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OBSERVATIONS ON CHICK EMBRYO LENS MORPHOGENESIS *IN VIVO* AND *IN VITRO*

by

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ABSTRACT

Ultrastructural observations on the six-day chick embryo lens reveal that fibre cell differentiation, which involves extensive cell elongation, is characterized by the presence of numerous oriented microtubules and by marked changes in intercellular relationships which are felt to be important for cell extension. Increased Golgi activity in cells initiating elongation appears to be related to the formation of intercellular junctions and the elaboration of new surface membrane.

Studies concerning the differentiation *in vitro* of anterior lens epithelia demonstrate that their cells elongate either to a great extent or to a limited extent, depending on factors deriving from the epithelial conformation. The conformation of an explanted anterior lens epithelium can be manipulated so that its cells elongate consistently to either a limited or a great extent. These cells, whether elongated to a limited or to a great extent, are ultrastructurally similar with respect to features reflecting cytoplasmic differentiation, including those important for morphogenesis. Their fine structure closely resembles that of fibre cells differentiating *in vivo*. The cells of anterior lens epithelia differentiating *in vitro*, whether elongating to a limited or to a great extent, are the same in terms of the nature and relative proportions of their soluble proteins as indicated by polyacrylamide gel electrophoresis. There is a change in the relative proportions of the soluble proteins accumulated by the

anterior lens epithelia when they differentiate *in vitro* that is similar to that which occurs in cells undertaking fibre cell differentiation *in vivo*. It is concluded that those factors deriving from the conformation of the anterior lens epithelium that affect the degree of elongation of its cells differentiating *in vitro* are physical in nature. Since such physical factors are important with regard to restraining or encouraging the expression of morphogenetic potential *in vitro*, it is suggested that similar physical forces are important in lens morphogenesis *in vivo*.

Observations concerning anterior lens epithelia elongating to a great extent *in vitro* demonstrate that their cells progressively elongate for only about three days. Well differentiated explants cultured for longer periods do not show greater elongation or further cytoplasmic differentiation. Their cells, at the ultrastructural level, resemble differentiating fibre cells *in vivo* rather than differentiated ones. It is concluded that the anterior lens epithelium has some capacity for differentiation independent of the ocular environment, but that the latter is essential for the expression of its full developmental potential.

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INTRODUCTION

The Vertebrate lens has long been studied by developmental biologists with a variety of points of view. Spemann's demonstration, at the turn of the century, of the role of the optic vesicle in inducing the lens is a classic example of induction (see Spemann, 1938). His work, as well as more recent investigations (Jacobson, 1966; McKeehan, 1954; Muthukkaruppan, 1965), have contributed much to the concepts of induction and determination. The adjustment of the growth of the lens to the rest of the eye has received some attention (Twitty, 1955; Coulombre and Coulombre, 1969). Biologists interested in the regulation of the cell cycle have also directed their attention to the lens (see recent review by Harding *et al.*, 1971).

Workers concerned with the problems of cell differentiation have been attracted to the lens for a number of reasons. The organisation of this structure is such that one can study, in a single lens, cells at different stages of differentiation. And it is possible to physically separate different regions of the lens that correspond to different stages of cell differentiation in order to do biochemical studies (Papaconstantinou, 1967). An additional advantage is that the lens is composed of cells of a single type derived from an initial ectodermal population, the lens placode. Furthermore, lens cell differentiation involves marked morphological and biochemical changes, the most striking of which are extensive cell elongation and the synthesis of large quantities of unique structural proteins, the lens crystallins. For all these reasons, considerable effort

has been directed toward describing lens differentiation, ultrastructurally and biochemically, with a view to learning something about the processes of cell differentiation and their control.

The lens also represents a structure interesting from the point of view of morphogenesis inasmuch as its cells are highly organized and form a symmetrical structure of particular shape, with a specific spatial relationship to other components of the eye, to which lens growth is appropriately regulated (Coulombre, 1969; Coulombre and Coulombre, 1969). The growth of the lens involves the generation, through mitosis, of new cells within the anterior lens epithelium and the coordinated differentiation of cells at the lens equator. Here, differentiation involves extensive cell elongation so that new fibre cells are added to the lens body uniformly, generating a functional lens of appropriate size and shape. What is of interest is the manner in which morphogenesis is achieved and how it is controlled.

The tissues of the eye interact with each other in some manner that is important for its development and integrity (Coulombre, 1969; Coulombre and Coulombre, 1963; Takeichi, 1970; McKeehan, 1951, 1954; Muthukkaruppan, 1965). It would be of interest to be able to distinguish the nature and relative importance of the roles the ocular environment and the properties of the lens play in lens development. A report relevant in this context is that of Coulombre and Coulombre (1971). These authors demonstrated that if the lens of a chick embryo were replaced with anterior lens epithelium, the latter

would form a lens with an epithelium, of appropriate size and orientation, and of a shape which, although not perfect, more or less resembled that of a normal lens. On the other hand, when placed *in vitro*, the cells of the anterior lens epithelium were reprted (Philpott and Coulombre, 1965, 1968) to elongate, in culture medium supplemented with serum, to only a limited degree. They did not elongate at all, however, in unsupplemented medium.

These findings raise a number of questions, first with respect to the reasons for limited rather than extensive cell elongation, and second concerning the relationship between cell elongation and cytoplasmic differentiation. They also indicated the possibility of manipulating lens development *in vitro* and thereby learning something about morphogenesis, cell differentiation and the relative roles of the lens and the ocular environment with respect to these processes.

Because of these considerations it was considered of interest to investigate the development of the chick embryo anterior lens epithelium *in vitro*. Since the published ultrastructural observations (Porte *et al.*, 1968; Beyers and Porter, 1964) appeared somewhat inadequate, my investigation was first concerned with the fine structure of the chick embryo lens in order to make observations relevant to morphogenetic considerations, and necessary for ultrastructural evaluation of lens differentiation *in vitro*.

It was apparent that most ultrastructural observations reported were concerned with cytodifferentiation of the lens. The loss of cytoplasmic organelles that accompanies lens fibre cell differentiation, and the features attending protein synthesis (prominent nucleoli, numerous polysomes, accumulation of cytoplasmic matrix) are fairly well documented for a number of Vertebrate lenses (Wanko and Gavin, 1959; Porte et al., 1968; Rabaey and Lagasse, 1965; Eguchi, 1964; Cohen, 1965; see Smelser, 1965). Only two fine structural studies concerning the chick embryo lens were performed using glutaraldehyde fixation (Beyers and Porter, 1964; Porte et al.,1968), and the former was concerned mainly with microtubules in the early lens rudiment.

With the exception of the possible role of microtubules (Beyers and Porter, 1964; Kuwabara, 1968) in fibre cell elongation, little consideration has been given to observations pertinent to lens morphogenesis. Although intercellular junctions (tight junctions and 'desmosomes') between fibre cells have been reported in a number of Vertebrate lenses, the literature does not provide a clear description of their development. Nor does the literature provide any observations concerned with the manner in which the new surface membrane required for cell elongation is elaborated, or very much information regarding the Golgi apparatus, an organelle considered to be concerned with membrane synthesis (Sjostrand, 1968; Whaley et al.,1971), in cells of the lens.

With respect to investigating *in vitro* differentiation of anterior lens epithelia, a preliminary experiment indicated that cells of the epithelia would undergo considerably greater elongation than that previously reported (Philpott and Coulombre, 1965, 1968). It subsequently became apparent that the epithelial conformation was important for this elongation, and that by manipulating the epithelial conformation in certain ways eother very limited or very great cell elongation would result. It was then of interest to undertake ultrastructural studies with respect to anterior lens epithelia differentiating *in vitro* from the points of view of morphogenesis and cytodifferentiation.

MATERIALS & METHODS

General in vitro procedures.

Fertilized White Leghorn eggs of six days incubation were obtained from the Department of Poultry Genetics (U.B.C.). The embryos were removed and transferred to a petri dish containing sterile Puck's Saline-G for dissection. The vitreous bodies with adhering lenses were plucked from the eye with watchmaker's forceps and tha adhering iris and vitreous separated from the lenses. The lens epithelia were separated from the lens body, and carefully transferred, by one of three methods, to either a Nuclepore (Winley-Morris) or Millipore (type TH) filter.The filter was supported by either a Plexiglass or stainless steel mesh raft on the surface of approximately 1 ml. of culture medium (Ham's F-10, North American Biological Co.) supplemented with 15% fetal calf serum (Grand Island Biological Co.) and antibiotics (100U/ml. penicillin, 0.25 mcg./ml.fungizone, 100 mcg./ml. streptomycin, Grand Island Biological Co.) in an organ culture dish (Falcon Plastics).

Transfer method 1. This method of transferring epithelia was one that involved no attempt to orient the explant, and resulted in the epithelia having a variety of conformations.

Transfer method 2. This method involved reversing the normal epithelial curvature so that the epithelia were folded with the lens capsule inside.

Transfer method 3. This method of explantation was such as to maintain the normal curvature of the epithelia so that the epithelia were folded with the lens capsule outside.

The cultures were incubated at 37°C. in an atmosphere of 96% air and 4% CO_2 , and a humidity approaching 100%, for different periods of time between 1-6 days, after which they were processed for microscopic examination or for electrophoresis.

Microscopy.

Lenses and explants were fixed in 3% glutaraldehyde in 0.1N sodium cacodylate (pH 7.4) at room temperature, washed in buffer (plus 10% sucrose) and post-fixed in cold OsO_4 (1% in phosphate buffer, pH 7.2). Subsequently the tissues were washed, dehydrated and embedded in Epon.

Sectioning was performed with a LKB ultratome. Thick sections (approximately 1um) for light microscope examination were stained with 1% Toluidine Blue (Fisher Scientific) in 1% sodium borate. Thin sections for electron microscopy were stained with methanolic uranyl acetate and Reynold's lead citrate.

Thick sections were examined and photographed with a Zeiss photomicroscope. From the blocks of embedded tissue examined in this manner, appropriate ones were chosen for thin sectioning. Electron microscopy was performed with the HU 7S, HU 11A, and AEI 801 electron microscopes.

Polyacrylamide gel electrophoresis.

Electrophoresis on 7% polyacrylamide gels was performed as outlined by Truman (1968). Lenses or explants were homogenized in 10mM phosphate (pH 7.2), the homogenate centrifuged (10,000 g. for 10 min.) and the supernatant (mixed in 40%

sucrose, 1:1), in appropriate quantity, layered on the stacking gel. Electrophoresis was carried out at 3 mA/tube for 2-4 hours. The gels were subsequently fixed (in 12% TCA), stained (in a mixture containing 45ml. of 0.2% Coomassie Brilliant Blue (Mann Research Laboratories), 45ml. absolute ethanol, 10 ml. glacial acetic acid), destained (in a mixture containing 65ml. water, 25 ml. absolute ethanol, 10 ml. glacial acetic acid), stored (in 10% acetic acid), and scanned with a Gilford spectrophotometer at 540 nm.

OBSERVATIONS

I- Organization of the 6-day chick embryo lens.

The 6-day chick embryo lens is illustrated in figures 1-4. The developing lens is a population of epithelial cells enclosed by a modified basement membrane, the lens capsule. The cells of the anterior portion (anterior lens epithelium) are undifferentiated and mitotically active. Those of the posterior part (facing the neural retina) are highly differentiated fibre cells. In the region of the equator (figures 1, 4), cells of the anterior lens epithelium elongate and contribute to the lens body. Here, one can observe cells at different stages in their remarkable transformation into lens fibres. Figures 1-4 illustrate how various regions of the 6-day lens have been defined, for purposes of discussion, as representing different stages in what is a continuous process of differentiation.

II- Morphology of the central anterior epithelium (region I).

The central anterior epithelium (region I, figure 2) of the six-day chick embryo lens is a simple cuboidal layer adherent to the lens capsule. The nature of the association between cells is reflected by the numerous intercellular spaces (figures 2, 7) and by the relationship between adjacent membranes which are not closely apposed, but the contours of which generally conform (figures 5,7,8). Typical epithelial junctional complexes are evident at the apical and basal boundries, each with associated microfilaments traversing the cytoplasm between junctions (figures 5,8). The apical junctional complexes are more prominent than the basal ones, and involve

regions of tight junction and zonulae adhaerentes, while the basal ones involve only the latter.

The mitochondria, although of general distribution, tend to be concentrated basally and apically (figures 5,7).They are often observed to be associated with lipid inclusions (figure 5) which are consistently found, usually in the apical cytoplasm. A moderately developed perinuclear rough endoplasmic reticulum is present (figures 5,6), and its elements always appear filled with dense material. Ribosomes, in addition to those associated with the endoplasmic reticulum, appear in the cytoplasm, though polysomes are not prominent. A moderately developed Golgi apparatus is consistently found in the apical cytoplasm (figure 6). Centrioles and cilia can also be observed in this region.

Microtubules are present (figure 8), but relatively sparse (except in mitotic figures), and are often randomly arranged. The number of microtubules and their degree of orientation increase towrd the lateral regions of the anterior epithelium. Microfibres (figure 8), approximately 100 Å in diameter, are consistently found in the central anterior epithelium. They are largely restricted in their distribution to the basal cytoplasm and show no particular orientation.

III- Morphology of region II.

The lateral anterior epithelium is multi-layered (figures 3-4). The intercellular spaces first become restricted to the basal regions and then disappear. Toward the equator, region II shows the initial features of differentiation that will be

progressively elaborated in regions III and IV, and the cells of region II (figures 9-11) illustrate a stage of differentiation intermediate between that of regions I and III. They contain a more active Golgi apparatus than those of the central epithelium. Cilia are more frequently observed in this region, though lipid inclusions are fewer. Some degree of cell elongation, accompanied by a reduction in intercellular spaces, is apparent. Microtubules are more abundant and are oriented with the long axes of the cells. Since these structures appear to occupy the peripheral aspects of the cytoplasm, their abundance can be appreciated in sections cut tangentially through the long axes of adjacent cells (figure 10).

IV- Morphology of region III.

Cells of region III (figure 4), immediately adjacent to young fibre cells, have started to elongate and contain oriented microtubules, particularly in the region between the nucleus and the apical surface. Cilia are frequently observed at the apical border of these cells (figure 16) and satellites, associated with basal bodies (figure 17) appear to be involved in the elaboration of microtubules. In the more basal regions of these cells, oriented microfibres are remarkably abundant (figure 12). The intercellular spaces of region III are few and basal. Adjacent cell membranes run more or less parallel (figures 12, 15. 18) and engage in junctions only toward their apical and basal surfaces.

The Golgi apparatus appears to be very active (figures 15, 27) and extends throughout the apical cytoplasm,

occasionally from two or three centres.Vesicles, apparently of Golgi origin, are particularly evident in the apical cytoplasm of cells in this region (figures 14-16). These vesicles appear empty and are frequently associated with the plasma membrane, occasionally appearing to fuse with it (figure 14). Elements of the rough endoplasmic reticulum, although tending to be concentrated just above and just below the nucleus, are often distriuted along the length of the cell. The quantity of rough endoplasmic reticulum (figures 13, 15) does not appear to be greater than in cells of region I. However, numerous free polysomes pervade the cytoplasm (figures 15, 18).

The nuclei of this region of the developing lens have an elliptical shape, prominent nucleoli, and many nuclear pores, all features which become more fully elaborated in young fibre cells (figures 22, 23).

V- Morphology of regions IV & V.

The cells of region IV are spectacularly elongated young fibre cells (figure 20) in which microtubules are remarkably plentiful (figure 19). However, in more mature fibre cells, which occupy a more central position in the lens body, microtubules are much less abundant (figures 21,25,26,28,29). Microfibres, so abundant in region III, are much less so in differentiating fibre cells of region IV, and in region V only scattered microfibres are observed (figure 25).

While the adjacent plasma membranes of cells in region III are not involved in junctions, except for apical and basal complexes, the relationship of adjacent cell membranes of

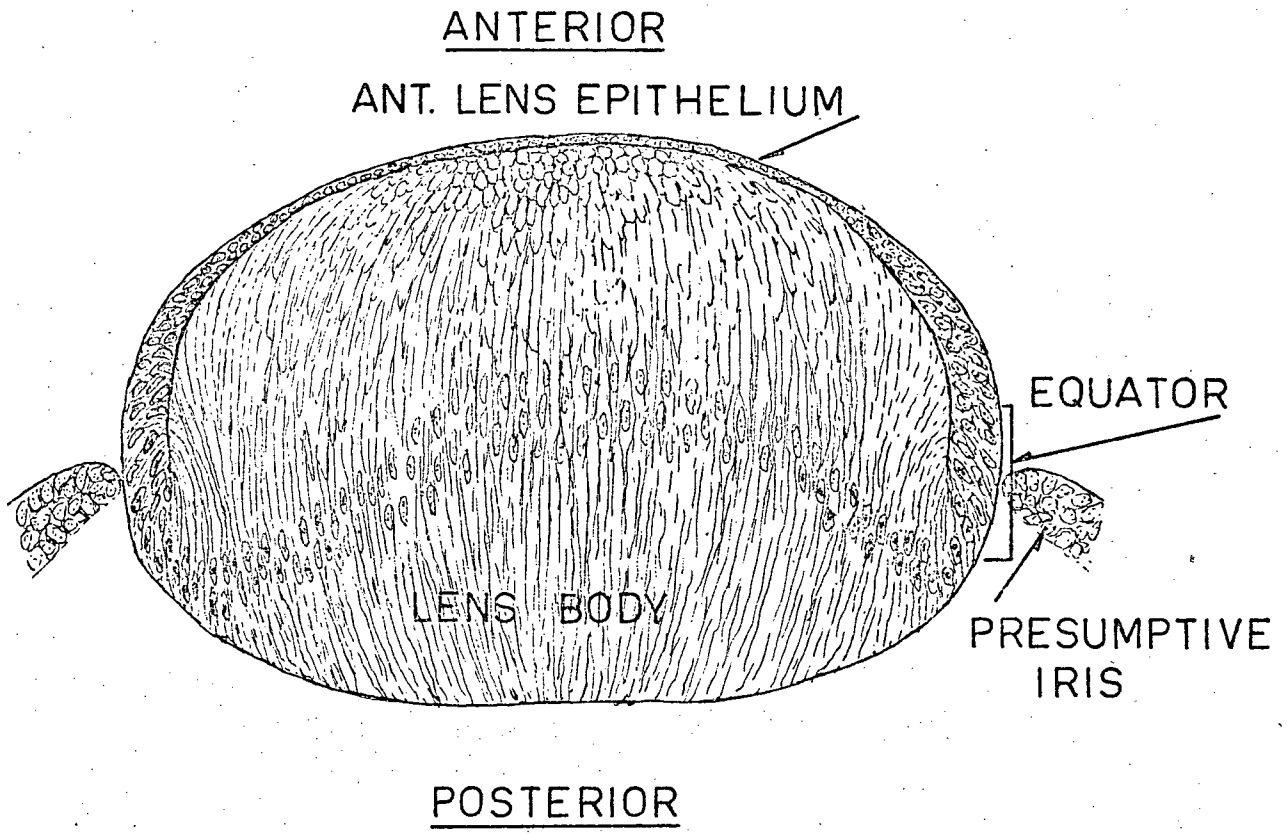
Young fibre cells is different in two ways. One modification is the association of plaques of dense material with the membranes of adjacent cells at various points along their lengths (figure 20). The intercellular space of these junctions contains amorphous material. Similar junctions in the chick embryo nervous system have been termed 'adhesion plaques' (Pannese, 1968). These junctions appear to be maintained as the cells differentiate, since they are evident in region V (figures 21,28,29). Porte et al. (1968) observed these junctions between fibre cells of the chick embryo lens and referred to them as desmosomes. However, in the intercellular spaces of these junctions amorphous material rather than the characteristic structure of desmosomes is evident. Coated vesicles are often observed in association with and fused with cell membranes at various points (figure 24) suggesting that these junctions may be derived from the Golgi. Another striking modification of young fibre cell membranes is the development of tight junctions. With differentiation, the elaboration of tight junctions appears more and more extensive, as illustrated by cell membranes in region V, where tight junctions are quite extensive, but not continuous (figures 21,28,29).

While the Golgi apparatus is well developed in cells initiating elongation, and many Golgi derived vesicles are observed in the apical cytoplasm of these cells (figure 20), Golgi activity appears to decline with further differentiation. In fibre cells of region V, a very reduced Golgi body is typically found in the apical cytoplasm (figure 26). The rough

endoplasmic reticulum also becomes reduced, the vesicles appearing to fragment, so that in region V only small, scattered elements are observed. Mitochondria also become reduced in number. Degenerating mitochondria can be observed (figures 28, 29). Some mitochondria undergo extensive elongation (figure 29), yet also show signs of degeneration. Polysomes, which are very abundant in young fibre cells, become progressively dispersed amidst an accumulating matrix with differentiation (figures 21,26,28,29) and decline in number. Cilia and basal bodies are often seen in the apical cytoplasm of cells in regions IV and V.

In summary, it may be considered that the ultrastructural differences between the cells of regions I, II, and III represent the changes that occur during the initial stages of lens cell differentiation. Between regions I and III the cells become moderately elongated, and intercellular spaces are eliminated. Oriented microtubules increase in number and abundant microfibres come to occupy the basal cytoplasm. Golgi activity increases. Nuclei become elliptical in shape, develop prominent nucleoli, and many nuclear pores. Polysomes increase in number. Marked elongation of cells in region IV is characterized by the presence of numerous oriented microtubules and the development of intercellular junctions, first adhesion plaques and then tight junctions. Nucleoli are prominent and nuclear pores numerous. Polysomes pervade the cytoplasm. Microfibres appear to be greatly reduced in number. Cells in region V illustrate that with fibre cell differentiation there is a progressive loss of organelles and the deposition of cytoplasmic matrix.

Figure 1.- Diagram of the 6-day chick embryo lens. Different regions of the lens are illustrated in figures 2-4.


ANTERIOR
ANT. LENS EPITHELIUM
EQUATOR
LENS BODY
PRESUMPTIVE IRIS
POSTERIOR

1

Figures 2-4.- Six day chick embryo lens in mid section showing different regions. These are defined (I-V) for reference to ultrastructural illustrations. x 300

Figure 2.- Anterior portion of the lens showing the central anterior lens epithelium (region I). Note the intercellular spaces.

Figure 3a.- Lateral region of the anterior epitjelium. Note that it becomes multi-layered.

Figure 3b.- That area of the lateral epithelium defined as region II. Note that the intercellular spaces become restricted to the basal area and then disappear.

Figure 4.- The equatorial region of the lens. Note cells at different stages of elongation. Regions III, IV and V are defined. Nucleoli in region IV are very prominent.

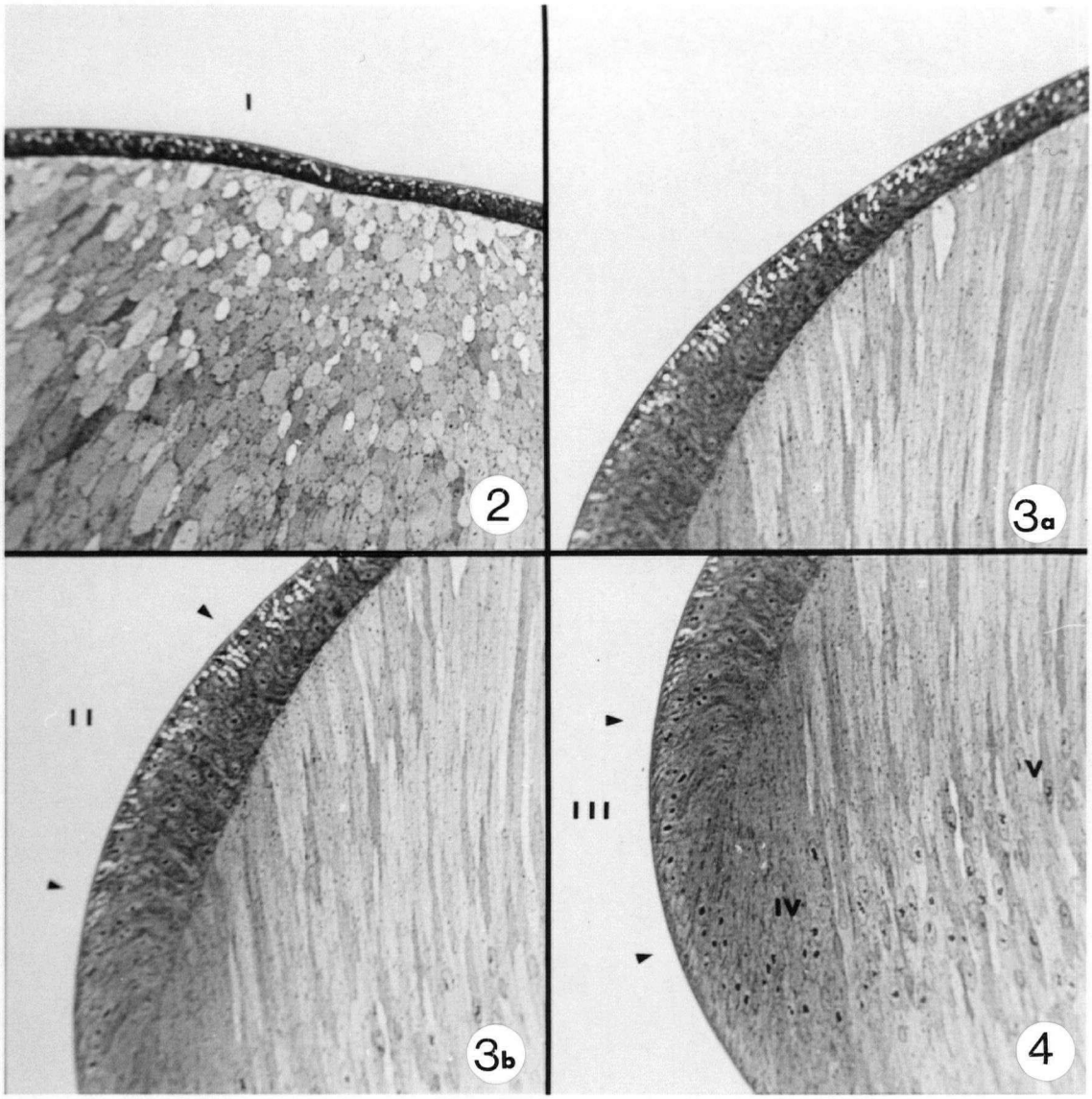

I
2
3a
II
3b
III
IV
V
4

Figure 5.- The apical portion of region I. Lipid inclusions are evident with associated mitochondria, Junction associated microfilaments can be seen adjacent to the plasmalemma. Note that the adjacent cell membranes are parallel with no intercellular junctions except at the apical border. x 25,000.

Figure 6.-Similar to figure 5. Note the rough endoplasmic reticulum filled with dense material and the elements of the golgi apparatus. The lower right hand corner is the apical portion of an apposed fibre cell.x36,000

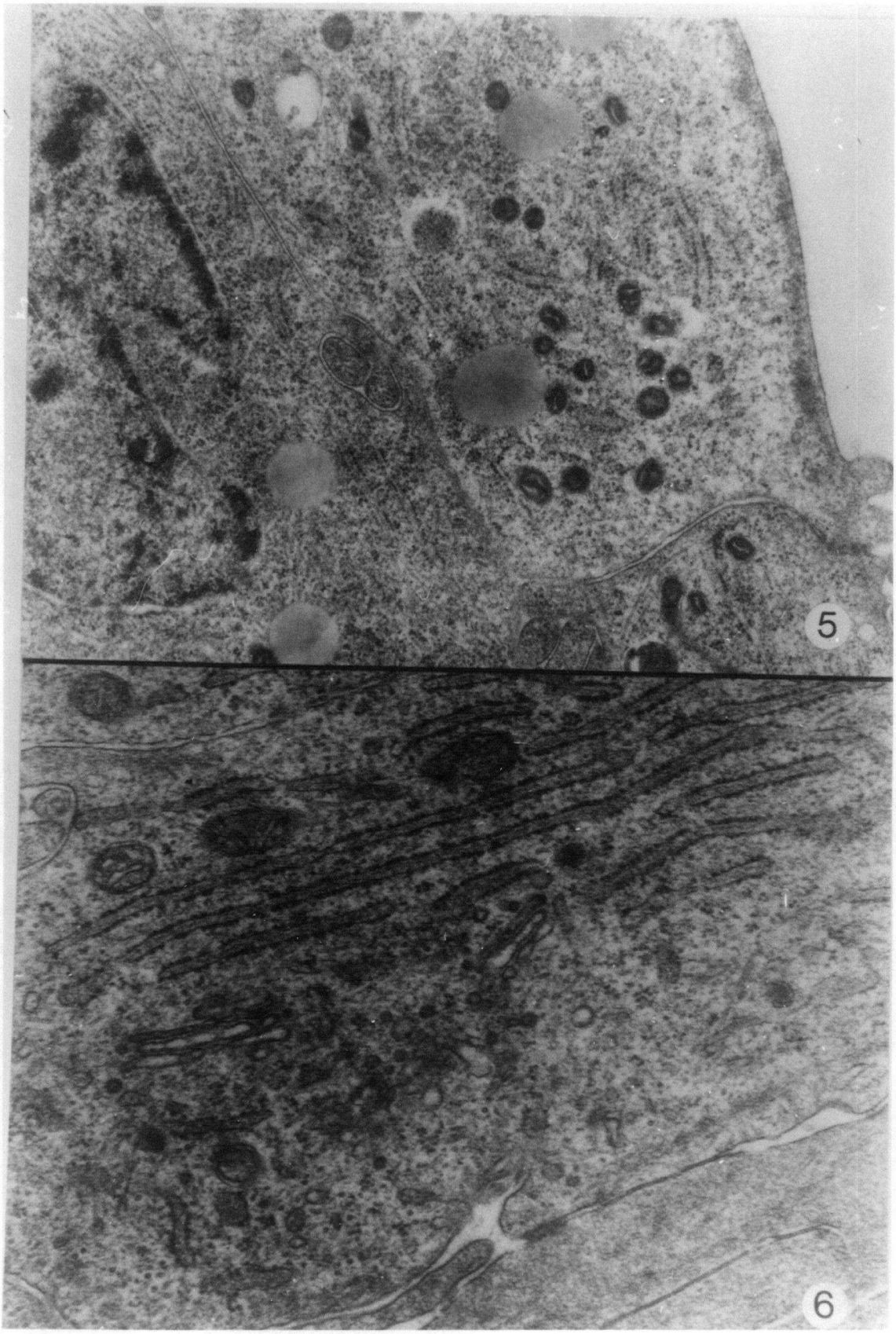

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Figure 7.- Low magnification of region I. Note the intercellular spaces and the distribution of mitochondria and rough endoplasmic reticulum. The lens capsule can be seen on the left and apposed fibre cells on the right. x 10,000.

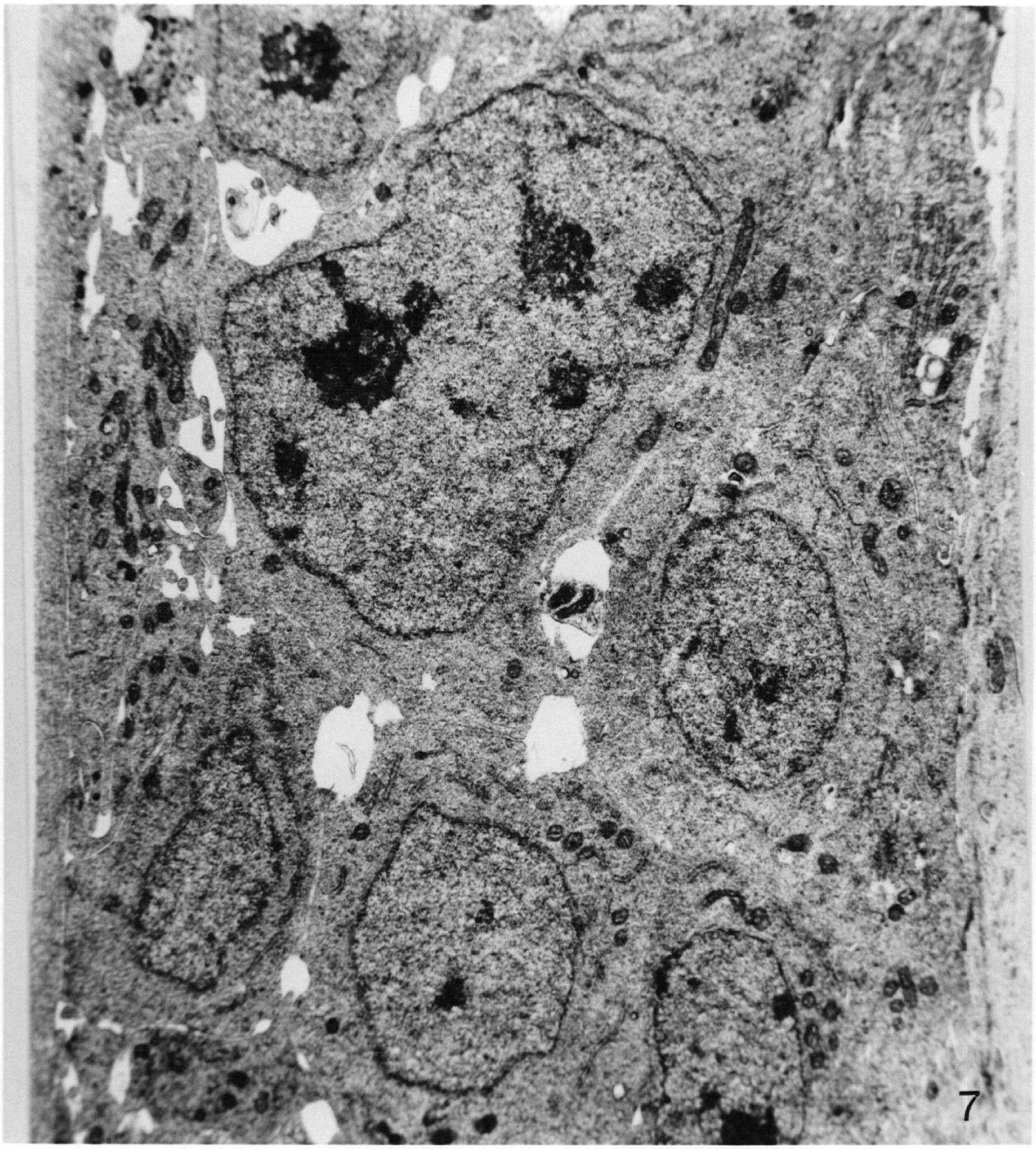
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Figure 8.- Basal aspect of a cell in region I. Note microfibres throughout cytoplasm. CAP, lens capsule. x 44,000.

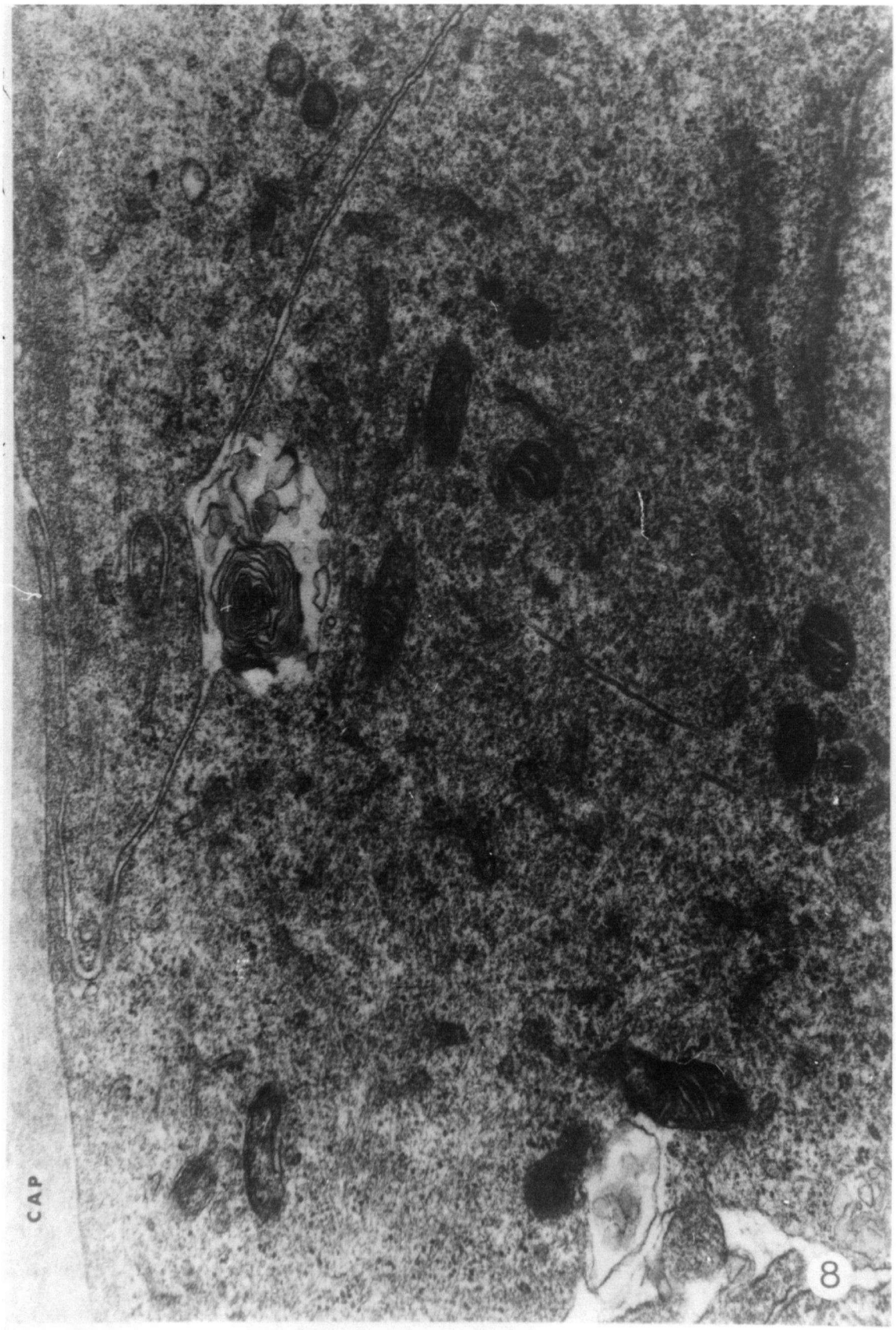
CAP
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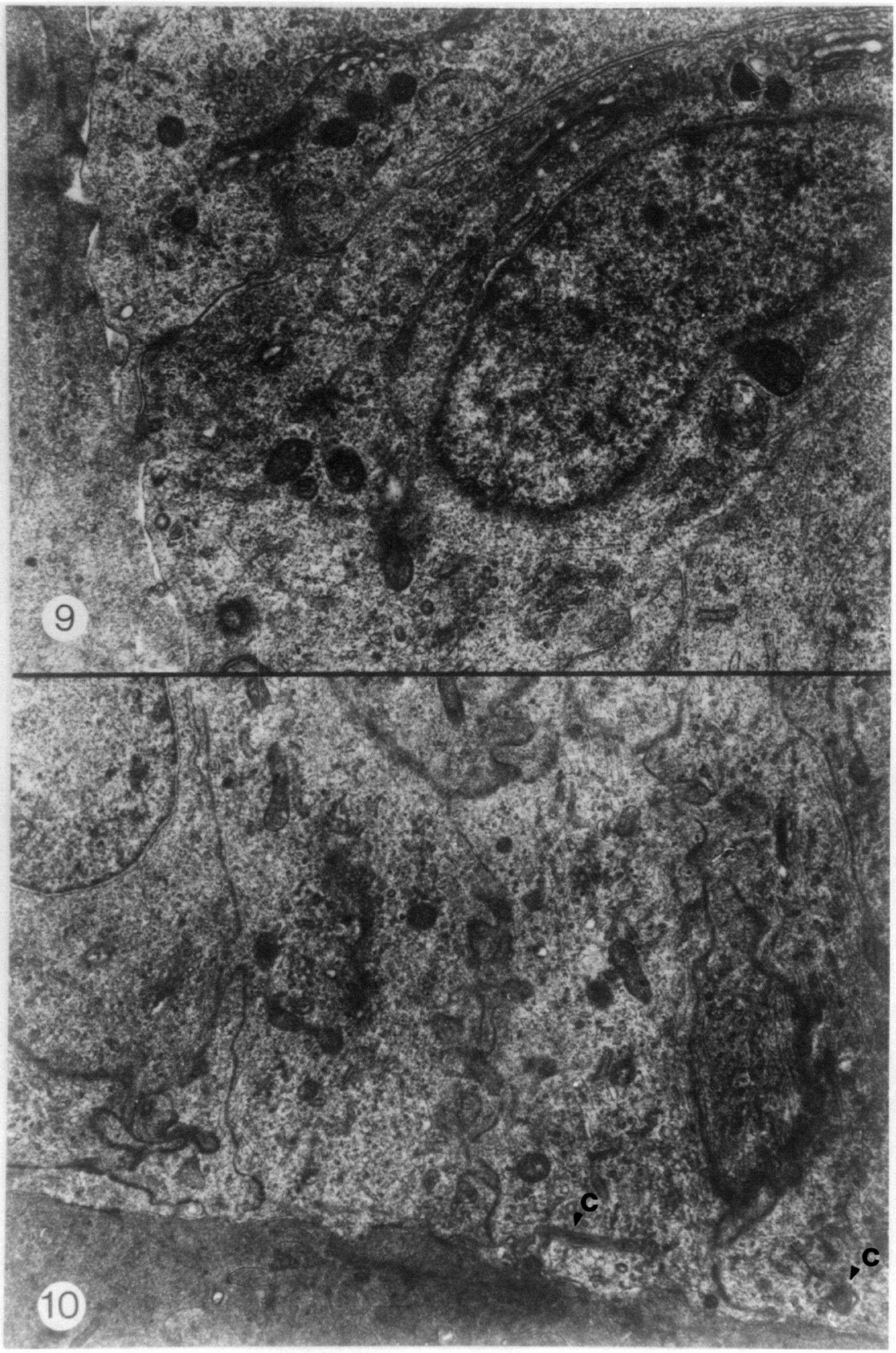
Figure 9&10.- Apical regions of cells in region II. Note oriented microtubules and the contrast between the cytoplasm of the epithelial cells and the adjacent apical portions of apposed fibre cells. The apical cell membranes of fibre cells and cells of the anterior epithelium can be seen to engage in junctions at certain points. Arrows in the lower right hand corner of figure 10 point to cilia. Golgi activity appears greater than in cells of region I. x 14,500.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c

Figure 11.- Similar to figures 9&10. Note the golgi and associated vesicles. x 32,000

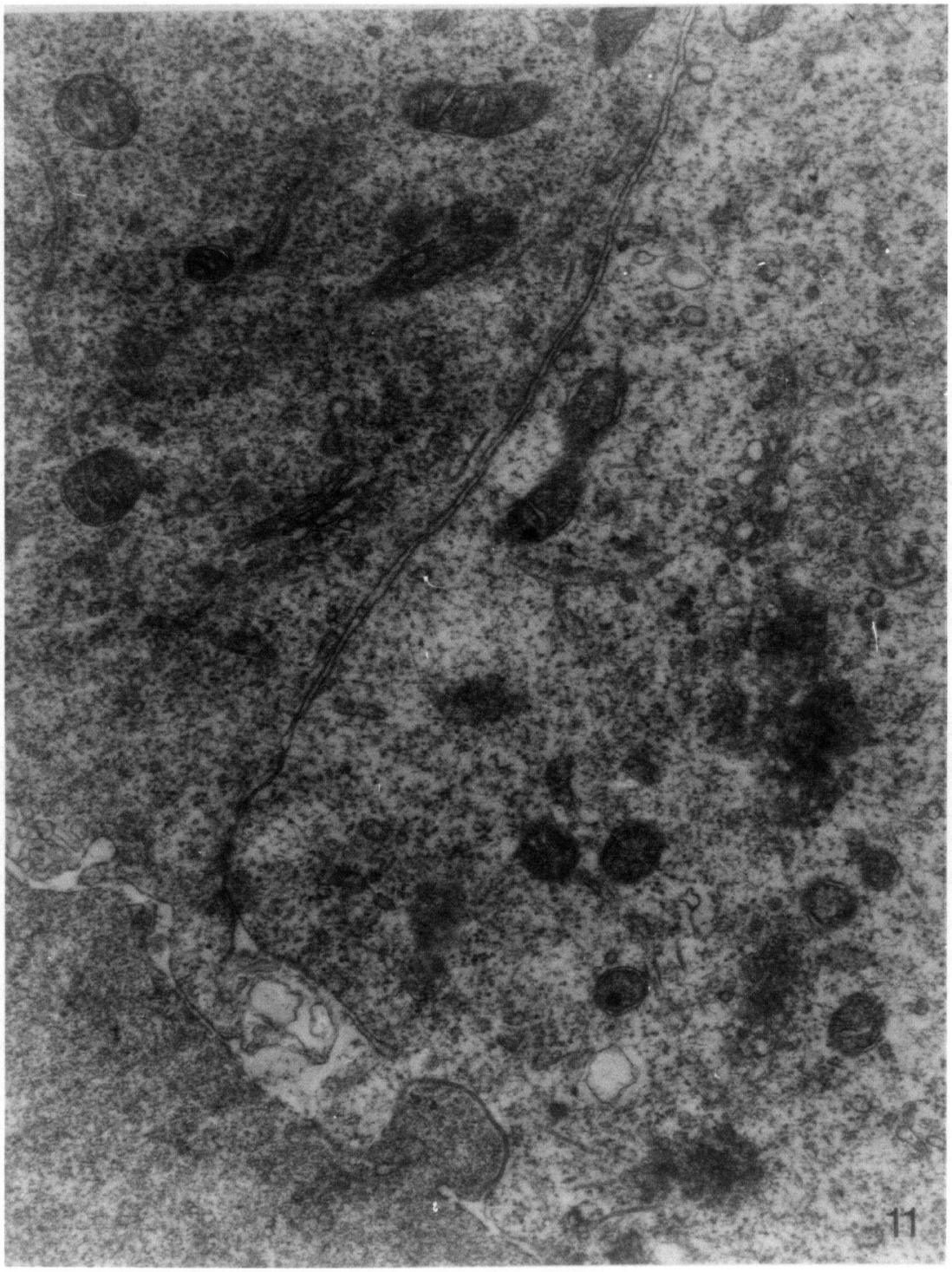
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Figure 12.-Basal aspect of cell of region III, illustrating numerous oriented microfibres and microtubules. x 48,000.

Figure 13.-Cell of region III showing rough endoplasmic reticulum. Also note microtubules adjacent to cell membranes x 48,000.

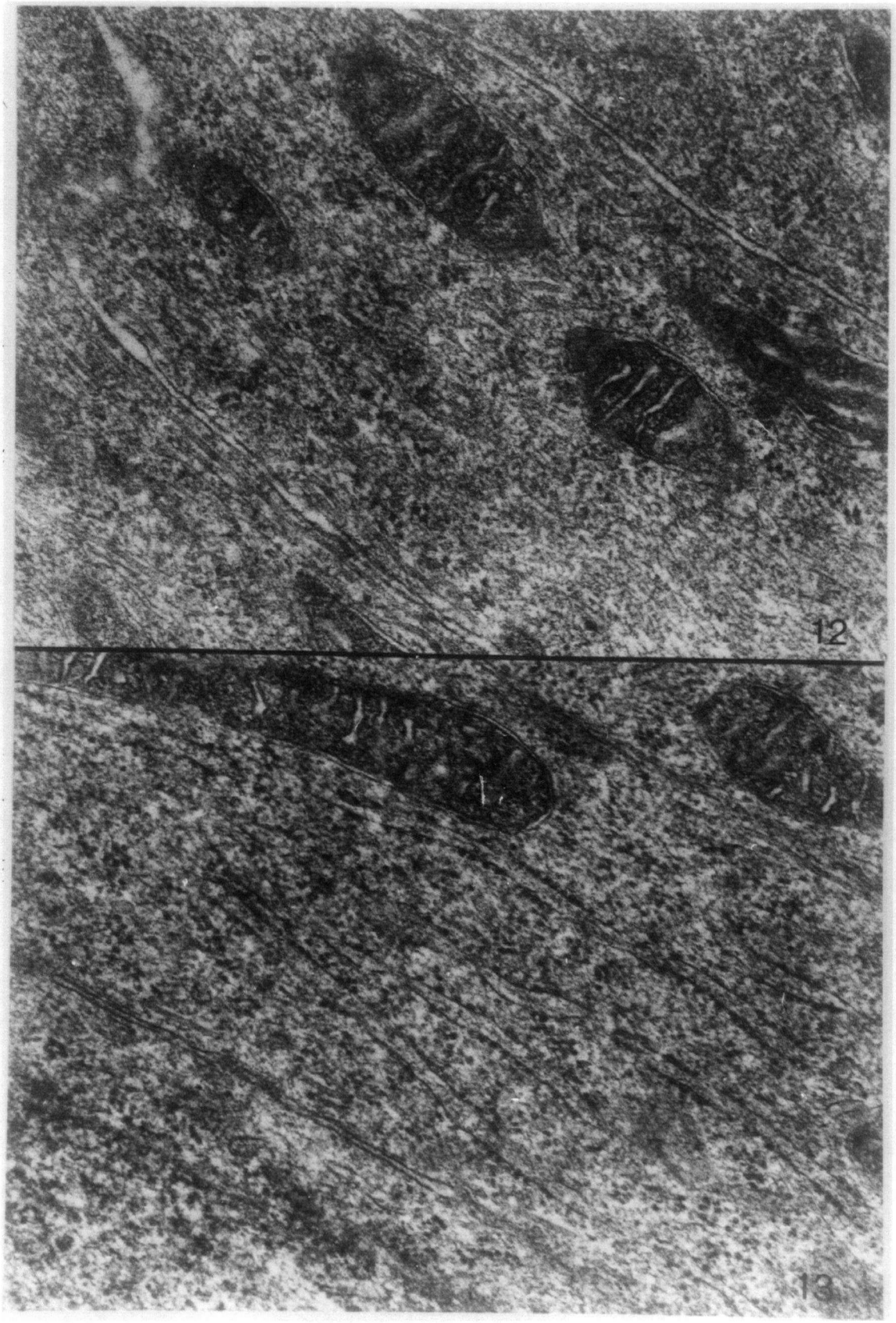
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Figure 14.-Apical border of cell of region III. Note apparent fusion of cytoplasmic vesicle with plasma membrane. x 70,000.

Figure 15.-Mid apical portion of a cell similar to that in figure 14 showing golgi and associated vesicles. Note aggregation of vesicles near lateral cell border. x 37,000

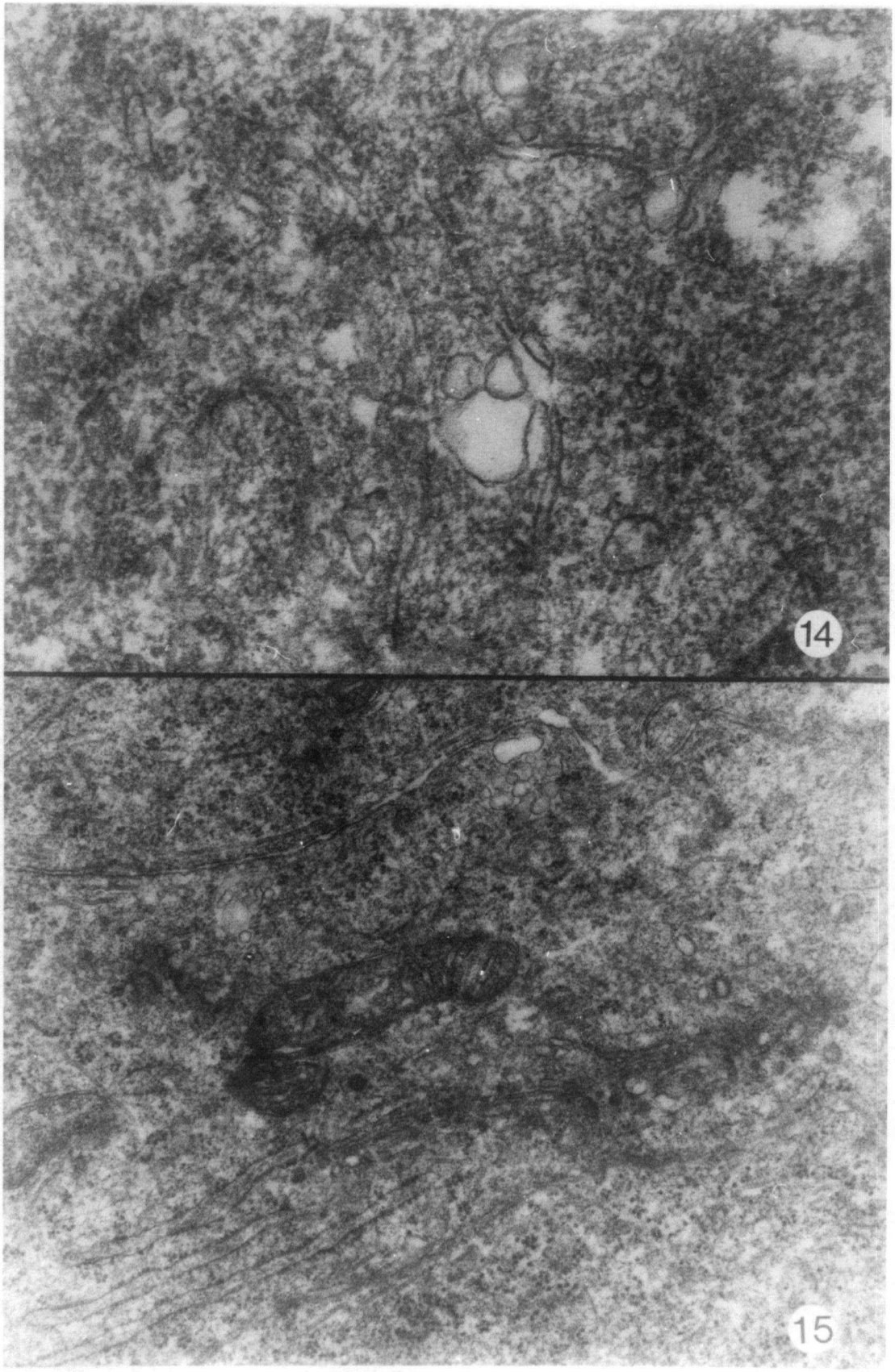
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Figure 16.-Apical portion of cell of region III illustrating cilium. Note also numerous cytoplasmic vesicles. x 64,000

Figure 17.- Apical portion of cell of region III showing part of a cilium, basal body and associated satellite and microtubules. x 64,000.

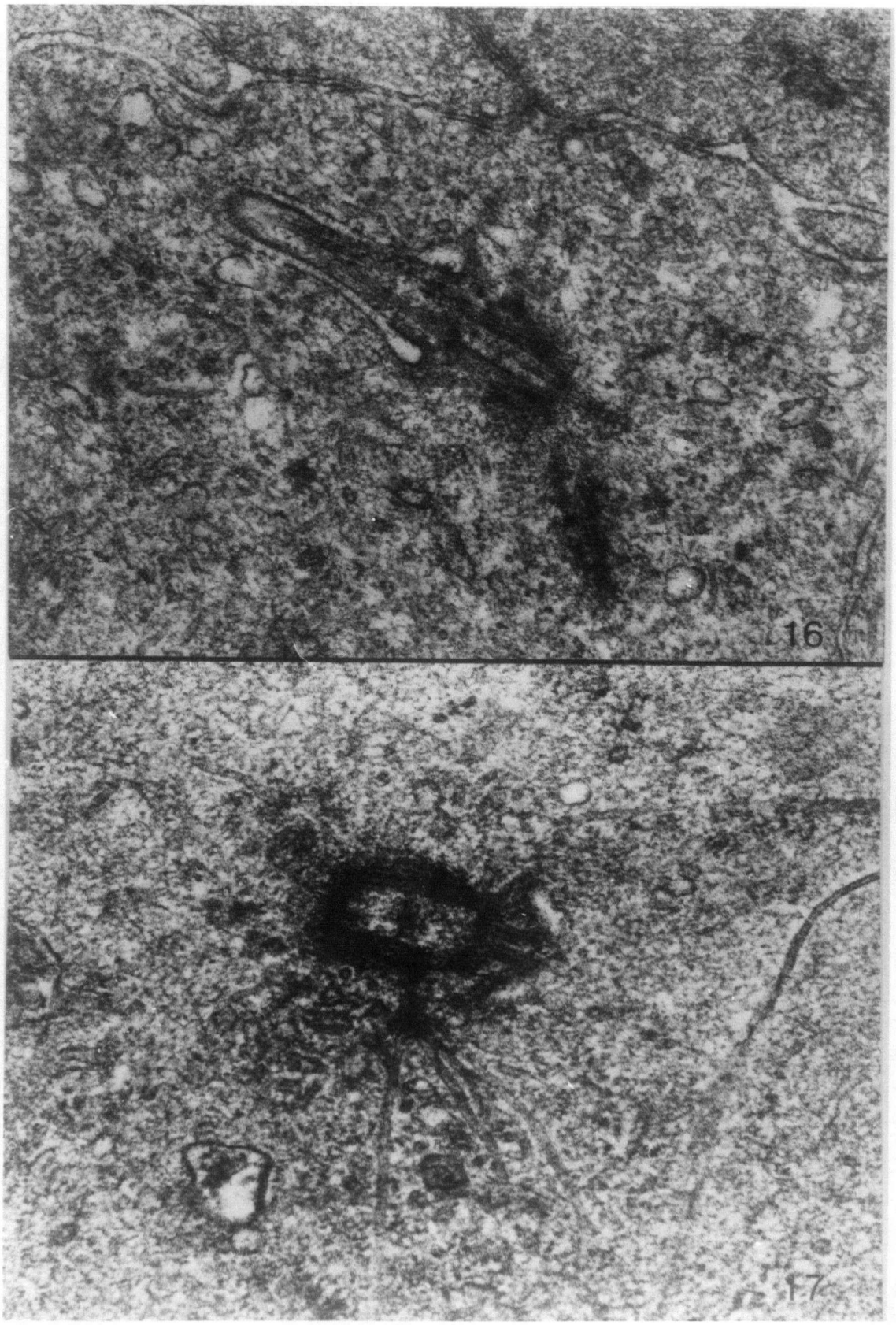
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Figure 18.-Mid basal portion of cell of region III. Note oriented microtubules and microfibres . x 45,000.

Figure 19.-Mid apical aspect of young fibre cell of region IV.Note microtubules. x 64,000.

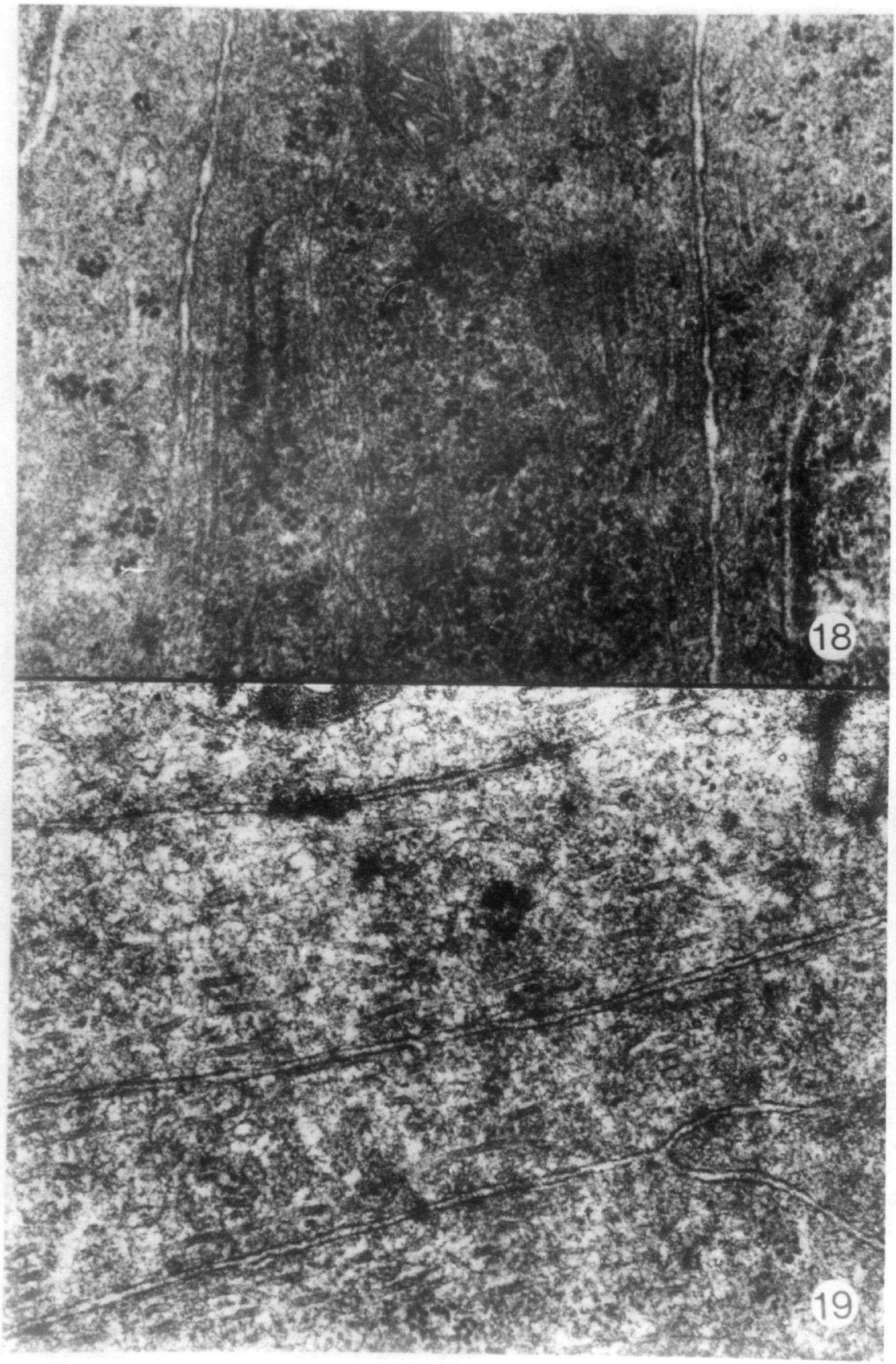
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Figure 20.- Zone of transition between regions III and IV showing apical portions of cells. Note the numerous microtubules in the young fibre cells as well as the polysomes and the dense material associated with adjacent cell membranes at various points (adhesion plaques). Note also the apical junctional complexes and the numerous cytoplasmic vesicles in the apical aspects of the cells of both regions. x 10,000.

Figure 21.- Young fibre cells between regions IV and V demonstrating numerous polysomes and extensive tight junctions. x 17,000.

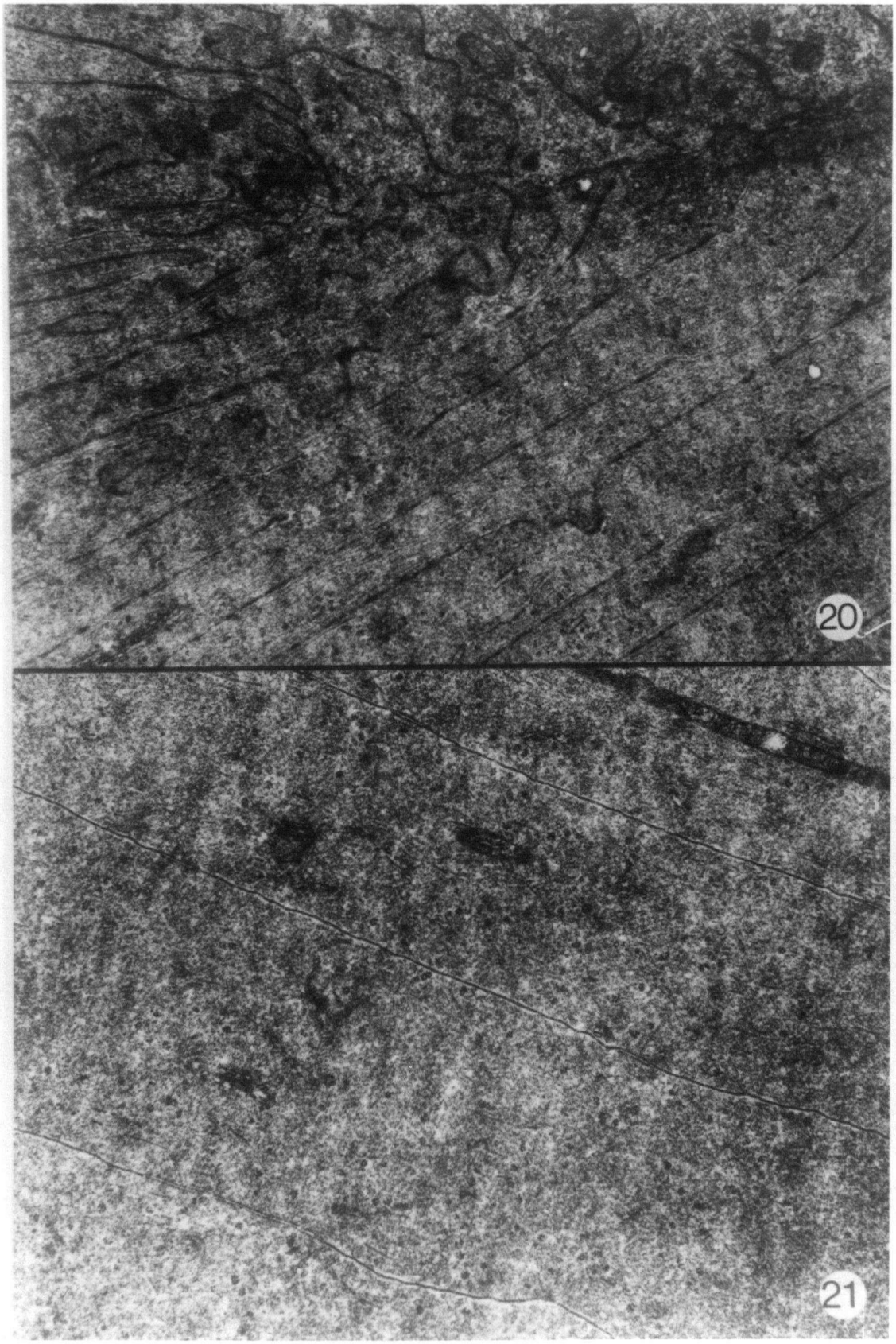
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Figure 22 & 23.- Young fibre cells of region IV showing prominent nucleolus and nuclear pores. x 38,000.

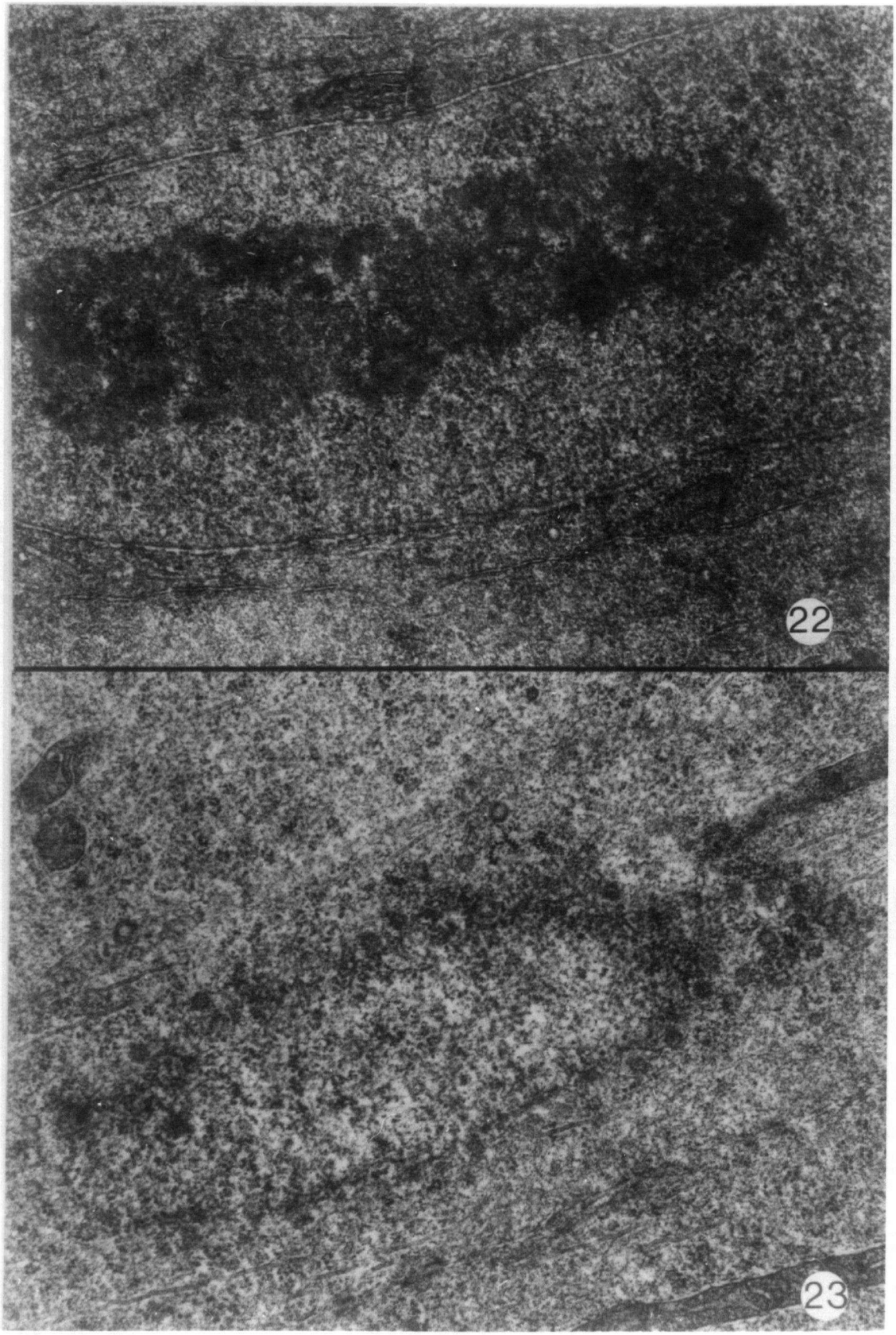
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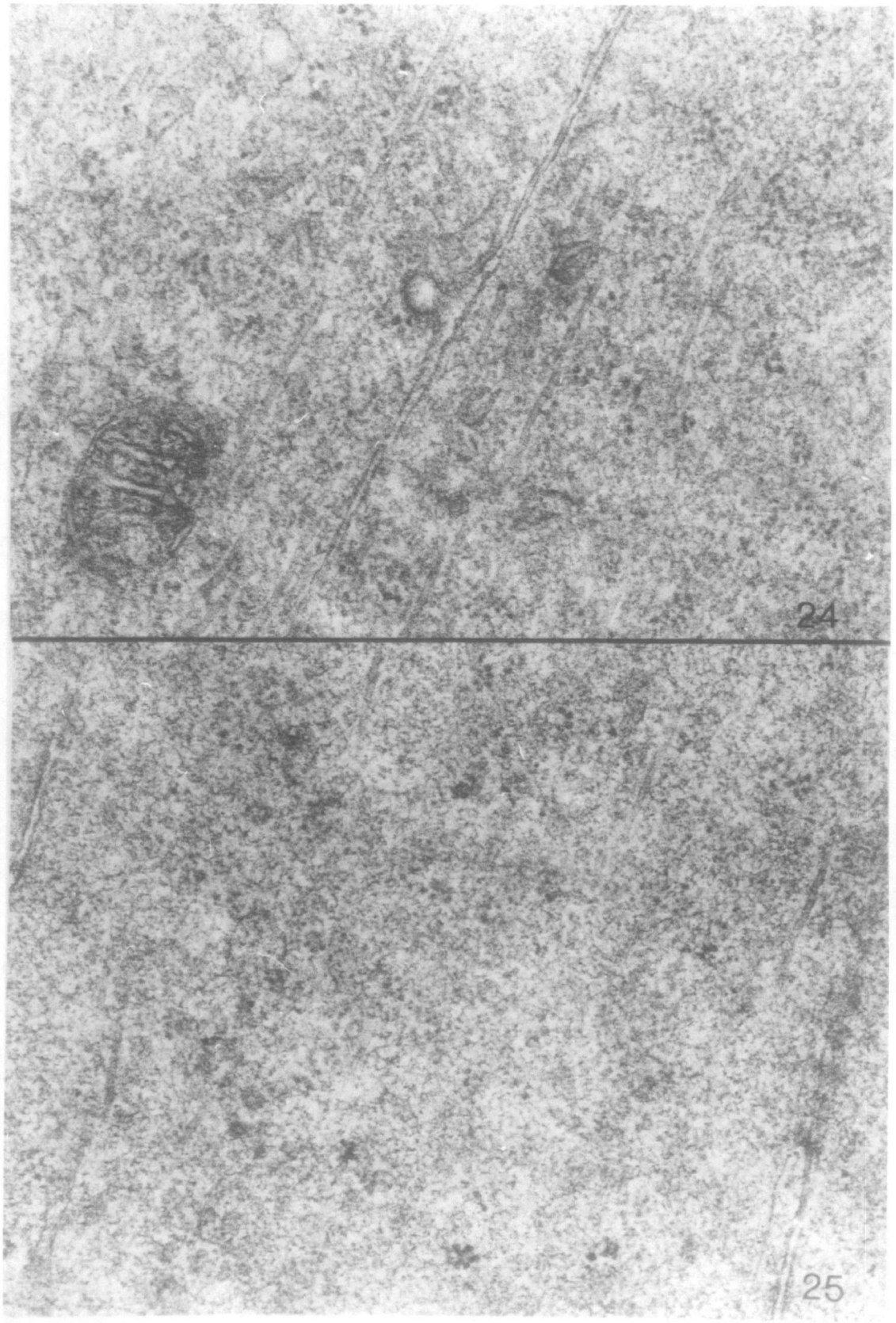
Figure 24.-Young fibre cell demonstrating apparent fusion with cell membrane of coated vesicle. x 64,000.

Figure 25.- A more mature fibre cell . Note relative sparsity of microtubules and microfibres and the dispersion of polysomes amidst the cytoplasmic matrix. x 64,000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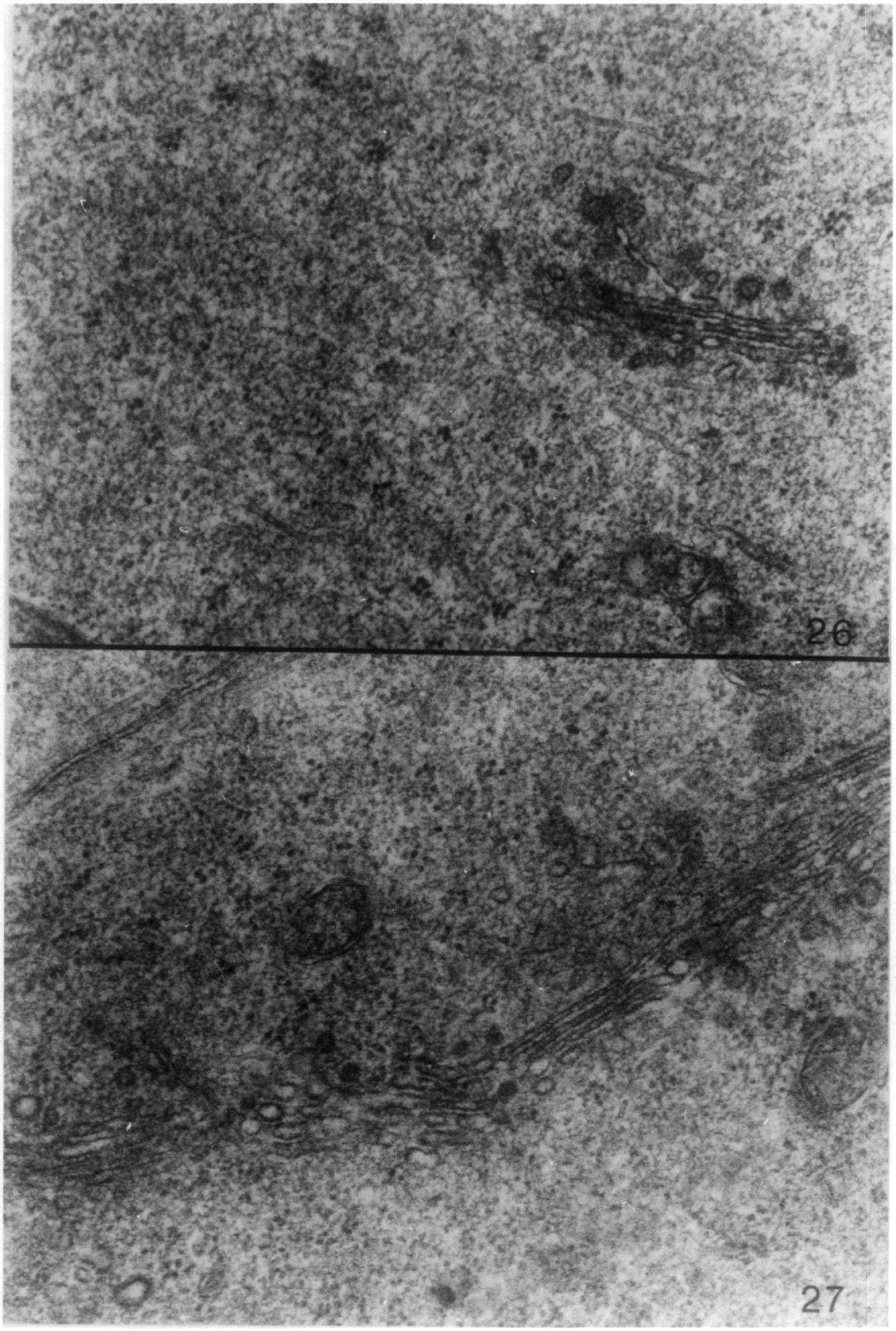
Figures 26 & 27.- Figure 26 is the apical portion of a fibre cell in region V showing reduced golgi apparatus compared to that in figure 27 which shows only a portion of the golgi in a cell of region III. x 57,000.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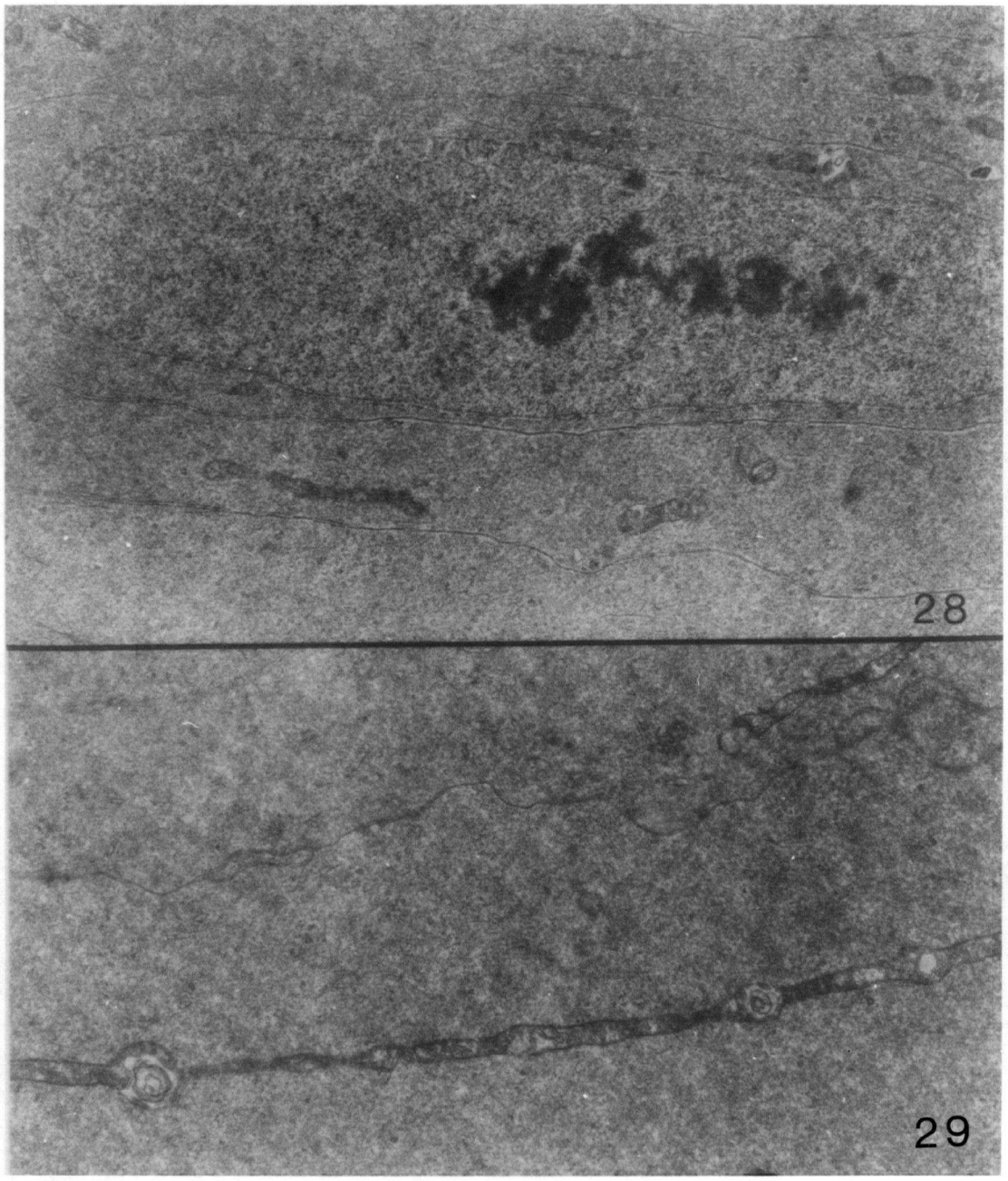
Figures 28 & 29.- Cells of region V showing elongation of nucleus, extensive regions of tight junction and greatly elongated mitochondria with apparent areas of degeneration. Note that some adhesion plaques are evident. x 12,000.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VI- Differentiation of anterior lens epithelia in vitro.

Examination of the 6-day anterior lens epithelia explanted by procedure 1 and cultured for 3,4,5, or 6 days demonstrated that in 87% (105/121) of the cases a significant portion of the explant was differentiated. Twenty-six explants were highly organized (figures 34-39). Sixteen were differentiated to a limited degree, the cells having elongated to the extent illustrated in figures 43-44. Another 16 were poorly differentiated. In 11 of the remaining 79 cases, a portion of the explant was evident in which the cells had elongated only to a limited degree. In all, 26 explants were highly organized, 47 (including the 16 with poor differentiation) poorly organized, and the remaining 48 organized to an intermediate degree.

Those cultures with a high degree of organization and histogenesis had a common feature: the epithelium was so oriented that its curvature tended to enclose the cells within the capsule. Similarly, in those explants with less organization, the regions of maximum differentiation tended to be in areas more or less enclosed by the lens capsule (see figures 33, 37). On the other hand, in those explants or regions of explants with limited differentiation, the arrangement was one of little epithelial folding, or with it so folded that the epithelium enclosed the capsule, as seen in figures 43 and 44.

The cells of the epithelia explanted by procedure 2, with the epithelium everted and the capsule folded on itself, consistently (15 cases) differentiated to a limited extent

(figures 43-44) during three days in culture.

However, when the epithelia were explanted by procedure 3, with the normal curvature maintained, their cells consistently elongated to a great extent (8 cases) during three days in culture (figures 45-47). Even after 1-1½ days in culture (7 cases) a high degree of cell elongation was apparent in epithelia explanted in this manner (figures 40-42).

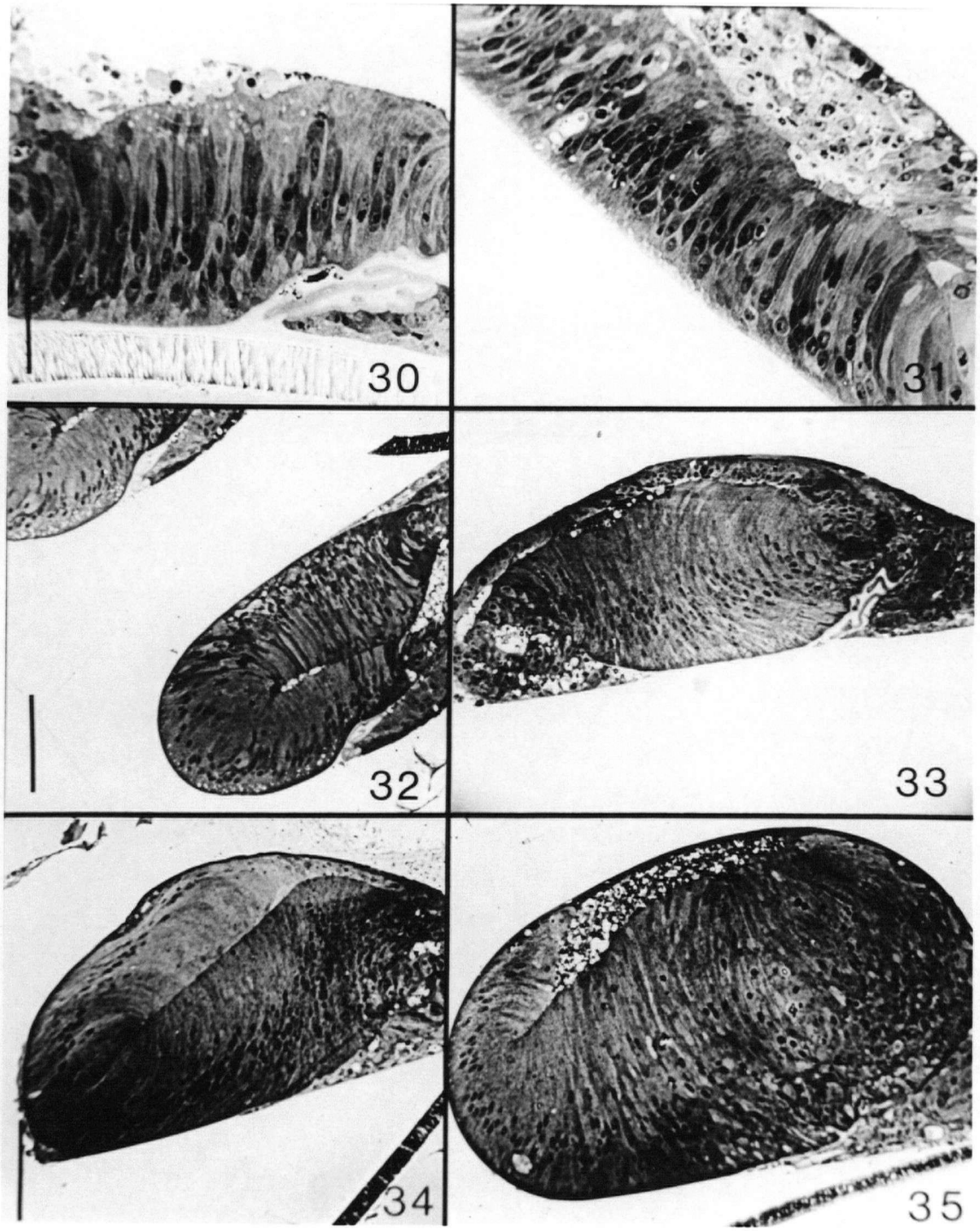
Examination of cultured epithelia fixed at different intervals (1-6 days) indicated that their cells elongate progressively for a period of about three days. The explants cultured for longer periods (see figures 38-39) did not show greater cell elongation. Elongation tended to be in a direction perpendicular to the raft, as though reflecting the polarity imposed by the medium-air interface.

In summary, 6-day chick embryo lens anterior epithelia will undergo lens histogenesis *in vitro*. The extensive cell elongation involved in this process appears to be encouraged by an epithelial curvature whereby the cells form the lesser curvature and the lens capsule the greater curvature. Lack of epithelial curvature, or curvature opposite to the above seems to discourage cell elongation.

Figure 30 & 31.- Anterior lens epithelia after 1 day *in vitro*. Explanted by method 1. Bar (vertical)represents 50 micra.

Figures 32 & 33.-Anterior lens epithelia after 2 days *in vitro*. Explanted by method 1, Bar represents 100 micra.

Figures 34 & 35.-Anterior lens epithelia cultured for 3 days. Explanted by method 1. Bar represents 100 micra.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Figures 36 & 37.- Figure 36 represents a lens anterior epithelium explanted by method 1 and cultured for 3½ days. Figure 37 is similar, being cultured for three days. Bar represents 100 micra.

Figure 38.-Anterior lens epithelium cultured for 5 days. Bar represents 100 micra.

Figure 39.- Same as figure 38, but cultured for 6 days.

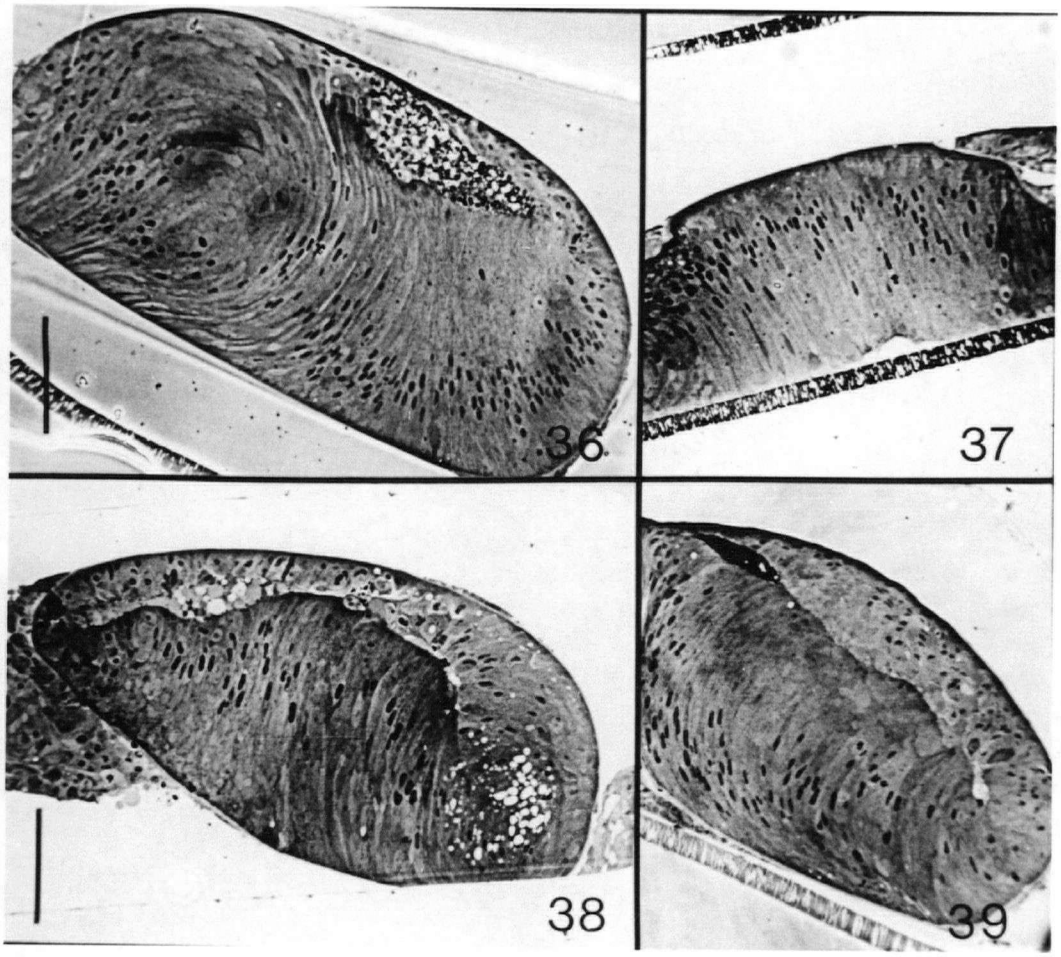
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Figure 40.-Anterior lens epithelium explanted by method 3 and cultured for 1½ days. Bar represents 50 micra.

Figure 41.- Enlargement of figure 40. Bar represents 50 micra.

Figure 42.- Similar to figure 41. Bar represents 50 micra.

Figure 43.- Anterior lens epithelium explanted by method 2 and cultured for three days. Bar represents 50 micra.

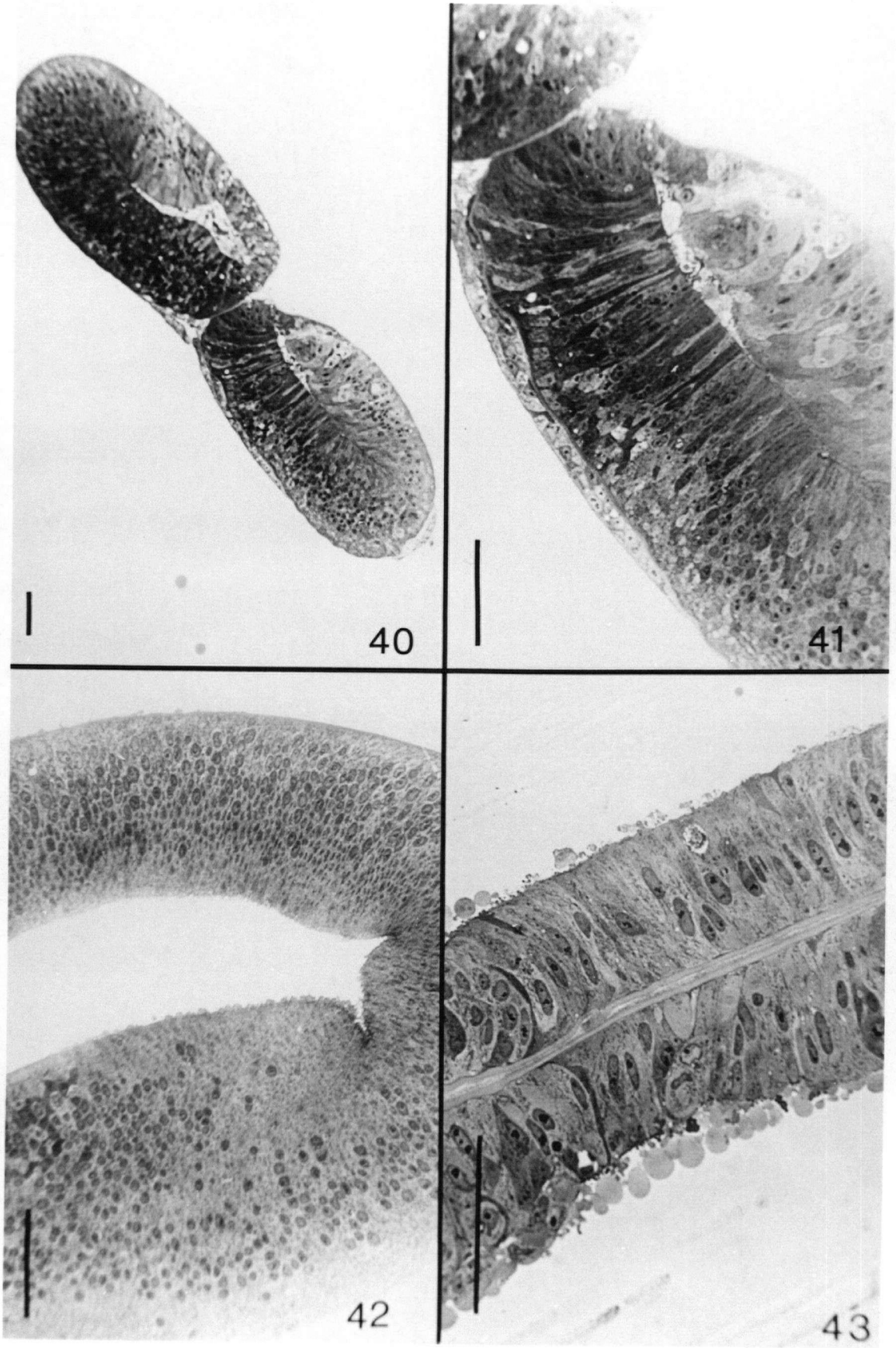
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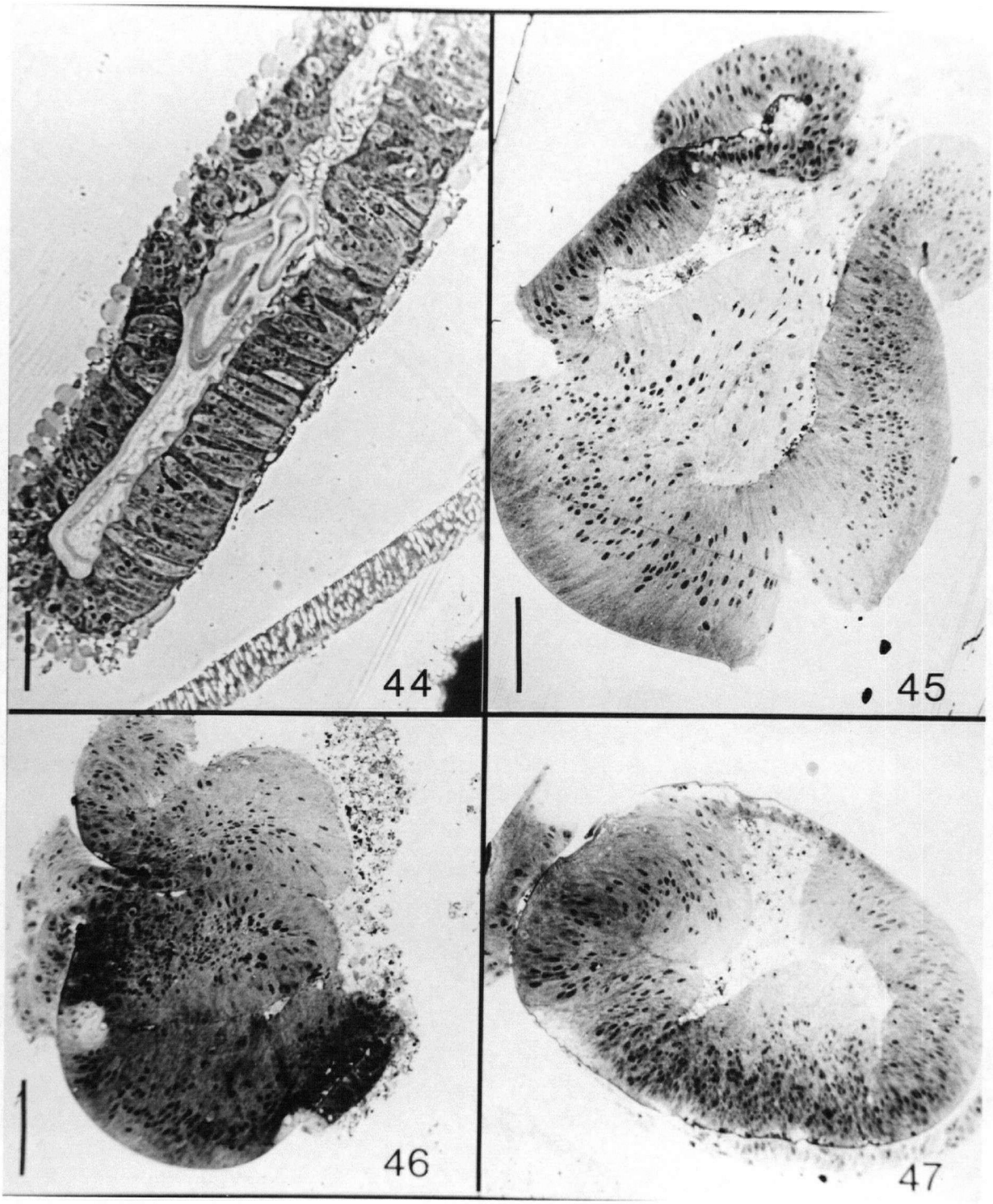
Figure 44.- Anterior lens epithelium cultured for 3 days. Explanted by method 2. Bar represents 50 micra.

Figures 45-47.- Anterior lens epithelia explanted by method 3 and cultured for three days. Bar represents 100 micra.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VII- Fine structure of anterior lens epithelia differentiating *in vitro*.

Those epithelia cultured for periods of 1-6 days that appeared to be well organized and differentiated were examined with the electron microscope in order to evaluate their fine structure in terms of that of cells in different regions of the normal embryonic lens. Ultrastructural examination of these explants illustrated a number of features central to their differentiation which, in general, correspond to those observed in normal lens fibre cell differentiation.

With differentiation, nuclei come to assume an elliptical shape, with their long axes aligned with those of the cells. As the cells elongate, their nuclei become more elongated and narrow, resembling closely those of differentiating fibre cells, with prominent nucleoli and numerous nuclear pores (figures 48,60). Polysomes, dispersed throughout the cytoplasm , are most prominent in cells cultured for two days (figures 50, 58), appearing to subsequently become somewhat less numerous. With differentiation, the cells accumulate a fine cytoplasmic matrix that appears similar to that of lens fibre cells (figure 63).

Cell elongation is accompanied by the presence of numerous oriented microtubules (figures 57-58) which tend to occupy the peripheral cytoplasm. They are typically more abundant apically than basally, particularly during the initial stages of elongation. As in the lens, they appear to be elaborated by satellites associated with basal bodies (figure 53).

Microtubules generally persist in well differentiated explants, even after six days *in vitro*, not declining in number to the extent they do in lens fibre cells of region V *in vivo*.

Oriented 100 A microfibres are evident in all the cultured epithelia examined, but differ in number and distribution at different stages of *in vitro* development. The appearance of cytoplasmic microfibres after 1 day in culture is illustrated in figures 48-49. They are much more numerous in cells of explants differentiating during their second and third day in culture (figures 50, 59). The relatively high concentration of microfibres shown in these figures is typical only of the cytoplasm basal to the nucleus. Examination of well differentiated explants cultured for three or more days indicated microfibres to decline in number during this period, although not to the same degree as in fibre cells *in vivo*, and assume a more general distribution. While in most sections the length of microfibres is indeterminate, in some it is evident they may be quite long (figure 64).

A relative increase in Golgi activity accompanies the initial stages of cell elongation (figure 55). As in the lens, the Golgi generates coated vesicles, some of which appear to be involved in the establishment of adhesion plaques (figures 49,50,52,55),and other vesicles which are often associated with the cell membrane (figures 52,54). The Golgi appears most developed in cells undergoing elongation during the second day *in vitro* (figure 55) and appears to subsequently decline in activity (figure 62).

Mitochondria are, in the initial stages of cell elongation, of a general distribution, During further elongation, the mitochondria appear to become somewhat reduced in number, and a considerable portion of the mitochondrial population occupies the basal cytoplasm (figure 59) immediately adjacent to the lens capsule. With initial cell elongation, the mitochondria tend to show moderate elongation. With further elongation of the cells, some mitochondria elongate to a great extent, as in fibre cell elongation *in vivo*. Mitochondrial degeneration, similar to that described in the lens , is also evident. However, apparently healthy mitochondria seem to be rather more numerous in differentiating fibre cells *in vitro* as compared to those *in vivo*.

A moderate rough endoplasmic reticulum is present at the initial stages of *in vitro* development. While elements of this organelle tend initially to be concentrated in the perinuclear region, they become dispersed throughout the cytoplasm, often aligned with the long axis of the cell (figures 50,59,60). As the cells differentiate , many elements of the endoplasmic reticulum appear to be quite small, as if representing fragmentation products of larger ones (figure 58). Although the rough endoplasmic reticulum becomes reduced in extent with cell differentiation, some elements generally persist, so that the organelle does not disappear as it does in the lens. With *in vitro* differentiation, intercellular relationships are modified. During the first day in culture, the intercellular spaces of the anterior lens epithelium are largely eliminated,

(figures 40-42). As the cells differentiate, adhesion plaques are formed between adjacent cells (figures 48-50), possibly by means of coated vesicles which are often observed in association with cell membranes (figures 49, 50, 52, 55). Also, regions of tight junction are elaborated (figures 59-60). However, they do not become as extensive as those between fibre cells *in vivo*. Basal junctional complexes are maintained and apical junctional complexes are prominent, with bands of microfilaments coursing between adjacent junctions (figures 51-52).

In summary, anterior lens epithelia differentiating *in vitro* undergo morphological changes similar to those evident in the lens. Cell elongation is accompanied by the loss of intercellular spaces, increased Golgi activity, and the formation of intercellular junctions. Tight junctions do not become as extensive as they do in the fibre cells of the lens. Microtubules, apparently elaborated from satellites in the apical cytoplasm, are numerous. Nuclei elongate and contain numerous pores and prominent nucleoli. Polysomes become abundant. Numerous oriented microfibres appear in the basal cytoplasm. With differentiation, the loss of cytoplasmic organelles is not nearly as marked as it is in the lens.

Those epithelia explanted by method 2, which elongated only to a limited extent during three days *in vitro* (figures 43-44) were examined first in order to determine if there were any ultrastructural features that might account

for their not elongating further, and second to compare their cytoplasmic differentiation with that of explants that elongated to a great extent *in vitro*. Ultrastructural examination of these explants, however, demonstrated them to be in no other way remarkably different from those that had elongated to a greater degree (see figures 65-66). The cells contained numerous microtubules, microfibres and polysomes. They had typical junctional complexes, with associated prominent microfilaments, and were typically ciliated. Intercellular relationships, involving adhesion plaques and tight junctions were very much the same as those in epithelia in which the cells had elongated to a much greater extent.

Figure 48.- Anterior lens epithelium after 1 day *in vitro*. Note microtubules and microfibres. Arrows point to nuclear pores of a tangentially sectioned nuclear membrane. x 20,000.

NOTE: Figures 48-64 are micrographs of epithelia cultured for various periods after having been explanted by method 1. The micrographs are of explants chosen for their high degree of organisation and differentiation and include those illustrated in figures 30-39.

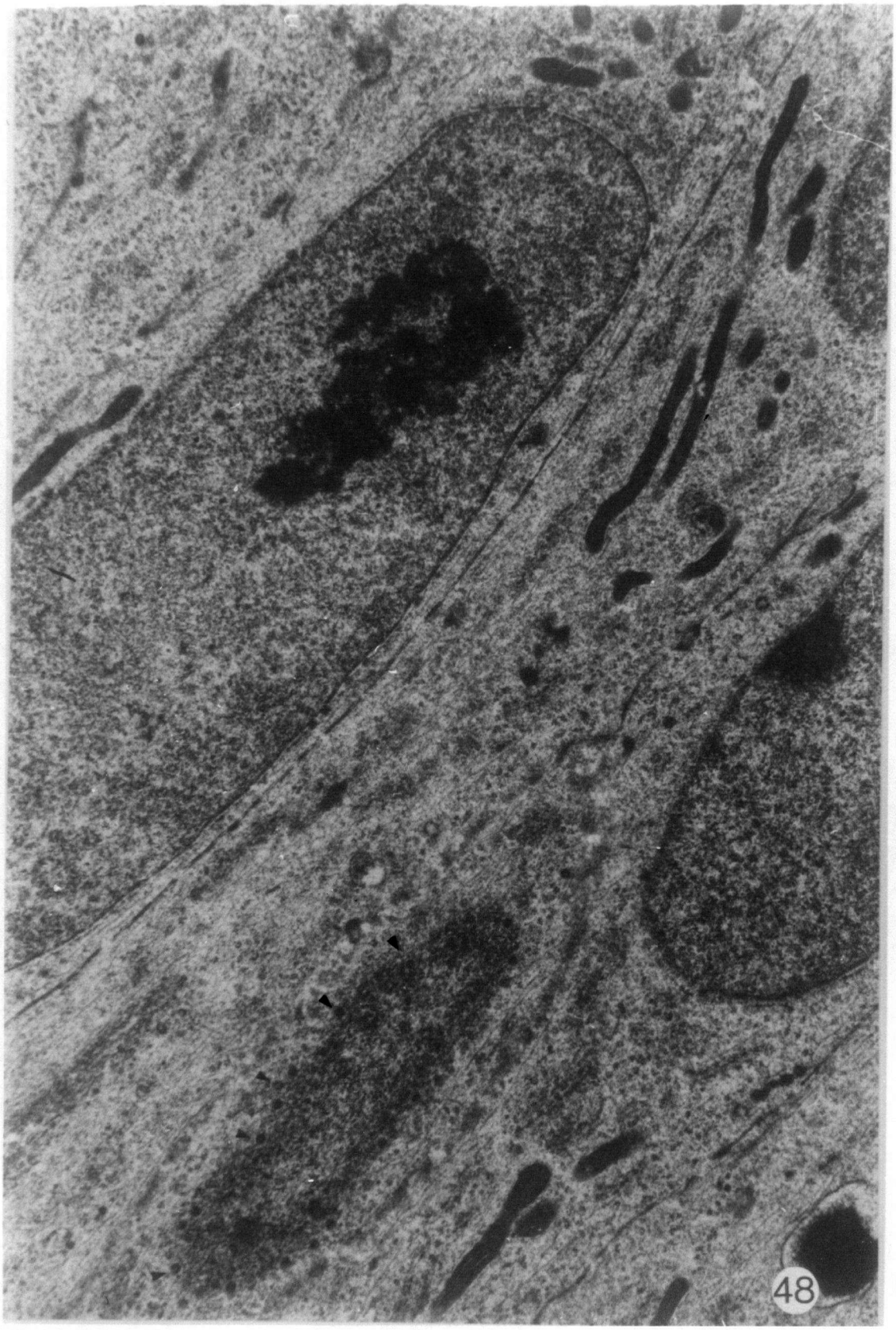
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Figure 49.- Similar to figure 48 showing mid basal aspect of cell. Note microtubules and microfilaments and dense material associated with adjacent cell membranes (arrows) termed adhesion plaques. x 20,000.

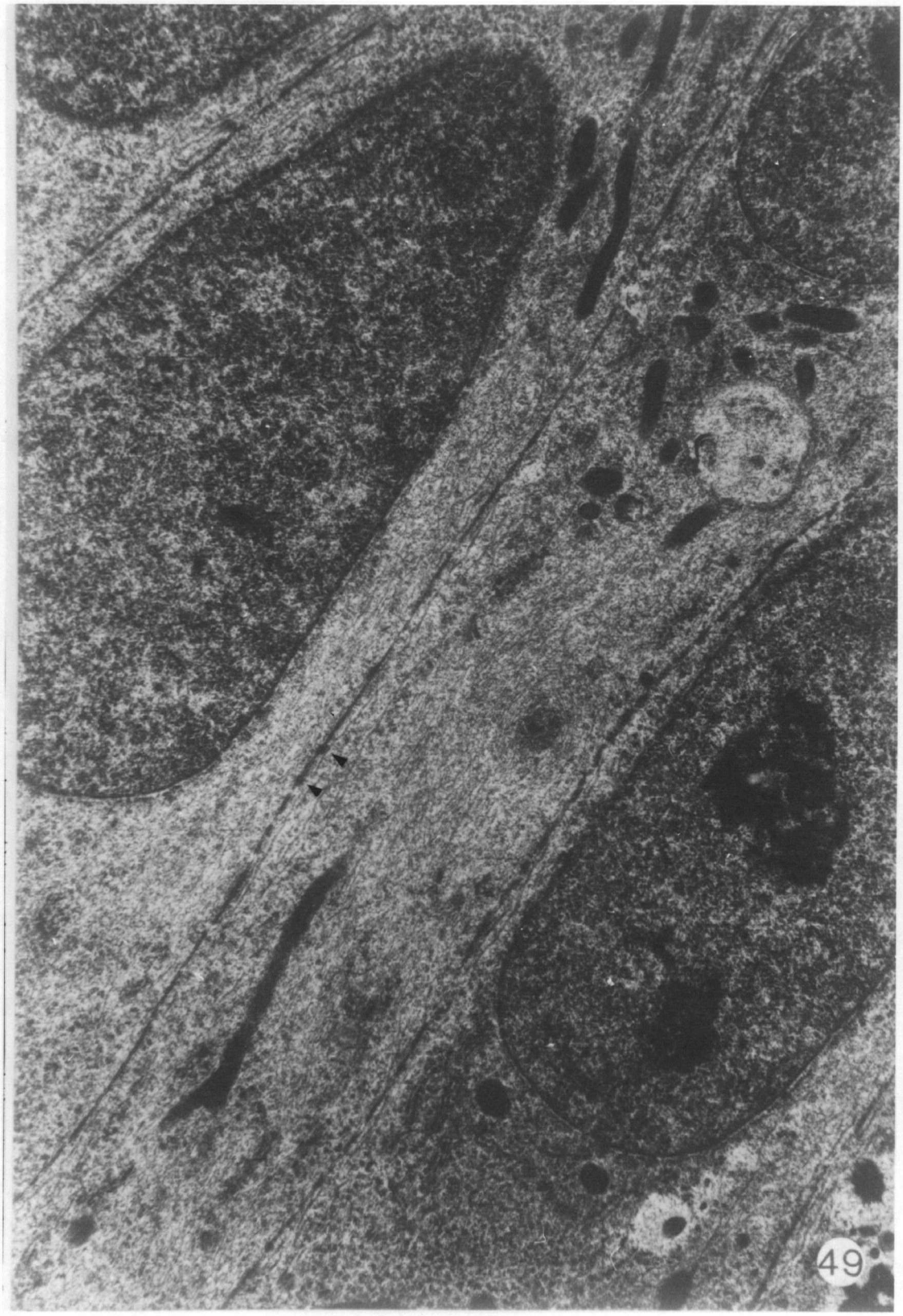
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Figure 50.- Cells of anterior lens epithelium after 2 days *in vitro*. Note numerous polysomes, and numerous microfibres. x 20,000.


50

Figure 51.- Apical region of cells of anterior lens epithelium after 1 day *in vitro* demonstrating prominent microfilaments extending between junctional complexes. x 9,000

Figure 52.-Enlargement of figure 51. Note the coated vesicles in what appears to be various stages of fusion with the plasma membranes. x 40,000

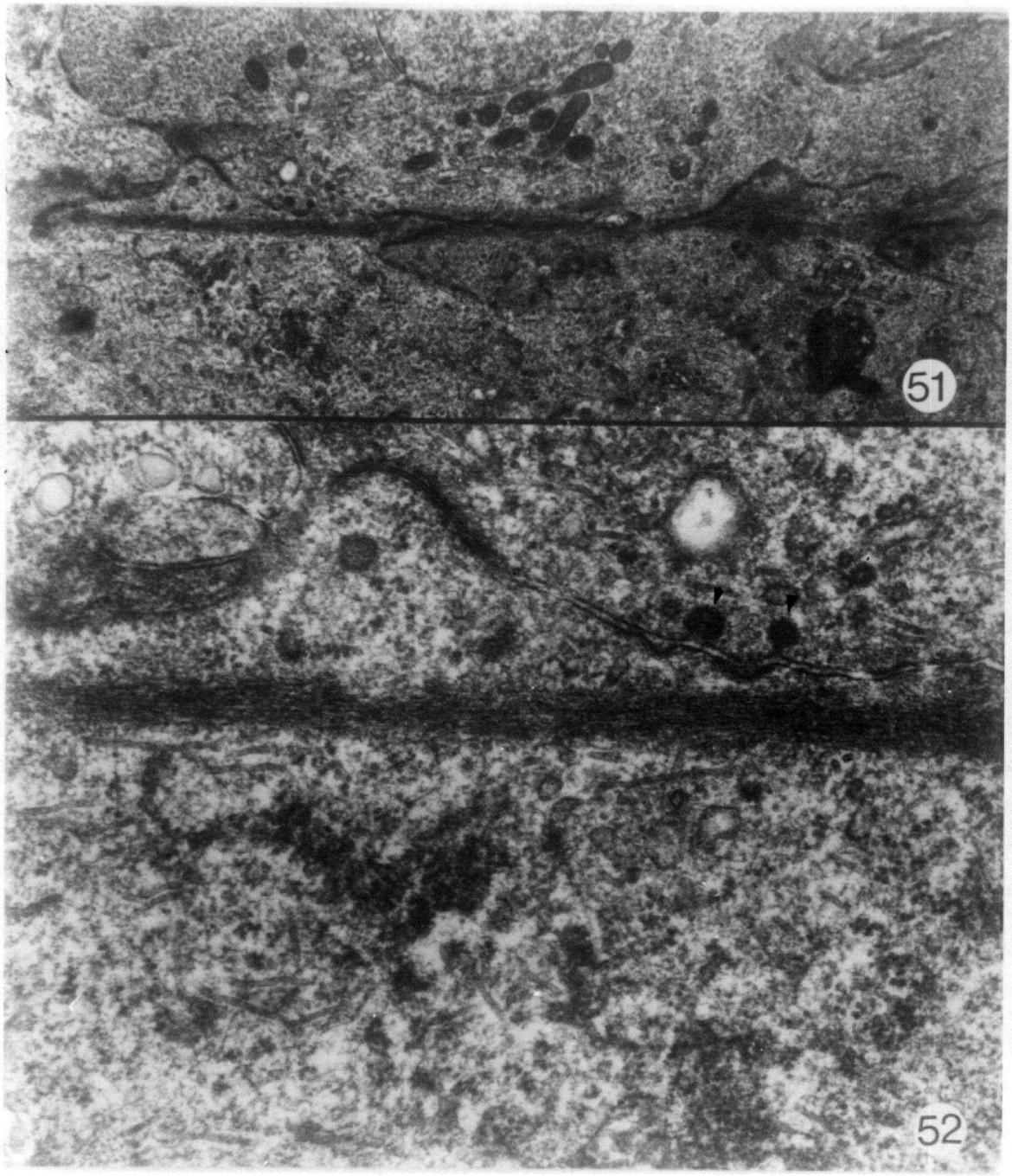
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Figure 53.- Illustration demonstrating cilium and satellite with associated microtubules at apical border of cell after 2 days *in vitro*. x 40,000.

Figure 54.- Apical region of cell after 1 day *in vitro*. Note arrangement of microtubules and microfibres. The arrows point to some of the numerous vesicles which occupy the cytoplasm toward the lateral cell border. x 20,000.

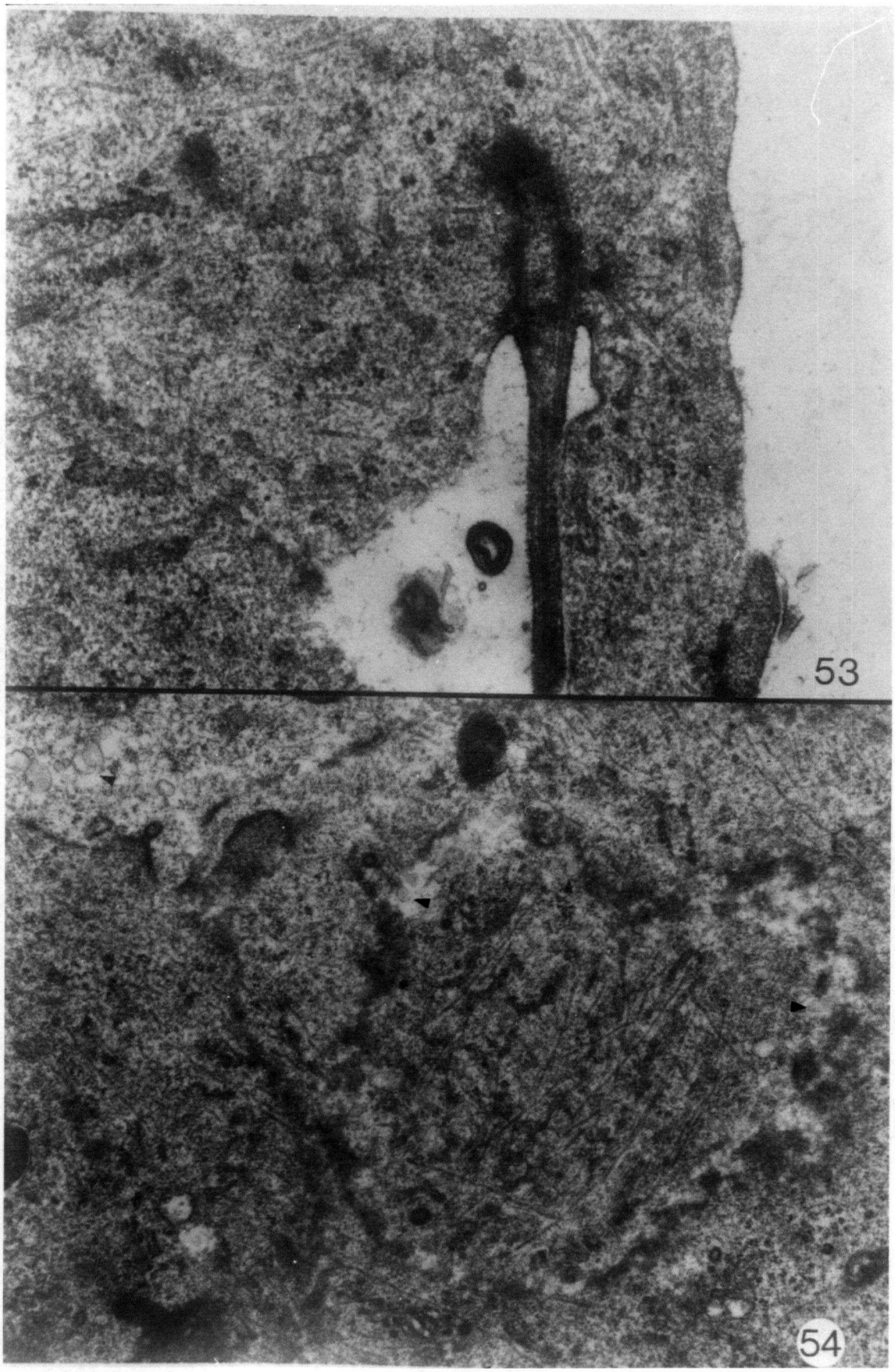
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Figure 55.- Illustration of part of golgi apparatus in elongating cell after 2 days *in vitro*. Note numerous microtubules. x 34,000

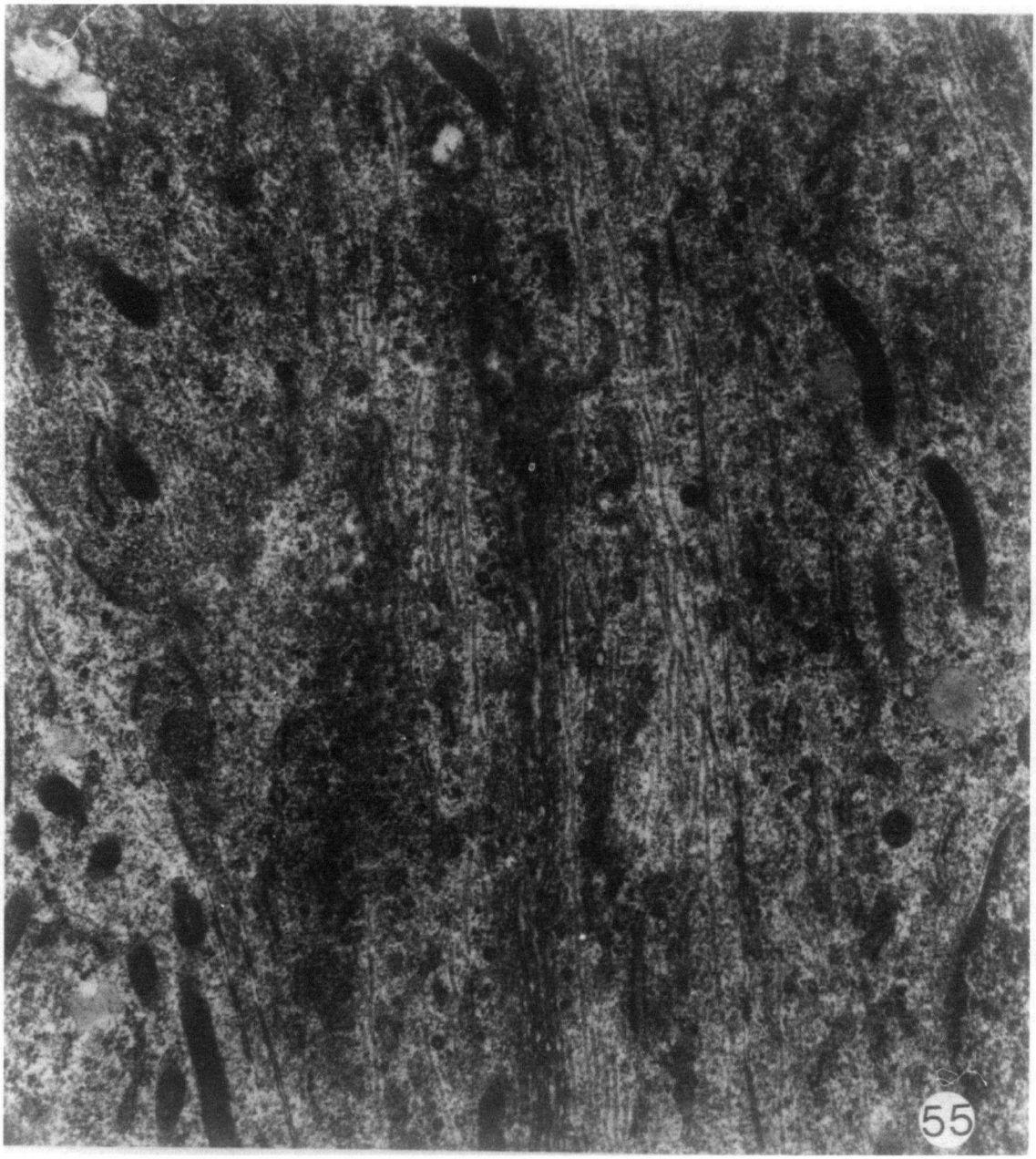
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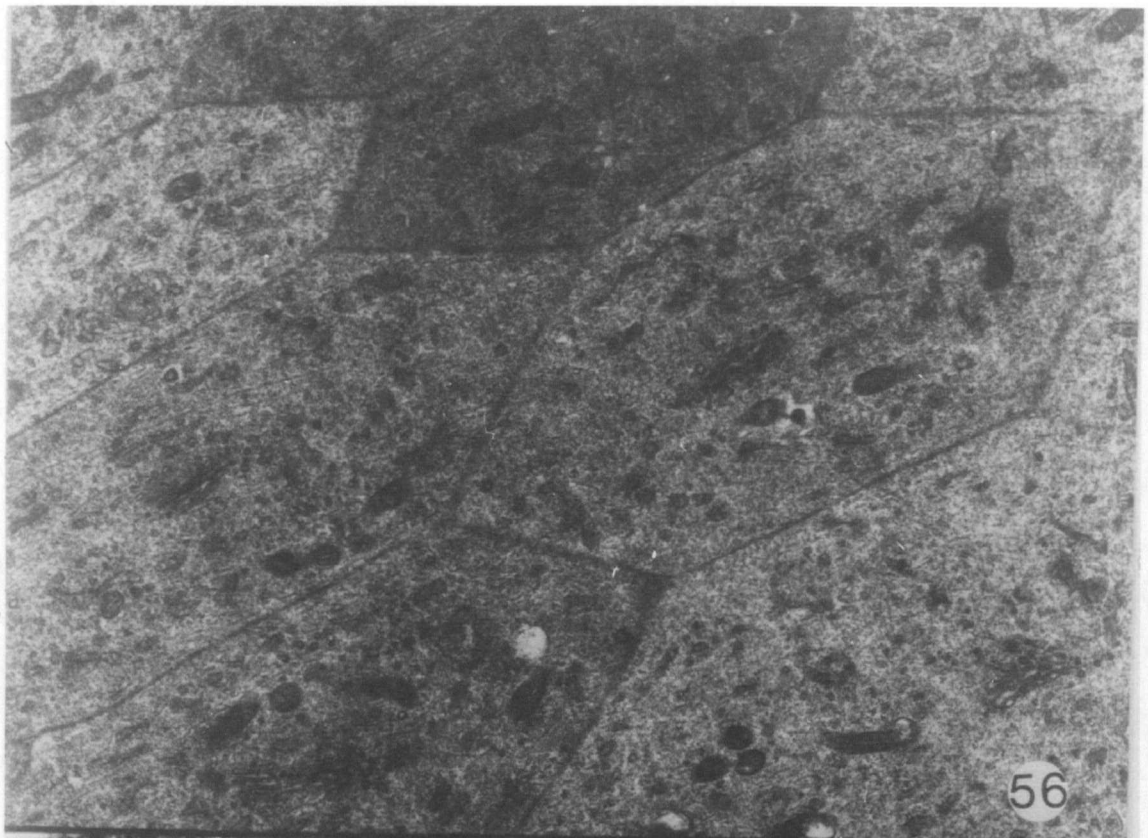
Figure 56.- Section through elongating cells after 1½ days *in vitro* showing hexagonal packing. x 10,000.

Figure 57.- similar to figure 56, but a more tangential section showing numerous microtubules occupying the lateral cytoplasm. x 40,000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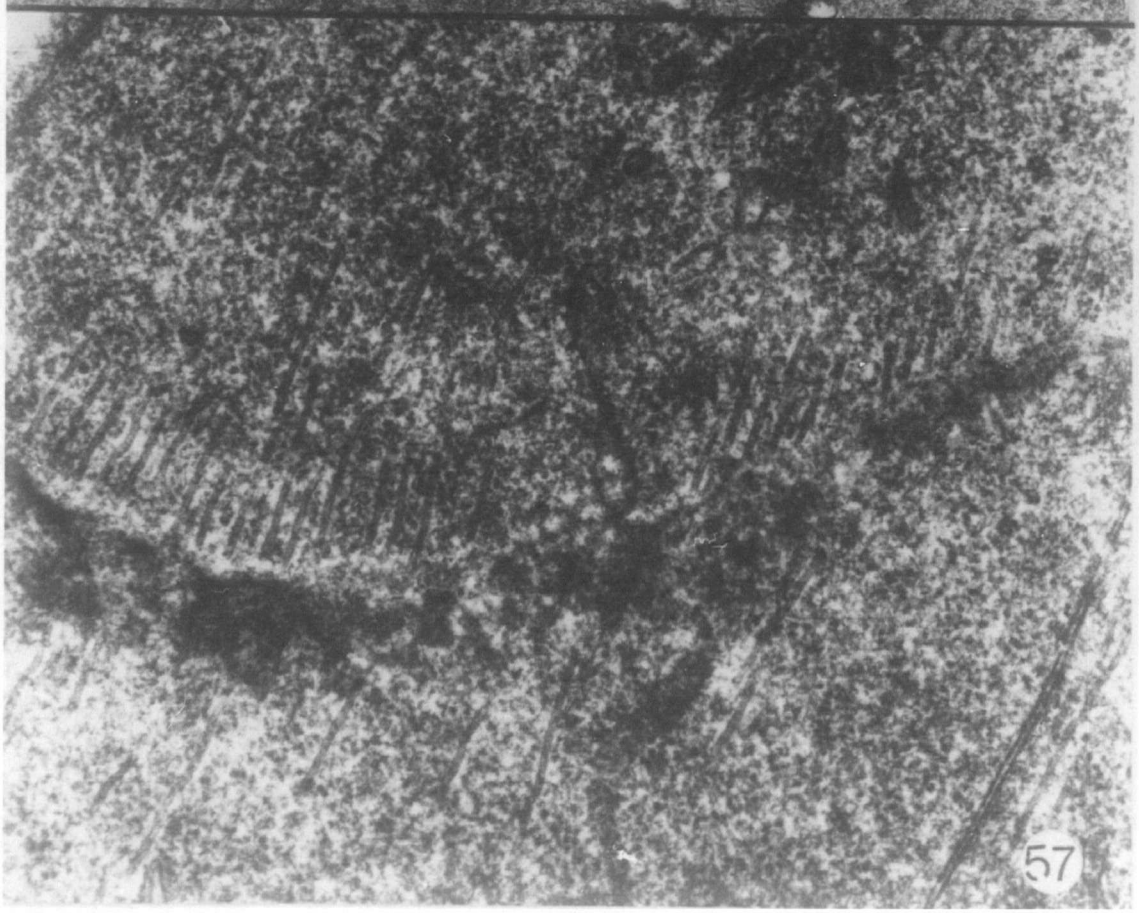
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Figure 58.- Apical portions of cells after 2 days in culture demonstrating microtubules and polysomes. x 20,000

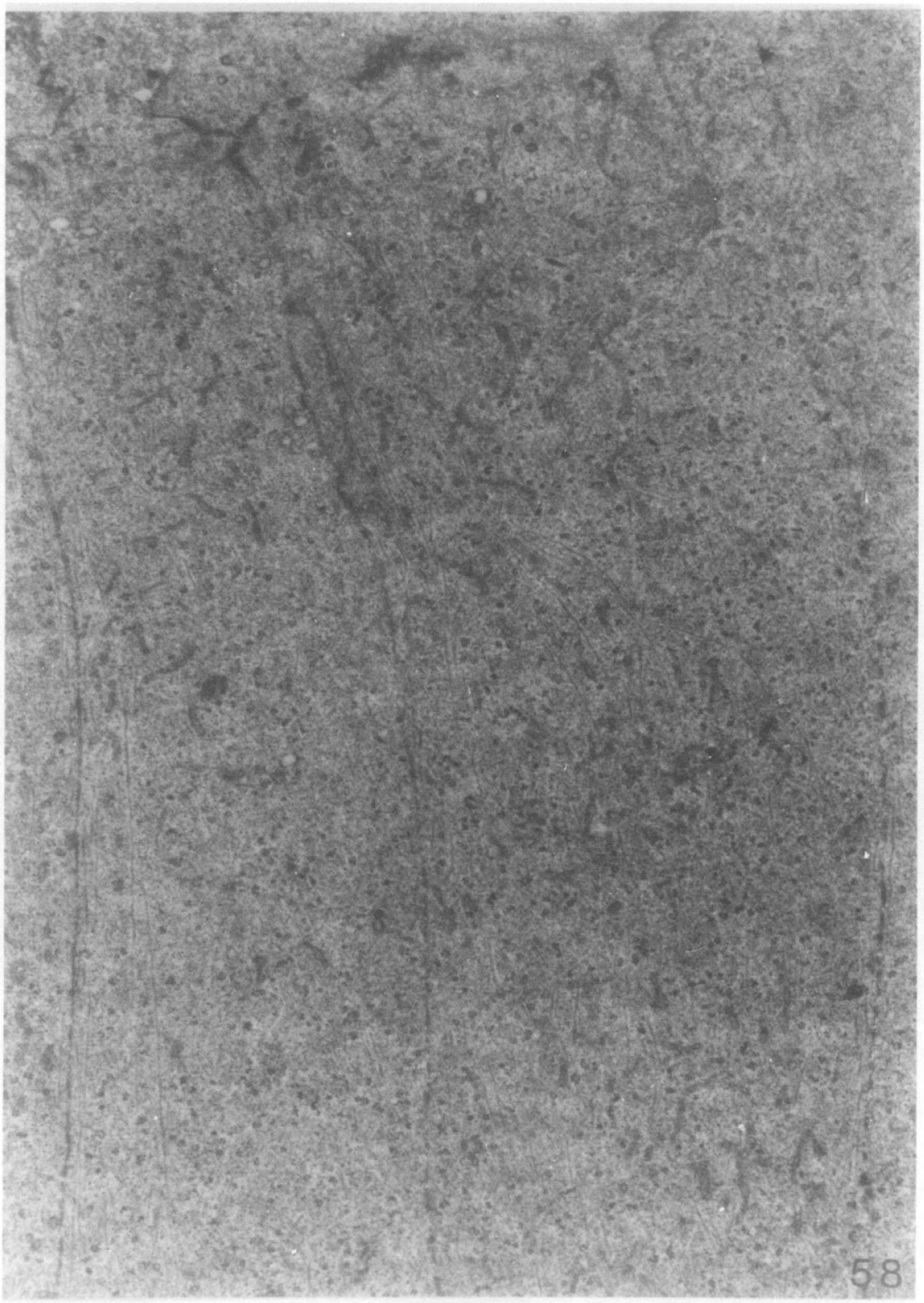
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Figure 59.-Basal aspect of cells after 3 days in culture. Note the numerous microfibres in the cytoplasm, the elements of endoplasmic reticulum and golgi, and the distribution of mitochondria. The lens capsule is in the lower right hand corner. x 20,000

Figure 60.- Cells after three days *in vitro* . Note region of tight junction. x 20,000


60

Figure 61.- Cells after 4 days in culture. Note the numerous microtubules and mitochondria.x 26,000.


61

Figure 62,- Apical region of cell after 3½ days in culture demonstrating the reduced extent of the golgi. x 30,000.

Figure 63.- Cells after 5 days *in vitro*. Note the cytoplasmic matrix, the microtubules, and the lack of tight junctions. x 35,000.

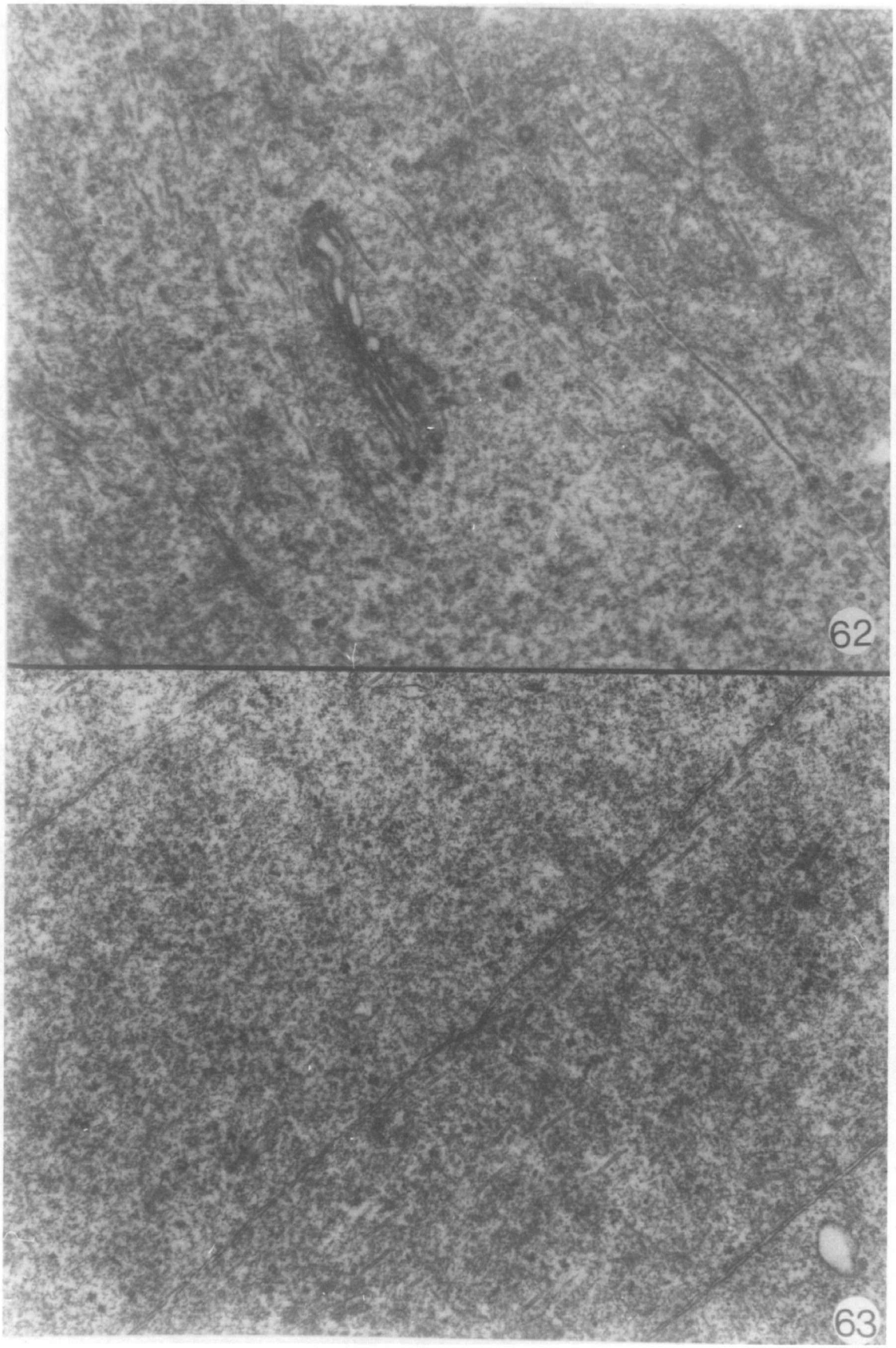
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Figure 64.- Cell of an explant cultured for 6 days. Note the microtubules and microfibres. x 54,000

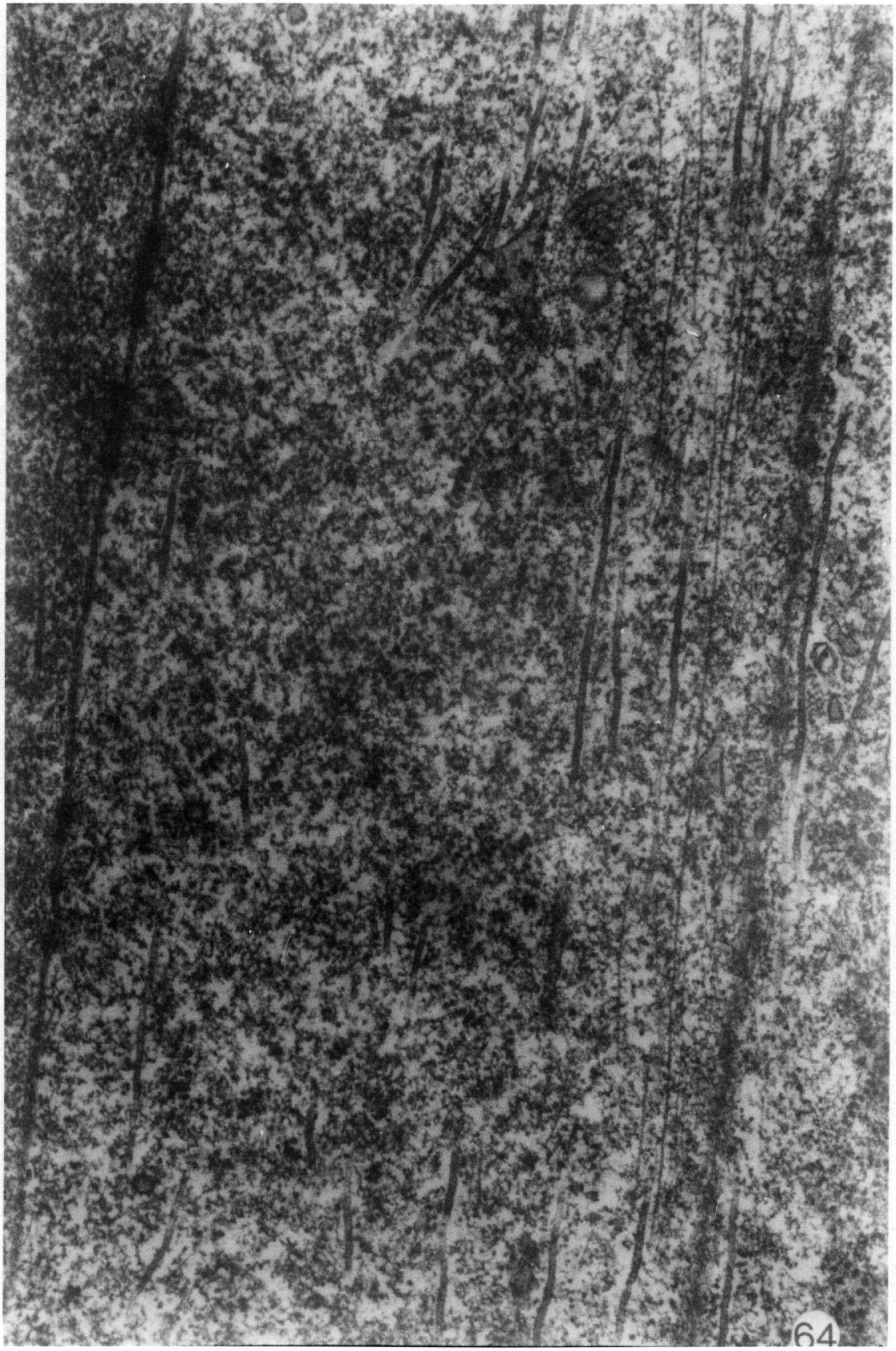
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Figure 65.- Region of an explant showing limited differentiation after three days *in vitro* after being explanted by method 2. Note polysomes and intercellular junctions. x 20,000.

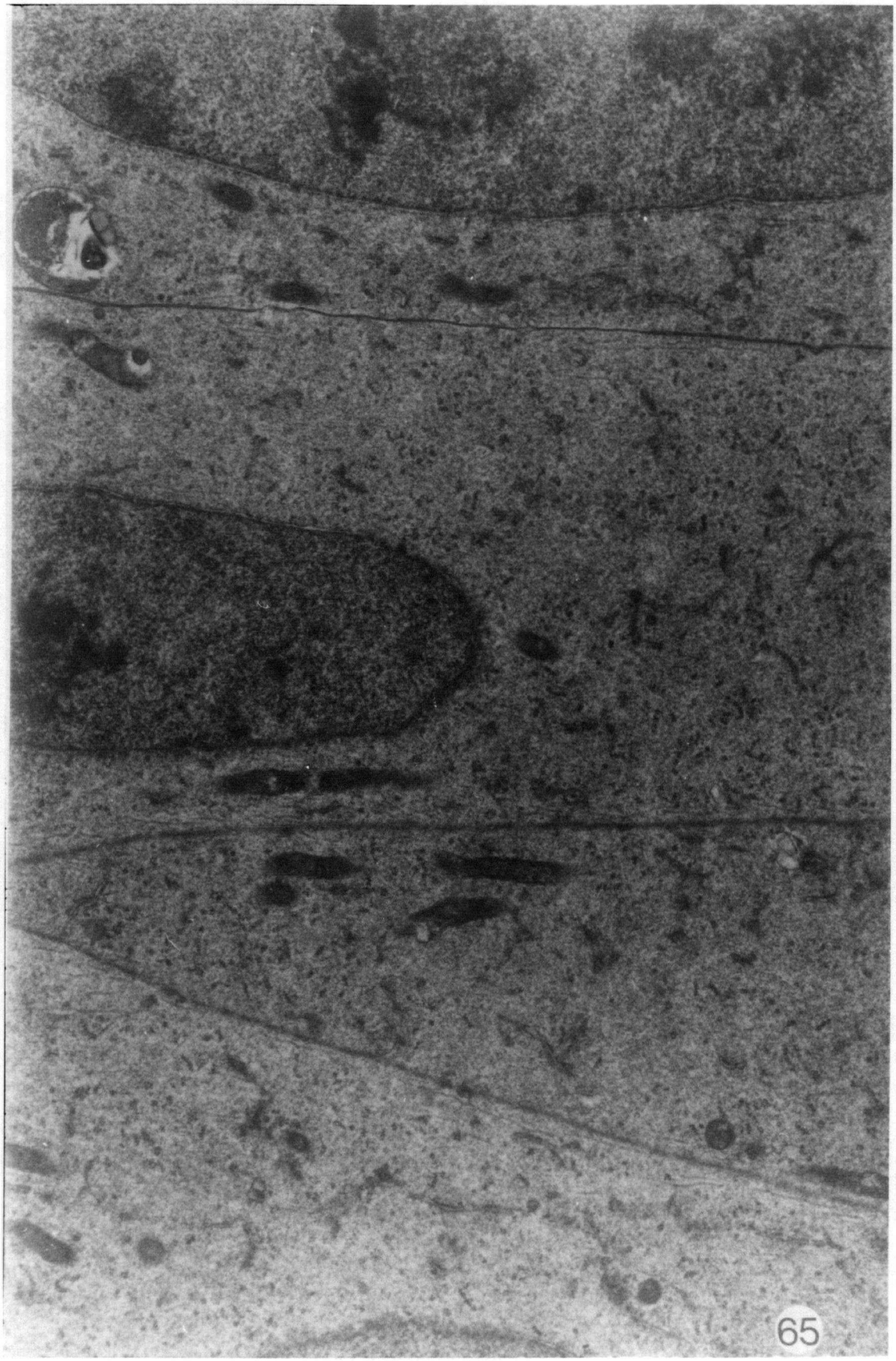
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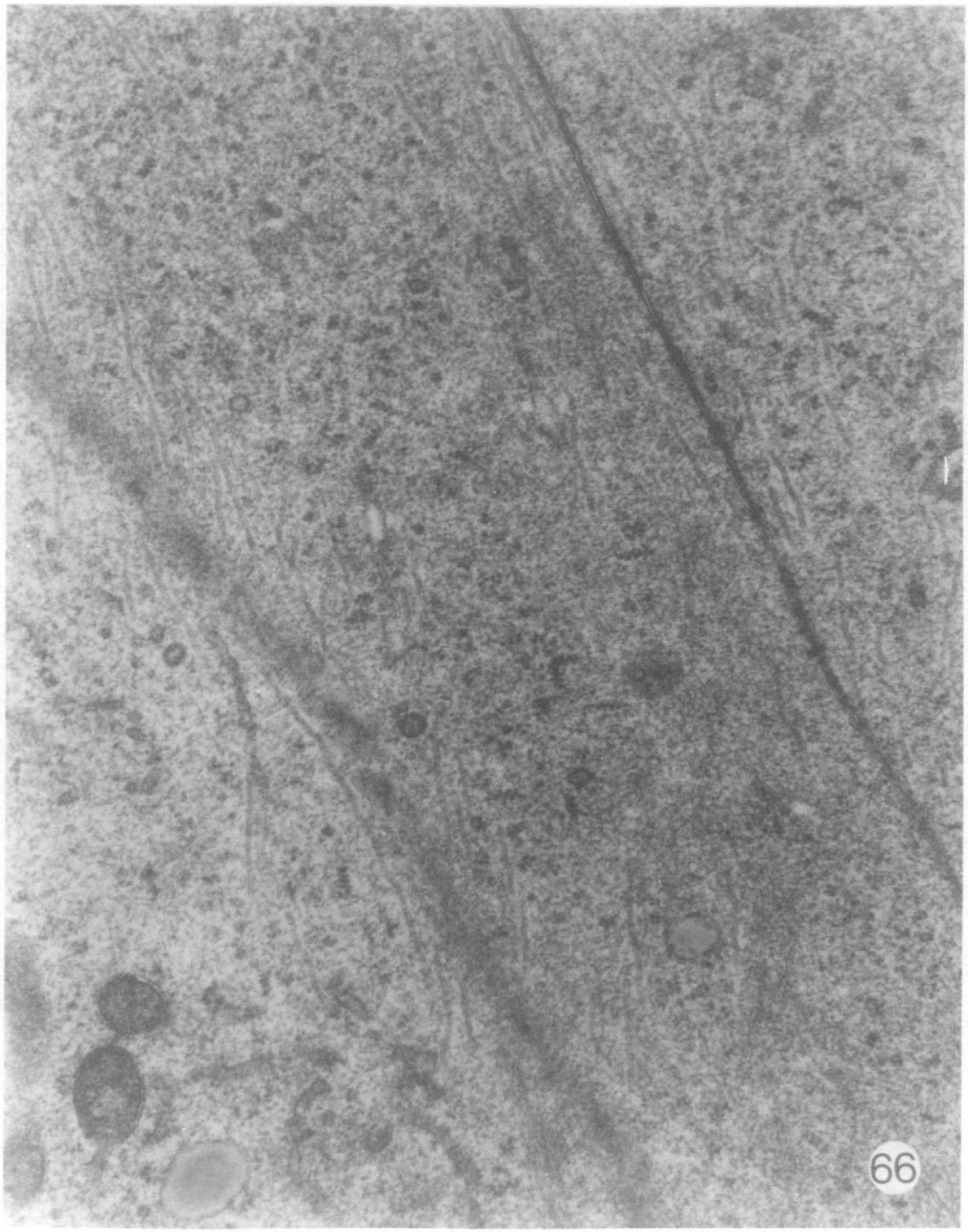
Figure 66.- Similar to figure 65. Note the numerous polysomes and microtubules.x 40,000.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VIII- Polyacrylamide gel electrophoresis.

Electrophoresis of the soluble proteins of cultured and uncultured anterior lens epithelia was performed to determine if cell elongation *in vitro* is accompanied by a change in the proportion of soluble proteins and to compare the soluble proteins of cells elongated to a great extent *in vitro* with those of cells elongated only to a limited degree.

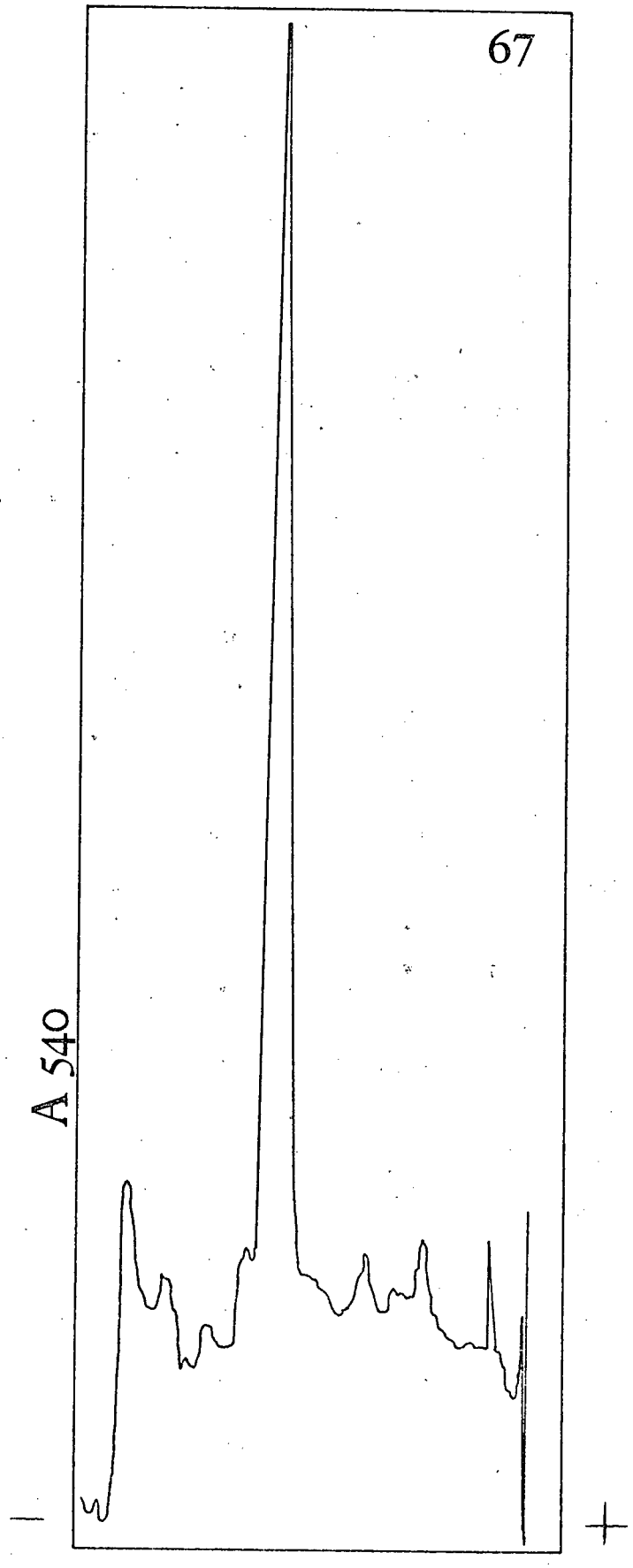
As pointed out in the introduction, one interesting feature of lens cell differentiation is the synthesis of large quantities of what are considered unique structural proteins, the lens 'crystallins'. The different lens crystallins have traditionally been defined by electrophoretic mobility. Most lens protein is lens crystallin protein. And in the 6-day chick embryo lens the only major crystallin is the First Important Soluble Crystallin (FISC) (Clayton, 1970; Yoshida and Katoh, 1971a,b). The essential difference between the cells of the anterior epithelium and the fibre cells, at this stage, with respect to their soluble proteins is that the fibre cells synthesize almost exclusively FISC, while the cells of the anterior epithelium synthesize additional proteins. (Clayton, 1970; Yoshida and Katoh, 1971a,b; Piatigorsky, 1972). Thus, the proportion of FISC relative to all the proteins is very high in fibre cells and relatively lower in cells of the anterior epithelium.

Electrophoresis of the soluble proteins of epithelia explanted by methods 2 and 3 and cultured for three days demonstrated these two groups to be the same by this criter-

ion (figures 68,69), since they showed identical profiles. The difference between cultured (figures 68,69) and uncultured (figure 67) epithelia with respect to their soluble proteins is that the major peak (which would be expected to include FISC) of the latter group comprises a considerably smaller proportion of the total protein on the gel in comparison to that of the former group. That the cultured epithelia have a higher proportion of their soluble proteins in the major peak relative to uncultured epithelia suggests them to be demonstrating a characteristic of fibre cell differentiation.

Figure 67.- Scan of polyacrylamide gel on which the soluble proteins of 6-day anterior lens epithelia have been electrophoresed. Note the proportion the major peak comprises of the total gel protein. Origin at the cathode.

Figures 68 & 69.- A similar scan comparing the soluble proteins of anterior lens epithelia cultured for 3 days after having been explanted by method 2 (figure 68) and by method 3 (figure 69). Equivalent amounts of sample, in terms of numbers of explants, were applied to the gel. Note that the proportion the major peak comprises of the total protein on the gel is considerably greater than that in figure 67.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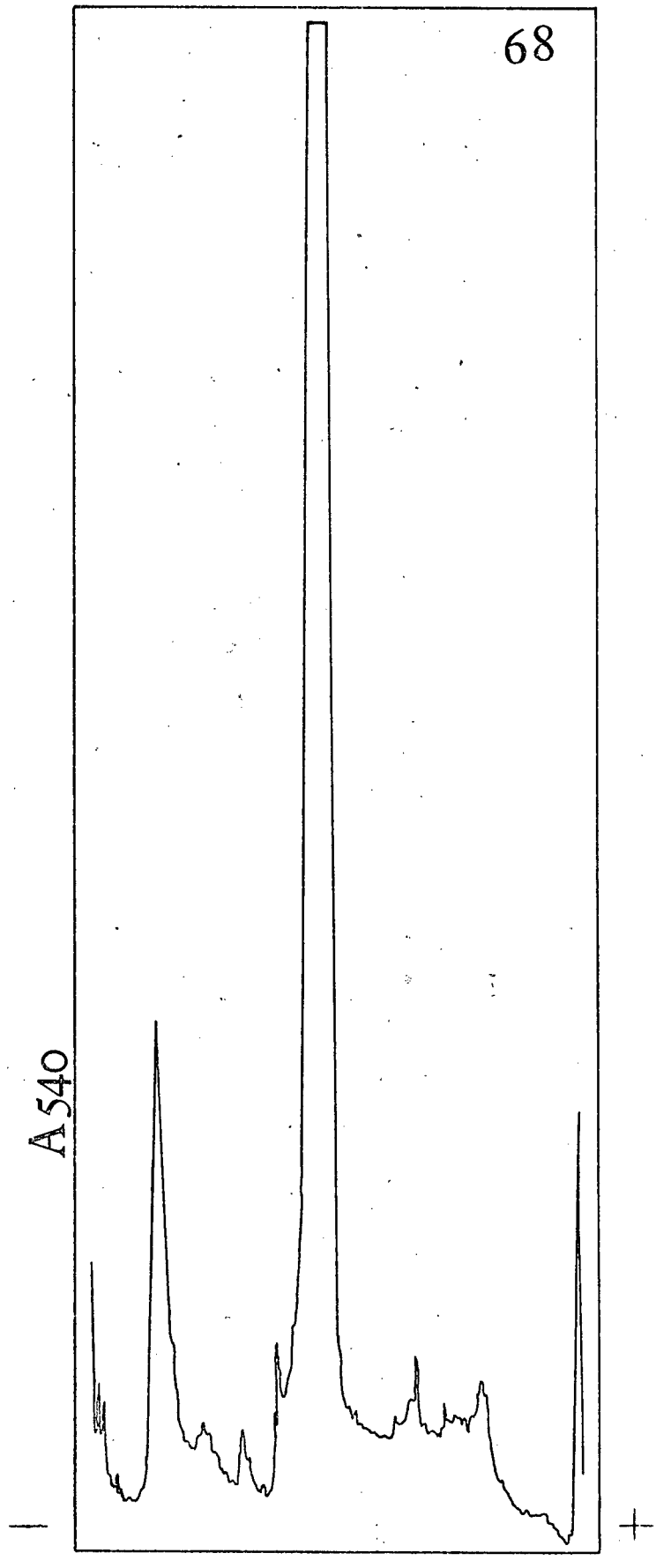
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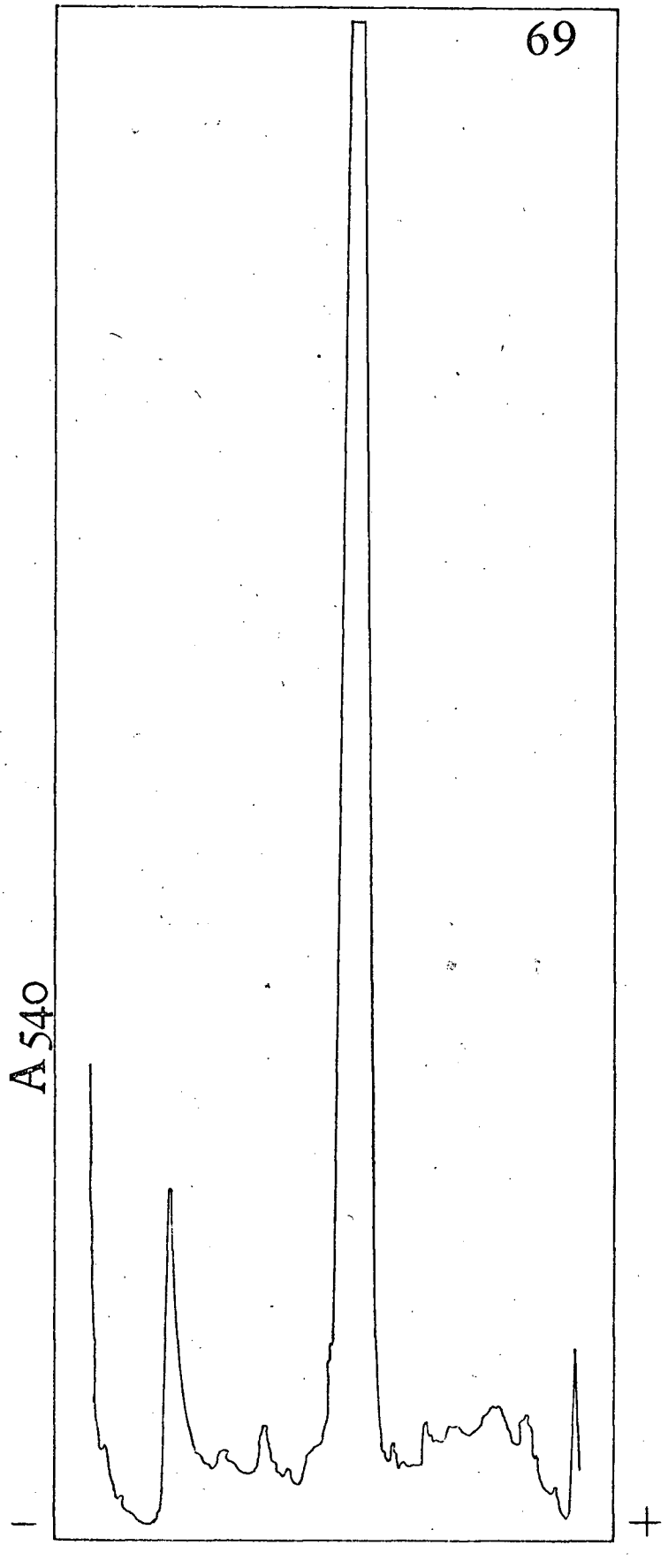

DISCUSSION

I- Chick embryo lens morphogenesis.

A. Plasma membrane.

Lens fibre differentiation involves extensive cell elongation. This change in shape requires the addition of new surface membrane and the question arises as to how this requirement is accommodated. Ultrastructural observations suggest the Golgi apparatus to be involved in the synthesis of new cell membrane. The Golgi increases in activity in those cells initiating elongation, and numerous cytoplasmic vesicles, probably of Golgi origin, are evident in these cells. These are often observed in association with cell membranes with which they sometimes appear to fuse.

Cytoplasmic vesicles have been demonstrated to contribute new cell membrane during mitosis in both plant (Whaley *et al.*, 1966) and animal (Buck and Tisdale, 1962; Hay and Low, 1972) cells. While Buck and Tisdale (1962) suggest that such vesicles are derived from smooth endoplasmic reticulum, their derivation from the Golgi in other systems is clearly established (Whaley *et al.*, 1971), and there are a few reports in the literature which directly relate Golgi activity to the addition of new plasma membrane. Hicks (1966) reported that in the rat transitional epithelium, membrane specializations (regions of asymmetrical thickening) were observed in the Golgi as well as at the cell surface, and that such membrane specializations appeared to be transported there from the Golgi apparatus in the form of fusiform vesicles. Falk (1969) assigned a similar

origin and fate to fusiform vesicles in certain Algae. Bonneville and Weinstock (1970) discussed what they termed 'surface forming vesicles' in the cells of the small intestine of metamorphosing Xenopus. These vesicles are apparently involved in the expansion of the apical surface and the elongation of microvilli. Whaley et al. (1971) observed the Golgi to be very active during pollen tube extension. Arnold (1967) reported that squid iridophore differentiation involves the elaboration of considerable new cell membrane which is contributed by Golgi derived vesicles. These appear empty, and are rather similar to those observed in the present investigation.

Thus, the available evidence has led Sjostrand (1968) and more recently Whaley et al. (1971) to view the Golgi apparatus as a possible centre of membrane differentiation. Indeed, Whaley et al. (1971) suggest,

> ...it seems likely that much plasma membrane is, in one form or another, transferred from the Golgi apparatus. (p. 28)

With regard to lens development, the observations described in sections IV and V suggest that the Golgi activity observed in cells initiating fibre differentiating is concerned with the synthesis of new membrane, and that cytoplasmic vesicles observed in these cells contribute to the plasmalemma, possibly through fusion with it. That the Golgi is moderately active in the cells of the anterior lens epithelium, and that vesicles similar to the above are seen in this region, are observations also consistent with the stated view, since these cells are mitotically active and must, therefore,

elaborate new membrane.

B- Cell junctions.

Other vesicles generated by the Golgi are coated vesicles. These have been studied in a number of tissues wherein they are considered to be involved in secretion and uptake (see discussion by Sheffield, 1970). Coated vesicles are of interest regarding lens morphogenesis since my ultrastructural observations suggest them to be involved in the formation of the 'adhesion plaques' (according to the terminology of Pannese, 1968; also termed 'maculae adhaerentia diminutae' by Hay, 1968; Sheffield, 1970; and Sheffield and Fischman, 1970) established between differentiating fibre cells. Sheffield (1970) hypothesized, on the basis of studies with embryonic neural retina, that coated vesicles form such junctions by fusing with the plasma membrane. My observations on fibre cell differentiation support such an hypothesis.

Junctions of this type have been observed in a number of tissues, both embryonic (Sheffield, 1970; Sheffield and Fischman, 1970; Pannese, 1968; Hay, 1968; Aoki,1967) and adult (Raviola and Raviola, 1967; Palay, 1967). They appear to represent points of strong adhesion between cells (Pannese, 1968), and their function is considered (Sheffield and Fischman, 1970; Aoki, 1967) to be one of stabilizing intercellular relationships during morphogenesis. It is of interest to note that the formation of adhesion plaques precedes the development of synaptic connections in the embryonic retina Sheffield and Fischman 1970) since, in the lens, the formation

of adhesion plaques precedes the development of tight junctions. Possibly adhesion plaques in the lens may serve to stabilize the relationship between differentiating fibre cells in a manner that facilitates the subsequent development of extensive regions of tight junction.

The role these junctions might play in lens cell elongation will be discussed in a later section. At this point, however, it may be pointed out that tight junctions may be important in another way in lens development. Lowenstein's (1968) work has demonstrated that tight junctions are permeable to smaller molecules so that to some extent the intracellular environment of a fibre cell is shared by its neighbours. Lowenstein has pointed out that such junctions may be one basis for the intercellular communication fundamental to developmental processes, and it is possible that this kind of communication facilitates the degree of uniformity of differentiation so very characteristic of the lens.

C. Microtubules.

This consideration of lens morphogenesis must include a reference to microtubules since the presence of abundant, oriented microtubules in the cells of the chick embryo lens at the stage at which they undergo great elongation implicates them in this process. Microtubules are ubiquitous organelles and their appearance and distribution correspond with changes in cell shape in a number of tissues (Tilney and Gibbons, 1969; Gibbons *et al.*,1969; Tilney, 1971) including the lens (Kuwabara, 1968; Porte *et al.*, 1968; Beyers and Porter, 1964). Yet the

circumstances and manner in which microtubules are organized is not entirely clear, though there is considerable evidence that they are composed of subunits that can be reversibly associated as postulated by Inoue and Sato (1967) (see Tilney, 1971).

The microtubules observed by me in presumptive and differentiating fibre cells appear to be elaborated by satellites associated with basal bodies. Satellites are small dense structures considered to serve as sites for initiating polymerization of microtubular subunits (Tilney, 1971). That microtubules are elaborated from a point in the apical cytoplasm may explain why microtubules are more abundant apically than basally during initial stages of cell elongation, and may help to account for their particular distribution and orientation in lens morphogenesis.

D. Cell elongation.

While microtubules are implicated in cell elongation, that intercellular junctions are formed by cells undergoing extension suggests these are also important in this process. There is good evidence that , in certain systems at least, intercellular relationshipsas well as microtubules are important in morphogenesis. Beyers and Porter (1964) reported oriented microtubules in the cells of the chick embryo lens placode, and suggested them to be responsible for the palisading of this presumptive structure. Pearce and Zwaan (1970) reported that the application of colcemid to the chick embryo lens

placode, while reducing significantly the number of microtubules observed in the cells, did not result in the loss of their columnar form. These authors suggested microtubules to be involved in the initiation, but not the maintenance of cell elongation.

Somewhat similar conclusions were reached by Tilney and his associates (see Tilney, 1971) on the basis of a number of elegant experiments involving the effects of calcium-free sea water, D_2O, hydrostatic pressure and colchicine on sea urchin morphogenesis. The primary mesenchyme cells of the sea urchin embryo undergo, at the time of gastrulation, a number of changes in cell shape that can be correlated with the distribution of microtubules. All the above treatments prevented the morphogenesis of these cells, the D_2O by stabilizing microtubules in their initial distribution and the others by disassembling them. While the colchicine caused only partial reduction of ectodermal cell elongation, treatment with calcium-free sea water, which not only disassembles microtubules but interferes with intercellular junctions and the integrity of the basement membrane and hyaline layer, resulted in their spherulation. The application of hydrostatic pressure, which effects the disassembly of microtubules and interferes somewhat with intercellular junctions and basement membrane integrity, resulted in partial spherulation of the cells. Tilney has concluded that while microtubules are required to produce changes in cell shape, additional factors (intercellular junctions and attachments to extracellular material) are important in its maintenance.

While microtubules appear to be involved in the process of cell elongation in the lens, they do not fully account for it. Nor do microtubules provide any explanation for the maintenance of cell elongation, particularly since the number of microtubules is greatly reduced in differentiated fibre cells. It is suggested here that intercellular junctions play a role both in cell elongation and its maintenance, and further that it is important to recognize the energetic stability of the lens body in the context of these processes.

Examination of the lens suggests that the lens body influences the degree of elongation of differentiating fibre cells. The great extent of tight junctions between fibre cells reinforces the view that the lens body is composed of cells that are tightly adherent to one another. The shape of the lens body is maintained in the absence of the lens capsule, which suggests it to have a stable conformation that tends, as the lens develops, toward the spherical. With continued growth of the lens, new fibre cells wrap around the lens body. They proceed to elongate somewhat more than those cells laid down previously since they have a larger circumference to attach to and over which extend in order to maintain the energetically stable conformation of the lens body as they become an integral part of the structure. It is suggested, then, that the lens body plays a role in determining to what degree new fibre cells elongate, i.e. that physical factors are important in lens morphogenesis.

While microtubules probably make a major contribution to lens cell extension, intercellular junctions are probably important to this process as well. Wolpert (1967) has pointed out on simple theoretical grounds the influence cell adhesions might have in the development of cell shape. If one considers a sheet of cells with basal and lateral attachments, the reinforcement of the latter and the reduction of the former will favour the elongation of the cells. This is precisely what happens in the lens, for as the young fibre cells differentiate, they become strongly adherent at their lateral aspects and lose their attachment to the lens capsule basally. That cells initiating elongation become laterally adherent might well be important in terms of the shape they undertake. These lateral adhesions may also serve to stabilize cell elongation as the latter proceeds. Thus both microtubules and intercellular junctions may be considered important for fibre cell elongation. After cell elongation has been achieved, intercellular junctions contribute to the maintenance of cell shape in the sense that they maintain each cell as an integral part of the lens body.

The essential suggestion here is that the intercellular junctions formed during lens cell elongation are not merely a reflection of the transformation of undifferentiated cells into differentiated ones. Rather, the intercellular junctions formed contribute to the process of cell extension.

In summary, my ultrastructural observations on the embryonic lens suggest the following view of fibre cell elongation in lens morphogenesis. New plasmalemma, derived from Golgi activity, is contributed to the cell surface. Microtubules, elaborated from satellites in the apical cytoplasm, contribute to cell elongation. This elongation is assisted through the formation of surface adhesions laterally with adhesion plaques and then tight junctions, while basal attachments to the lens capsule are lost. These developing lens cells extend along other previously differentiated cells to an extent determined by the degree to which the latter have elongated. This extended state is in turn influenced by the stability of the overall conformation of the lens body.

E. Cilia.

That cilia have been observed in this investigation in cells of all regions of the lens may be of interest with respect to the cell cycle. Fonte *et al.* (1971) have reported that while all cells are mitotically active in the chick embryo limb bud (st. 19), a high proportion are ciliated, and they conclude that the cells in the G_1, S, G_2 phases of the cell cycle may have cilia. Similarly, in the lens, ciliated cells have been observed in the present investigation in regions in which all the cells are reported (Persons and Modak, 1970) to be mitotically active . The presence of a cilium may reflect a lens cell in something other than the M phase of the cell cycle so that in the cells of regions II-V cilia

would be expected in those cells involved in elongation, since the cells in mitosis round up near the apical border. The fact that cilia appeared to be more frequently observed in cells of region II as compared to region I may be the result of having examined the apical borders of cells of the multi-layered lateral epithelium, a method that would select for those cells not in mitosis. The frequent observation of cilia in cells of regions III-IV, cells which have entered the terminal cell cycle (Modak and Purdue, 1970), suggests that as the cells complete their final mitosis they become ciliated and proceed to differentiate. Therefore, that some cells of the anterior lens epithelium are ciliated is consistent with the view that the presence of cilia reflects the status of the cell cycle (Fonte et al., 1971) rather than mitotic activity (Rash et al., 1969).

When columnar cells of the lens placode enter the mitotic phase of the cell cycle, they round up near the apical surface, undergo division, and subsequently re-elongate (Zwann, 1969). The appearance and distribution of cytoplasmic microtubules correspond to this change in shape, Thus the cell cycle imposes a constraint on the organization of cytoplasmic microtubules, and therefore on morphogenesis. Many cells of the lateral anterior lens epithelium (region II) have a tendency to be somewhat elongated, with an appropriate distribution of microtubules, but since these cells undergo division,

they undergo recurrent changes in cell shape. It would be of interest to know if the tendency of the cells of region II to be somewhat elongated is a reflection of a greater duration of the growth phase of their cell cycles as compared to that of the cells of region I. Cells of region III correspond to those demonstrated (Persons and Modak, 1970) to be mitotically inactive. Thus it seems fairly clear that lens cells show their tendency to elongate only when in an appropriate phase of the cell cycle. However, whether or not cytoplasmic microtubules are formed in a sufficient quantity and organized in a manner as in the extensively elongating cells of region III and IV may depend on other factors in addition to the cell cycle. Such additional factors would seem to derive from the position of the cells relative to the ocular environment, particularly the neural retina (Coulombre, 1969). Yet, considering that one essential theme of lens development is the regulation of lens growth by means of regulating the cell cycles of the cells of the anterior lens epithelium, it is very tempting to speculate that the influence mediated by the neural retina affects the cell cycle of the cells of region III such that the latter are 'locked' in the growth phase of the cell cycle and thereby permitted to differentiate. That is, rather than attributing the inability of the cells of region III to divide to their commitment to differentiation, it may be more profitable to view their commitment to differentiation as a result, at least in part, of cell cycle regulation.

F. Microfibres.

It is not clear whether the 100 A microfibres are involved in morphogenesis or cytodifferentiation. Their appearance in large numbers in an orientation coincident with that of cell elongation during the initiation of cell elongation could be interpreted to signify morphogenetic import, or the orientation could be merely a reflection of cell polarity, the abundance of microfibres reflecting cytoplasmic differentiation. Porte et al. (1968), who observed these fibres, speculated that they might represent a specific lens protein, and that their subsequent disappearance might be due to transformation into cell matrix, i.e. lens 'crystallin' protein. On the other hand, the 100 A microfibres are often found together with microtubules (which also subsequently disappear), and there is some circumstantial evidence (Wisniewski and Terry, 1968; Wisniewski et al., 1968) indicating that microtubules may reversibly convert to 100 A microfibres when treated with mitotic inhibitors, as though demonstrating a common basis to these units. At the moment, the question of whether these microfibres represent the 100 A class of fibre found in many cells, or whether they are in some way unique to lens cells, is not resolved. The problem and these structures warrant further study.

II- Chick embryo lens morphogenesis in vitro.

The *in vitro* differentiation of six-day anterior lens epithelia indicates that their cells can undergo greater elongation than the 'limited elongation' previously reported (Philpott and Coulombre, 1965; 1968). This is probably due to my different method of explantation, for the cited authors cultured the epithelia flattened out on the bottom of a Petri dish. It would seem from the present examination that the epithelium has a capacity to differentiate in supplemented medium, but that the degree of elongation depends on conditions relating to the epithelial conformation, a view supported by the observation that the epithelium shows either limited or greater cell elongation according to whether it is explanted by the third or second method. This view is also consistent with the observation that those epithelia explanted by the first method show both limited and greater cell elongation, since these end up in various conformations.

Although the lens epithelium seems to be capable of greater differentiation *in vitro* than reported by Philpott and Coulombre (1965; 1968), my results are consistent with their observation that elongation takes place for only about three days. Of further interest is their observation that epithelia cultured in unsupplemented medium progressively lost over a period of three days the ability to differentiate when subsequently placed in supplemented medium. Apparently the isolated epithelium lost a factor necessary for differentiation, but the loss could be compensated for, in their work, if

the epithelium were subsequently placed in the eye of a lentectomized host, or in association with embryonic mesoderm in culture.

Ultrastructural observations on the cells of anterior lens epithelia that differentiated to a high degree *in vitro* demonstrate them to have features essentially similar to those of lens fibre cells differentiating *in vivo*. Cell elongation is accompanied by the presence of numerous oriented microtubules; a modification of intercellular relationships involving adhesion plaques, tight junctions and apical junctional complexes with associated prominent bands of microfilaments; the appearance of oriented microfibres in the more basal cytoplasm; a relative increase and subsequent decline in Golgi activity; the elaboration of numerous free polysomes and a cytoplasmic matrix similar in appearance to that of lens fibre cells. Thus, observations at the levels of both the light and electron microscopes indicate that the cells of the anterior lens epithelium undergo lens fibre cell differentiation *in vitro*.

The differences at the ultrastructural level between fibre cell differentiation *in vitro* and *in vivo*, involving lesser degrees of tight junction formation and of loss of cytoplasmic organelles in the former, do not detract from this conclusion. Rather, they may be interpreted to signify that the cells differentiating *in vitro* come to resemble *differentiating* fibre cells more than *differentiated* ones. This interpretation is consistent with the report (Piatigorsky et al., 1972) that lens epith-

elia differentiating *in vitro* show a pattern with respect to polypeptide synthesis intermediate between that of anterior lens epithelia and fibre cells of six-day embryonic lenses.

The cells of explants elongate in culture for only about three days. These cells, at the ultrastructural level, do not show further cytoplasmic maturation after this time in terms of developing more extensive tight junctions and almost completely losing cytoplasmic organelles, as fibre cells do in the lens. This suggests a common basis to these observations. Possibly, the loss of the hypothetical factor of Philpott and Coulombre (1965; 1968) previously referred to during three days *in vitro* affects not only the ability of the cells to further elongate, but also their ability to undergo further cytoplasmic differentiation that would result in the characteristic morphology of the mature lens fibre cell. It is suggested, then, that whatever is lost during a period of time in culture actually concerns processes fundamental to cell differentiation rather than only those involved in cell elongation.

Considering this, the reason the anterior lens epithelium differentiates *in vitro* is not because lens development is independent of the ocular environment's influence, or because the influences of the ocular environment are somehow duplicated *in vitro*, but because the lens epithelium has acquired, through its developmental history, some measure of capacity to differentiate independently of these influences (cf. McKeehan, 1954). A certain period of isolation, however, demonstrates a certain measure of dependency.

This is further illustrated by the observation (Coulombre and Coulombre, 1971) that when the anterior lens epithelium is placed in the ocular environment, it does not immediately undertake to differentiate, as it does *in vitro*. Rather, it becomes mitotically active and forms a vesicle that develops into a lens, with fibre cells posteriorly and a germinative epithelium anteriorly. Thus the ocular environment is essential for its influence on the lens with respect to mitotic activity, which permits growth, and to the encouragement of continued differentiation (cf. McKeehan, 1951, 1954; Muthukkaruppan, 1965).

The ultrastructural observations regarding those explants in which the cells had elongated only to a limited degree were made to determine if there were any morphological features that might account for their not elongating further. However, in terms of the ultrastructure of these moderately elongating cells, there proved to be nothing remarkably different from that of those elongating to a great extent. Neither was there any difference apparent between these groups with respect to the polyacrylamide gell electrophoresis profiles of their soluble proteins. These observations are consistent with the view that an explanation of limited cell elongation is probably related to physical factors resulting from the conformation of the epithelium. It is necessary, then, to consider how to interpret the relationship between an epithelium's conformation and the degree to which its cells extend.

In order to elongate, cells generally must have a substrate to adhere to and over which to extend themselves. For lens cells, the substrate is other cells, and it has been pointed out previously in this discussion that in the lens new fibre cells extend over those previously laid down, wrapping around the lens body so that the newer fibre cells deposited during growth ultimately become longer than the older ones, having to extend over a greater circumference. Since during growth new fibre cells are continuously added, there exists, at a given time at the periphery of the lens body, a number of associated differentiating fibre cells with a progression of lengths. Each cell, then, is somewhat shorter than the one preceding it and marginally longer than the one following. This suggests that elongation takes place in an incremental fashion, so that as the 'substrate' extends, the next younger cell can also extend, and so on.

One can conceive that cell elongation <u>in vitro</u> may well depend on the establishment of a substrate of one or a few cells which at a given time are marginally more elongated than their neighbours, so that the cells can elongate along each other, yet more or less together. In such a view, differentiating cells could alternate between serving as substrata and elongating in association with neighbouring cells so that all the cells could elongate more or less together. Also, a group of cells proceeding in this way could serve continuously as substrata for cells at their periphery, giving the appearance of centres of relatively greater elongation surrounded by

areas of relatively less elongation.

It is possible that my third method of explanting epithelia resulted in a high degree of cell elongation because the epithelial conformation was such that the cells converged and substrata were generated. On the other hand, explanting epithelia by the second method resulted in only limited cell elongation, and may have done so because with a flat epithelium cells are not convergent, and when the epithelium is curved,with the capsule forming the lesser curvature, the cells would tend to diverge. In other words, a certain amount of lateral tension on the sheet of cells would work against cell elongation, while a reduction in lateral tension would encourage cell elongation.

This investigation indicates that epithelial conformation is important for fibre cell morphogenesis and suggests that the tendency of the lens primordium to form a vesicle during normal development is important, not only for the construction of a germinative anterior epithelium, but also for the morphogenesis if fibre cells in the posterior part.

SUMMARY

1. A study with the light and electron microscopes of the *in vitro* differentiation of 6-day chick embryo lens anterior epithelia was undertaken to evaluate lens cell differentiation and to ascertain what factors might be involved in cell elongation.

2. It was found necessary to examine the ultrastructure of the normal (*in vivo*) 6-day chick embryo lens to properly evaluate *in vitro* lens cell differentiation. This study revealed that initial stages of lens cell differentiation *in vivo* are accompanied by increased Golgi activity. It is suggested that this activity is related to the synthesis of new surface membrane required for cell elongation. Extensive cell elongation in the lens is accompanied by the presence of numerous oriented microtubules and by the development of junctions (adhesion plaques and tight junctions) between cells along their lengths. It is suggested that microtubules and intercellular junctions may be important for cell elongation.

3. *In vitro* studies demonstrated that cells of the anterior lens epithelium undergo either extensive or limited elongation, depending on factors deriving from the epithelial conformation. Experiments were performed that involved explanting epithelia with their normal curvature either retained or reversed. The former procedure consistently resulted in extensive cell elongation while the latter always result-

ed in limited cell elongation, indicating these conformations encouraged or discouraged cell elongation. Examination of explants in which the cells had elongated extensively during 1-6 days *in vitro* indicated that cell elongation progresses for only about three days.

4. Fine structural studies on lens epithelia that elongated extensively *in vitro* demonstrated that their cells were, in general, ultrastructurally similar to lens fibre cells differentiating *in vivo*. However, they did not come to resemble, even after six days *in vitro*, fully differentiated fibre cells in terms of the extent of tight junction formation and the loss of cytoplasmic organelles. Indeed, they did not appear to show further cytoplasmic differentiation, in these terms, after three days *in vitro*.

5. Ultrastructural studies regarding cells of the anterior lens epithelium that elongated only to a limited degree *in vitro* demonstrated them to have features similar to those that elongated extensively *in vitro*.

6. Anterior lens epithelia explanted with reversed or normal curvature were the same in terms of their soluble proteins as indicated by polyacrylamide gel electrophoresis.

7. It was concluded that continued lens development depends on as yet undisclosed influences of the ocular environment, but that the degree of lens cell elongation appears to be dependent upon physical factors inherent in the epithelial conformation and enhanced by microtubules and

intercellular junctions. It is suggested that the normal curvature of the *in vivo* lens is important in this regard.

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