MORPHOLOGY OF THE HUMAN AMNION CELL

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by

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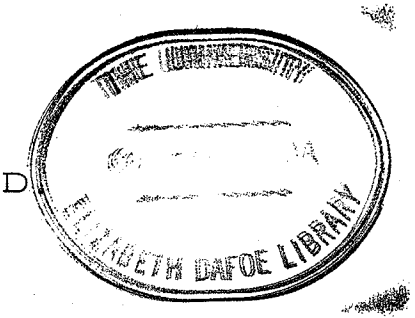

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ABSTRACT

Cine-phase and electron microscopy were used in an effort to discover the meaning of intracytoplasmic vacuolation in human amnion cells.

Cine-phase films indicated that nonvacuolated amnion cells develop monolayers better than vacuolated cells. It also showed the manner in which these monolayers developed, maintained themselves and degenerated. Several miscellaneous observations were recorded and illustrated using photomicrographs and a 16 mm cine-film taken by the cine-phase camera.

The ultrastructure study confirmed much of the previous work done on the fine structure of the human amnion cell, and the observations indicated that these cells were anatomically capable of secretory and absorptive roles. The proximity of lipid vacuoles, which are surrounded by a unit membrane, to the nucleus, endoplasmic reticulum, Golgi complex and mitochondria suggests that the vacuole contains a secretory product, which is known to include cholesterol among other lipids. The amnion cells observed were divided into types "A" to "C", with "A" and "B" being nonvacuolated and vacuolated cells and type "C" showing signs of degenerative change.

These observations are the basis for an hypothesis of the human amnion cell secretion <u>in vivo</u> and <u>in vitro</u>. This hypothesis suggests the amnion cell has a secretory function

in vivo, producing cholesterol and possibly related hormones. The hypothesis also explains why vacuolated cells grow poorly in tissue culture because the accumulation of secretory products blocks cell dedifferentiation, therefore reducing its ability to adapt to a new environment following trypsinization. The limited life span of the primary amnion cell in vitro is explained by the accumulation of large amounts of secretory products in the cytoplasm.

It was concluded that there is some evidence for the belief that the human amnion cell has secretory function, but further work will be needed in the area of radioautography, biochemistry and pharmacology before this can be proven.

DEDICATION

To my wife for her love,
understanding, and support,
which made this work possible.

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INTRODUCTION

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INTRODUCTION

The human amnion cell is capable of replacing many other tissue culture cells used for virus studies. This cell is very suitable for virus work because it is readily available, relatively easily cultured, and responds to many viruses in a predictable manner. Unfortunately the human amnion cell is poorly understood and much research is needed in morphology, biochemistry, physiology, and tissue culture before we can understand its basic functions. When the physiology of the amnion cell is known, researchers will be able to produce tissue cultures more readily, interpret the effects of viruses better, and understand the role of the amnion epithelium in the support of the fetus.

The cell. The epithelial cells of the human amnion line the inner surface of the placental membranes of the human conceptus. The apical surface of the cell is bathed in amniotic fluid and is closest to the fetus, and the basal surface is adherent to the basement membrane and closest to the chorionic tissue of the fetal membranes. A detailed account of the human amnion cell will be described in the review of the literature to follow.

I. THE PROBLEM

Statement of the problem. Wilt and Miller (1965) reported that human amnion cells that were markedly vacuolated did not grow as well as amnion cells that had few or no cytoplasmic vacuoles. Are these vacuoles, as seen through the phase contrast microscope, the cause of poor cell growth in tissue culture, or do they only mirror some other defect in cell metabolism?

Purpose. The purpose of this thesis is to discover the meaning of vacuolation in the human amnion cell by studying its morphology. The main areas of concern will be the morphology of the human amnion cell and its vacuoles, in vivo and in vitro.

II. THE APPROACH

The first phase of this research is a study of trypsinized human amnion cell suspensions while they developed monolayers in Rose multipurpose tissue culture chambers (Rose chambers) (Rose, 1954). This activity was recorded by a time-lapse, phase-contrast, photomicrographic, (cine-phase) 16 millimeter (mm) camera, which made it possible to observe the dynamics of cells developing tissue culture monolayers in vitro.

The second part of the study concerns the in vivo

ultrastructure of the amnion cell as indicated by biopsies of the amnion epithelium. Pellets of trypsinized amnion cells were studied through the electron microscope to visualize their early appearance *in vitro*. Further, a technique was developed to obtain thin sections of tissue culture cells for ultrastructure study.

Finally, these observations were combined with the results of other studies from the literature to form an hypothesis of absorption and secretion by the amnion cell. The hypothesis is a modification of the current thinking about the role of the amnion cell *in vivo*, and it is hoped that it will stimulate further research in the field.

CHAPTER II

REVIEW OF THE LITERATURE

CHAPTER II

REVIEW OF THE LITERATURE

The human amnion cell is discussed under three main headings: (1) the morphology of the cell as seen by the light and electron microscopes, (2) a review of the information about the human amnion cell as it is grown *in vitro*, and (3) the hypotheses concerning the function of this cell *in vivo*.

I. MORPHOLOGY OF THE HUMAN AMNION CELL *IN VIVO*

Bourne (1962) in the most recent and authoritative monograph about the human amnion and chorion has discussed the morphology of the human amnion cell in great detail. This review relies heavily upon Bourne's monograph and, if not otherwise specified, will refer to it directly.

The human amnion membrane. The human amnion is traditionally divided into two main areas for the purpose of study: (1) the placental membrane which is directly adherent to the chorion covering the placenta proper, and (2) the reflected membrane which is adherent to the chorion directly overlying the decidua of the uterine wall. The amniotic membrane is composed of five layers: (1) the epithelium, (2) the basement membrane, (3) the compact layer of connective

tissue, (4) the fibroblast layer, and (5) the spongy layer of connective tissue adherent to the chorion. The remainder of this review discusses the epithelium of the human amnion, with occasional reference to the basement membrane and the compact layer of connective tissue.

The amnion cell shape and size. The shape of the human amnion cell varies from squamous and cuboidal on the reflected membranes to columnar on the placental portion. (Colpi, 1898) Some authors have suggested that the columnar cells are a result of contraction of the membranes after birth, but as Bourne points out, it is very unlikely that the placental contract more than the reflected membranes. According to Bourne post partum contraction occurs primarily in the chorion, while the spongy layer separating the two allows the amniotic membrane to fold on itself, maintaining its surface area and cell shape.

When viewed from the outer or lumenal surface of a biopsy of the amnion, the cells appear irregular and polygonal shapes in a mosaic pattern. They often overlap each other and are firmly joined by many well defined intercellular bridges.

Danforth and Hull (1958) reported that the amnion cell, when measured in stained sections were 0.01 to 0.02 mm wide and 0.02 to 0.03 mm high.

The apical surface. On the apical surface of the cell, irregular microvilli are present (Mandl, 1905, Bourne and Lacy, 1960), and these structures appear to fulfil the definition of a "brush border" as defined by Bloom and Fawcett (1962). "Free surfaces of cells specialized for the absorption of substances are characterized by the presence of thousands of minute cylindrical processes which collectively form the brush border or striated border."

The electron microscope shows that the microvilli are covered with the unit membrane as defined by Robertson (1962), as is the rest of the cell. These microvilli may be interspersed with cilia-like structures, although no one has been able to show convincingly the "nine plus two" arrangement of fibrillar bundles normally seen in cilia (Bourne, 1962, Thomas, 1965). However, fibrillar bundles were noted by Bourne and Lacy (1960), passing from the microvilli into the cytoplasm for a length of up to one-half micron. He assumed these made up canals with an internal diameter of 70 angstroms (A), but cross sections of the microvilli show structures that are only suggestive of these canals.

The lateral cell border. The convex-shaped apex of the amnion cell unites with other cells at its lateral borders, and is joined by interdigitating, fingerlike cytoplasmic processes and desmosomes (Thomas, 1965, and Fawcett, 1966). The

lateral intercellular canal formed at the junctions is 100 to 200 angstroms in diameter, tortuous and frequently dilated. These periodic dilations produce a series of irregularly shaped vacuoles which invaginate the cell, giving the impression of being intracellular in position. The degree of intercellular canal dilation is thought to be greatly increased in amnion cells obtained following normal delivery, as opposed to those resulting from Caesarian section (Thomas, 1965).

Bourne reported that these intercellular vacuoles, which often have thin cytoplasmic processes projecting into them, communicate with the amniotic fluid via the intercellular canals. The intercellular vacuoles closest to the base of the cell contain material similar to the basement membrane (Edwards and Fogh, 1959).

Thomas (1965) noted very small vacuoles on the cytoplasmic side which are adherent to, or indentations of the unit membrane making up the lateral cell wall. They are present also at the base of many of the apical microvilli and in the walls of the basal processes. These small vacuoles are bounded by a double-layered membrane and contain particulate matter of moderate electron density.

The cell base and basement membrane. The base of the cell was noted by Mandl (1905) to have processes which appear to enter the basement membrane. Danforth and Hull

(1958) called this basal portion of the cell a brush border, but Bourne pointed out that this is improper terminology for the base of the cell. The basal processes, as confirmed by Bourne, are of varying thickness and length and are intimately associated with the connective tissue of the basement membrane. Further, he noted fine canals which open into the intracellular vacuoles as well as the extracellular space. This observation has not been confirmed and may be a result of early imperfect fixing and staining methods. Thomas (1965) noted the presence of half desmosomes and the previously mentioned small vacuoles on the borders of the basal processes, which also contain cytoplasmic fibrils of a coarser nature than found elsewhere in the cell. She described the basement membrane as having three main constituents: (1) very fine fibrils in a dense "felted" area close to the cell, (2) larger more loosely arranged fibers, and (3) large, collagen type fibrils with marked periodicity in the deeper layers.

The nucleus. The human amnion cell usually has one nucleus, but multinucleated cells have been noted frequently using the Gomori stain (Colpi, 1898, Schmidt, 1956, Danforth and Hull, 1958, Schwarzacher and Klinger, 1963). Some authors (Schmidt, 1956, and Bourne, 1962) suggest these cells are giant cells rather than several confluent uninucleated cells, because they are usually two or three times larger than the

normal cell and the nuclei are regularly distributed in the cytoplasm.

The ovoid nucleus is usually fairly large and may be seen at the apex, center, or base of the cell. The nucleus is surrounded by a double-layered membrane which contains pores and often several deep folds into the nuclear body. Vacuoles seen in these clefts have given the impression of intranuclear vacuoles, but true intranuclear vacuoles have not been found. The nucleus usually contains one or more nucleoli irregularly placed in a background of granules of varying size and electron density (Edwards and Fogh, 1959).

Mitotic figures have been noted in the human amnion epithelium during the early periods of gestation, but Schwarzacher and Klinger (1963) in a study measuring the increase in desoxyribonucleic acid (DNA) pointed out that few or no mitoses occurred 120 days after conception until term. They attempted to show, although inconclusively, that the cells in the last 60 days of gestation multiply mainly by amitosis.

The cytoplasm. The basic substance of the human amnion cell cytoplasm was shown by Bourne and Lacy (1960) to contain many paired parallel lines. They believed that these lines were fine canals with a 100 A wide lumen and membranes 60 A thick, which passed through the

cell plasma membrane. Other studies, which used different specimen preparation techniques, did not confirm this (Edwards and Fogh, 1959, Thomas, 1965).

Fibrils of 20 to 25 A thick which are present throughout the cytoplasm, show no periodicity or axial orientation and are especially plentiful in the areas of the microvilli, basal processes and nucleus. Thomas (1965) has divided human amnion cells into two categories, partly on the basis of these fibrils: (1) the "fibrillar type" cell, with many coarse, electron dense fibrils, but with few elements of the Golgi complex, and (2) the "Golgi type" cell which has few fibrils, but also has increased amounts of the Golgi complex.

Numerous granules of ribonucleic acid (RNA) 100 to 150 A in diameter are present singly or in rosettes throughout the cytoplasm. Although they are usually free, these granules may be seen lining the cytoplasmic surfaces of the endoplasmic reticulum (Edwards and Fogh, 1959). Small, dilated cisternae of endoplasmic reticulum were reported to contain finely fibrillar to granular, moderately electron dense material (Thomas, 1965 and Edwards and Fogh, 1959).

Small, oval mitochondria were reported as being few in number by Edwards and Fogh (1959) and Bourne and Lacy (1960). Thomas (1965) noted mitochondria with the "usual" ultrastructure; more being present in the "Golgi type" cell

than in the "fibrillar" cell.

The Golgi apparatus was first reported by Edwards and Fogh (1959) who described it as a "concentrated group of vesicles and lamellae" usually near the nucleus as a single organelle. The Golgi apparatus was not seen by Thomas (1965) in the "fibrillar type" cell, but in the "Golgi type" cell it was noted to be common and widely distributed, consisting of lamellar and vesicular elements.

Vacuoles. As the human amnion matured toward term, Colpi (1898) noted increasing numbers of intracellular lipid granules or vacuoles. Mandl (1905) showed that the vacuoles were actually empty areas from which lipid had been removed by the solvents used during the fixation of the tissues.

An early developmental study by Bondi (1905) of various mammalian placentas showed that a few, small lipid granules were first seen at the third month in human amnion cells, and at term the granules had increased to many large and small perinuclear lipid droplets. The increase of lipid droplets toward term occurs in spite of a decrease in the lipid content in per cent dry placental weight (Watanabe, 1923, and Needham, 1963). Polano (1905) thought the lipid droplets were limited to the placental portion of the amnion, but he agreed with Bondi that the amount of lipid increased until term. Contrary to Polano's findings, vacuoles and lipid

droplets were described by Danforth and Hull (1958) in both placental and reflected amnion cells. Incidentally, these authors suggested that the reflected amnion cells were more mature and probably developed over the placenta and were displaced toward the periphery of the reflected amnion during maturation.

Recent studies by Wilt and Miller (1965), using specialized staining methods and phase-contrast micrography, showed that those cells which contained Sudan IV positive lipid bodies two to four microns in diameter, were highly vacuolated when seen by the phase contrast microscope. Those cells which showed no Sudan IV positive lipid droplets have few or no vacuoles when observed by the phase contrast microscope. Moreover, the size, morphology and intracellular distribution of the lipid droplets were the same as the vacuoles seen in phase-contrast. This suggests very strongly that the sudanophilic, lipid droplets and the vacuoles seen by the phase-contrast microscope are different views of the same object.

Bourne (1962) believed that the majority of these vacuoles were dilated intercellular canals, with the remaining intracellular vacuoles being in basal, perinuclear and apical positions. However, Wilt and Miller (1965) have photographed cells removed by digestion from highly vacuolated epithelia and have shown that they remain highly vacuolated, although the

trypsin had removed the intercellular canals by separating the cells. These workers have noted also that slightly vacuolated cells seen in biopsy before preparing for digestion become devacuolated upon trypsinization, forming small, round, dark cells. This supports Bourne's belief that intercellular vacuoles are present, but in very vacuolated amnions the intracellular vacuoles predominate.

Although many workers have shown that the amnion cell usually contains considerable numbers of lipid droplets, Bourne (1962) stated that the proportion of lipid containing vacuoles is very small. His electron micrographs of these vacuoles show a moderately electron dense content which may rule out the high lipid content usually associated with very electron dense material but not a substance of a lower lipid concentration. The ultrastructure studies by Edwards and Fogh (1959) and Thomas (1965) showed a relatively high frequency of "fat droplets" within the amnion cell cytoplasm.

In his studies on the possibility of meconium transport by the amnion cell, Bourne suggested, with few supporting facts, that many of the vacuoles in the cytoplasm contain meconium. Using biochemical tests for bile, an attempt to confirm the presence of meconium in these vacuoles was made by Wilt (1966), without success.

Finally, Bourne (1962) described basal vacuoles which

contained material identical to the basement membrane, but to this observer these appear to be cross section views of projections of the basement membrane between the basal processes.

II. MORPHOLOGY OF THE HUMAN AMNION CELL IN VITRO

The human amnion cell was first reported as a source of cells for large scale tissue culture by Zitcer, et al., in 1955. Since then considerable work has been done by many researchers, most recently by Wilt and Miller in 1965 and 1966, in order to simplify culture techniques, to lengthen the primary cell life in culture, and finally to establish the normal appearance of these cells following trypsinization. This portion of the review will discuss the immediate post-trypsinization appearance and the morphology of primary human amnion cell, in tissue culture.

Freshly trypsinized human amnion cells. The first investigation on this type of cell was done by Danforth and Hull (1958) using lipid stains and phase-contrast microscopy. Smears of trypsinized cells contained cells 0.01 to 0.02 mm long and 0.02 to 0.03 mm wide, with filamentous projections at the base of the cell, occupying 10 per cent of the total cell area. Structures resembling canaliculi extend up to the

area near the apex of the cell from the filamentous projections at the cell base. The authors believed that these intracellular canaliculi are either true channels or filamentous chondriosomes, and related to the possible secretory or absorptive functions of the cell. Irregular cell borders were noted on trypsinized cells; these may be the remains of the intercellular bridges.

Trypsinized cells stained by Sudan IV contained varying amounts of fat droplets; according to Danforth and Hull (1958) Page 544:

> In some cells the fat is present in large clumps; in others it is finely dispersed, either throughout the cell or only in one or another portion of the cell. As a general rule the fat droplets predominate in the perinuclear zone, but are concentrated on the side of the nucleus toward the free border of the cell.

Phase-contrast micrography confirmed this and in addition irregularly dispersed "secretory granules" of 1.0 to 2.5 microns in diameter were seen in the cytoplasm between the nucleus and the cell base. Mitochondria were seen and differentiated from the granules by their dumb-bell or thin centered disc shape. In an effort to discover which amnions would yield good cells for tissue culture, biopsies of human amnion epithelia were examined by Wilt and Brambilla (1966) and graded from I to IV, with grade I amnions having no vacuoles and grade IV having many vacuoles. Further, these workers were able to show that trypsinized cells were of six

different cell types, based on vacuole concentration, cell outline and refractility. Grade I amnions, when treated with 0.25 per cent trypsin, produce mostly small, round, dark, nonvacuolated "type 1" cells which grow well in tissue culture. On the other hand a grade IV amnion, when trypsinized, produces mainly "type 3" (markedly vacuolated) cells which grow poorly in tissue culture, as well as producing a few "type 2" (slightly vacuolated) and "type 1" cells. The other three types of cells are characterized by increasing refractility and irregularity of shape which is probably due to a deleterious trypsin effect. The original illustration of the classification of amnion cells by Wilt and Brambilla is in Appendix B, Figure 65, page 181. Suspensions of amnion cells containing predominantly types "1" and "2" cells grow well in tissue culture, but suspensions of types "3" to "6" cells grow poorly or not at all.

The only published work on the ultrastructure of trypsinized cells was done by Edwards and Fogh in 1959. In an effort to determine which cells were suitable for cultivation, amnions were studied after being digested in 0.25 per cent trypsin in Hank's solution at 37 degrees centigrade (37°C) for one hour. Some cells maintained the usual structure seen in biopsies while others showed incomplete plasma membranes surrounding a few cytoplasmic fragments.

Between these extremes trypsinized cells showed the following changes: (1) rounding to produce an ameboid type cell without polarity, (2) swollen microvilli with constricted bases, (3) a change from granular to filamentous cytoplasm, (4) dispersal of the tonofilament bundles, (5) a probable increase in lipid droplets, (6) the formation and later degradation of small, (0.23 to 0.17 microns) ovoid mitochondria, especially near the nucleus, (7) hypertrophy of the endoplasmic reticulum and Golgi complex, with the later stages showing almost complete cytoplasmic replacement by this "endomembrane" system, (8) nuclear lobulation and degeneration, and (9) nucleolar hypertrophy. Edwards and Fogh (1959) pointed out that many of these changes are similar to the morphology observed in some malignant cells. From this they concluded that these changes allow the ameboid primary human amnion cells to survive in vitro as a unicellular organism and hence "making it a potential malignant cell".

The morphology of human amnion cells in tissue culture. Only brief mention of this aspect of the amnion cell is made in the literature. Bourne (1962) showed two photomicrographs of amnion cells dividing in a monolayer after being prepared from a 16 week old amniotic membrane. Descriptions of primary amnion cells infected by viruses have been made (Rosan, et al., 1964), but in each case the description of

the uninfected culture cell has been very sketchy. In fact, the normal morphology of trypsinized primary human amnion cells in tissue culture is relatively unstudied in the areas of both light and electron microscopes.

III. POSSIBLE FUNCTIONS OF THE HUMAN AMNION CELL

Possible secretory and absorptive roles of the human amnion cell. A rather philosophical paper by Cane in 1888 speculated that the human amnion secreted amniotic fluid. Lipid granules in the human amnion cell were seen by Colpi (1898), but he considered these the result of degeneration. Mandl (1905) countered this opinion with the fact that cells showing signs of degeneration in biopsies of the amnion seldom contain fat droplets. He saw vacuoles at the apex of normal amnion cells, apparently being extruded into the lumen. Mandl pointed out that the morphology of the amnion cell parallels that of a secretory cell, and for these reasons believed it to be secretory. Bondi (1905) supported the view that the amnion epithelium produces amniotic fluid, by showing that as the amount of amniotic fluid increases toward term, so the lipid granules increase in the cell cytoplasm. Polano (1905) supported both of the above findings and reaffirmed their theories as to the secretory role of the human amnion

epithelium.

A strong argument supporting the theory that lipid granules in amnion cells are released to produce vernix caseosa was made by Keiffer (1926). His work showed that the vernix covering the fetus is not likely due to the secretion of the relatively few sebaceous glands in the fetal skin. Keiffer assumed the amnion cell lipid droplets are the source of vernix, since they stain similarly with Sudan IV.

The general belief that the amnion epithelial cells are secretory is supported by the morphological studies of Danforth and Hull (1958). They also discussed the relative merits of other theories designed to explain the absorption and production of amniotic fluid, namely swallowing of the fluid by the fetus and urinary excretion into the amniotic fluid by the fetal kidneys. Jeffcoate and Scott (1959) suggested that probably both these factors plus the amnion epithelium are responsible for the secretion and absorption of amniotic fluid. The average volume of amniotic fluid present at term in a normal pregnancy is 800 milliliters (ml) according to a recent study by Charles, et al., (1965). Using heavy water isotopes, the water exchange in the amniotic fluid was calculated to be 34.5 per cent, or 280 ml per hour (Vosburgh, et al., 1948 and Plentl and Hutchinson, 1953). Although some ions turn over at a much slower rate, e.g. sodium at 7 per cent per hour,

(Vosburgh, et al., 1948), the water transport is quite large and it is unlikely that the fetus could absorb and secrete one half that much fluid per hour, thus placing a considerable amount of the absorptive and secretory work upon the amnion epithelium. The fact that the sodium ions of the amniotic fluid are replaced at one-fifth the rate of water, suggests that the amnion epithelium is able to absorb selectively.

Jeffcoate and Scott (1959) stated that the fetal respiratory tract, because of its contracted state before birth, is an unlikely source of amniotic fluid. Makepeace, et al. (1931), noted that the urea content of the amniotic fluid is similar to that of the maternal serum up to the twentieth week of gestation, at which time the urea content in the amniotic fluid increases. Since about the twentieth week of gestation is the time when the fetal kidneys are first capable of secretion, it is believed that the increase in amniotic fluid urea content is due to the increasing role of the fetal kidneys in amniotic fluid production.

Normal amounts of amniotic fluid have been found in cases of fetal renal agenesis, proving that the amniotic fluid may be produced without the fetal kidneys (Jeffcoate and Scott, 1959, Shaw and Marriott, 1949). This suggests that the amnion epithelium has a very active role in the production of amniotic fluid either as an active secretor or transporter

of the fluid and its constituents (Needham, 1963). The amnion epithelium is permeable to many different ions and water, with facilitation of soluble lipid transfer being shown in Garby's study (1957). He used portions of stripped amnion epithelium in Tyrode's or Ringer's solution and radioactively labeled molecules.

From the above information we may assume that the amniotic fluid undergoes fairly rapid change, that the fetus participates in the absorption and secretion of this fluid and that the amnion epithelial cells probably play a major role in the production and absorption of the amniotic fluid and its constituents.

Possible secretory products of the human amnion cell. Previous studies of the amniotic fluid indicate that it has many constituents (Needham, 1963), but only substances of interest are referred to here. Relatively large amounts of nonspecific gamma globulin are found in the amniotic fluid in a form which differs from both the maternal and fetal globulins, suggesting that it may be a result of amnion secretion (Villee, 1960). Lambert and Pennington (1964) isolated oxygenated steroids including 20-hydroxy derivatives of 6-hydroxycortisol and 6-hydroxycortisone, assumed to be products of fetal urine, but which may result from hormone production within the placenta. Cholesterol is also present (Needham, 1963) and vernix

caseosa, which has a very high content of cholesterol (Keiffer, 1926) is readily seen floating in the amniotic fluid.

Cholesterol is present also in placental tissue, one third being free and the rest esterified (Watanabe, 1923).

Chorionic gonadotrophin was produced *in vitro* by cultures of minced, whole placental tissue, but evidence of estrogen production at the same time was inconclusive (Jones, *et al.*, 1943). The *in vitro* production of chorionic gonadotrophin does not prove that the hormone is present in the amniotic fluid, but does show that the placental tissues are capable of producing it.

Cholesterol is associated with the production of progesterone, testosterone and estradiol and is probably a precursor of these hormones in its esterified form (White, *et al.*, 1963). Ville (1960) noted that steroids are soluble in organic solvents; they orient themselves on an oil-water interface which causes birefringence in the polarizing microscope, and they also fluoresce. All these characteristics parallel those of the lipid droplets found in organs producing steroids, e.g. the corpus luteum and theca interna of the ovary, the Leydig cells of the testicle, and the cells of the zona fasciculata and zona glomerulosa of the adrenal gland (Deane, *et al.*, 1948). Using specific histochemical techniques, Dempsey and Wislocki (1944) were able to show that the lipid droplets

in the syncytial trophoblasts of the chorionic villi of the placenta probably contain "steroidal substances". Progesterone and some other steroids have been isolated from finely ground placentas analysed by paper chromatography (Salhanick, et al., 1952).

The foregoing information suggests that the placental tissues contain steroids and probably hormones, and that these molecules may be related to the lipid droplets which are seen throughout the placenta and amnion epithelium during the later stages of gestation. This discussion concerning the significance of the lipid droplets in the placenta is summarized by Wislocki and Padykula (1961) page 936:

> There is considerable justification for associating the formation of placental steroid hormones with the sudanophilic, birefringent, lipid droplets present in the syncytium, but there is no evidence that steroid hormones are liberated from cells, in the adrenal glands or elsewhere, in a visible sequence of liquifying granules and discharging vacuoles.

The lipid droplets or vacuoles in the human amnion epithelium show the same characteristics of sudanophilia, birefringence and fluorescence as the syncytial trophoblasts and therefore may contain similar constituents (Wilt, 1966a).

An hypothesis of human amniotic fluid absorption. Working from a purely morphological basis, Bourne and Lacy (1960) developed an hypothesis describing how the human amnion cell absorbs the amniotic fluid. They conjectured that the

amniotic fluid entered the cell at the base of the microvilli and via the intercellular canals at the lateral edges of the cell. Once in the cell, the absorbed fluid entered the many intracellular canals to the base of the cell, where it passed into basal vacuoles (protrusions of the basement membrane into the cell area) and intercellular vacuoles which contain some basement membrane material. From here the fluid passed down into the connective tissue layer through the network of collagen fibers.

This is the first detailed hypothesis describing an active absorptive role for the human amnion cell. It is relatively uncomplicated, but based on a morphological picture of the amnion cell which appears distorted when compared with more recent electron micrographs prepared using more modern preparative techniques.

In summary, there are four main observations that should be emphasised in this review. First, the morphology of human amnion cells has been well studied and it suggests an absorptive and secretory role for the cell, although not enough is known of the ultrastructure to say how these cells secrete or absorb. Second, the lipid granules or droplets in the human amnion cell cytoplasm are probably the vacuoles seen through the phase-contrast microscope, and these vacuoles may be a secretory product which appears to influence cell growth

in tissue culture. Third, the morphology of the amnion cell in tissue culture is little known. Finally, the research work concerning the amnion cell secretory products may be nearing the point of fruition, but much work is needed before we obtain adequate knowledge of the function of human amnion cells.

CHAPTER III

MATERIALS AND METHODS

CHAPTER III

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This chapter describes the preparations for the study of: (1) the cine-phase record of human amnion cells developing into tissue culture monolayers, and (2) the ultrastructure of human amnion cells in membrane biopsies and pellets of recently trypsinized cells. A preliminary study of the ultrastructure of primary human amnion cells taken from tissue culture monolayers is also included.

I. THE CINE-PHASE STUDY

Cell source and preparation

The human amnion membranes used for cell preparations were obtained from the labour floor of The Women's Pavilion, Winnipeg General Hospital, Winnipeg, Manitoba. Only placentas with healthy membranes were used; those which were stained with meconium, contaminated, or in contact with antiseptics were discarded.

Shortly after delivery the selected placenta was placed in a sterile, liter beaker containing 500 ml of cold Hank's balanced salt solution, and this was transported to the laboratory for processing usually within one hour. The solutions and techniques used to process these amniotic membranes for

tissue culture cells are described in a paper by Wilt et al. (1964). The details of these techniques and solutions used are in Appendix A, page 178.

Briefly, the amnion membrane was biopsied and graded, and if suitable, stripped from the placenta, washed and predigested in versene for ten minutes. The membrane was then placed in trypsin at 34 degrees centigrade (34°C), removed after one hour and placed in a propagating medium wash, while the cells were being removed from the trypsin solution by centrifugation. The membrane was removed from the wash, discarded, and the propagating medium used to resuspend the cells removed from the trypsin. The resuspended cells were classified according to type, diluted as necessary and usually dispensed into roller tubes.

Rose chamber techniques

The techniques of assembling and filling the Rose chamber were described in the original paper by Rose in 1954. Figure 1, page 31, describes the techniques used in this laboratory for assembling and filling of Rose chambers. Cleaning and sterilizing techniques are described in Appendix B, page 193. Modifications of the original Rose chambers made for this study included; stainless steel outer covers and silicone gaskets lightly coated with silicone grease.

Rose chambers used for the cine-phase study contained

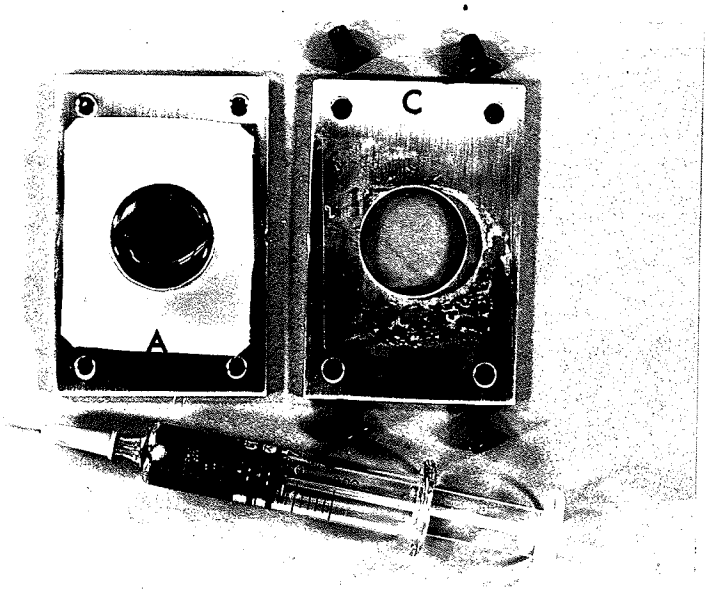

Figure 1a. A dismantled Rose chamber. The silicone gasket (A) is normally covered on both sides by the glass coverslips held firmly in place by the stainless steel plates, (C), which are held together with the screws shown. This recently dismantled chamber shows some medium on the lower, carbonated coverslip in preparation for selecting specific monolayer cells for ultrastructure study.

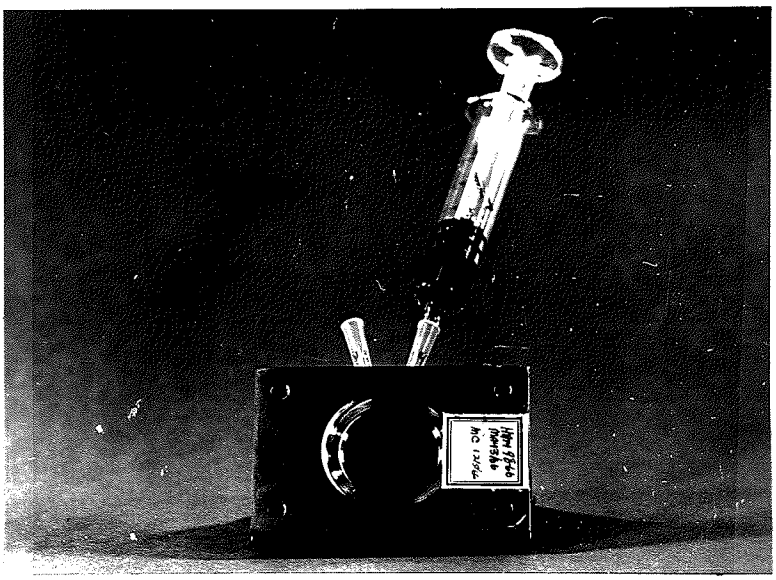

Figure 1b. A labeled and assembled Rose chamber with two 25 gauge disposable needles inserted into the chamber. The left hand needle serves as an air exhaust and the other is used to inject the cell suspension from the syringe.

about 1.5 ml of trypsinized cells suspended in propagating medium to a concentration of 5×10^4 cells per ml. The Rose chamber was then strapped on the stage of the inverted microscope used for the cine phase study. The suspended cells within one hour fell to the lower coverslip of the chamber, and the photographing of the chosen cells was begun immediately. For the Rose chambers to be used later for ultrastructure studies, the lower coverslip was coated with a thin layer of carbon and then sterilized by dry heat before use. The details of technique and the apparatus used for this are described in Appendix B, page 192.

During the time the cells were being photographed in the Rose chamber the pH would often drop to below pH 7, which was considered harmful to the cells. A perfusion apparatus was devised to change the medium in the Rose chamber when the pH dropped to 6.8, as read visually comparing the color of the phenol red indicator with the LaMotte Chemical Standards, depicted in Figure 2, page 33. The perfusion apparatus consisted of a five milliliter disposable, glass syringe, polyethylene, non-toxic tubing with Luer adapters, four 25 gauge needles, and a universal bottle, as can be seen in Figure 3, page 34. When the medium required a change, the perfusion apparatus was attached to the Rose chamber, as seen in Figure 3, page 34, and five milliliters of fresh

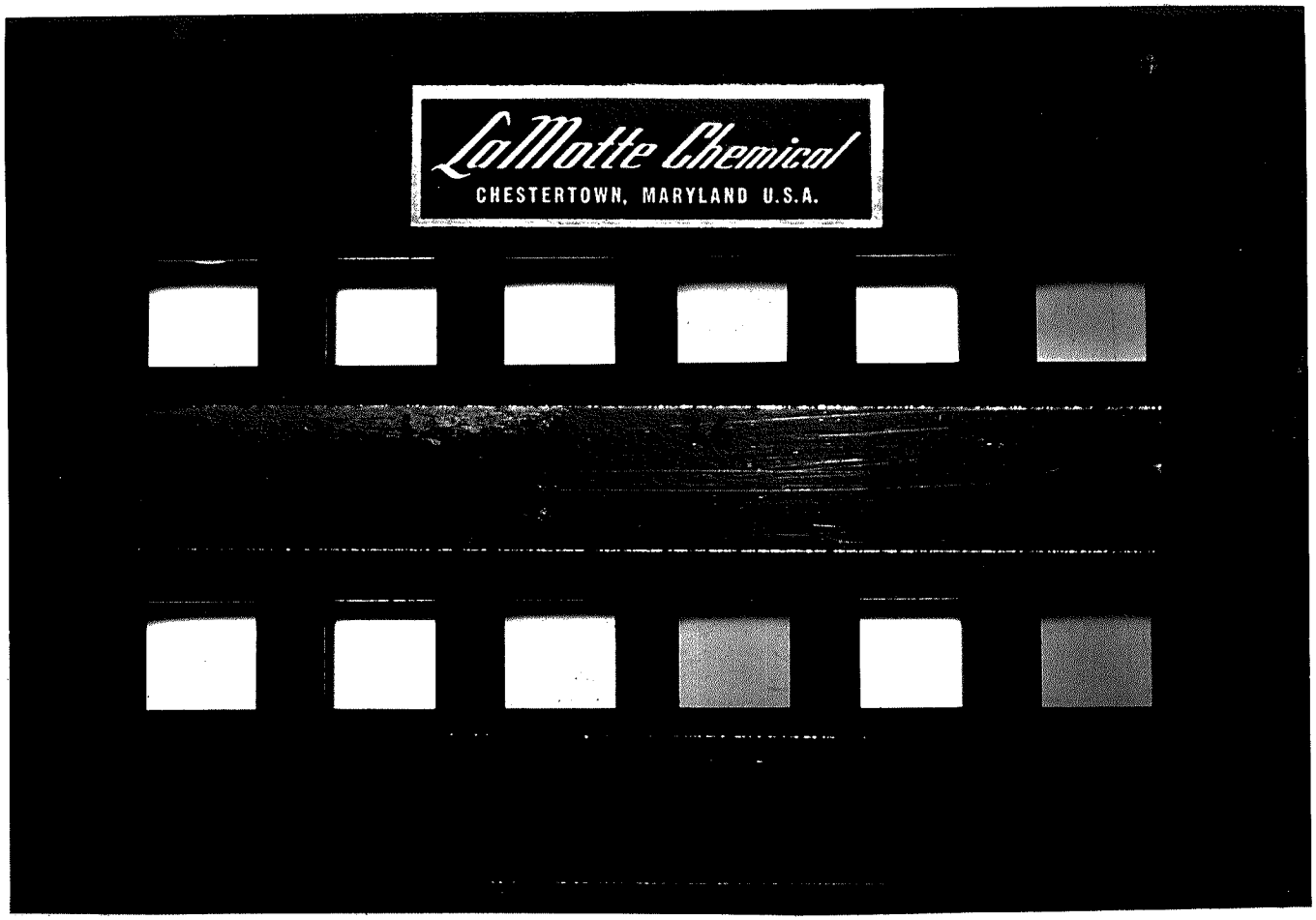

Figure 2. The LaMotte Chemical pH standard. The optimal pH for amnion cells is in the range of 7.0 to 7.2. The medium in Rose chambers was usually adjusted upward if it reached pH 6.8

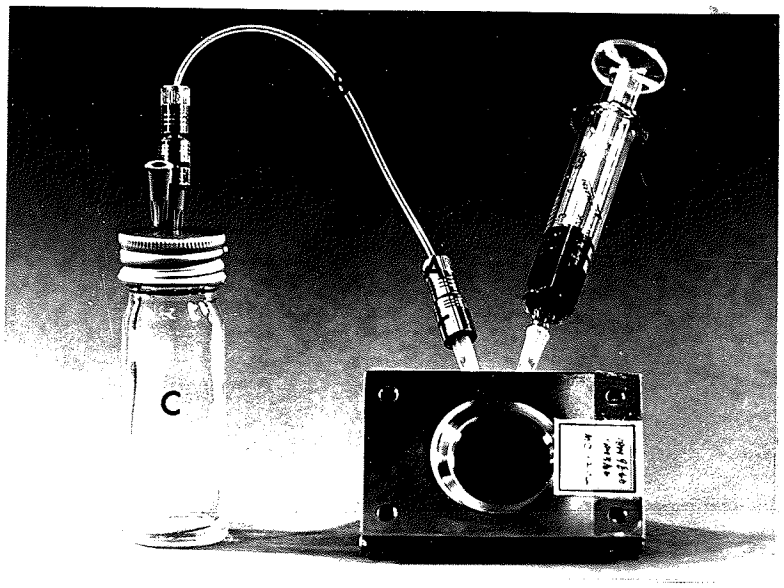

Figure 3a. The sterile perfusion apparatus. This is similar to Figure 1b, except that the exhaust needle is attached to a Luer adapter (A) and polyethylene tube (B) to allow the excess to run off into the universal bottle (C) which it is attached to by another Luer adapter and needle. The other needle inserted into the bottle top is an air exhaust.

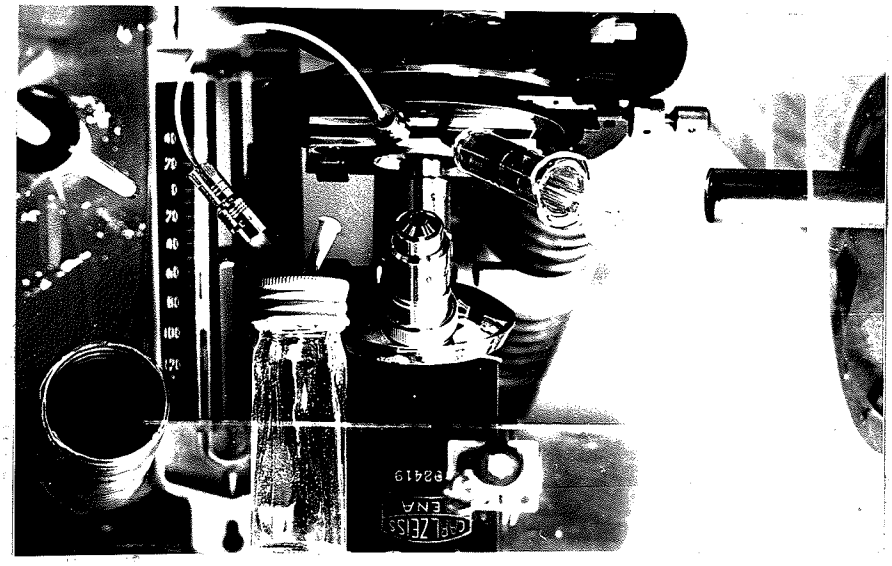

Figure 3b. The sterile perfusion apparatus as seen in use in the cine phase apparatus.

medium were mixed with and partially replaced the medium in the chamber to bring the pH up to 7.2. A medium change was required about once every five days in chambers with actively developing monolayers. Once the medium change was complete, the perfusion apparatus was removed immediately to reduce vibration.

Construction of the cine-phase apparatus

A cine phase apparatus was designed to observe amnion cells developing into monolayers on the bottom coverslip of the Rose chamber. The design was complicated by the fact that the microscope had to be inverted to observe these cells at all stages of development and yet use conventional equipment where possible. A photograph of the completed unit may be seen in Figure 4, page 36. Details of the equipment may be found in Appendix B, page 185.

The microscope. An older type Carl Zeiss Jena microscope was converted to phase-contrast optics using a Zeiss IS condenser and neofluar objective lenses. The microscope was inverted and a clamp was attached to the arm of the fine focus, which was ineffective in the inverted position. The clamp allowed the coarse focus to be clamped in position, once in focus (Figure 5, page 37). The microscope was attached to a plywood board by three bolts and the board was inverted

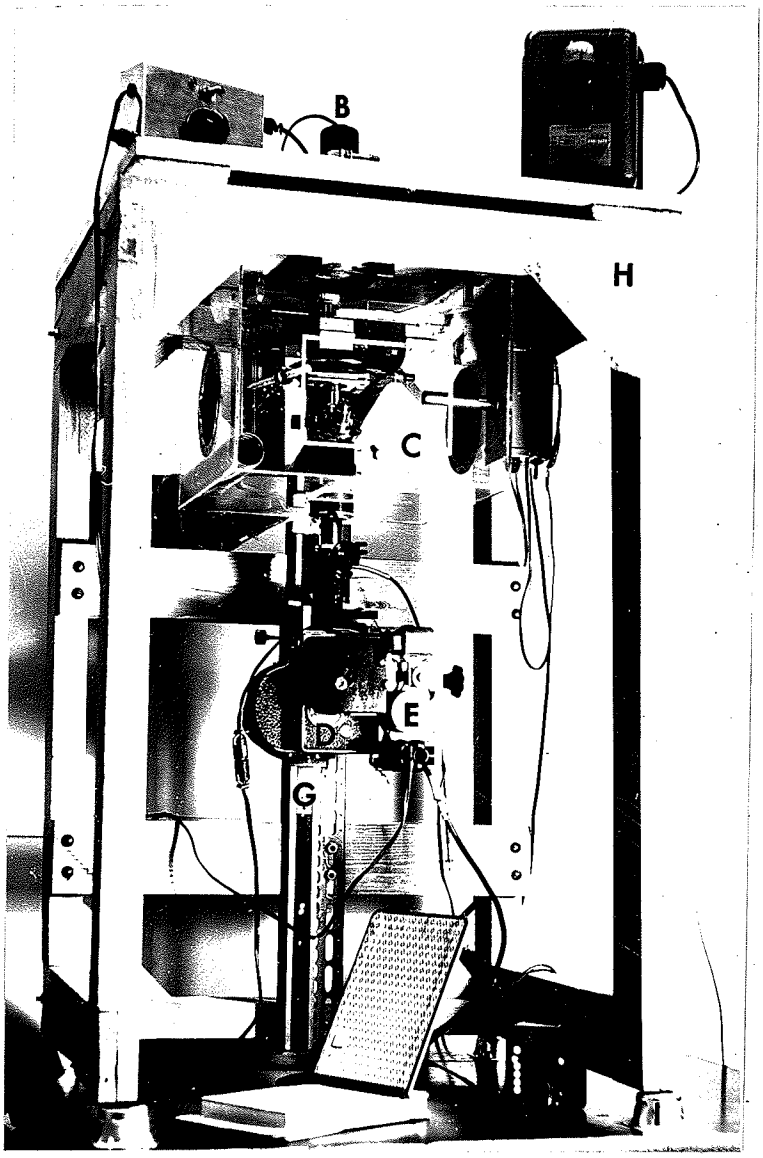

Figure 4. The cine-phase apparatus. This is a picture of the inverted microscope (A), the light system which includes light, transformer and relay unit (B), the incubator (C) with heating unit (D), camera, motor and timer (E), periscope (F), supporting slide rail (G), stand (H), and Lord mounts (I).

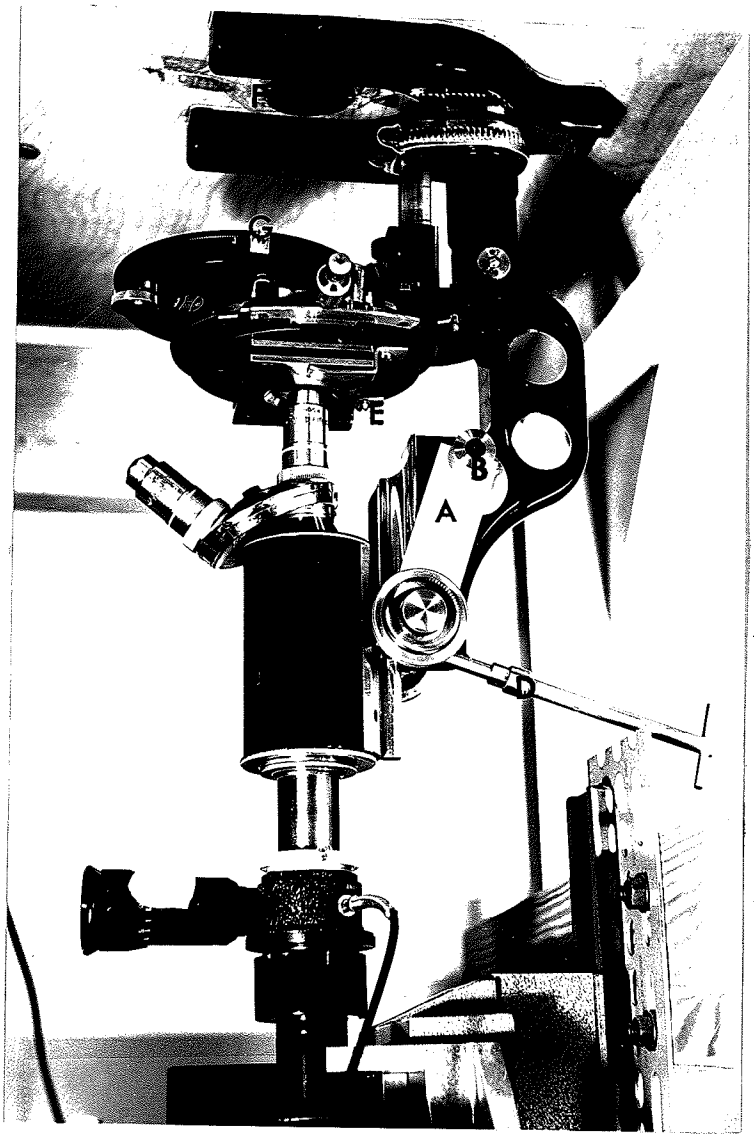

Figure 5. The microscope coarse focus clamp (A) is attached to the fine focus arm (B) and is adjusted by a wrench (D). The clamps (E) on the microscope stage hold the Rose chamber firmly in place. The base board to which the microscope is bolted contains a hole (F) through which the rays from the light source reach the condenser (G). This hole is covered with a glass coverslip (H) to reduce heat loss from the incubator and dust contamination.

and rested on the upper edges of the main stand, thus suspending the microscope in an inverted position. A hole 1.75 inches in diameter was drilled in this base board and a Leitz Wetzlar, six volt, tungsten, coil filament lamp was mounted over the hole and clamped in direct line with the microscope's optical system (Figure 5).

Initially it was found that light bulbs burned out within 48 hours because of the constant off and on action. A relay system was designed[1] to turn the bulb current to very low instead of off, yet allowed the bulb to achieve full power rapidly during full illumination; thus prolonging bulb life to two months. Details of this device may be seen in Appendix B, Figure 66, page 187.

A prismatic Leitz Wetzlar periscope viewer, with a 10 X ocular and a camera adapter, was attached to the tube of the microscope. This viewer was equipped with a prism-removing mechanism and cable so that all the available light was directed toward the film emulsion when the periscope was not in use. The camera adapter was designed to shield the film emulsion from outside light without touching the microscope, so that there would be no transmitted vibration from

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[1]The light relay mechanism was designed and constructed by Mr. G. McLaren, Electronics Technician, Department of Bacteriology and Immunology, Manitoba Medical College, Winnipeg, Manitoba.

the camera to the microscope.

Since the calibrated stage was inverted with the microscope, two clamps were screwed onto the stage to hold the Rose chamber firmly in place. These clamps were loosened and tightened as needed when the Rose chambers were changed (Figure 5, page 37).

The incubator. The microscope was encased in an incubator clamped onto the base board and made of thin perspex with side arm holes and a front hinged door. The exact measurements of the incubator and details of its components are in Appendix B. A hole for the heat input tube was made in the lower left corner and another in the right upper corner for the air exhaust tube. A Thermoregulator thermostat was installed on the right side of the incubator with the probe at the same level as the microscope stage and one inch to the side. This thermostat was set for 37°C and coupled with the fan and heating element found in the heat input tube. When the temperature dropped below 36.5°C, the heater was turned on with the fan which sucked air out of the incubator and then forced it through the heating element back into the incubator. The fan which was fixed to the wall, connected to the input and output tubes by loosely folded polyethylene, thus reducing the transmission of vibration from the fan to the incubator, The incubator was equipped with a

maximum-minimum thermometer to check temperature fluctuation, which was normally about 0.5 degrees centigrade. Figures 6 and 7, page 41, show most of these features.

The camera and timer. A Bolex H 16 M, 16 mm camera, an electric motor drive with variable transformer and a lens adapter to fit the periscope made up the camera unit. The camera was bolted to one of two adapter clamps which slid up and down on a rail from a Leitz Aristophot stand. The rail was bolted to an angle iron secured to the two cross braces on the back portion of the main stand.

A Stevens electric, 110 volt, constant-motor drive timer was used to turn on the light and activate the camera mechanism at the desired intervals. The usual interval between pictures was one minute, although 30 second intervals were used occasionally. Figure 8, page 42, illustrates how this unit was assembled.

Kodak, plus-X negative film was used throughout and was developed and printed by a local firm noted in Appendix B.

The stand. Dried spruce wood, two by four inches, was used to construct the stand which supported the microscope, incubator, camera, timer and lighting systems. The corners of the stand were fitted, glued, and bolted with carriage bolts to make a very firm frame, as seen in Figure 4,

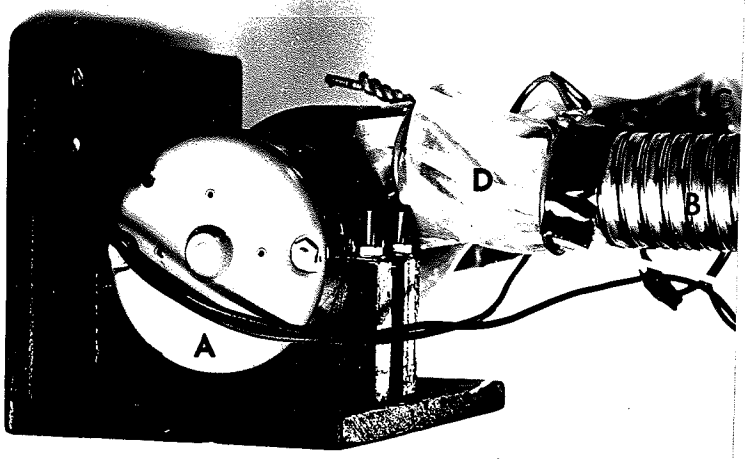

Figure 6. The heating unit, with the fan (A), attached to the wall, the metal tube containing the heating element (B), the flexible fiberglass air outlet tube (C), and the polyethylene sheeting used to connect the fan and the tubes (D).

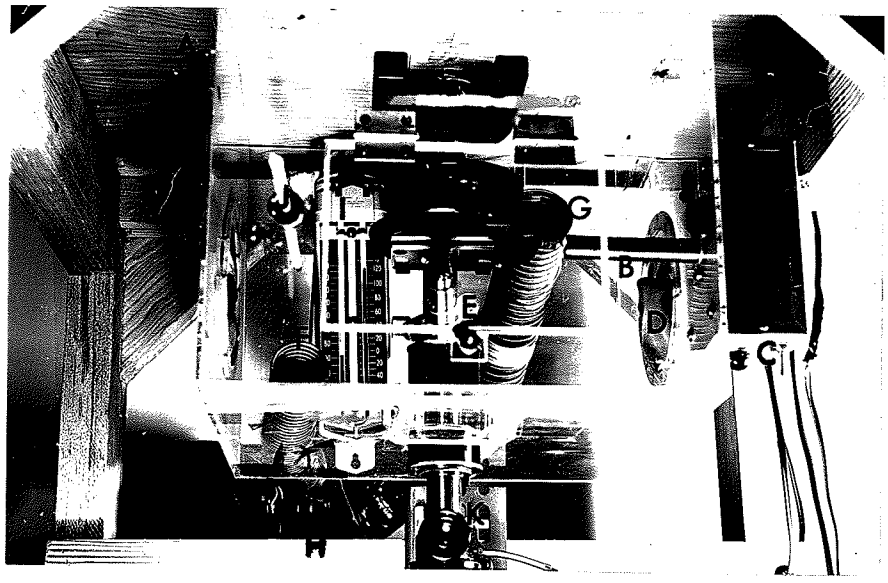

Figure 7. The incubator with the base board securing clamps (A), thermoregulator (B), and its switch and indicator light (C), side arm holes covered with latex sheet rubber (D), front door (E), heat inlet (F), air outlet (G), fan (H), maximum-minimum thermometer (I), and a centigrade thermometer at the Rose chamber level (J). The periscope may be seen as well (K).

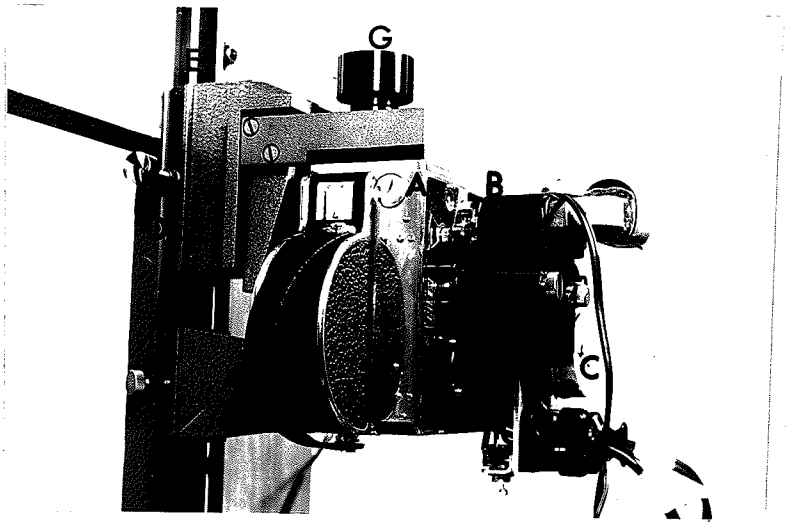

Figure 8a. The camera (A), motor (B), timer (C), unit supported by an angle iron (D), to which the sliding rail (E) is bolted on which the camera clamps (F) slide. The camera-periscope adapter can be seen (G).

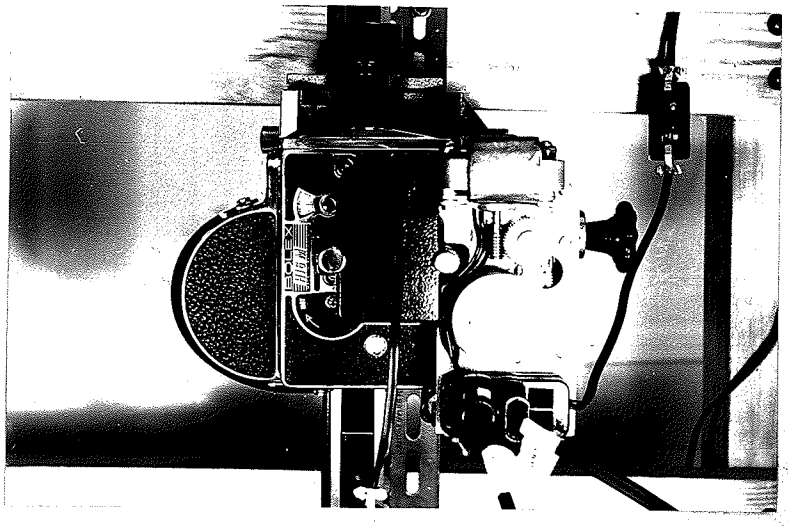

Figure 8b. The same unit seen from the side.

page 36. The plywood board holding the light, microscope and incubator was separated from the stand by four one inch thick fiberglass pads, which rested on the four upper corners of the stand. This precaution was necessary to reduce the vibrations of the camera motor which would otherwise be transmitted to the microscope.

Design of the cine-phase study

The advantages of using cine-phase techniques in morphology are that they produce a permanent record on 16 mm film and that the slow movements of living cells can be recorded at one minute intervals and then speeded up in the projection of the film to show the activity and interaction of cells. Using these advantages, the activities of amnion cells were studied in two situations: (1) while developing into monolayers, and (2) while in established monolayers. Special attention was paid to the behavior of amnion cells in groups and that of individual cells and their contents while growing in vitro. A total of 17 amnions were observed, seven were mainly type "1" cells, six were mostly type "2" and four were of predominantly type "3" cells. A total of five different amnions were photographed for 67 hours or less, eight were photographed from 67 hours to 9.5 days, and four were photographed from ten to 40 days. Appendix B, page 194. contains detailed information concerning these amnions and

their cell types are summarized in table IV.

In an attempt to test the validity of the implications of the cell types defined by Wilt and Brambilla (1966), over 100 amnion cells were observed to determine how each of the different cell types developed as the monolayer formed. This was done by following each of the many cells of a microscope field, using time-lapse photography, until the monolayer was relatively complete. The three amnions used for this particular study were of predominantly types "1" and "2" cells, and selected because only these three developed satisfactory monolayers in Rose chambers.

There are no techniques for proving cell death in a Rose chamber, without adding undesirable variables to the experiment. For this reason cells which did not enter into monolayer formation, that is "unhealthy" or "dying" cells, were considered those which showed no ameboid movement, produced no processes, and had no fluctuating membranes. Unhealthy cells also had at least two of the following three properties: (1) the inability to stick firmly, or at all, to the glass coverslip, (2) a spherical shape with an irregular surface, and (3) a very refractile appearance as seen through the phase-contrast microscope. On the other hand, healthy cells stuck to the coverslip readily, were not refractile, showed ameboid movement, produced processes and a fluctuating membrane,

and participated in monolayer formation within seven days.

Abstracts of cell action reported in this thesis were made using photomicrographs reproduced from the original cine-phase 16 mm film. These abstracts are composed of photomicrographs placed in sequence and labeled according to the time interval in an effort to describe the cell activity. This method is incomplete in its portrayal of the movement of cells and their contents. For this reason a 16 mm film depicting all the sequences described in chapter IV is included in Appendix F, page 204.

II. THE ULTRASTRUCTURE STUDY

Specimen source. Human amnion cells were obtained from the source described in the early part of this chapter. After inspection of the test biopsy, and just before stripping the amnion from the placenta, 2 centimeter (cm) biopsies from each of the placental and reflected membranes were taken and placed immediately in bijou bottles. Veronal buffered osmic acid was added as a fixative and left for at least 1.5 hours.

Trypsinized cells were obtained from the cells suspended in the propagating medium used as the post-trypsinization wash. A volume of six to ten milliliters was removed before the cells were diluted for dispensing, and was centri-

fuged at 1000 rpm for ten minutes to form a pellet of about 0.5 ml of packed cells.

Specimens for the preliminary study of the monolayer cell morphology were cells grown in Rose chambers for at least one week. These cells were grown on thin layer carbon-coated coverslips to permit easy removal after embedding.

Specimen preparation for ultrastructure study

Biopsy preparation. All amnion biopsies were prepared for ultrastructure study using vestopal embedding techniques (Pease, 1964). The detail of this technique is found in Appendix C, page 196, but an outline is given below. The biopsy was fixed in veronal osmic acid, washed in deionized water, placed in uranyl acetate to post-stain, dehydrated in alcohols, prepared for embedding by the use of styrene, and vestopal and finally embedded in activated vestopal and polymerized in an oven for 48 hours. Following this the blocks were trimmed and sections were cut at about 90 millimicrons (Mμ) thick on a Porter-Blum Ultramicrotome, floated onto 200 mesh copper grids stained and viewed through the Philips EM 100 electron microscope.

Pellet preparation. Those amnion cells used for the post-trypsinization ultrastructure study were prepared using the agar and vestopal embedding technique (Pease, 1964). The

details of this technique are found in Appendix C. In general terms, the pellet of cells was fixed in veronal osmic acid, washed in deionized water, post-fixed in uranyl acetate, dehydrated in alcohols, coated with agar at 45°C, chopped into minute pieces, further dehydrated in alcohol, prepared for embedding styrene, then vestopal, embedded in activated vestopal and finally polymerized at 65°C for 48 hours. Grids were prepared as described above.

Monolayer cell preparation. After dismantling the Rose chamber, a 2.5 ml disposable syringe with a 26 gauge by 0.5 inch disposable needle was used to remove the medium covering the lower, carbon-covered coverslip. A thin layer of medium was left on the coverslip to avoid premature dehydration, and as this evaporated it was replenished by the medium in the syringe (Figure 1a, page 31). Using a standard Reichert phase contrast microscope and an inverted Leitz microscope, cells were selected and isolated from their neighbors by scratching a circle around them through the carbon layer, using the needle on the syringe. The selected cells were photographed in a phase contrast microscope (Figure 9, page 48), and the coverslip with the chosen cells was immersed in 5.5 per cent glutaraldehyde in isotonic Tyrode's solution following the technique of Robbins and Gonatas (1964). This technique is described in detail in Appendix C, page 198. In general,

Figure 9. An example of the phase-contrast appearance of monolayer cells prepared for processing for the ultrastructure study. The cells of interest are marked "A", and the isolated island of carbon is labeled "B". Magnification, 1000x, amnion number, 67-66.

after being fixed in glutaraldehyde, the cells were post-fixed in osmium tetroxide, dehydrated in alcohols, prepared for embedding by placing in vestopal and finally embedded in activated vestopal with the open end of the capsule containing the vestopal inverted over the selected cells. The capsule was held in place by a specially designed capsule clamping apparatus and polymerized for 72 hours. The capsule was then snapped off the coverslip, trimmed and sectioned and prepared for viewing in the usual manner.

The capsule clamping apparatus for holding the embedding capsule over the selected area was designed in this laboratory[2]. The clamping apparatus was made of two sheets of one-eighth inch thick perspex, 2.5 by 2.0 inches, and four number 6-32, 1.5 inches long flat head machine screws which, together with four knurled nuts held the "sandwich" together firmly yet allowed easy separation by hand (see Figure 10, page 50). The capsules were held in place by perspex blocks, 0.5 by 0.5 by 0.75 inches with a hole 0.375 inch in diameter drilled 0.25 inch deep in one end to accommodate the capsule so that it protruded 0.25 inch from the block. This block allowed easy positioning of the capsule and gave it good stability.

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[2]The author would like to acknowledge the assistance of Mr. Walter Jones, Machinist, Manitoba Medical College, Winnipeg, Manitoba, in the design and construction of this apparatus.

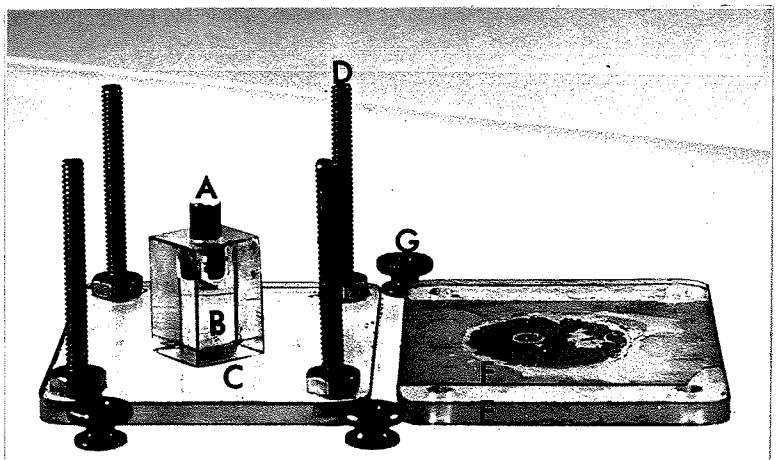


Figure 10a. The capsule clamping apparatus, dismantled. The gelatin capsule (A) and supporting perspex block (B) are resting on the base plate (C) which contains the four flathead machine screws (D). The top plate (E) supports the coverslip (F) and the knurled nuts (G) are placed at each end of the clamp.

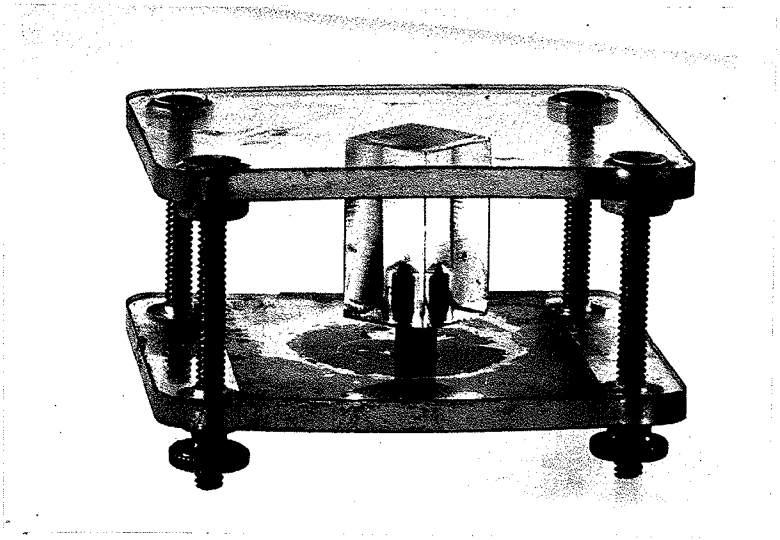


Figure 10b. The capsule clamping apparatus, assembled.

Before using the apparatus it was necessary to coat the entire device with silicone grease to reduce the amount of sticking and marking of the plastic caused by the activated vestopal or acetone.

Staining of the microsections

All the microsections used for this ultrastructure study were stained by the Karnovsky lead stain technique as described by Pease (1964). The details of this procedure are in Appendix C, page 199.

Design of the ultrastructure study

The experimental design of the ultrastructure study was to observe vacuolated and nonvacuolated human amnion cells in membrane biopsies, and a centrifuged pellet of cells that had just been trypsinized. A total of 15 amnions were studied, and where possible, biopsies from the placental and reflected portions of the amnion were obtained, as well as a pellet of the same amnion. Table I, page 52, shows how many amnions of each cell type were studied and the number of various specimens from these amnions. A further breakdown of information about the amnions used for this study may be seen in Appendix C, table VI, page 200.

The preliminary study of human amnion cells as they grow in tissue culture was designed to correlate the phase

TABLE I

THE NUMBER OF AMNIONS AND SPECIMENS OF EACH CELL TYPE IN THE ULTRASTRUCTURE STUDY OF THE HUMAN AMNION CELL

Predominant Cell Type	Number of Amnions Studied	Portions Studied		
		Placental Biopsy	Reflected Biopsy	Pellet
1	7	6	5	6
2	2	2	2	2
3	5	5	4	5
4	1	1	1	1

contrast picture with the ultrastructure appearance. To date only a few monolayer cells have actually been observed through the electron microscope. Cells chosen for observation included those in monolayer, containing vacuoles and intercellular bridges.

Photographic techniques

All techniques for the processing of photographic films and the materials used in the ultrastructure study are included in Appendix D, page 201.

Histochemical stains

Sections and smears of amnion cells, and some microsections that were cut for the electron microscope were stained with Sudan IV. The details of this staining technique are included in Appendix E, page 203.

CHAPTER IV

RESULTS

CHAPTER IV

RESULTS

Introduction

This chapter describes firstly the appearance of amnion cells in tissue culture as observed by cine-phase, and secondly the fine structure of amnion cells as revealed by the electron microscopic studies of biopsies, pellets of trypsinized cells, and cells in tissue culture monolayers.

Generally, morphological studies of this kind involve descriptions of many often unrelated observations, adding to the sum total of information about the described cells, but producing a somewhat disjointed story. In an effort to develop some continuity, the cine-phase study has been divided into three main sections, they are:

(1) The studies of cell suspensions developing monolayers, which includes observations on the ability of cells of various types to develop monolayers, and a description of the changes which occur as suspensions of cells develop into monolayers, as well as those which did not.

(2) The morphology of the cells once developed into monolayers, including a general description of the living cell and its various parts, such as the membranes, nucleus, mitochondria and cytoplasm, and some of their activities, as well

as a description of apparent degeneration of monolayer cells.

(3) A final section devoted to the cine-phase findings concerning cytoplasmic vacuoles, their size, shape and movements within the cell.

The observations made on the ultrastructure of human amnion cells were divided into three sections and they are:

(1) The largest section which concerns the ultrastructure of cells in biopsies of human amnions, involving detailed descriptions of size and shape, peripheral appendages of the cell membranes, nucleus, cytoplasm and its various constituents, with emphasis placed upon cytoplasmic lipid vacuoles.

(2) Observations made on pellets of trypsinized cells, comparing them with the previous results.

(3) A preliminary report of the initial observations on cells obtained from monolayers grown in Rose chambers.

I. CINE-PHASE

Cell suspensions to monolayers

The system of typing human amnion cells developed by Wilt and Brambilla (1966), is based on the belief that types "1" and "2" grow in tissue culture better than types "3" to "6". This belief can be confirmed by observing the development of monolayers from suspensions of amnion cells, using cine-phase techniques.

A total of seven cell suspensions, each from a different amnion, were photographed (Appendix A). Only those amnion cell suspensions which developed monolayers could be used to determine which type of cells grew best, so that the observations are limited to three amnions. The development of one of these monolayers in Rose chamber number 133-66, is recorded photographically in Figure 11, page 58. (An example of amnion cells which did not develop into a monolayer may be seen in Figure 16, page 68.) Figure 11 shows the amnion cell suspension at low power initially, and then follows the development of the monolayer at a higher magnification. Each cell was carefully observed as it developed from a suspension to monolayer cell, except those which migrated from the microscopic field. Those cells which did not develop into a monolayer were considered unhealthy or dying. The results of the analysis of the three amnions studied are summarized in table II, page 59. On the average type "1" cells grew into monolayers 92 per cent of the time, type "2" cells grew 83 per cent of the time, while only 38 per cent of type "3", and none of types "4" and "5" grew into a monolayer. Types "3" to "5" were poorly represented in this study as any cell suspensions with high proportions of these cells would not develop monolayers in a Rose chamber. Measurements of all the cells recorded in table 3 were made. The average diameters of these cells in microns (μ) are; type "1", 14.4μ , type "2",

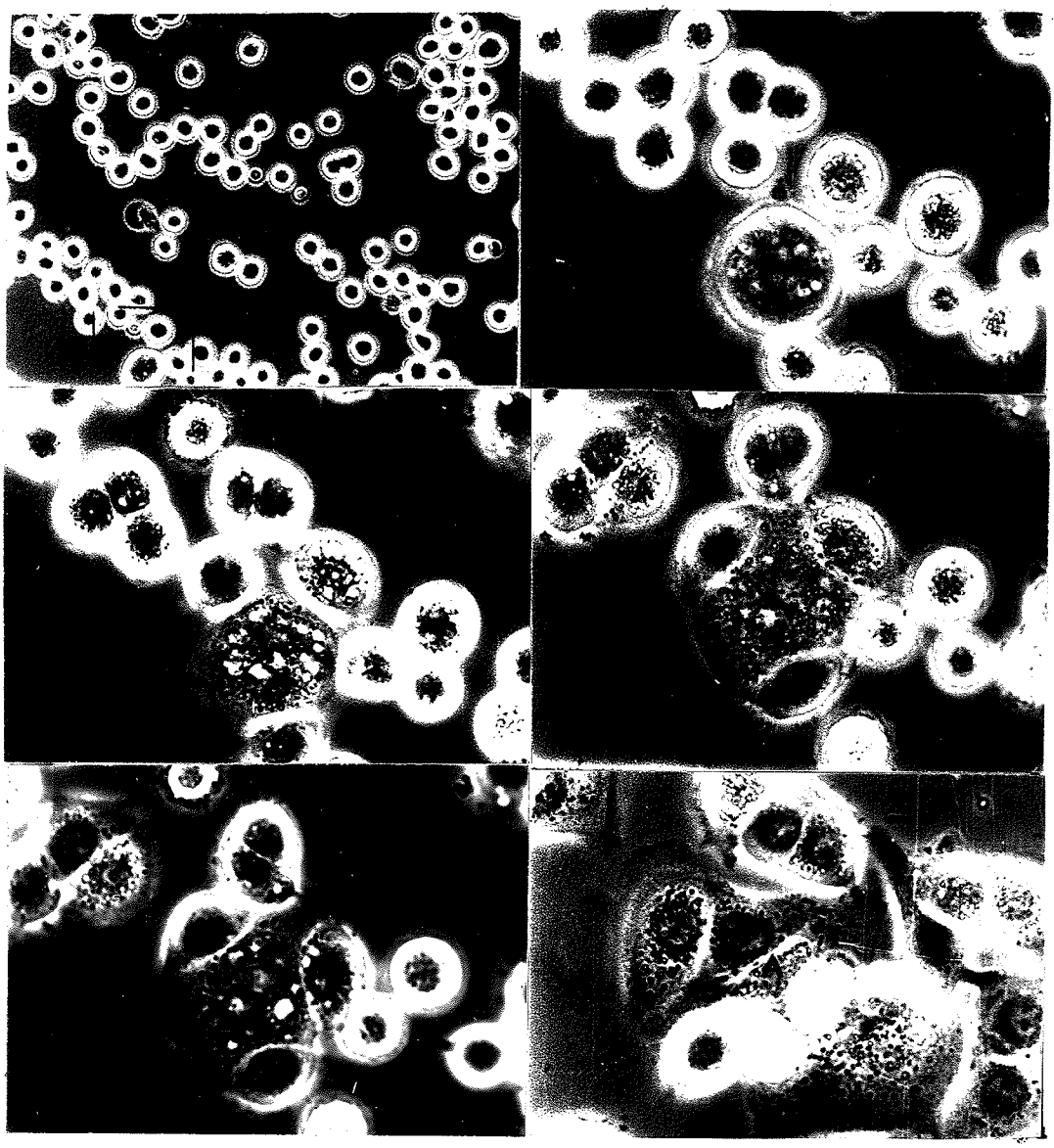


Figure 11a-f. Monolayer development. Amnion 133-66, magnification, (a) 250x, (b)-(f), 630x. The box in photomicrograph (a) denotes the area seen in (b)-(f). The cells developed into a complete monolayer by 124 hours. Cell "A" became very vacuolated in a 16 hour period (b)-(e), yet remained an active part of the monolayer.

TABLE II

THE PER CENT OF THE VARIOUS TYPES OF AMNION CELLS WHICH DEVELOPED MONOLAYERS SUCCESSFULLY IN ROSE CHAMBERS

Rose chamber number	Per cent growth of cell type				
	1	2	3	4	5
231	93 (14/16)*	73 (22/28)	57 (3/7)	(0/0)	(0/0)
269	92 (12/12)	89 (8/9)	(0/0)	0 (0/2)	0 (0/1)
133-66	90 (9/10)	100 (3/3)	0 (0/1)	(0/0)	(0/0)
Average per cent growth	92	83	38	0	0

* The figures in parenthesis represent the number of cells which grew into monolayers, over the total number of cells of that type observed in the microscopic field.

15μ , type "3", 16μ , type "4", 16μ , type "5", 10 by 20 μ (in the only cell measured).

The morphology of monolayer development. As soon as the suspended cells had fallen through the medium to the bottom coverslip of the recently seeded Rose chamber, the cine-phase apparatus was used to photograph the chosen field. Some settling of the cells occurred during the first ten hours, requiring an adjustment of the focus.

The first type of movement seen in the Rose chambers was a rather irregular, apparently random, Brownian-type movement. The next remarkable activity was the gradual clumping of cells into groups of two or more (Figure 11b, page 58). Within 24 hours the sharply defined borders of the spherical cells became less distinct and appeared to have melted into the cell periphery for a distance of about five microns (Figure 11c, page 58). The fluctuation of this membrane was seen to be very active in the cine-phase film, even when the picture interval was decreased from one minute to 30 seconds. Some cells developed processes, much like fibroblasts, at one or two poles and used them to make contact with cells up to 30 μ away. Often the processes seemed to take part in a gliding movement toward neighboring cells (Figure 11e, page 58). These two activities resulted in the union of cells to form a continuous sheet or monolayer (Figure 18, page 72) within 87

hours. In the early stages when the cell was spherical, the nuclei and cytoplasmic granules could not be distinguished. Within 24 hours, vacuoles and granules could be seen readily, and by 30 hours nuclei with their nucleoli could be seen easily. As the monolayer development reached completion, the intracellular contents became more easily seen and remained this way throughout the life of the cell.

Cells from two of the amnions studied took longer to develop into a monolayer, and these cells illustrated the variety of morphological shapes that the amnion cell is able to assume. Figure 12, page 62, shows the variety of forms two ameboid cells assume over a 39.7 hour period. Cell "A" contains a nucleus, nucleolus, few vacuoles and many granules, and similar to a fibroblast, has two long processes, one measuring 80μ long. Cell "B" has similar contents to cell "A", but is smaller initially and pinocytotic vacuoles may be seen in its fluctuating membrane (Figure 12c, page 62). This cell was able to move at a rate of 20 μ per hour, and illustrates very well the tendency of amnion cells to change from spherical to ameboid to fibroblastic forms.

Another amnion, Figure 13, page 63, contained cells which assumed fibroblast-like shapes and a parallel alignment. In this sequence of photomicrographs, phagocytosis by one of the cells occurred as it appeared to ingest material produced

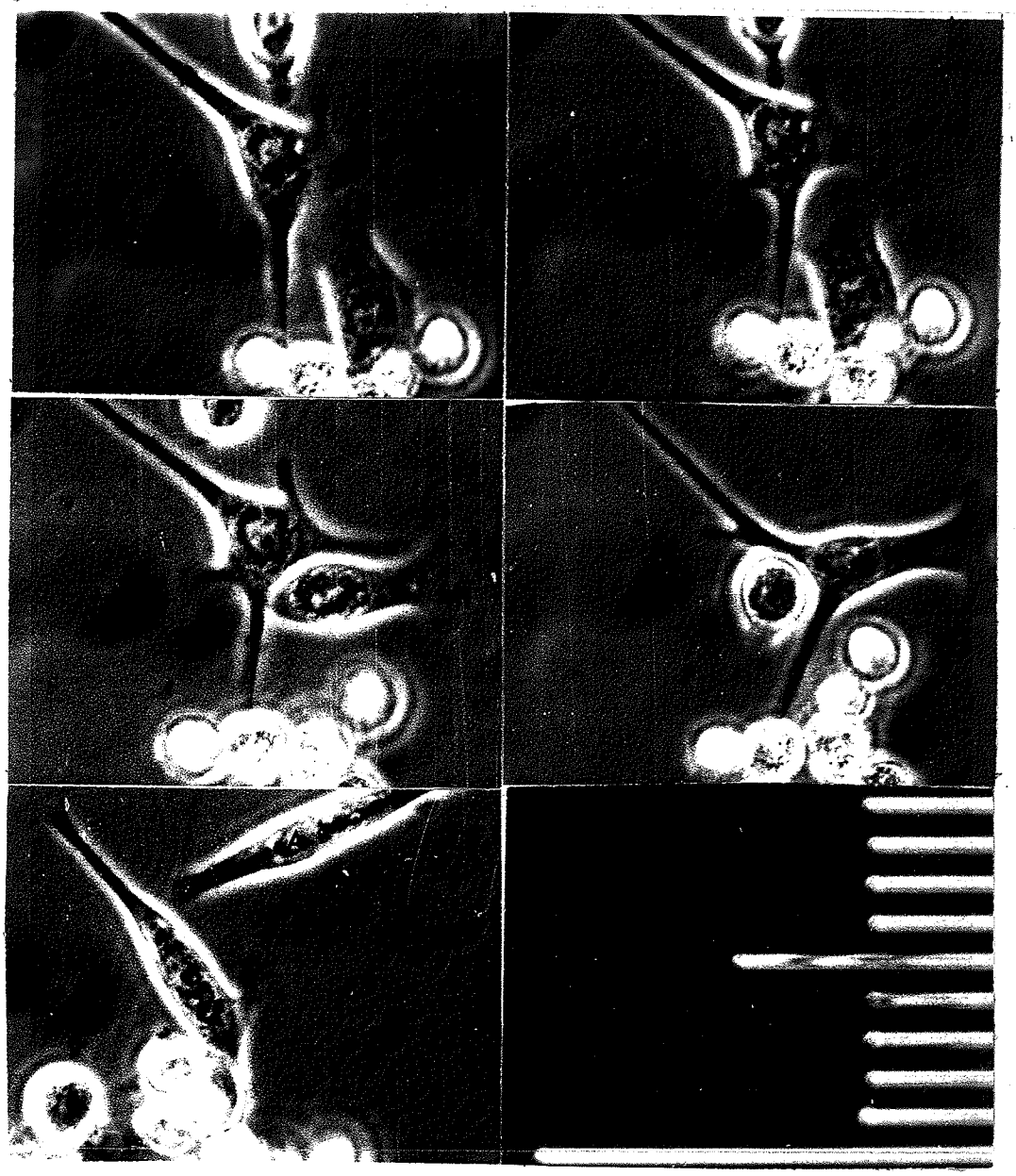


Figure 12a-f. Ameboid movement and pinocytosis. Amnion 225, magnification 545x. These photomicrographs illustrate the ameboid shape and movement present in the early stages of human amnion monolayer development. In (c), cell "B" contains pinocytotic vacuoles (pv) in its fluctuating membrane (fm). Cell "A" has the long processes (p) which typify the ameboid-type cell. The smallest unit of the stage micrometer (f) is 0.01 mm.

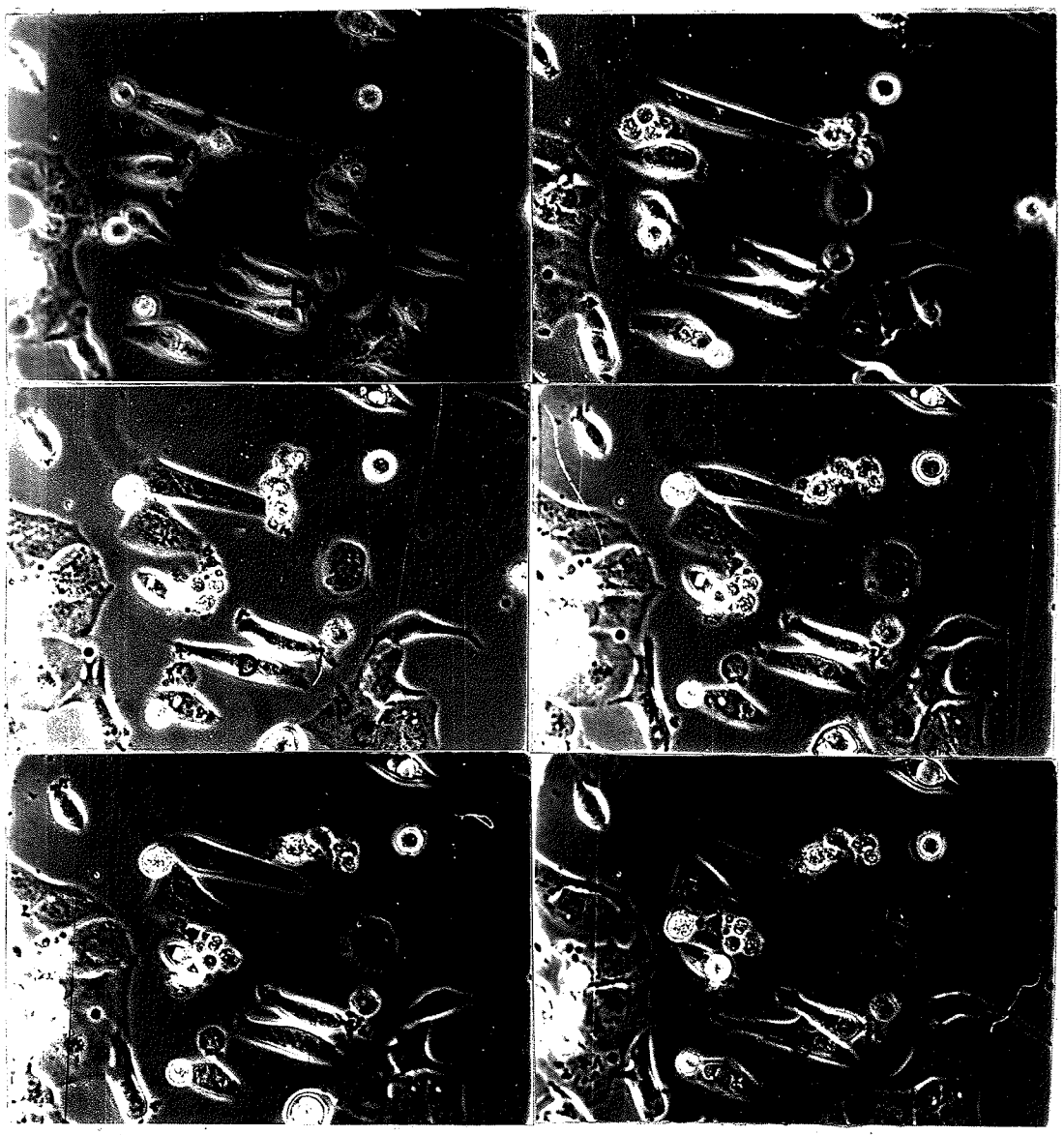


Figure 13a-f. Fibroblast-like human amnion cells, illustrating parallel alignment, phagocytosis and the later development of epithelioid cells. Amnion 5-66, magnification, 300x. In (c) the ingested particle (ip) is seen developing at the edge of cell "C"; in (d) the particle is being ingested by cell "D". Cells "A" and "B" are fibroblast-like in (a), but have become epithelioid in (f).

by a neighboring cell.

Those cells which did not develop into monolayers showed characteristic activity as well. The early movement of the spherical cells was the same as in any fresh Rose chamber suspension. Occasionally a pseudopod developed, but within 12 hours those cells which would not form monolayers, produced long thin, pointed, microextensions or macrospikes, up to 78 μ in length and two microns wide. The term macrospike, used to describe these very long microextensions, was decided upon because they are over four times larger than any of the microspikes previously described (Weiss, 1962, and Taylor and Robbins, 1963). Weiss defined a microspike as a microextension with a rigid core. These microextensions took up to 40 hours to develop, but only one hour to retract into the cell. Figures 14 and 15, pages 65 and 66, illustrate the long narrow appearance of the macrospikes in unhealthy human amnion cells in vitro, and the rapidity with which they retract. These macrospikes either touched and adhered to other cells, or moved from side to side in a pendulum-like motion before retracting. Figure 15, page 66, also shows that the process is able to go over the top of the neighbouring cell, or possibly pass right through it to reach another point. This type of activity often lasted until the fifth day after trypsinization, when most of the macrospikes had retracted back into the cell.

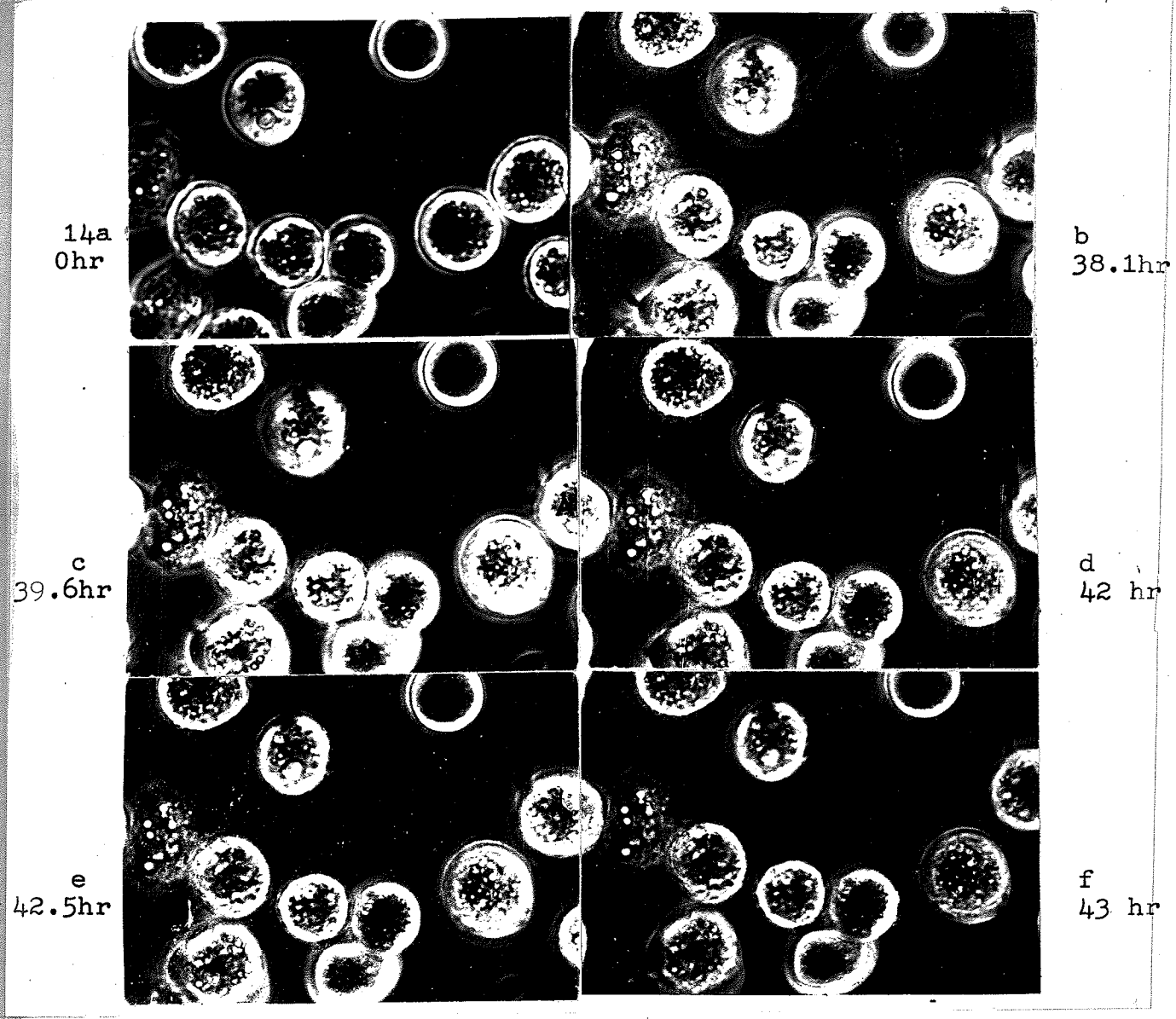


Figure 14a-f. Development and retraction of amnion cell macrospikes (ms). Amnion 255, magnification 300x. The arrow points to the developing and retracting macrospike. Cell "A" moved several microns toward cell "B" after the cells were joined by the macrospike in (e), but moved back again after retraction in (f). Blebs (bl) have appeared between (e) and (f). (22 minutes.)

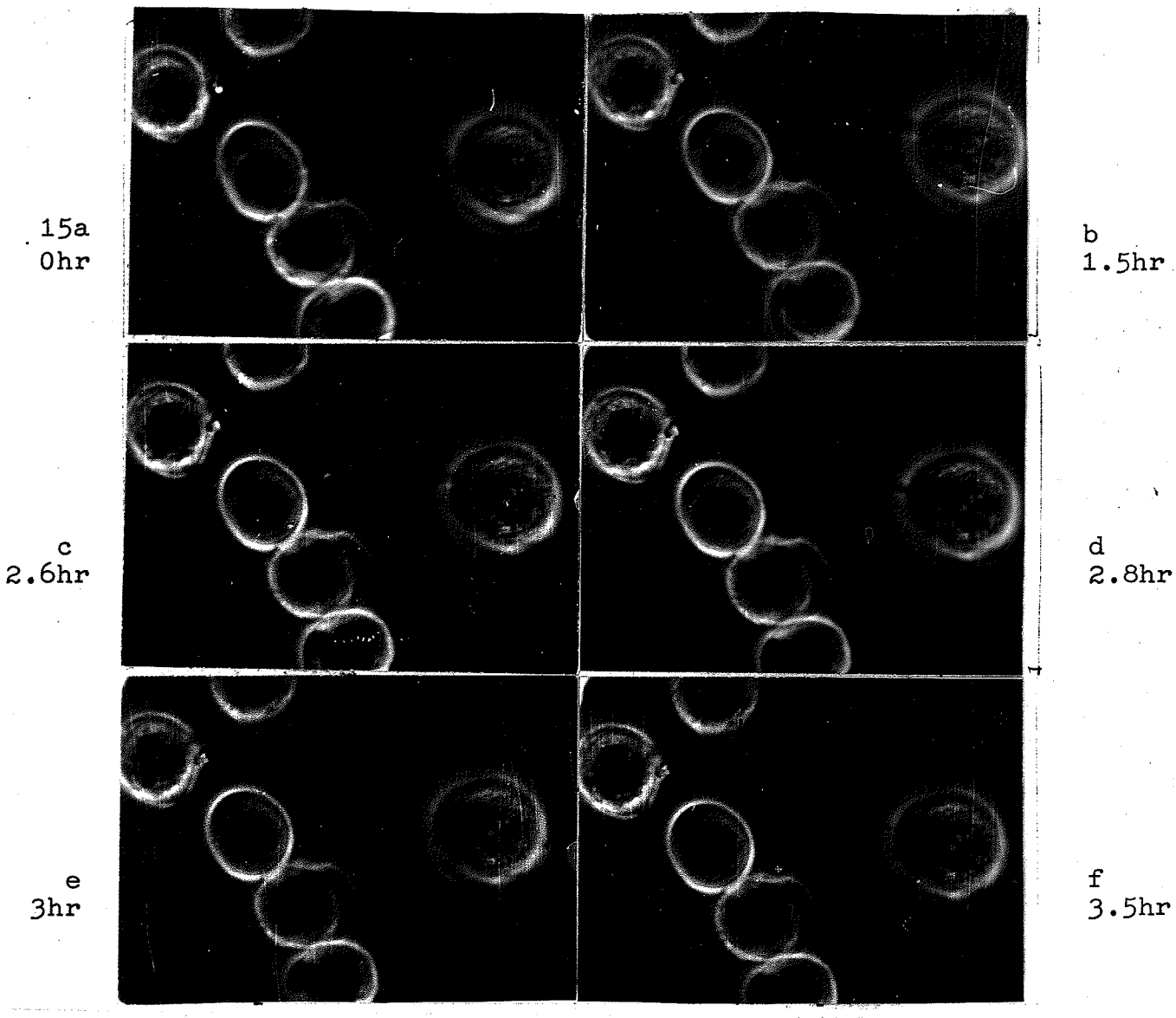


Figure 15a-f. The retraction of an amnion cell macrospike. Amnion 161, magnification 630x. The long spike-like appearance of the macrospike (ms) is depicted. (Some other macrospikes are present.) The retraction of this long process took 216 minutes (a)-(f). Blebs (bl) may be seen throughout this series.

At this time the unhealthy amnion cells gradually became more refractile as the vacuoles became more prominent. The smooth, spherical surface of the cells began to wrinkle, and many of the cells produced round blebs which often became as large as some of the cells. Figure 14 and 15, pages 65 and 66, illustrate the appearance of these early, small blebs.

Figure 16, page 68, depicts a group of cells which did not form a monolayer. It shows most of the above described characteristics except for the macrospikes which are poorly seen. This Figure also contains photomicrographs of the apparent union of two cells to form one. Superimposition of the cells is unlikely, as both cells remain in focus during the process. After union the cell became active, producing many tiny spherical zeiotic blebs (Costero and Pomerat, 1951), one after the other, much like cells which have just undergone mitosis. Following this the cell put out some processes in the direction of the neighboring cells, which the separate cells had done only briefly some hours before union. Each time one of these processes contacted another cell, the united cell appeared to undergo a temporary burst of activity. The phenomenon of cell union has been recorded by the cine-phase camera in this study on only one occasion.

Morphology of the human amnion monolayer cells

Cell size and shape. Before developing into a monolayer,

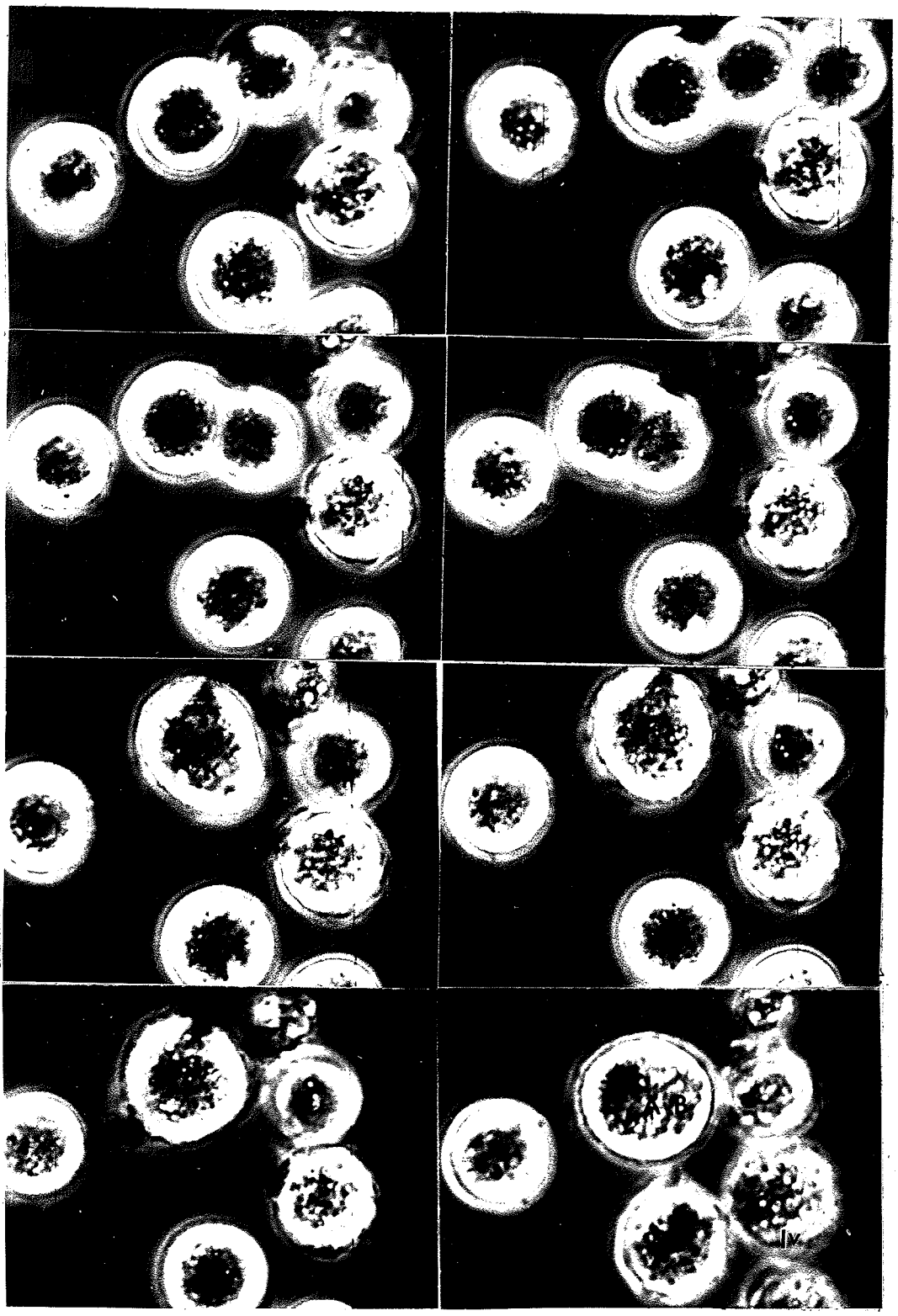


Figure 16a-h. Dying amnion cells which did not develop a monolayer. Amnion 251, magnification 1050x. The described features of cells which do not develop into monolayers may be seen. Cells "A" and "B" unite to form one cell. Macrospikes are poorly seen. Other objects seen are membrane wrinkling (mw), vacuoles (lv), macrospikes (ms) and cell process (p).

the cell shape may be fibroblastic or ameboid, but when in a monolayer, the shape is epithelioid. Epithelioid cells may be narrow and elongated, or wide and polygonal, similar to the cells seen in Figure 18, page 72. The width ranges from 10 to 100 μ and up to 110μ long, but only the multinucleated cells achieved the larger sizes, for example, Figure 17, page 70. The large epithelioid cell borders were usually moving, resulting in a constantly changing shape for the intercellular boundaries. On occasion, the epithelioid cell was observed to migrate out of the microscopic field overnight, indicating movement of a fairly large section of the monolayer to accommodate this motion.

The cell membrane, phagocytosis and pinocytosis. Once in a monolayer, the cell membrane bordering other cells can be seen readily in photomicrographs, Figure 17, page 70. This series shows the boundaries between cells, as well as the dark areas of the upper free membrane superimposed upon the lower or closer cytoplasm. This membrane appeared to sweep back and forth over a portion of the cytoplasm, like the ebb and flow of tidal waters. There may be several areas producing this motion at the same time, especially well seen in multinucleated cells, and often they appear to originate in the paranuclear area. Films of this membrane movement indicate that the membrane appears related to some cytoplasmic

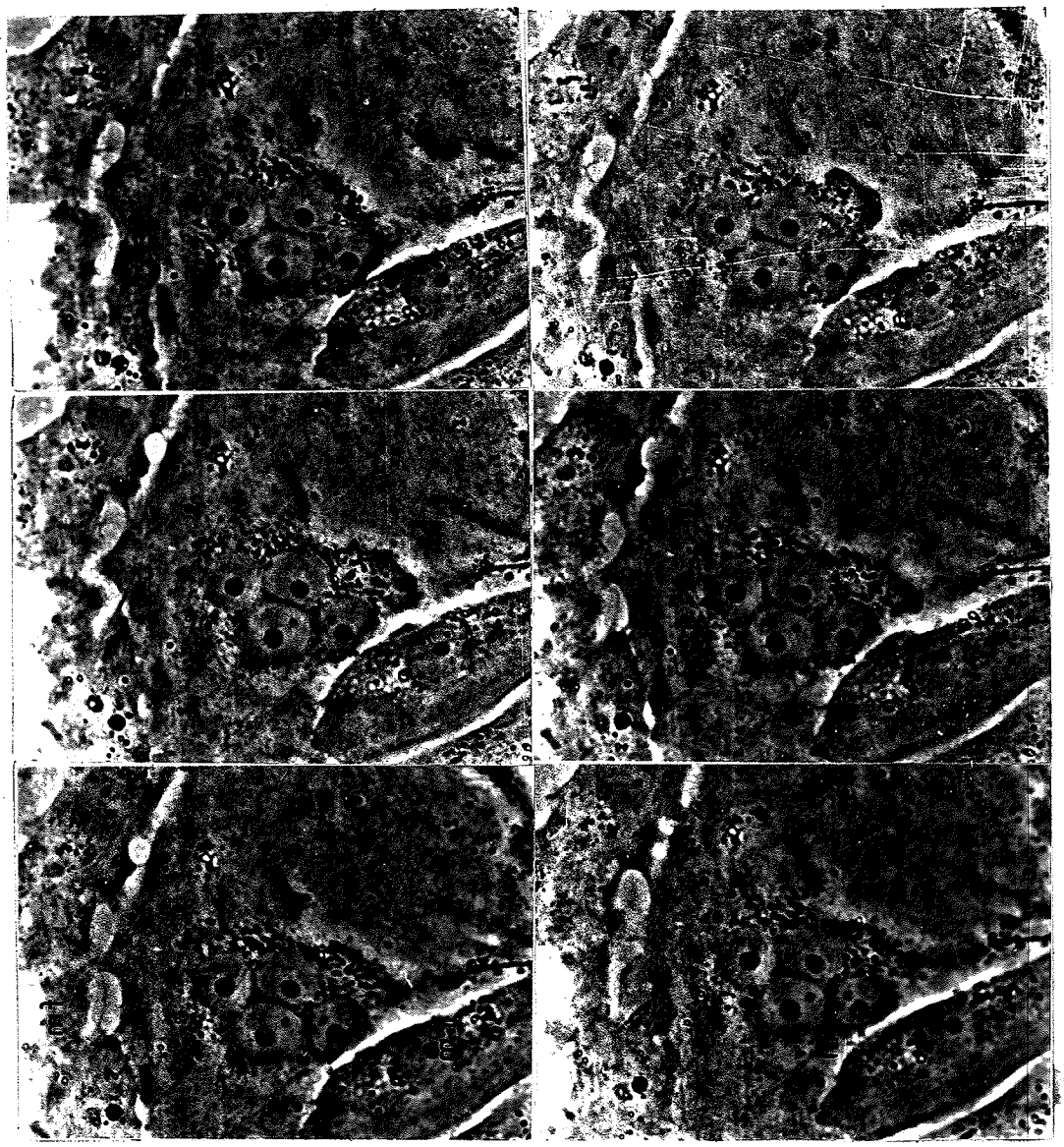


Figure 17a-f. A fluctuating membrane in a monolayer cell. Amnion 74-66, magnification 630x. The darker wave-like area labeled (fm) is the fluctuating membrane, and the area of movement can be seen in photomicrographs (a)-(f). The adjacent vacuoles (1v) appear to be contained between the nucleus and the membrane, suggesting some relationship between the latter and the vacuoles.

vacuoles, especially those in the perinuclear area, and when movement occurred some of the vacuoles appeared trapped in it.

When the edge of a cell was not in contact with another cell, it was noted to be very active, often engulfing particles by phagocytosis and fluid by pinocytosis. Phagocytosis by a human amnion cell is recorded in Figure 18, page 72. This sequence shows the original distance between the cell and the irregular, extracellular particle, the ensnaring of the particle, along with some fluid, and finally the presence of the rounded particle within the cytoplasm of the ingesting cell. The actual phagocytosis of the particle was done in 58 minutes. Figure 19, page 73, illustrates the pinocytosis of fluid by the free membrane of a multinucleated cell. This process occurred in ten minutes and the fluid was dispersed into the cell cytoplasm within one hour of ingestion.

An interesting occurrence is recorded in Figure 20, page 74, where massive pinocytosis took place when a neighboring cell, after a flurry of activity, contracted, became refractile, and floated off the glass coverslip. Also associated with the degeneration of this cell was the production of many tiny zeiotic blebs in the two uninucleated neighboring cells. Further, the general movement of all cells nearby increased greatly and the two uninucleated cells moved into the degenerated cells place beside the multinucleated cell.

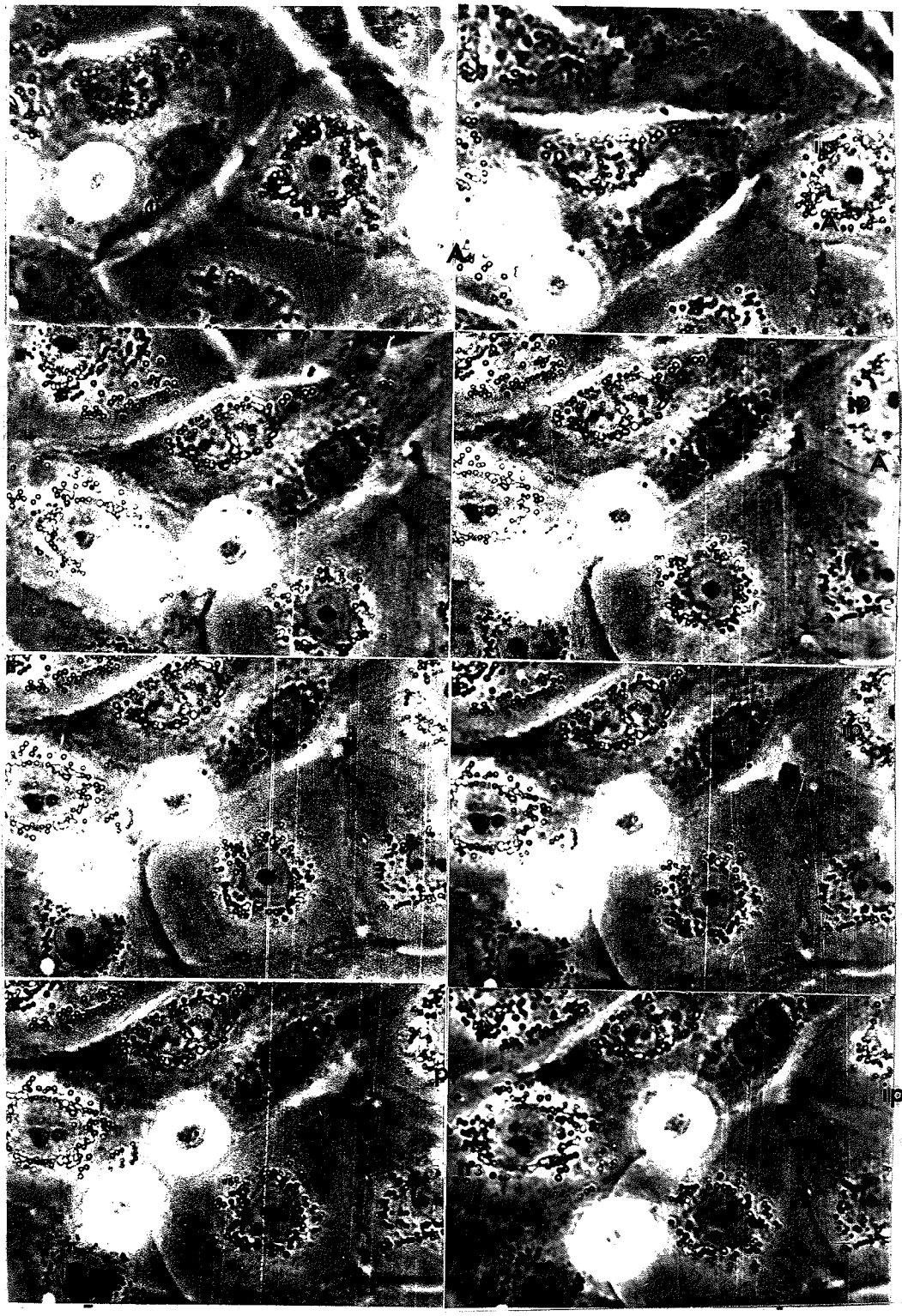


Figure 18a-h. Phagocytosis by a monolayer cell. Amnion 133-66, magnification 850x. The particle (ip) is well separated from the ingesting cell "A" in (a). Fluid (pf) is ingested with the particle in (f). Nucleoli of irregular shape (nl), touching the nuclear membrane (n2), and close to a nuclear fold (n3) may be seen.

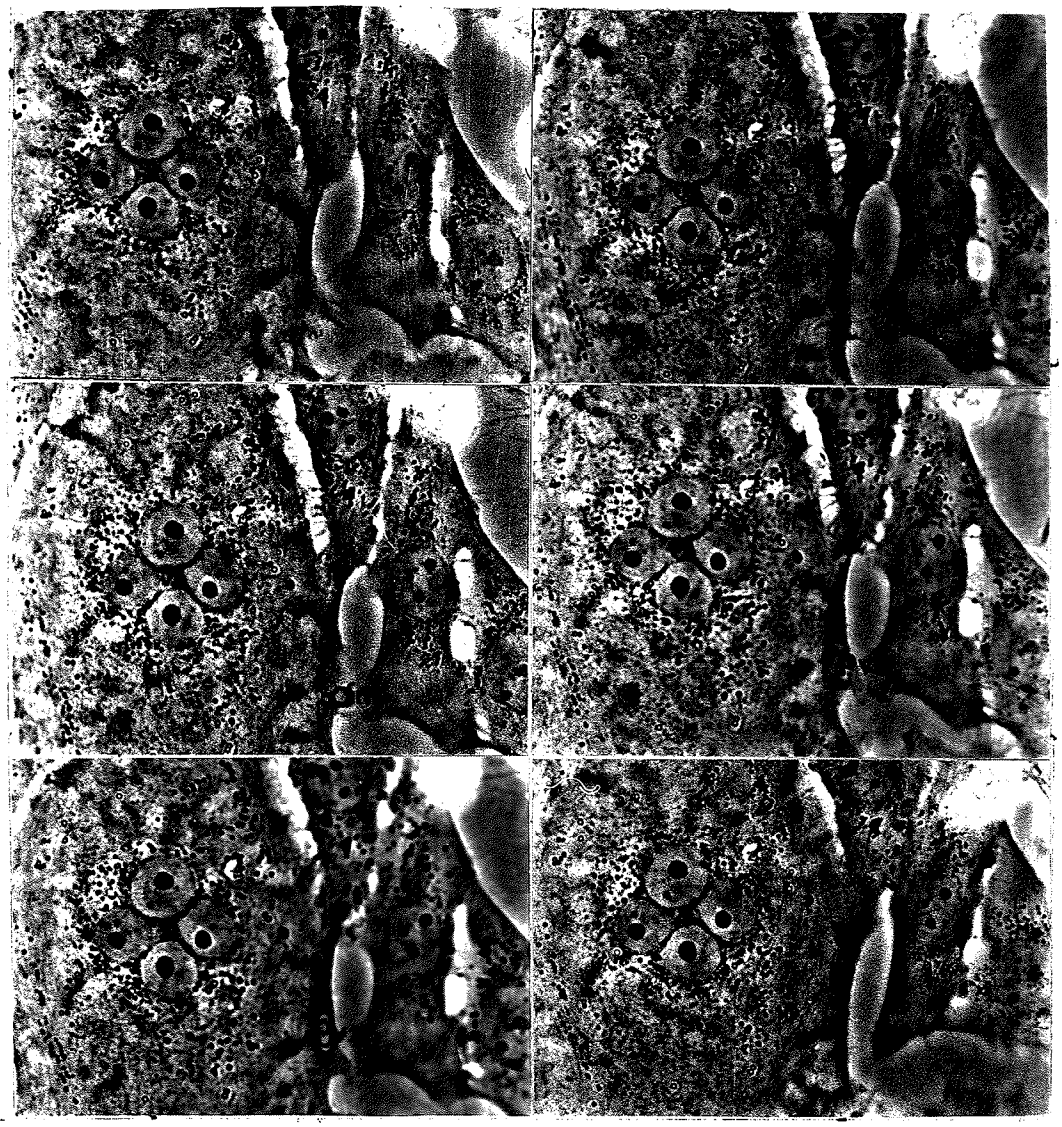


Figure 19a-f. Pinocytosis by a monolayer cell. Amnion 74-66, magnification 630x. The fluctuating membrane (fm) formed three pinocytotic vacuoles (pv) of fluid in (c). These vacuoles (pv1) are united in ten minutes to form a vacuole (pv2) about five microns in diameter, seen in (e). Within 57 minutes this material was dispersed into the cytoplasm.

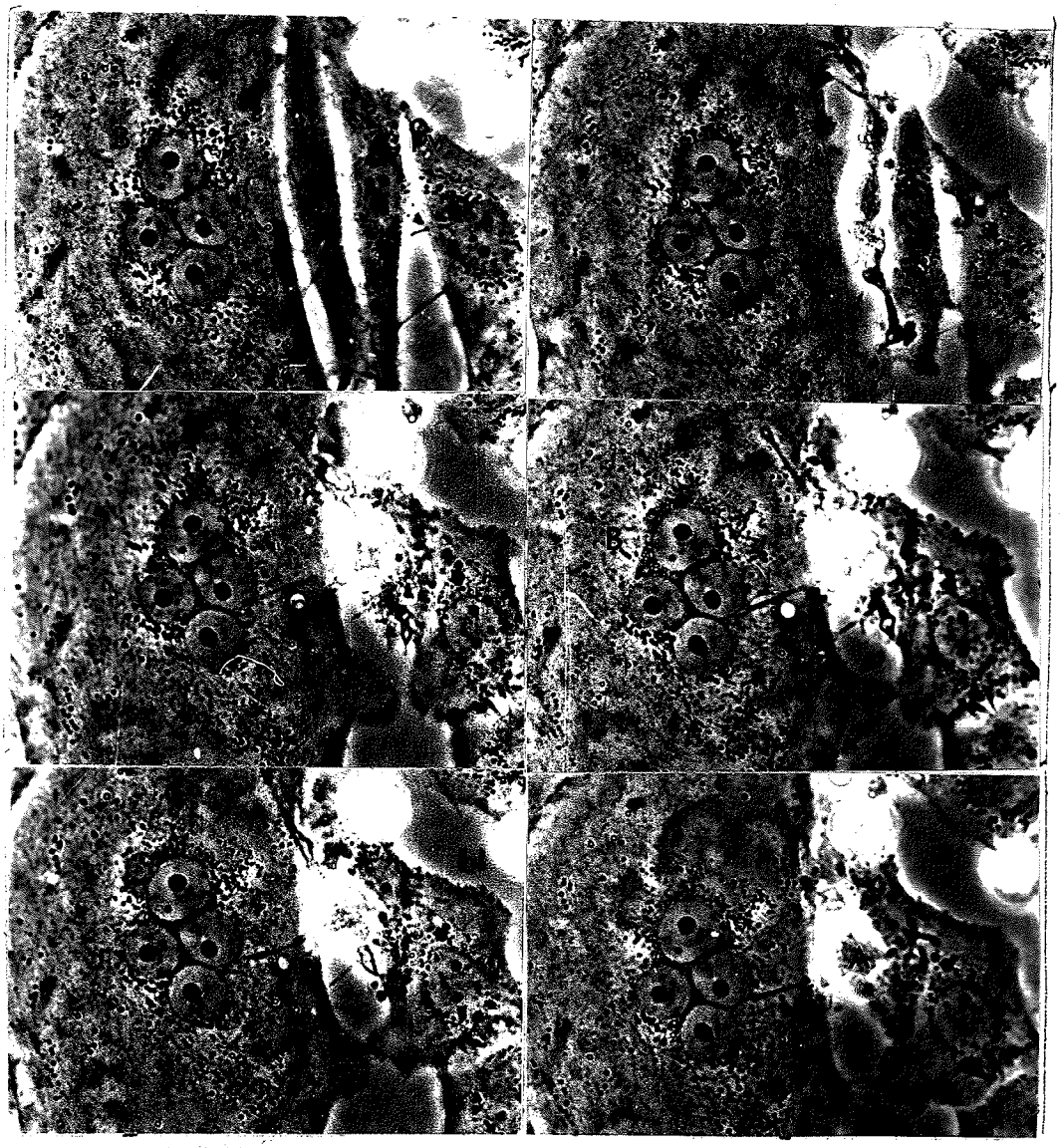


Figure 20a-f. Pinocytosis associated with the degeneration of a neighboring cell. Amnion 74-66, magnification 630x. Cell "A" retracted and became very refractile (a)-(c). The fluctuating membrane of cell "B" engulfed fluid (pv) within five minutes of retraction in (d), and cell "D" did likewise in 30 minutes (e). Cells "C" and "D" showed very active, minute blebbing (bl) for the next ten hours after the death of cell "A" (d)-(f).

A process from the degenerated cell appeared to stick to the multinucleated cell, and it was in the area of this process that the pinocytosis of very large droplets of fluid took place.

The cytoplasmic contents of the human amnion monolayer cell. Aside from the ground substance, which is made up of tiny, regular granules, there are three major components of the cytoplasm that are visible through the phase-contrast microscope: 1) granules, 2) vacuoles, and 3) mitochondria. Vacuoles and granules under five microns in diameter will be described in the final section of this chapter.

Vacuoles larger than five microns found in the cytoplasm were usually of two varieties; those vacuoles associated with pinocytosis which disappeared as rapidly as they were formed; and those large vacuoles found in the cytoplasm periodically, which disappeared as slowly as they developed. Figure 23, page 80, shows a cell containing one of these vacuoles which remained in the cytoplasm for over two weeks. The vacuole in this figure is six microns in diameter, but often this type of vacuole is larger and may be seen in groups of two or three. Ultimately the large vacuole will gradually decrease in size and disappear from the cytoplasm. Pinocytotic vacuoles have been seen as large as five microns, although they are usually smaller (three to four microns) before they coalesce

and disappear. The pinocytotic vacuoles usually form and are dispersed into the cytoplasm within one hour (Figure 19, page 73).

Mitochondria, as recorded by the cine-phase camera, appear to be thin, filamentous and motile. They are about 0.5μ thick and form long, interconnected networks in the cytoplasm. Figure 21, page 77, is a photomicrograph of three monolayer cells containing one, three and four nuclei. The cell with four nuclei contains a large network of mitochondria, which can be seen on close inspection of the photomicrograph. The cell with one nucleus contains a geometrical pattern, similar to the mosaic cells described by Rose in 1963. The mosaic pattern may be due to very prominent mitochondria or dilated endoplasmic reticulum; whatever its cause, the pattern disappeared after several days and did not return.

In Figure 23, page 80 , rotation of the cytoplasmic contents has been observed associated with the rotation of the nuclei. The vacuoles and granules of the cytoplasm near the center of the cell appear to rotate, while the peripheral vacuoles do not. During this rotation, which is 360 degrees counter-clockwise, no increased activity of the cell border was noted.

The nucleus and nucleolus. Amnion cells in tissue culture monolayers may contain from one to seven nuclei. The

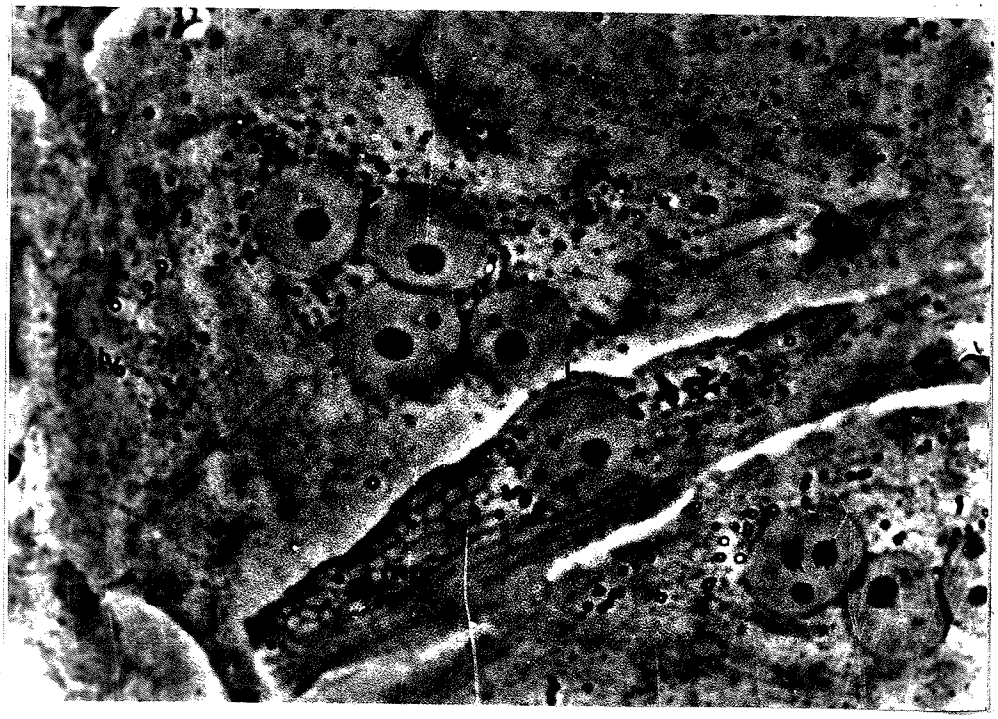


Figure 21. The cytoplasm of the cultured human amnion cell in monolayer. Amnion 74-66, magnification 1,100x. The mitochondrial network is labeled (mn). The cytoplasm ground substance appears to be finely granular, with some coarser granules (g) and vacuoles (lv) found in it. The fluctuating membrane (fm) can be seen in several areas of the multinucleated cell. One of the nuclei of this cell is touching the cell membrane.

A mosaic cell is also depicted with its mosaic pattern (mp). The closeness of the nucleus to the edge of the mosaic cell has produced a bulge (b) in the cell border.

A nuclear fold (nf) extending in toward a nucleolus (nl) can be seen in the cell with two visible nuclei (n).

nucleus is usually round, about 14μ in diameter, and although seven nuclei have been seen in one cell, the most common situation is one or two nuclei per cell. Generally, the greater the number of nuclei, the larger the cell. The nucleus is usually located near the center of the cell, but the position can vary greatly.

Nuclei are mobile in the human amnion cell and Figure 22, page 79, records the movement of a group of four nuclei moving from one side of the cell to the other in 12.6 hours. In the last photomicrograph of this sequence, one nucleus appears to be in direct contact with the membrane of the cell, which in turn appears to be touching the neighboring cell membrane.

Another type of nuclear movement is a revolving motion within the cytoplasm, so that the four nuclei appear to be revolving 360 degrees as a unit, around a single point. Figure 23, page 80, illustrates this movement, as well as the rotation of 360 degrees of a nucleus around its own axis. The Figure shows that a nucleus is able to rotate around its own axis once in 64.6 hours, and the group of nuclei are able to rotate around a single axis in 107 hours. This Figure also shows that the perinuclear cytoplasm seemed to accompany the group of nuclei as it rotated. All of the types of rotation described occurred in a counter-clockwise direction.

The nucleus contains from one to three nucleoli which

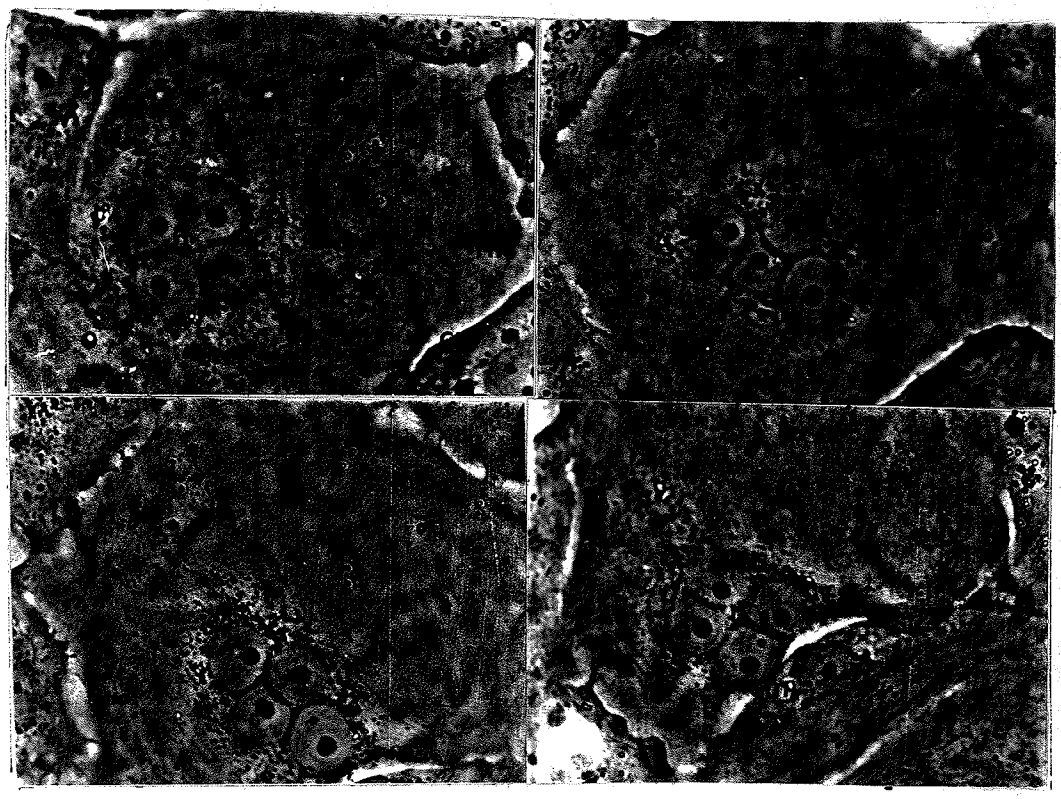


Figure 22a-d. Linear nuclear movement within the amnion cell. Amnion 74-66, magnification 630x. The nuclei (n) have moved from the left side of the cell to the right side (a)-(d) where one nucleus touches the cell membrane (cm), which in turn is in contact with the cell membrane of the neighboring cell (d). Note the fluctuating membrane (fm).

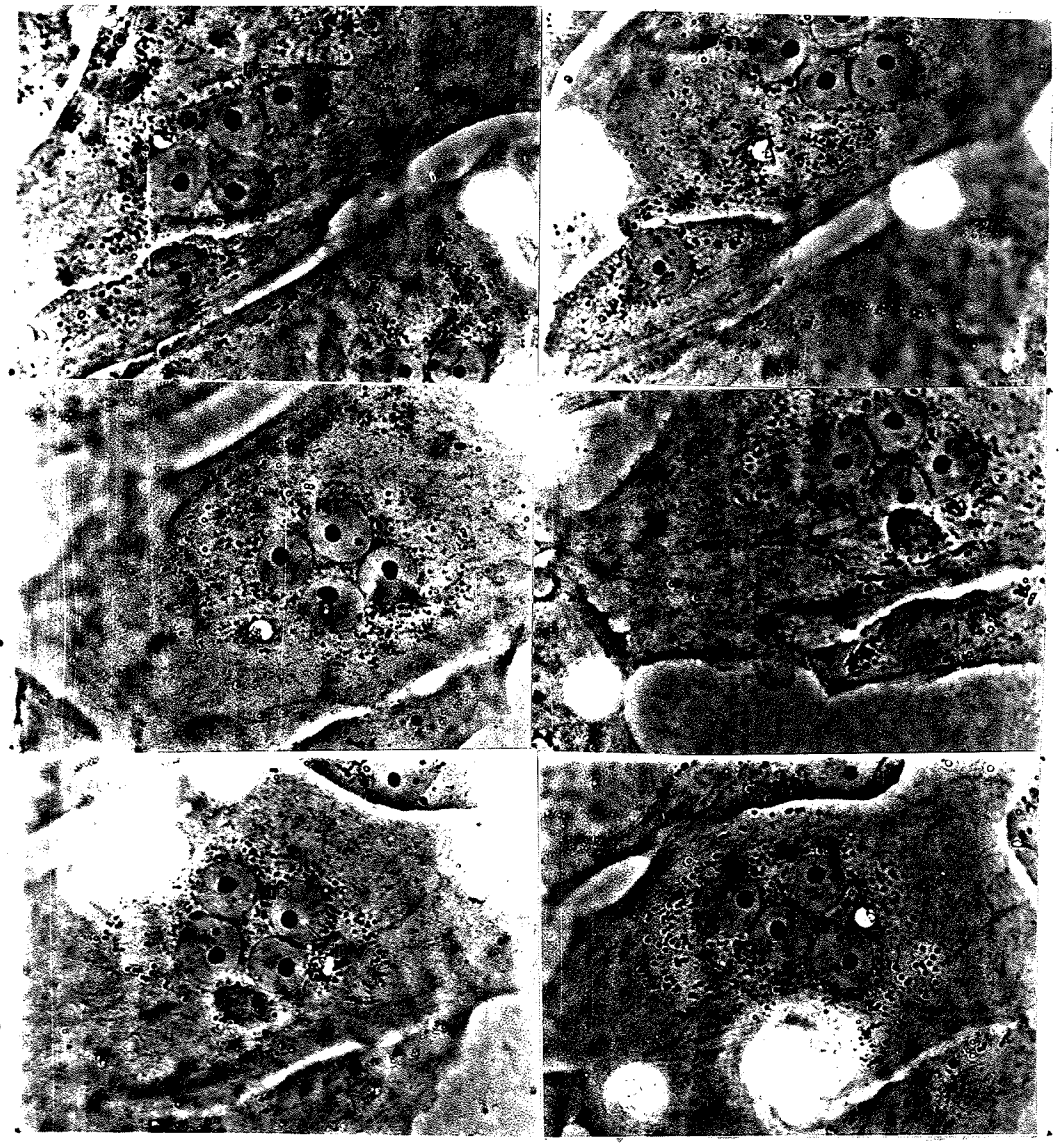


Figure 23a-f. Nuclear and cytoplasmic rotation. Amnion 74-66, magnification 630x. In 64.5 hours nucleus "A" has rotated 360 degrees (a)-(d). All four nuclei (n 1-4) as a group have rotated 360 degrees in 107 hours (a)-(f). The large vacuole (lv), along with much of the cytoplasm in the center of the cell appear to rotate with the nuclei, although the cytoplasm at the periphery of the cell does not. All rotation is counter-clockwise. A light area in one of the nuclei is probably a nuclear vacuole (nv)

are usually round, dense and four microns in diameter. The nucleolus often touches the nuclear membrane and is frequently in close proximity to a nuclear fold. Where three nucleoli are present, one is often larger than the others and occasionally one will have an irregular outline. Figure 18, page 72, illustrates most of these observations.

Figure 23, page 80, also shows a light area in one of the nuclei. This area appears to be in a constant position, in relation to the nucleolus, over a long period of time. It appears to rotate with the nucleus, and is probably a nuclear vacuole.

Nuclear folds, or invagination of the nuclear membrane, are commonly present in the nuclei of human amnion cells, and may be associated with the nucleolus. Nuclear blebbing has been observed in degenerating monolayer cells and will be described in the following paragraphs.

Indications of degeneration in human amnion monolayer cells. In chapter III it was stated that there is no certain means of detecting cell death and some criteria for separating healthy from unhealthy cells were given. Figures 24 and 25, pages 82 and 83, contain cells which show irreversible degenerative changes and the following description of these degenerating monolayer cells may be added to the previous description given of cells in suspension.

The first indication of an adverse change within the

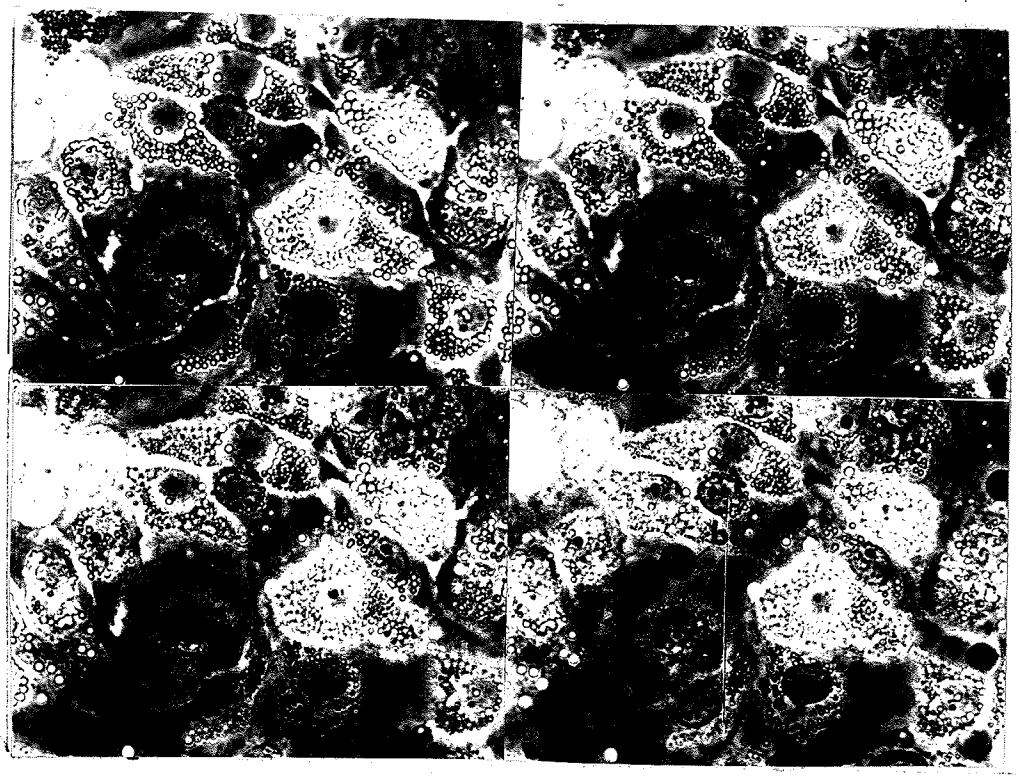


Figure 24a-d. Monolayer cell degeneration. Amnion 133-66, magnification 630x. Initially some cells are markedly vacuolated, others are not, and this does not appear to change appreciably in this particular field during degeneration. The nuclei labeled (n) show the nuclear granulation or nuclear stippling (ns) and darkening of the nuclear membrane. Blebs (bl) show an increase in size from (c)-(d), (four hours), and some of them burst later (not shown).

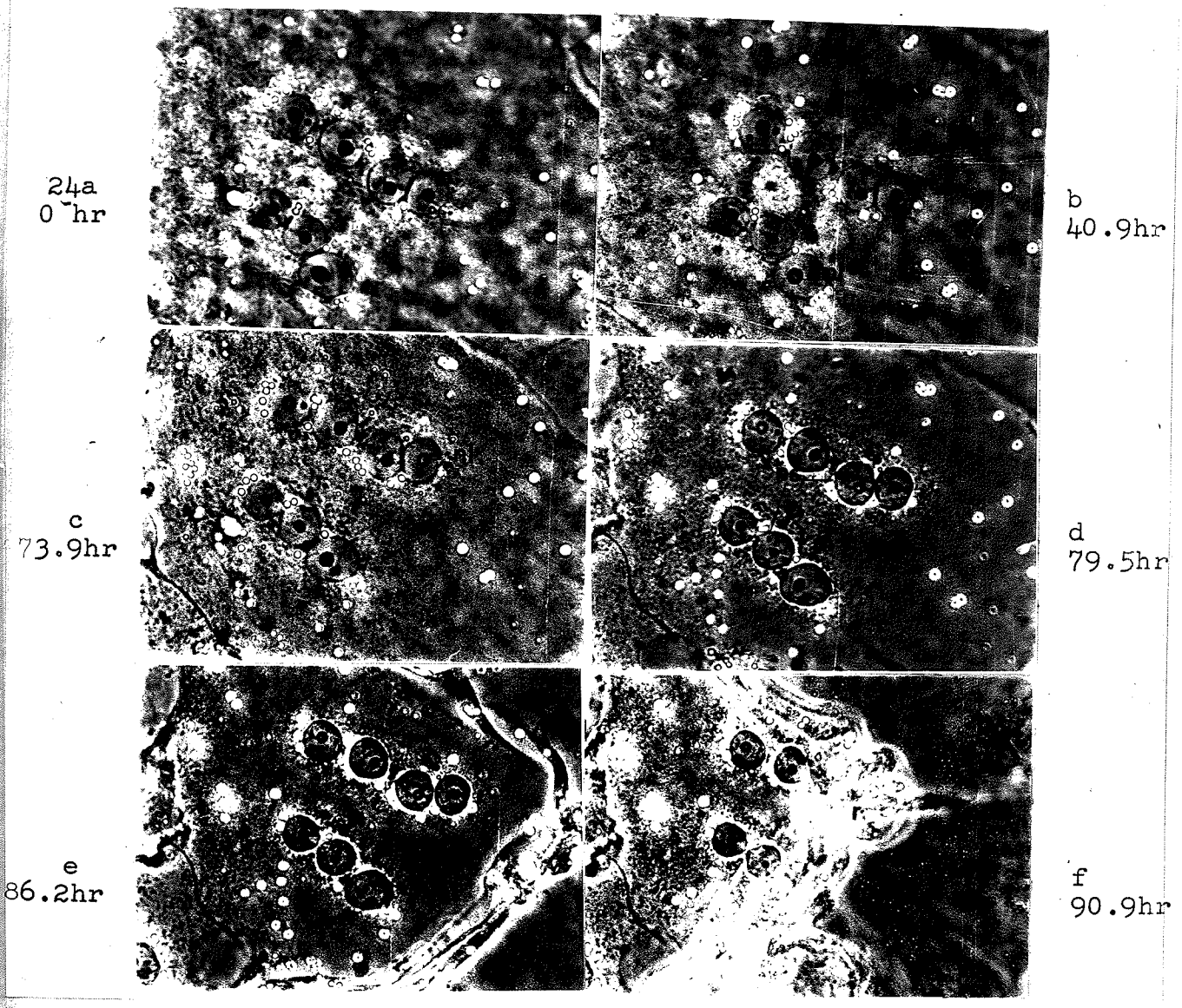


Figure 25a-f. Degeneration and retraction in a multinucleated cell. Amnion 74-66, magnification 630x. Nuclear stippling (ns) (d)-(f), the lightening of the nucleolar centers (nl) (b)-(f), the retraction (r) of the degenerated cell are seen (e)-(f). At this magnification some increase in the granularity of the cytoplasm is noted.

cell was the appearance of a light area in the center of a nucleolus, and this was followed by an increase in the density of the nuclear folds. At this point the peripheral cytoplasm and cell membrane abruptly stopped all forms of motion, quickly followed by the cessation of perinuclear activity. Within minutes the nuclear membrane became very dense, the nucleolemma developed very coarse granulations and small blebs formed on one of the nuclei, as can be seen in Figure 26, page 85. Blebbing at the cell borders completed the degenerative process. In Figure 24, page 82, the described changes may be seen in all but the very heavily vacuolated cells, and it is of interest to note that the changes began to occur almost simultaneously throughout the microscopic field, and were completed in one hour. Figure 25, page 83, shows these changes in a multinucleated cell, although this cell was the only one in the area to undergo degeneration. This Figure also illustrates cell contraction following degeneration and the immediate replacement with the cytoplasm of other cells in the monolayer. The process of degeneration in this cell took one hour. Figure 26, page 85, is a sequence taken between the time the photomicrographs 25c and 25d were taken, in order to show the detail of nuclear blebbing associated with cell degeneration.

Granules and vacuoles in the cytoplasm of human amnion monolayer cells. The presence of granules and vacuoles is a

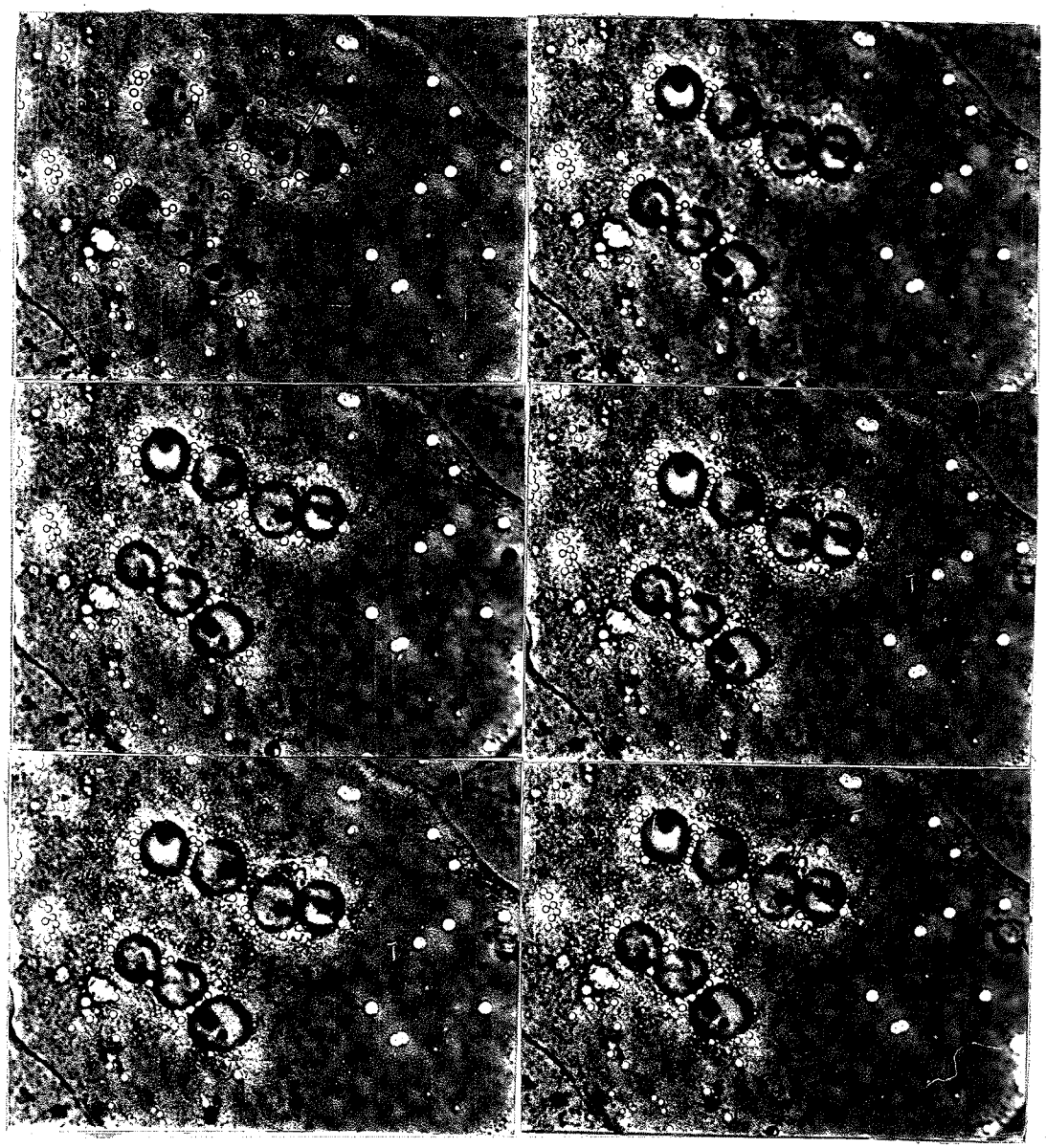


Figure 26a-f. Nuclear blebbing during cell degeneration. Amnion 74-66, magnification 630x. The cell before degeneration is seen in (a). Nuclear blebs (nb) began to develop in the nuclear membrane (nm) in (d), and they became larger by (f). This development occurred in 13 minutes. (This series represents a sequence of events which occurred between the time that photomicrographs 25c and 25d were taken, page 83.)

very remarkable characteristic of the cytoplasm of the human amnion cell in vitro. The granules are dark and spherical, measuring up to one micron in diameter, and the vacuoles are also spherical, but measure from one to three microns in diameter. The separation of these two entities is probably artificial, as the phase-contrast microscope tends to portray anything under one micron as a solid object. On close inspection of the photomicrographs of amnion cells containing granules and vacuoles, the larger granules appear to have less dense centers, similar to vacuoles. Further, it appeared that the greater the number of cytoplasmic vacuoles, the fewer the granules, although an accurate count of these objects could not be done. These observations suggest that the granule and the vacuole are different sizes of the same entity.

Vacuoles may be seen in freshly trypsinized cells as well as in biopsies of the amnion membrane and in cells of the tissue culture monolayer. In Figure 11c-e, page 58, a relatively non-vacuolated cell appeared to produce many vacuoles in a ten hour period as it developed into an epithelioid cell, and by 18 hours it was markedly vacuolated. Amnion cells in tissue culture which do not develop into a monolayer may become more vacuolated (Figure 16, page 68).

The position of the granules or vacuoles is generally tightly perinuclear in early monolayer cells, and as the cytoplasm spreads outward, the vacuoles become increasingly active.

Granular or vacuolar movement is usually irregular, almost Brownian-type movement, and it is most active in the perinuclear area. Some vacuoles aggregate in the fluctuating membrane when it passes over them, and then the vacuoles move for a time with the membrane as it sweeps back and forth through the cell. Vacuoles over three microns in size often migrate to the periphery of the cell where they become less active. Once in the periphery of the cytoplasm the vacuoles line up in rows of four to eight, and can be seen in the long, thin processes of some cells, for example, Figure 27, page 88.

Cells which are in the ameboid form have relatively few vacuoles (Figure 12, page 62), and those cells which are packed with vacuoles tend to be immobile. Multinucleated cells have relatively fewer vacuoles considering the total size as compared with those cells with only one nucleus (Figure 24, page 82). Figures 24 and 25, pages 82 and 83 show that cells which undergo degeneration are not necessarily vacuolated, and that both vacuolated and relatively nonvacuolated cells undergo degeneration in a similar way.

On a few occasions vacuoles have been observed moving from the cytoplasm of one cell to the cytoplasm of the neighboring cell. Figures 28 and 29, pages 89 and 90, show two different examples of vacuole transport from one cell to another.

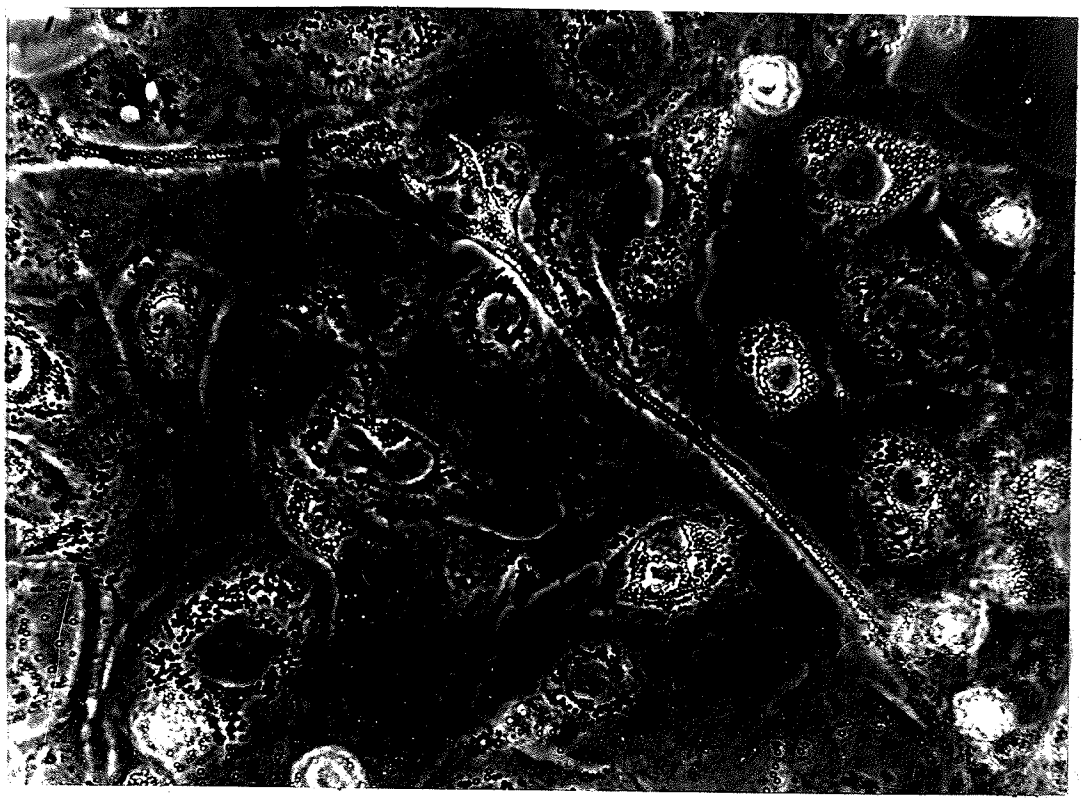


Figure 27. Peripheral vacuole arrangements. Amnion 5-66, magnification 900x, 35 mm film. Some vacuoles (lv) are in linear arrangements in the cytoplasm at the cell periphery, and a considerable number are in an exceptionally long (100 μ) cell process (p).

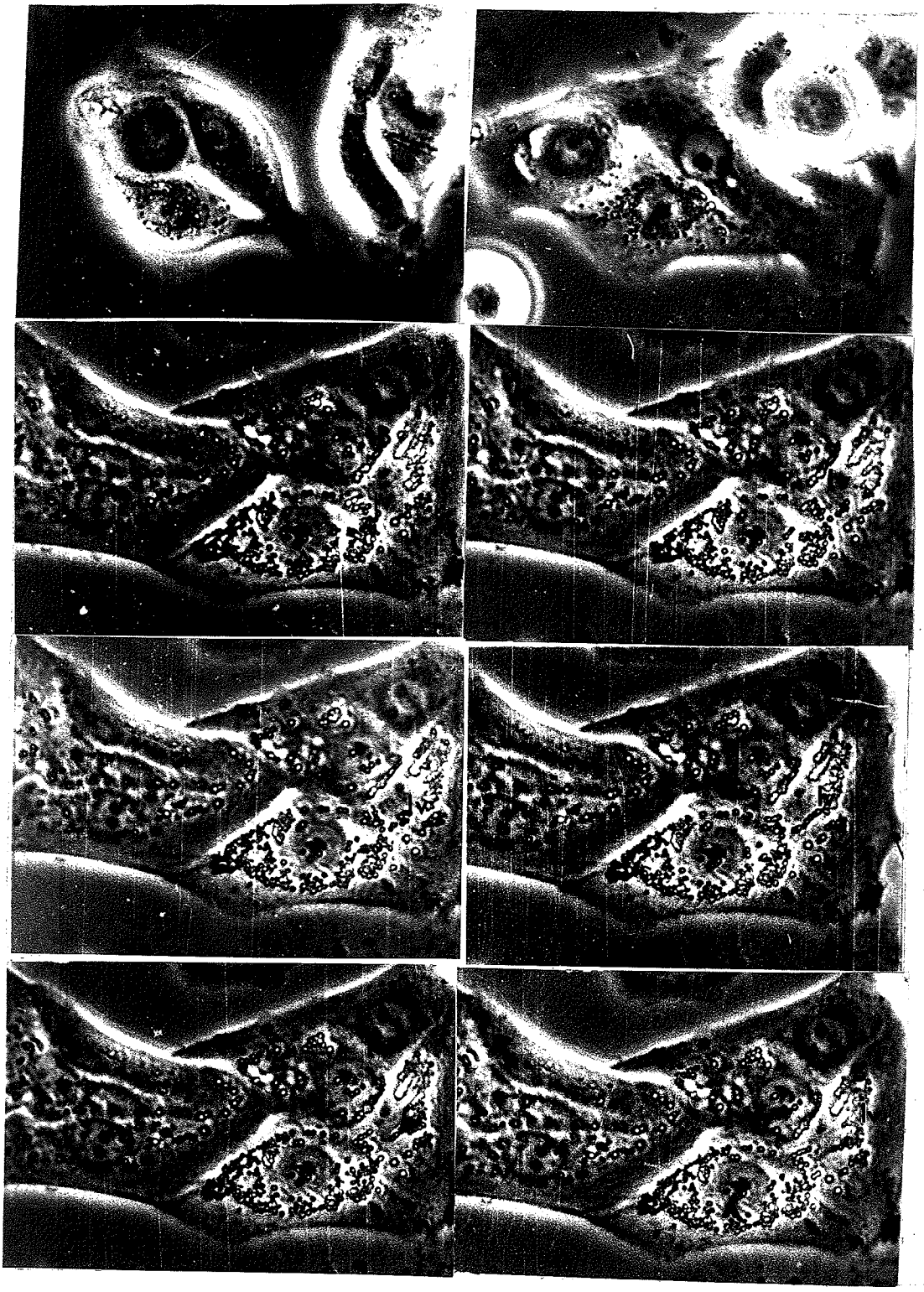


Figure 28a-h. Intercellular vacuole transport. Amnion 1-66, magnification 850x. The early development of these cells is seen in (a), and (b). Vacuoles (lv) pass across the cell membrane (cm) from cell "A" to "B" in the remaining photomicrographs, over a period of 100 hours.

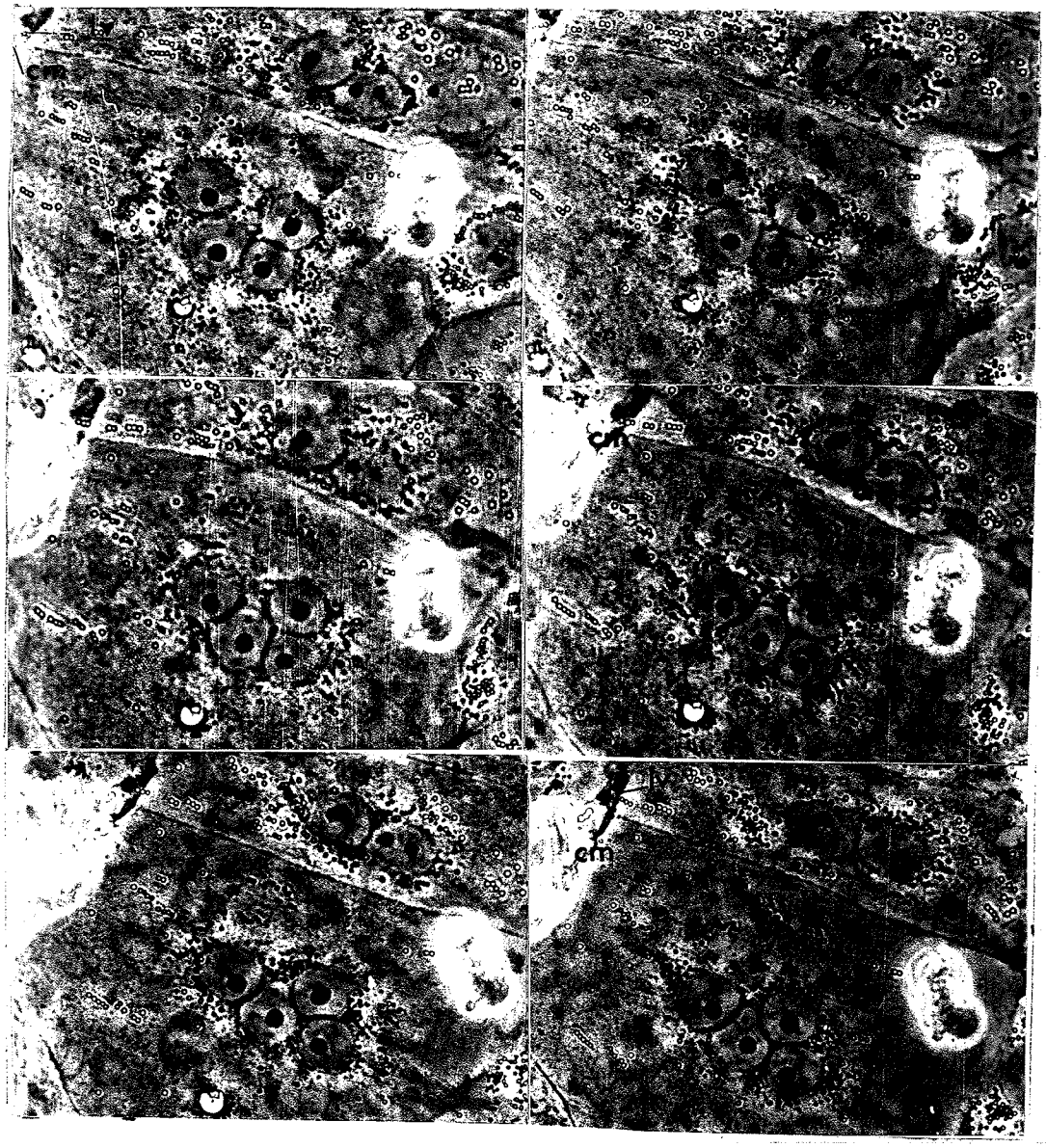


Figure 29a-f. Intercellular vacuole transport. Amnion 74-66, magnification 630. The vacuoles (lv) pass through the cell membranes labeled (cm). The movement of the multinucleated cell toward the bottom of the microscopic field represents a movement of seven microns in 27 hours.

Vacuoles may move across membranes singly or in rows of two to four, in from one to 16 hours.

To date, the cine-phase camera has not recorded the expulsion of vacuoles from cells, other than the transport of vacuoles from one cell to the other. This may be related to the fact that the photomicrographs are of the inferior surface of the cell, and any secretory product would probably leave the cell from its free, superior surface, and therefore be out of focus.

As the monolayer ages, those cells which were vacuolated initially usually become packed with vacuoles, whereas multinucleated cells and some others are never more than sparsely vacuolated, even when they die after long periods of culture (Figure 24, page 82).

In summary, observations of human amnion cell suspensions developing into monolayers, and those which did not, have been recorded. The general morphology of amnion cells in tissue culture monolayers has been described, with special emphasis placed upon the vacuoles of these cells.

II. ULTRASTRUCTURE

The ultrastructure of cells in biopsies of human amnions.

The major portion of the ultrastructure was obtained by the studies of biopsies of the reflected and placental portions of the human amnion. Figures 30 and 31, pages 93 and 94, are low power electronmicrographs of biopsies of reflected and placental portions of different amnions. There were no consistent differences between the reflected and placental biopsies of the same membrane in the overall study. Figure 30, page 93, illustrates the typical features of amnion cells seen in biopsies; and they are: 1) columnar cells about 12 by eight microns, 2) microvilli at the cell apex, 3) the complex interdigitation of microvillous-like processes on the sides of the cells forming a tortuous intercellular canal which frequently were dilated to form large openings, 4) basal processes which are about 0.6 wide and project 1.1 into the basement membrane, 5) a nucleus with a nucleolus, 6) oval lipid vacuoles, 7) dilated endoplasmic reticulum, and 8) some areas of the Golgi complex. Cuboidal-shaped epithelial cells were seen almost as commonly as columnar, but flat, squamous-shaped cells were seen only once in the epithelium. Figure 31, page 94, differs from this description as it has material adherent to the microvilli, longer and thinner basal processes with deeper invagination of the basement membrane material into the cytoplasm, fewer vacuoles

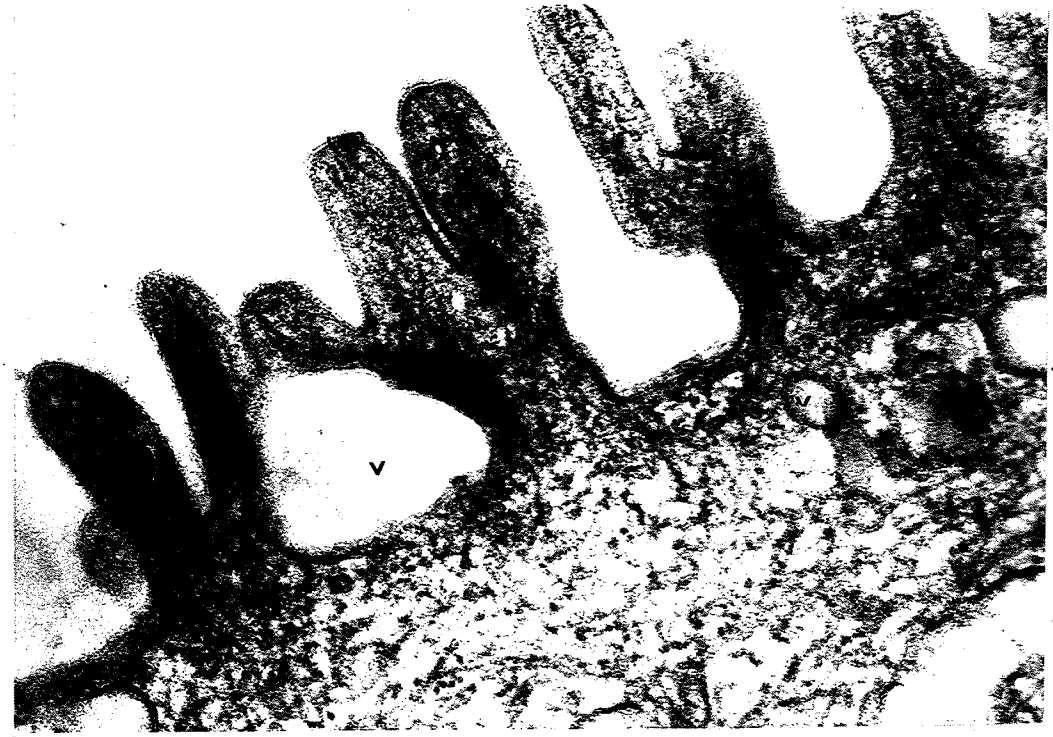
Figure 30. A typical biopsy specimen of the reflected amnion. Amnion 6-66, magnification 7,000x. Included are the microvilla (mv), dilated intercellular canals (icc), desmosomes (d), basal processes (bp), basement membrane (bm), nucleus (n), nucleolus (nl), dilated endoplasmic reticulum (er), Golgi complex (gc), and lipid vacuoles (lv).

Figure 31. A biopsy specimen of the placental amnion. Amnion 10-66, magnification 9,600x. Included are the microvilli (mv), dilated intercellular canals (icc), desmosomes (d), basal processes (bp), basement membrane (bm) extending deeply into the cytoplasm, nucleus (n), and lipid vacuoles (lv).

and areas of dilated endoplasmic reticulum, a lacy appearance of the cytoplasm and an irregularly shaped nucleus.

The microvilli. The microvilli are usually cylindrical projections of the cytoplasm, 0.6 μ tall and 0.1μ wide, and situated at the apex of the cell. They may be simple or branched and covered with the unit membrane (Robertson, 1962) which is composed of outer and inner electron dense layers, each about 35 A thick, and separated by a less dense layer about 30 A thick. These measurements of the unit membrane were representative of the measurements of all the triple-layered membranes throughout the cells observed in this study. The microvilli contain fine fibrils and granules of about 60 A in diameter, and small vacuoles of up to 0.1 μ in diameter are seen often at their bases. Occasionally the tips of the microvilli were seen united as if pinching off material to form a vacuole, Figure 32a, page 96. Figure 32b, page 96, shows dark staining material which is often found on the outer surface of the microvilli, obscuring the definition of their tips, as well as the small vacuoles present at their bases. A large mass of homogeneous material may be seen separated from the tips of the microvilli in Figure 33, page 97, and may be a result of material produced by the cell.

The intercellular canal. The intercellular canal area



32a

32b

Figure 32. Two views of microvilli.
a) Amnion 108-66. Magnification 57,750x. These microvilli are free of material and have a distinct unit membrane (um), contain fibrils (f) and what may be pinocytotic vacuoles (v).
b) Amnion 10-66. Magnification 46,750x. These microvilli are surrounded with material (sm) which partially obscures their tips. Very small vacuoles (v) may be seen at their bases.

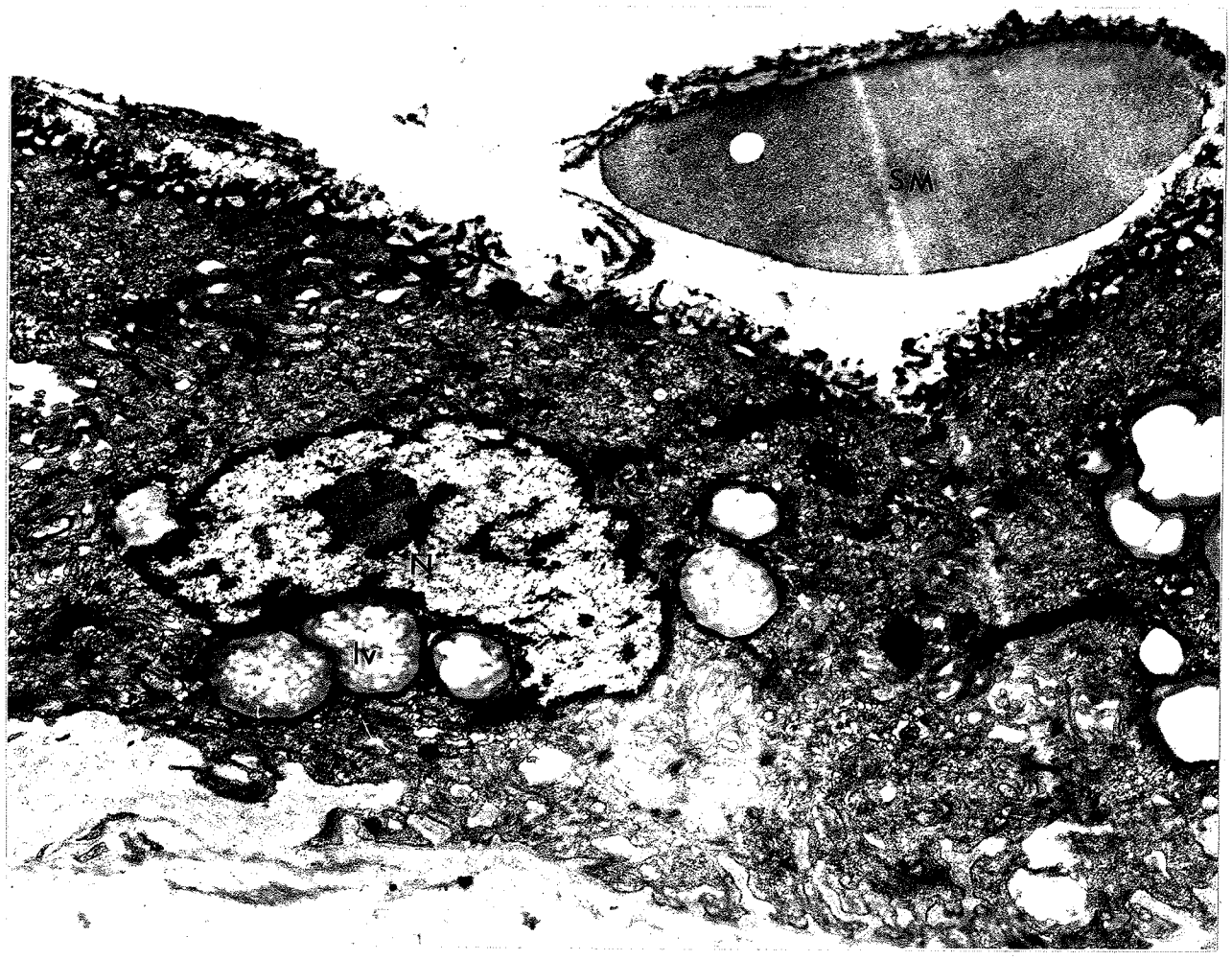


Figure 33. Cuboidal vacuolated cells with an homogenous mass at the tips of the microvilli. Amnion 268, magnification 5,250x. The separation of the material (sm) appears to have occurred during preparation. Lipid vacuoles (lv) may be seen indenting the nucleus.(n).

marked on Figure 30, page 93, is shown at a higher magnification in Figure 34, page 99. The intercellular canal is formed by the approximation of the outer cell membranes of two cells, joined by the interdigitation of microvillous-like processes and cemented by desmosomes. In some areas the cell borders separate irregularly and form dilated intercellular spaces of various sizes. The unit membrane forming the edges of these canals and the outer edges of the cell, contains many small vacuoles 0.11 μ in diameter which seem to be forming into the cell cytoplasm, with an opening into the canal. Figure 35, page 100, depicts a biopsy of cells with very dilated intercellular canals, as well as marked cytoplasmic vacuolation. This electron-micrograph is not typical, as very few cells had such widely dilated intercellular canals, scanty cytoplasm, fine microvilli or thin basal processes.

The basal processes and basement membrane. In Figure 34, page 99, typical basal processes enclosed in the unit membrane, penetrate into the basement membrane which lies next to this portion of the cell. The basement membrane is darker staining and composed of finer fibers than the deeper connective tissue which consists of collagen fibers about 46 mμ in diameter, with regular periodicity and a hollow structure, Figure 36, page 101. The appearance of the basement membrane between the basal processes is often foamy, as if composed of many tiny droplets.

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Figure 34. The basal processes, intercellular canals and basement membrane. Amnion 6-66, magnification 16,500x. Note the interdigitation of basement membrane (bm) and basal processes (bp) and the small vacuoles (sv) seen in the unit membrane (um) of the dilated intercellular canal (icc) and basal processes. The presence of desmosomes (d), fibrils (f), mitochondria (m), dilated endoplasmic reticulum (er) and a lipid vacuole (lv) may be seen.

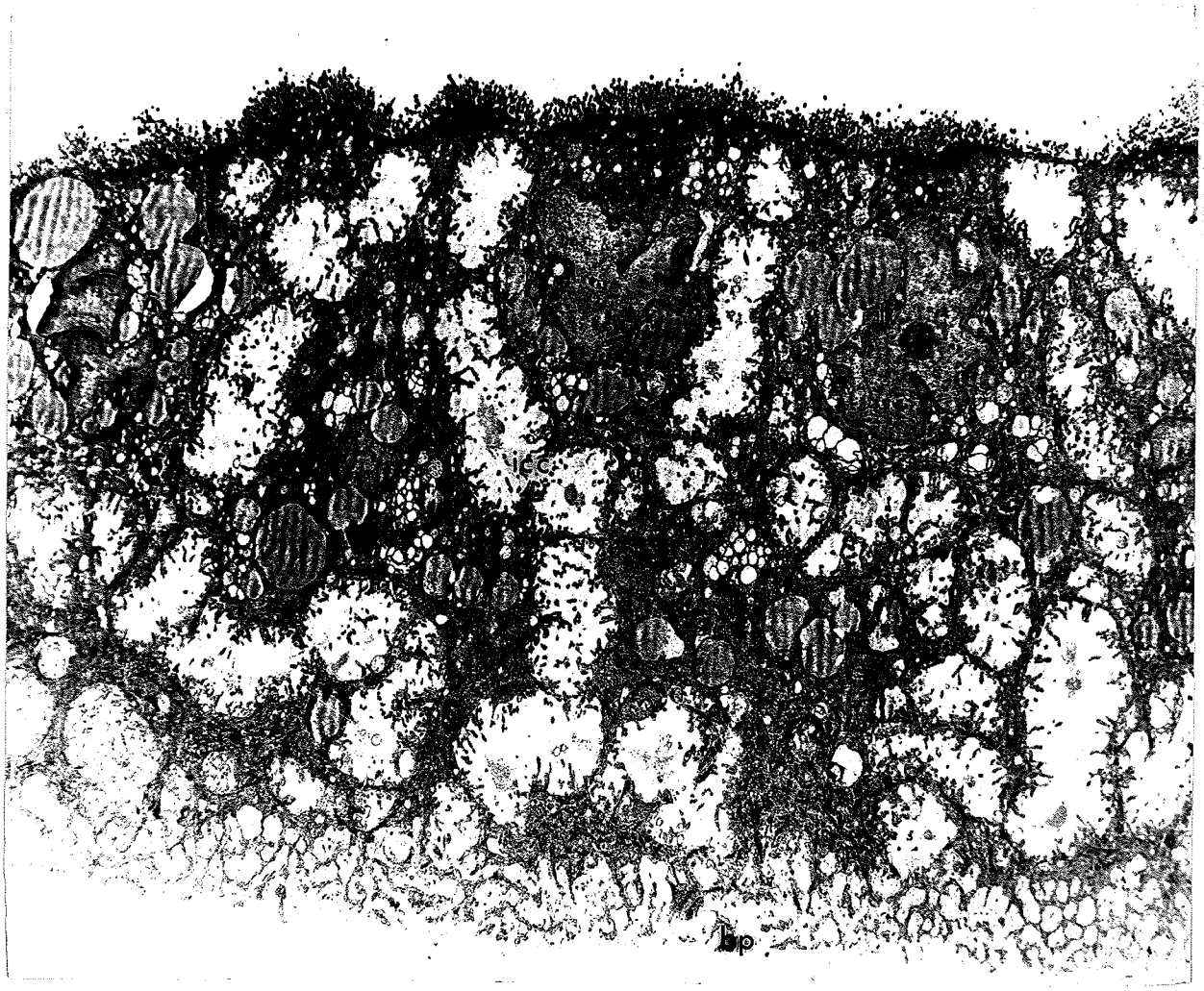

Figure 35. A biopsy of a reflected amnion with widely dilated intercellular canals. Amnion 83-66, magnification 3,500x. The widely dilated intercellular canals (icc), are accompanied by long basal processes (bp), thin microvilli (mv), many lipid vacuoles (lv), dilated Golgi complexes (gc) and nuclei (n) with many folds (nf).

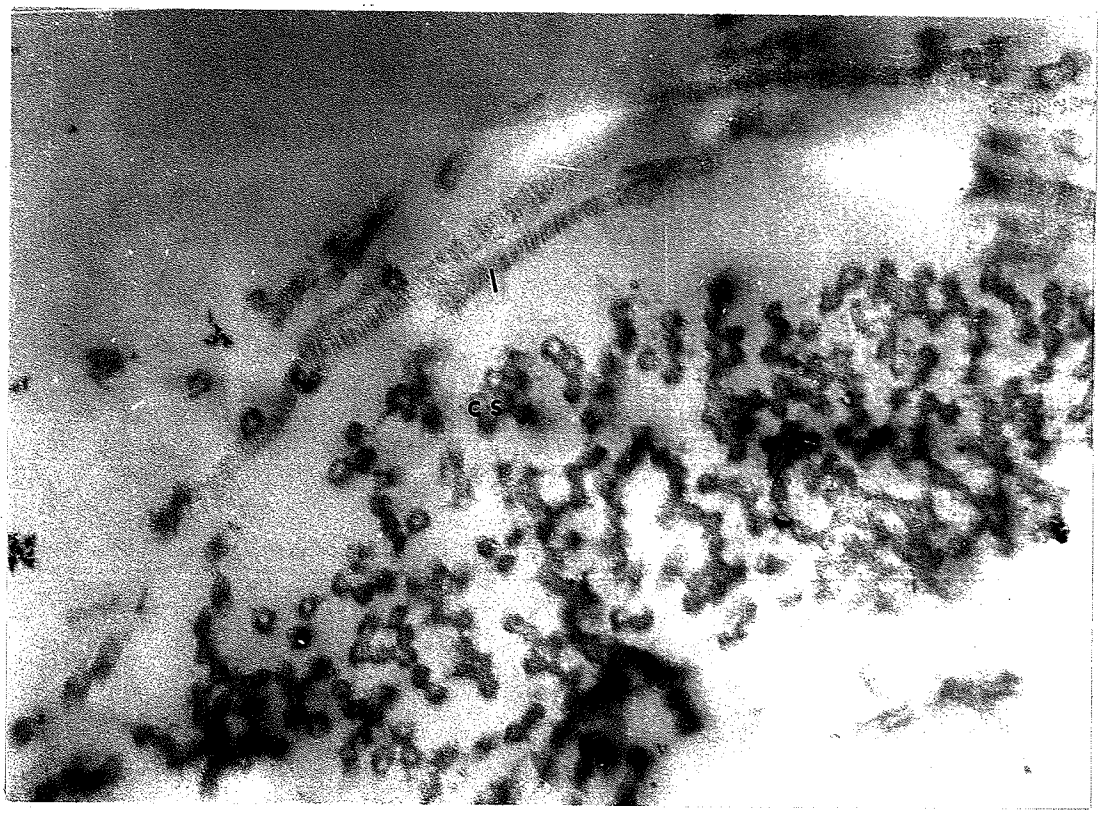

Figure 36. Collagen fibers of the connective tissue layers. Amnion 16-66, magnification 57,750x. Longitudinal views (l) of these fibers show a regular periodicity and longitudinal sections show a tubular structure superimposed on this periodicity. Cross sections (cs) of these fibers demonstrate the hollow structure.

Minute vacuoles, similar to those in the unit membrane bounding the intercellular canals, may be seen in the membrane of the basal processes. These processes contain many fibrils about 80 A in diameter and granules up to 200 A in diameter, many small vacuoles 0.1 μ in diameter, several mitochondria and some dilated cisternae of endoplasmic reticulum. Figure 34, page 99, contains an enclosed area of basement membrane, which is probably a cross section of a projection of basement membrane into the cell between the basal processes. This Figure also illustrates how the basement membrane material enters the dilated intercellular canals present at the base of the cells.

The nucleus and nucleolus. Previous electronmicrographs (Figures 30, 31 and 35, pages 93, 94 and 100) have shown that the nucleus of the human amnion cell assumes many shapes, measures up to six microns in diameter, contains at least one nucleolus, has a coarse, dark staining nucleolemma enclosed in a five-layered membrane, and can be found in different areas within the cell. Figure 37, page 103, is a higher power view of the nucleolemma, which appears to be composed of coarse granules, no smaller than 80 A, surrounded by a nuclear membrane which contains a pore. The proximity of the nucleus to the intercellular canal should be noted; the perinuclear cytoplasm will be discussed later. The portion of a nuclear

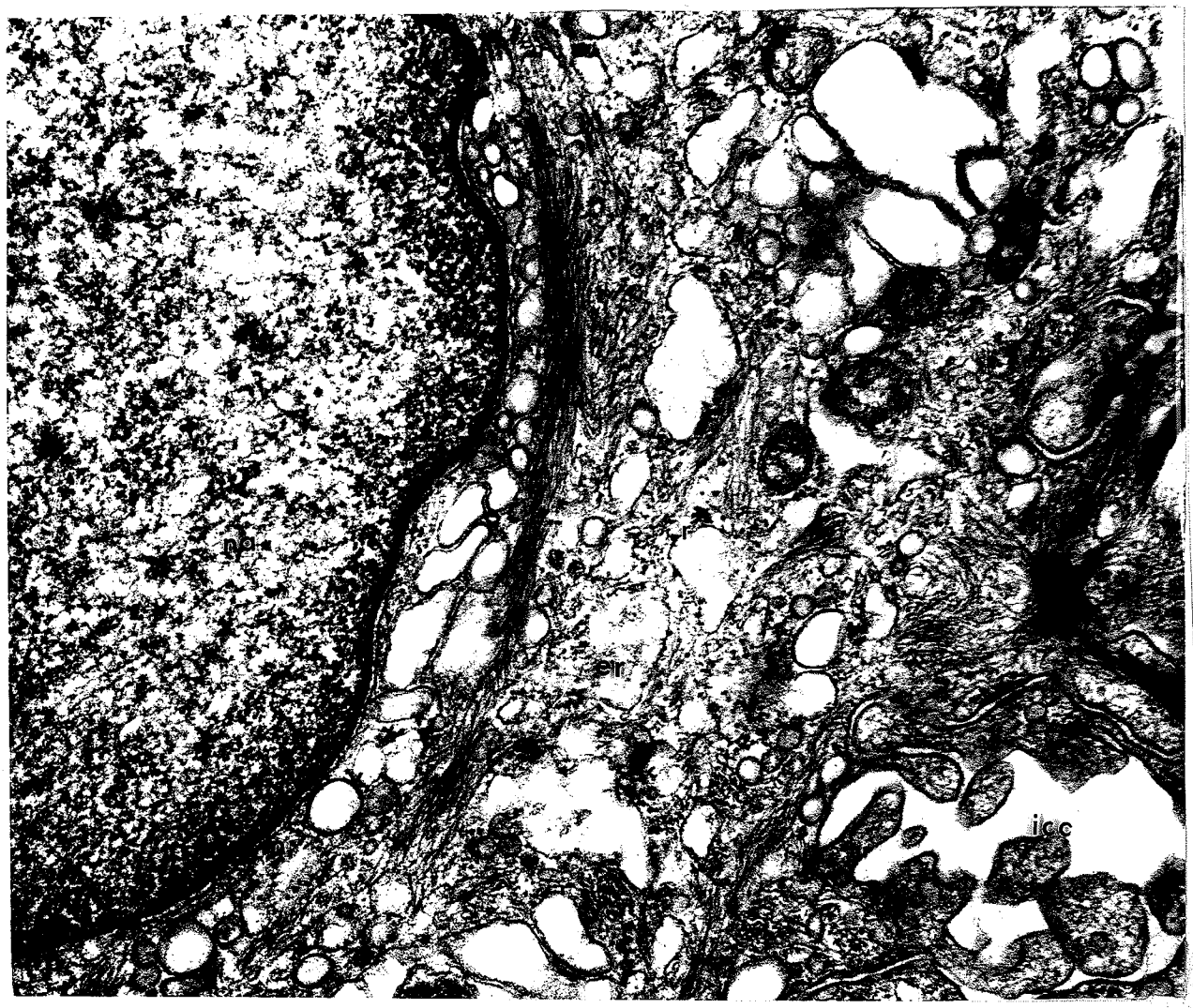

Figure 37. The nucleolemma, nuclear membrane and perinuclear cytoplasm. Amnion 108-66, magnification 30,250x. The coarse nucleolemma (na) is enclosed in the five layered nuclear membrane (nm) in which there is a nuclear pore (np). Fibrils (f) and small vacuoles (v) approximate the nucleus which is situated near the dilated intercellular canal (icc), mitochondria (m), dilated Golgi complex (gc), ribosomes (r), and dilated endoplasmic reticulum (er).

membrane containing a pore is further magnified in Figure 38, page 105; the outer portion of the membrane is trilaminated with the outer and inner layers being electron dense and 83 A thick and separated by a less dense band about 42 A thick. This outer membrane is separated from what appears to be an inner condensation of dark staining nucleolemma, usually greater than 165 A thick, by a layer 498 A thick and composed of moderately electron dense material which is granular in nature. The pore appears to have a covering about 250 A thick and composed of similar material to that of the trilaminated membrane. Occasionally the outermost layer of this membrane is absent when it is close to small perinuclear vacuoles (Figure 37, page 103).

Deep folds in the membrane are common in the nucleus of the human amnion cell. Figure 39, page 106, shows indentations of the nucleus by large vacuoles and one fold extends deeply into the nucleolemma, touching the nucleolus. These folds occasionally contain mitochondria or small vacuoles, and in one case the cytoplasm appears to be in the process of pinching off nuclear material, as illustrated in Figure 40a and b, page 107.

The nucleus may contain up to three nucleoli which are usually oval, dark staining areas of up to three microns in diameter, as seen in Figures 33, 35 and 39, pages 97, 100 and 106. The nucleolus appears to be composed of round, densely

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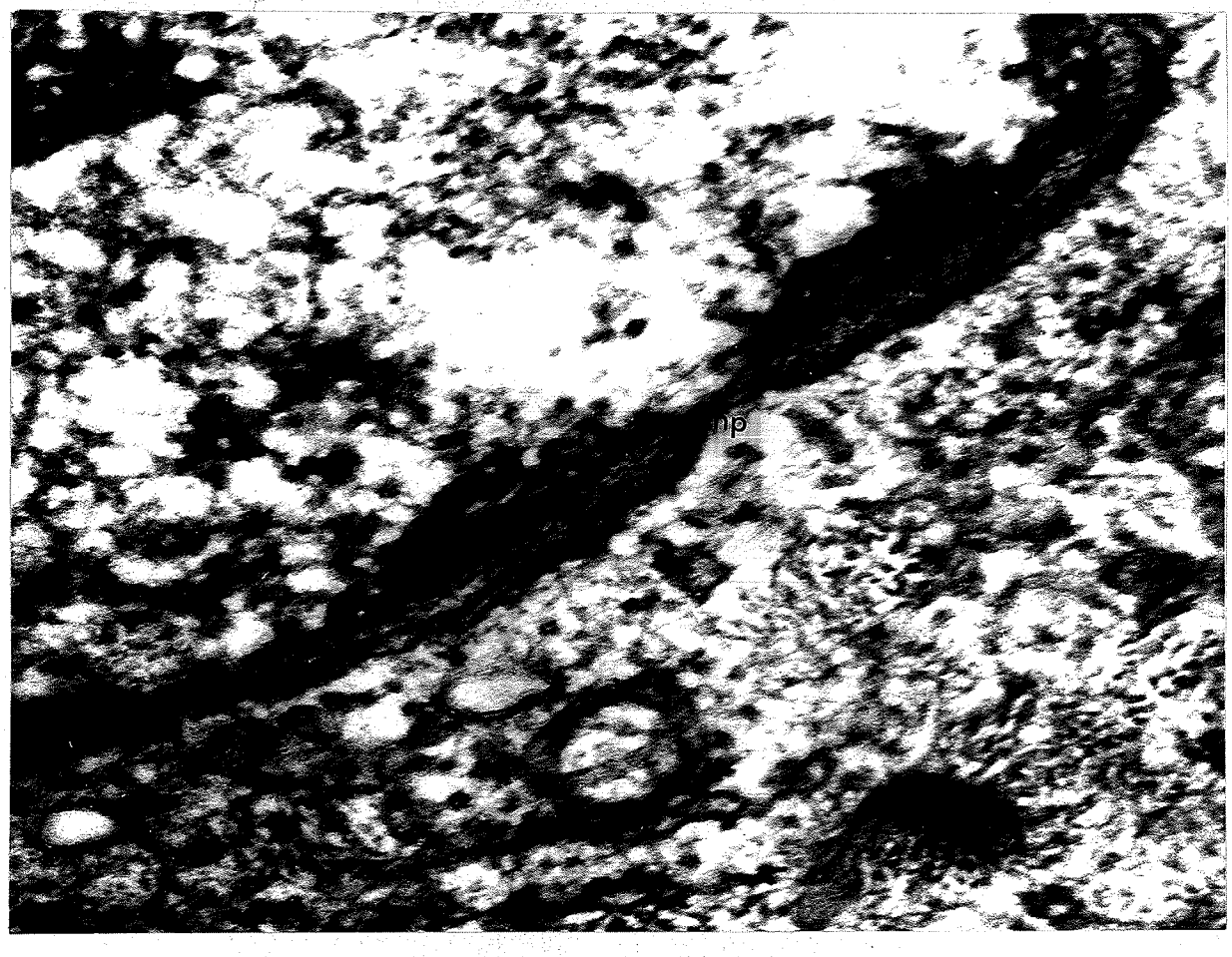


Figure 38. The nuclear membrane and a nuclear pore. Amnion 6-66, magnification 93,500x. The five-layered nuclear membrane (nm) contains a pore (np) with a covering which seems to be a condensation of the outer four layers of the nuclear membrane.

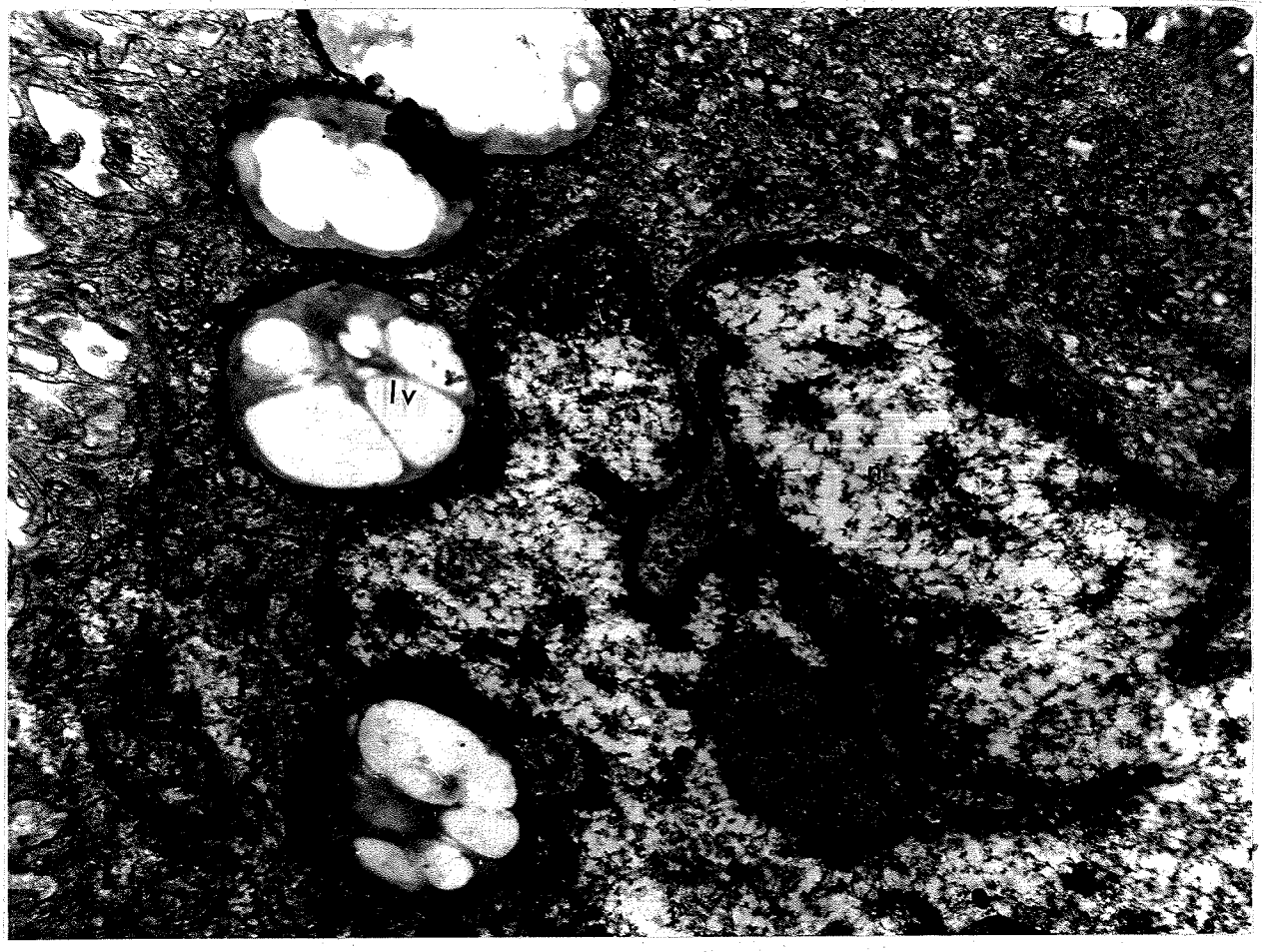
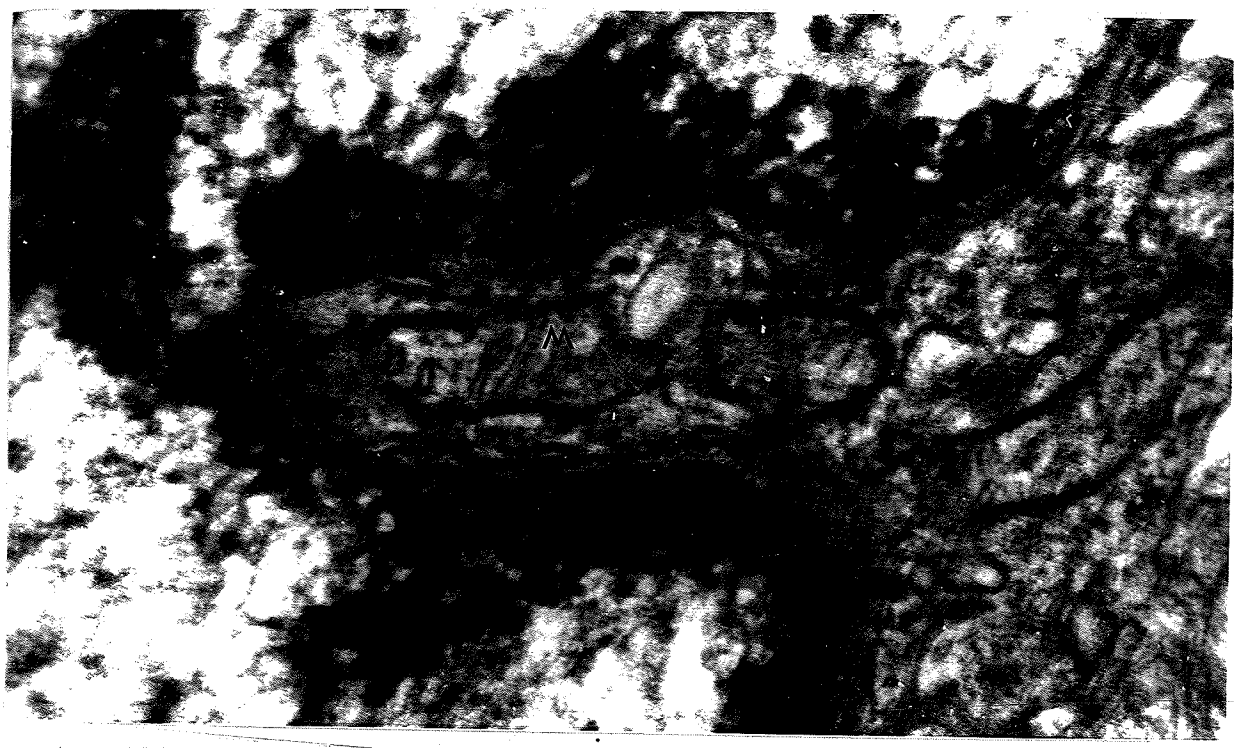
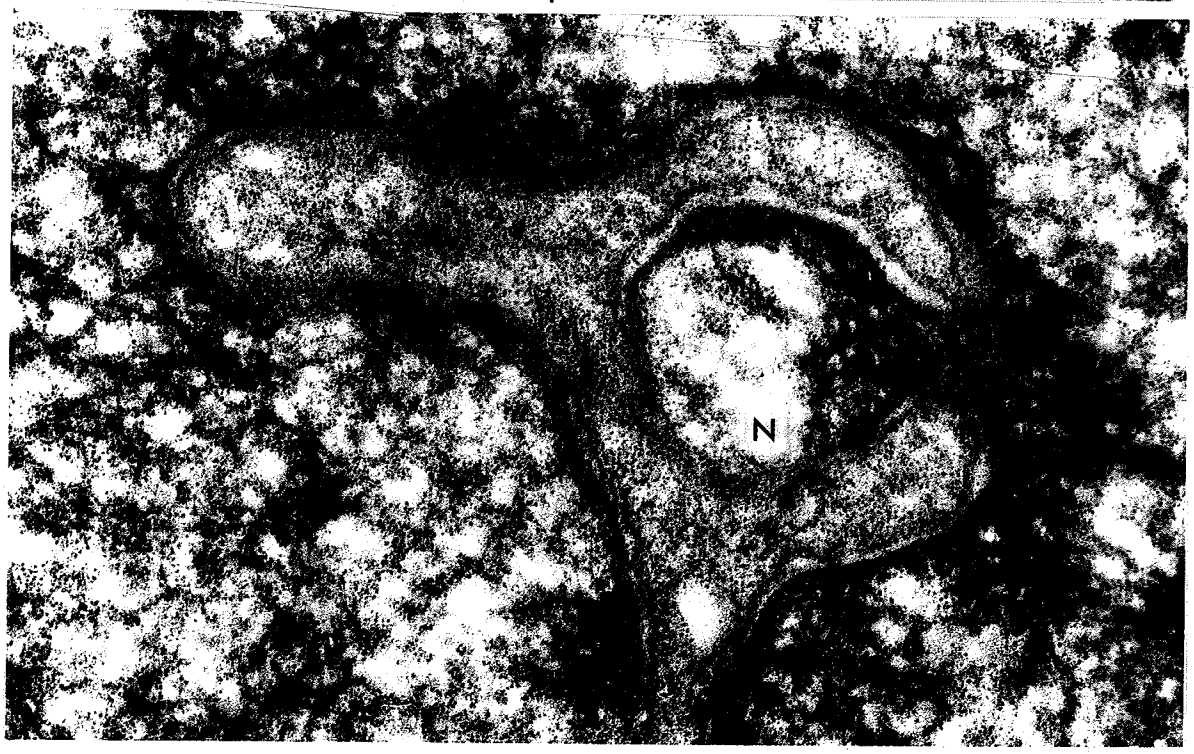


Figure 39. A vacuolated cell containing a nucleus with nuclear folds. Amnion 268, magnification 24,750x. The nuclear fold (nf) containing cytoplasm (p) extends into the center of the nucleus (n) and touches the nucleolus (nl). Lipid vacuoles (lv) appear to indent the nucleus.



40a



40b

Figure 40. Folds in the nuclear membrane.
a) Amnion 268, magnification 101,750x. This nuclear fold contains a mitochondrion (m).
b) Amnion 267, magnification 67,200x. The cytoplasm (c) in this fold appears to be in the process of pinching off a portion of the nuclear material (n).

packed, dark staining granules of up to 150 A in diameter. In some biopsies the nucleolus contains circular areas which were less dense than the granules and up to 0.12μ in diameter. These lighter areas were usually associated with cells which contained very vacuolated cytoplasm, as is illustrated in Figure 42a and b, page 110. The nucleolus, although usually in the middle of the nucleus, may be very eccentrically placed, touching the inner layer of the nuclear membrane at the end of a nuclear fold, Figure 39, page 106, or at the outer edge of the nucleus as in Figure 41, page 109. In one case, a nucleolus was seen in the cytoplasm, Figure 42a, page 110. A closer view of this nucleolus in Figure 42b, page 110, shows the typical structure with four lighter staining areas, with the whole body being surrounded by a unit membrane which it does not possess when inside the nucleus.

The cytoplasm. The smallest and one of the more abundant measurable entities in the cytoplasm of the amnion cell is the ribosome, which is dark staining and up to 200 A in diameter (Figure 37, page 103). Many fibrils of varying length and about 110 A in diameter may be seen throughout the cytoplasm in differing linear and circular arrangements as illustrated in Figures 37 and 43, pages 103 and 111. Small vacuoles of up to 500 A, surrounded by a unit membrane, are usually present in large numbers throughout the cytoplasm, but are especially

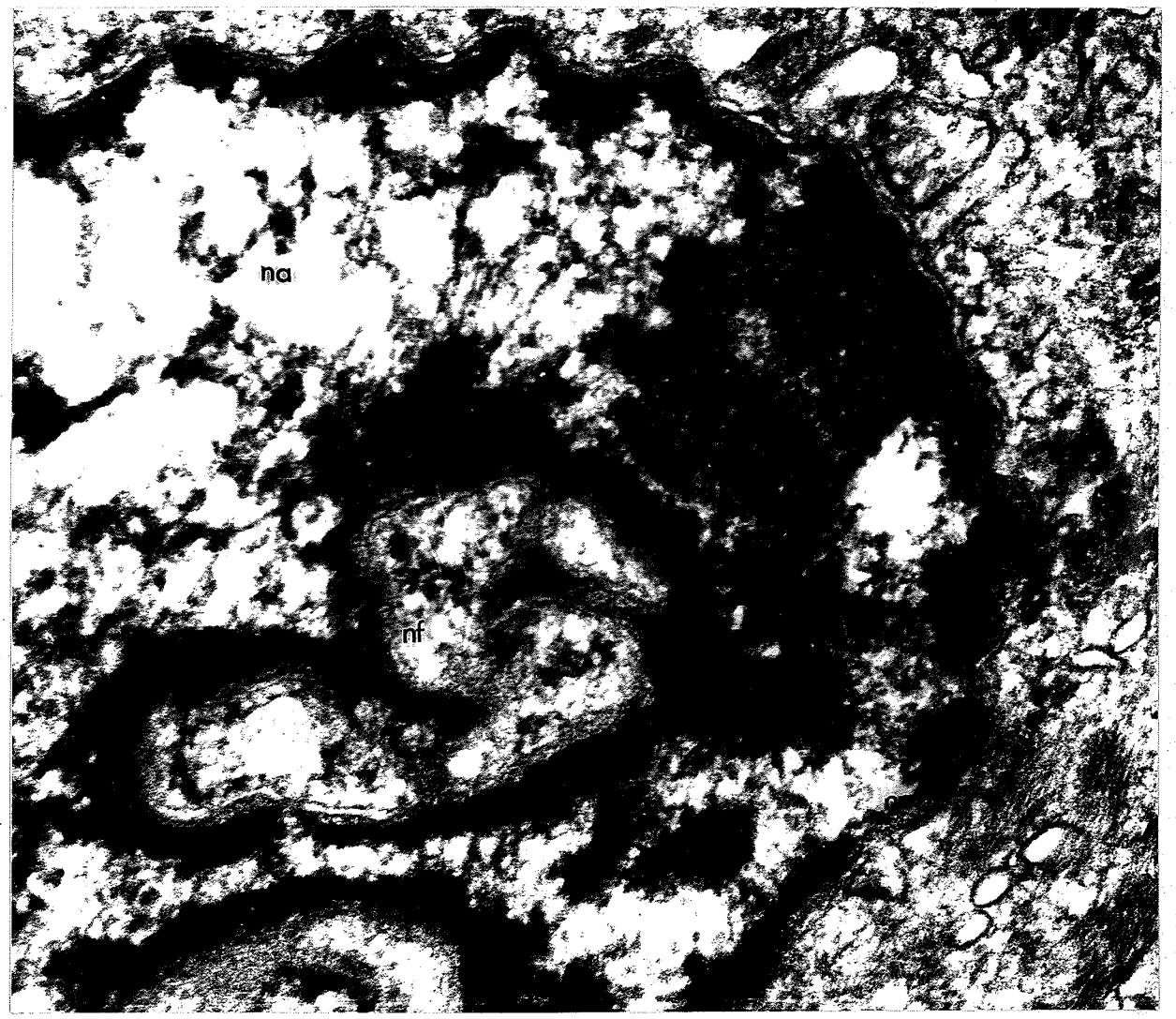
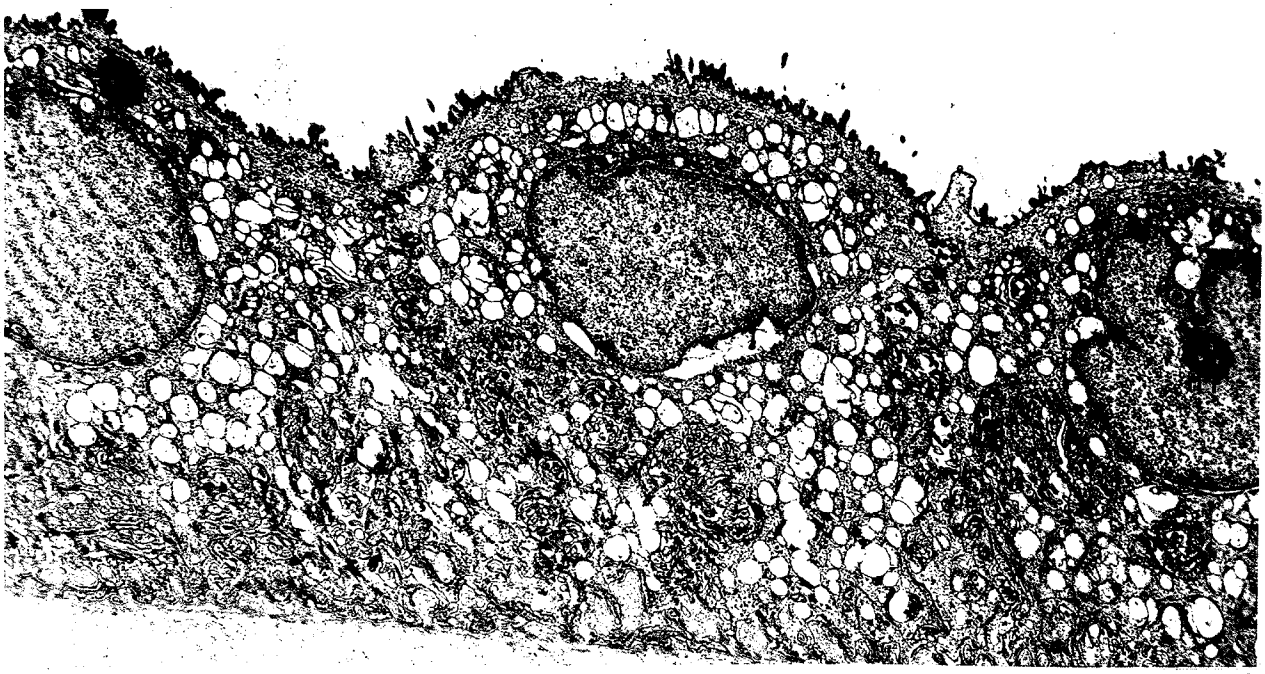
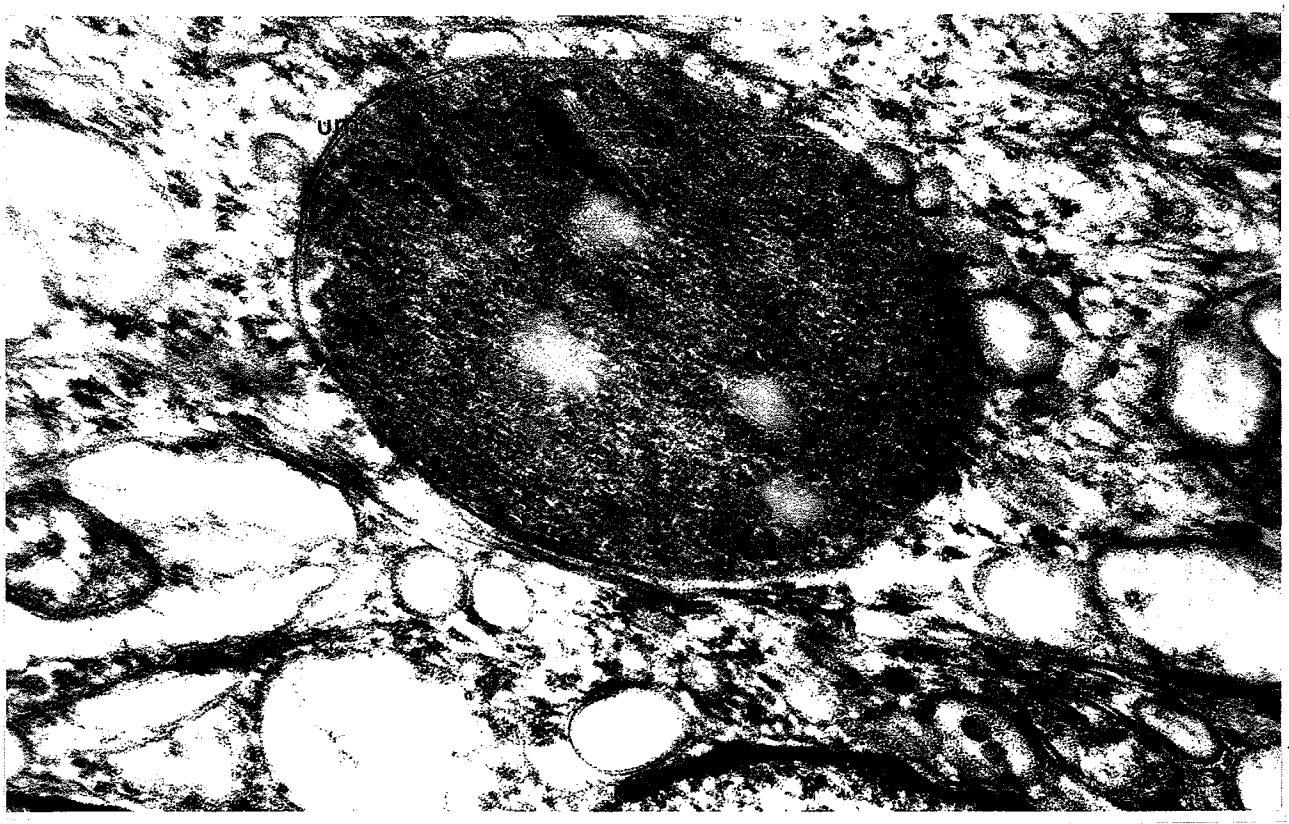


Figure 41. A nucleolus touching the nuclear membrane. Amnion 10-66, magnification 48,000x. The nucleolus (nl) is typical in appearance and contains a light area near its center. A portion of a nuclear fold (nf) is seen in the nucleolemma (na) which is surrounded by a nuclear membrane (nm).



42a



42b

Figure 42. A placental amnion biopsy of vacuolated, cuboidal cells and a nucleolus in the cytoplasm. Amnion 116-66.
a) Magnification 4,000x. The two nucleoli (nl) in this biopsy appear the same, except one is in the cytoplasm.
b) Magnification 51,000x. This cytoplasmic nucleolus is surrounded by a unit membrane (um) and contains the typical granular material of a nucleolus, plus four light areas.

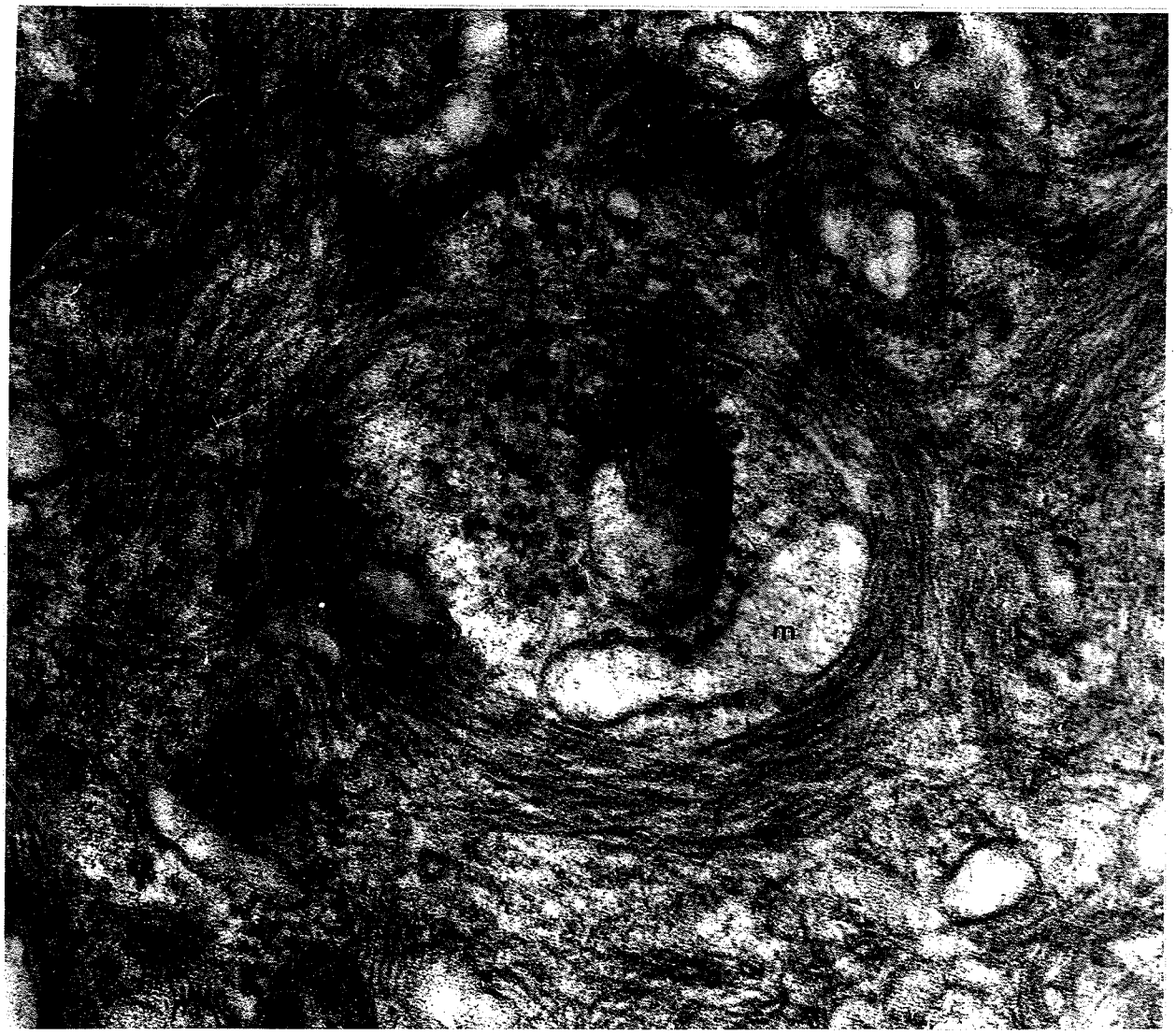


Figure 43. Cytoplasmic fibrils. Amnion 99-66, magnification 73,500x. The fibrils (f) are in circular and linear alignments, close to the nucleus (n) and surrounding what appears to be a mitochondrion (m).

prevalent around the nuclear membrane, the areas of the Golgi complex, and the periphery of the cell.

Mitochondria. Mitochondria are numerous in the human amnion cell, as seen in Figure 44, page 113, and are usually long, filamentous and frequently branching, as in Figure 45, page 114. Figure 46, page 114, shows the usual structure of the mitochondria which, when seen in a 90 mμ thick section, is an oval shape, of varying size, surrounded by a unit membrane, containing fine granular material and double-walled cristae mitochondriales, which often extend from one side of the mitochondrion to the other. Each electron dense layer of the cristae is about 88 mμ thick and separated from the other layer by a less dense area 114 mμ thick. Dilations at the tips of the free ends of some cristae are not uncommon. Figure 47, page 115, shows a normal mitochondrion beside a swollen one which, from time to time, can be seen in a great number of the mitochondria in some cells (Figure 44, page 113). The position of the mitochondria in the cell is not limited to any one area, but is frequently in the perinuclear area and deep in the basal processes. The width of the mitochondria is usually about 0.5μ, which is the same measurement as that in living cells as seen through the phase-contrast microscope.

The Golgi complex. The Golgi complex is usually perinuclear and quite readily seen in its undilated form in relatively

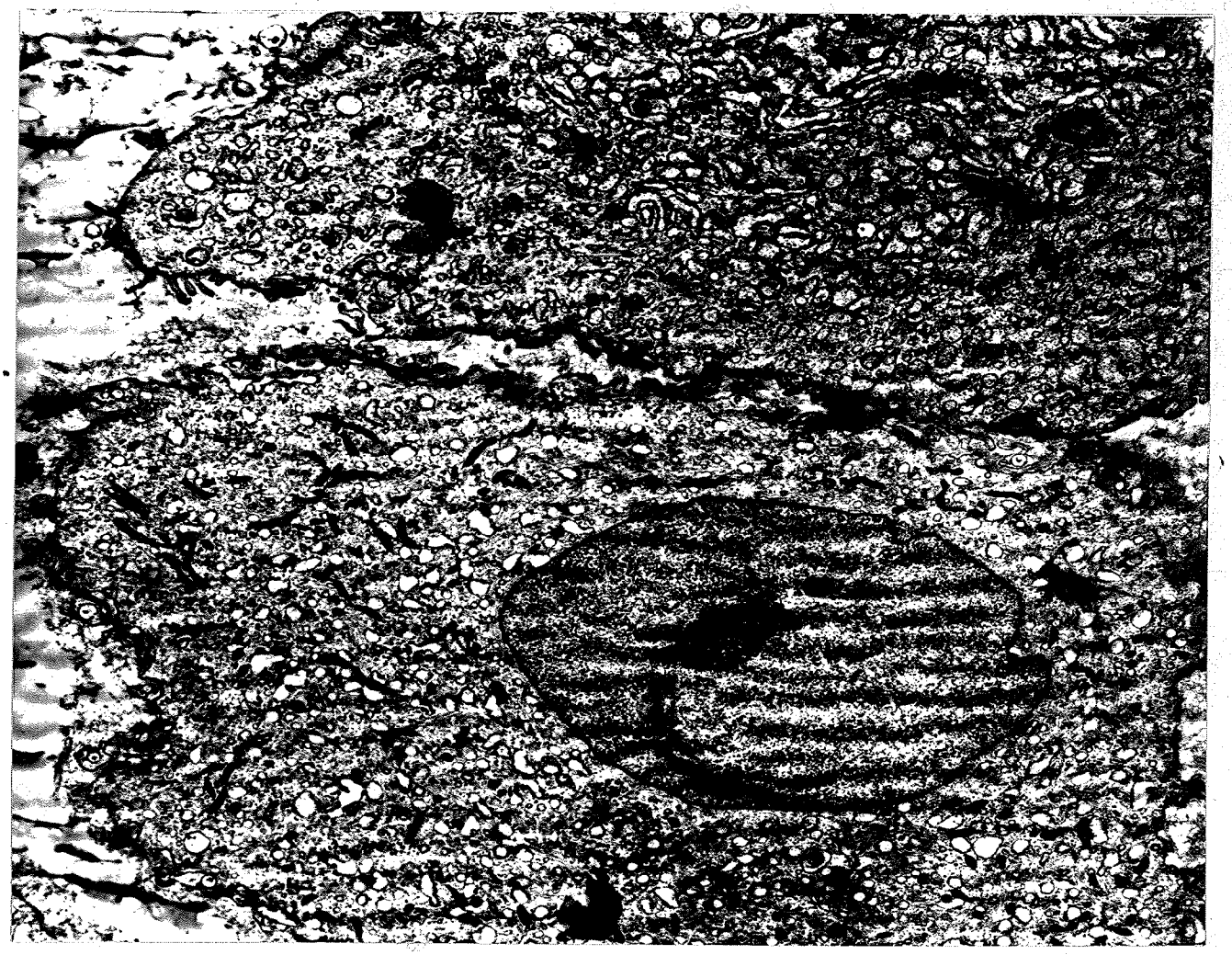


Figure 44. A biopsy of reflected amnion containing many mitochondria and much dilated endoplasmic reticulum. Amnion 116-66, magnification 5,500x. The long thin mitochondria in cell "A" are very dark, while in cell "B" they are quite swollen in many areas. The dilated endoplasmic reticulum is more abundant in cell "B" than in cell "A".

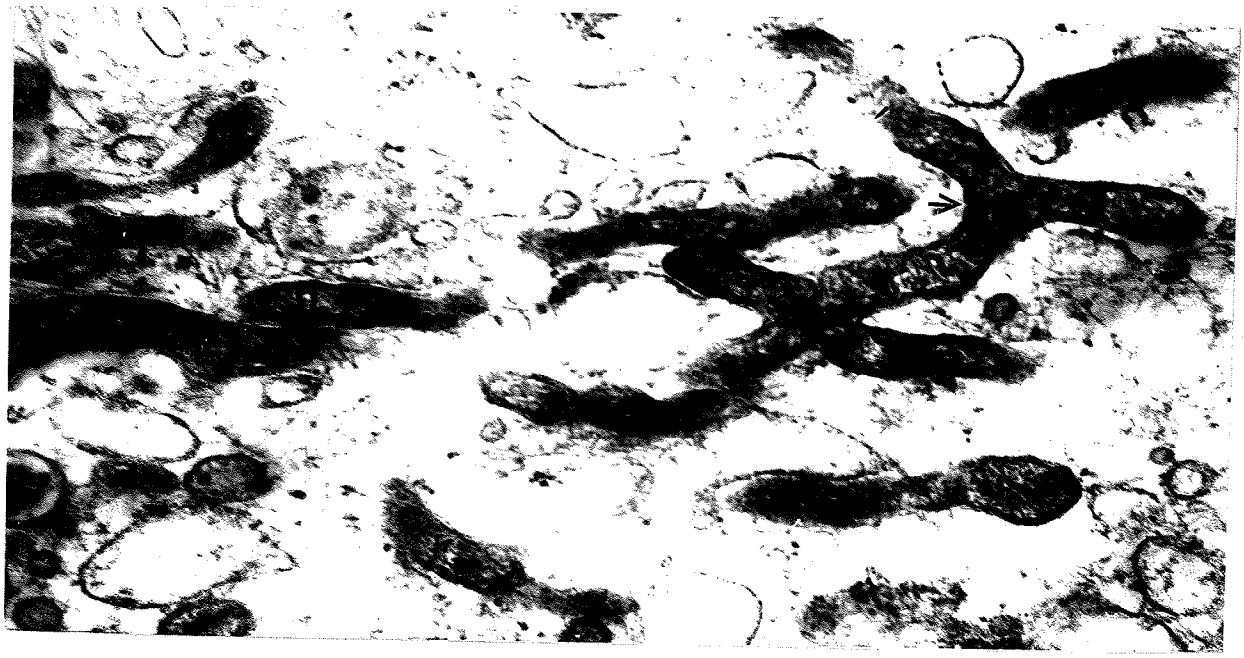


Figure 45. Filamentous mitochondria. Amnion 166-66, magnification 67,000x. The mitochondria (m) are branched (arrow), long, thin and form a kind of network.

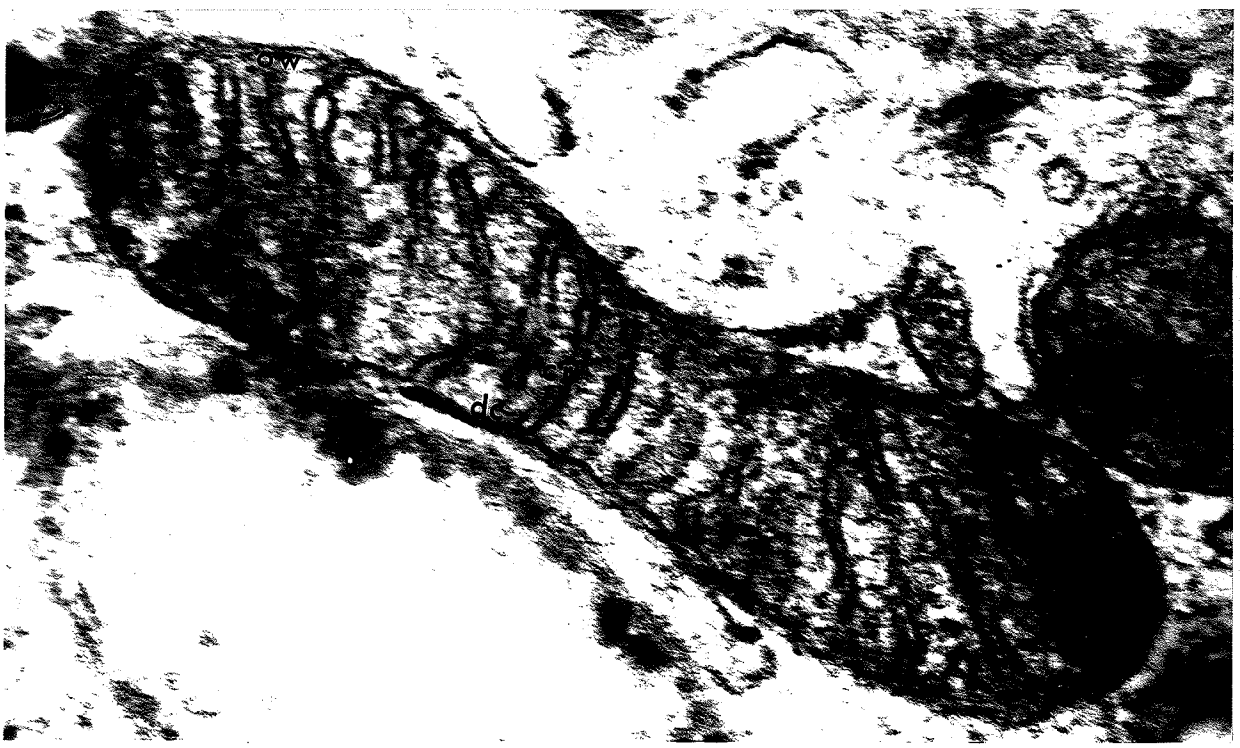


Figure 46. A typical mitochondrion. Amnion 166-66, magnification 67,000x. The outer wall (ow) of the mitochondrion is double-layered, as are the cristae (cr). A dilation (dc) at the end of one of the cristae is noted.

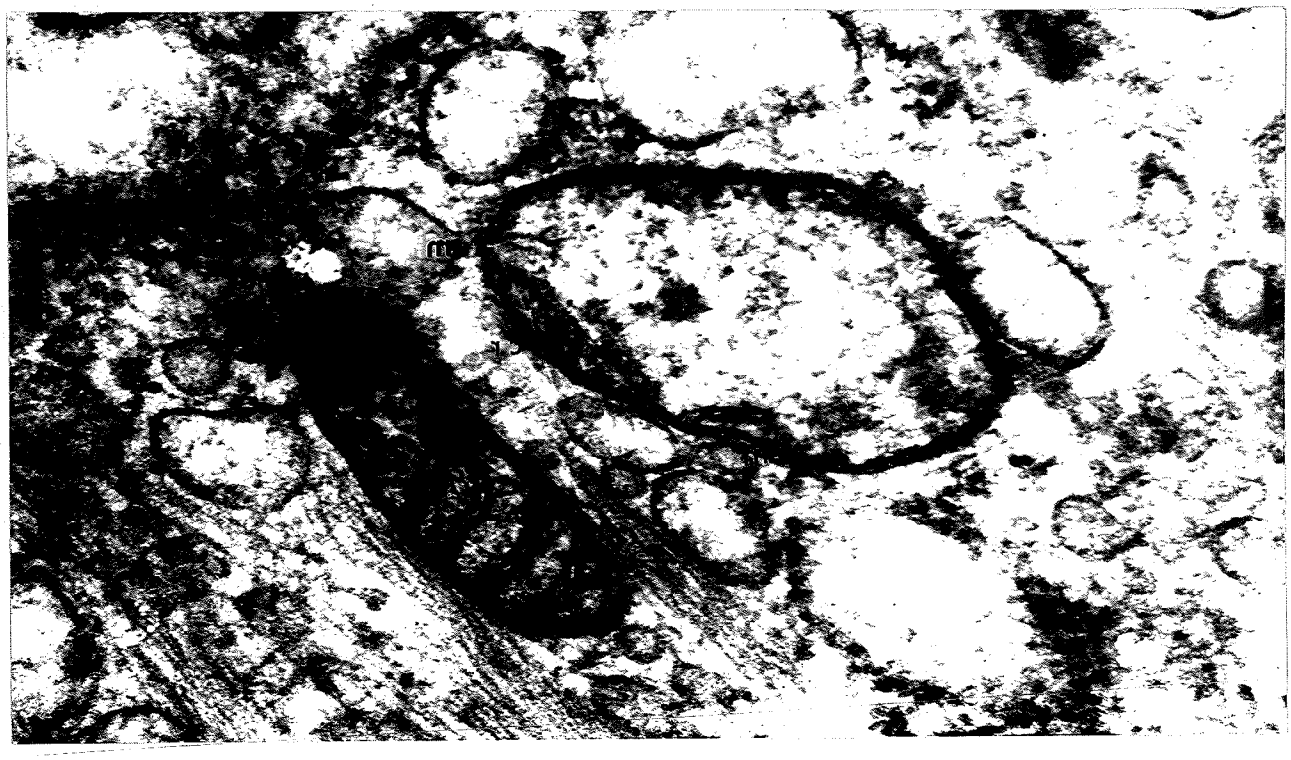


Figure 47. Normal and swollen mitochondria. Amnion 116-66, magnification 67,300x. The normal mitochondrion (m) is unremarkable except for its proximity to a swollen mitochondria (sm), which can be identified only by the few remaining cristae (cr).

nonvacuolated cells. It consists of elongated, flattened, laminated areas of unit membranes of over 1.3μ long and 0.012μ thick, the tips of which approximate tiny vacuoles as seen in Figure 48, page 117. The dilated Golgi may be seen in Figure 49, page 118, which also illustrates the proximity of the Golgi complex to the mitochondria and the dilated rough endoplasmic reticulum. In cells with very large lipid vacuoles, the Golgi complex is usually seen very close to the vacuoles (Figures 48 and 54, pages 117 and 124).

The endoplasmic reticulum. The endoplasmic reticulum of the human amnion cell is usually of the rough or granular type, having ribosomes of up to 200 A in diameter attached to its triple-layered α-cytomembrane (Sjostrand, 1964). The dilated cisternae of endoplasmic reticulum contains a fairly granular, moderately electron dense material, as seen in Figures 50 and 51, pages 119 and 120. The dilated endoplasmic reticulum may be seen throughout the cell (Figures 30 and 34, pages 93 and 99), often very close to the nucleus and frequently touching the large lipid vacuoles as seen in Figures 51 and 54, pages 120 and 124.

The cytoplasmic lipid vacuoles. Large cytoplasmic vacuoles, measuring up to four microns in diameter, are usually found in a perinuclear position, and often indent the nucleus,

Figure 48. The Golgi complex. Amnion 6-66, magnification 44,000x. The undilated Golgi complex (gc) may be seen in three areas, one touching a lipid vacuole (lv), and the proximity of these structures to the nucleus (n) is noteworthy.

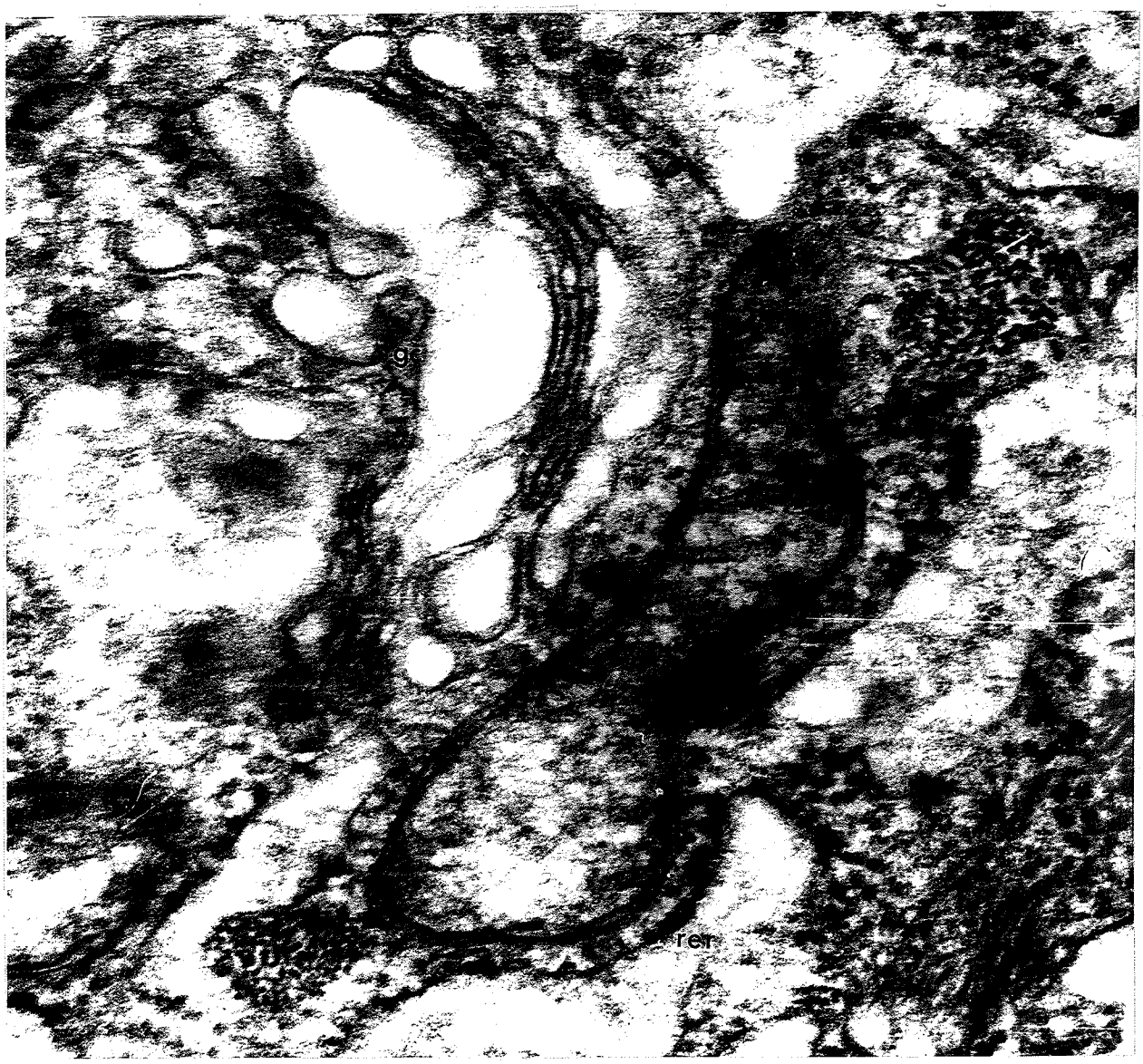


Figure 49. A dilated Golgi complex. Amnion 112-66, magnification 80,500x. The dilated Golgi complex (gc) is close to a mitochondrion (m) and both are close to areas of dilated rough endoplasmic reticulum (rer).

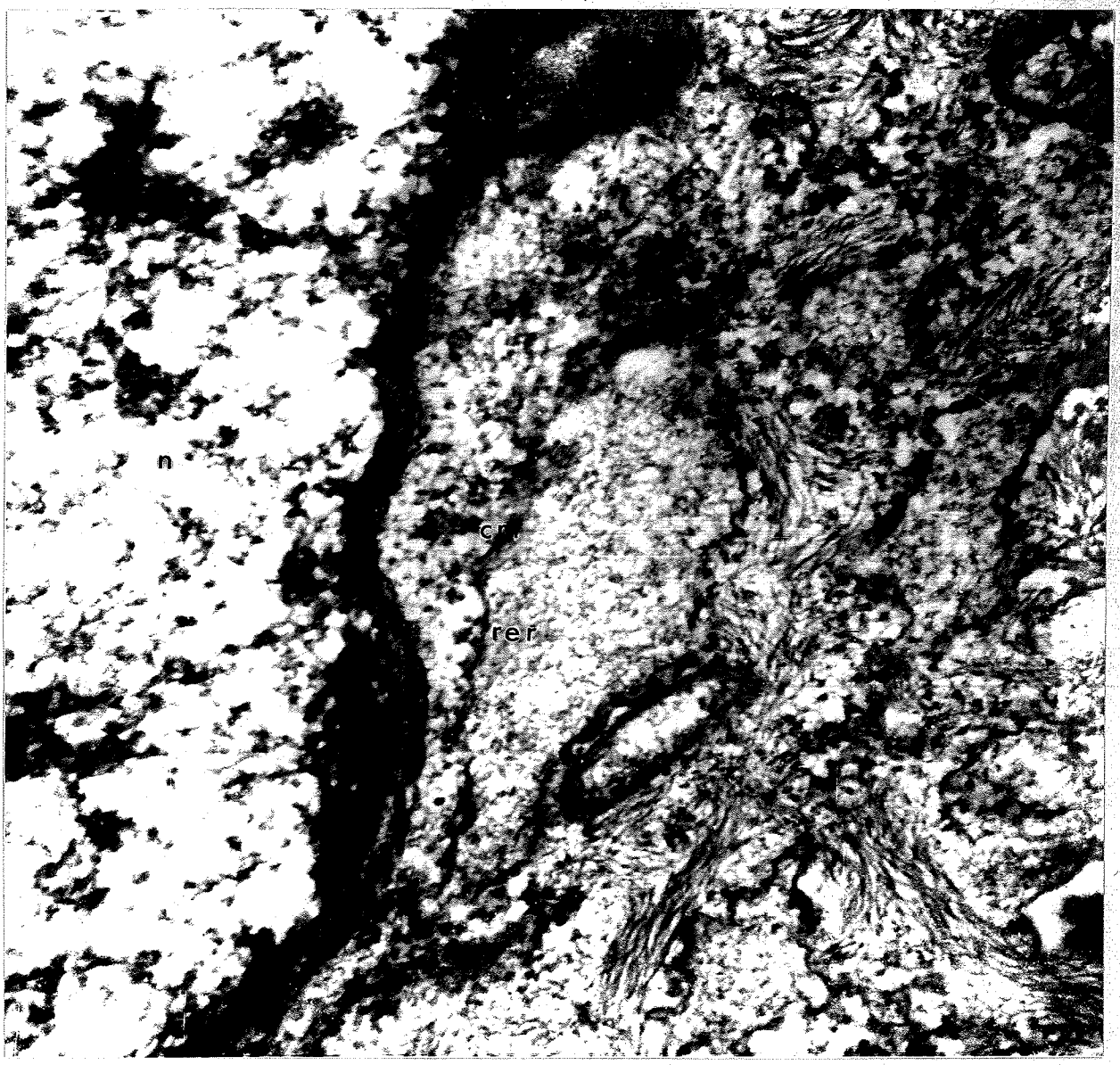


Figure 50. Dilated rough endoplasmic reticulum. Amnion 6-66, magnification 50,400x. A granular material is seen within the α-cytomembrane (cm) of the dilated rough endoplasmic reticulum (rer), of which several areas are present near the nucleus (n).

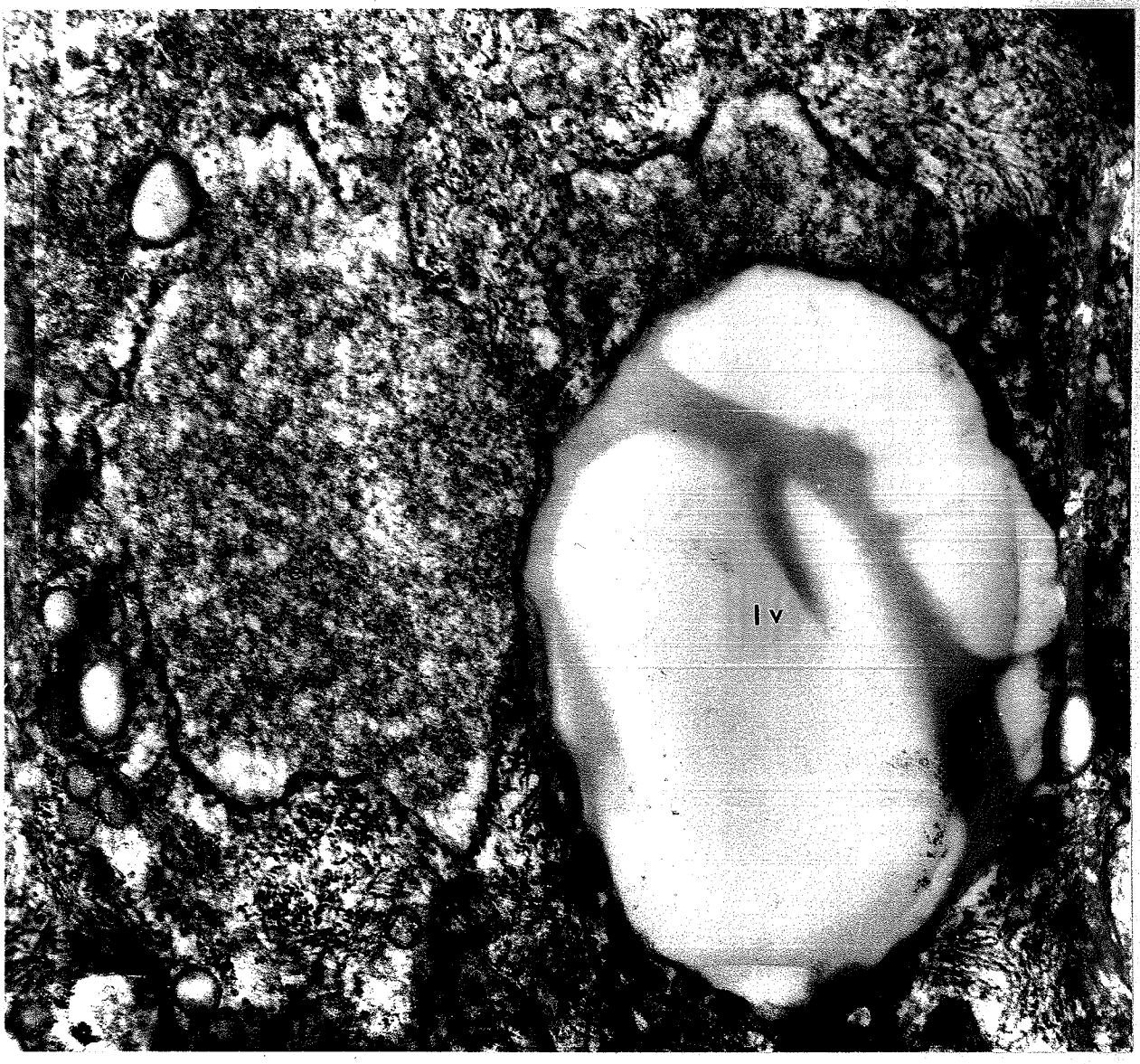


Figure 51. Rough dilated endoplasmic reticulum associated with a lipid vacuole. Amnion 108-66, magnification 39,200x. The lipid vacuole (lv) appears to be in direct contact with two different areas of dilated rough endoplasmic reticulum (rer) which contain a moderately dense granular material. Considerable retraction of the vacuolar material has occurred.

Figures 33, 35 and 39, pages 97, 100 and 106. They are usually surrounded by a doubled triple-layered membrane which resemble unit membranes, Figure 52, page 122, but where the lipid stains very heavily the inner layer is obliterated, Figure 53, page 123. The contents of these vacuoles have the smooth and light staining characteristics of saturated lipids in osmium tetroxide (Fawcett, 1966), but in many cases the lipid retracts, producing large clear areas in the vacuole contents. Figure 54, page 124, shows two lipid vacuoles slightly indenting the nucleus and in direct contact with both a dilated Golgi apparatus and dilated endoplasmic reticulum. These vacuoles are often so large and numerous that they take up a very large part of the cell, similar to the picture seen in vacuolated cells viewed through the phase-contrast microscope.

The lysosome. A lysosome is shown in Figure 55, page 125, situated near a dilated intercellular canal. Its contents of granular material, a portion of laminated triple-layered membrane and a small vacuole are enclosed in a unit membrane. These bodies were seen rarely in the series of biopsies viewed for this study.

The ultrastructure of trypsinized amnion cells.

Figure 56, page 126, illustrates that there are few differences between trypsinized and nontrypsinized human amnion cells, except for the absence of the basement membrane and its

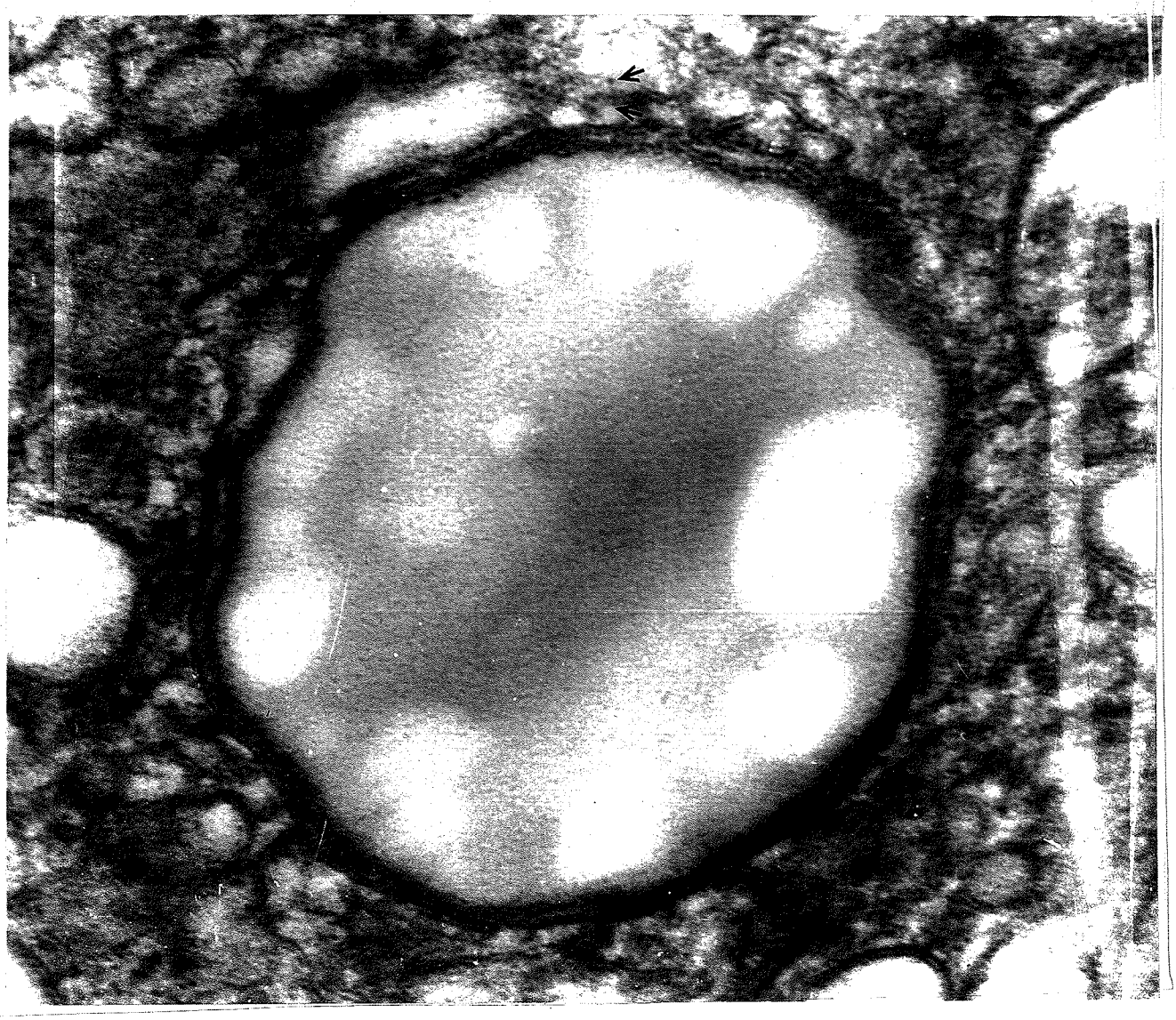


Figure 52. A lipid vacuole with a doubled, unit membrane. Amnion 108-66, magnification 119,000x. The two layers of unit membranes are indicated by arrows. Some retraction of the lipid content of the vacuole has occurred, producing lighter areas in the contents near the inner membrane of the lipid vacuole.

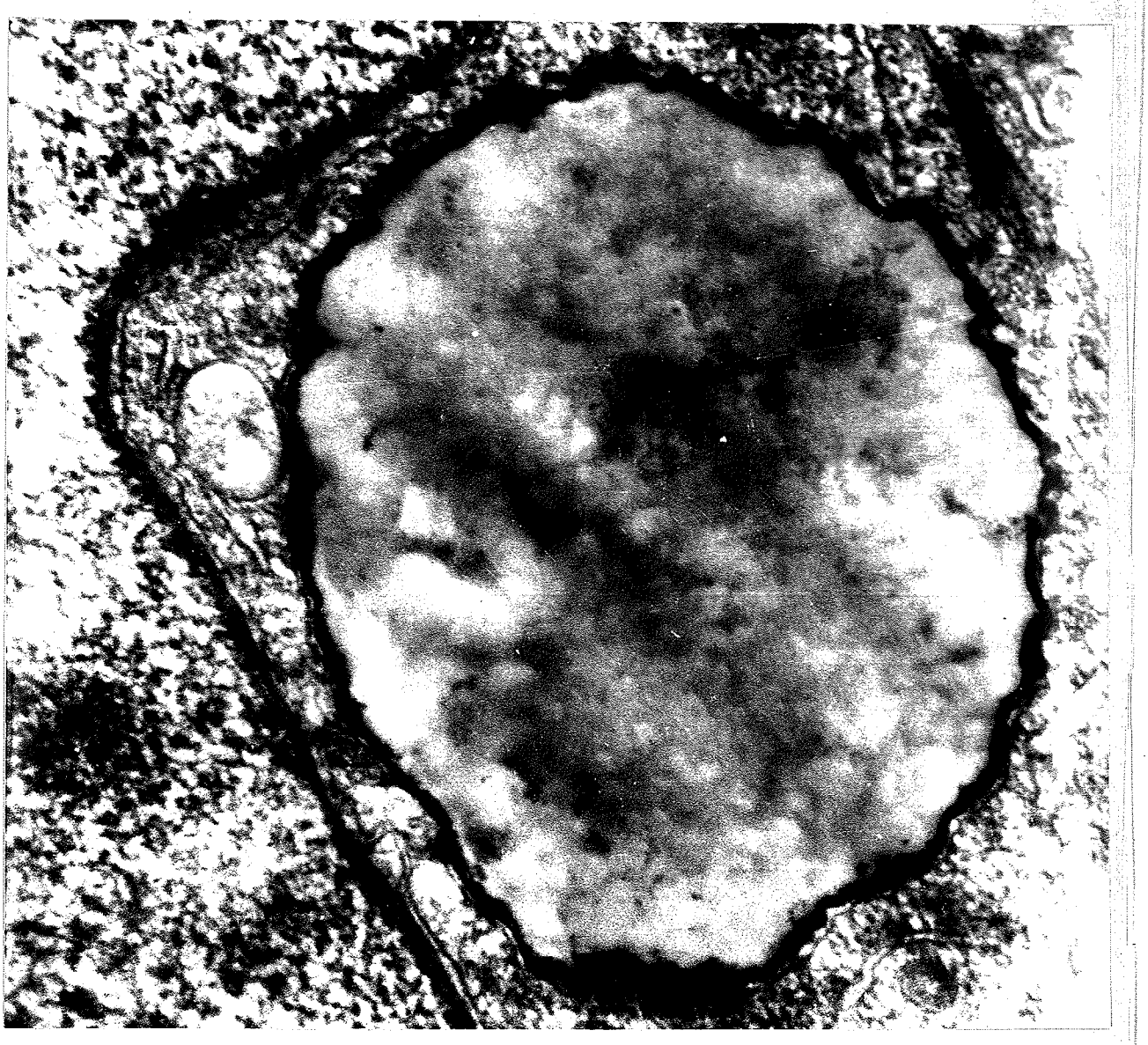


Figure 53. A lipid vacuole close to the nuclear membrane. Amnion 83-66, magnification 64,000x. The contents of this vacuole stained irregularly and the inner unit membrane is obliterated by a typical dark staining inner ring (ul) of unsaturated fat.

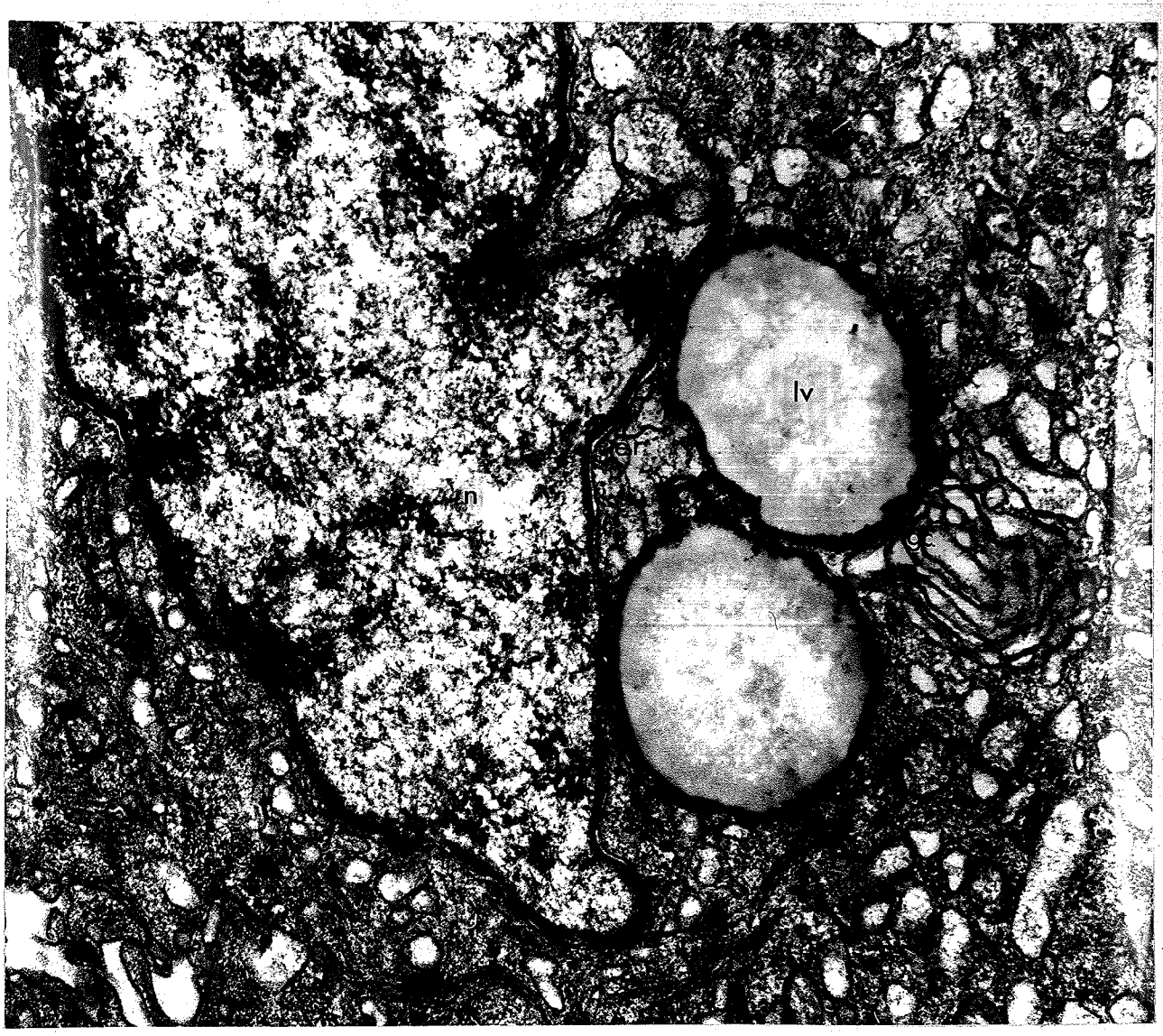


Figure 54. Lipid vacuoles and related structures. Amnion 267, magnification 28,800x. The lipid vacuoles (lv) are close to the nucleus (n), dilated endoplasmic reticulum (er), the dilated Golgi complex (gc) and mitochondria (m).

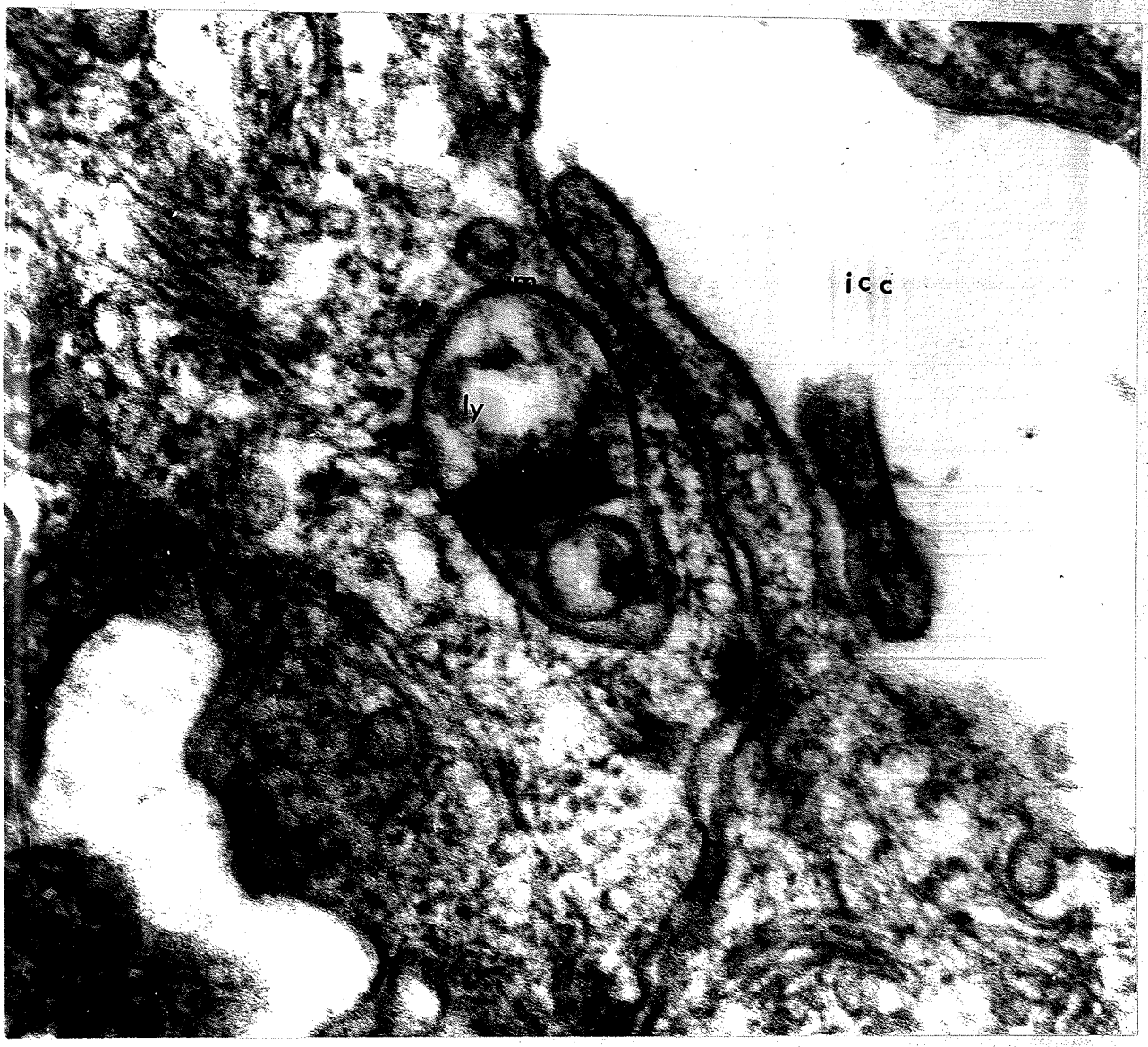


Figure 55. A lysosome. Amnion 108-66, magnification 84,000x. Surrounded by a unit membrane (um) and containing a small vacuole (v) and laminated membrane material (lm), the lysosome (ly) is situated next to a dilated intercellular canal (icc).

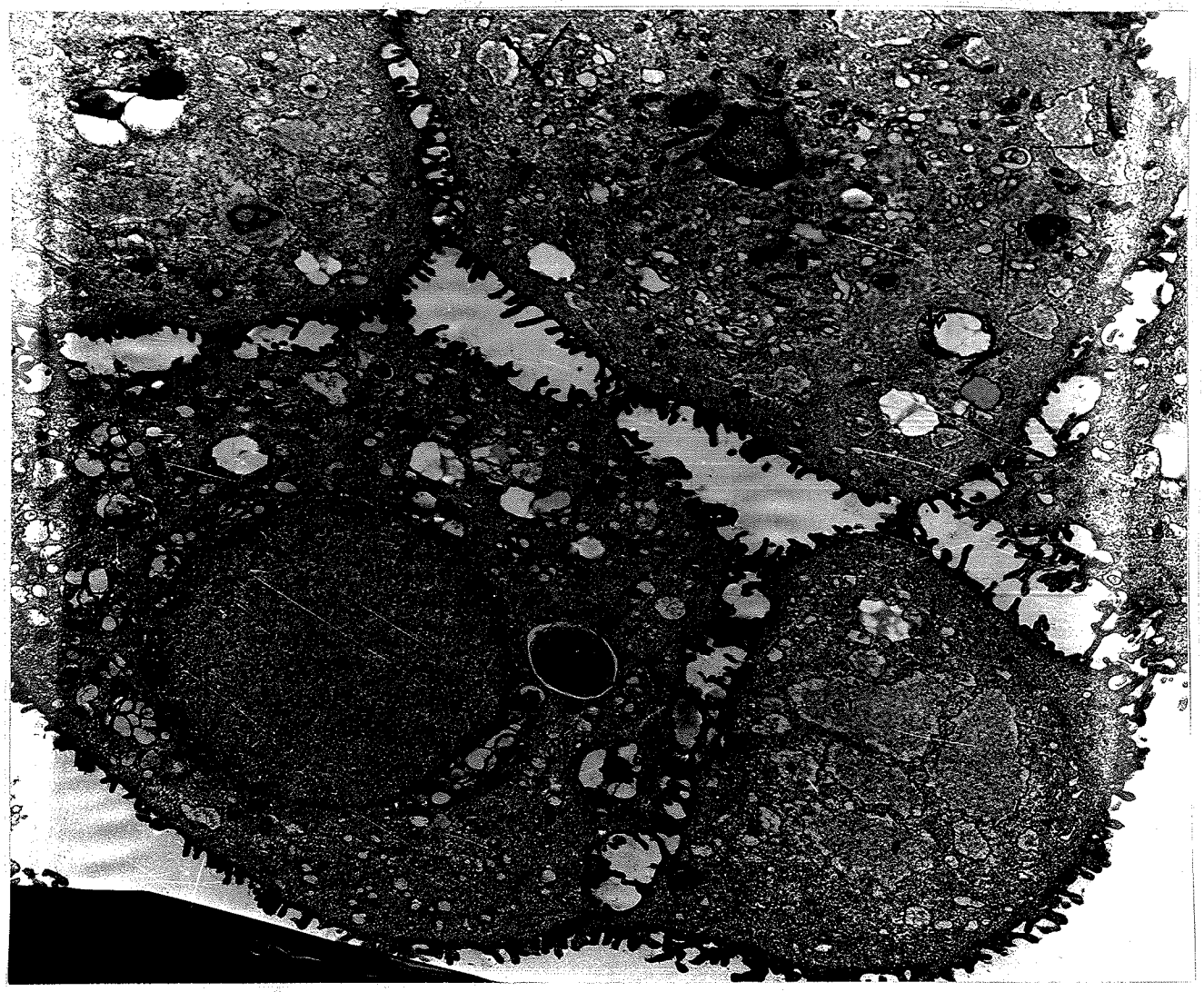


Figure 56. Trypsinized cells sectioned from a pellet. Amnion 108-66, magnification 6,875x. Most of the features of biopsy cells are present, such as microvilli (mv), desmosomes (d), nuclei (n), mitochondria (m), lipid vacuoles (lv), a myelin laminated body (mb) and in some cases very widely dilated endoplasmic reticulum (er).

appendages. These cells are not completely separated and contain some desmosomes, but are otherwise typical of properly trypsinized cells and show the features of the cells seen in biopsies. Other trypsinized cells are seen in Figure 57, page 128, where the cells are completely separated, and a portion of one of the cells is bulging outward in large blebs, probably due to excessive trypsin. These cells have lost a portion of their membranes and have become extremely lacey in appearance, probably due to the dilation of the Golgi and endoplasmic reticulum. Little recognizable normal structure remains, except for one or two large lipid vacuoles and the nuclei with nucleoli. The nuclei shown are less dense, with one showing a stalked projection of nuclear material which may be a nuclear bleb.

Myelin laminated bodies (Shultz, 1959) may be seen occasionally in biopsy and trypsinized cells, and an example of one of these bodies may be seen in Figure 58, page 129. This body appears to be composed of a coiled unit membrane about 150 A thick and very similar to the myelin sheath present around nerve fibers (Fawcett, 1966).

The ultrastructure of human amnion monolayer cells.

Many difficulties were encountered in obtaining sections of monolayer cells from the coverslips of Rose chambers, however some electronmicrographs of fair quality were made. The monolayer cell which was in vitro for one week, Figure 59, page 130, is surrounded by a unit membrane, has very few microvilli,

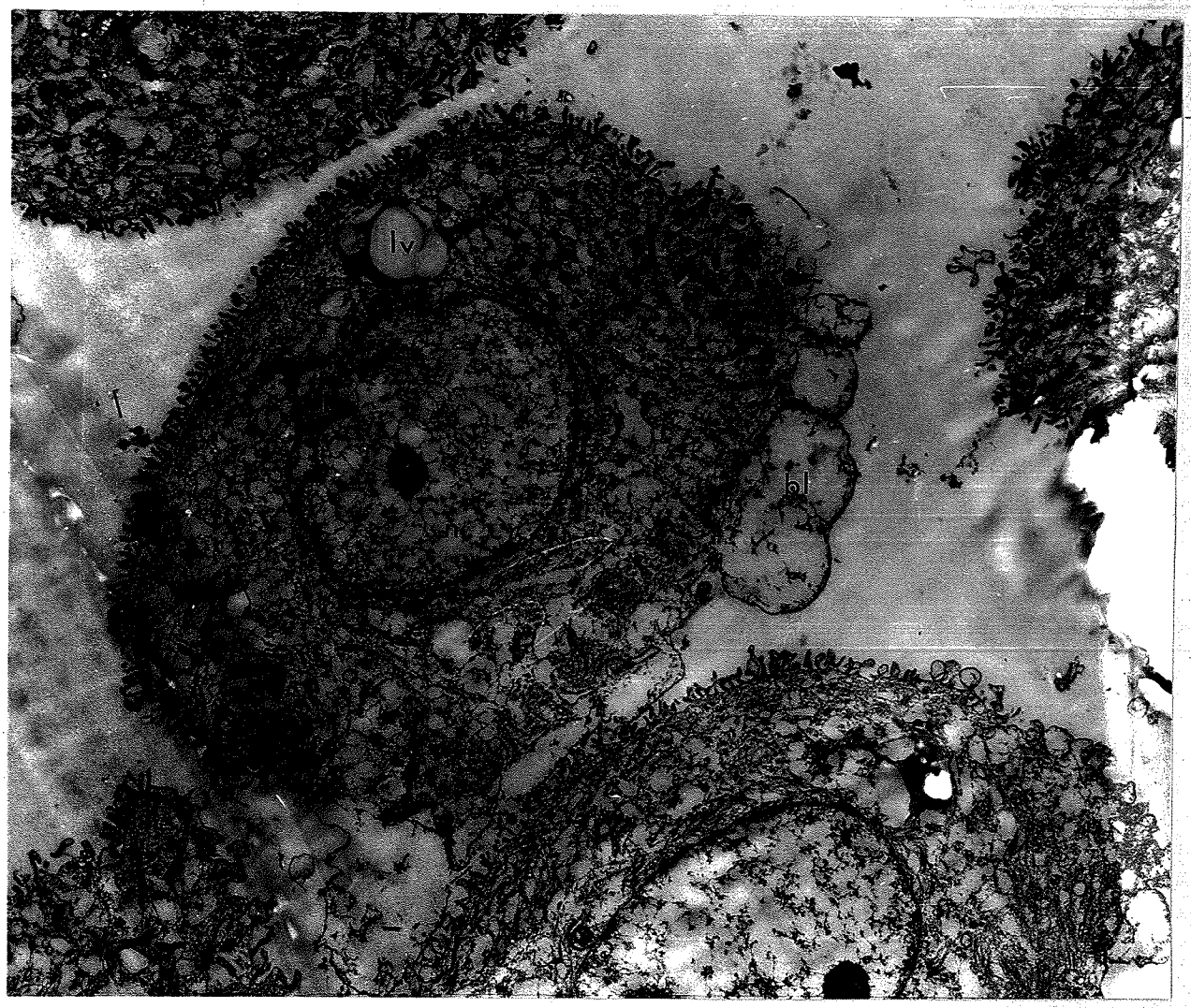


Figure 57. Trypsinized cells sectioned from a pellet. Amnion 4-66, magnification 5,500x. The cells show some loss of the unit membrane, with large blebs (bl) appearing in those areas of the cell surface. The cytoplasm is very vacuolated, and little normal anatomy can be seen except for a few lipid vacuoles (lv) and nuclei (n) with nucleoli (nl). One nucleus has a nuclear bleb (nb) which may be related to those seen in Figure 26a-f, page 85.

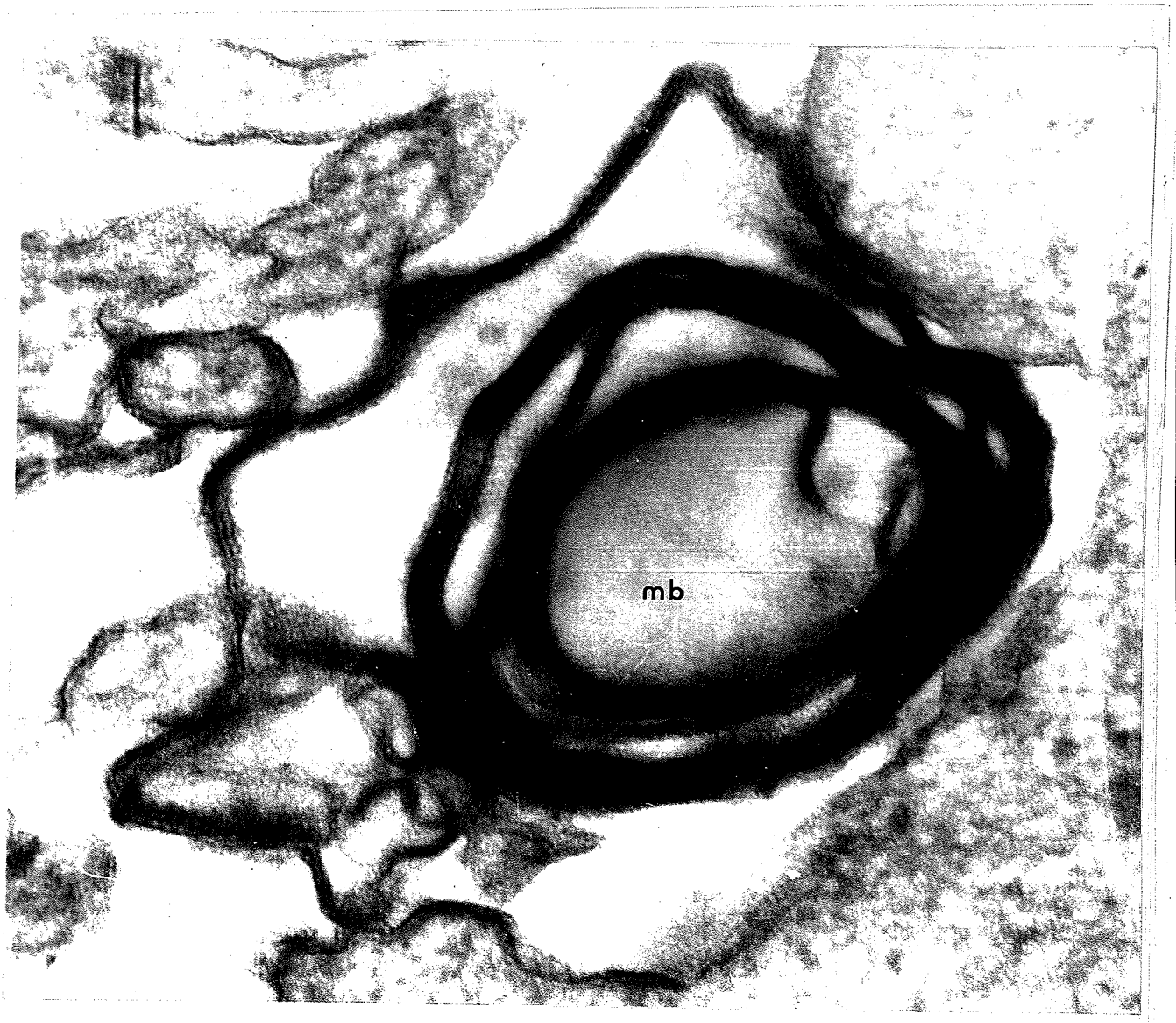


Figure 58. A myelin laminated body. Amnion 270, magnification 147,000x. This body (mb) was seen on the outer surface of a trypsinized cell.

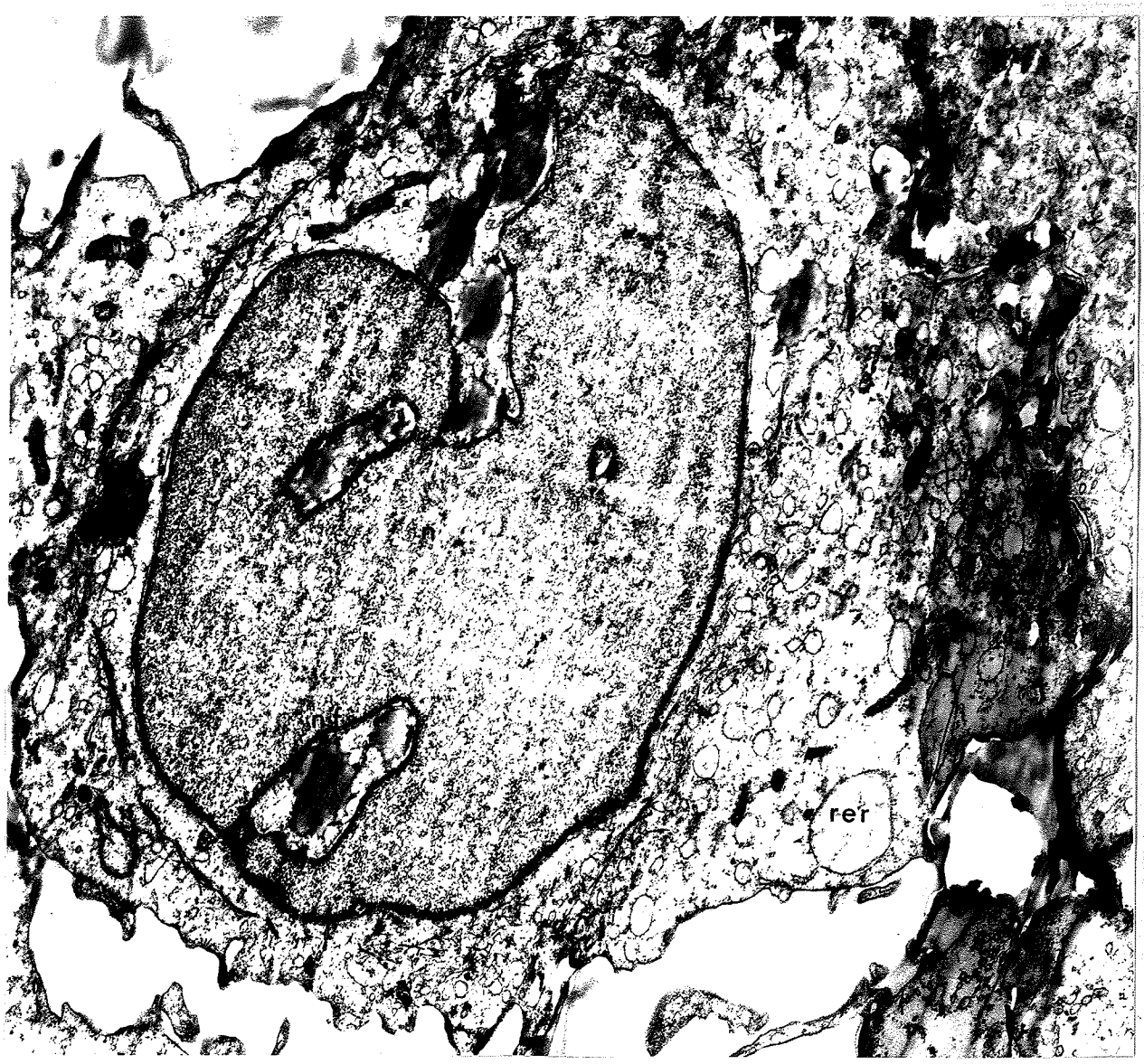


Figure 59. A monolayer cell after one week in vitro. Amnion 137-66, magnification 13,475x. This portion of the cell contains a normal appearing nucleus (n), with lipid vacuoles (lv) in its folds (nf). Some fibrils are seen in the perinuclear position, as are some irregularly shaped mitochondria (m) and areas of very dilated rough endoplasmic reticulum (rer) with little stained contents. The surface of the cells have very few microvilli (mv) and no desmosomes are seen at the intercellular canals (icc).

and those which are present appear to interdigitate with a neighboring cell, similar to those seen in biopsies, but without the desmosomes. The nucleus is unremarkable, except that in one of the cells some contracted lipid vacuoles are seen in the nuclear folds, best illustrated by Figure 60, page 132.

The cytoplasm shows a marked decrease in density compared with the cells in biopsy material due to the sparsity of granules, very small perinuclear vacuoles, and an almost complete lack of fibrils, except in the perinuclear area. Some normal appearing mitochondria are present, as well as many widely dilated areas of rough endoplasmic reticulum, with very little staining material present in them. A few lipid vacuoles are seen in the cytoplasm, but no areas of the Golgi complex can be identified.

All membrane measurements appear similar to those of the cells seen in biopsies, except for the lipid vacuoles, where it is impossible to detect a unit membrane. This may be a result of the marked contraction of the lipid material or due to the change in environment.

Figure 61, page 133, is an electronmicrograph of a two week monolayer cell showing the presence of microvilli, dilated intercellular canals and many perinuclear lipid vacuoles. This is a typical appearance for older monolayer cells, with the vacuoles packed tightly in the cytoplasm.

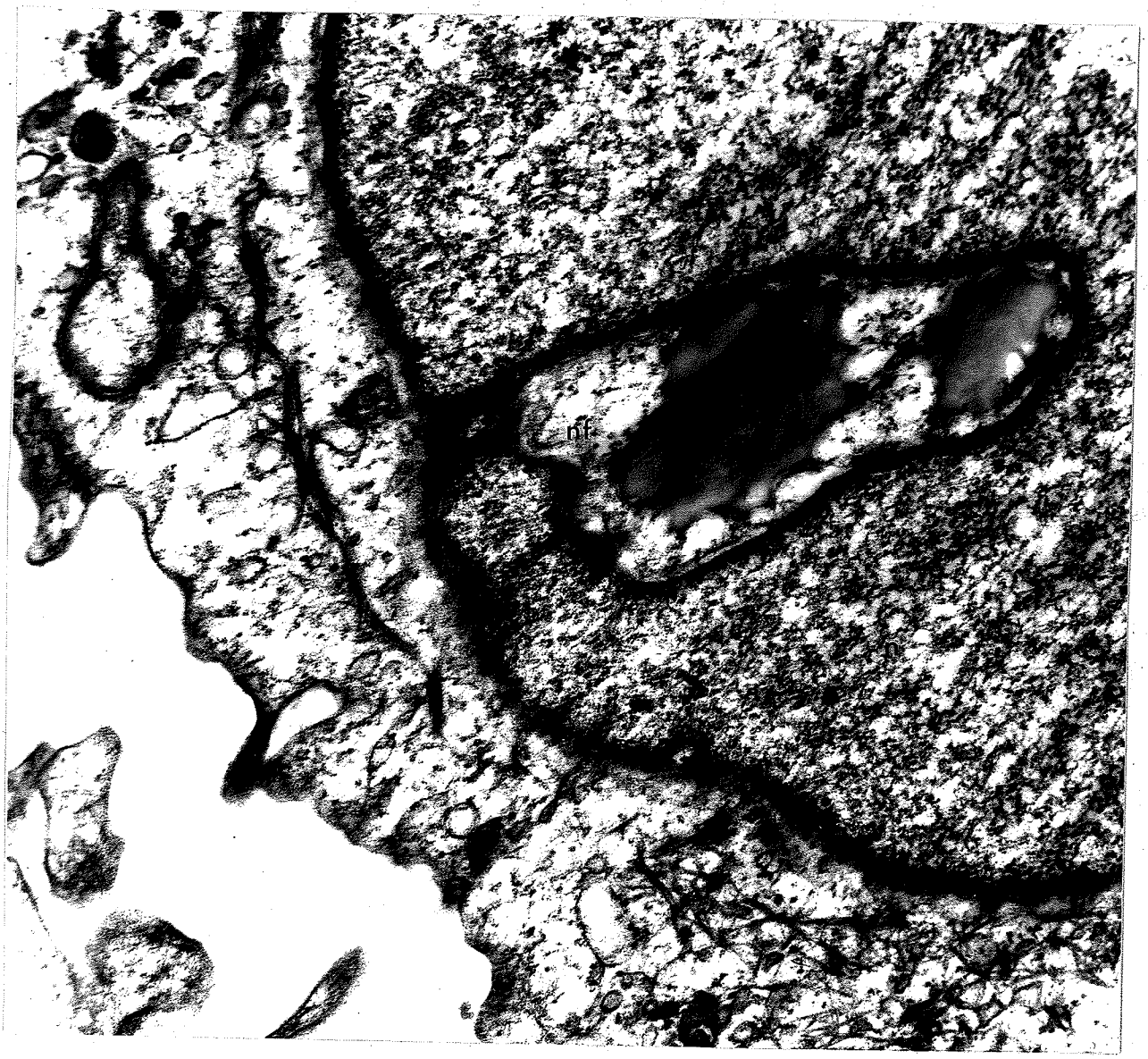


Figure 60. The nucleus of a monolayer cell. Amnion 137-66, magnification 33,000x. Lipid vacuoles (lv) are in the nuclear folds (nf) and a unit membrane cannot be seen on the outer surface of the vacuoles. The nucleus (n) appears similar to the nuclei in cells from biopsies.

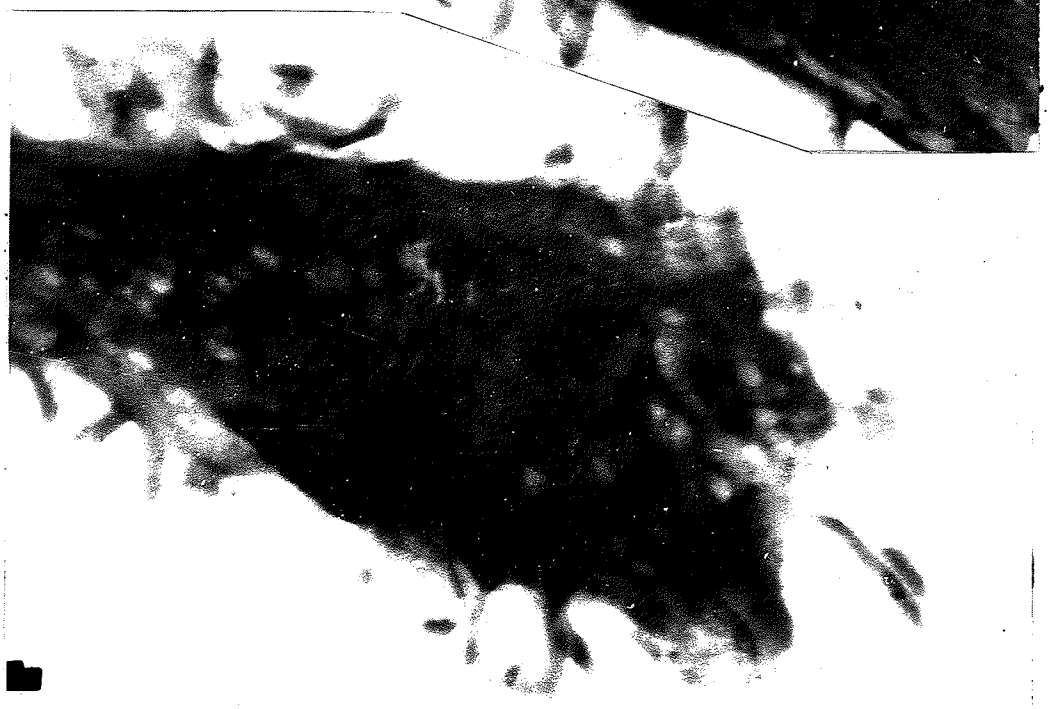
Figure 61. Monolayer cells after two weeks in vitro. Amnion 67-66, magnification 5,800x. Lipid vacuoles are packed in the perinuclear area, and microvillus processes (mvp) are present in the dilated intercellular canal (icc) separating the neighboring cells. Vacuoles are also present in the small portion of a cell process (p) and microvilli (mv) are present on the surface of the cell.

A cell process from a different cell in the same Rose chamber is depicted in Figure 62a to c, page 135. The inset, Figure 62a, shows the entire process with microvilli on its surface, a triangular-shaped tip and a stalk of uniform diameter, 0.7μ, except for the broadening at the base and tip. The electronmicrographs of greater magnification, Figure 62b and c, show that the stalk is composed of long, tightly packed fibers which originate in the cytoplasm and end within a curved shape in the tip which also contains what are probably pinocytotic vacuoles.

In summary, the ultrastructure of the human amnion cell has been described as seen in biopsy material, pellets of trypsinized cells and in the monolayer cells from tissue cultures grown in Rose chambers.

62a

62b



62c

Figure 62. A cell process in a monolayer cell after two weeks in vitro. Amnion 67-66.
a) Magnification 3,500x. The entire process with the areas (b) and (c) demarcated.
b) Magnification 42,000x. Microvilli (mv) are present on the surface and on the inner aspect fibrils (f), which originate in the cell body, extend along the process.
c) The end of the process contains the curved ends of the laminated fibrils (cf) small vacuoles (v) in the cytoplasm and microvilli on the surface.

CHAPTER V

DISCUSSION

CHAPTER V

DISCUSSION

Introduction

The discussion of results will be divided into four sections: 1) the cine-phase results, 2) the ultrastructure observations, 3) those findings which can be related to both cine-phase and ultrastructure studies, and 4) the consideration of an hypothesis of the function of human amnion cells *in vivo* and *in vitro*. It is hypothesized that the amnion cell is both absorptive and secretory, producing a lipid material which may include steroid hormones. Where possible, the various aspects of the results will be discussed under headings similar to those used in chapter IV.

I. CINE-PHASE

Cell suspensions to monolayers

The response of various cell types in tissue culture. The results presented concerning the system of typing amnion cells, show a difference in the development of monolayers between type "1" and "2" cells and type "3" cells. Types "1" and "2" cells develop into monolayers more readily than type "3" cells, thus supporting the belief that type "1" cells are more desirable for tissue culture (Wilt and Brambilla, 1966). The cine-phase

technique appears to be ideally suited for this type of study, and further work may provide more definitive results.

Morphology of amnion cells developing into monolayers. This is the first record of the events occurring in amnion cells developing into monolayers. The primary human amnion cell develops in a similar way to other cells in tissue culture (Gey, 1954), but the more significant phenomena are discussed below.

Pleomorphism. The fact that amnion cells often assume fibroblast and ameboid-like shapes before developing the epithelioid cell, substantiates the belief of Weiss (1958) that a cell's origin cannot be determined by its shape.

In their discussion on the ameboid appearance of trypsinized amnion cells, Edwards and Fogh (1959) reason that if the cells were able to survive as unicellular organisms in a dedifferentiated form in a foreign environment, they had common properties with malignant cells. This theory could not be proven, nor disproven, as tumors could not be produced in conditioned rats. It is unlikely that this is a malignant transformation, but more probably a nonmalignant dedifferentiation to a more self-sufficient cell. Thus trypsinization changes a cell from a differentiated to a dedifferentiated, spherical, unicellular organism, which gradually differentiates once again from the fibroblast to the ameboid to the epithelioid form as it adapts to the new environment. If the environment is toxic, the cell dies in its spherical

form, or if the environment is partially suitable, the cell may develop as far as the ameboid form before dying.

Macrospikes. Those cells which developed macrospikes invariable did not develop monolayers and this type of process may be a result of the cell's final effort to obtain nutrition in an environment which was to prove fatal. Processes or microextensions of this type have been described by Weiss (1962), Taylor and Robbins (1963) and Taylor (1966). These authors described microspikes, which are minute projections from the cell membrane, 15 by 0.5μ , usually of uniform diameter and maintaining a straight and stiff appearance while waving back and forth through the medium. These processes retract by folding back into the plasmalemma or by collapsing without retraction. Occasionally the plasma membrane advanced along one of these microspikes to form a new cell process. The above observations were made on human liver, conjunctiva and HeLa cells as well as chick embryo liver and kidney cells.

The macrospike differs from the microspike primarily in size, the former being as large as 78 by two microns, and in shape, with the macrospike being pointed at the tip and wider at the base. The two processes are similar in their movements and form of retraction, although the macrospike does not always undergo the pendulum-like movement. Bends and curves were often seen in the macrospike, and frequently it became fixed

to neighboring cells for a period of time after which it was retracted into the cell. The macrospike has never been observed associated with the development of a new process, and to date it has been seen only in the primary human amnion cell in tissue culture. Furthermore, its presence almost certainly indicates that the cells in that suspension of cells will fail to develop a monolayer.

Cell union. Cell union or fusion has been described by Roizman (1962) as a rare event. The cell union observed in this study (Figure 16, page 68) may be fairly common as many binucleated cells are observed in tissue culture monolayers. Once union occurred, the united cell showed an increase in membrane activity and several cytoplasmic processes touched nearby cells, although no permanent associations were made. This suggests that the union allowed the cell to become more active, possibly by combining the resources of the two cells. This process and that of the post-trypsin dedifferentiation may be a mechanism of cell protection in an unfavorable environment.

Zeiosis. The process of zeiosis, or "the blebbing or bubbling-like activity of the cell membrane" (Rose, 1964), appears to be limited to the development of small, transient, spherical blebs in the amnion cells in vitro. This activity was associated with the cell union, and also with cells next to those undergoing

degeneration (Figure 20, page 74). Generally this occurs in other types of cells which have just undergone mitosis, but mitosis was never seen in this study and Schwarzacher and Klinger, (1963) reported that the amnion cells at term do not undergo mitosis.

Morphology of the human amnion monolayer cells

Phagocytosis and pinocytosis. There is no previous record of phagocytosis and pinocytosis occurring in amnion cells in tissue culture. The association of pinocytosis and increased movement in cells surrounding a neighboring cell which has recently undergone degeneration (Figure 20, page 74), suggests that nearby cells obtained material from the degenerating cell, thus increasing it's own activity.

Nucleus and nucleolus. The nuclei of multinucleated amnion cells are usually very motile. The movement of four nuclei around a single axis accompanied by the perinuclear cytoplasm has not been recorded previously, nor has the simultaneous rotation of all nuclei around their own axes. The significance of these activities is unknown, although Pomerat (1953), in his paper on nuclear rotation in nerve tissue, suggests it may be related to nutrition and the distribution of nuclear material in the cytoplasm. The movement of nuclei across the cell to establish contact with the cell membrane which is close to the nucleus of the neighboring cell (Figure 22, page 79) may be due

to cell nutrition or the interchange of nuclear material between the nuclei of two cells.

The mosaic cell. The mosaic cell (Figure 21, page 77) first seen by Rose (1962) was observed in the amnion cell in vitro. Rose does not speculate as to its composition, but suggests it is a result of cellular differentiation.

Human amnion monolayer cell degeneration. It is impossible to prove the death of cells while observing them in vitro. Studies by Mizutani, et al., (1961) gave the only record of the description of dying amnion cells in vitro, when they described amnion cells which rounded off and left the glass coverslip following lethal doses of radiation. For the purpose of this study, all monolayer cells which showed sudden cessation of movement, pyknosis of the nuclei, nuclear and cell blebbing, and retraction and detachment from the glass coverslip were considered either degenerating or dead. After this they were no longer of use for the study as they had left the microscopic field.

II. ULTRASTRUCTURE

The ultrastructure of human amnion biopsy cells

The description of the ultrastructure of human amnion cell biopsies confirms most of the findings of Bourne (1962) and Thomas (1965). This study did not confirm the observations of Thomas (1965) concerning the "fibrillar" and "Golgi" type cells, however, three different types of cells, types "A", "B" and "C", were observed in biopsy and pellet specimens.

The type "A" cell is darkest staining, generally rich in all the cytoplasmic elements such as fibrils, ribosomes, mitochondria, undilated Golgi complex, moderately dilated areas of endoplasmic reticulum, and very few large lipid vacuoles, Figure 30, page 93. The type "B" cell, seen in Figure 54, page 124, contains all these elements, but the Golgi complex is dilated, the endoplasmic reticulum is more dilated and the lipid vacuoles are more numerous and are three to four microns in diameter. The type "C" cell, seen in Figure 42a, page 110, has a relative paucity of ribosomal and fibrillar material, few or no lipid vacuoles or normal mitochondria, but does have a widely dilated Golgi complex and endoplasmic reticulum, giving the cytoplasm a very vacuolated or lacey appearance. The description of this latter cell is similar to those cells that have been adversely affected by trypsin, (Figure 57, page 128) which suggests a degenerating process, not unlike that seen in other cells (Cameron, 1964).

Type "A" and "B" cells appear similar to the types "1" and "3" cells described by Wilt and Brambilla (1966). It is possible that these two types of cells are the extremes of a secretory cycle, with the "A" cell being pre-secretory and the type "B" cell being near the end of the secretory phase. Further discussion of the possible secretory process will be considered later in this chapter.

The cell membranes. All the membrane measurements done in this study are approximate; this and the osmium tetroxide staining method may account for the increase in thickness of 40 A over Robertson's 75 A measurements (1962). The measurement of the unit membrane throughout the cell appears to be fairly constant, indicating its consistency.

The microvilli. No definite pattern was noted in the interior of the microvilli of human amnion cells, contrary to the suggestions of Bourne (1962) and Thomas (1965) that there was a pattern similar to the nine-plus-two configuration of cilia. Vacuoles found at the bases of the microvilli suggest absorptive activity and the union of their tips to form enclosed areas could be due to the process of pinocytosis, which has been observed in tissue culture.

The dark staining material intermingled with the microvilli, Figure 31, 33 and 34, pages 94, 97 and 99, suggest the elaboration of a lipid secretion as suggested by Mandl (1905) and

Keiffer (1926). How the microvilli could provide a surface which can absorb and secrete cannot be answered.

The basal processes and basement membrane. There are several aspects of this portion of the amnion cell, seen in the biopsy material, which suggests an absorptive function for this cell. The projection of processes into the basement membrane, the presence of tiny pinocytotic vacuoles in the cytoplasm and outer membrane of these processes (Fawcett, 1962), and the presence of mitochondria and endoplasmic reticulum, all parallel the characteristics of very active absorptive cells of the salt gland of the bird (Fawcett, 1962) and the cells of the human renal tubule (Ham, 1965). Although the processes are usually not as long as these classic examples of very absorptive cells, nor the mitochondria as many, this similar appearance suggests some absorptive capabilities of the basal processes of the amnion cells.

Bourne (1962) described vacuoles of basement membrane material in the area of cytoplasm next to the basement membrane. This has been seen during the present work, but only near basal processes, suggesting that these are cross-sectional views of the invaginations of basement membrane at the base of the cell.

Deep to the basement membrane, and making up the lower part of it, are collagen fibers of regular periodicity,

which on cross section appear to be hollow. This type of structure has been noted by Kennedy (1955) who described collagen fibers as being tubular. It is possible that this structure could assist the transfer of products to and from the amnion cell and the deeper tissues.

The cytoplasmic constituents. Most of the findings of Thomas (1965) concerning ribosomes, fibrils, mitochondria, endoplasmic reticulum and the Golgi complex have been confirmed in this work. The fine canals which Bourne and Lacy (1960) described as the main constituents of the cytoplasm, were not seen in this study or by Thomas (1965), and they have not been seen in other human cells. This suggests that these fine tubules were probably an artefact produced by the staining technique.

The presence of the above constituents supports the theory of secretory activity by the cell, especially when related to the large cytoplasmic vacuoles, to be discussed later.

Lysosomes are seen rarely in the amnion cell cytoplasm, as is the case of myelin lamellar bodies. The presence of both these bodies in the amnion cell cytoplasm has not been recorded previously, and their significance is uncertain, although it has been suggested that they are associated with degenerative processes (de Duve, 1963 and Shultz, 1959).

The nucleus and nucleolus. The configuration of the

amnion cell nucleus was described as irregular by Bourne (1962), but more than this it has very deep folds into the nucleolemma, occasionally touching the nucleolus and often containing small vacuoles and mitochondria. This is a very characteristic feature of the nucleus of this cell, in biopsies and in the tissue culture specimens, and is probably related to nucleocytoplasmic transport.

Bourne (1962) described the nuclear envelope as being a "double membrane", but this study has shown that the nucleus has a five-layered membrane. The nucleolemma is unremarkable and no nuclear vacuoles were seen, as was Bourne's observation (1962).

The nucleolus surrounded by a unit membrane in the cytoplasm was an incidental finding and its significance is unknown. Since this occurred in a cell which contained some degenerative changes and because the nucleolus contained lighter areas similar to degenerating cells seen in cine-phase, this may be a product of cell degeneration (Cameron, 1964).

Ultrastructure of trypsinized amnion cells

Some of the observations of Edwards and Fogh (1959) have been confirmed in this study, however, it is believed that some of the bizarre shapes seen in their work may have been due to trypsinization. Some of the findings of Edwards and Fogh (1959) were not seen in this study, such as nuclear hypertrophy, increase in lipid droplets, formation of ovoid

mitochondria, and dispersal of "tonofilaments" (presumably fibrils) associated with trypsinized cells. Other findings are probably due to excess trypsinization, such as swollen microvilli, hypertrophy of the "endomembranes" and nuclear lobulation, as was the case in this work.

III. CINE-PHASE AND ULTRASTRUCTURE

The intercellular canals and vacuolation. Bourne (1962) described the dilatated intercellular canals (Figure 35 and 36, pages 100 and 101) as being lateral vacuoles. When trypsinized, the intercellular canals are removed, yet amnion cells in biopsies containing lipid vacuoles do not appear less vacuolated after trypsinization when seen through the phase-contrast microscope. This intercellular configuration may contribute to the picture of vacuolation of a biopsy of the amnion membrane, but only in a minor way in heavily vacuolated amnions.

The mitochondria. The overall structure of the mitochondria as seen in cine-phase was confirmed by the ultrastructure study as being long, filamentous and branched (Figure 45, page 114). Movement of the mitochondria seen in cine-phase was considerably less, and the appearance much lighter than the mitochondria seen in time-lapse films produced by Chevremont and Fredric of the University of Liege, and by Pomerat, et al., of the Pasadena Foundation of Medical Research. The ultra-

structure of the mitochondrion does not differ basically from the usual descriptions as found in standard texts (Fawcett, 1966).

The perinuclear cytoplasm. In both cine-phase and ultrastructure studies the cytoplasm contains small vacuoles which are very active and close to the nuclear membrane. The significance of these vacuoles is unknown, but it is possible that they may be part of a secretory process. The Golgi complex is usually seen in the perinuclear area as well, and these vacuoles may be a portion of this complex.

The nucleus and nucleolus. Nuclear folds, and nuclear eccentricity in the cell were seen using both types of microscopy. Nucleolar shape, size and deviation from the usual central position to touch the membrane at the nuclear edge or nuclear fold, were noted in both the cine-phase and ultrastructure studies. These may be related to transport of nucleolar material to the cytoplasm, especially in the presence of mitochondria which have been seen in the nuclear folds (Moses, 1964).

The nuclear vacuole seen in Figure 23, page 80, was not seen in any of the cells viewed through the electron microscope. Similar vacuoles have been reported previously in cine-phase studies of HeLa cells by Gonzalez-Ramirez, (1963).

The nuclear blebbing reported in the cine-phase study during the degeneration of a multinucleated monolayer cell

(Figure 26, page 85) is supported by the finding of blebs in the nuclear membrane in cells which have been exposed to too much trypsin (Figure 57, page 128). The significance of this event is uncertain, but it is probably associated with a degenerative process (Cameron, 1964).

Human amnion monolayer cells. The most striking change in the ultrastructure of monolayer cells from that of biopsy cells, is the marked decrease in cytoplasmic elements and the dilation of the endoplasmic reticulum with material which does not stain with osmium tetroxide. This reduction of cellular elements and dilation of the endoplasmic reticulum may be due to the gradual starvation of the cell in an incomplete medium or the widespread distribution of the cytoplasm in the epithelioid monolayer cell. However, Cameron described cell degeneration as being accompanied by the dilation of the mitochondria and endoplasmic reticulum, probably from water imbibition. This is the picture seen in the monolayer cell in Figure 59, page 130, and therefore these changes may be those of degeneration.

Some cells in tissue culture contained many large vacuoles, and when seen through the electron microscope, they are tightly packed in the cytoplasm and dark staining with osmium tetroxide.

Cell processes seen in cine-phase studies of amnion cells

developing monolayers, when observed for their ultrastructure have microvilli on their outer surface and fibrils which appear to originate in the cytoplasm of the cell and pass as a laminated bundle along the process and end in the distal portion of the process. Small vacuoles seen in the tip of the process were probably pinocytotic vacuoles of medium engulfed by the fluctuating membrane, as seen in the cine-phase films. The material in these vacuoles is likely transported to the main body of the cell via the fibrillar structures.

Cytoplasmic vacuoles.

The amnion cell has three major types of vacuoles as seen by the phase-contrast microscope: 1) small and large short-lived pinocytotic vacuoles, 2) transient vacuoles of over five microns in diameter which remain in the cytoplasm for some days, but eventually disappear, and 3) those perinuclear vacuoles which enlarge to about four microns in diameter, and then often migrate to the cell periphery. The first type of vacuolation is self explanatory, while the second is unexplained. The third type is made up of those vacuoles which Wilt and Brambilla (1966) have used to establish their typing system and which have been a cause for study and speculation for over 60 years.

Those vacuoles which stain positively for lipid in histochemical studies are the only large enough to produce the vacuolated appearance in the phase-contrast microscope, and

are considered to be one and the same by Wilt and Miller (1965). On close examination of the cine-phase photomicrographs, the larger granules of up to one micron in diameter contain a light center similar to the vacuole, but on a smaller scale. Added to the fact that the granules decrease as the number of lipid vacuoles increase, this evidence indicates that the granules seen in the phase-contrast photomicrographs are actually developing lipid vacuoles. Specimens of amnion cells stained with osmium tetroxide and viewed through the electron microscope, support both the histochemical and phase-contrast pictures.

Since Colpi's paper in 1898, it has been argued that vacuoles in amnion cells are a result of degeneration. This is unlikely as Figure 11, page 58, shows the development of marked vacuolation in a type "1" cell within 18 hours, yet during this period of time the cell was more active than its nonvacuolated neighbors, judging from the degree of membrane and intracellular movement during the period of vacuolation. This vacuolated cell remained a healthy part of the monolayer despite the vacuoles, during the time that it was photographed by the cine-phase camera.

In the cine-phase study, the granules and vacuoles were seen most frequently as active moving particles in the perinuclear position. In electronmicrographs of amnion biopsy cells vacuoles were often seen indenting large areas of dilated endoplasmic reticulum and the nucleus, and in direct contact with

the dilated Golgi complex, suggesting that these portions of the cell take part in vacuolar production.

The typical staining characteristic of saturated lipids, using osmium tetroxide, is a smooth staining material of moderate electron density, while unsaturated lipids stain black (Fawcett, 1966). The fine structure of most vacuoles shows a smooth staining material of moderate electron density, which is probably saturated fat, surrounded by a doubled, unit membrane. The inner unit membrane may be obliterated by a ring of dark staining material which is probably unsaturated lipid.

It was noted that the vacuoles in the monolayer cells, as seen through the electron microscope, were darker than the vacuoles in cells from biopsies. Fawcett (1966) has pointed out that the darker the lipid stains, the higher the content of unsaturated lipids.

In a study to be reported, Pritchard, et al., (1966) have disrupted the membranes of several suspensions of cells from different amnions using a teflon grinder, and removed the vacuolar fraction by ultracentrifugation. These fractions were analysed by chromatography, and found to contain cholesterol and other lipids, thus proving that vacuoles contain lipids.

Although the granules usually maintain a perinuclear position, the vacuoles, having reached a size of four microns in diameter, often migrate to the periphery of the cell. This suggests that the vacuoles which have reached a certain size

are moved away from the area of synthesis to be expelled from the cell. Because the expulsion of vacuoles from the cell was not observed in this work, this does not mean that it did not occur.

The movement of vacuoles from one cell to the next is very interesting. Similar transport of cytoplasmic contents from one cell to the other has been recorded in fibroblasts and other cells in vitro by Rose (1960, 1963). In vivo the amnion cell has a gradient produced between its apex and base by the presence of amniotic fluid at its apex and the basement membrane at its base. Trypsinization removed this gradient, with the possible result that the heavily vacuolated amnion cell passed its vacuoles to a less vacuolated cell.

The final type of vacuolar movement in amnion monolayer cells, is that associated with the fluctuating membrane. The movement to and from the nucleus while associated with the membrane, may be a result of attachment to the cytoplasm by the vacuoles, or it may be a method of increasing the content of the vacuoles by obtaining nourishment from various portions of the cell.

The relative lack of vacuoles in ameboid or fibroblast-like cells, and the presence of many vacuoles in epithelioid cells suggests that the former two types of cells are less differentiated and perhaps unable to produce vacuoles. Epithelioid cells however, are more differentiated and therefore able to

produce lipid vacuoles.

In summary, the related morphological observations of the cine-phase and ultrastructure have been discussed. The result of these observations appear to: 1) support the processes of absorption and secretion, and 2) indicate that the vacuoles seen in the light, phase-contrast and electron microscopes contain lipid.

IV. AN HYPOTHESIS OF AMNION CELL FUNCTION

The hypothesis suggested by Bourne and Lacy states that the function of the amnion cell is to absorb material from the amniotic fluid, with meconium collecting in the large cytoplasmic vacuoles. The water in the amniotic fluid has a very rapid turnover of about 35 per cent per hour, although ions such as sodium are considerably less active at 7 per cent per hour (Vosburgh, et al., 1948). In vitro the amnion is permeable to many different ions and water, with lipids showing increased permeability (Garby, 1957).

The fetal gut and respiratory tract are considered inadequate for the absorption of the amount of fluid that is produced each hour (Jeffcoate and Scott, 1959) so the epithelium is considered the likely area for fluid absorption. The microvilli present on the apex of the cell, forming a type of brush border, are capable of absorbing fluid in a manner similar to the pinocytosis observed in the cine-phase studies.

The fact that amniotic fluid is present before the fetal kidneys are able to secrete, and may be found in normal amounts in cases of renal agenesis, indicates that the amnion epithelium either transports or synthesizes amniotic fluid. To do this the amnion cell probably absorbs material from the basement membrane via the small pinocytotic vacuoles which enter the cytoplasm through the unit membrane of the basal processes and the dilated intercellular canals. The secretory-like material seen in some electronmicrographs of the apices of amnion cells would be the probable area of discharge for this material.

All living cells must absorb and secrete material to maintain life, so the above processes could be an intensification of this type of activity to keep the potential space in the amniotic sac filled with fluid.

The vacuolation of human amnion cells has been considered a degenerative process because it is often present in the epithelium after the placenta is expelled from the uterus and is then in an unsuitable environment. The in vitro aspect of this study of amnion cells has shown that degenerating cells were not always vacuolated and that vacuolated cells may be as active as nonvacuolated monolayer cells. In degeneration the cell usually becomes filled with vacuoles and dies, but in many monolayer cells some vacuoles may be seen in the cytoplasm throughout the cell life in vitro.

There is good evidence to support the belief that vacuolation is a result of amnion cell secretory activity. Mandl (1905) published drawings of amnion cells discharging fat staining "granules", in a fashion similar to apocrine glands. Keiffer (1926) observed that vernix and the "granules" in amnion cells stained in an identical way using fat stains, and he also noted that vernix contained cholesterol. Since then little new evidence has been produced to support the amnion cells produce a secretion.

Evidence supports the belief that the steroids produced by the adrenal cortex are associated with cholesterol vacuoles or droplets. When the adrenal cortex is stimulated to produce hormones, these lipid droplets disappear from the cells involved, and when the cortex is not stimulated for a period of time, the droplets reappear. These observations have led biochemists to believe that cholesterol is a precursor of the steroid hormones, especially progesterone and the estrogens (White, et al., 1963). The lipid vacuoles of the human amnion cell have been shown to contain cholesterol (Pritchard, et al., 1966), minced placental tissues contain progesterone, and some other steroids (Salhanick, et al., 1952) and when sliced placental tissues were grown in vitro chorionic gonadotrophin was produced (Jones, et al., 1943). The lipid vacuoles in amnion cells parallel the physical characteristics of birefringence and fluorescence of the lipid bodies in cells secreting hormones in the ovary, testis and adrenal cortex.

Further, Pritchard, et al., (1966) have noted that isolated vacuoles contained a large proportion of esterified cholesterol, as is the case in vernix.

All this evidence supports the belief that the vacuolation in human amnion cells is the result of a secretory product, which may produce vernix and be associated with steroid hormone synthesis. The accumulation of this product in vitro may be due to the fact that the medium is unphysiological and this could cause the cell to be unable to excrete the material. If too much of the secretion collects in the cytoplasm, the cell may be unable to dedifferentiate or adapt to the new environment after trypsinization, thus suggesting one reason why the type "3" cells do not grow well in vitro.

In their work on cell typing and biopsy interpretation, Wilt and Brambilla, (1966) noted that one biopsy of the amnion is usually representative of the entire membrane. Thus, if one part of the membrane is vacuolated, the rest of the amnion will usually be vacuolated, indicating that most of the cells in the membrane are synchronized for vacuolation or nonvacuolation. Under these circumstances it would be reasonable to hypothesize that those cells which were nonvacuolated were in an early pre-secretory phase, while vacuolated cells were in a late secretory phase.

The hypothesis of secretion in the human amnion cell is summarized in Figure 63, page 160. This diagramatic represen-

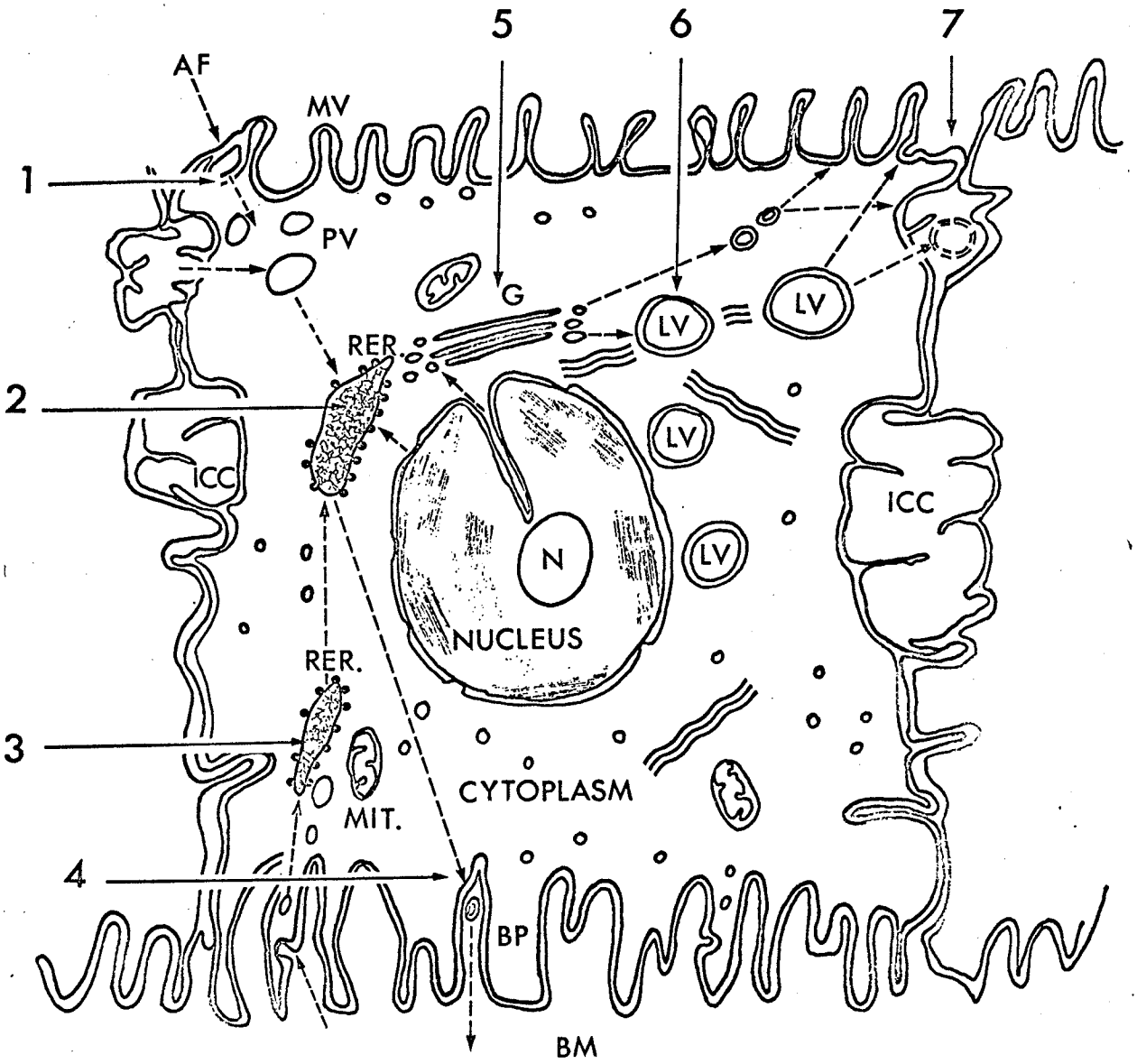


Figure 63. A schematic representation of a human amnion cell in vivo summarizing the secretory hypothesis. Step "1"- pinocytosis of amniotic fluid (AF), step "2"- accumulation in the rough endoplasmic reticulum (RER), step "3"- the accumulation of material from the basement membrane (BM) via the basal processes (BP), step "4"- discharge of waste material into the basement membrane, step "5"- the development of the secretory product in the Golgi complex (G) after receiving material from the endoplasmic reticulum, the nucleus and nucleolus (N), step "6"- the accumulation of the secretory product into lipid vacuoles (LV), step "7"- the expulsion of the secretory material through the dilated intercellular canals (ICC) or through the microvilli (MV).

tation of an amnion cell in vivo has the steps leading to secretion numbered. Step "1" is the engulfing and passage into the cytoplasm of amniotic fluid and its constituents from the amniotic cavity and the dilated intercellular canals. In step "2" the absorbed material is collected in the endoplasmic reticulum, as is the material absorbed from the basement membrane in step "3". Step "4" involves the discharge of waste products into the basement membrane, from where it is transported to the connective tissues and eventually the maternal circulation. Before involving the Golgi complex or, while in it, the developing secretory product receives material from the nucleus and nucleolus via nuclear pores and folds, and during step "5" the secretory product is elaborated. Step "6" involves the accumulation of cholesterol and other secretory products in the vacuoles. Once the secretory product is concentrated, the vacuole is expelled from the cell upon the proper hormonal stimulation, either into the dilated intercellular canals or through the microvilli.

In vitro this sequence of events would be disrupted by an artificial environment. Figure 64, page 162, is a diagramatic representation of a trypsinized human amnion cell which is suspended in propagating medium and about to develop into a monolayer cell. Steps "1" and "2" are unaffected by the new environment, but steps "3" and "4" are blocked due to the absence of the basement membrane and its nutrients. Steps

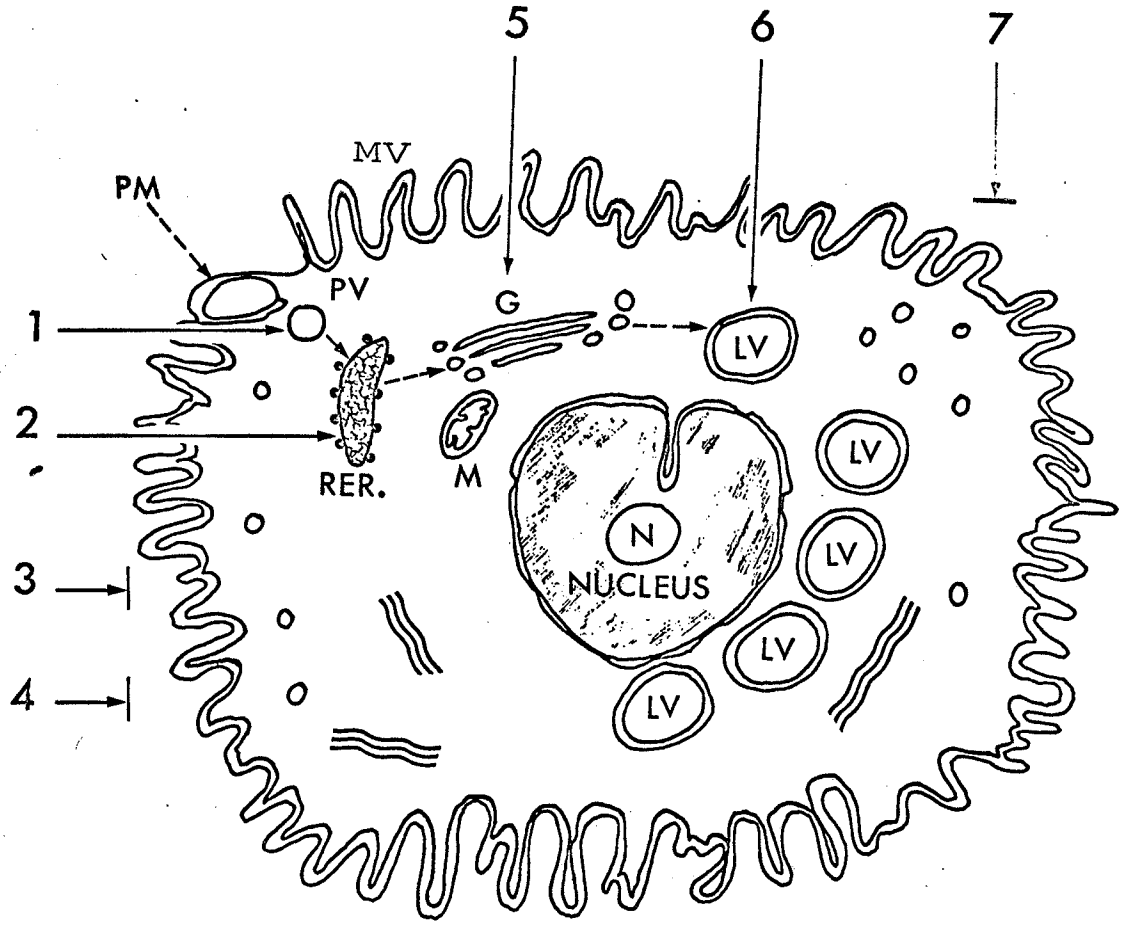


Figure 64. A schematic representation of a trypsinized human amnion cell in vitro, summarizing the secretory hypothesis. Step "1"- pinocytosis of the propagating medium via the microvilli (MV), step "2"- accumulation in the rough endoplasmic reticulum (RER), steps "3" and "4"- blocked because of the absence of the basement membrane, step "5"- the development of the incomplete secretory product in the Golgi complex (G) after receiving incomplete material from the endoplasmic reticulum, nucleus and nucleolus (N), step "6"- the accumulation of the incomplete secretory product in lipid vacuoles (LV), step "7"- blocked so that the lipid vacuoles accumulate in the cytoplasm.

"5" and "6" continue, although altered, because of incomplete materials, to produce a modified secretory product, and hence the more unsaturated lipid seen in the vacuoles of monolayer cells. Step "7", or expulsion of the secretory material is blocked perhaps for several reasons, such as an incomplete secretory product, an altered cell surface due to trypsinization, or a lack of the proper hormone to stimulate the release of the product. The inability of the cell to expell the secretory product in vitro causes the cytoplasm to become packed with vacuoles, thus reducing the amount of cytoplasm able to metabolise for the cell and resulting eventually in cell death. This could explain the limited life of primary amnion cells in vitro, as well. Cells which have many vacuoles before trypsinization may not be able to dedifferentiate, and consequently are unable to adapt to the new environment, and do not survive in tissue culture. Those cells which had just expelled their secretory products before trypsinization probably have had insufficient time to enter the secretory phase, and by dedifferentiating avoid the problems of the vacuolated cell.

This hypothesis answers several important questions including the function of the amnion cell in vivo and the reasons for the nonvacuolated cell surviving better in vitro. However, the supporting evidence for the hypothesis is primarily morphological and much work in the areas of biochemical analysis for hormones and pharmacological stimulation of secretion in these

cells must be done before the ideas expressed above can be considered more than an hypothesis.

In summary, an hypothesis concerning human amnion cell secretion is submitted. This hypothesis explains the function of the human amnion cell *in vivo*, the reason for nonvacuolated cells growing better *in vitro* than vacuolated cells, and the probable reason for the limited life of primary amnion cells in tissue culture.

CHAPTER VI

SUMMARY AND CONCLUSIONS

CHAPTER VI

SUMMARY AND CONCLUSIONS

This thesis was designed to discover the meaning of vacuolation in the human amnion cells. The problem was approached from the morphological aspect using cine-phase and electron microscopy.

A relatively simple and inexpensive cine-phase apparatus using an inverted microscope and standard equipment, where possible, was designed. A simple clamp was designed for embedding monolayer cells in vestopal for the ultrastructure study.

The belief that nonvacuolated amnion cells grow better in vitro than vacuolated ones was tested by watching their response to the new environment, as recorded by the cine-phase camera.

A detailed description of human amnion cell suspensions of various cell types developing into monolayers, and the degeneration of these cells was recorded to establish a base line for further studies on these cells using viruses and other agents.

Trypsinized amnion cells developing from suspended cells to monolayer cells, appear to become progressively differentiated by developing from spherical to fibroblast, to ameboid, to epithelioid forms. Fibroblast and ameboid-like cells are usually nonvacuolated, whereas epithelioid cells often are

vacuolated, suggesting that vacuoles usually develop in the more differentiated form, if they were not present in the cell previous to trypsinization.

The degeneration of amnion monolayer cells involves the cessation of all cell movement, lightening of the center of the nucleolus, pyknosis of the nucleus, nuclear blebbing followed by cytoplasmic blebbing, and cell retraction.

Cytoplasmic vacuoles were of three main types: 1) pinocytotic vacuoles, 2) large vacuoles over five microns in diameter which persist for several days and then disappear, and 3) the vacuoles which correspond to lipid vacuoles. The granules seen in the amnion cell cytoplasm and the lipid vacuoles are probably different sizes of the same entity. These vacuoles are usually mobile and perinuclear until they reach four microns, when they often migrate to the periphery of the monolayer cell, where they are often seen in a linear alignment. These peripheral vacuoles were seen to pass from one cell to the next in a kind of intercellular communication.

Other cine-phase findings include; counterclockwise rotation of single nucleus and of a group of four nuclei with their surrounding cytoplasm around a single axis, the presence of long, thin processes called macrospikes, cell union or fusion, the presence of a mosaic cell, fluctuating membranes, as well as the processes of phagocytosis and pinocytosis.

The ultrastructure study indicated there are three

main types of cells seen in biopsy and pellet material. Type "A" cells are nonvacuolated and rich in the usual cytoplasmic constituents, type "B" cells are vacuolated with many and large lipid vacuoles, but the cytoplasm contains some dilation of the remaining portions of the Golgi complex and endoplasmic reticulum. This classification suggests that amnion cells are either nonvacuolated (type "A") or vacuolated (type "B") with degrees of vacuolation occurring between these two groups. Type "C" which is probably a degenerating cell with dilated endoplasmic reticulum, a widely dilated Golgi complex and possibly swollen mitochondria, but with a marked paucity of lipid vacuoles.

The ultrastructure of the human amnion cell is such that it is potentially capable of absorption and secretion. The anatomical findings which suggest this are the presence of: 1) microvilli or a type of brush border, 2) small pinocytotic vacuoles in the lateral and basal unit membranes, 3) basal processes invaginating the basement membrane, 4) the presence of mitochondria, many tiny vacuoles and dilated endoplasmic reticulum in the basal processes, 5) endoplasmic reticulum, a Golgi complex, mitochondria and lipid vacuoles in the perinuclear area, and 6) a nucleus with a mobile nucleolus, and nuclear folds which contain cytoplasm and occasionally mitochondria.

Trypsin can have very deleterious effects on human amnion cells, disrupting the cell membrane, the nuclear membrane

and causing dilation of the mitochondria, Golgi complex and the endoplasmic reticulum.

Relating both cine-phase and ultrastructure observations, mitochondria were seen in both studies as a long, thin network of filamentous structures. Characteristic nuclear folds were seen in both studies and related to nucleocytoplasmic activity, as was the presence of the nucleolus at the edge of the nucleus. Nuclear blebbing was considered a characteristic associated with the degenerating cell and seen using the phase-contrast and electron microscopes.

The fine structure of the monolayer cell in tissue culture was related to the cine-phase portrayal and it was noted that the monolayer had a marked depletion of cytoplasmic contents, which could be a result of cell starvation or dilution due to the extensive spreading of the epithelioid cell cytoplasm in the monolayer.

Cytoplasmic processes contain many parallel fibrils, which probably maintain the structure of the process and assist in the transport of material from the distal to the proximal end of the process.

Lipid vacuoles in biopsy and trypsinized human amnion cells were usually surrounded by one or two layers of unit membrane and most often situated initially near the active components of the cytoplasm, such as the Golgi complex, endoplasmic reticulum and mitochondria. The perinuclear, cytoplasmic

granules seen in the phase-contrast microscope are probably small vacuoles which later enlarge to about four microns and then spread into the periphery of the cytoplasm. Vacuoles were seen to move in the cytoplasm with the fluctuating membrane and were also seen moving from one cell to the next.

Trypsinized cells are believed to be dedifferentiated amnion cells which become more differentiated as they develop into monolayers. The ability to dedifferentiate allows cells to adjust to new environments in vitro.

An hypothesis of human amnion cell function in vivo and in vitro, as presented in this thesis, explains the meaning of vacuolation. The hypothesis states that the amnion cell is both absorptive and secretory and the lipid vacuoles contain the secretory product which contains cholesterol amongst other lipids. The presence of a precursor of hormones, cholesterol, suggests the possibility of hormone secretion by the human amnion cell.

The main evidence used to support this hypothesis is based on the morphology of the human amnion cell. Therefore further work in the radioautography and biochemistry of the secretory product and the pharmacological stimulation of secretion in the amnion cell will be needed before the hypothesis can be supported with more definite evidence.

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APPENDIXES

A, B, C, D, E, F

APPENDIX A

I. PROCEDURE FOR PROCESSING HUMAN AMNION CELLS FOR TISSUE CULTURE (Wilt, et al., 1964)

Collection

Placentas were collected in one liter beakers containing 500 ml of cold Hank's balanced salt solution. The placenta was transported from the labor floor of the hospital to the laboratory as quickly as possible (one to four hours).

Selection

A biopsy of the placental portion of the amnion was obtained under sterile conditions, placed on a slide, and observed through a phase contrast microscope. If the biopsy classified as grade I to III, the amnion was processed; if grade IV, it was discarded. Other amnions that were discarded were those which were meconium stained, obviously contaminated, and those which had contacted antiseptics.

Processing

Stripping. In a sterile cabinet the amnion was stripped off the chorion, using a sterile flat pan and forceps. Excess mucous and blood were removed from the amnion by partially closing one of the sterile forceps and by pulling the membrane through the constricted area between the teeth of the forceps.

Washing and pre-digestion. The stripped amnion membrane was placed in a bottle containing 300 ml of 0.1 per cent diaminoethanetetracetic acid, disodium salt, (EDTA. or versene) which was heated to 34°C. The bottle was then attached to a wrist action shaker and shaken for ten minutes. Any excess mucous or blood attached after washing was removed by scraping the membrane with sterile forceps.

Trypsinization. The pre-digested membrane was placed in 300 ml of 0.25 per cent trypsin, which was warmed to 34°C, then shaken briefly and placed in a water bath at 34°C. After one-half hour, the membrane and trypsin were shaken vigourously, replaced in the water bath for another half hour, removed, and shaken briefly again.

Post-trypsinization. The membrane was placed in 200 ml of propagating medium and then removed after being shaken vigorously. The membrane was then discarded. While the membrane was being washed, the cells suspended in trypsin were centrifuged for ten minutes at 1,000 revolutions per minute (rpm). Immediately after centrifugation, the trypsin was removed from the cell sediment by suction and the cells were resuspended in the 200 ml of propagating medium in which the amnion membrane had been washed just after trypsinization.

Cell dilution for tissue culture. A drop of the cell

suspension was placed on a hemocytometer and one drop was placed on a clean glass slide and covered with a coverslip. The number of cells per milliliter of suspension was calculated from a double count in the hemocytometer counting chamber. The predominant cell type was then determined by doing a differential count on the first 100 cells seen through the phase contrast microscope in the suspension on the glass slide. The criterion used to determine the cell type was that devised by Wilt and Brambilla (1966) as reproduced in figure 65. The formula used to calculate the volume of propagating medium required to dilute the post-trypsin cell suspension to the proper concentration or "X", based on the cell type, is as follows:

$$\frac{\text{present concentration of cells}}{\text{desired concentration of cells}} \times \text{present volume} = \text{"X"}$$

II. SOLUTIONS

Hank's balanced salt solution

Stock solutions were prepared in 400 ml amounts to be diluted ten times before use.

Solution A

Sodium chloride (NaCl)	320 grams
Potassium chloride (KCl)	16 grams
Magnesium sulphate ($MgSO_4 \cdot 7 H_2O$)	8 grams

VARIATIONS IN HAM EPITHELIUM

1	2	3	4	5	6
DARK	SHADED	BRIGHT	TRANSLUCENT	TRANSLUCENT	TRANSLUCENT
SMALL	SMALL TO MEDIUM	MEDIUM TO LARGE	LARGE. OFTEN CLUMPED	SMALL TO MEDIUM	SIZE VARIES. SPHERICAL

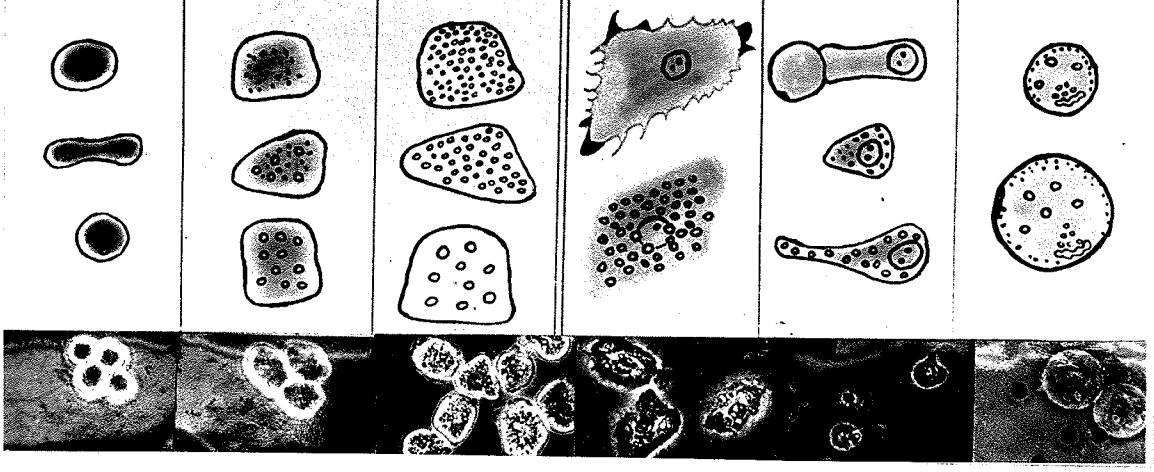

Figure 65. The classification used to determine the various cell types found in suspensions of recently trypsinized human amnion cells, Wilt and Brambilla, 1966.

Solution B

Sodium Phosphate (Na_2HPO_4-anhydrous). . . . . 1.92 grams

Potassium Phosphate (KH_2PO_4). . . . . 2.4 grams

Each group of salts listed in solutions "A" and "B" were dissolved in less than 40 per cent of the final volume of water distilled in glass (about 152 ml each).

Solution C

Calcium Chloride ($CaCl_2$). . . . . 5.6 grams

This salt was dissolved in 20 per cent of the final volume of water distilled in glass (about 76 ml). About 20 ml of a 0.4 per cent solution of phenol red was added to the concentrate.

Solutions "A" and "B" were first mixed together making up about 80 per cent of the final concentrate and then solution "C" was added along with the phenol red to make up the final volume of solution.

As a preservative, two milliliters of chloroform were added, and the solution was stored at four degrees centigrade.

When required for use, one part of the above solution was added to ten parts of glass distilled water and then autoclaved at ten pounds pressure for ten minutes at 110°C in 500 ml quantities. A sterility test was done on one sample from each batch.

Propagating medium

Propagating medium was prepared by combining the following constituents:

70 per cent Hank's balanced salt solution.

20 per cent tryptose phosphate broth.

10 per cent local pooled calf serum.

The calf serum was prepared by forcing it by positive pressure through a millipore filter (HAWP 142 50, HA 0.45 microns) and heating to 56°C for 30 minutes to inactivate complement. The amount of calf serum varied from 10 to 20 per cent, with the other constituents being adjusted proportionately.

Other constituents of this medium are listed in the following table.

TABLE III

OTHER CONSTITUENTS OF PROPAGATING MEDIUM AND THE AMOUNTS USED

Ml used per 100 ml solution,	Concentration	Material added
2.0 ml	10 per cent	glucose
1.0 ml	200,000 I.U. per ml	penicillin (Ayerst),
2.0 ml	10 mg per ml	streptomycin sulphate (B.D.H.),
1.0 ml	5 mg per ml	chloromycetin (Parke, Davis),
0.5 ml	10,000 U. per ml	mycostatin (Squibb),
1.5 ml	0.1 N	HC1 (sufficient to obtain pH 6.9),
6.0 ml	1.4 per cent	$NaHCO_3$.

APPENDIX B

I. DATA RE: CINE PHASE APPARATUS

Camera

Bolex H 16 M, 16 millimeter with Bolex electric motor, MC 17 with Transformer. An exposure interval of 0.7 seconds was obtained using the time exposure setting.

Timer

Stevens Cine Timer M1 6-2255
Stevens Engineering Co.,
340 N. Newport Blvd.,
Newport Beach, California.

Microscope

Carl Zeiss, Jena, Nr. 92419
Phase condenser - IS 465220
Objectives - Neofluar Ph 2, 40/0.75
Neofluar Ph 2, 16/0.40
Neofluar Ph 3, 63/0.90
Periscope viewer - Leitz Wetzlar prismatic viewer with 10X ocular and prism removal mechanism.

Light source

Bulb- ribbon filament, 6 volts.
Transformer and light socket-
Ernst Leitz Wetzlar GMBH

Redyx type, 6 volts, 5 amperes.

Light relay- an electronic device to maintain minimal heat in the light bulb during the off cycle, to lengthen bulb life from 48 hours to two months. See Figure 66 for wiring plan.

Incubator

Constructed of one-eighth inch thick Perspex (plexiglass), $12\frac{1}{2}$ x $13\frac{1}{4}$ x $11\frac{1}{4}$ inches.

Openings- Front door, 7 x $4\frac{1}{2}$ inches
Side hand holes $4\frac{1}{2}$ inches in diameter and covered with sheet latex rubber
Heat input, 1 3/4 inches in diameter
Heat output, 2 inches in diameter

Heat source- G.E. hair dryer heater element 1xH1074, 62 ohms, 230 watts set in flexible metal input tube attached by polyethylene to the fan

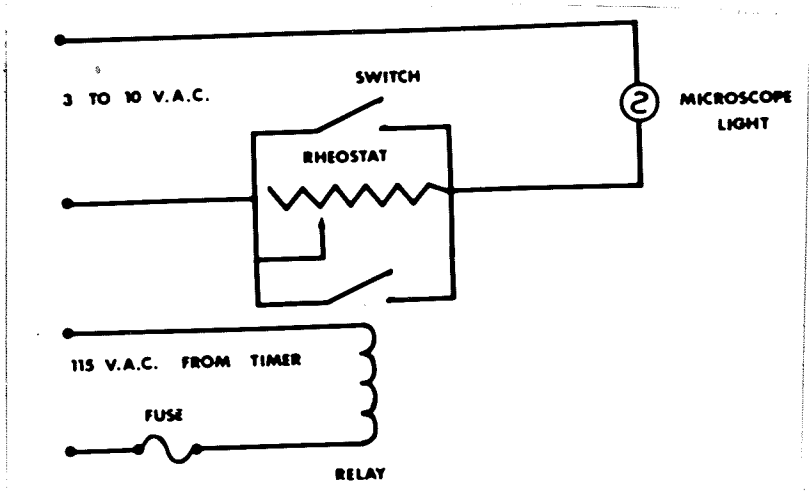

Figure 66. the wiring plan for the light-relay unit as designed and constructed by Mr. G. McLaren, Electronics Technician, Department of Bacteriology and Immunology, Manitoba Medical College, Winnipeg, Manitoba.

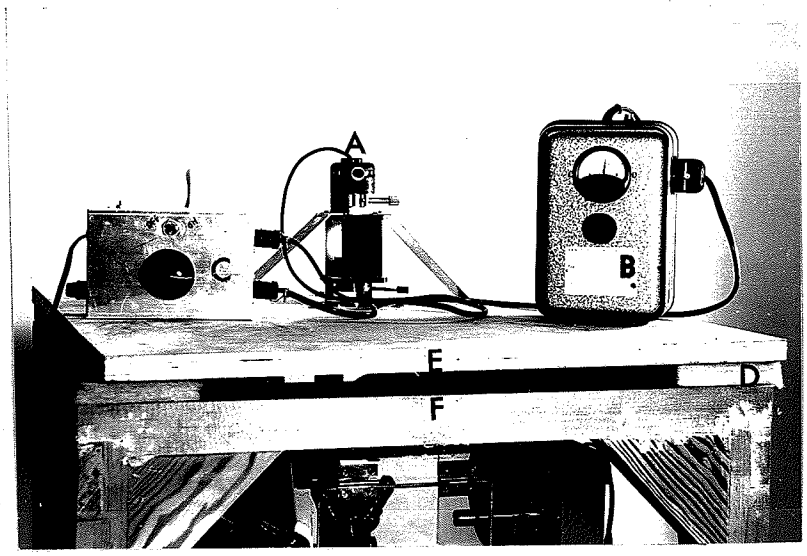

Figure 67. a photograph of the light system, with the light and its supports (A), transformer (B) and the light-relay unit (C). The fiberglass pads (D) separating the board (E) on which this system rests, and the main stand (F) may be seen.

output.

Fan- Dayton, model 2C782, H.P. 1/250 RPM. 3160, Dayton Electric Co., Chicago 48, Illinois. Intake attached to the flexible fiberglass exhaust from the incubator by polyethylene.

Thermostat- Thermoregulator Flange Head Type, Fischer number 15-178, 115 volts, 15 amperes, size $3\frac{1}{2}$ x 5/8 inches, range, -100 to 400 degrees farenheit.

Stand

Material- first grade pine wood, 2 x 4 inches. Carriage bolts, 6 x $\frac{1}{4}$ inches.

Size- 24 x 24 x 36 inches, with fitted and glued corners.

Braces- at each angle and two vertical braces at the back, 7 and 17 inches from the base.

Mounts- four Lord mounts were used to support the stand to reduce

vibration: number 156H-16, obtained from Railway and Power Engineering Corporation, Limited, Winnipeg Office, Manitoba.

Camera supporting rail- — from a Leitz Aristophot stand, (27 inches long) and bolted to 26 inches of angle iron, which was bolted to the two back, vertical cross braces.

Microscope Base — Plywood, 0.75 x 24 x 24 inches with a 1.75 inch hole drilled for the light to shine on the microscope condenser. The light holder was screwed onto the upper surface, and the microscope bolted to the lower surface in optical line with the light source. The base rested on four fiberglass pads, 4 x 4 x 1 inches, and this rested on the four corners at the top of the stand.

Light meter — Photovolt Light Meter, Model 501 M with a special adapter to read light

through the periscope-camera attachment.

Power supply	110 volts, 60 cycles, attached to the timer, from which the camera motor and light received power at the appropriate time intervals.
Recording system	Punch cards as obtained from the Pasadena Foundation for Medical Research, Pasadena, California. See Figure 68, page 191.
Film	Kodak, plus X negative 16 millimeter movie film.
Development	Ken Davey Productions Limited, 695 Sargent Avenue, Winnipeg, Manitoba.
Rose chamber covers	Made of machined and finely polished stainless steel by: Mr. T.J. Reichert, Microdar of Canada Limited, 1085 Winnipeg Avenue, Winnipeg, Manitoba.
Silicone gaskets	These gaskets were made in this laboratory from bulk, non-toxic silicone

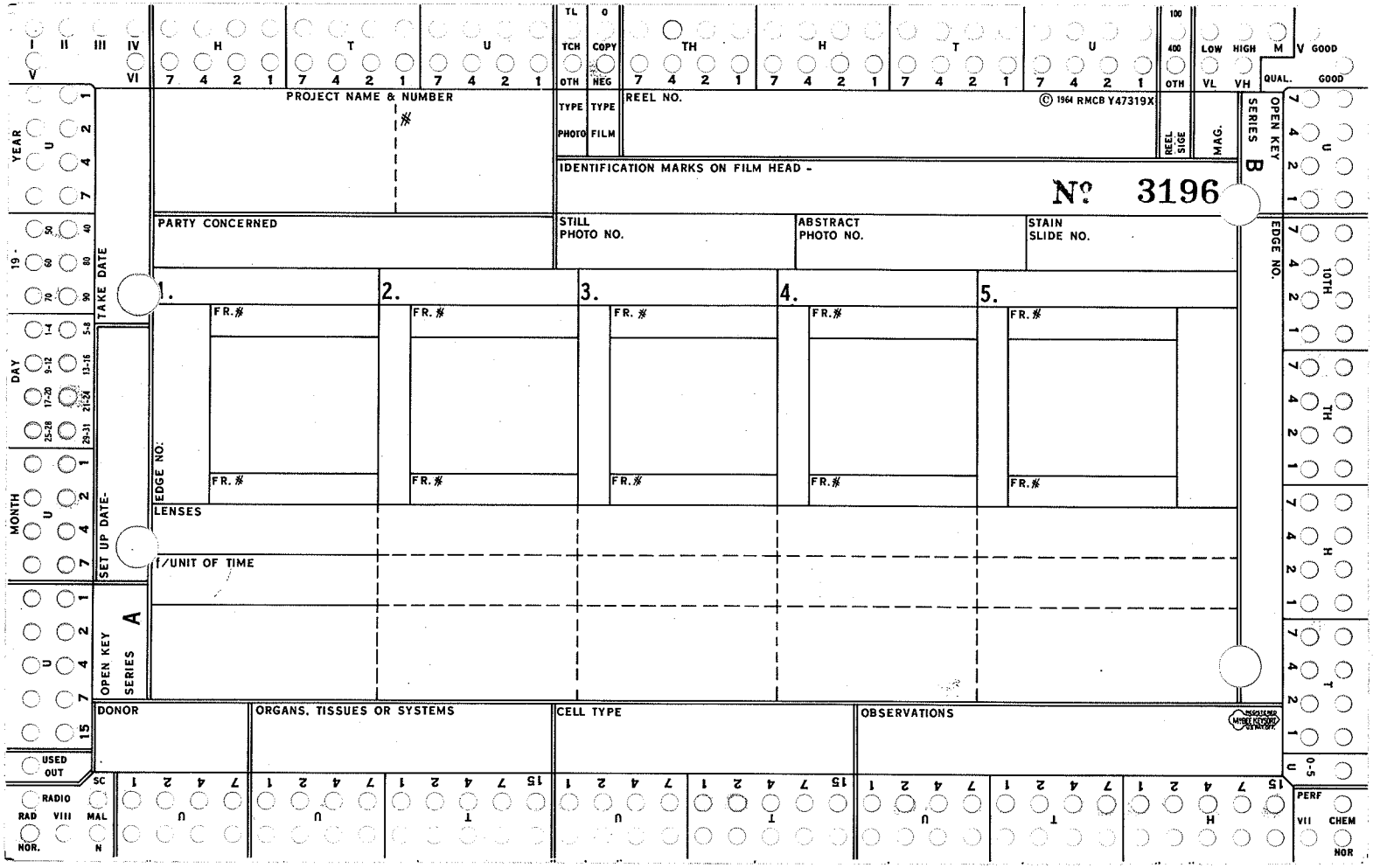

Figure 68. A sample of the punch cards used as the recording system. These cards were designed by the Pasadena Foundation for Medical Research, specifically for cine phase recording.

obtained from: Hadbar, Incorporated, 733 S. Fremont Avenue, Alhambra, California, The silicone grease used to coat the gaskets was from Dow Chemical, Limited.

Coverslips — Corning coverslips, number 1, Size 45 x 50 millimeters.

pH color guide — LaMotte Chemical, Chestertown, Maryland (Figure 2, page 33).

Carbon coating — This process was done using the Edwards High Vacuum Carbon Coating Unit, model 12E6/658.

Perfusion apparatus — Comprised of Intramedic polyethylene tubing, PE 200 with matching Luer-lock or Luer-slip adapters, 25 gauge 5/8 inch disposable needles, a universal bottle and a 5 ml syringe (Figure 3, page 34). The tubing and adapters were manufactured by the Clay-Adams Company, Incorporated, New York.

II. CLEANING OF ROSE CHAMBER COMPONENTS

Coverslips

The coverslips for Rose chambers were cleaned in the following manner:

(1) Dipped in acetone for three minutes and without drying placed in

(2) 100 per cent ethanol for four minutes,

(3) step (2) repeated,

(4) coverslips dried with lint free cloth,

(5) sterilized in a dry oven at 180°C for 24 hours, or autoclaved at 120 degrees at 15 pounds for 20 minutes.

Gaskets

The cleaning of previously unused gaskets was done as follows:

(1) Boiled for one hour in fresh distilled water and repeated three times in fresh water,

(2) Coated lightly with silicone grease,

(3) Placed in petri dishes and autoclaved at 120 degrees centigrade at 15 pounds for 20 minutes.

Gaskets that had been used previously were cleaned in the same way, except that they were boiled only three times in step (1).

TABLE IV

ROSE CHAMBER NUMBERS, FOOTAGE OF FILM TAKEN, AND CELL TYPES OF AMNIONS USED FOR CINE-PHASE STUDIES

Rose chamber number *	Feet of film in hundreds **	Cell types 1	2	3	4	5	6	7
130	1	11	22	36	20	-	1	10
161	1	27	35	34	12	-	1	1
170	1	4	37	19	34	4	2	-
200	1	9	36	38	11	3	3	-
205	1	96	-	-	-	3	1	-
225	3.5	22	57	10	6	1	4	-
231	3.5	12	62	16	6	1	3	-
251	2	68	14	-	-	9	3	6
255	4	21	32	23	20	-	1	-
269	2	67	10	1	14	6	2	-
1-66	4.5	55	30	2	3	3	6	1
5-66	12	75	1	-	20	-	3	1
67-66	3	1	16	68	15	-	-	-
80-66	4	24	53	11	9	-	3	-
74-66	15	75	2	-	23	-	-	-
133-66	7	86	-	-	8	-	6	-
190-66	2	-	53	32	14	-	-	-

* These numbers correspond to the processing numbers of the amnions from which the cells used were obtained.

** 100 feet of film is equivalent to about 67 hours of filming when the usual interval of one minute between pictures was used.

Cell type "7" - cells which were clumped.

TABLE V

CELL TYPE AND GROWTH SUCCESS OF AMNIONS IN THE ROSE CHAMBER MONOLAYER DEVELOPMENT STUDY

Number of Rose Chamber	Cell types in per cent						Growth
	1	2	3	4	5	6	
231	32	51	14	3	0	0	Yes
251	30	57	0	13	0	0	No
269	52	36	0	8	4	0	Yes
67-66	0	9	77	9	5	0	No
80-66	17	42	29	8	4	0	No
133-66	68	25	7	0	0	0	Yes
190-66	5	35	48	9	3	0	No

This number is the per cent of cells of that type in the microscope field studied by cine-phase as opposed to the per cent of cell types as calculated after trypsinization and before distribution (see table IV.)

APPENDIX C

DETAILS OF THE TECHNIQUES FOR THE ULTRASTRUCTURE STUDY

Vestopal processing

(1) Tissue fixed in veronal buffered osmic acid for 1.5 to two hours.

(2) Tissue washed in five changes of distilled or de-ionized water.

(3) Tissue post-fixed in 2 per cent uranyl acetate for 25 minutes.

(4) Tissue dehydrated in alcohol:

Two changes of 30 per cent in five minutes

Two changes of 50 per cent in five minutes

Two changes of 70 per cent in five minutes

Two changes of 95 per cent in five minutes

Two changes of absolute alcohol in 25 minutes.

(5) Tissue immersed in two changes of styrene in 15 minutes.

(6) Tissue placed in vestopal and rotated overnight.

(7) Tissue placed in vestopal with 1.0 per cent initiator and 0.5 per cent activator and rotated for 1 hour.

(8) Tissue embedded in a capsule of the above vestopal and polymerized at 65°C for 48 hours.

(9) The tissue block was then trimmed and microsection

of about 60-90 mμ thick cut using the Porter-Blum ultramicrotome, "MT-1".

Agar embedding

(1) The pellet of cells was fixed in veronal buffered osmic acid for one to 1.5 hours.

(2) Washed in deionized water for 10 minutes.

(3) Post-fixed in 2 per cent uranyl acetate for 20 minutes.

(4) Dehydrated in alcohol:

30 per cent for 15 minutes then

70 per cent for 15 minutes.

(5) Coated with agar at 40 to 45 degrees centigrade, cooled, and cut into very small pieces.

(6) Dehydrated further in alcohol:

90 per cent, two changes in 15 minutes

100 per cent, two changes in 15 minutes.

(7) Placed in styrene, two changes in 15 minutes.

(8) Rotated in vestopal overnight.

(9) Placed in vestopal to which were added 1.0 per cent initiator and 0.5 per cent activator and rotated for 1.5 hours.

(10) Embedded in the above vestopal solution in capsules and polymerized at 65°C for 48 hours.

(11) The block was then trimmed and microsections were

cut (90 mμ) using the Porter-Blum ultra-microtome.

Selected cell technique for isolating monolayer cells from Rose chamber coverslips (Robbins and Gonatas, 1964)

Preparation of coverslips for this study of monolayer cells was done in the following manner:

(1) Coverslips were cleaned by the method described in Appendix B, page

(2) A fairly heavy coat of carbon was applied by spraying the coverslips for one second in the Edwards carbon coating apparatus.

(3) The coverslips were placed in petri dishes and sterilized in a hot air oven for 48 hours at 180°C.

The processing of monolayer cells for ultrastructure studies after being selected and isolated was done in the following manner:

(1) The cells were fixed in 5.5 per cent glutaraldehyde in isotonic Tyrode's solution for 15 minutes.

(2) Post-fixed in osmium tetroxide for two hours.

(3) Dehydrated. (See dehydrating technique under "vestopal processing" page 196.)

(4) Placed in vestopal for 12 hours.

(5) Placed in vestopal with 1.0 per cent initiator and 0.5 per cent activator by clamping the vestopal containing

capsule over the selected cell using the "capsule-clamping apparatus".

(6) The material was polymerized at 65°C for 72 hours.

(7) Following the polymerization, the capsule clamp was removed and the capsule snapped off the coverslip.

(8) The block was then trimmed down to the circle etched in the carbon coat, which was at this point embedded with the selected cells in the vestopal.

(9) The sections were cut at about 90 mμ thick using the Reichert "OMUS" automatic ultra-microtome.

Staining of thin sections for ultrastructure study. (Karnovsky's lead stain)

The staining solution consists of 12 ml of boiled, distilled water and 0.2 ml of stock lead stain. This solution was centrifuged at 3,000 rpm for 15 minutes, before use.

The method is as follows:

(1) A drop of staining solution was placed on a clean wax surface in a staining cabinet.

(2) The section-containing grid was placed on the drop of staining solution, and left for 12 minutes.

(3) The grid was then removed and rinsed immediately in distilled water.

(4) The grid was then dried using absorbant paper.

The grids used throughout this work were of the Athene, 200 copper mesh type.

TABLE VI

THE AMNION NUMBERS, CELL TYPES AND SPECIMENS OBTAINED IN THE ULTRASTRUCTURE STUDY

Amnion number	Cell types							Specimens		
	1	2	3	4	5	6	7	P.B.	R.B.	P.
267	3	24	43	25	-	4	-	*	*	*
268	5	12	51	15	15	1	1	*		*
272	5	54	37	1	2	1	-	*	*	*
270	23	18	10	44	3	2	-	*		*
1-66	55	30	2	3	3	6	1	*		*
4-66	26	57	9	8	-	-	-	*	*	*
6-66	38	20	30	8	-	2	-	*	*	*
7-66	68	18	1	9	-	4	-	*	*	*
10-66	47	25	4	20	-	5	-	*		
16-66	56	21	1	17	-	5	-		*	*
83-66	12	25	58	1	-	4	-	*	*	*
99-66	-	33	61	6	-	-	-	*	*	*
108-66	-	24	60	15	-	1	-	*	*	*
112-66	51	16	11	19	-	3	-	*	*	*
116-66	Grade I i.e. no vacuoles in biopsy.							*	*	*

* The asterisk signifies that a specimen was obtained.

The underlined cell types were considered the main cell type for that amnion.

P.B. - placental biopsies.
R.B. - reflected biopsies.
P. - pellet of trypsinized cells.

APPENDIX D

PHOTOGRAPHIC TECHNIQUES AND MATERIALS

Kodak fine grain positive 35 mm film was used for all pictures of ultrastructure, and Kodak Kodabromide paper was used for the prints. The method for developing the 35 mm film is as follows:

(1) The film was placed in a film developing tank and developed in Kodak Dektol (2:1, Dektol: water) for four minutes, agitating once every minute.

(2) Rinsed in Stop Bath for two minutes.

(3) Fixed in Edwal Quick Fix for five minutes.

(4) Washed in running water at 68°C for 30 minutes.

This was done using an OA Kodak filter as a light source.

The photographic prints were prepared in the following manner:

(1) The paper was exposed to the illuminated negative for the appropriate time, using the Beseler Enlarger, model 45 MCX.

(2) The print was then developed in a one part Dektol to two parts water solution for two minutes.

(3) The print was then washed in Stop Bath for two minutes.

(4) The prints were fixed in Edwal Quick fix for five minutes.

(5) The prints were washed in running water at 68°C for 30 minutes.

This was done using an AO Kodak filter as a light source.

This printing technique was used also for the abstract prints taken from the 16 mm film obtained in the cine phase study.

Panatomic X film was used for still pictures of amnion cells which were photographed through a Reichert phase-contrast microscope.

The kodak Company of Canada supplied all the materials, except the Edwal Quick Fix which was supplied by the Edwal Scientific Products Corporation, Chicago, Illinois.

APPENDIX E

STAINING PROCEDURES

Sudan IV

Sudan IV was suspended in isopropyl alcohol (one gram in 100 ml of alcohol) and this solution was diluted with four parts of water to six parts of solution and filtered before use. The method of staining is as follows:

(1) The specimen was fixed on the glass slide using egg albumen.

(2) Stained in the Sudan IV solution for 30 minutes.

(3) Rinsed in distilled water.

(4) Counter-stained in Erlich's 50 per cent aqueous hematoxylin for five minutes.

(5) Washed in tap water.

(6) The specimen was mounted in glycerin jelly and sealed with nail polish.

APPENDIX F

This appendix is a log of all the sequences of cine-phase, time-lapse film from which Figures 11 to 29 were abstracted.

TABLE VII

FILM LOG

Thesis Figure Number	Rose Chamber Number	Film Number	Time Covered	Observations
11	133-66	63 & 64	67 hr	Vacuolation of type "1" cell and monolayer development
12	225	9	39 hr	Ameboid movement
13	5-66	28	134 hr	Fibroblast-like amnion cells and phagocytosis
14	255	16	43 hr	Macrospikes and cell movement without monolayer formation
15	161	3	3.5 hr	Retraction of a macrospike
16	251	14 & 15	103 hr	Cell fusion and cells which did not develop a monolayer
17	74-66	60	70 min	Fluctuating membrane in a multinucleated cell

TABLE VII (Continued)

Thesis Figure Number	Rose Chamber Number	Film Number	Time Covered	Observations
18	133-66	65	13 hr	Phagocytosis by a monolayer cell, also variety of nucleolar shape and position
20	74-66	59	110 min	Pinocytosis associated with neighboring cell death
19	74-66	59	104 min	Pinocytosis by a monolayer cell
22	74-66	60	30 hr	Nuclear movement from one side of a cell to the next and a mosaic cell
23	74-66	61 & 62	107 hr	Nuclear and cytoplasmic rotation
24	133-66	69	10 hr	Monolayer cell death
25	74-66	55	91 hr	Degeneration and retraction in a multinucleated cell
26	74-66	55	26 min	Nuclear blebbing during cell degeneration
28	1-66	25	100 min	Intercellular vacuole transport
29	74-66	57	27 hr	Intercellular vacuole transport