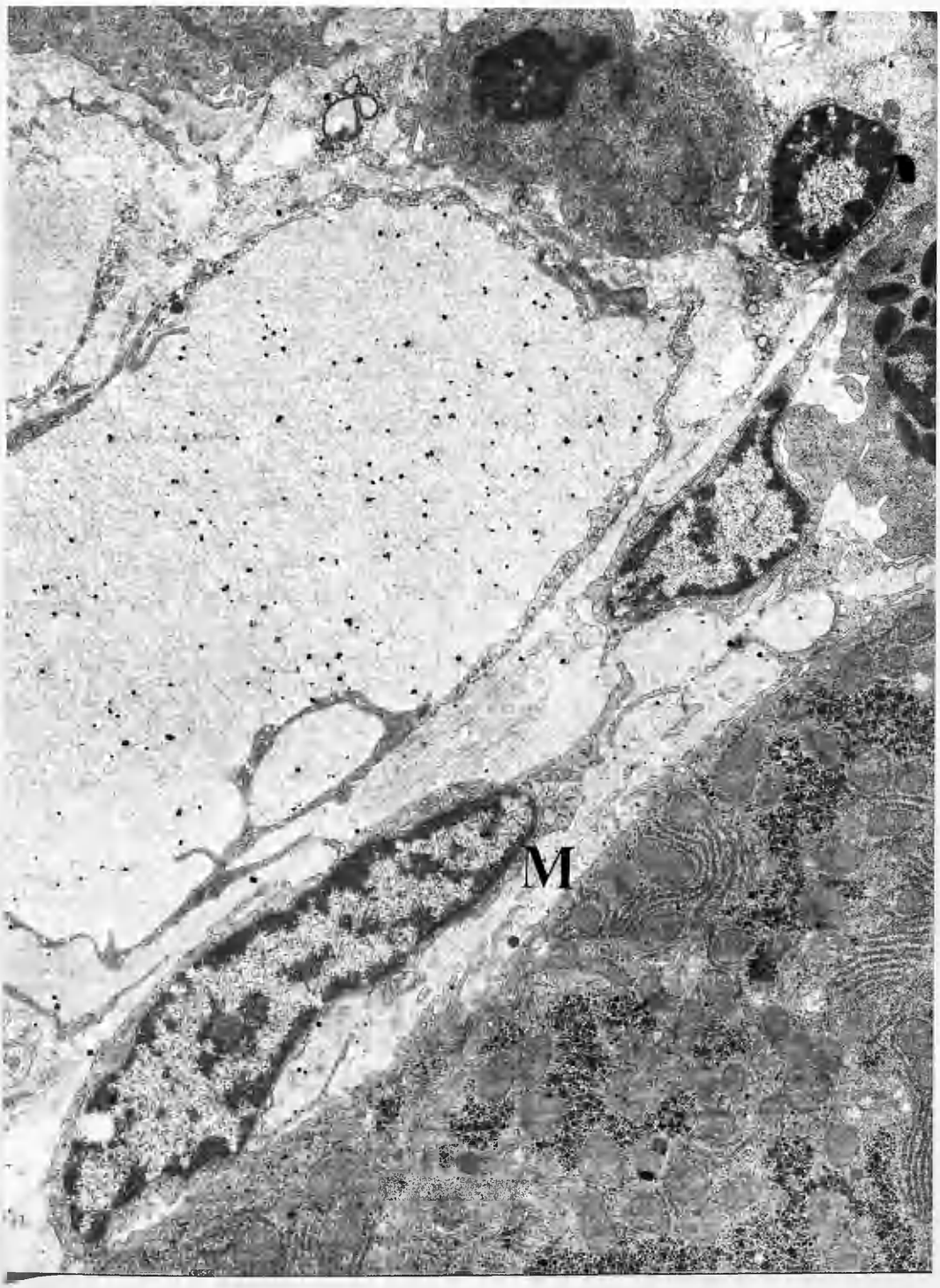

M

Fig. 61

Shows Pontamine Sky Blue particles and chylomicrons in the sinusoidal lumen.

Note: A - a cluster of various sizes of chylomicrons
B - a large chylomicron
C - a cluster of small chylomicrons in the space of Disse

x 11760

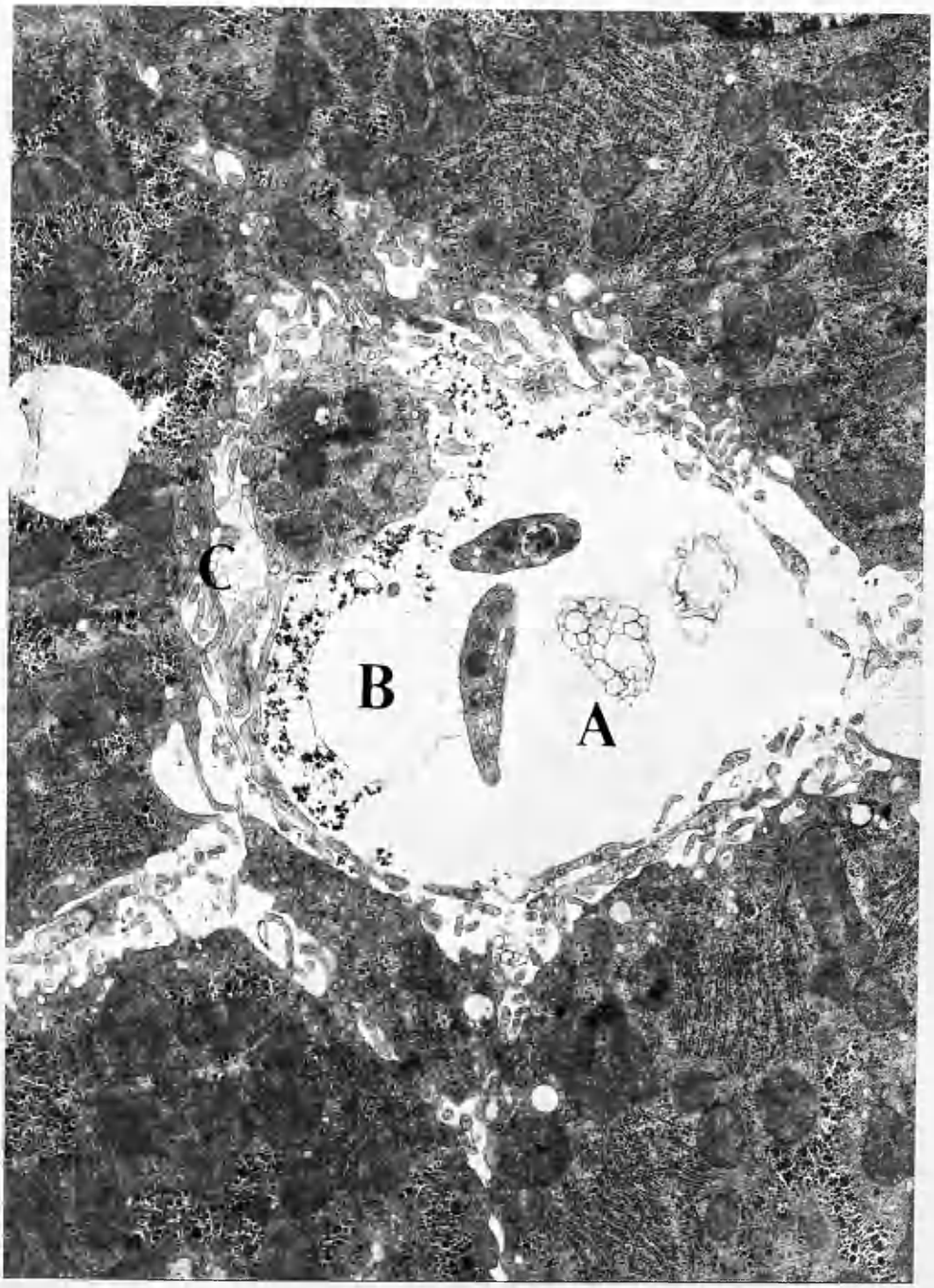

C
B
A

Fig. 62

Shows chylomicrons (arrows) in a longitudinal sectional profile of a fibroblastic channel.
x 28000

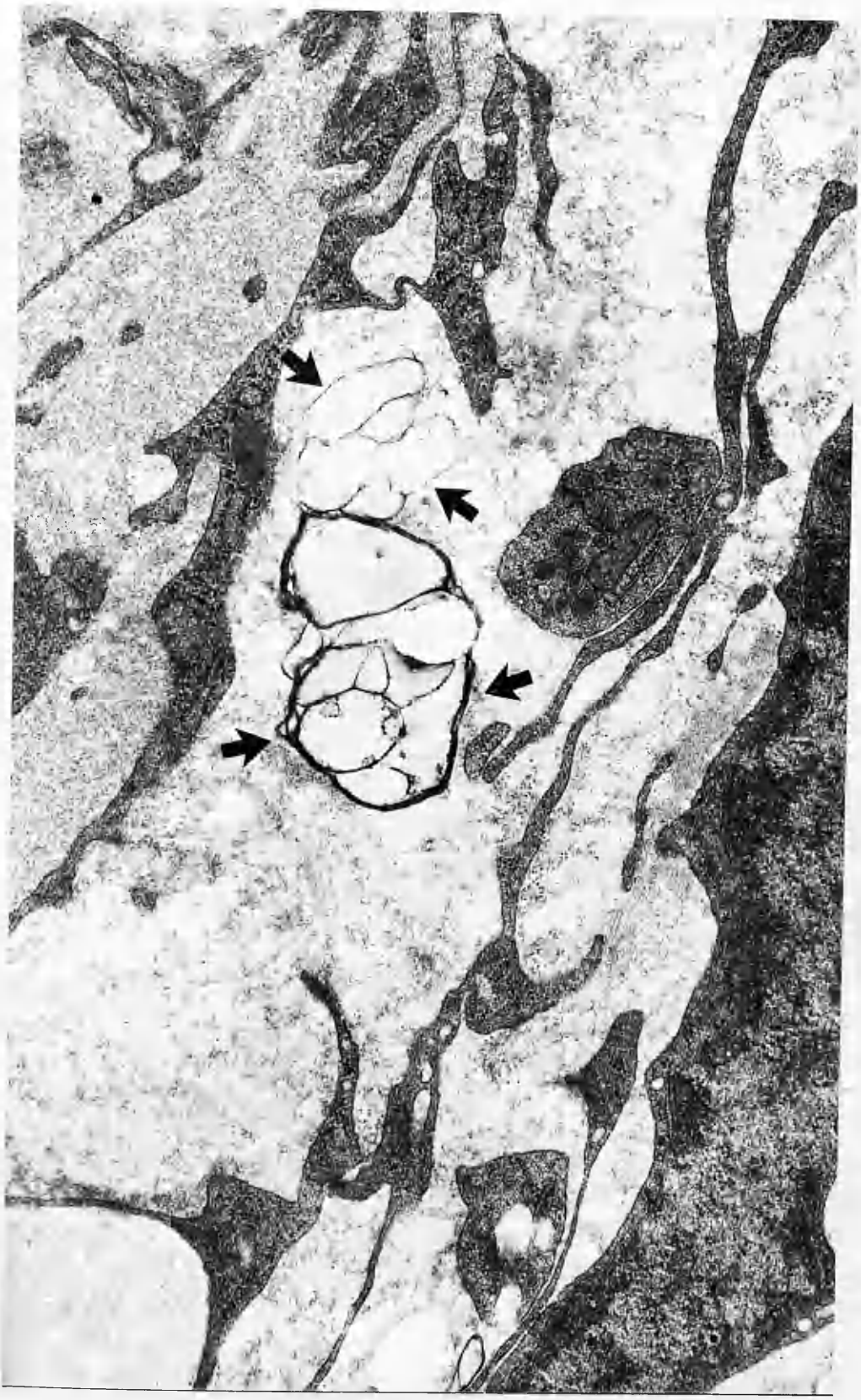
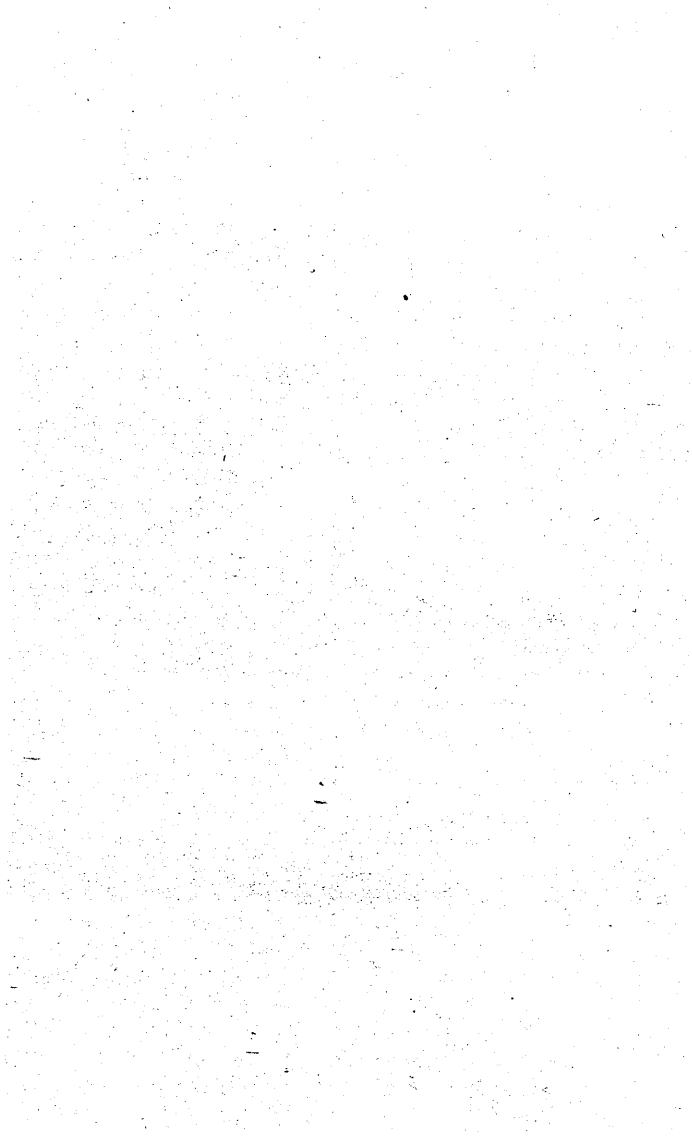


Fig. 63

Shows chylomicrons (arrowed) in the interstitium of a portal tract and in a lymphatic vessel.
x 7840



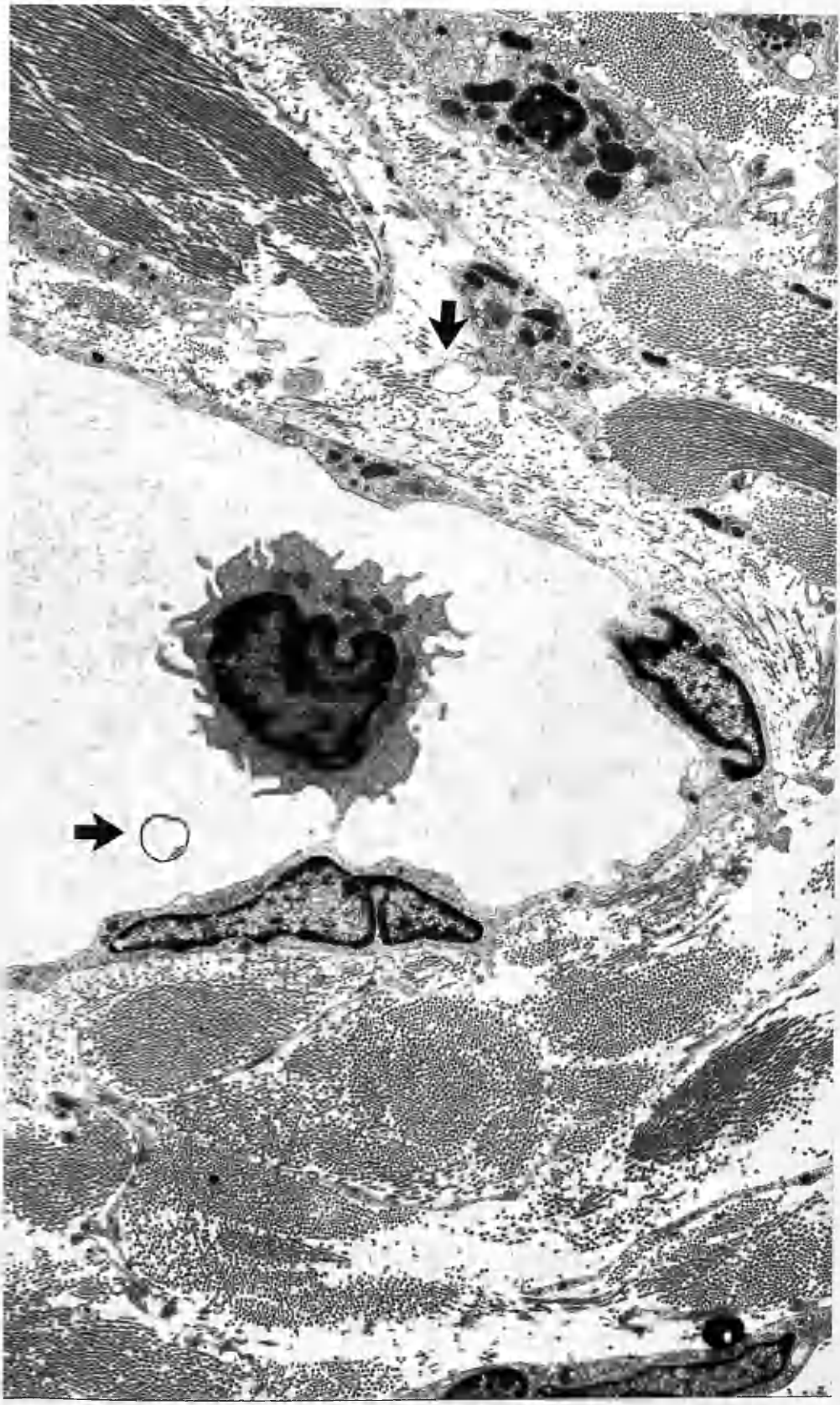


Fig. 64

Shows lipoprotein particles in the sinusoidal lumen (S) and in space of Disse (D).
x 11760

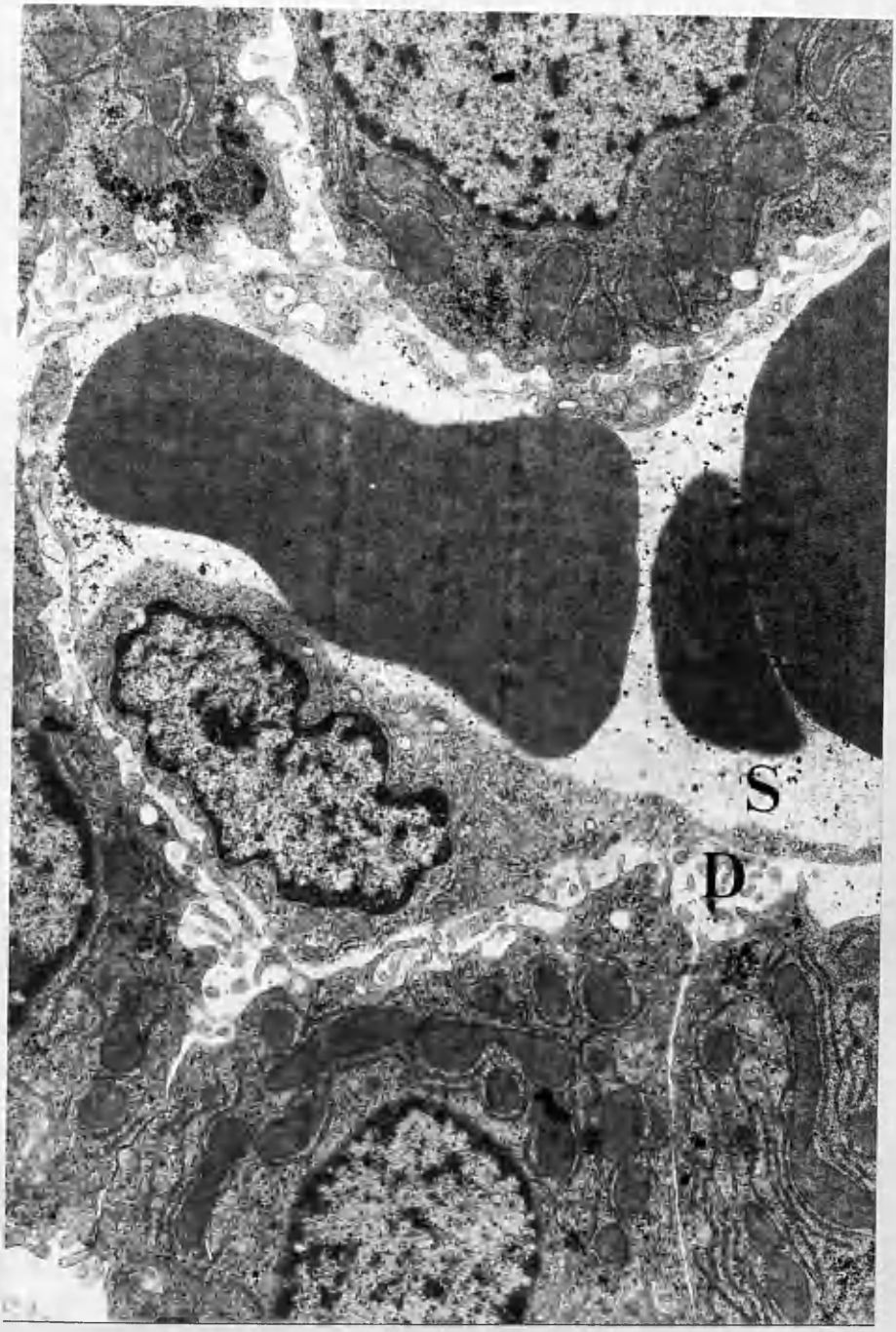

S
p

Fig. 65

Shows lipprotein particles in various spaces of the portal tract interstitium and in a lymphatic (L). x 7840

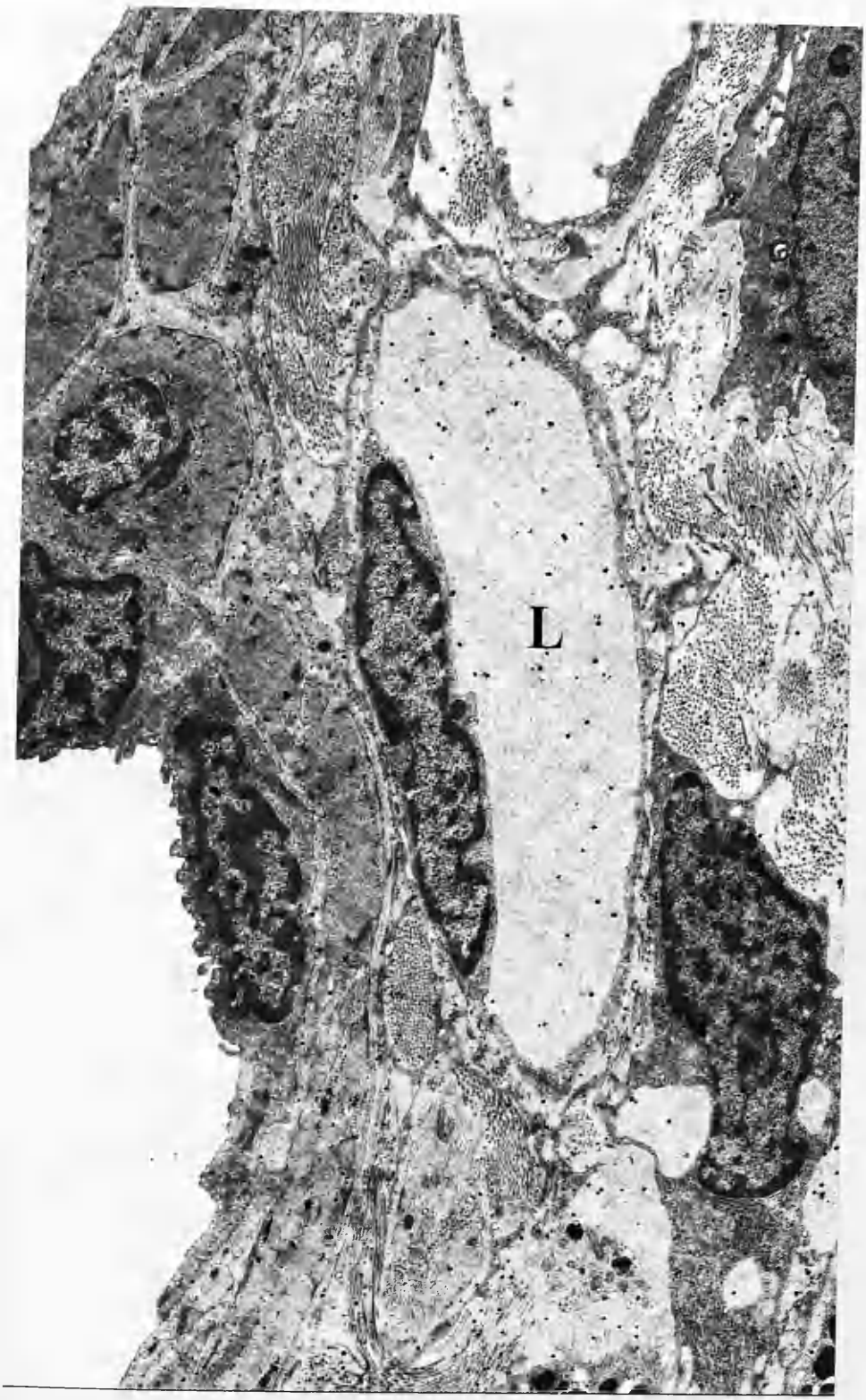

L

Fig. 66

Shows Kupffer cells full of Monstral blue particles.
x 320

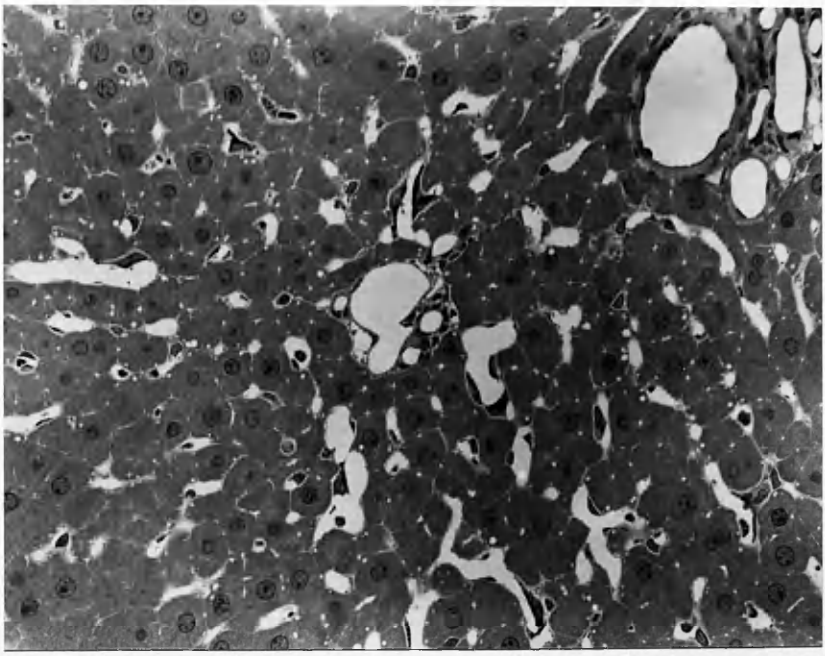
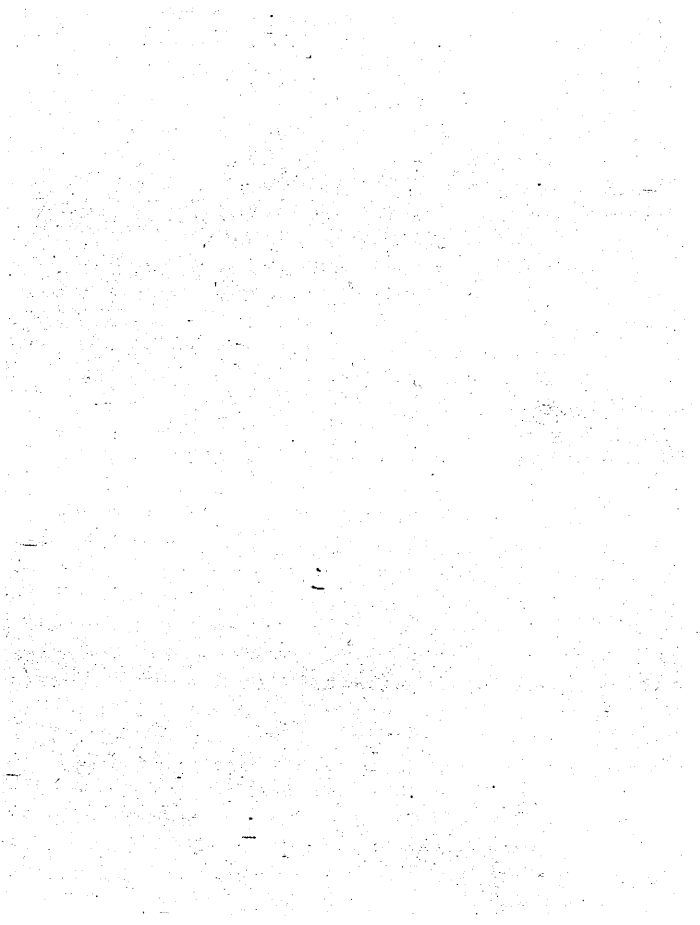


Fig. 67

Shows many Monstral blue particles adherent to the sinusoidal surface of the Kupffer cell which avidly phagocytosed the particles.
x 19600



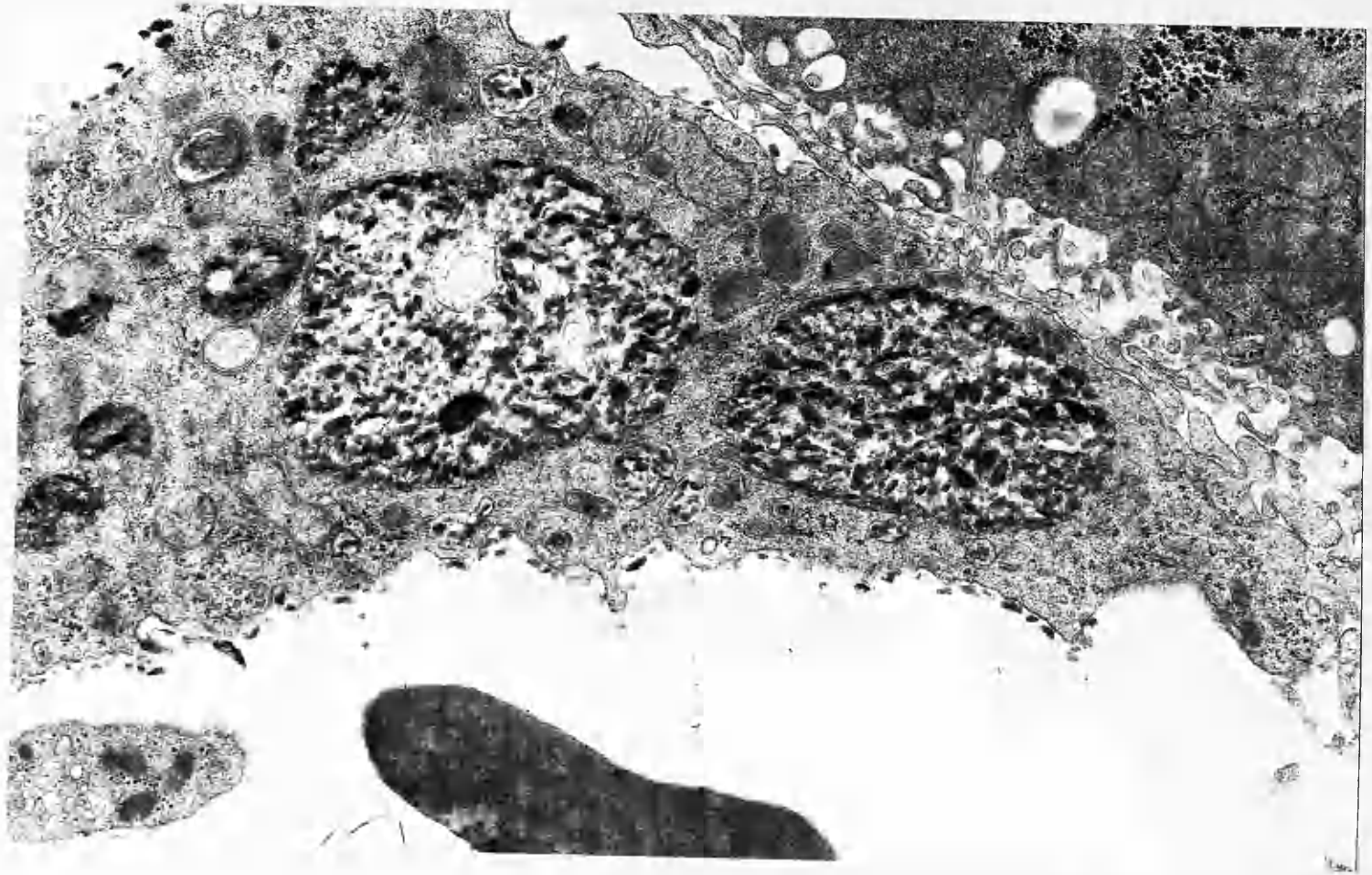


Fig. 68

Shows a few Monastral blue particles passing through interendothelial overlapping junctions.
x 39200

Fig. 69

Shows that only a few Monastral blue particles reached the space of Disse (D).
x 50400

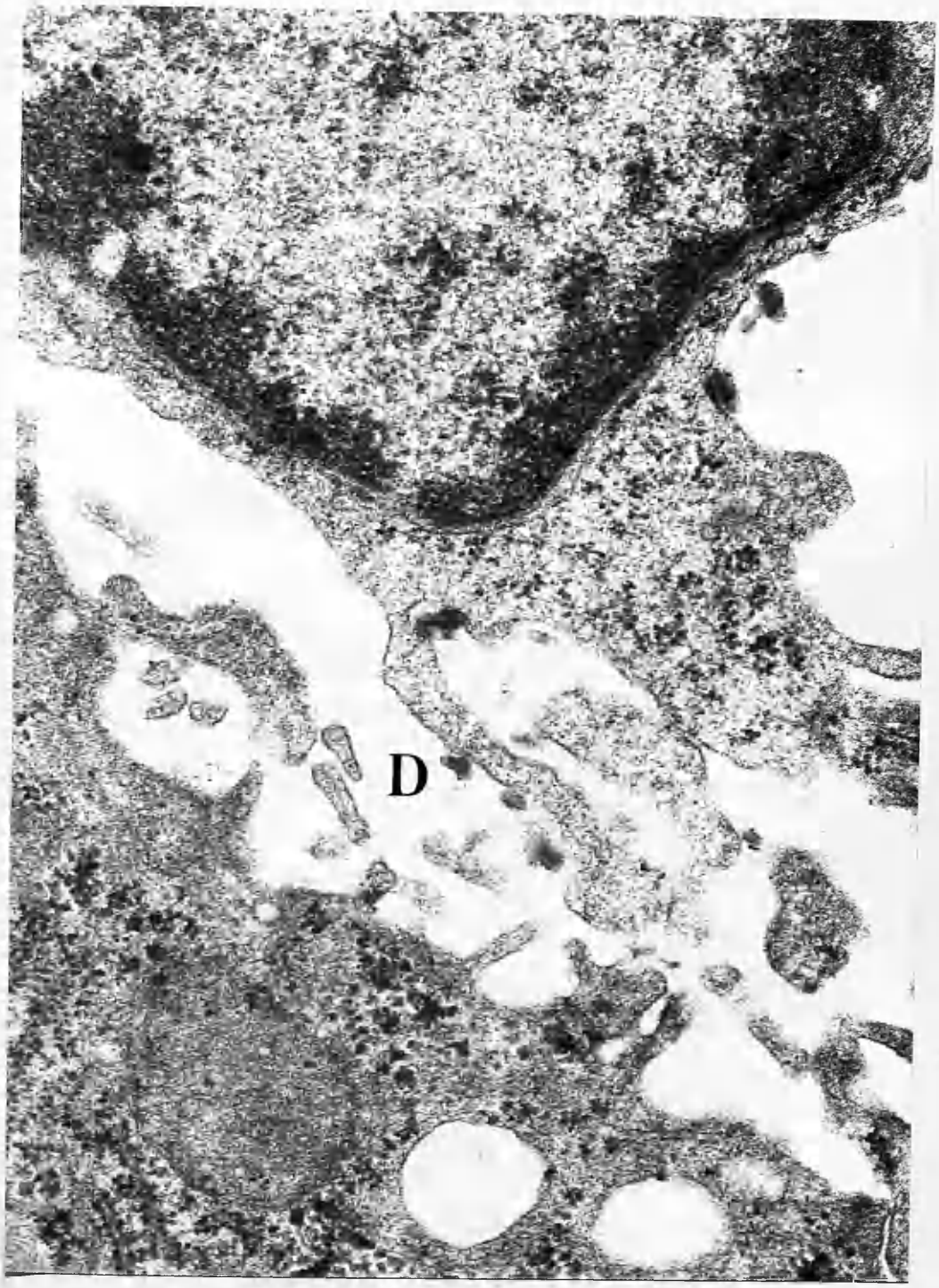

D

Fig. 70

Shows a rare sight of a small cluster of Monastral blue particles in the interstitium of a portal tract and even within a lymphatic.
x 28000

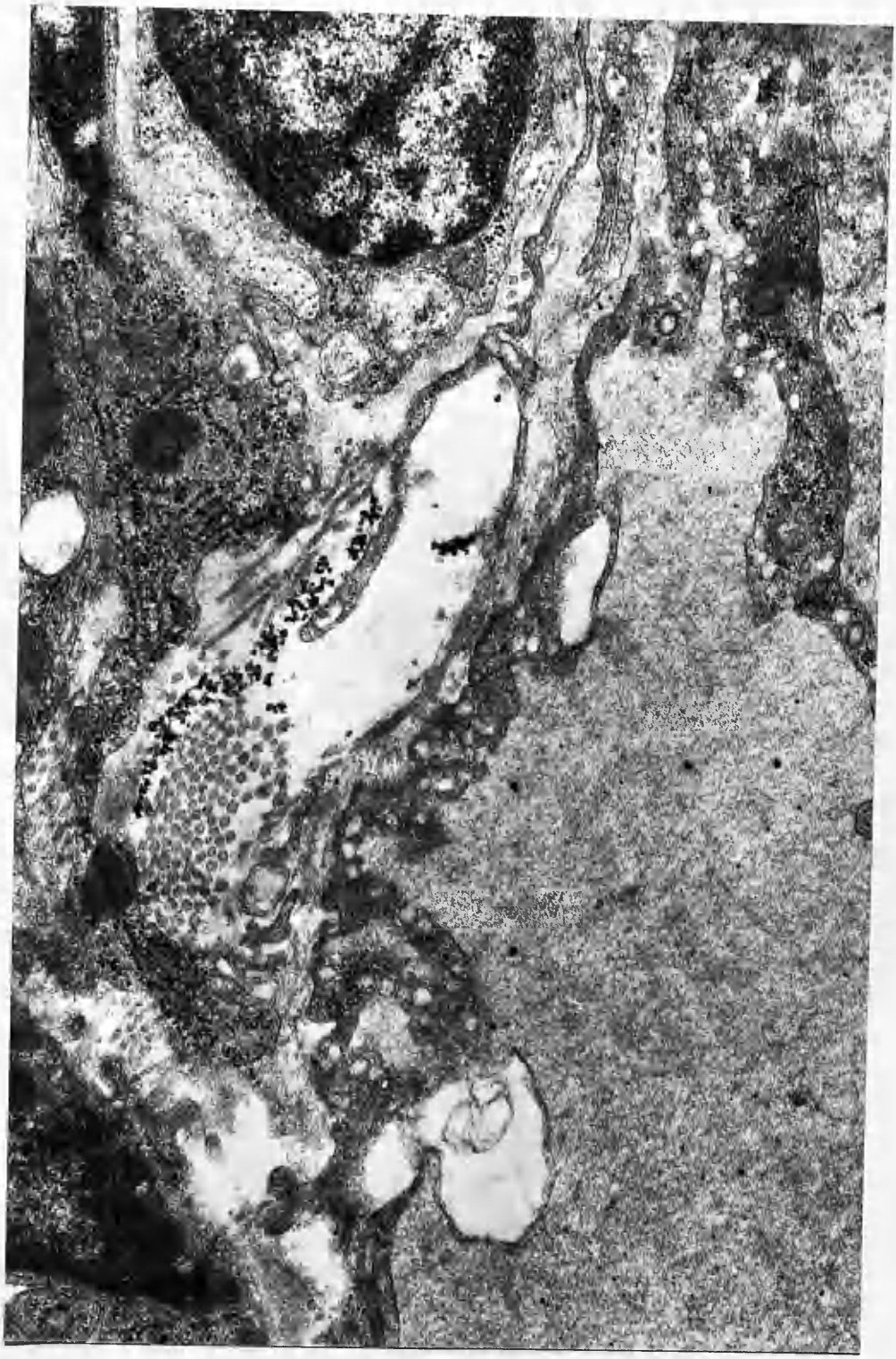


Fig. 71

Shows sectional profile of a large portal tract whose accompanying portal vein branch diameter is greater than 180 μm.

Note: a single lymphatic (arrowed) in the portal tract

24 hr old animal.

x 225

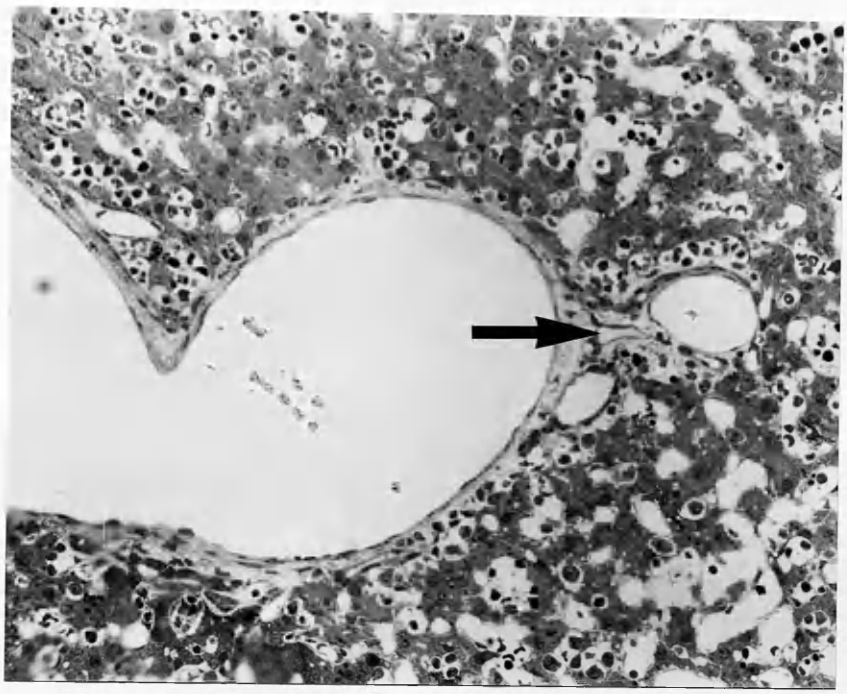


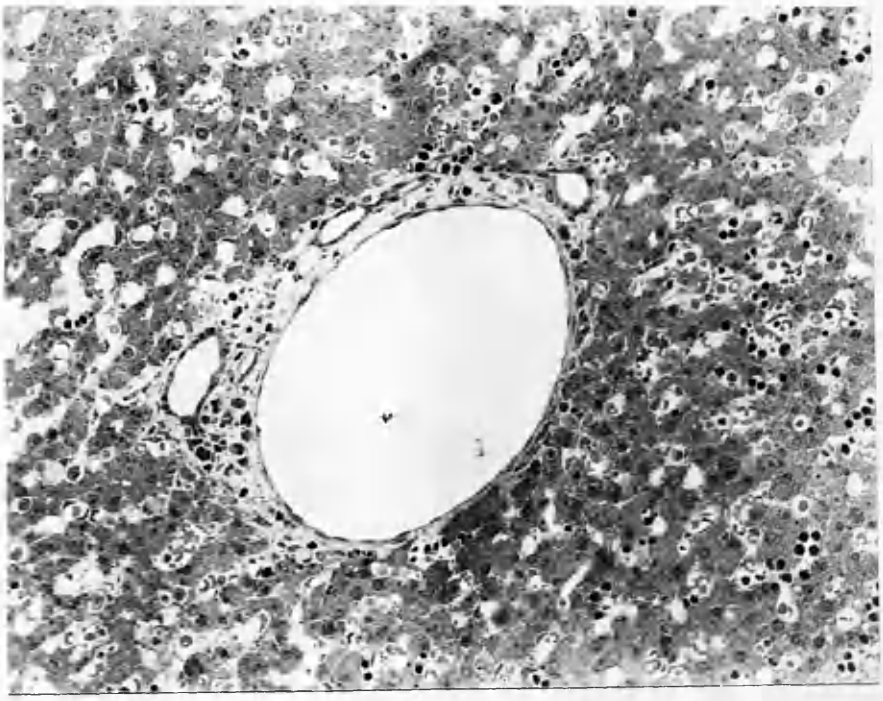
Fig. 72

A & B

Shows sectional profiles of more peripheral portal tracts, with portal vein branch diameters of less than 180 μm, lacking lymphatics.

24 hr old animal.

x 360



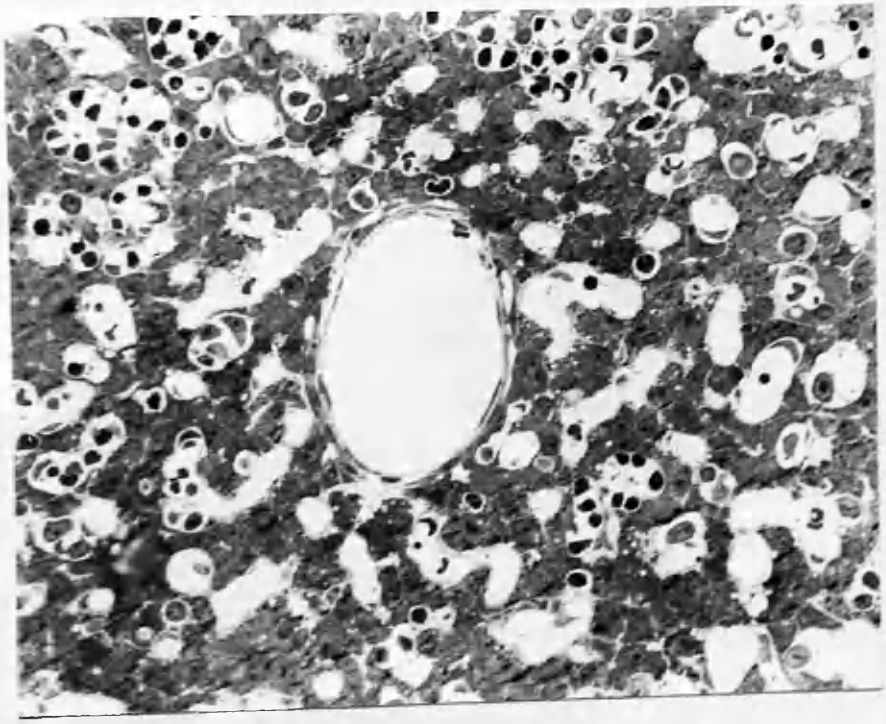
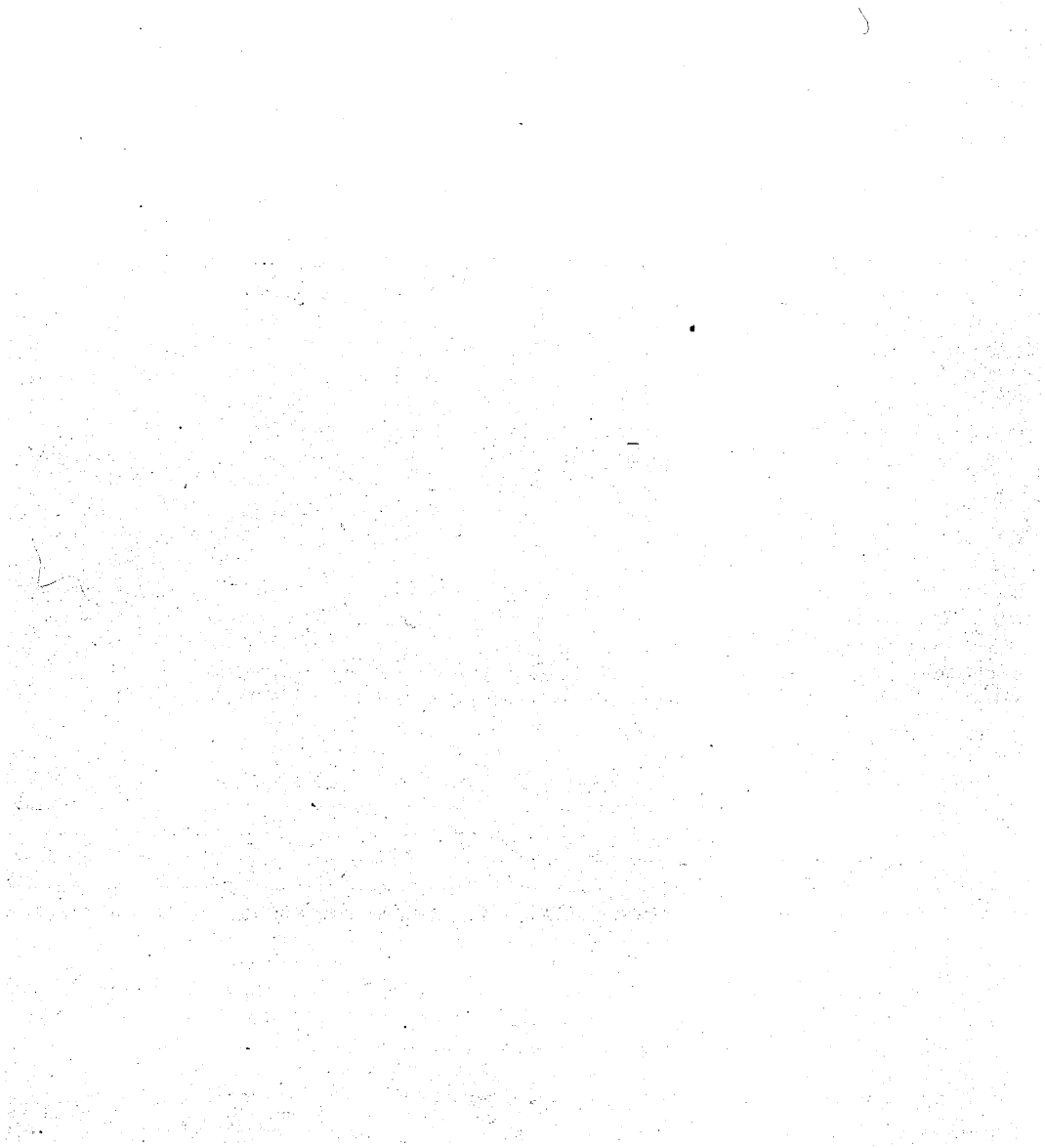


Fig. 73

In this semi-thin sectional profile of a developing lymphatic in a 2 weeks old animal, the lymphatic (L) shows two loculi on the luminal and abluminal sides of the wall.
x 320



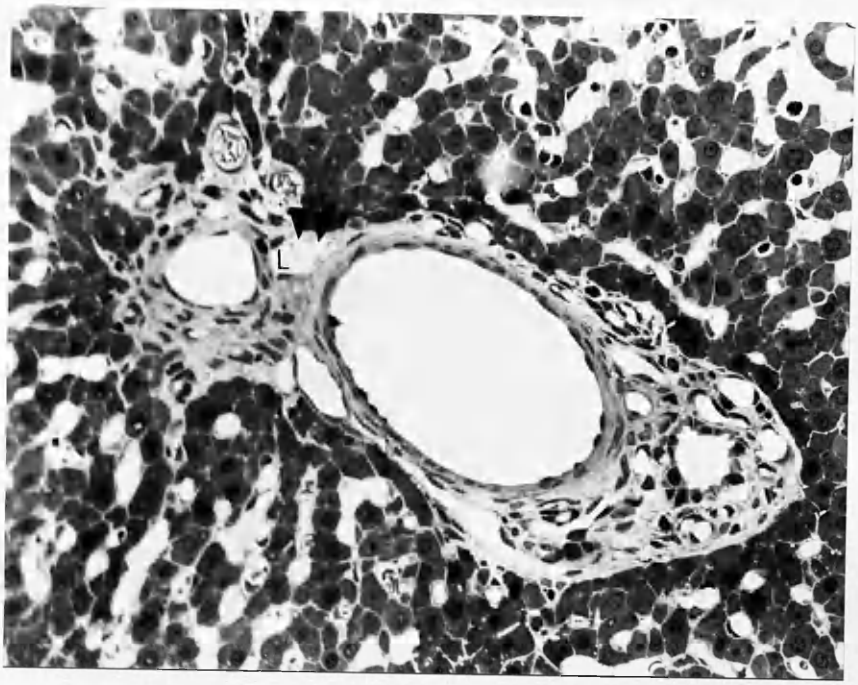

L

Fig. 74

The electron microscopic appearance of lymphatic capillaries shows considerable developmental variations from its "typical" patterns depending upon the age of the animal and the position of the lymphatic within the liver.

In a 2 weeks old rat:

Note: The lining is continuous, but at (A) shows typical endothelial characteristics, and at (B) shows characteristics intermediate between mesenchymal cells and endothelial cells.

x 5940

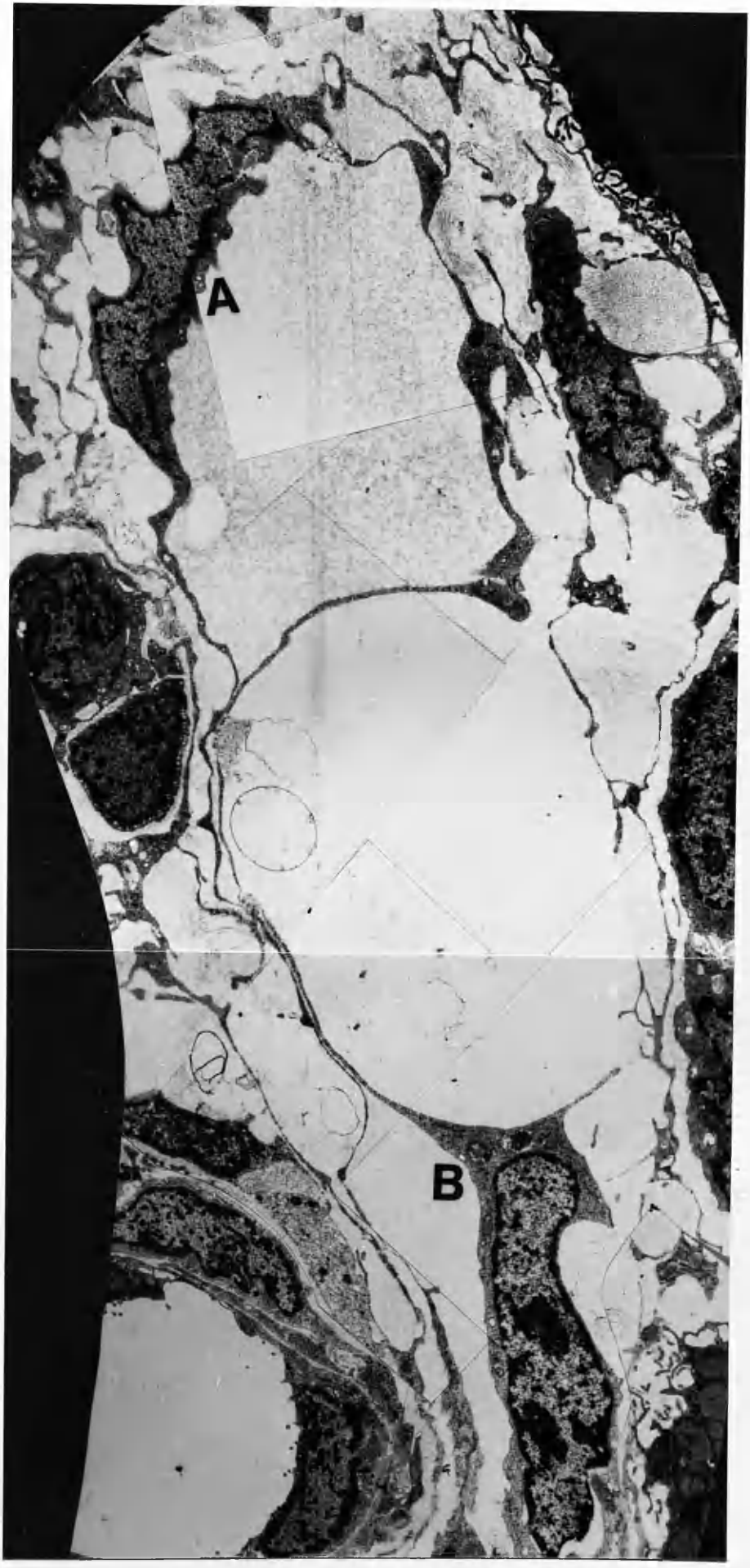

A
B

Fig. 75

Shows two cells A & B which contribute to the wall of an irregular loculated channel in a 24 hour old animal. Both cells retain some mesenchymal characteristics.
x 3920


B
A

Fig. 76

Shows a lymphatic capillary profile in a 2 week old animal.

Note: both cells A & B have "mesenchymal" characteristics.

x 5880

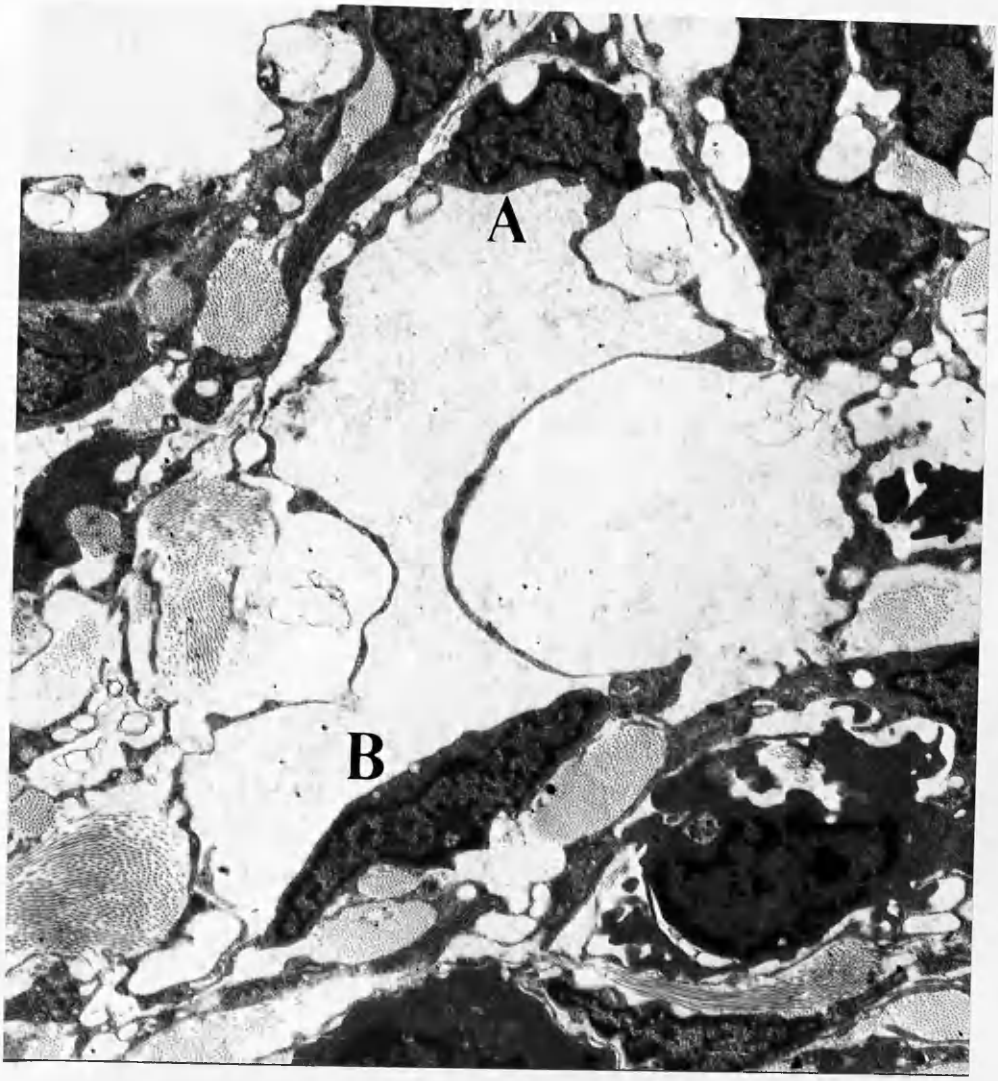
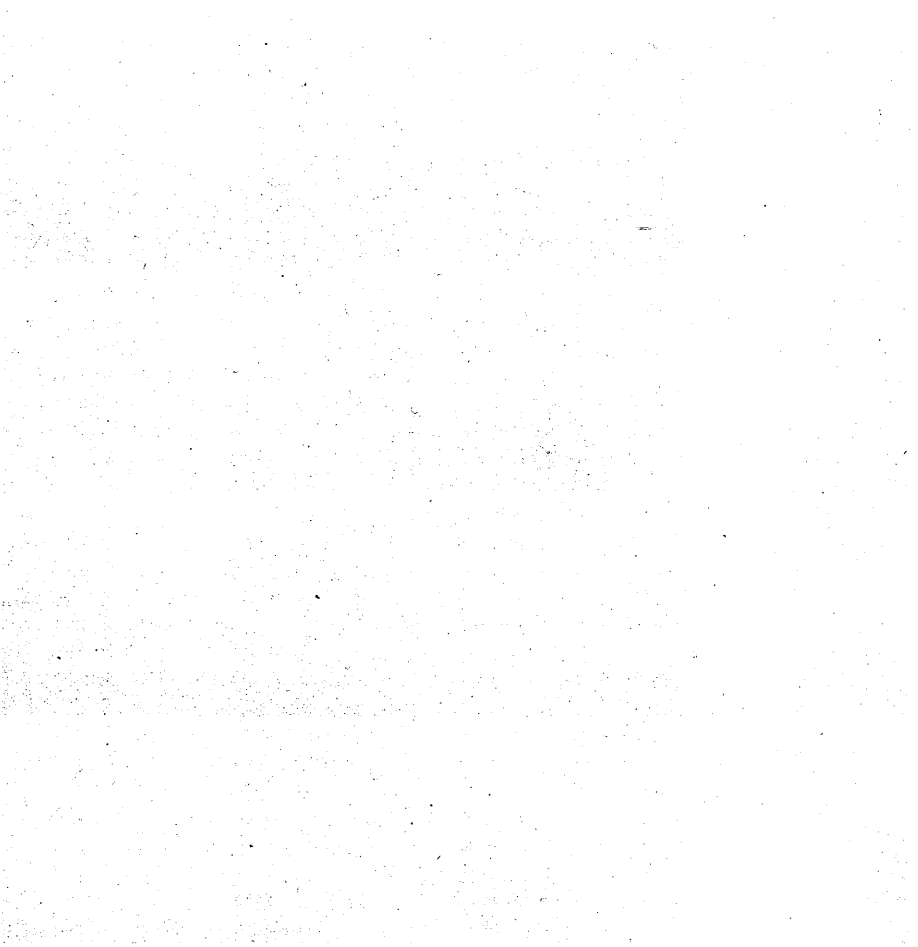

A
B

Fig. 77

Shows a lymphatic sectional profile where the lining cell A has more mesenchymal characteristic compared to cell (B) which looks more like a "characteristic" endothelial cell.
x 3920



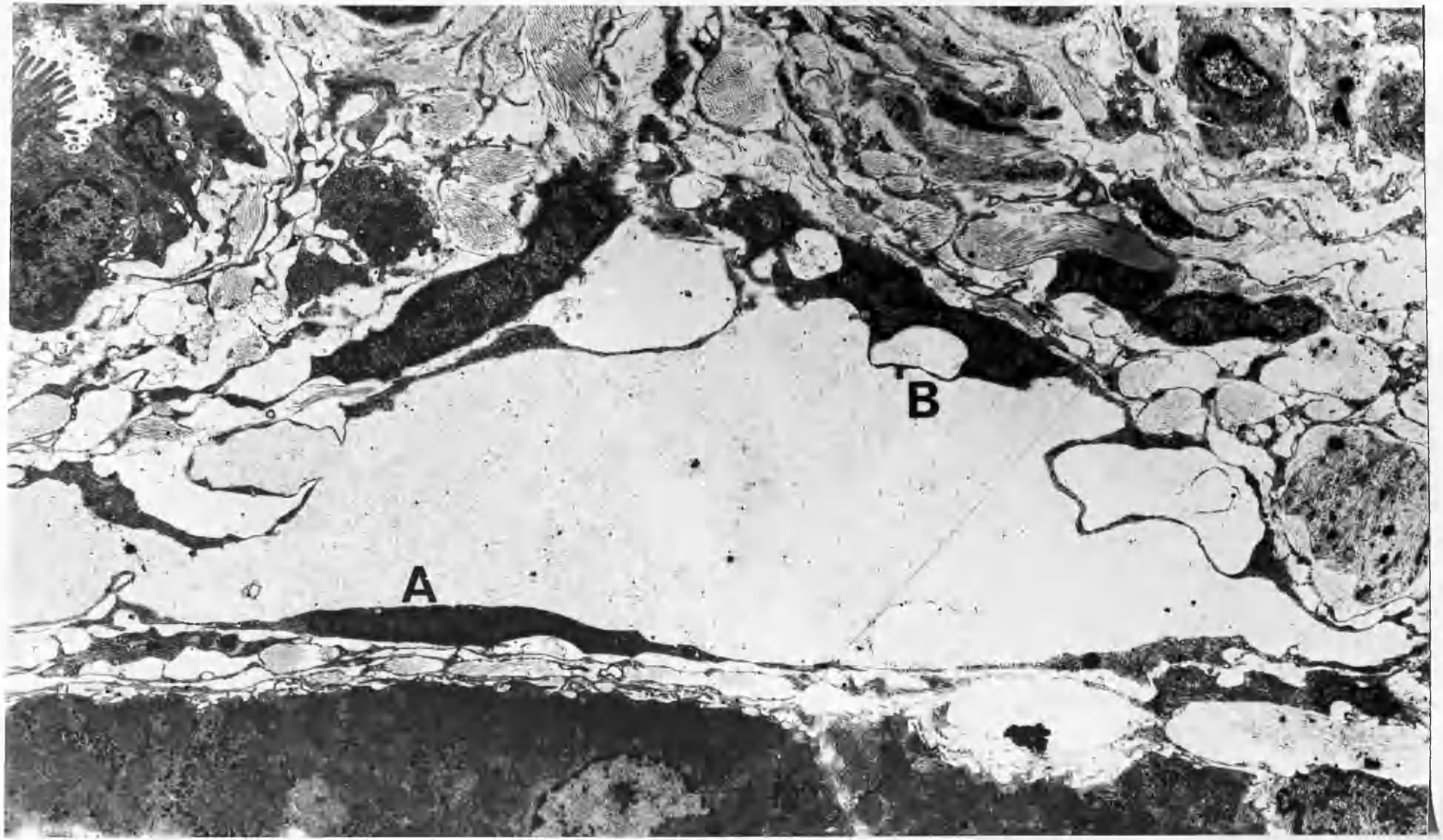

B
A

Fig. 78

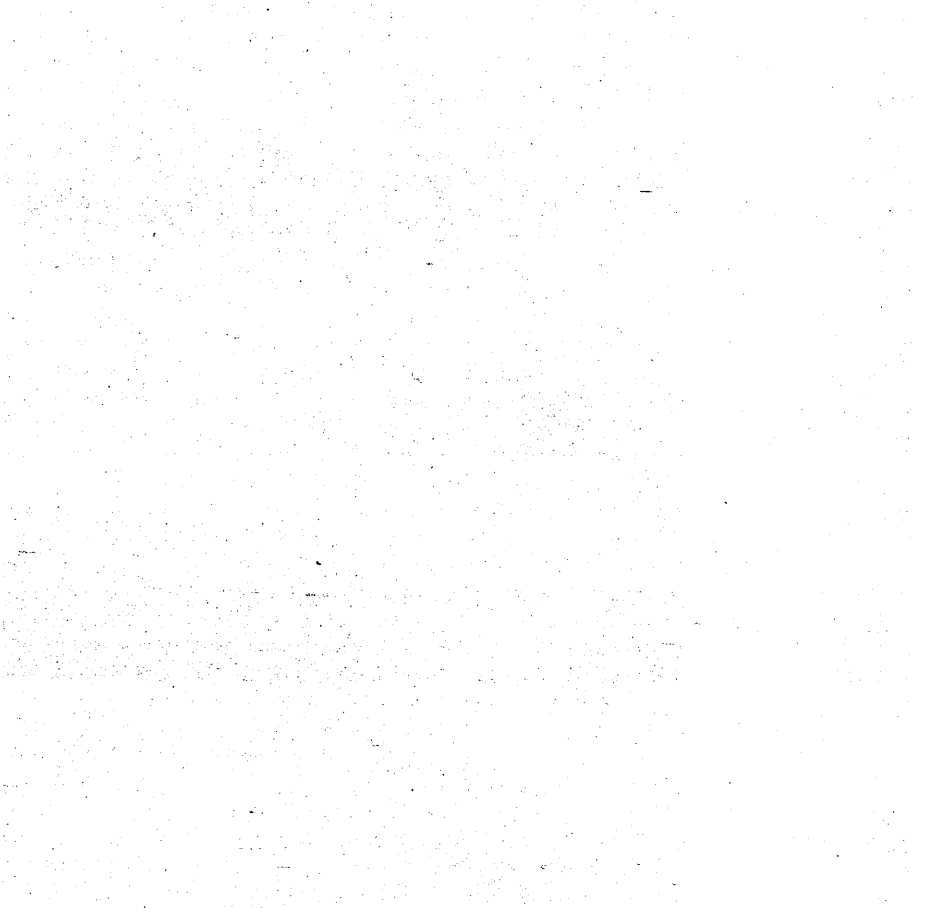
A, B & C

A series of 3 serial sectional profiles of lymphatic capillaries lined by cells which differ in appearance fom typical endothelium. From their abluminal surfaces, cytoplasmic processes extend into continuity with typical mesenchymal cells, with which they help to enclose spaces in the interstitium.

A x 3920
B x 3920
C x 3920

Fig. 79

The lining cell (A) of the lymphatic vessel, in a 2 week old rat, bounds a vacuole which opens into the interstitium.
x 7056




A