

AN ABSTRACT OF THE THESIS OF

Spencer Hiroshi Hamada for the degree of Doctor of Philosophy
in Zoology presented on August 15, 1975

Title: THE MORPHOLOGICAL EVENTS DURING THE
SELF-REASSEMBLY OF THE SEA URCHIN EMBRYO IN
CULTURE

Abstract approved Redacted for Privacy
Patricia Harris

The gastrula of the sea urchin Strongylocentrotus purpuratus was dissociated into single cells. In culture these cells appeared apolar, but reassembled into quasi-normal embryos composed of structures with polarized cells. In this study emphasis was placed on those morphological events coincident with the re-establishment of a polar orientation within the epithelial cells. Using both time-lapse dark-field-phase microscopy and electron microscopy, the major morphological events followed in detail include: (a) the migration of lysosome-like vesicles to specific margins within the epithelial cells; (b) the migration of the epithelial cells during the re-construction of the epithelium; and (c) the reformation of the cell junctions between the epithelial cells.

Evidence indicates that:

(1) The orientation resulting from the migration of the lysosome-like vesicles is transient and may not determine any lasting

polarity of the epithelial cell. The sequential migration of the lysosome-like vesicles first to the epithelial cell to cell interface, then to the basal margin and finally to the apical margin of the epithelial cell was coincident with and apparently in response to the presence of cell debris often trapped at these sites. The lysosome-like vesicles formed tight clusters along the epithelial cellular margins where phagocytosis of cell debris and resorption of specialized structures occurred. In contrast, the mesenchyme cell had smaller numbers of phagosomes and lysosome-like vesicles and was observed to participate to a more limited degree in phagocytosis and resorption.

(2) The orientation resulting from the formation of a leading edge during the migration of the epithelial cell is transient. The sequence of cytological events associated with epithelial cell migration included the formation of: (a) hyaloplasmic blebs along the cell free margin; (b) filopodia and microvilli; and (c) lamellae with a number of lamellipodia at the leading edge. In contrast, the mesenchyme cell formed filopodia on a smaller scale and was observed to participate in migration only to a limited degree. Coincident with the cessation of epithelial cell migration was the enlargement of the lamella between the epithelial cells into a hyaloplasmic sheet which forms the blastocoel wall.

Embryonic cells from reassembling aggregates were cultured as monolayers on glass treated with an adhesion enhancing factor derived

from the incubation medium. Cords of cells, composed of both epithelial and mesenchyme cells migrate away from the aggregate and leave in their wake clusters of cells. Epithelial cells were found at the leading margins of these cell cords and were observed forming cytoplasmic blebs, filopodia and hyaloplasmic leading edges during the migration of the cell cord. No apparent coordinate orientation was detectable within the epithelial cells of the migrating cell cord or stationary cell cluster.

A hypothesis is presented suggesting a role for resorption during epithelial cell migration.

(3) Cell contacts formed during reaggregation become more adhesive as development proceeds. The sequence of fine structural events associated with cell contact formation include the formation of (a) a non-junctional cell apposition, which was characterized by parallel apposed membranes; (b) an incipient continuous junction, which was characterized by parallel apposed membranes; and (c) a continuous junction, which was characterized by the presence of an electron dense region both superjacent and subjacent to the plasma membrane at the site of the junction.

The cell appositions between premigratory cells were easier to disrupt mechanically than the later forming continuous junctions. The forming continuous junction was first focal in extent and later zonal in girth around the apical margin of the epithelial cell.

Concurrent with the formation of the strongly adhesive continuous junction by the lamella was a cessation of epithelial cell migration, followed by an expression of an apical-basal polarity within the epithelial cell.

A hypothesis is presented suggesting a role for cell contacts during the coordinated orientation of the epithelia during the formation of the blastocoel wall.

The Morphological Events During the
Self-Reassembly of the Sea
Urchin Embryo in Culture

by

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A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

June 1976

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August 15, 1975

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THE MORPHOLOGICAL EVENTS DURING THE
SELF-REASSEMBLY OF THE SEA URCHIN
EMBRYO IN CULTURE

I. THE GENERAL EVENTS DURING REASSEMBLY

A. Introduction

One of the earliest investigations in experimental embryology was the disassembly of the sea urchin embryo by Curt Herbst (1900). He determined that calcium ions have a role in the maintenance of a confluent epithelium.

Recently a number of workers (Giudice, 1962; Millonig and Giudice, 1967; Timourian, et al., 1973; Hinegardner, 1975; Raff, 1975; Spiegel and Spiegel, 1975) have re-investigated this phenomenon emphasizing the reassembly of the sea urchin embryo from dissociated cells.

Electron microscopic studies (Millonig and Giudice, 1967; Giudice and Mutolo, 1970) have shown that with the exception of an occasionally acentric nucleus, the dissociated sea urchin cell of the mesenchyme blastula is apolar. There is no apparent localization of mitochondria, yolk, or vesicles. After the onset of reassembly, however, cell junctions are apically positioned, thus re-establishing cellular polarity. Not only is cellular polarity re-established but the normal animal-vegetal polarity of the embryo as well. The animal pole, which is determined in oogenesis by the position of the polar

body, has been experimentally disrupted to demonstrate the plasticity of the embryonic cell (Lallier, 1964).

The absence of hyaloplasmic projections when fixed for electron microscopy (Giudice and Mutolo, 1970) contrasts with the findings of Timourian et al. (1973) who find numerous hyaloplasmic projections in dissociated cells when cultured on untreated glass slides and slides coated with extracts of the hyaline layer.

It is postulated by Timourian et al. (1974) that hyaline synthesis play an important role as an aggregation factor. Tonegawa (1973), however, has isolated and chemically characterized an adhesion enhancing factor, which is a mucopolysaccharide-protein and is apparently different from hyaline or ovacquenin (Kondo and Sakai, 1971).

The purpose of this paper is to investigate the early events of self-reassembly in the sea urchin gastrula and determine whether micro-extensions are detectable during reassembly, and if there exists a relationship with the adhesion enhancing factor as isolated by Tonegawa (1973) and the cell surface.

B. Materials and Methods

Embryo Culture

The West Coast purple sea urchins, Strongylocentrotus purpuratus, were collected during the months of December through

February, which are the months the sea urchins are gravid along the Oregon coast. Gametes were shed after the injection of 0.5M KCl into the coelomic cavity. Sperm were collected in a dry beaker, while eggs were collected into a beaker filled with sea water. The eggs were washed three times in sea water.

Synchronous populations of embryos were obtained upon fertilization. Embryonic cultures were incubated at 10°C in filtered (Millipore, 0.22 μ pore size) sea water, to which 0.01% each of streptomycin and penicillin had been added.

Disaggregation of Embryos

Embryos at the appropriate stage were harvested with a hand-powered centrifuge, washed once in sterile sea water, and three times in calcium free sea water (Horstadius, 1950). Five milliliters of packed embryos were suspended in 15 ml of a solution containing 0.44 M sucrose, 0.05 M citrate, and no EDTA (Giudice, 1965). The embryos were mechanically disaggregated by 30-80 gentle excursions of a Potter homogenizer with no rotation of the pestle.

The resulting cells were monitored for completeness of disaggregation and viability by their ability to exclude eosin (Eaton et al., 1959; Geczy and Baumgarten, 1970).

Aggregate Culture

Cells were reaggregated in 200 ml of sterile sea water at a cell concentration of 0.1×10^6 cells/ml to 1.5×10^6 cells/ml, depending upon the size of the aggregates desired. A cell concentration of 0.1×10^6 cells/ml results in small aggregates with an average size of 5 cells after the first 30 minutes and 8 cells after 90 minutes. Cultures were continuously stirred with a plexiglass paddle at 60 rpm and incubated at 10° C.

For dark-field-phase observation, aggregates were cultured in either a Sykes-Moore chamber or under a paraffin sealed cover slip.

Isolation of an Adhesion Enhancing Factor

Freshly disaggregated cells were incubated in calcium free sea water (CaFSW) for two to six hours with constant stirring at 60 rpm. The cells were then gently centrifuged by hand and the supernatant centrifuged at $1,000 \times g$ for 20 minutes (Tonegawa, 1973). The pellet of cells and cell debris was discarded, the supernatant then treated with normal sea water and an adhesion enhancing factor allowed to precipitate overnight at 4° C. The precipitate was then dissolved in CaFSW. Glass slides or coverslips were coated with the adhesion enhancing factor by dipping first into the concentrated factor dissolved in CaFSW followed by a dip in normal sterile sea water.

Phase-light Vesicle Isolation

Nine milliliters of aggregates were homogenized in three milliliters of a solution containing 0.44 M sucrose 0.05 M citrate, and 0.001 M phosphate buffered at pH 7.2. The crude homogenate was layered on a sucrose step gradient composed of 0.88 M sucrose and CaFSW in the following CaFSW: sucrose proportions: 1:8 and 1:0 with the dense layer at the bottom. The gradient with the crude homogenate was then centrifuged at 16,000 X g for 30 minutes at 4°C using a HB-4 Sorvall swinging bucket head. The top two fractions were pooled and recentrifuged at 10,000 X g for one hour in a sucrose step gradient constructed in the following proportions: 1:5; 1:10; 1:5; 1:3; 1:2; 1:0.

The top three fractions were collected, checked for purity with dark field phase microscopy and then fixed for 30 minutes in OsO₄ buffered with 0.5 M phosphate at pH 7.2.

Electron Microscopy

Aggregates were gently hand centrifuged and fixed in 3-5% glutaraldehyde with 0.25 M sucrose and buffered at a pH of 7.2 by 0.5 M phosphate. After 30 minutes of fixation, the samples were washed in 0.5 M phosphate buffered at pH 7.2.

Samples were dehydrated in a graded ethanol series and flat embedded in Araldite. Aggregates of the proper size and orientation were photographed, mounted, and sectioned with a Sorvall MT-1 ultramicrotome. Sections were stained in saturated uranyl acetate and lead citrate (Venable and Coggeshall, 1965) and viewed with a RCA 3-H electron microscope.

For cytochemical staining, alcian blue was purified according to the method of Scott (1972). Alcian blue (.1%) was added to a fixative composed of 3% glutaraldehyde and 0.35 M $MgCl_2$ buffered at a pH of 6.5. Samples were then post fixed in a solution containing 1% OsO_4 , 0.35M $MgCl_2$, adjusted to pH 6.5.

C. Results

The early gastrula (Figure 1) was dissociated into 90% (\pm 3%) single viable cells (Figure 2b). Viability was determined by the cell's ability to exclude eosin Y (Eaton et al., 1959; Geczy and Baumgarten, 1970). The epithelial cells were characteristically ciliated and spherical, while the mesenchyme cells were non-ciliated and twice the diameter of the epithelial cells.

Giudice (1962) first showed that dissociated cells of the gastrulae from Paracentrotus lividus can reassemble into quasi-normal embryos. In this study, the dissociated cells from Strongylocentrotus purpuratus, like those of Paracentrotus lividus reaggregate immediately upon being

placed into normal sea water (Figure 3). The aggregated cells of Strongylocentrotus purpuratus segregate into clusters of like cells, with the epithelial cells forming the outer cluster and the mesenchyme cells forming the internal cluster. The epithelial cells of Strongylocentrotus purpuratus form the blastocoel wall and gut (Figure 4), while the mesenchyme cells form the pigment cells, muscle, and skeleton.

To determine the cellular distribution of the adhesion enhancing factor, which was characterized chemically as a mucopolysaccharide-protein by Tonegawa (1973), aggregates at different stages were stained with a dye detectable under both the electron and light microscope. Alcian blue 8-GX contains an electron dense copper atom and is reportedly specific for a number of glycoproteins (Quintarelli *et al.*, 1964; Scott and Dorling, 1965; Tice and Barrnett, 1965; Behnke and Zelander, 1970), depending upon the conditions during fixation.

Alcian blue positive material can be detected by light microscopy on the surfaces of the epithelial cells during the first 50 hours of reaggregation (Figure 6).

Electron microscopy of freshly dissociated cells show patches of electron dense alcian blue positive material along the cell surface (Figure 7) and especially at the endings of microvilli, which are fixed at pH 6.5 (Figure 8). The diameters of these alcian blue positive

regions range between 50 - 1,000 Å. The heterochromatic regions of the nucleus is the only other region of the cell which contains alcian blue positive areas.

Controls, with $MgCl_2$ deleted and the pH adjusted to 7.2 show no alcian blue reaction in the nucleus and only a slight alcian blue reaction along the cell surface (Figure 9).

Since Tonegawa (1973) has shown that the adhesion enhancing factor is soluble in CaFSW, cells treated in a calcium free medium should show a decrease of the adhesion enhancing factor their surfaces. When cells are treated for 2 to 6 hours in a calcium free medium, there is a decrease in the amount of alcian blue positive material at the cell surface but no detectable decrease within the nucleus (Figure 10). Along with the decrease in the alcian blue positive material, there is also a decrease in the rate of reaggregation for the incubated dissociated cells. Reaggregation begins only after 90 minutes, when cells treated in calcium free medium for 2 to 6 hours are returned to normal sea water compared with the immediate reaggregation for controls not incubated in CaFSW.

The viability of the sea urchin embryonic cells is only slightly affected by a short incubation in CaFSW. Two to three percent of the cells are eosin positive before incubation; 3 to 4% after a six hour incubation in CaFSW; and 12% (± 4) after a 24 hour incubation.

The calcium free incubation media can then be centrifuged at 10,000 X g for 30 minutes, the precipitate suspended in CaFSW, and then reprecipitated overnight according to the method of Tonegawa (1973). The isolated factor is alcian blue positive and promotes adhesion to foreign objects.

When studied in greater detail, the early morphogenic events following reaggregation clearly show migrations of both cells and organelles, which may play a role in the reformation of polarity in the reassembling sea urchin embryo.

One hour after the onset of reaggregation, phase-light vesicles begin to migrate to the cell-cell interface of the apposed epithelial cells (Figure 6b). This event occurs during the first hour for small aggregates (2-15 cells in size) and the second hour for large aggregates (30 or more cells in size).

Two hours after the onset of aggregation, active epithelial cell migration occurs simultaneously with the formation of a hyaloplasmic projection at the leading edge (Figure 5c). The mesenchyme cells, however, form only limited hyaloplasmic projections and do not actively migrate. The migration of the epithelial cells may continue until the sixth hour of reaggregation in the smaller aggregates (2-15 cells in size).

Five hours after the onset of aggregation, hyaloplasmic sheets enlarge, forming the wall of the blastocoel (Figure 5d). This marks

the first reformation of an embryonic structure. Frequent overlapping of the enlarging hyaloplasmic sheets is observed.

Fifteen hours after the onset of aggregation, the blastocoel may be fully formed in the smaller aggregates (Figure 5e). The recovery of a polar orientation in the reassembled embryo is evidenced by an animal pole composed of squamous epithelium at the anterior margin and a vegetal pole composed of cuboidal epithelium at the posterior margin. Characteristic of the epithelial cells of the vegetal plate are the hyaloplasmic filopodia, which can be detected radiating from these cells into the blastocoel. The mesenchyme cells often differentiate into highly refractive pigment cells and are positioned unusually close to the animal pole only in the small aggregates.

D. Discussion

There are two major problems involved in the self-reassembly of an embryo from disaggregated cells: (a) the reaggregation of the dissociated cells and (b) the recovery of the normal embryonic form.

Reaggregation may be enhanced by factors similar to those isolated and characterized from the embryos of Pseudocentrotus depressus by Tonegawa (1973). An adhesion enhancing factor with similar properties was isolated from the disaggregated cells of Strongylocentrotus purpuratus and tentatively identified as an alcian

blue positive material on the cell surface apparently concentrated at the ends of microvilli.

The recovery of normal embryonic form appears to be a more difficult problem to understand because of the types and numbers of cells that can be involved. By decreasing the numbers and types of cells in the reforming aggregate the problem can be simplified.

Though epithelial-mesenchyme cell interactions play an important role during the development in the sea urchin (Hynes et al., 1972) and in many other systems (Abercrombie and Middleton, 1968), by selecting aggregates with only epithelial cells, the embryonic sea urchin system could be simplified even more.

Thus during the formation of the blastocoel by the epithelial cells there appear to be three morphological events occurring in small aggregates: (a) the migration of phase-light vesicles to the cell-cell interfaces of the epithelial cells; (b) the active migration of the epithelial cells with the concurrent formation of a hyaloplasmic leading edge; and (c) the reformation of the blastocoel by enlarging the hyaloplasmic sheet of the epithelial cells.

The formation of extensive hyaloplasmic projections and cell migration calls for caution (Steinberg, 1970) in the interpretation of cell behavior during cell segregation. Without consideration of cell migration, the aggregate would morphologically resemble an onion, if quantitative differences in adhesion was the only operating factor during

segregation. The presence of hyaloplasmic projections suggests great caution in interpreting various cell movements as being "directed" as Spiegel and Spiegel (1975) alluded to in their work on cell aggregation between interspecies hybrid aggregates.

Cells may still make random contacts with their extensive cytoplasmic projections, which increase the cells' effective sphere. Contraction of these cell projections when attached to another cell may give the impression of "directed" movements if the resolution of the microscope is low.

It is far from clear what roles these morphological events play during embryonic reformation, however, more detail study of these events may lead to an understanding of the mechanisms which control and coordinate reassembly.

II. PHASE-LIGHT VESICLE BEHAVIOR AND FUNCTION
DURING POLARITY DETERMINATION OF
REAGGREGATING SEA URCHIN
EMBRYONIC CELLS IN
CULTURE

A. Introduction

Membrane bound vesicles play important and varying roles in exocytosis (Jamieson and Palade, 1967) and endocytosis (Friend and Farquhar, 1967) especially during epithelial regeneration (Odland and Ross, 1968; Anderson and Fejerskov, 1975; Buckley, 1974). Recent studies elucidate vesicle formation (Dauwalder, et al., 1972; Whaley, et al., 1972) and vesicle behavior (Schramm et al., 1972; Buckley, 1974) with major emphasis during the cellular events of phagocytosis (Deter, 1971; 1975) and secretion (Simson, et al., 1974).

The explanation of movement and positioning of membrane bound vesicles is still in doubt. Microtubule and microfilament involvement is well documented (Porter, 1966; Freed and Lebowitz, 1970; Allison et al., 1971; Bhisey and Freed, 1971). Experimental studies using colchicine, vinblastine and vincristine show a coincidental and perhaps a causal relation between the disappearance or reappearance of microtubules and the cessation or initiation of vesicle migration (Wisniewski, et al., 1968; Karlsson, et al., 1971; Wikswo and Novales, 1972; Wagner and Rosenberg, 1973). Cyclic-AMP and ATP involvement is also suspected (Schramm, et al., 1972).

Recent thin-section (Gabbay, et al., 1975) and freeze fracture (Orci, et al., 1973) EM analyses reconstruct the possible events occurring during vesicular binesis (i. e. vesicular fussion). Gabbay and coworkers (1975) suggest that the vesicles tandemly juxtaposition themselves and then form vesicular projections or lingula and simultaneously form a complementary vesicular furrow, just before fusing.

Vesicle behavior and function have been studied in the embryonic cells of chick (Buckley, 1974), rat (Buckley and Porter, 1967) and to a lesser extent in the sea urchin (Dan, 1960).

In the sea urchin, vesicle-like cortical granules migrate to the cell margin and participate in the formation of the fertilization membrane (Endo, 1961). Other vesicles with pigment also migrate to the cell margin, however, their function is unknown (Parpart, 1953; Belanger and Ross, 1970). No study on the sea urchin embryonic cells has been done on the phase-light or lysosome-like vesicles, which play important roles during the epithelial regeneration in other animals (Odland and Ross, 1968; Andersen and Fejerskov, 1974). Three vesicle types have been reported in the primary mesenchyme cells of the sea urchin embryo. Often in the pseudopodium of the migrating primary mesenchyme cell, large (100-400 μm diameter) vesicles, coated vesicles and "double" vesicles are seen (Gibbins, et al., 1969).

Giudice and coworkers (1962, 1967) have shown that the disaggregated sea urchin cell is initially nonpolar and during reaggregation becomes polar.

This paper details the behavior and structure of the phase-light vesicles during the formation of polarity in the epithelial cells of the reaggregating sea urchin embryo.

B. Results

Vesicular kinesis was followed in the living cell through the use of phase microscopy and vesicular fine structure detailed through the use of electron microscopy. As a necessary background, the structure of the living epithelial cell will be described as it appears during reaggregation in culture.

Similar to rat (Buckley and Porter, 1967) and chick (Buckley, 1974) embryonic cells in culture, the epithelial cells of the sea urchin in culture has an ovoid to round nucleus, acentrically positioned in the cytoplasm (Figure 11). Also within the cytoplasm are clearly visible granular organelles. Lipid droplets appear as spherical inclusions with characteristic phase-dark margins and phase-light centers. This appearance is due to the high refractive nature of the droplet. Yolk granules are round inclusions with a uniformly phase-dark appearance due to the opaque carotenoid pigment present. As in the rat and chick cells, lysosome vesicles are round to ovoid with

either a uniform phase-light or phase-gray appearance. Typically the lysosome vesicles are two or three times as large as either yolk or lipid droplets and often occur in tight clusters with their common margins apposed to each other.

The phase appearance of these structure in the disaggregated cell is similar to their appearance in the cells of the intact embryo (Figure 12) during the clustering of the primary mesenchyme cells.

Unlike the chick and rat embryonic cells, the smaller mitochondria of the embryonic sea urchin cell cannot be detected with phase microscopy. Under certain conditions hyaloplasmic regions of the cytoplasm may occur with no phase-visible organelles present.

During reassembly (0-30 minutes after the onset of reaggregation), no polarity is detectable in the epithelial cells either with phase-microscopy (Figure 13) or with electron microscopy (Figure 14). Typically the nucleus is acentrically positioned with no apparent polar distribution of organelles.

While there is no strict synchrony of vesicle behavior in the cells of large aggregates (16 - 30 cells in size) there is fair synchrony in smaller aggregates (2 - 15 cells in size).

As reassembly occurs there is a displacement of the nucleus to the apical surface of the cell and of the lysosome-like vesicles to the cell-cell interface (Figure 15 and 16). Lysosome-like vesicles occur in large groups at the cell-cell interface with very little cellular

ground substance separating the clustered vesicles. The vesicles usually contain amorphous electron detectable material (Figure 17). While a few of the vesicle-like structures may actually represent indentations of the plasma membrane, those vesicles with amorphous material most probably are truly vesicular in nature, while those without any material most probably are surface indentations of the plasma membrane.

When offered a number of alternate interfaces with glass, cotton, nylon or cellulose fibers the phase-light vesicles prefer the cell-cell interface (Figure 23).

The lysosome-like vesicles often occur either at sites where the cell is phagocytizing the cellular debris trapped between the aggregating cells (Figure 18) or where the cell is reabsorbing differentiated organelles, like its cilia (Figures 19 and 21b). Phagosomes can often be seen in these regions (Figures 18 and 19). Membranes observed within the lysosome-like vesicles are most probably indentations of adjacent structures like the plasma membrane (Figure 18) or other lysosome-like vesicle (Figure 19). Yolk granules can be seen in many of these lysosome-like vesicles probably being reabsorbed.

Often in close proximity to these lysosome-like vesicles are golgi apparati (Figure 20) and myeloid bodies (Figure 21). Myelin figures most probably represent tangential sections of massive

indentations from adjacent structures (Figure 22b).

In the reassembling sea urchin embryo, the blastocoel begins to be reformed when the differentiating cells form apical junctions. The lysosome-like vesicles at this stage of reassembly migrate to the basal margins of the cell, especially in regions that have accumulated cellular debris in the forming blastocoel (Figure 24).

In a few cells the growth of heterophagosomes can be followed under phase microscopy. In these fortuitous observations lipid and yolk granules with lingula projections are found in these phagosomes, while lysosome-like vesicles tend to be distributed along the margins of these phagosomes (Figures 22a, 22b, 25). Electron microscopy of these cells shows lipid, yolk granules, and remnants of membranous organelles (Figure 25). Lysosome-like vesicles can be seen around these phagosomes.

During this early stage of blastocoel reformation vesicles are formed in both outer cluster and inner cluster cells (Figure 26). In the epithelial cells of the outer cluster, lysosome-like vesicles seem to be formed from the fussion of saccules of the golgi apparatus. This is in contrast to the cells of the inner cluster, which produce vesicles from the cisterna of the golgi apparatus (Figure 26). The production of these vesicles does not seem to be localized to any site within the cell, since other sections through the same cell often show vesicles being formed from golgi apparatus on opposite sides of the

nucleus with the mature faces of the golgi apparatus in different directions (compare Figure 26 with 27).

After the epithelial cells of the outer cluster have formed cell junctions and a blastocoel cavity is well formed, the lysosome-like vesicles are localized at the apical margins of the cells at the site where they are normally located (Figure 28). The yolk granules, lipid droplets, and mitochondria are not found in a specific site in the cells at this time.

Vesicular kinesis occurs concurrently with another phenomenon, vesicular binesis. Similar to binesis in other cells, the juxtaposed vesicles of the sea urchin form vesicular lingula and complementary vesicular furrows (Figures 17, 18, 22b, 26). While there are suggestions of vesicular lingula in a number of phase-light vesicles in living epithelial cells (Figure 11), there is no unequivocal phase microscope observation of lingula formation between tandem phase-light vesicles.

When the three types of membrane bound vesicle are isolated in a sucrose medium, no vesicular lingula or furrow can be detected between lipid droplets (Figure 30a), or phase-light vesicles (Figure 30b). While the resolution of phase microscopy is too low to determine the presence of vesicular lingula between yolk granules, electron microscopy show only an occasional lingula projection of one vesicle into another (Figure 29).

C. Discussion

One of the first indications of successful self-reassembly is the acquisition of polarity by the epithelial cells in the reforming sea urchin embryo. The manifestations of this polarity as detected with light and electron microscopy are the concurrent basal positioning of the nucleus and the apical localization of the phase-light vesicles, followed by the apical formation of cell junctions.

In the following discussion the possible relation between vesicular kinesis with the acquisition of polarity and with other events during reaggregation will be ascertained.

That the distribution of phase-light vesicles is an expression of cellular polarity is suggested by its peculiar position at a physiologically active margin of the cell. No other inclusion shows any discernible distribution pattern during reaggregation.

The early localization of the phase-light vesicles at the cell-cell interface appears to be specific, since cell interfaces with some foreign substances do not show a preferential accumulation of vesicles.

This polarity defined by the vesicles is, however, transient and shifting as suggested by the distribution of the vesicles first at the cell-cell interface, then at the basal margin, and finally at the apical margin of the aggregating cell.

That vesicular kinesis is most probably due to external conditions rather than an intrinsic determination of embryonic polarity, is strongly suggested by the coincidence of vesicular positioning and cellular debris, and by the lysosome-like appearance and behavior of the phase-light vesicles.

Polarity of vesicles during binosis has been suggested by Gabbay and coworkers (1975), based on their observations that only one vesicular lingula forms from any given vesicle. Electron micrographs presented here tend to support their observations. Nevertheless, both phase microscopic observations of phase-light vesicles in living cells and isolated vesicles in sucrose medium, tend to suggest that vesicular lingula formation between tandem vesicles, at least in the sea urchin, may be an artifact of electron microscopic preparation. It is noteworthy that all observations of vesicular lingula formation between tandem vesicles have been in aldehyde-fixed material. It would be interesting to see if vesicular lingula are still formed in OsO_4 - fixed cells, as was done here for isolated vesicles.

The suggestion may still be valid, however, that vesicular lingula may represent a polarity in the vesicles. It could represent a weakening of the vesicular membrane just before binosis. It would be perilous to attribute modes of amoeboid activity to vesicular kinesis based on vesicular pseudopodia formation, as some have done (Schramm et al., 1972), until the fixation has been corrected.

The reformation of the sea urchin epithelium, like the regeneration of epithelia in other animals, forms phagosomes, myelin figures, and localizes lysosomes at the site of endocytosis. With the resorption of cilia, loss of microvilli and polarity, the epithelial cell takes on the appearance of a less developed cell or "de-differentiated" cell.

Since lysosome kinesis may not determine any meaningful polarity in the early reaggregating cell, the study of the other morphogenic events may prove to be more fruitful. The role of cell migration and cell junction formation during self-reassembly need to be investigated.

III. THE MIGRATION OF SEA URCHIN CELLS DURING REASSEMBLY AND IN MONOLAYER CULTURE

A. Introduction

Cell migration seems to play an important role in the development of the chick neural crest cells (Weston, 1963), the mouse primordial germ cells (Trinkaus, 1969), and the sea urchin mesenchyme cells (Dan, 1960; Gustafson and Wolpert, 1961; Gibbins, et al., 1969; Tilney and Gibbins, 1969).

Many of the cellular migrations during development appear to be due to the formation of a ruffling leading edge rather than of an amoeboid pseudopodium. There is no evidence of either cytoplasmic streaming or plasma sol-gel conversion during these migrations.

The sequence of morphological events which characterizes cell spreading and migration includes: (a) the formation of cytoplasmic blebs; (b) the formation of microvilli-like projections; (c) the centrifugal growth of filopodia; (d) the formation of cytoplasmic webbing with the concurrent flattening of the cell; and (e) the formation of the ruffling leading edge for locomotion (Witkowski and Brighton, 1971; Fogh and Sykes, 1972; Rajaraman, et al., 1974).

Electron microscopic study by Gibbins and coworkers (Gibbins, et al., 1969; Tilney and Gibbins, 1969) have determined the morphological events during the onset of migration for the primary mesenchyme cells in the intact embryo. Before the primary mesenchyme cell

migrates from the vegetal plate into the blastocoel it forms cytoplasmic blebs, which contain ribosomes and ER. The structures within the cytoplasmic blebs are uniformly distributed. Gibbins and co-workers (1969) suggest that cytoplasmic blebs function as a possible storage mechanism for ribosomes and ER. Soon after the formation of the cytoplasmic blebs, hyaloplasmic filopodia are formed containing well developed microtubules. These hyaloplasmic filopodia are thought to contract and thus pull the cell across the blastocoel cavity to form the mesenchyme ring.

During reaggregation (Shimanda, et al., 1974) and epithelial regeneration (Krawczyk, 1971; Andersen & Fejerskov, 1974) both microvilli-like projections and lamellipodia are thought to play a role in cell segregation and cell migration (Abercrombie, et al., 1971). Concurrent with the onset of migration, during epithelial regeneration, is a specific localization of lysosome vesicles and lipid droplets at the leading edge in migrating epithelial cells (Gibbins, 1968). Gibbins has suggested that there is a relationship between the phenomenon of phagocytosis and cell migration.

In culture, the migration of sea urchin embryonic cells isolated from the 16 cell stage is reported to be directed, when cells of two species are mixed (Spiegel and Spiegel, 1975).

B. Results

In the early reassembling sea urchin embryo (0 - 60 minutes) hyaloplasmic blebs appear along the cell free surface of the epithelial cell both before and during cell migration (Figure 31). Typically there are no phase detectable organelles within these cytoplasmic blebs. Within the cell body, the phase-light vesicles occupy no specific sites and the nucleus is acentrically positioned.

The formation of these hyaloplasmic blebs can be reconstructed from a series of electron micrographs taken at the onset of cell migration. Because the synchrony (\pm 15 minutes) in a population of even the smallest reagggregates is not accurate enough to allow a definitive reconstruction of this short-lived event (5 minutes) the sequence of formation presented here is necessarily tentative.

Early cytoplasmic blebs appear along the cell free surface in many epithelial cells just prior to and during phagocytosis (Figure 33).

Cytoplasmic blebs are believed to be formed by the formation of a cytoplasmic pit on the cell free surface of the epithelial cell (Figure 33 and 37b). Peculiar to these early cytoplasmic blebs is an electron dense region composed of fine fibers immediately subjacent to the plasma membrane and an electron dense region composed of coarse fibers in its center (Figure 33).

As the bleb enlarges the coarse fibers in the central region become farther spaced (Figures 37b; 34; and 35).

A fully formed cytoplasmic bleb is characterized by a well developed furrow which usually divides part of the electron lucid center of the cytoplasmic bleb from the rest of the cytoplasmic ground substance (Figure 35). In contrast to the closely packed coarse fibers in the center of early cytoplasmic blebs, the coarse fibers in the center of fully formed cytoplasmic blebs are widely separated from each other (Figure 35).

Under phase microscopy, the hyaloplasmic blebs can be seen to subside back into the cell body within two minutes and in a few cases the hyaloplasmic bleb may fragment from the cell body (Figure 42b).

The hyaloplasmic blebbing which occurs before the onset of cell migration continues actively through out the migration phase and ceases when cell migration halts (Figure 38).

The epithelial cell begins migration by flattening itself against a more non-mobile neighboring cell and is crescent shaped when viewed from the side (Figures 32a; b; and c). Concurrently, there is the formation of several hyaloplasmic projections of different sizes.

Hyaloplasmic lamellae are formed only by migrating cells (Figures 32a; b; and c), while microvilli-like projections are formed by both migrating and non-migrating cells in the smaller aggregates.

The microvilli-like projections appear to "investigate" the surrounding fluid media as well as the cell surface of the neighboring cell, however, unlike cilia they move asynchronously and may occasionally contain a cytoplasmic inclusion. The numerical density per epithelial cell and distribution of the microvilli-like projections appear to be the same for both migratory and non-migratory cells (Figures 32a; b; and c).

The hyaloplasmic lamella is only found at the anterior margin of the migrating cell and is the site of firmest attachment to the apposed cell (Figure 32a; b; and c). From the anterior edge of the leading edge an undulating hyaloplasmic projection can be detected under phase microscopy. This hyaloplasmic projection, the lamellipodium, does not seem to be in direct contact with the apposed cell.

Typically in the migratory epithelial cell the phase-light vesicles migrate to the cell-cell interface at this time and the nucleus is apically positioned.

When fixed for electron microscopy the microvilli-like projections are not preserved (Figures 36a and b). Due to the thinness and irregular outline of the lamella and lamellipodium only the base of the lamellipodium can usually be seen in thin sections (Figure 36b). The lamella of the leading edge has two hyaloplasmic lamellipodium. The superior lamellipodium makes a 30° - 40° angle with the substrate while the inferior lamellipodium is parallel to the substrate. The separation between the leading edge and the apposed cell is most

probably an artifact due to shrinkage during polymerization.

After cell migration ceases, the hyaloplasmic lamella is not only retained by the post-migratory epithelial cell but enlarged into a hyaloplasmic sheet, thus separating the cell bodies of the epithelial cells from each other (Figure 38). Like fibroblasts cultured on Araldite (Heaysman and Pegrum, 1973) there may be an overlapping of the lamellipodium of one cell with that of another. The hyaloplasmic lamella first enlarges forming a rectangular shaped blastocoel (Figure 38) and then curves convexly to form the typical spherical blastocoel (Figure 39). The overlapping of the hyaloplasmic lamella occurs along the apical margin of the epithelial cells and is the first indication of a permanent polarity in a stable epithelial sheet.

Both the simultaneous enlargement of the hyaloplasmic sheet and the synchronous beating of the cilia suggests a functional coupling of the epithelial cells at this time.

In order to investigate cell migration in greater detail cells were grown on glass slides. Sea urchin aggregates adhere poorly to clean glass, therefore glass microscope slides were coated with an adhesion enhancing factor derived from the incubation medium (Tonegawa, 1973). Spiegel and Spiegel (1975) using an unidentified protein film found that a coated glass slide was conducive to successful cell culture of embryonic sea urchin cells on glass, while Timourian and coworkers (1973) found that cells adhere poorly to

hyaline extract from embryonic sea urchins.

Large aggregates (15 cells or larger) with migrating epithelial cells were allowed to settle on the treated microscope slides or cover slips (Figure 40). Many of the epithelial cells formed filopodial attachments with the coated cover slip within five minutes of being introduced into the Sykes-Moore culture chambers. Similar filopodial formation has been observed during the attachment of other cells (Rajaraman, et al., 1974) and in the sea urchin (Spiegel and Spiegel, 1975). In some cases apparent apatulate endings could be seen at the sites of attachment as described by Fogh and Sykes (1972) in human amnion cells.

After the attachment sites are formed the filopodia grow centrifugally from the aggregate. The crescent shaped cells of the aggregate begin to flatten as they migrate onto the coated glass. Unlike the behavior of other cells (Rajaraman, et al., 1974) there is no apparent cytoplasmic webbing as the sea urchin embryonic cell spreads over the coated glass substrate (Figures 41a; b; and c).

Generally only one to three epithelial cells form well developed filopodium capable of pulling the cells of the aggregate into cell cords (Figures 42a; b; and c). Only the epithelial cells are observed to be actively migratory (Figures 40 and 42) and to be at the leading margin of the migrating cell cord. Endocytosis frequently occurs at the leading edge of the epithelial cells (Figures 41a; b; and c; 42a; b; and c).

Pigment cells derived embryologically from the mesenchyme cells maintain their spherical appearance for long periods of time and appear to be passively moved by the epithelial cells to which they are attached (Figures 42a; b; and c).

The apparent locomotive structure is the hyaloplasmic leading edge, which is well developed in the anterior most epithelial cells of the cord (Figures 41 and 42).

Hyaloplasmic filopodia are also seen at the posterior margin of the epithelial cells and often snap back as if under tension, as the cell cord migrates anteriorly. Many times "foot-prints" of hyaloplasmic fragments may be seen on the coated glass when the cell fails to disengage its attachment site (Figures 43 and 44).

As the cord of epithelial cells migrates away from the aggregate, patches of epithelial cell clusters are left in its wake (Figures 43 and 44). These epithelial cells are firmly anchored to the substrate by hyaloplasmic filopodia (Figures 43 and 44). There is no apparent localization of organelles within the cytoplasm of the epithelial cells, with the possible exception of the phase-gray vesicles at those positions which were once the leading edge (Figure 43).

Often pigment cells in these clusters develop hyaloplasmic filopodia, but they remain limited in size and there is little or no migration observed by these cells.

No reformation of an embryo occurs from these cell cultures grown on glass coated with the adhesion enhancing factor. This contrasts with the observation of Spiegel and Spiegel (1975) who observed reassembly occurring in monolayers of embryonic cells isolated from the 16 cell stage embryo.

C. Discussion

The morphogenic events occurring in the reaggregating sea urchin embryo before the onset of cell migration are similar to those observed in the cells of other systems (Witkowski and Brighton, 1971; Fogh and Sykes, 1972; Rajaraman et al., 1974). When cultured either in aggregate or monolayer, the embryonic epithelial cell of the sea urchin initiates migration in the following four sequential steps: (a) formation of cytoplasmic blebs through cytoplasmic furrowing; (b) formation of microvilli-like projections which may function by investigating the immediate region about the cell; (c) formation of a hyaloplasmic filopodia which anchor the cell to its substrate; and (d) formation of a lamellipodium at the anterior margin or the leading edge.

The only detectable difference is the apparent absence of cytoplasmic webbing by the sea urchin embryonic cell.

That there is at least an indirect relation between cytoplasmic blebbing and cell migration is suggested by the coincidence and

antecedence of cytoplasmic blebbing to cell migration both in the intact embryo and the reassembling embryo. The post-migratory epithelial cells display no blebbing activity on coated glass. It has been suggested that cytoplasmic blebbing serves as a storage mechanism for surface materials (Witkowski and Bright, 1971) or ribosomes and ER (Gibbins et al., 1969), based on the presence of these structures within the blebs. However, due to the paucity of ribosomes and ER in the cytoplasmic blebs formed during cell migration, it is doubtful that these blebs serve as storage mechanisms for these structures.

A similar blebbing activity is also observed in a cell about to divide or in a cell placed in calcium free medium (Tilney and Gibbins, 1969). In contrast to the uniformly formed cytoplasmic blebs occurring in cells treated with calcium free medium, the cytoplasmic blebs formed during reaggregation are composed of two distinct regions.

The fine filamentous layer subjacent to the plasma membrane in the region of the cell furrow and pit formation, may represent a contractile ring which initiates blebbing. The separation of the coarse fibers in the center of the cytoplasmic bleb may be due to an influx of water. Dornfeld and Owczarzak (1958) have suggested that a change in the hydrostatic pressure due to the movement of calcium ions may play a role in the formation of cytoplasmic blebs.

The microvilli-like projections which extend one cell diameter move in what appears to be a "searching" mode during reassembly in the sea urchin and in other systems (Taylor and Robbins, 1968). The increased surface area resulting from the large number of microvilli-like projections has been thought by some (Witkowski and Bright, 1971) to increase the efficiency of nutrient absorption. This is doubtful as the embryonic sea urchin cell at this time is well supplied with yolk and may have numerous heterophagosomes. The incubation medium has no nutritional value, hence the increased surface area in this case would not result in an increased nutrient absorption.

The ability to detect these microvilli-like projections in smaller aggregates and not in the larger aggregates agrees with O'Neill and Follett's (1970) observation that there is an inverse relation between the number of cells and the number of microvilli.

It is suggested here that these microvilli-like projections effectively increase 13-fold the area, which may influence the behavior of the cell. The numerical density and distribution of the microvilli-like projections appear to be similar in both migratory and non-migratory epithelial cells.

The hyaloplasmic filopodia anchor the cell to its substrate through modified apatulate endings. The adhesion is firm enough to cause fragmentation of the filopodial projection if the cell fails to disengage them before migrating. They do not seem to guide the cell

as suggested by Witkowski and Brighton (1971) since they frequently occur at places other than the anterior margin of the cell, and they do not display an "investigative" behavior when observed under time-lapse.

The lamellipodia at the leading edge appear to develop from the hyaloplasmic filopodia without passing through the cytoplasmic webbing stage. The hyaloplasmic filopodia and the leading edge in monolayer culture exhibit a tendency to form hyaloplasmic sheets. This tendency is also observed during the formation of the blastocoel in the reassembling embryo. It is suggested here that since the hyaloplasmic lamella is involved in the spreading activity during migration, it may also be involved in the spreading activity of the hyaloplasmic sheet during blastocoel reformation. If the cell junctions formed at this time are firm, the enlarging of the hyaloplasmic filopodium would result in the formation of a hollow sphere. A firm cell junction formed between epithelial cells would decrease the ability of the cell to migrate within the aggregate, thus explaining the cessation of migration just before blastocoel formation.

While the problem of migration cessation is important in the understanding of the formation of the stable epithelial sheet, the problem of migration initiation is important in the understanding of cell segregation and positioning.

Abercrombie's (1961) suggestion that it is the availability of a suitable free edge that triggers cell migration is supported by the migration of the epithelial cells from the forming aggregate onto the mucopolysaccharide-protein coated slide. The coated slide most probably presents a large concentration of an adhesion enhancing factor to the epithelial cells of the aggregate. The cell free surface of the epithelial cell is capable of forming a leading edge, when presented with a suitable free edge.

Gibbins' (1968) suggestion that phagocytosis plays a role during cell migration is supported by the large number of phagosomes and lysosomes observed in the pre-migratory and migratory cells. In contrast to Gibbins' (1968) observations in rat cells, no specific localization of lysosome vesicles and phagosomes was observed at the leading edge of the migrating epithelial cell in the sea urchin aggregate. The distribution of lysosome-like vesicles and phagosomes is influenced by the presence of cell debris which often accompanies epithelial disruption. The migrating epithelial cell of the rat may first contact cell debris at its leading edge and this would explain the presence of lysosome vesicles and phagosomes at this region.

If phagocytosis plays a role during epithelial cell migration it is doubtful that it plays an exclusively nutritive role (Gibbins, 1968),

since the embryonic sea urchin is well supplied with lipid and yolk inclusions.

It is suggested here that phagocytosis allows the cell to differentiate into a less developed state through the resorption of its specialized structures. This less developed state would permit the cell to migrate. Cells which do not resorb their specialized structures predictably would not migrate in culture during reassembly. Mesenchyme cells which do not resorb their pigment granules form only limited filopodium and do not migrate extensively in culture during reassembly.

This migratory advantage of the epithelial cells over the mesenchyme cells may allow the epithelial cells to successfully complete for the cell free surface region of the aggregate and thus passively segregate the less mobile mesenchyme cells into the interior region. This is not a new suggestion (Townes and Holtfreter, 1955; Steinberg, 1962; Trinkaus and Lentz, 1964), nevertheless, it does again emphasize the importance of cell migration and cell morphology during segregation despite proposals to the contrary (Armstrong and Parenti, 1972).

It is suggested here that the segregation of the epithelial cells to the outer cluster and the mesenchyme cells to the inner cluster could be a function of differential motility as well as differential adhesion.

That the leading edge plays a role in determining cellular polarity is suggested by its position at the anterior margin of the migrating epithelial cell; by its apical position in the post-migratory epithelial cell; and by its differentiation into the enlarging hyaloplasmic sheet at the apical margins of the epithelial cell. The cell contacts are formed between the hyaloplasmic sheets along the apical margins of the epithelial cells.

It is far from clear how the sequence of morphological events studied above controls cell migration. More detailed knowledge of these events would be of great value in understanding the nature of cell migration in the reforming epithelium and healing epithelium.

IV. CELL CONTACT FORMATION BY THE EPITHELIAL CELL DURING REASSEMBLY

A. Introduction

Cell junctions allow physical contact between apposing cells through specializations of the plasma membranes and cell surfaces. The events observed during cell junction formation in the embryonic tissues of chick (Trelstad, et al., 1967), fish (Lentz and Trinkaus, 1971), and the sea urchin (Wolpert and Mercer, 1963) parallel a number of morphogenic and physiological events. These diverse events include cell adhesion (Satir and Gilula, 1973), electrical coupling (Loewenstein and Kanno, 1964; Gilula, et al., 1970), short range cell-cell communication (Bennett and Trinkaus, 1970), and transepithelial permeability (Flower, 1971; Dan, 1960).

The formation of desmosome (Overton, 1962), septate junction (Gilula, 1973), gap junction (DeHahn and Sachs, 1972), and tight junction (Trelstad, et al., 1967) has been followed during normal embryogenesis. The common feature of most of these studies is the quantal increase in the elaboration and extensiveness of these junctions. The junctions become structurally more complex by an accretion of specializations to the plasma membrane of already well developed junctions. Electron dense areas are increased both superjacently into the intercellular space and subjacently into the cytoplasm. Many

junctions originate as small foci or plaques and increase in extent to become zones or belts, completely girdling the apical edges of a cell.

In morphologically stable invertebrate tissues one of the characteristic junctions formed between homologous as well as heterologous cells is the septate junction (Wood, 1959; Satir and Gilula, 1973).

Peculiar to the septate junction is a series of parallel sheets each of which is perpendicular to the apposing plasma membrane. These septate sheets are 50 A thick and extend across the 150-200 A interspace between the cells. Functionally, the junction may play a role in adhesion (Wood, 1959), in transepithelial permeability by acting as a sealing band about the apical end of the cell (Flower, 1971), and intercellular communication (Gilula, et al., 1970) since it may allow ionic coupling between cells (Loewenstein and Kanno, 1964). The primary difficulty with these studies is the concurrent presence of another junction with the septate junction, thus making it difficult to ascribe an exclusive function to the septate junction.

In many invertebrates, the septate junction may be supplanted occasionally by a pleomorphic form called the zonula continua. This continuous junction is a hybrid between a septate junction and a tight junction (Satir and Gilula, 1973). Though thin-sectioned, glutaraldehyde-fixed, and uranyl-lead-stained continuous junctions do not show septa, freeze fracture electron microscopy clearly demonstrates them. In thin sections the interspace of the continuous junction

is filled with a glyco-protein (Dallai, 1970) which when preserved obscures the septa.

B. Results

Through the use of phase microscopy, the living epithelial cells can be tracked as they actively migrate over the more stationary mesenchyme cells. If slight pressure is applied either through a cover slip or by surface tension, the cells of an aggregate can be partially separated.

When pressure is applied to an aggregate in which no active migration has yet taken place and one hour after the onset of reaggregation, the normally straight and angular cells maintain their boundaries even as they are separated (Figure 45).

When pressure is applied to an aggregate three hours after the onset of reaggregation and in which active migration has been observed, the leading edge can be drawn out into a broad hyaloplasmic projection (Figure 46). The region of coherence is limited to the leading edge and the junction is zonal in extent.

While there is no strict synchrony of cell migration and cell contact formation in the larger aggregates (20 or more cells in size), fairly synchronous aggregates can be obtained from populations of small aggregates (2 to 10 cells in size). The living cells were carefully monitored with dark-field-phase microscopy by photographing the

aggregate once every 15 or 60 seconds. From these populations of aggregates monitored by phase microscopy, parallel samples were taken for electron microscopy. Araldite embedded aggregates were photographed before thin sectioning, only if they were of the proper size and correct orientation.

Electron microscopy of pre-migratory and migratory epithelial cells show that these cells form non-junctional cell appositions with both the mesenchyme cells (heterologous appositions) and epithelial cells (homologous appositions) (Figure 49). In thin sections these cell appositions are characterized structurally by an irregular membrane surface causing varying intermembrane distances ranging between 75 - 200 A. At the site of apposition, the plasma membranes are characteristically non-parallel. The wave-like undulations of the apposing membranes appear to be out of phase, so that the points of contact are the apex of each wave. Unlike cell junctions, however, there is an apparent absence of any specialization of the plasma membrane or the intermembrane space. Under the electron microscope no detectable difference can be ascertained between cell appositions of premigratory and migratory epithelial cells (Figure 50). That these cell appositions, nevertheless, play a role in cell recognition is suggested by their temporal coincidence with the phenomenon of segregation.

As the epithelial cells make contact with other like cells they cease their migration and thus begin to form epithelial sheet. When slight pressure is applied to an aggregate at this time in reassembly, the normally straight or curved cell boundaries are drawn into numerous thin cytoplasmic projections (Figure 47). These thin projections suggest that cell coherence at this stage (5 hours after the onset of aggregation) is enhanced by a junction associated with localized regions on the plasma membrane of the epithelial cells and that the junctions in these regions are focal in nature rather than zonal. Very few of these localized regions of coherence occur between the outer epithelial cluster and the inner cluster (Figure 48).

A tentative sequence of cell junction formation can be reconstructed from EM samples taken at successively later stages of reassembly, however, because the junctions are localized plaques, they are observed in only a few thin sections, especially during early embryo reassembly.

On the surface of the aggregate, the epithelial cells contact each other along the leading edge of their undulating membrane. The filopodia of the leading edge usually come to lie subjacent to the edge of the neighboring epithelial cell (Figure 51). The cell contacts appear to be similar to cell appositions, except that the apposing membranes now are parallel at the sites of contact. No similar behavior is seen between heterologous cells.

The incipient junctions are initially seen at the apical border of the epithelial cells (Figure 52). Characteristically, the membranes are about 120-150 Å apart and are parallel to each other at the site of contact. An electron dense material is seen in the interspace between the parallel membranes especially at the apical borders of the epithelial cells. It seems justifiable to consider such regions as incipient continuous junctions, since they occur with greatest frequency in the early stages (10 - 15 hours after the onset of reaggregation) of reassembly and are in the appropriate position.

The electron dense junction increases in extent and is often found at the bottom of a "V"-shaped indentation formed between the conjoined cells (Figure 53).

During the latter stages of continuous junction formation, localized accumulation of fine texture material of intense density is seen in the cytoplasm subjacent to the junction (Figure 54). This material is alcian blue positive thus indicating that it could be a glycoprotein. The continuous junction may be asymmetrical at this time with the electron dense plaque often appearing at the apical edge of the more squamous of the two adjacent cells.

There is a lack of marked membrane interdigitation or microvilli formation during the time of cell contact reformation. The plasma membrane of the two apposing epithelial cells may diverge from each other subjacent to the region of cell junction formation and

hence outline an irregular cleft 200-1,000 A across. In this region there is a suggestion of small plaques of material attached to the plasma membrane, which may be similar to the material seen along the cell's free surface (Figures 50, 52, 53, and 54).

Occasionally mitotic figures can be seen during the reassembly process and therefore it is possible that some of the junctions observed could have been between daughter cells rather than junctions in the process of reforming between reaggregating cells. Aggregates treated with colchicine (10^{-5} gm/ml) for 5 - 30 hours form cell junctions similar to aggregates not treated with colchicine.

C. Discussion

When the zonula continua was first observed by Noirot and Noirot-Timothee (1967) along the apical edge of the intestinal epithelium in a number of insects, they postulated that tissue renewal was related to the presence of these junctions.

The present discussion further details the role of the continuous junction during the regeneration of the sea urchin's epithelium. In the following discussion three proposals will be developed: (a) that the cell contacts formed during reassembly play a role in the adhesion of the cells; (b) that this adhesion contributes to the mechanical stability of the reformed tissue sheet; and (c) that the cell junctions formed during reassembly play a role in coordinating the polar

orientation of the epithelial cells within the blastocoel wall.

Based on adhesive strength and time of occurrence there seems to be two functional types of non-junctional cell appositions. The cell appositions of the premigratory cell is weak in adhesion since cells can be separated without serious disruption to their angular shape during this time. In contrast, the cell appositions of the migratory cell is much more adhesive, since when separated the site of adhesion is maintained, even though the cell shape is seriously distorted.

The cell appositions of the premigratory cells are found between both homologous as well as heterologous cells and in this sense they are non-specific, on the other hand, the cell appositions of the migratory and post-migratory cells are found primarily between homologous cells. While heterologous epithelial-mesenchymal interactions play important roles during differentiation (Grobstein, 1968) and wound healing (Tarin and Croft, 1970) they do not seem to be a necessary condition for epithelium regeneration in the embryonic sea urchin, since many small aggregates of two or three cells complete the formation of a polarized epithelium without the presence of a mesenchyme cell. Thus while the epithelial cell resorbs much of its specialized structures, the cell still retains its ability to form a polarized epithelium.

The duration of each cell apposition is expected to be transitory in the actively locomoting cell. As in the intact embryo (Gustafson

and Wolpert, 1961), it is postulated here that the migrating cell extends its hyaloplasmic filopodia, anchors itself by a firm cell apposition, and then contracts the filopodium thus pulling the cell forward. This scheme would require the breaking and reformation of cell appositions during locomotion or the fragmentation of the anchoring structure. The latter could explain the "foot-prints" of hyaloplasmic fragments left in the wake of a migrating epithelial cell, when it is cultured on glass coated with mucopolysaccharide-protein layer.

Since cell appositions are coincidental with the events of cell segregation and cell recognition, they could play a role during these events. If they do, the similarity of cell appositions between both homologous and heterologous cells indicate that cell recognition and segregation are not dependent upon different cell contact types but rather to the possible differences in adhesion, duration or number of these cell appositions.

When the epithelial cells contact each other along the surface of the aggregate, they eventually cease locomotion. During this stage (3 - 5 hours after the onset of reaggregation) when cell appositions are still numerous, incipient cell junctions begin to appear. Unlike the earlier cell appositions, the cell appositions in the post-migratory stage are formed by apposing membranes which are parallel to each other. This prepares the contact region for cell junction formation, which requires parallel apposing membranes. These apical parallel

areas are proposed to be the site of incipient cell junctions. Not too surprisingly a similar parallel-nonparallel shift occurs during cell junction formation in the reaggregating chick cell (Lesseps, 1963).

Characteristically, the incipient continuous junction occurs between homologous cells. The cell junction is first recognized by the deposition of an electron dense material in the interspace between the two apposed membranes, and then by the formation of an electron dense plaque in the cytoplasm subjacent to the junctional region. This electron dense cytoplasmic material is similar to the electron dense apical material that forms during the development of the cell junction in the chick (Lentz and Trinkaus, 1971).

This early continuous junction would not aid in the regulation of transepithelial permeability, since during the early stages of its formation it is focal and only later during reaggregation does it become zonal.

The ability to draw out cells held by these junctions may be an indication that either the junctions are strongly adhesive or the cortical cytoplasm subjacent to the cell junction is more deformable than the rest of the cytoplasm. That it is a change in the adhesiveness of the junction is suggested by the observation of increased electron dense material in the interspace of the cell junction and no detectable change that would weaken the cortical cytoplasm.

The continuous junction forms gradually. Earlier work indicated that the septate junction (Wolpert and Mercer, 1964) and other junctions (Overton, 1962; Lentz and Trinkaus, 1971) were formed in a quantal fashion, that is in large steps and nearly completely formed. This discrepancy may not be difficult to understand if recent evidence is correct that cell junctions may form within 20 - 60 seconds under ideal conditions (Heaysman and Pegrum, 1973), while they may require 5 - 60 minutes to form under aggregate conditions (Johnson et al., 1974). The ability to detect finer gradations in junctional formation could be due to the slower formation of the cell junctions among disrupted cells.

The incipient continuous junction is formed between overlapping lamella along the margins of the leading edge. Since the lamella is the site of substrate attachment for the epithelial cells during migration, the incipient continuous junction occurs at the margin along the substrate or the basal margin.

During the enlargement of the hyaloplasmic sheet the basal lamellae abut and no longer overlap each other. During this time the cell body bulges into the blastocoel cavity coincidental with a convex curling of the hyaloplasmic sheet. The once basal lamella becomes apically positioned due to these two events. The lamellae are conjoined by mature continuous junctions, which are characterized by electron dense junctional regions.

If there is to be any coordinated orientation of cellular polarity in a reforming tissue, common reference points are necessary. Throughout reassembly a number of reference points can be detected. Early during reassembly, the cell free surface serves as a reference point for the segregation of the epithelial cells towards the external surface. The cell-cell interface due to trapped cell debris, serves as a reference region for the migration of lysosome vesicles. These references are relatively transient when compared to the more stable cell junctions.

While structures other than cell junctions (e. g. vesicles, cilia, and microvilli) also show an apical-basal polar distribution, it is suggested here that the cell junctions play a role in coordinating the polarity of all conjoined cells within a tissue perhaps due to the junction's ability of ionically coupling cells conjoined by septa (Loewenstein and Kanno, 1964).

The absence of mesenchyme cells in many reassembling embryos with coordinated oriented epithelia suggests that epithelial-mesenchyme cell interactions are not required for polar orientation during reassembly. Continuous junctions are not observed between the polar mesenchyme cells. These cells do not coordinate their polar orientation in the same manner that epithelial cells do.

The actual agents which communicate polarity within the cell is

not known, perhaps more study of cell junctions during the orientation of polarity would help our understanding and discovery of these agents.

V. SUMMARY

To determine the morphogenic forces involved in re-establishing an epithelium whose component cells are oriented in a coordinated fashion, a number of morphological events were studied in detail.

These morphological events include:

- (a) the migration of lysosome vesicles to specific cell margins;
- (b) the migration of epithelial cells to specific positions in the aggregate;
- (c) the formation of a number of hyaloplasmic projections during cellular migration; and
- (d) the formation of cell junctions.

The migration of vesicles to specific cell margins results in the first observable apical-basal polarity. This orientation is transient and probably, does not determine a lasting polarity in the epithelial cell, however, the vesicles themselves may play a role during re-assembly. These golgi produced lysosome vesicles are involved in the phagocytosis of cellular debris and resorption of cell structures. It is hypothesized that the resorption of specialized cell structures enables the cells to migrate by changing the differentiated state of the cell.

The migration of epithelial cells to the cell free surface is associated with a number of cytological events. The events include:

- (a) cytoplasmic blebbing;
- (b) formation of microvilli;
- (c) formation of a hyaloplasmic lamellae at the leading edge;
and
- (d) the formation of a number of hyaloplasmic filopodia by each epithelial cell.

While the distribution of the cytoplasmic fibers subjacent to the plasma membrane changes during cytoplasmic blebbing, structural changes in the lamella and filopodium during cell migration were not observed. The active epithelial cell migration which occurs during cell segregation, may play a role in positioning the epithelial cells at the outer surface of the aggregate.

The lamella overlap and initiate cell junction formation between epithelial cells. The structural events involved during the establishment of the cell junction include the formation of:

- (a) a non-junctional cell apposition, which is characterized by non-parallel apposed membranes;
- (b) an incipient continuous junction, which is characterized by parallel apposed membranes;
- (c) an early continuous junction, which is characterized by the presence of electron dense material in the intermembraneous space; and

- (d) a mature continuous junction, which may have an electron dense region subjacent to the junctional area. The forming continuous junction is first focal in extent and then gradually becomes zonal. Coincident with the formation of the continuous junction is the expression of an apical-basal polarity in the epithelial cells forming the wall of the blastocoel.

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APPENDIX

Figure 1. Intact early gastrula stage of the embryonic sea urchin. The embryo at this time is polar, with an animal pole (AP) at the anterior and a vegetal pole (VP) at the posterior. Two general cell types can be distinguished: the epithelial cells (E), which form both the ectoderm and the endoderm; and the mesenchyme cells (M) which form at this stage the mesenchyme ring in the blastocoel (B). (860 X)

Figure 2. Disaggregated and single cells of the early gastrula stage of the sea urchin.

(a) Dark-field phase contrast micrograph of a disaggregated epithelial cell. Nucleoli (arrows) can often be distinguished within the nucleus. (1,800 X)

(b) Low magnification showing the nearly complete disaggregation of the early gastrula. Seven percent of the cells are incompletely dissociated and occur as pairs. Mesenchyme cells are generally two times larger than the epithelial cells at this time (arrow) (360 X)

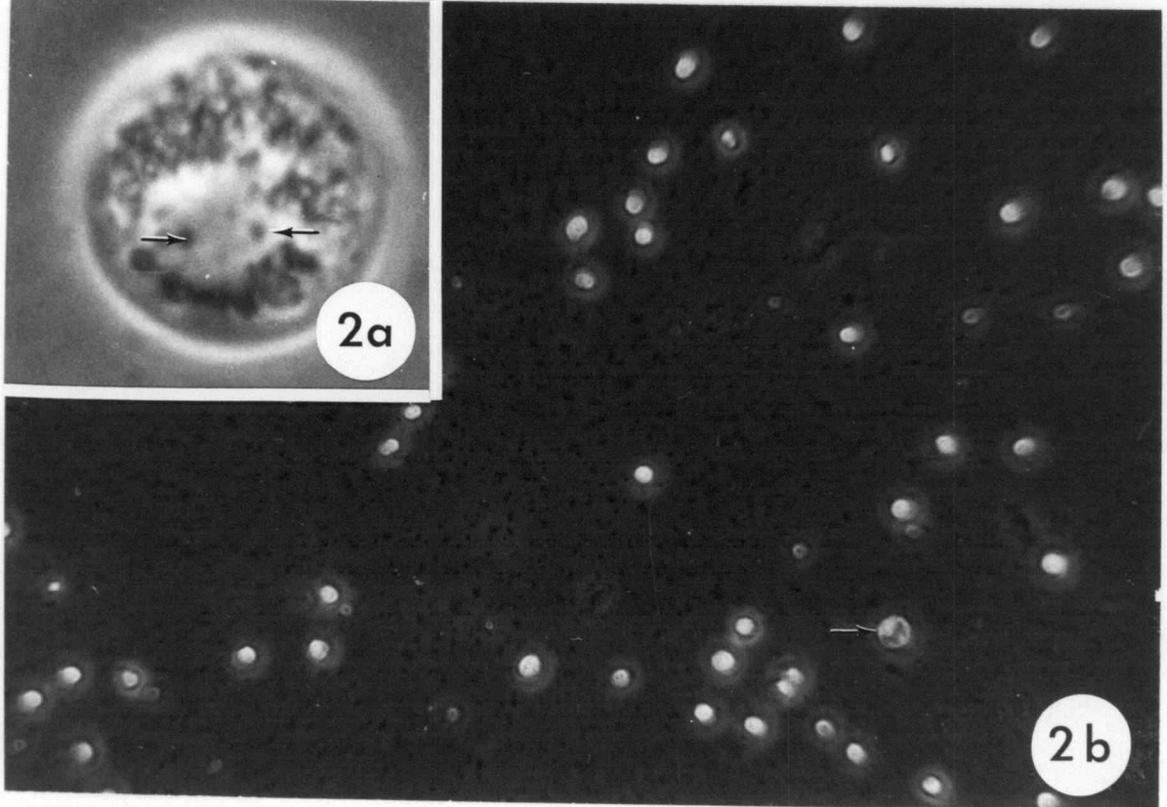
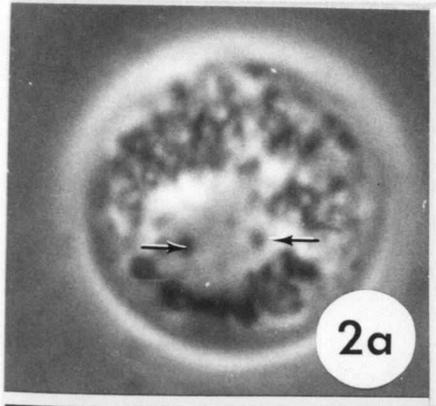
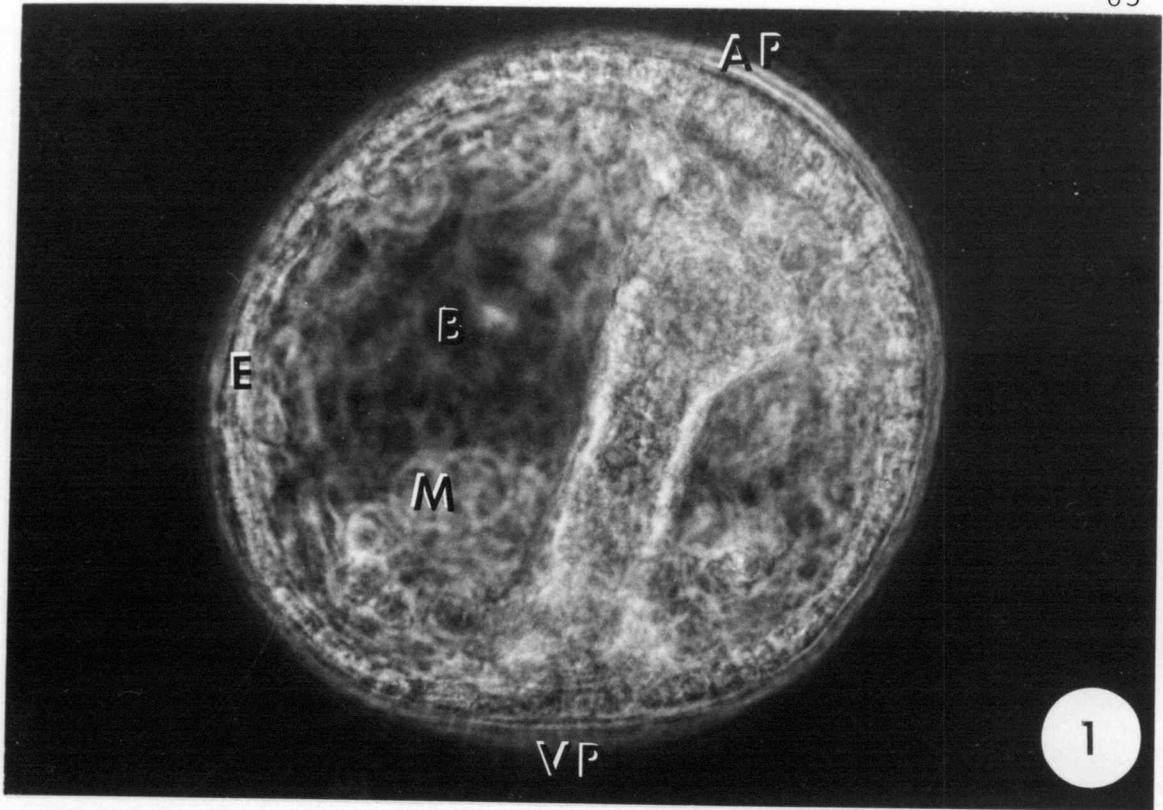


Figure 3. Three major stages of sea urchin reaggregation can be seen here: (a) initial clumping of cells; (b) migration of aggregated cells; and (c) the initiation of morphogenesis with the reformation of the blastocoel. (360 X)

Figure 4. Self-reassembled sea urchin gastrula with a well formed foregut (F), midgut (M), and hindgut (H). Within the blastocoel hyaloplasmic filopods (fl) can be detected. The embryo has regained its polar orientation with a distinct anterior (AP) posterior (VP) orientation. (1,430 X)

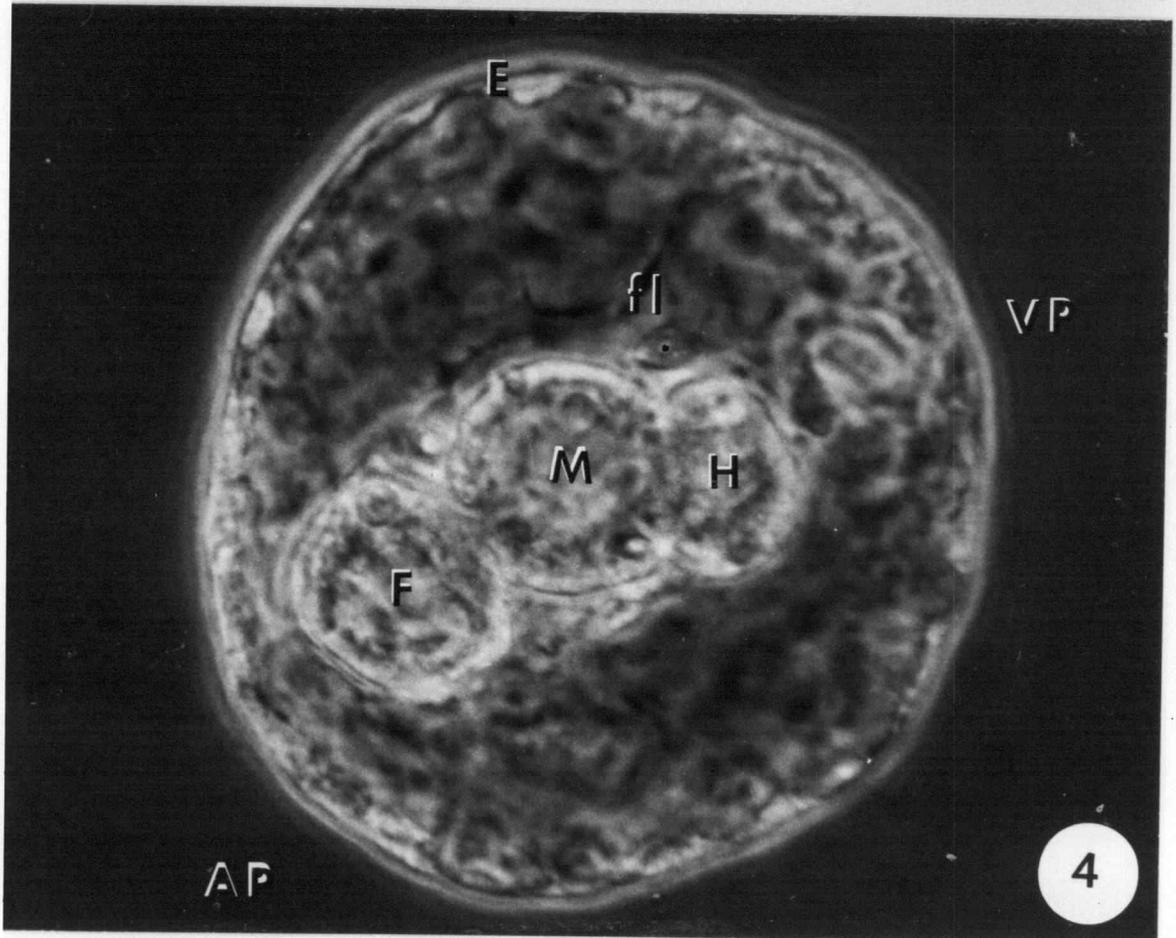
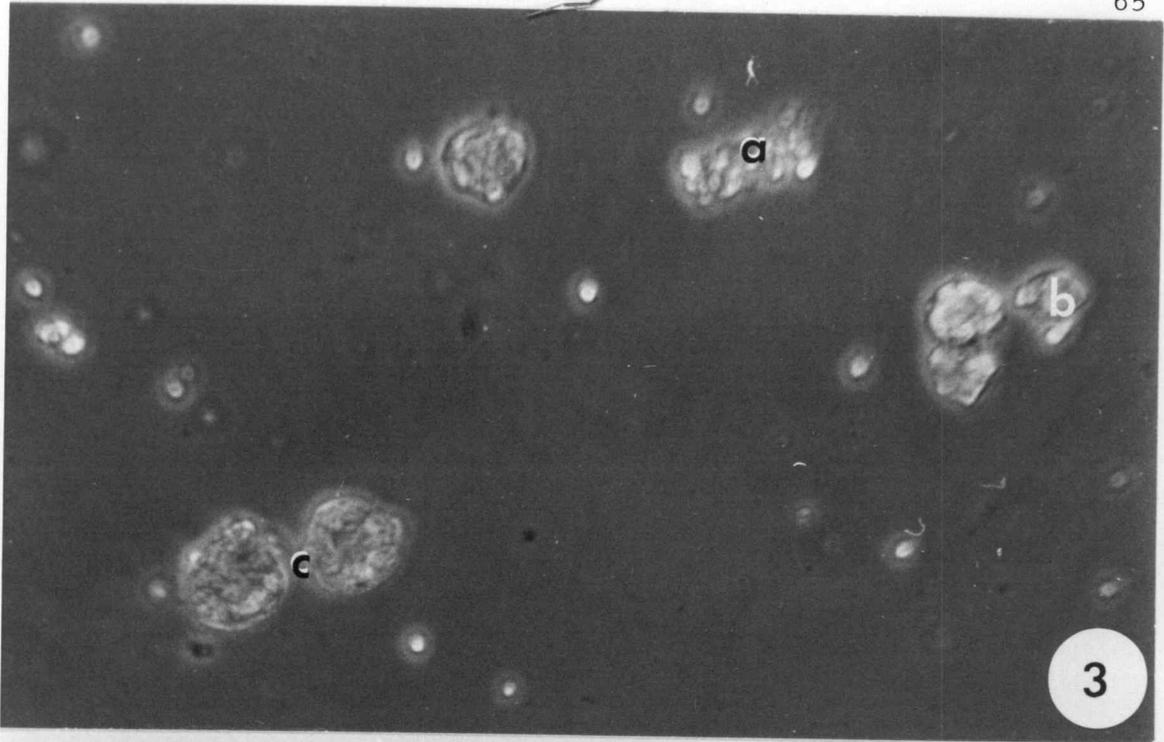


Figure 5. A time lapse sequence showing the five major events occurring during the self-reassembly of the sea urchin gastrula from dissociated cells in rotation culture. (3,020 X)

(a) A reaggregate +1 hour after the onset of initial aggregation. Between 0 - 3 hours single cells may still adhere to the forming aggregate.

(b) Two hours after the onset of reaggregation phase-light vesicles within the epithelial-like cells migrate to their cell-cell interfaces. In small aggregates (2 - 15 cells) this event occurs between +1-2 hours.

(c) Four hours after the onset of reaggregation active migration of the epithelial-like cells (me) are characterized by a hyaloplasmic leading edge (arrow). Mesenchyme cells (P), which form an internal cluster show no active migration during this time. In small aggregates (2-15 cells) this event occurs between +2-6 hours.

(d) Eight hours after the onset of reaggregation the blastocoel is reformed by the enlargement of hyaloplasmic sheets (hs), which frequently overlap at this time. The mesenchyme cells (P) maintain their position within the forming blastocoel cavity. In small aggregates (2-15 cells) this event occurs between 5-24 hours.

(e) Sixteen hours after the onset of reaggregation the blastocoel is well formed by a convex curving of the epithelial-like cells. The embryo at this time has a distinct polar orientation with an anterior margin (AP) composed of cubodial epithelial. The cubodial epithelial cells project numerous filopodia (arrows) into the blastocoel. The pigment cell (P) fully differentiates from a mesenchyme cell of the early aggregates. In small aggregates (2-15 cells) this event occurs between 15-36 hours.

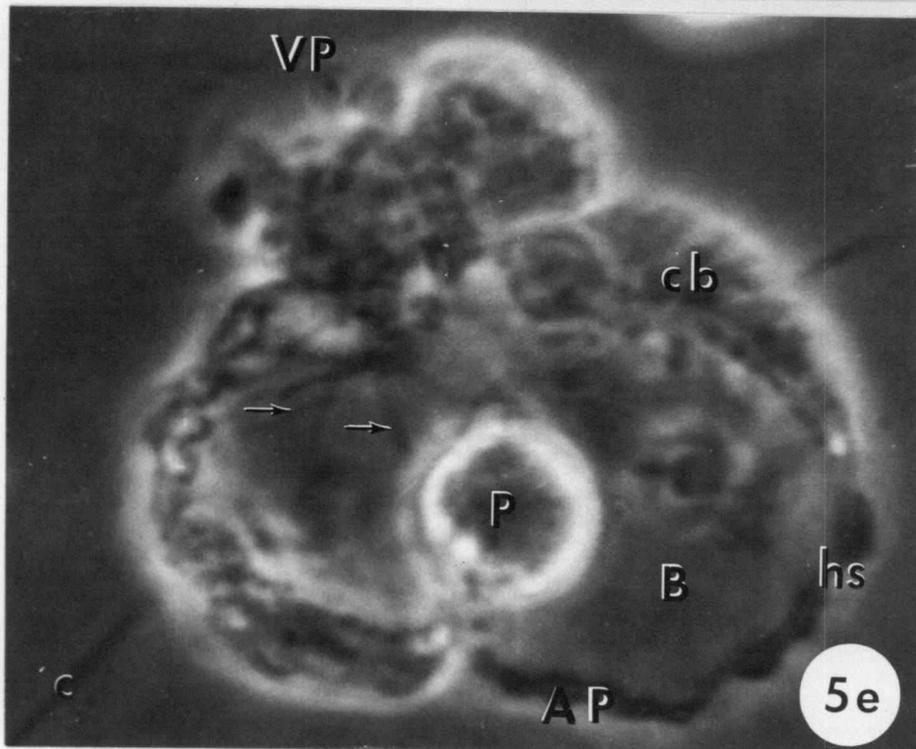
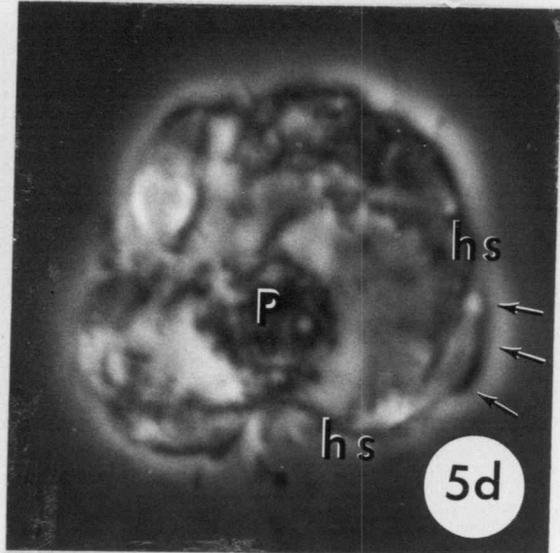
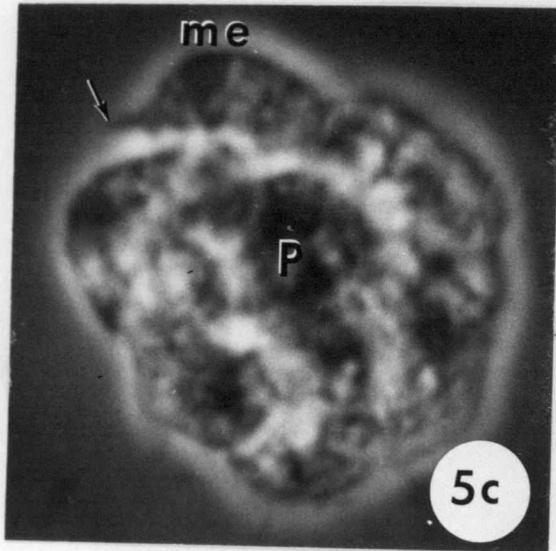
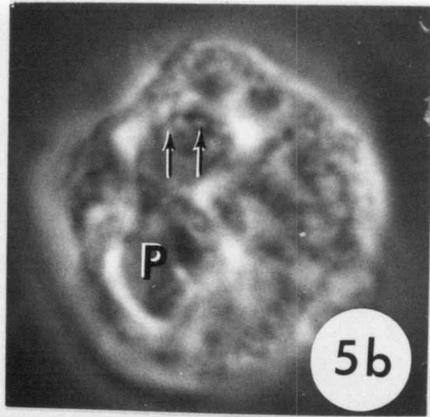
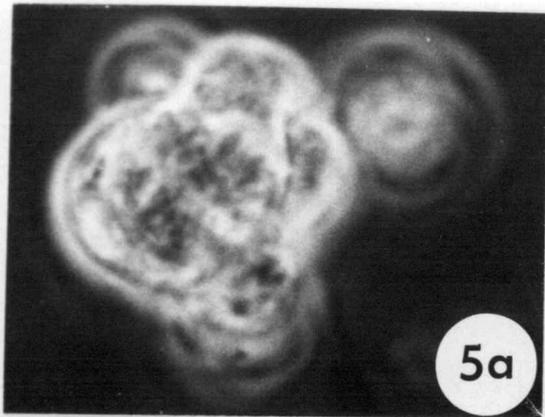
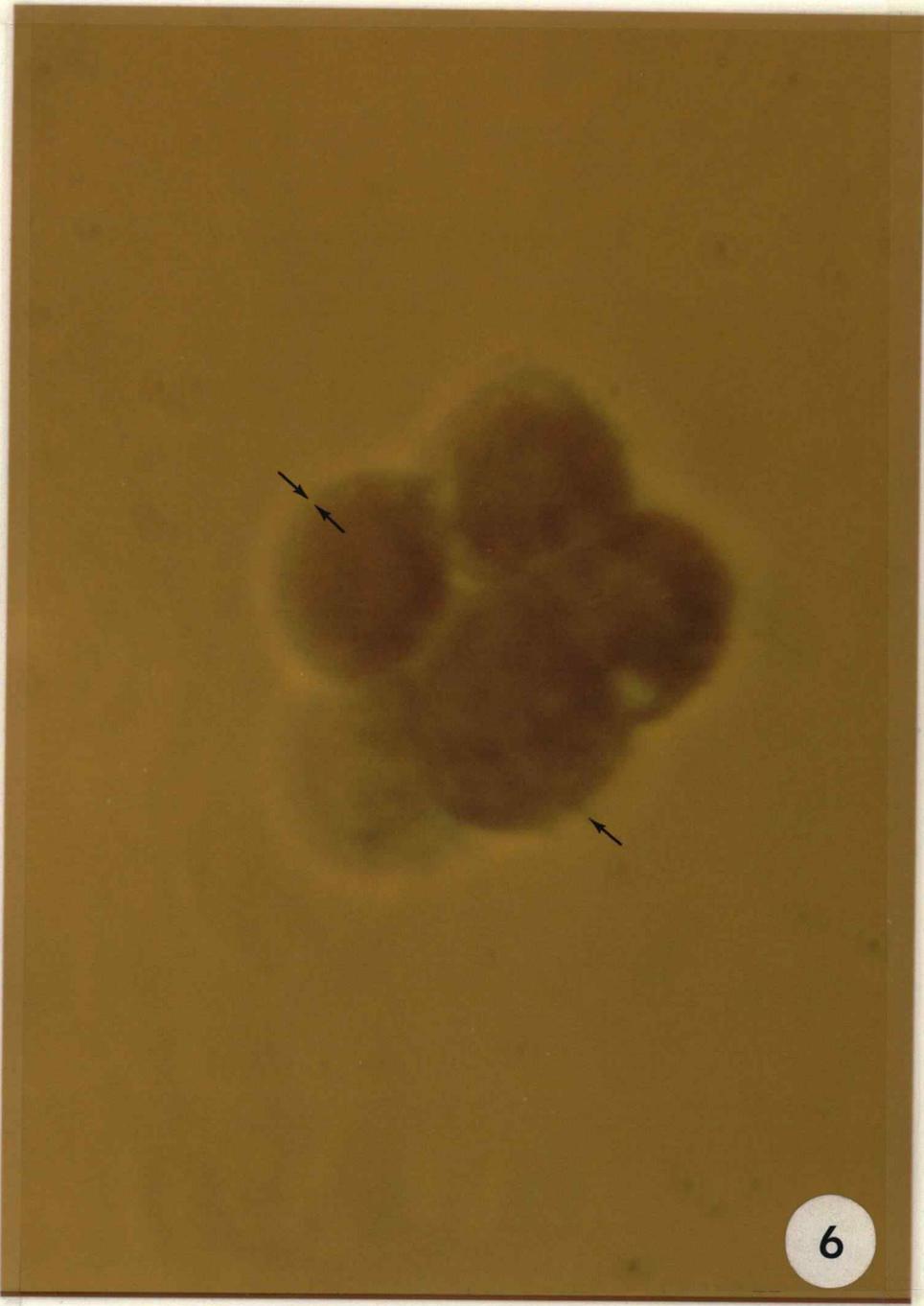


Figure 6. An aggregate fixed for electron microscopy 15 minutes after the onset of aggregation. Alcian blue positive regions can be detected (arrows) at the surface of the epithelial cells. (4,000 X)



6

- Figure 7. Freshly dissociated sea urchin embryonic cell. Alcian blue positive areas can be detected both in the nucleus (n) and along the cell surface (arrows). (4,000 X)
- Figure 8. Section through the same cell pictured in Figure 8. Along the apical margin of the epithelial cells microvilli with alcian blue positive areas can be detected (arrows). (4,000 X)
- Figure 9. Freshly dissociated sea urchin embryonic cell fixed at pH 7.2 and without MgCl₂ added to the fixative. There is no apparent alcian blue reaction within the nucleus (n), and only scattering areas of reaction along the cell surface (arrows). (7,000 X)
- Figure 10. Dissociated sea urchin cell cultured in calcium free sea water for 6 hours and then fixed for electron microscopy. While areas within the nucleus (n) stain with alcian blue, only limited areas of the cell surface show any reaction (arrows). (7,000 X)

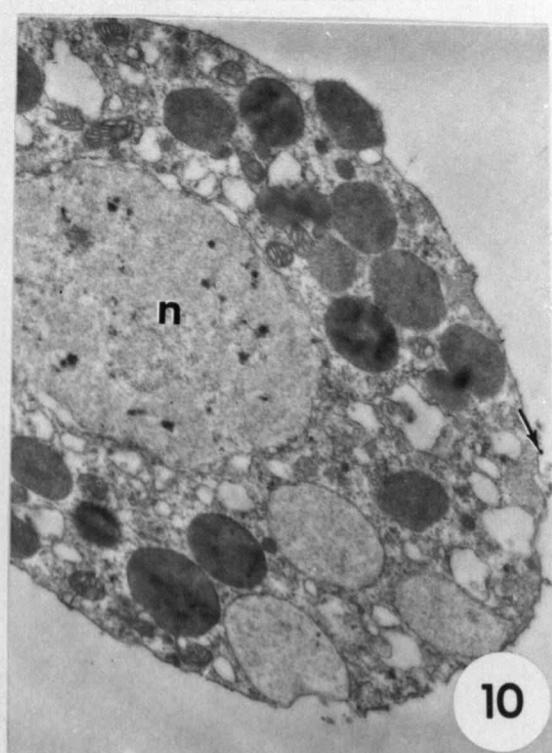
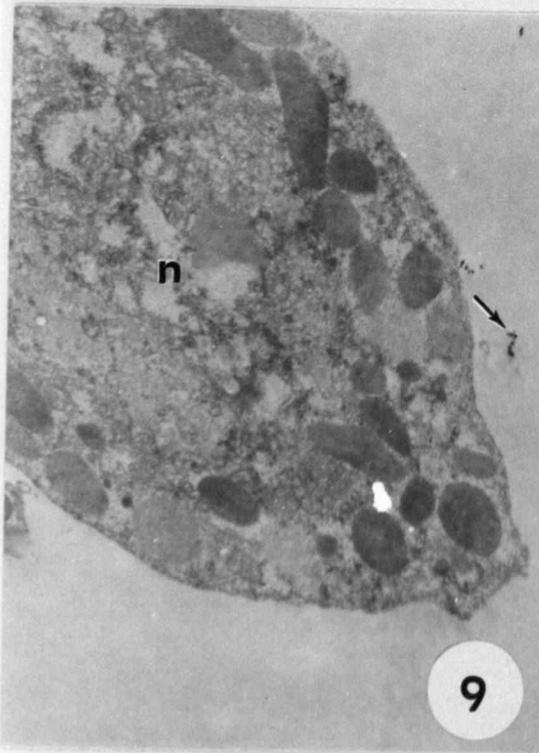
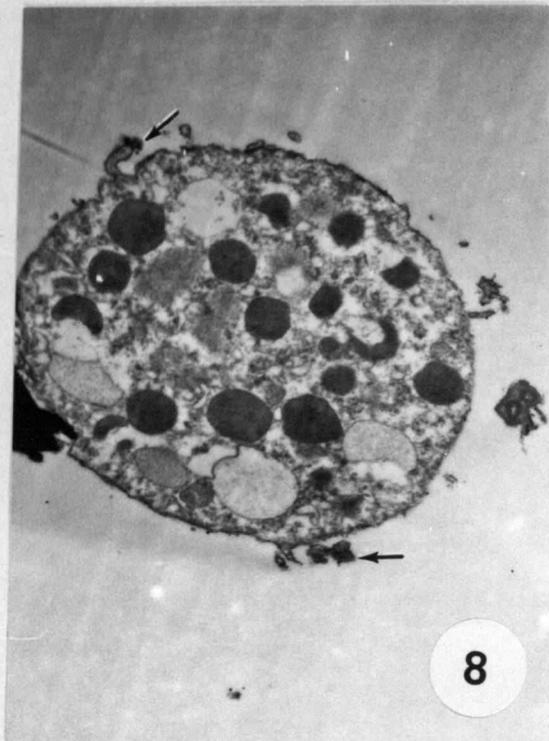
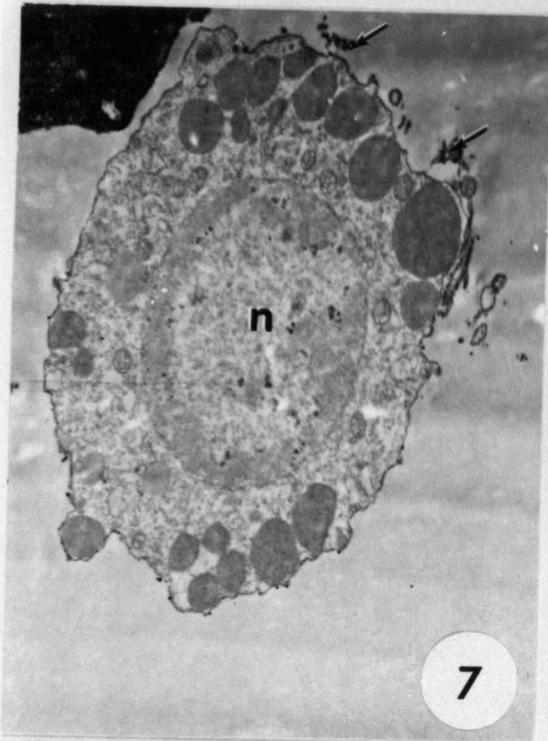


Figure 11. Two epithelial-like cells that have been slightly flattened for phase microscopy. Characteristically the 7-10 μ nucleus (N) is acentrically positioned in the cell. Within the cytoplasm numerous 1-3 μ lysosome-like vesicles (LV), 1 μ lipid droplets (L) and 0.5 - 1.0 μ yolk (Y) can be identified. Arrows indicate possible sites of vesicular lingula formation. (5, 100 X)

Figure 12. Two apposed mesenchyme cells with lysosome-like vesicles (LV) at the cell-cell interface during skeleton formation in the intact embryo. (3, 110 X)

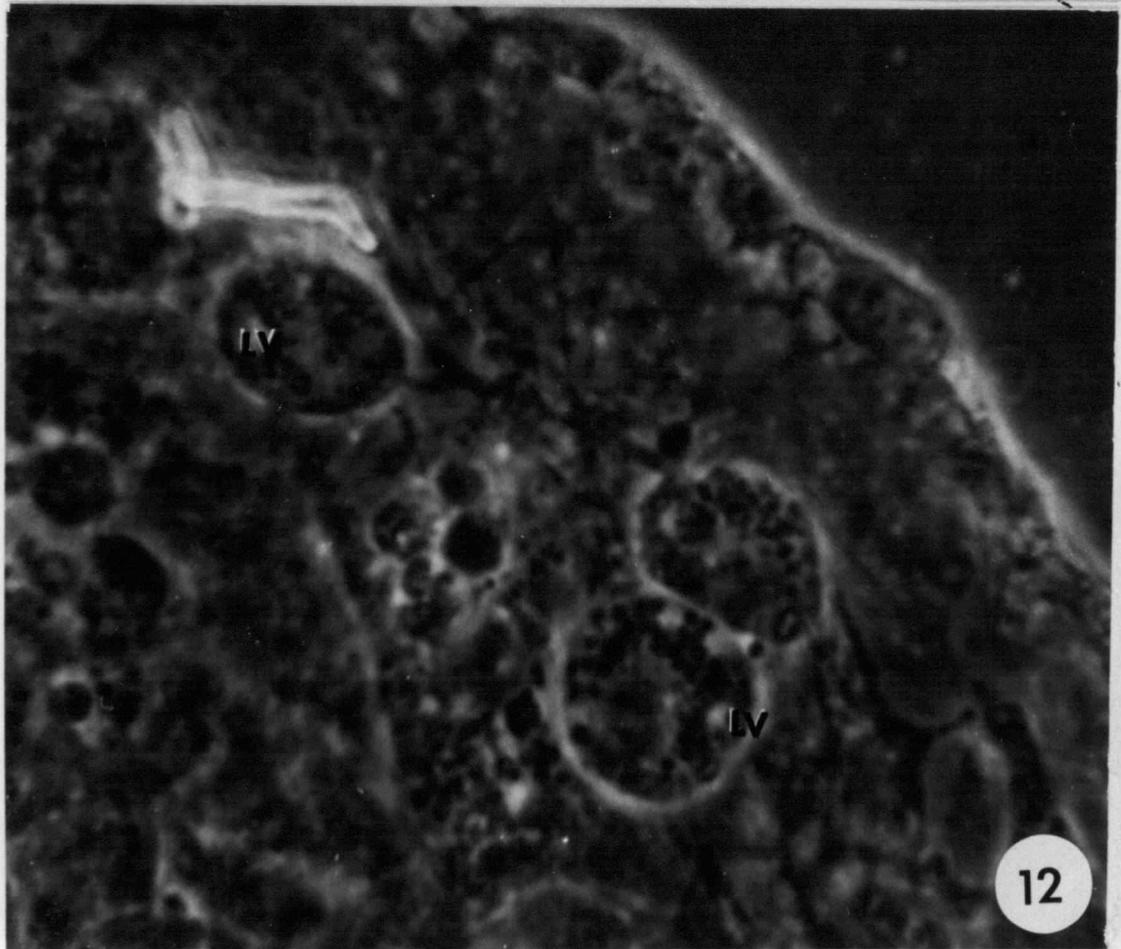
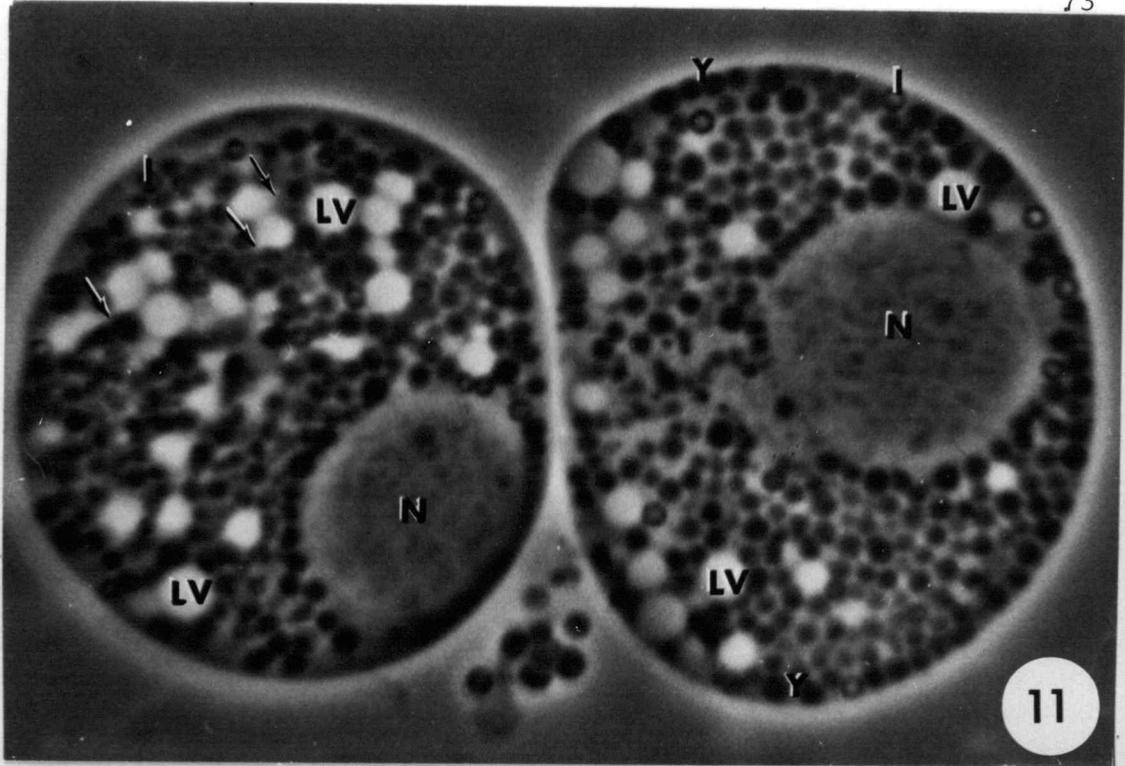


Figure 13. A small aggregate of three epithelial-like cells 30 minutes after reaggregate. There appears to be an even distribution of lysosome-like vesicles (LV), yolk granules (y), and lipid droplets (l). The nucleus (N) is usually acentrically positioned within the cell. At this time frequent cytoplasmic blebs (CB) can be seen along the cell free margin of the aggregate. (4,710 X)

Figure 14. A slightly larger aggregate fixed for electron microscopy and taken from the same population as the aggregate in Figure 13. There appears to be a slight localization of the lysosome-like vesicles (LV) at the cell margins. Yolk (Y) and lipid (l) granules, however, appear to be evenly distributed. Cell debris (d) appears to be trapped between the cells of the aggregate. (5,300 X)

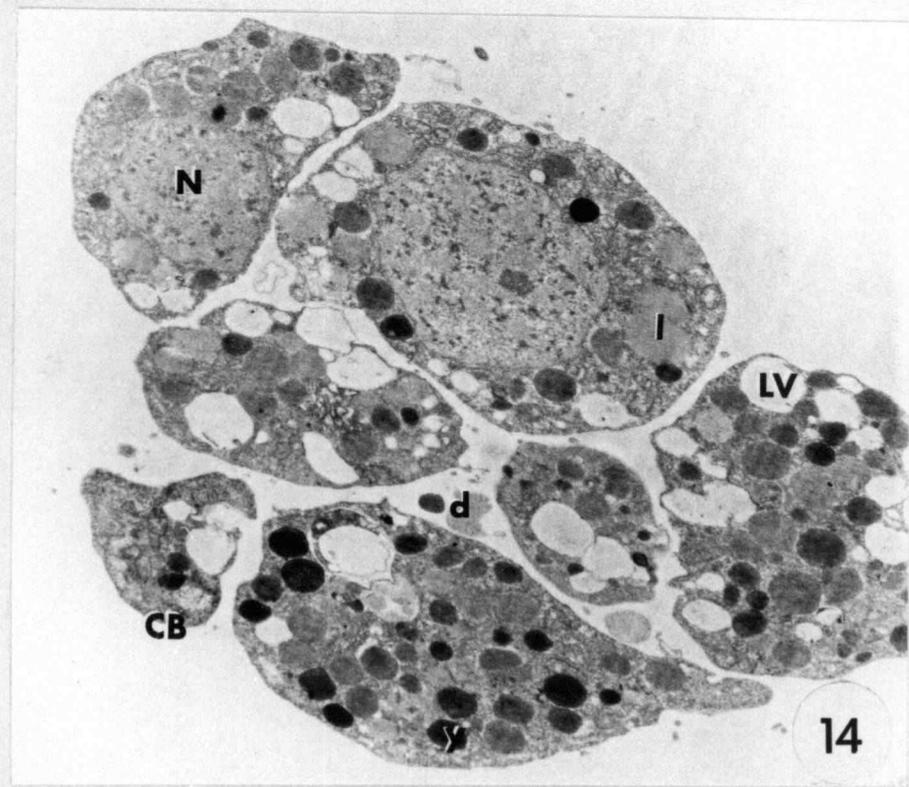
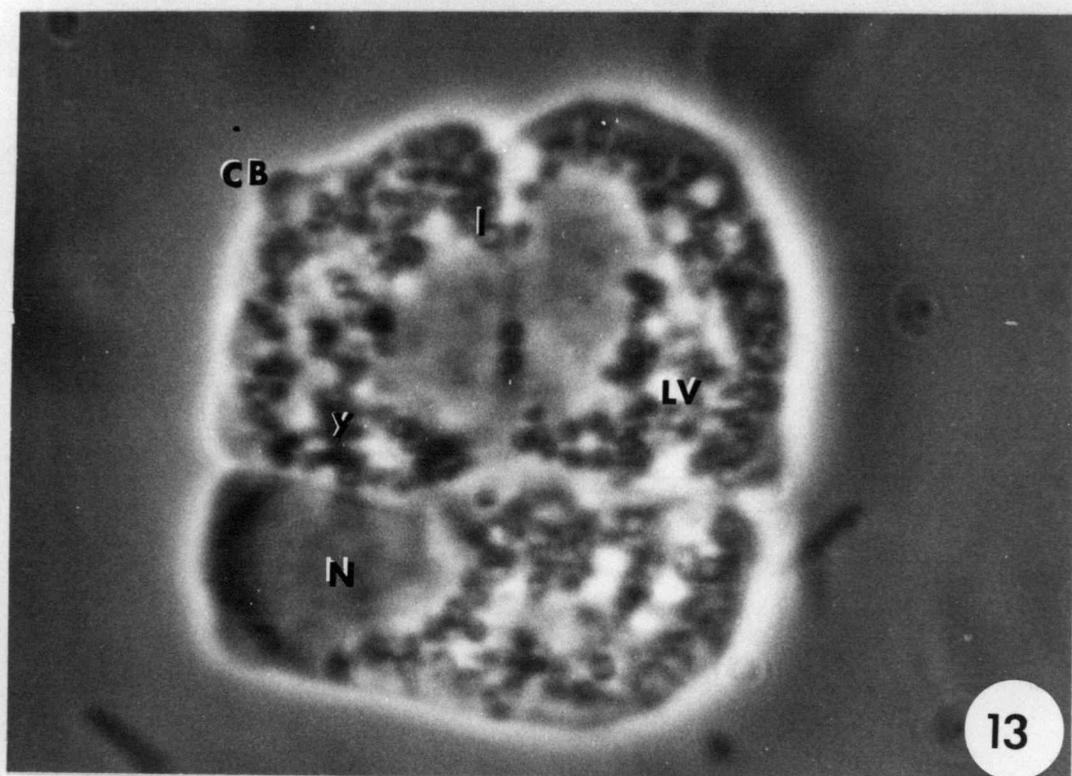
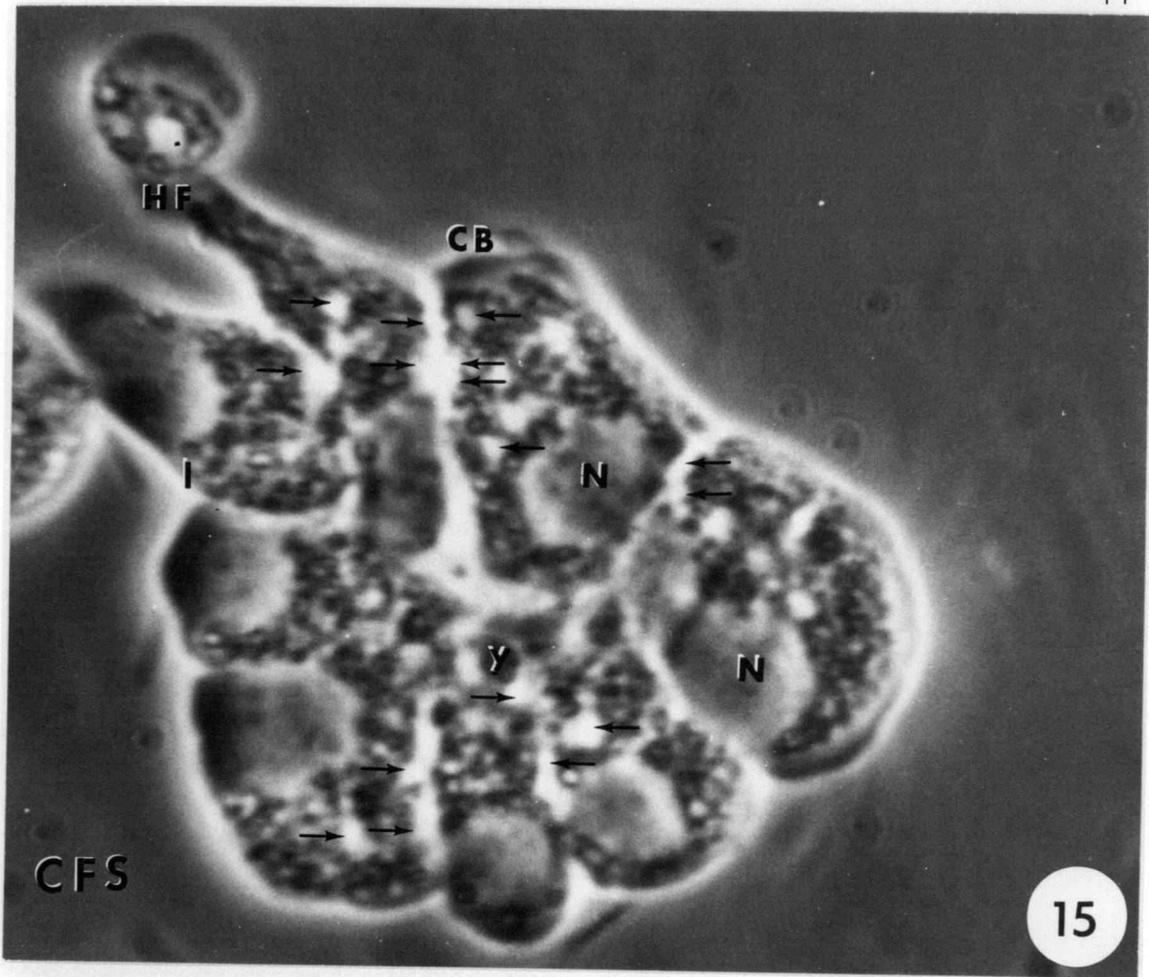
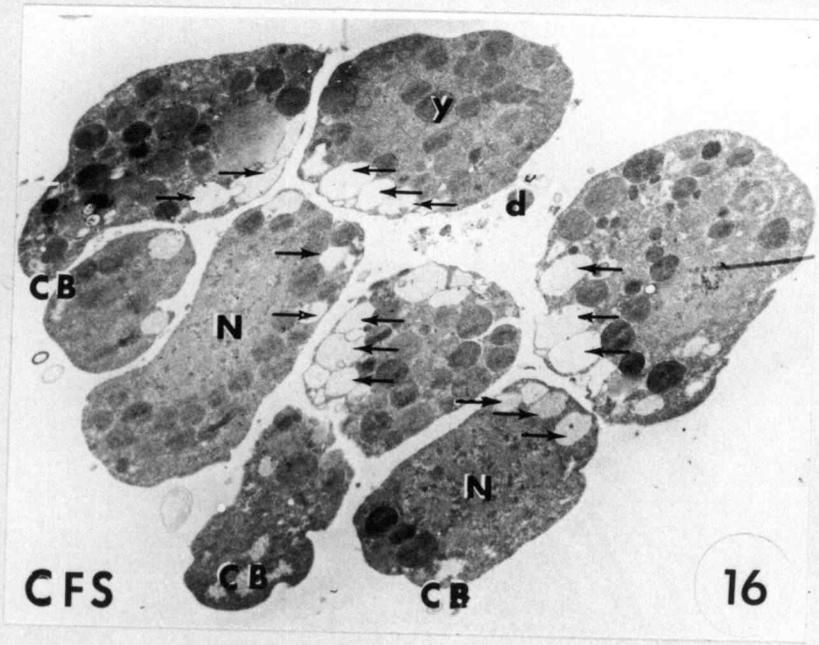


Figure 15. A small aggregate of 10 epithelial-like cells 95 minutes after reaggregation. While there appears to be a marked localization of the lysosome-like vesicles (arrows) at the cell-cell interface, the yolk (y) and lipid (l) granules still appear to be evenly distributed. The nuclei (N) tend to be positioned at the apical margin of the cells. Cytoplasmic blebbing (CB) still continues along the cell free surface (CFS) and frequently hyaloplasmic filopodia (HF) begin to be seen between epithelial-like cells. (4,850 X)

Figure 16. A small aggregate taken from the same population as the aggregate in Figure 13 and fixed for electron microscopy. The marked localization of the lysosome-like vesicles (arrows) contrasts with the uniform distribution of the yolk granules (y). Cell debris (d) appear to be trapped between the epithelial-like cells. Cytoplasmic blebs (CB) at different stages of activity can be seen at the cell free surface (CFS). (5,250 X)



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Figure 17. The cell-cell interface of two epithelial-like cells in a small aggregate fixed 60 minutes after the onset of reaggregation. The lysosome-like vesicles (LV) occur in clusters at the cell-cell interface. No similar distribution of yolk granules (Y) or mitochondria (M) can be detected. (31,700 X)

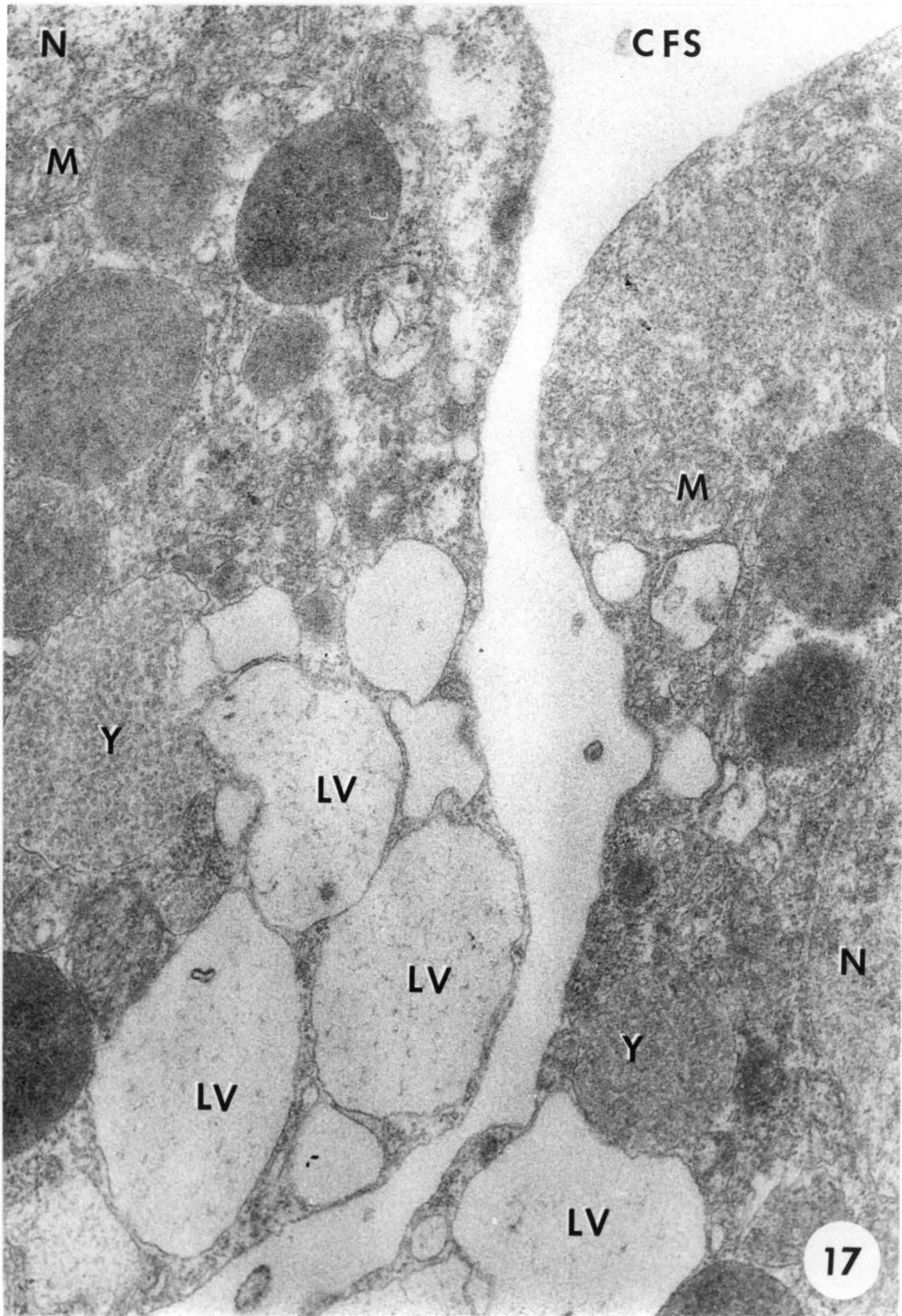


Figure 18. The phagocytosis (Ph) of cell debris (d) trapped between the interface of two epithelial cells can frequently be observed 2-4 hours after the onset of reaggregation. At these sites are clusters of lysosome-like vesicles (LV) and numerous phagosomes (PV). Yolk granules (Y) and mitochondria (M) appear to be evenly distributed while the nucleus (N) generally is positioned apically in the epithelial-like cell. Occasionally lingula (arrow) from the plasma membrane furrows into a lysosome-like vesicle. (41,600 X)

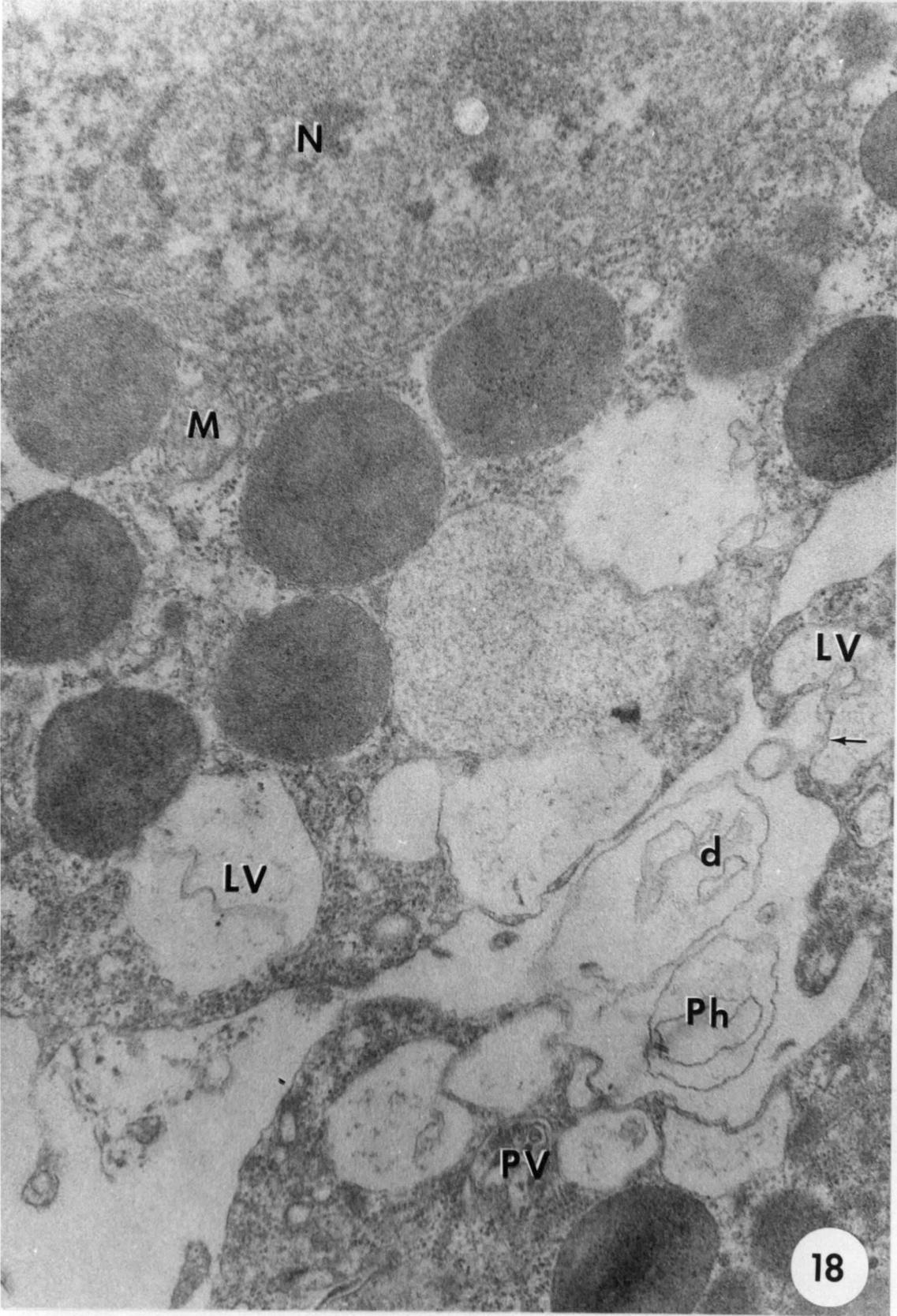


Figure 19. While phagocytosis of cell debris (d) often occurs at the basal margin of the epithelial-like cell, reabsorption of specialized structures like cilia (C) frequently occurs at the cell free surface (CFS). At these sites both lysosome-like vesicles (LV) and phagosomes (P) are numerous. Cytoplasmic blebbing (CB) may coincidentally occur adjacent to resorption along the cell free surface. Mitochondria (M). (18,500 X)

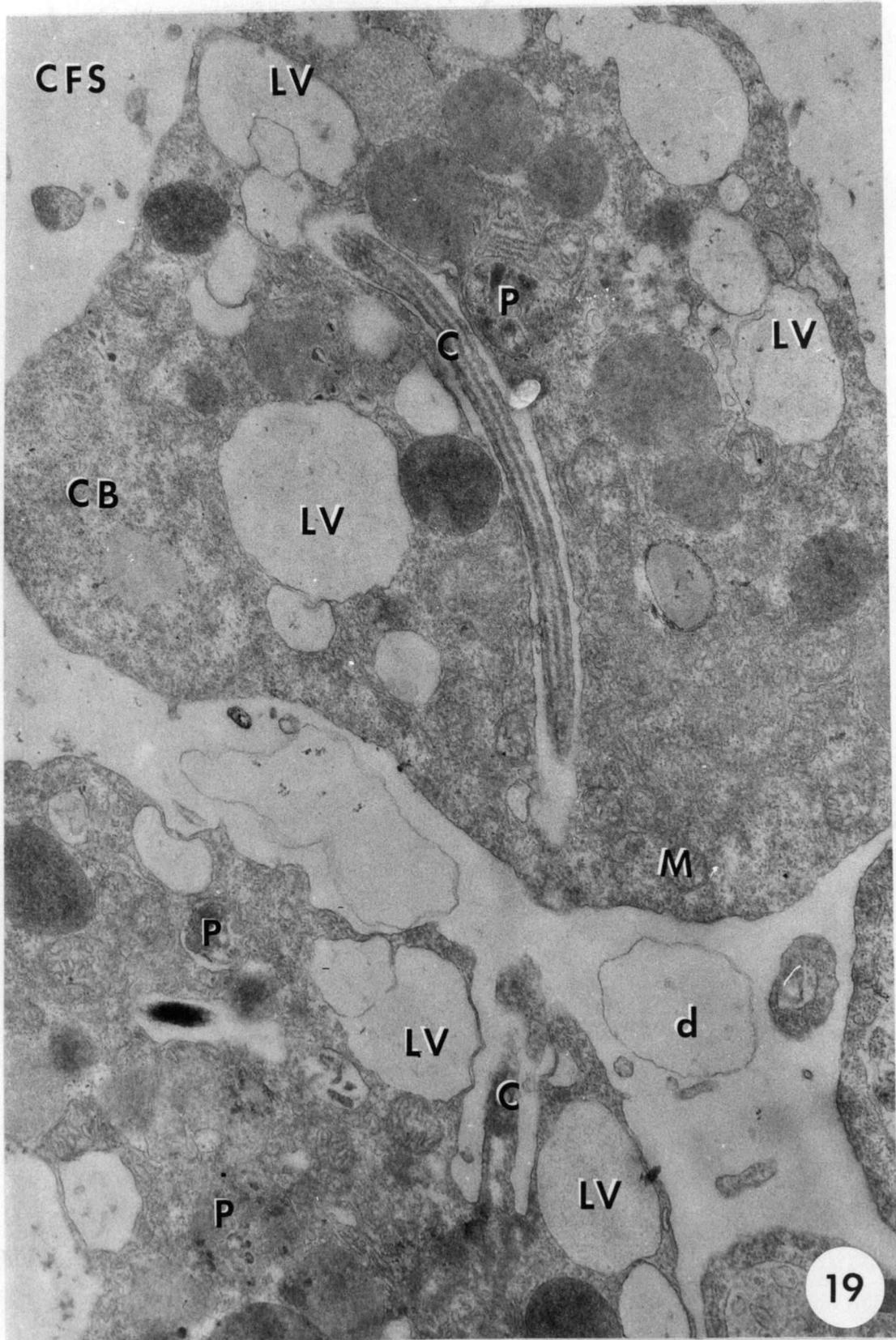


Figure 20. The absorption of yolk granules (Y) by the lysosome-like vesicles (LV) usually occurs near the cell free surface (CFS). A few of the lysosome-like vesicles appear to have vesicles within them, characteristic of a multi-vesiculate body (MVB). Mitochondria (M) and golgi bodies (G) are evenly distributed throughout the cell. Lipid droplets (LD) are few in number. (38,300 X)

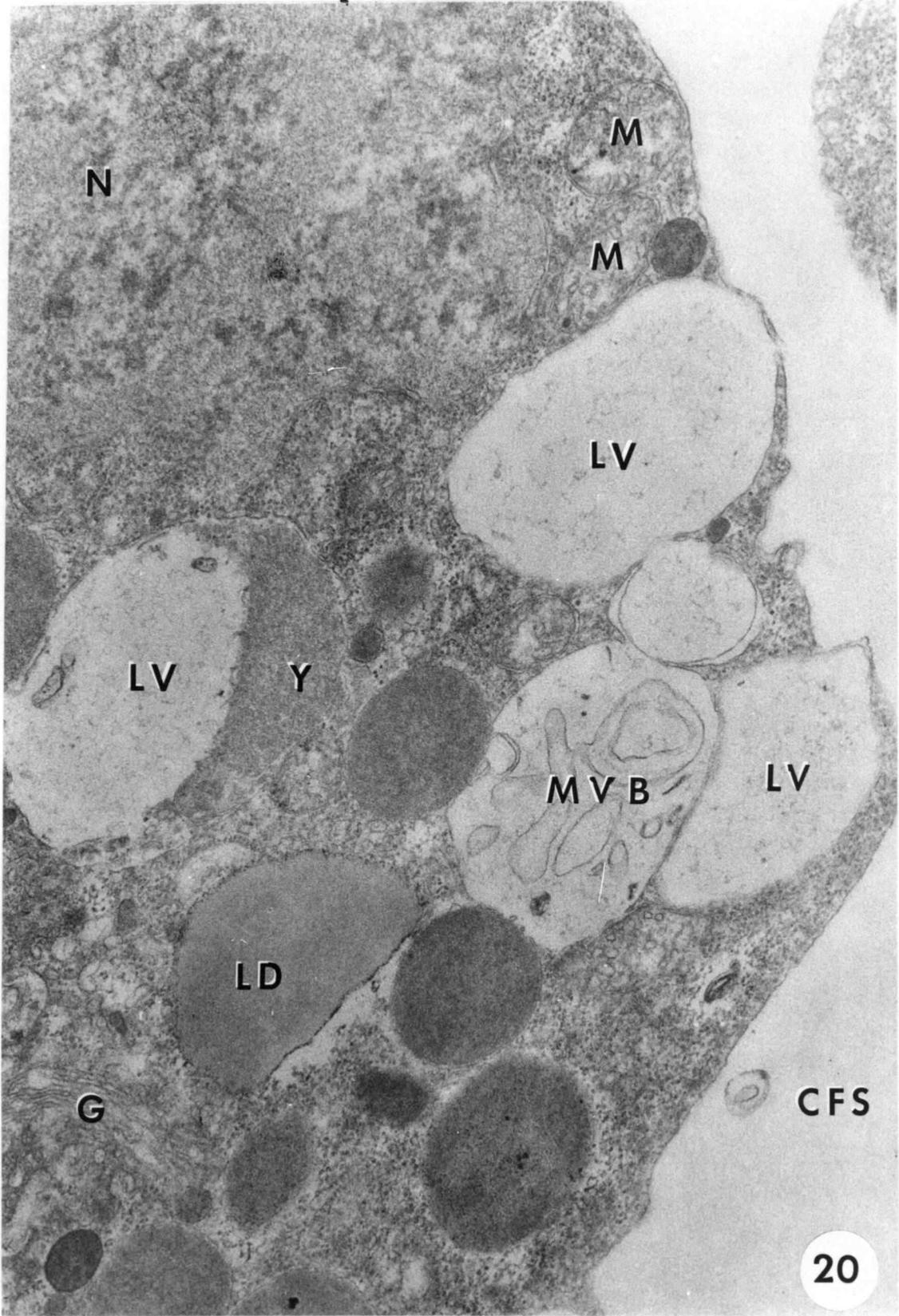


Figure 21a. Araldite embedded three-cell aggregate before sectioning. The site of cilia reabsorption (arrow) is marked by a cluster of lysosome-like vesicles at the apical margin of the epithelial cell. The area included by the rectangle approximates the electron micrograph of Figure 21b. (2,090 X)

Figure 21b. Two hours after the onset of reaggregation extensive reabsorption in the epithelial cells is evidence by the presence of myelin figures (MF) associated with lysosome-like vesicles (LV); by the presence of dense bodies (arrow) which are also associated with lysosome-like vesicles (LV); and by the presence of cilia (C) resorption. Yolk (Y) and mitochondria are evenly distributed within the cell while the nucleus (N) is at the basal margin. Cell debris (d) can only occasionally be observed between epithelial-like cells. CFS, cell free surface; CB, cytoplasmic bleb. (33,100 X)

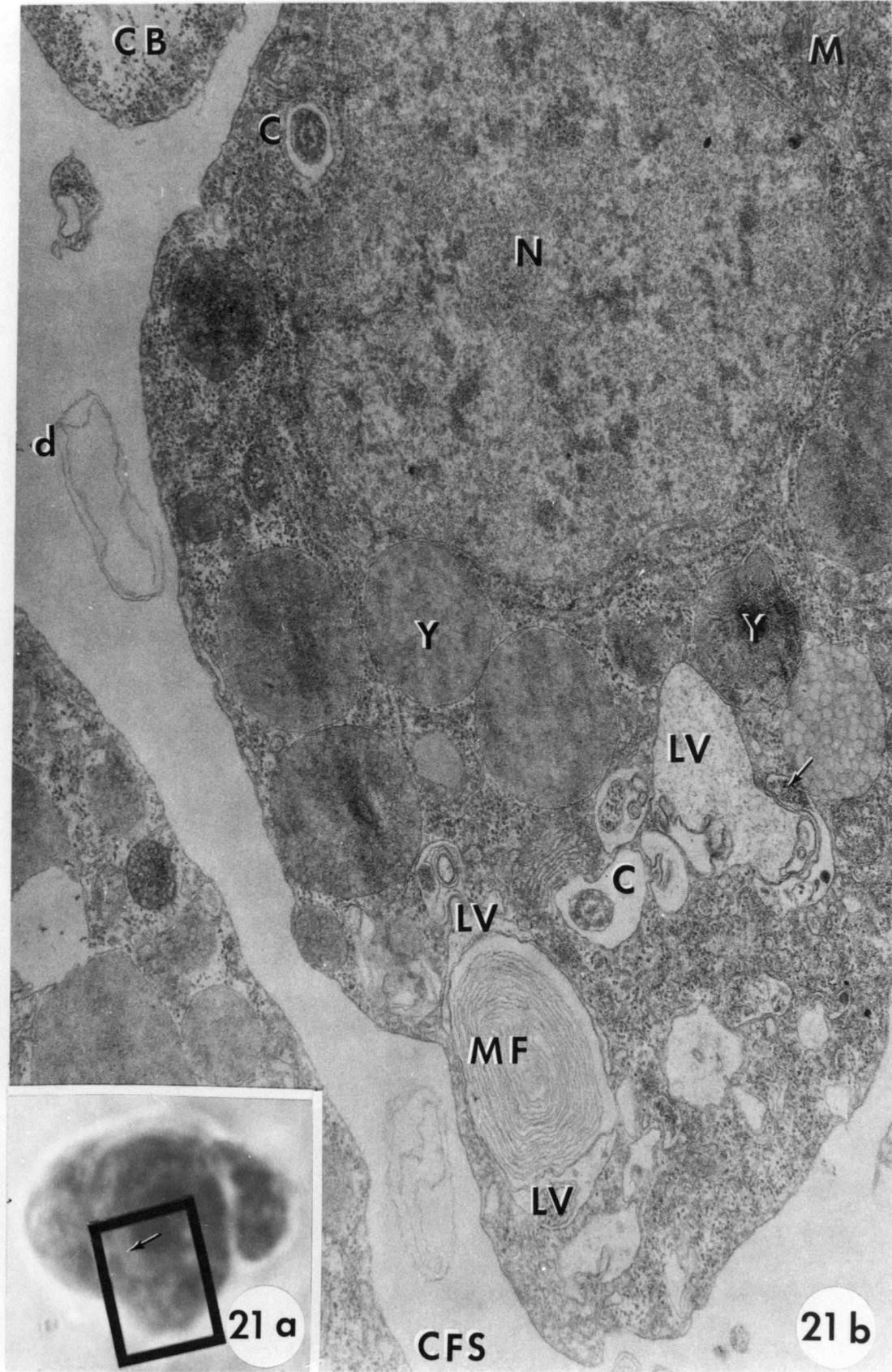


Figure 22a. Within large phagosomes (ph) distorted yolk granules (arrows) are being phagocytized. (2,450 X)

Figure 22b. In addition to yolk granules (Y) lysosome-like vesicles (LV) and cytoplasmic ground substance (gs) are also present in these large phagosomes (Ph). Myeloid bodies (MB) are most probably massive indentations of membrane into a lysosome-like vesicles (*). Cytoplasmic lingulae and furrows (arrow) form between the large phagosome and lysosome-like vesicles. Lipid droplets (LD), mitochondria (M), and rough endoplasmic reticulum (RER) are evenly distributed within the cytoplasm. (38,300 X)

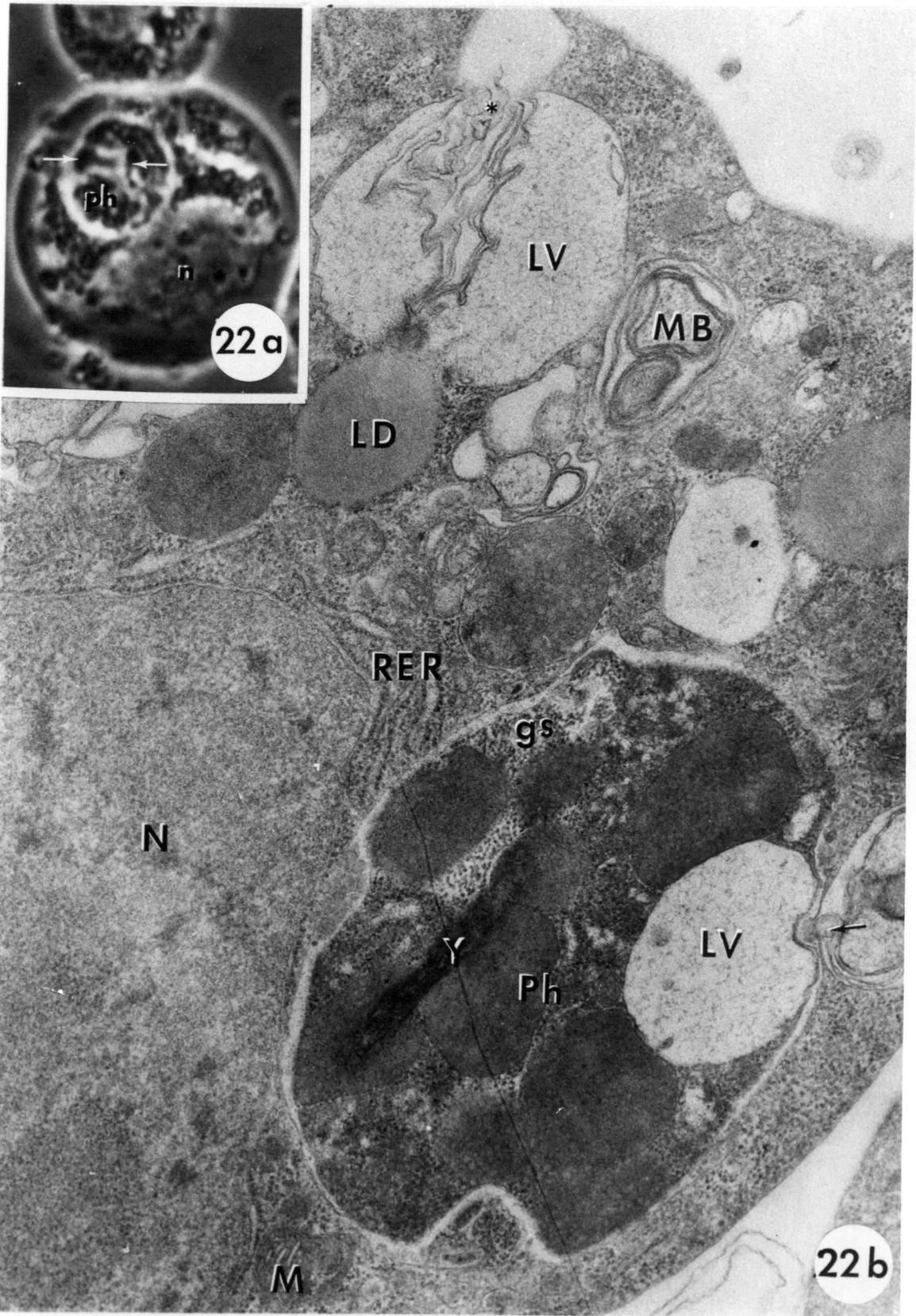


Figure 23. Small aggregate of six cells attached to a cotton fiber (CF). The lysosome-like vesicles (arrows) migrate to the cell-cell interface and none can be detected at the cell-cotton interface. Cytoplasmic blebs (CB) occur along the cell free margins. (2, 450 X)

Figure 24. Twelve hours after the onset of aggregation the lysosome-like vesicles (LV) are localized at the basal margins of the epithelial-like cells and usually associated with phagosomes (p). Apparent vesicular binosis (fusion) can be observed between the lysosome-like vesicles of a cluster (arrows). Debris can be seen in the presumptive blastocoel (pB). (11, 400 X)

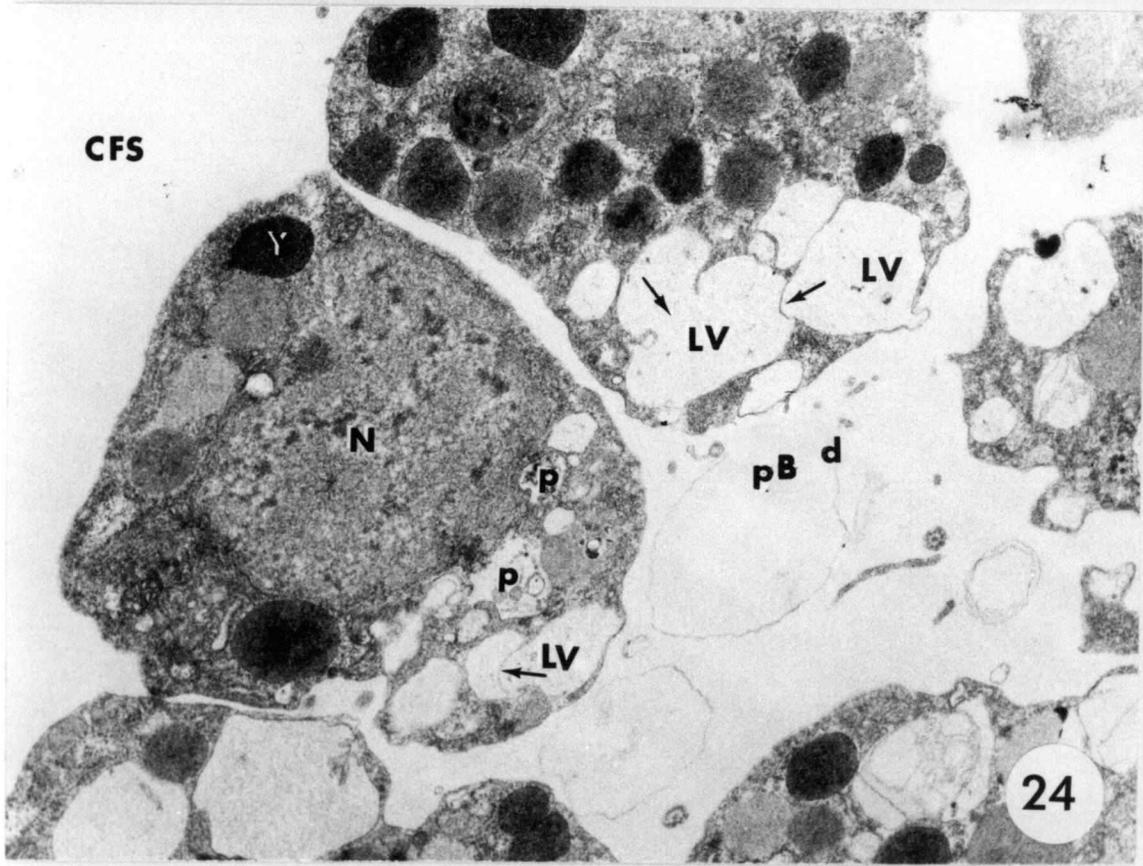
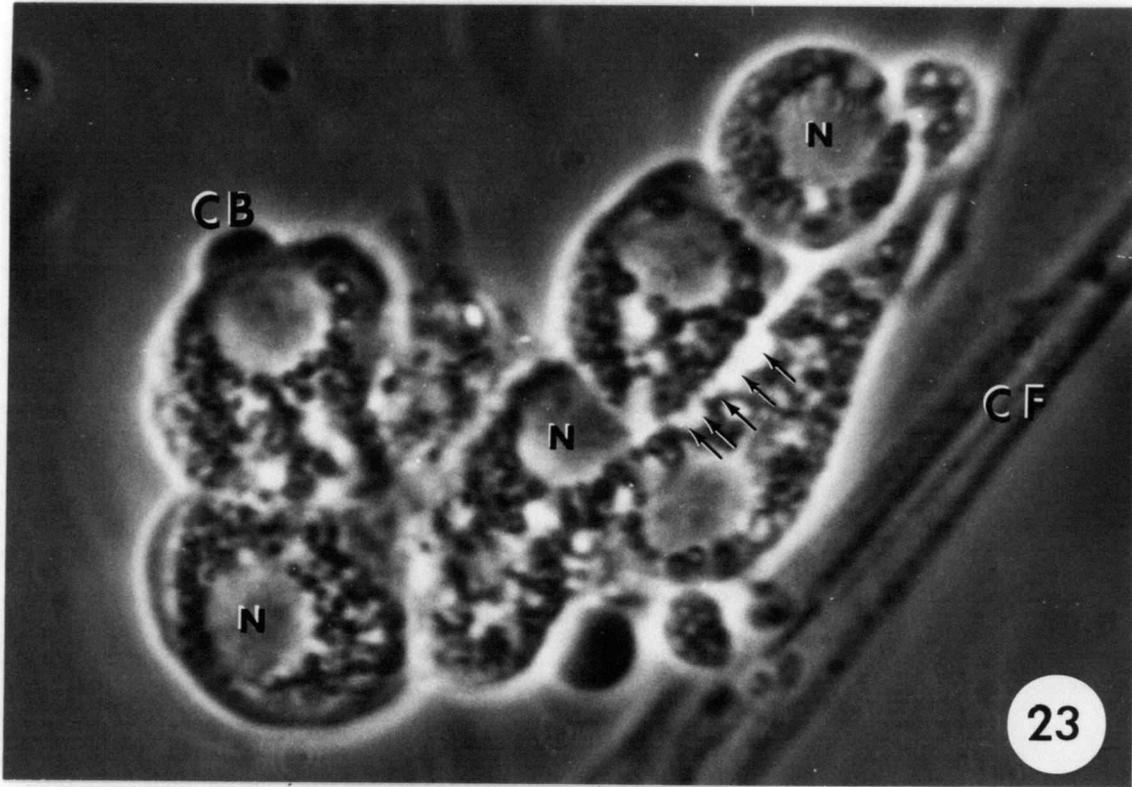


Figure 25. Yolk debris (yd), lipid debris (l d) and membrnous debris (md) can often be seen associated with large phagosomes at the basal margin of epithelial cells 3 hours after the onset of reaggregation. Lysosome-like vesicles (LV) are usually associated with these phagosomes. (38,300 X)

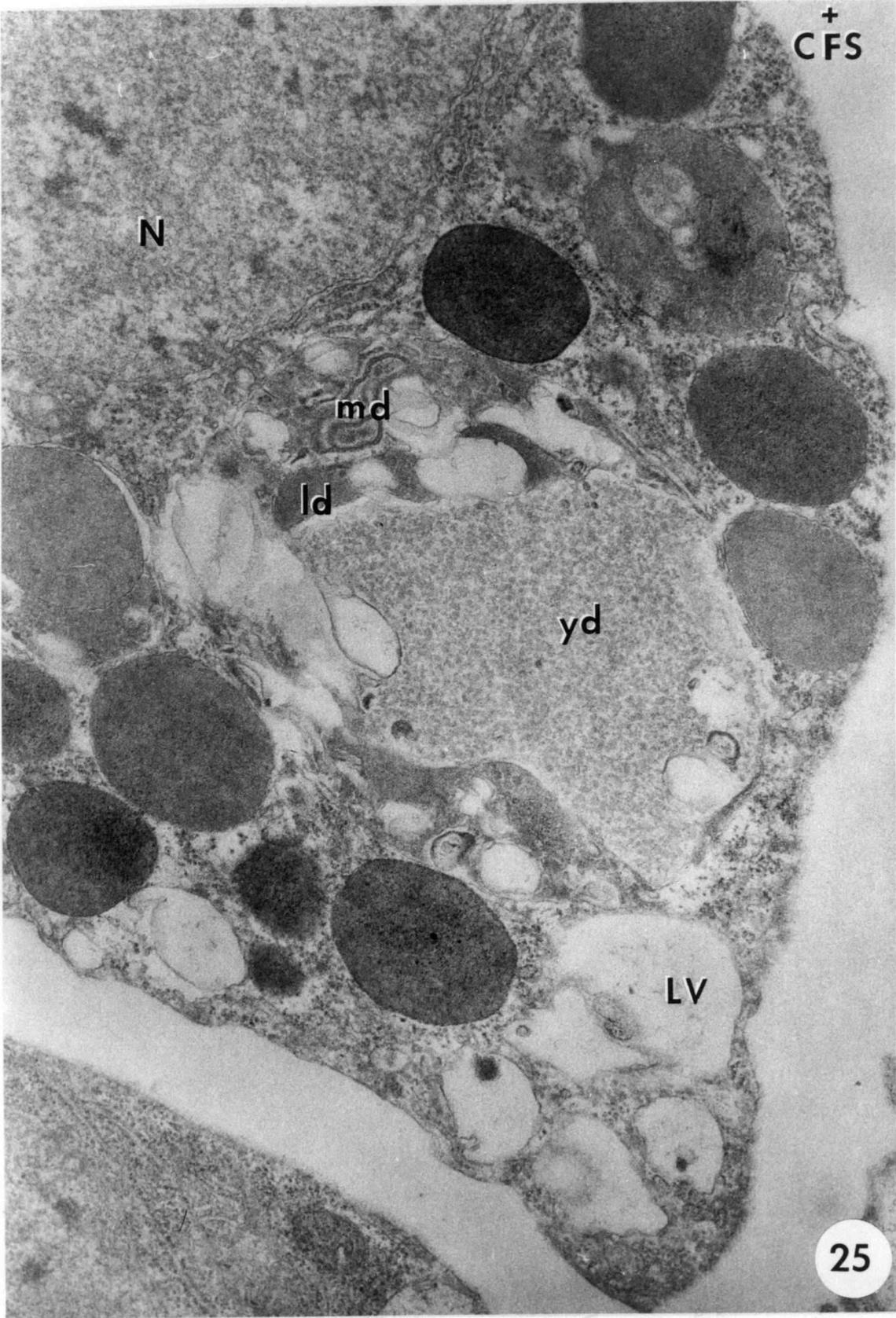


Figure 26. Within the presumptive blastocoel (pB) the presumptive mesenchyme cells (pM) form vesicles (V) by the direct enlargement of the golgi's (G) cisterna (c). There is little or no evidence of active phagocytosis in these cells. On the other hand, the presumptive epithelial cells (pE) at the cell free surface (CFS) characteristically have many phagosomes (P & P') often associated with lysosome-like vesicles (lv'). Vesicle formation in these cells is through the fusion of the golgi's saccules (arrow '). (20,600 X)

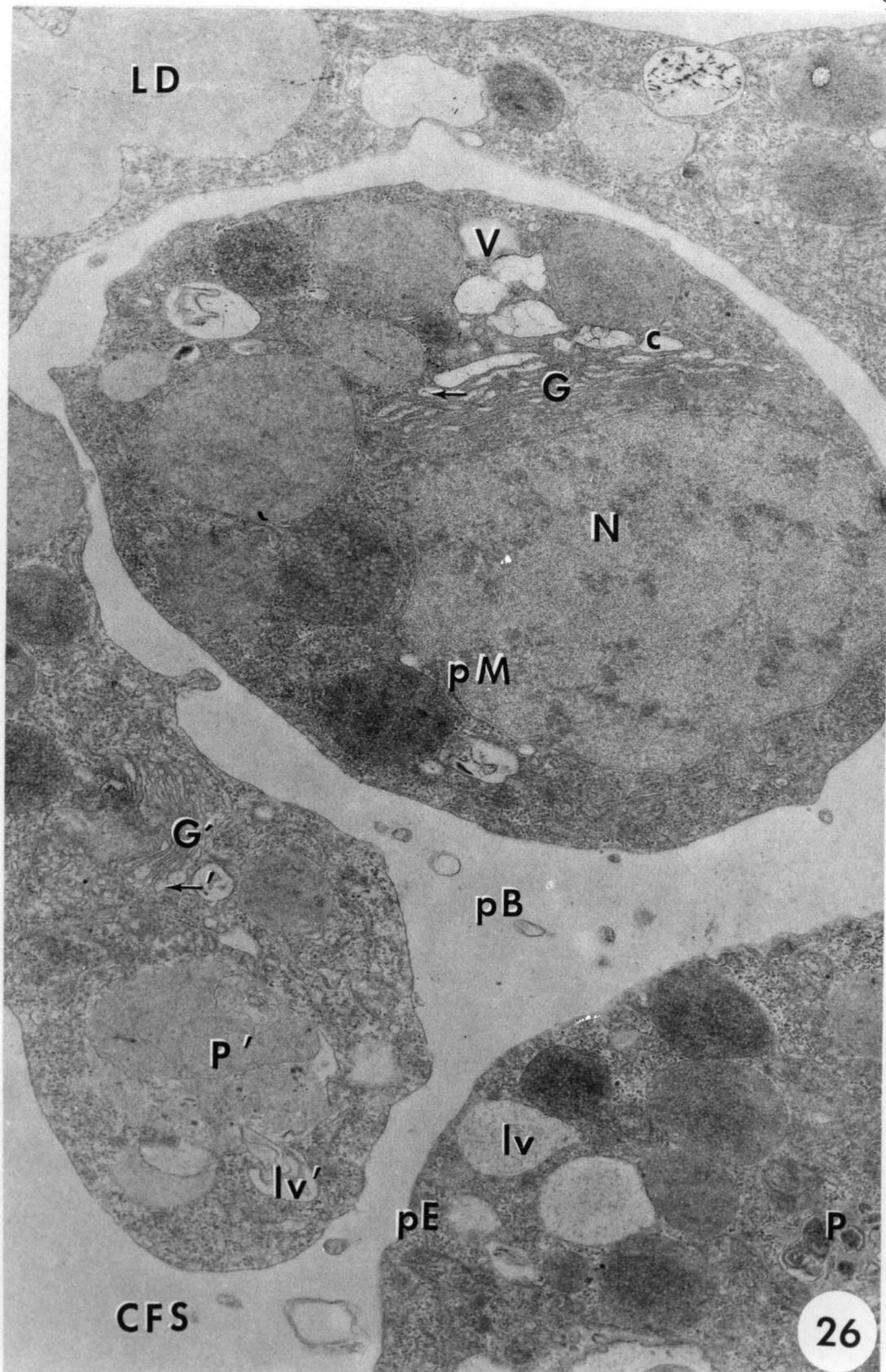


Figure 27. A different section of the aggregate pictured in Figure 26. The mature face of the golgi body (G) within the presumptive mesenchyme cell (pM) is 90° to the mature face of the golgi pictured in Figure 26. Vesicles are formed from the swelling of the cisternae (c) and not from the fussion of saccules (arrow). There is little or no debris in the presumptive blastocoel (pB) in many aggregates. Within the presumptive eithelial cells (pE) mitochondria (m), lysosome-like vesicles (lv), yolk granules (y) and rough endoplasmic reticulum (rer) are evenly distributed. There is little or no cell debris along the cell free surface (CFS) at this time. (37,600 X)

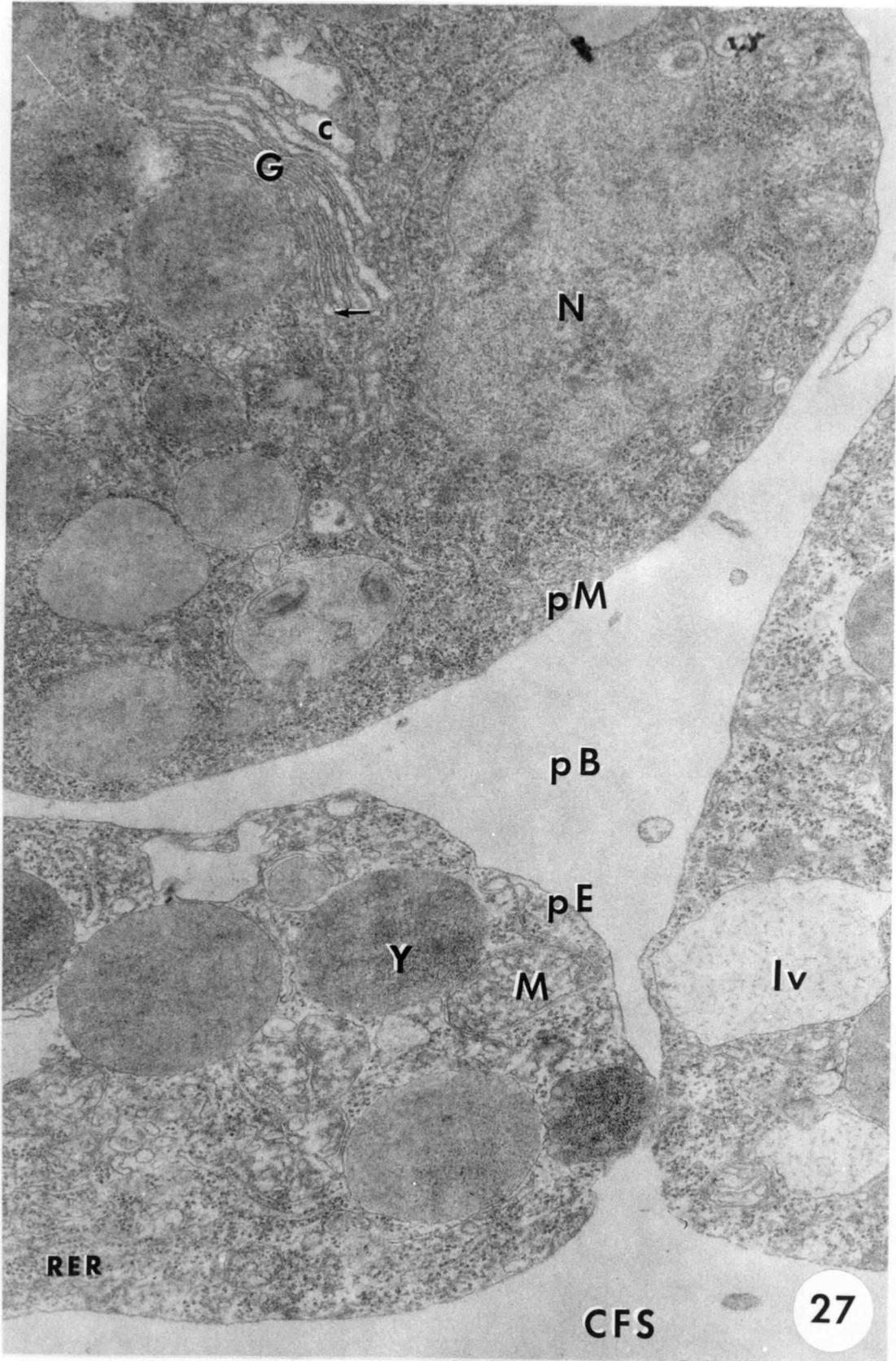
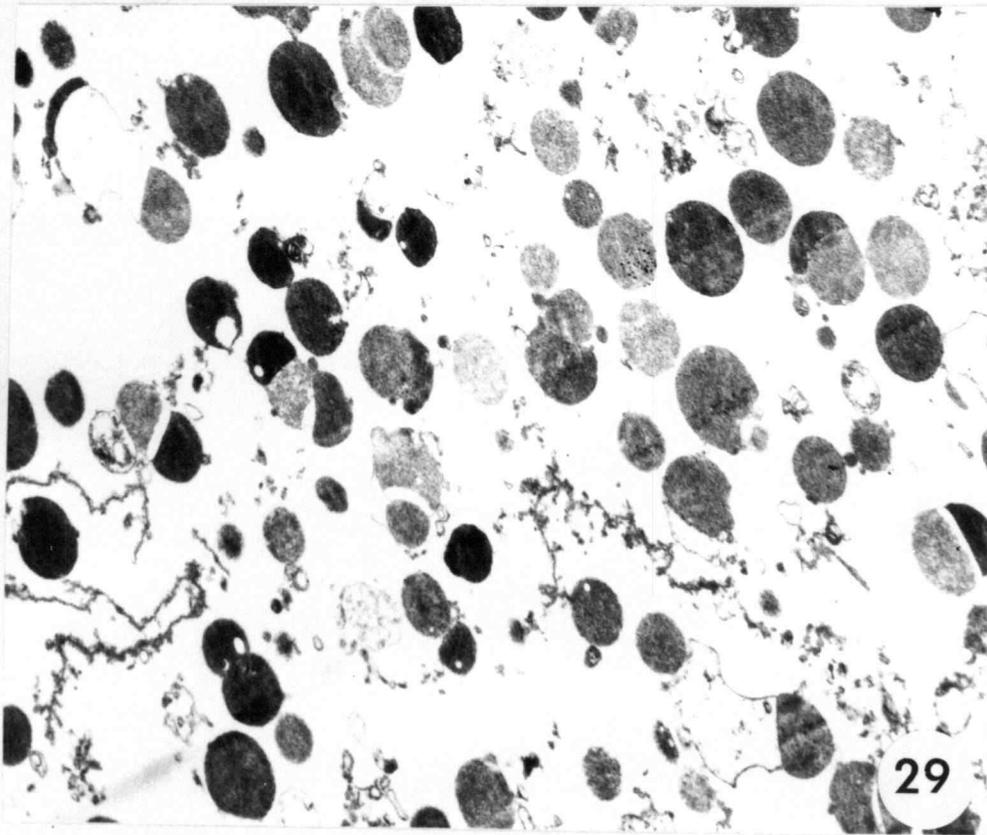
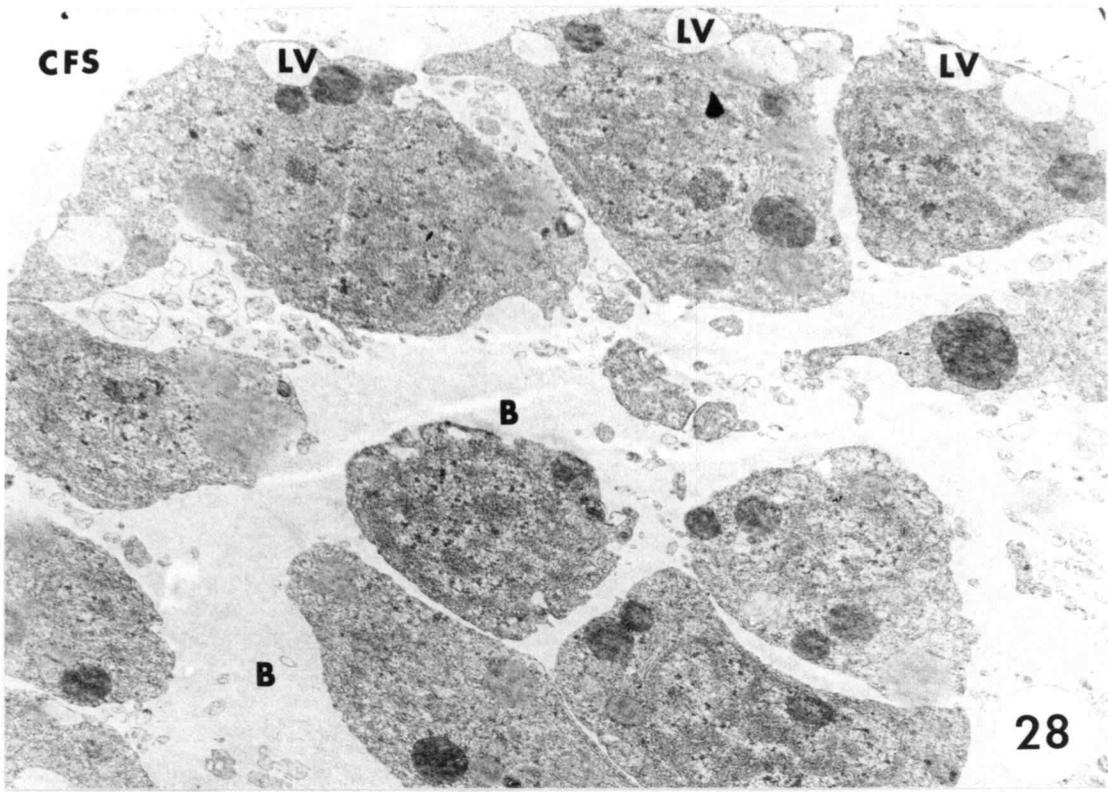


Figure 28. Thirty-six hours after the onset of reaggregation the lysosome-like vesicles (LV) are localized at the apical margin of the epithelial cells. (7,900 X)

Figure 29. A yolk fraction isolated in a sucrose gradient and fixed in osmium for electron microscopy. Though yolk granules often occur in clusters no apparent vesicular lingua or furrow can be detected between them. (5,500 X)



- Figure 30a. No apparent vesicular lingulae or furrows can be detected between lipid droplets, which form the first layer (lightest density) in a sucrose gradient. (2,850 X)
- Figure 30b. No apparent vesicular lingulae or furrows can be detected between lysosome-like vesicles, which form the second layer in a sucrose gradient. (2,850 X)
- Figure 30c. Yolk granules, which form the third layer in a sucrose gradient occur in clusters. Phase microscopy does not have the resolution to detect vesicular binesis. (2,850 X)

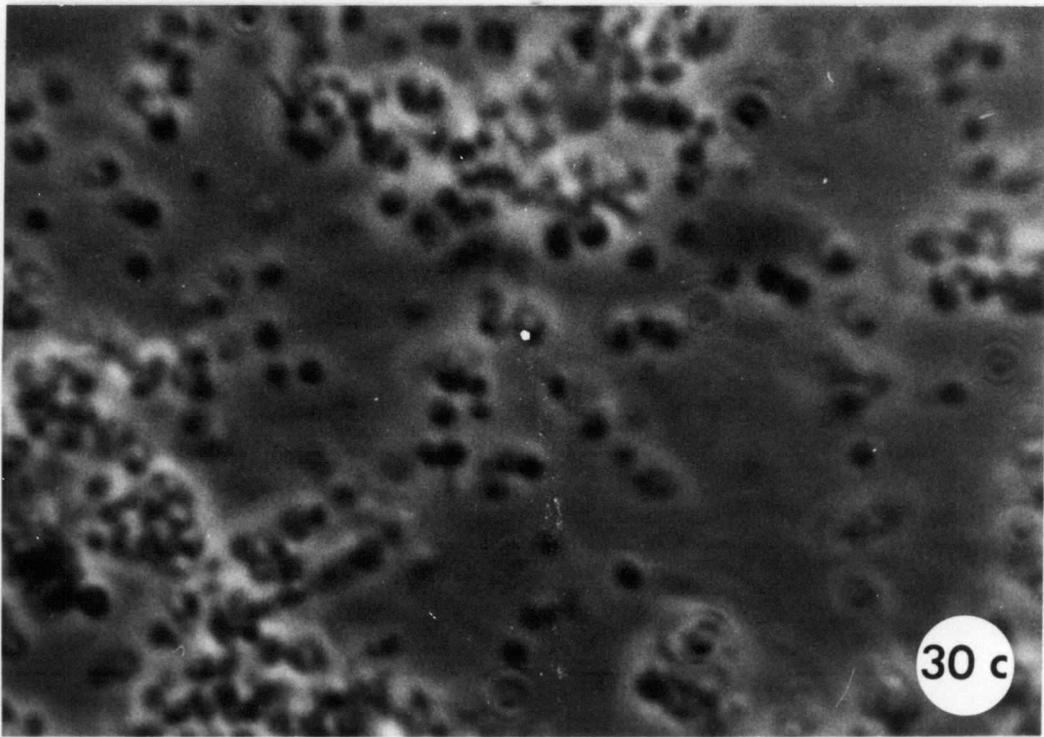
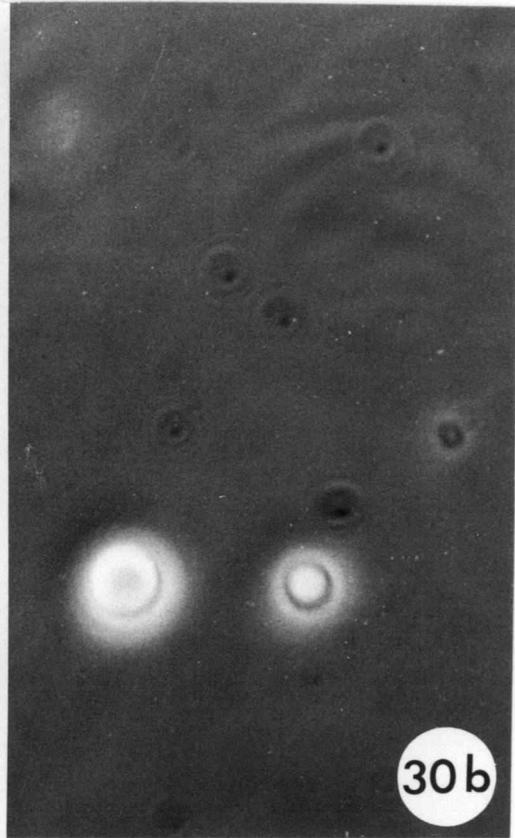
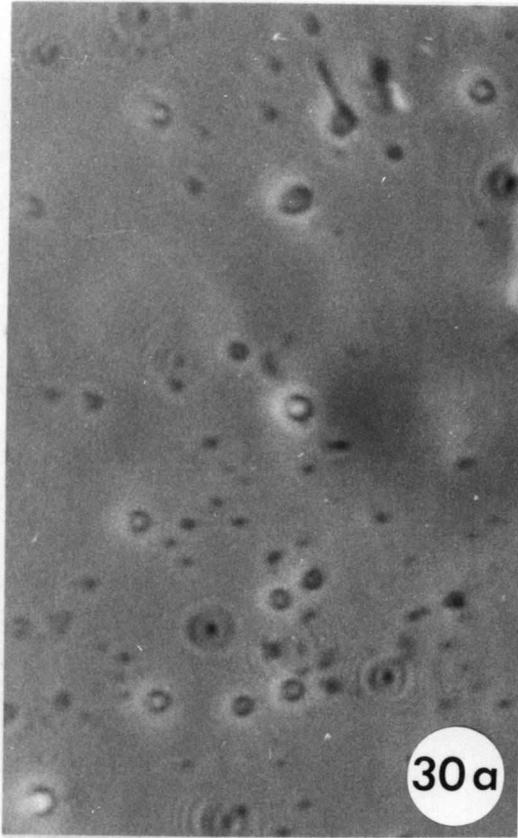


Figure 31. Cytoplasmic blebs (CB) frequently occur at the cell margins during the onset of cell migration. The lysosome-like vesicles (lv), yolk granules (y), and lipid droplets (l) are evenly distributed within the cytoplasm. (3,200 X)

Figure 32. (a) Time lapse sequence taken at 1 minute intervals. Characteristically, the migratory cell (m) has a marked localization of lysosome-like vesicles (lv) at its cell-cell interface and lamellipodia (outline by arrow heads) at its leading edge. Microvilli-like projections (outlined by the white lines) are evenly distributed in both migratory and non-migratory (nm) cells. The direction of migration for cell m is indicated by the heavy arrow. (3,800 X)

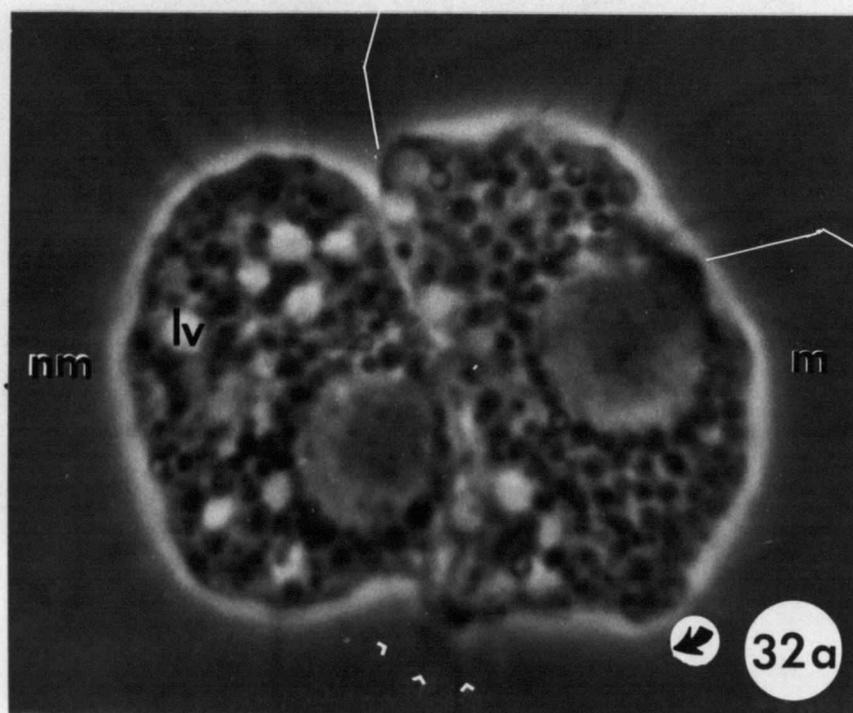
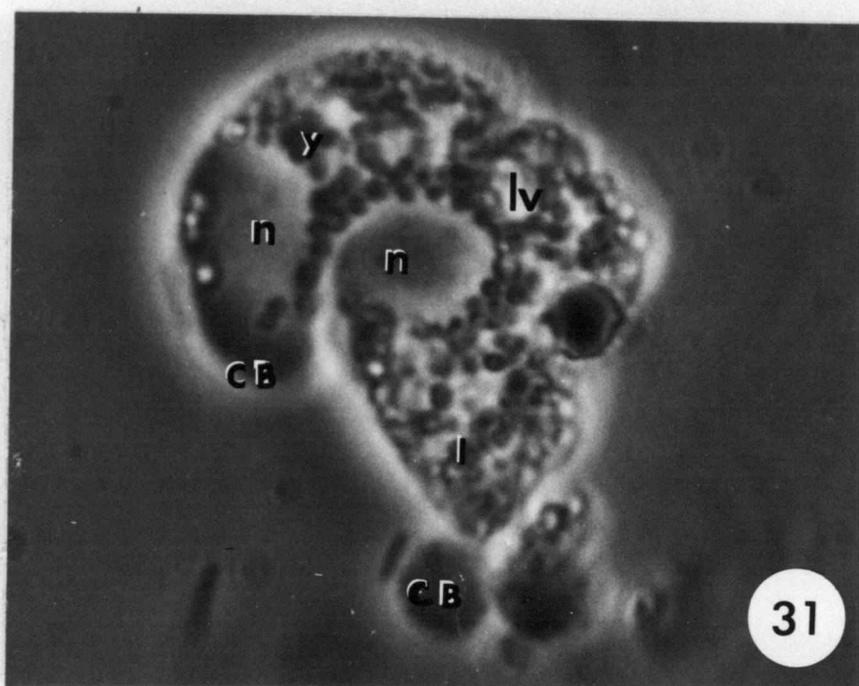


Figure 32. (b) Phase micrograph at time +1 minute, slightly under focused and printed on Agfa F-6 high contrast paper to show the microvilli to advantage.

(c) Phase micrograph at time +2 minutes and printed on polycontrast paper with #3 filter.

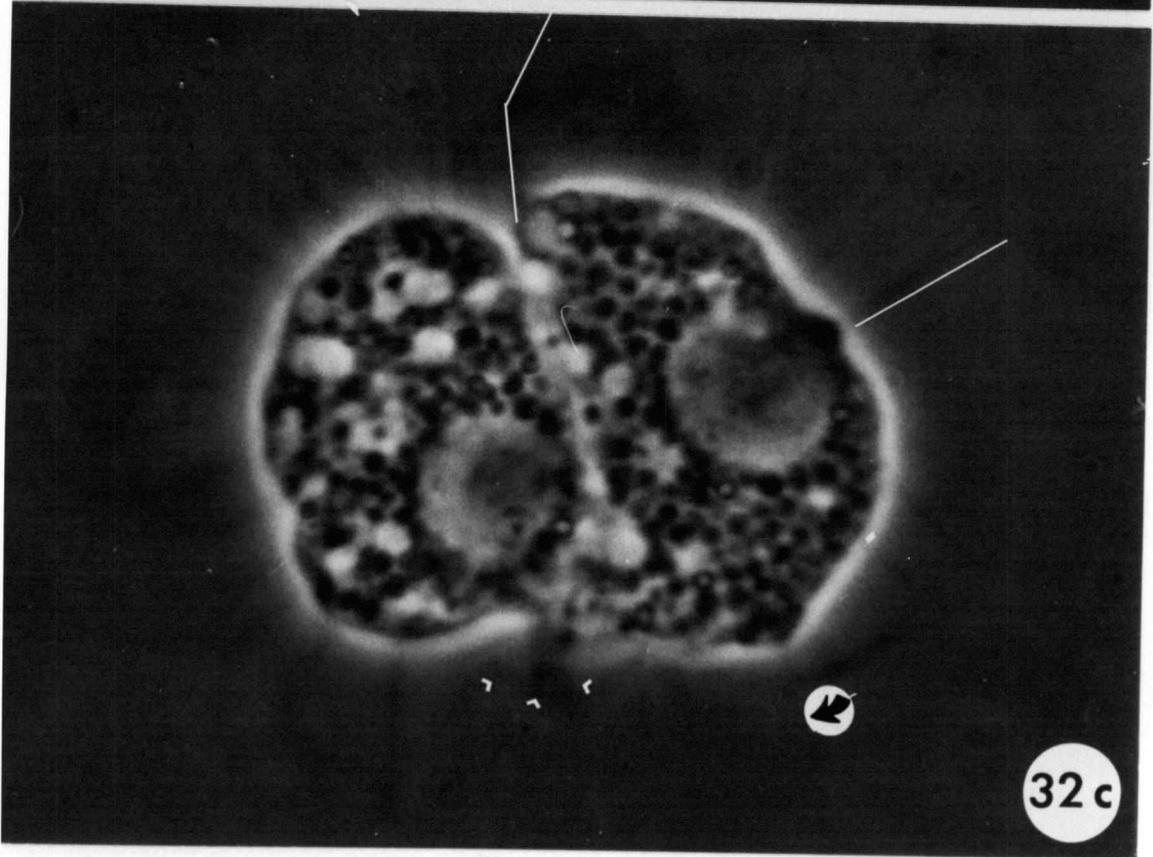
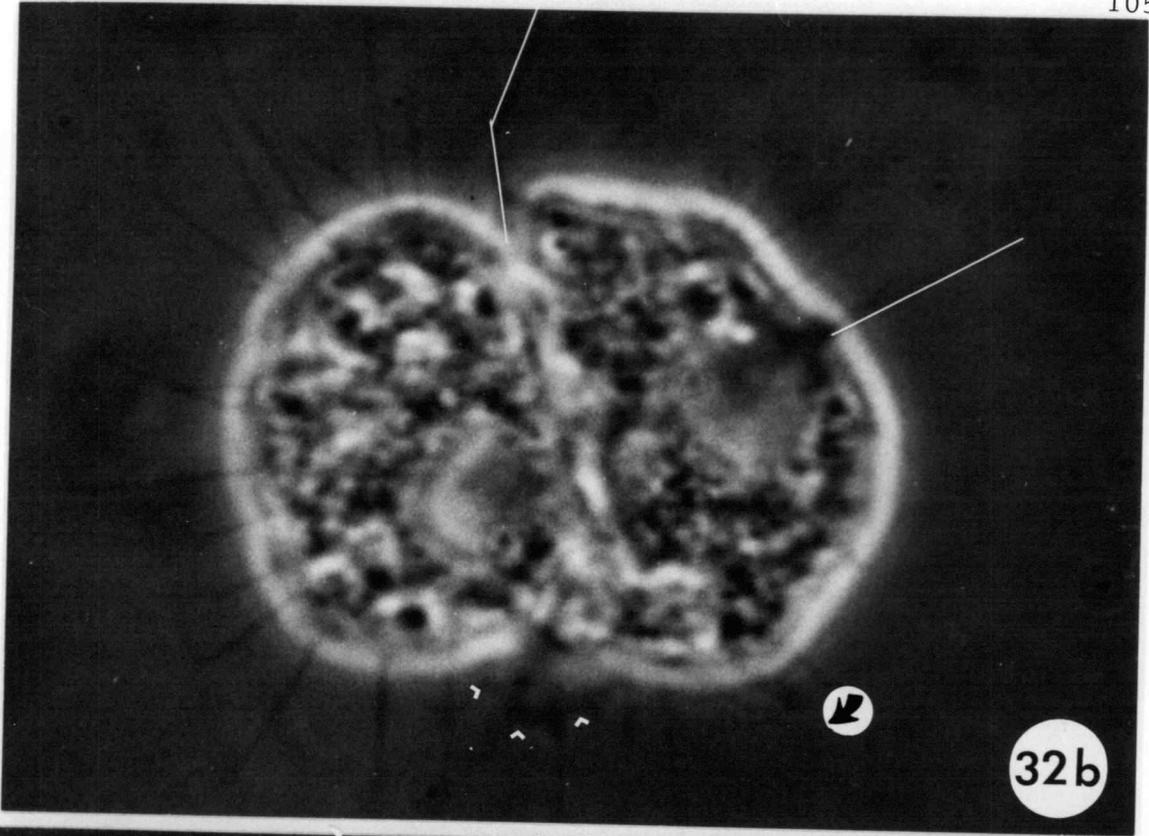


Figure 33. The formation of two cytoplasmic blebs (CB & CB*) by an epithelial cell along its cell free margin. Coarse fibers (arrow) which are compactly packed in the less developed of the two blebs (CB*) have begun to separate in the more developed of the cytoplasmic blebs (CB). Cytoplasmic pits (CF) are often found at the bases of the less developed cytoplasmic bleb. Lysosome-like vesicles (LV) and phagosomes (P) are localized at the basal margins of the epithelial cells. Cell debris (d) can frequently be found at the basal regions of cells. Golgi bodies (G) appear to be forming vesicles through binesis in many epithelial cells. Yolk (Y), mitochondria (M), and lipid droplets (LD) appear to be evenly distributed in the cytoplasm at this time. (16,300 X)

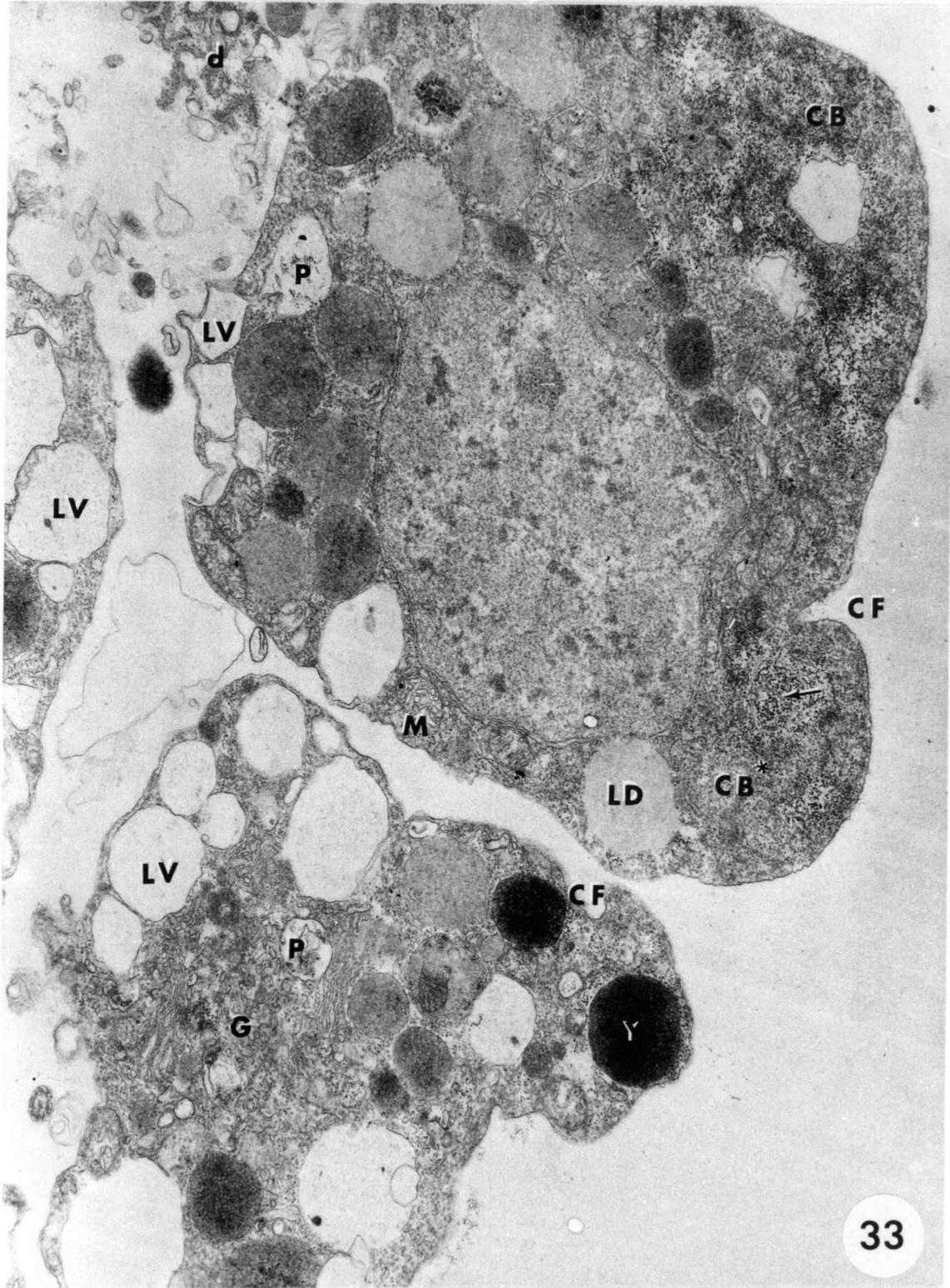


Figure 34. Two regions can be differentiated in the cytoplasmic blebs (CB): a dense fine fibrinous region (df) immediately subjacent to the cytoplasm and a lucid coarse fibrinous region (lf) in the center. While lysosome-like vesicles are localized at the basal margins of the epithelial cell, yolk (Y) and mitochondria (M) appear to be evenly distributed in the cytoplasmic ground substance. (23,000 X)

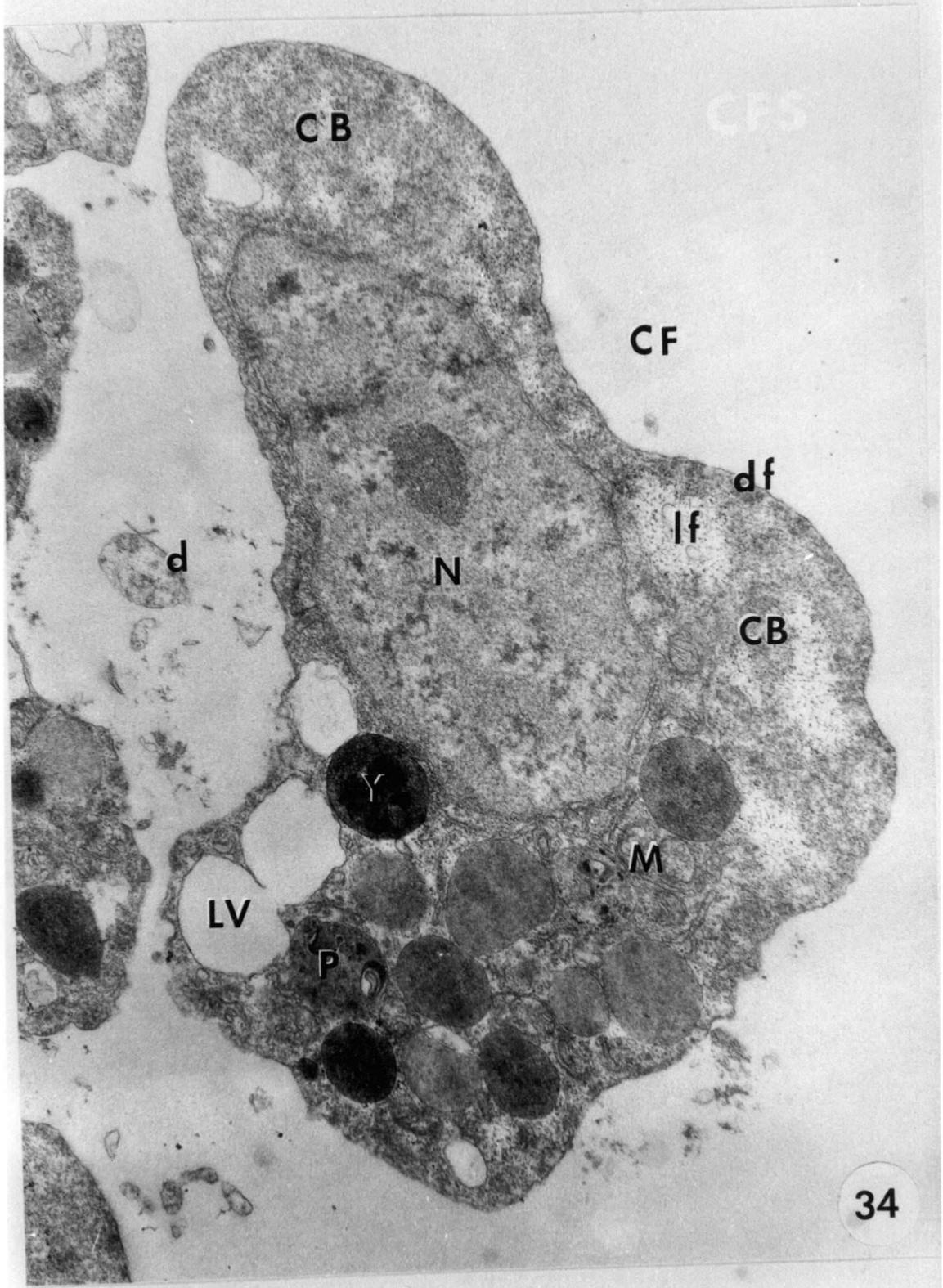
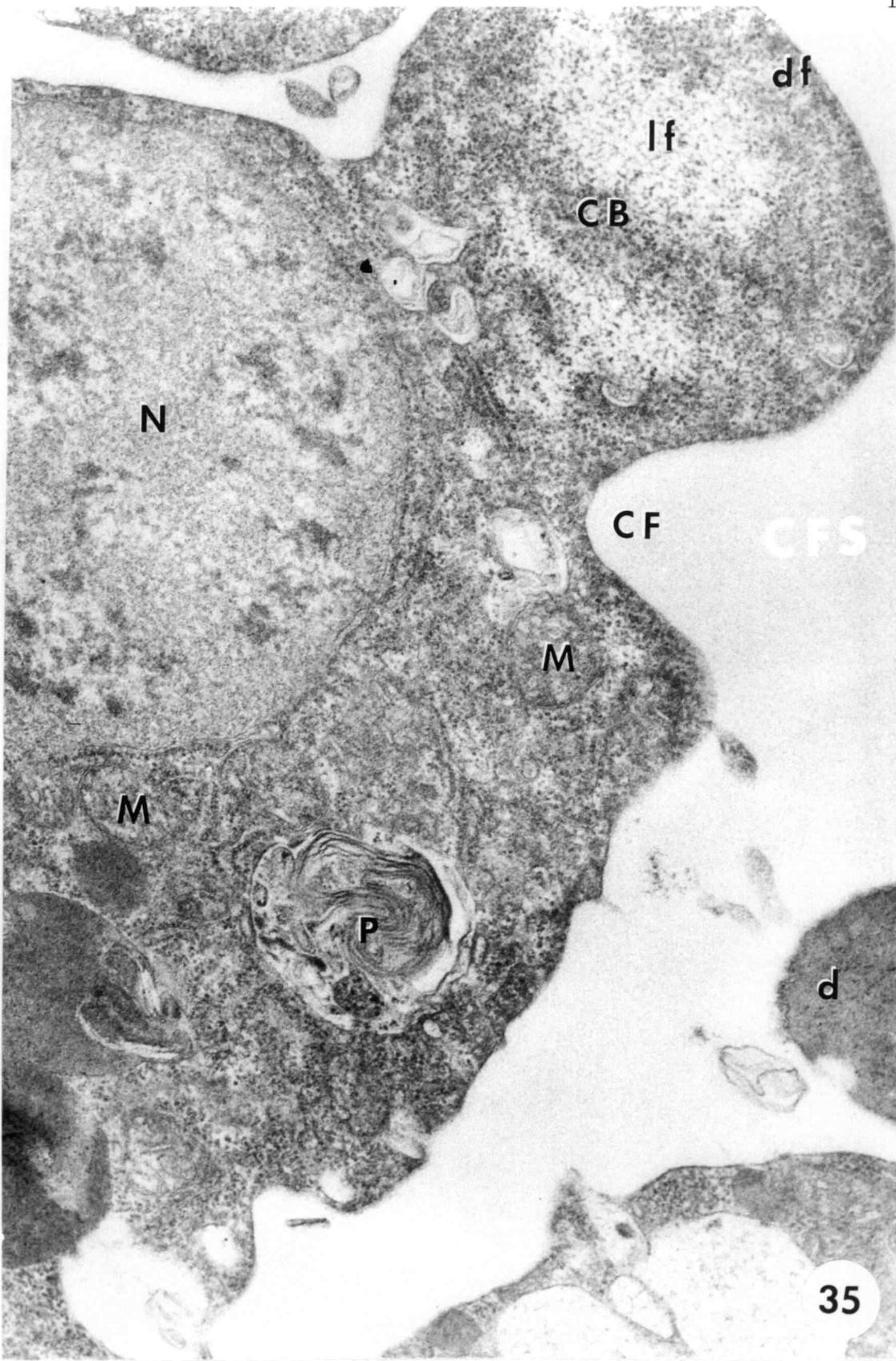


Figure 35. The dense fine fibrinous region (df) and the lucid coarse fibrinous region (lf) are markedly differentiated in the well developed cytoplasmic bleb (CB). Cytoplasmic pit has widened into a well formed furrow (CF). Mitochondria (M) and phagosomes (P) are present in the cytoplasmic ground substance but never in the cytoplasmic bleb. Cell debris (d). (39,000 X)



- Figure 36a. Araldite embedded three cell aggregate before sectioning. The area included by the rectangle is represented by the electron micrograph of the leading edge in Figure 36b. The probable direction of cell migration is indicated by the heavy arrow. Cilia reabsorption is indicated by the small arrows. (2,090 X)
- Figure 36b. Two lamellipodia (LP) appear to extend from the leading edge (LE). Yolk (Y), mitochondria (M), and phagosomes can be seen in the cytoplasmic ground substance posterior to the leading edge. The heavy arrow indicates the direction of cell migration. Cell free surface is indicated by CFS. (48,700 X)

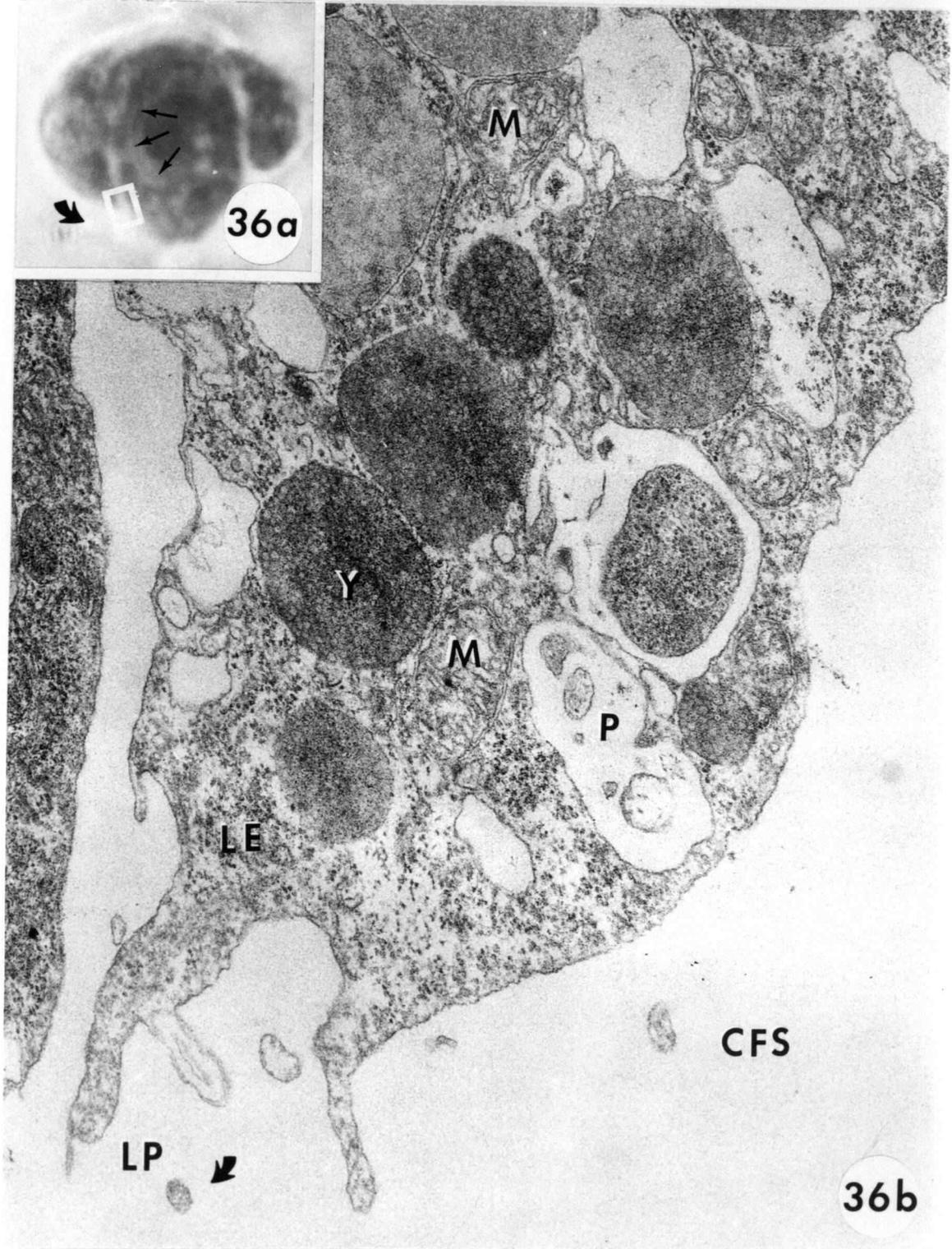
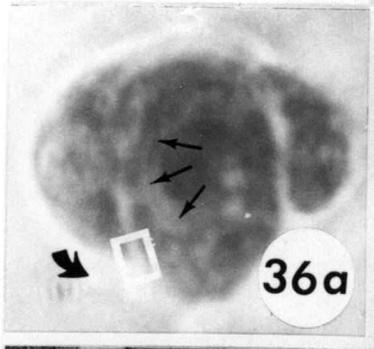


Figure 37a. Araldite embedded three cell aggregate before thin sectioning. The area included by the rectangle presents the electron micrograph of the leading edge in Figure 37b. The characteristic crescent shape of the migrating cell is clearly evident. (2,090 X)

Figure 37b. Cytoplasmic blebs (CB) with well formed cytoplasmic pits (CF) can frequently be found on the cell free surface (CFS) margin of migrating cells. Yolk granules (Y), mitochondria (M), and phagosomes are evenly distributed throughout the cytoplasmic ground substance. (48,700 X)

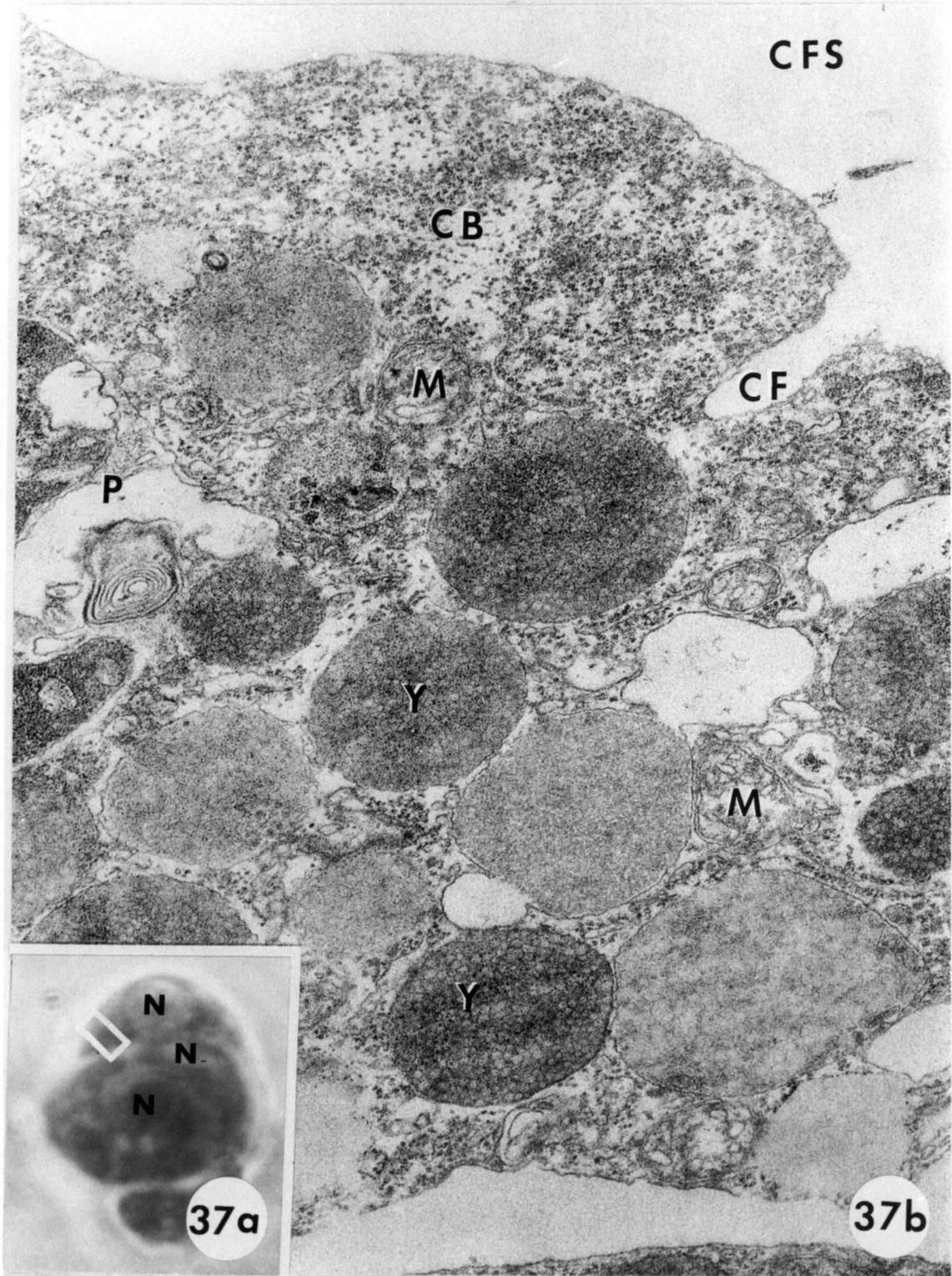


Figure 38. Reassembling embryo twenty four hours after the onset of reaggregation. The hyaloplasmic filopodia along the margin of the epithelial cell elongate into a hyaloplasmic sheet (HS) which often overlaps with the edges of the apposed hyaloplasmic sheet (arrow). With the formation of the blastocoel (B) the epithelial-like cells become distinctly polar in appearance. (3,100 X)

Figure 39. Reassembling embryo thirty five hours after the onset of reaggregation. The hyaloplasmic sheet (HS) curves to form a convex blastocoel wall. There are no mesenchyme cells within the blastocoel (B) of many well formed aggregates. (3,100 X)

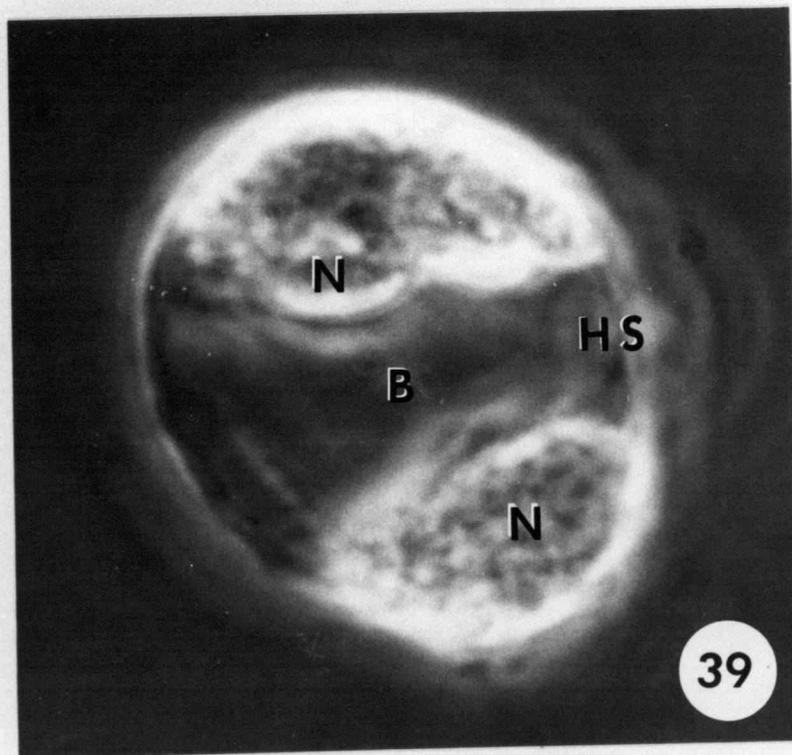
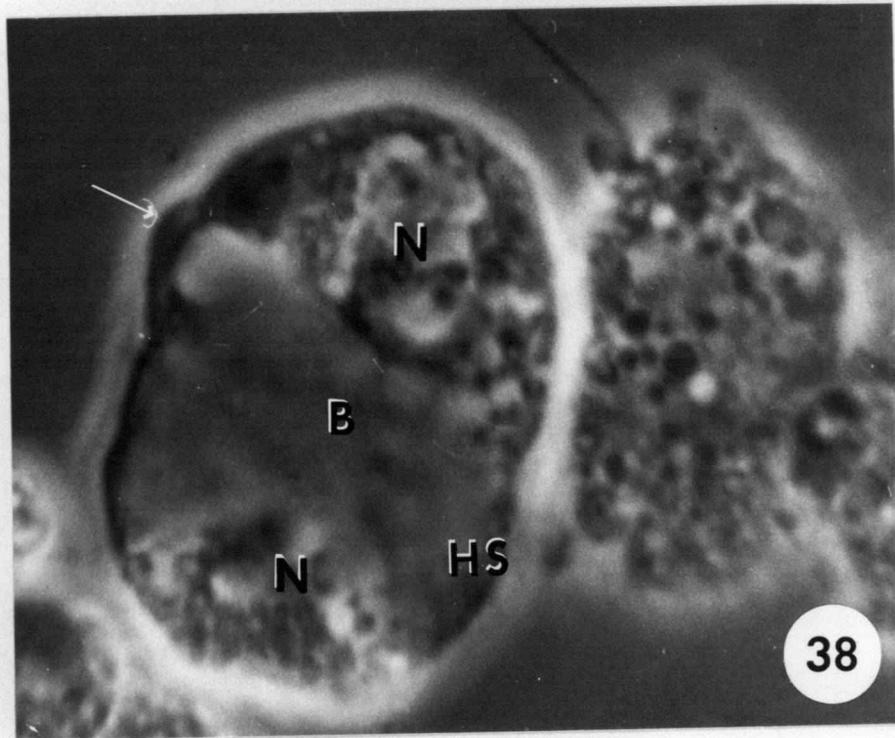


Figure 40. Hyaloplasmic filopodium (f) growing centrifugally from the aggregate onto a glass slide and making firm attachment with apatulatate endings (ae). (1,700 X)

Figure 41. Time lapse with three minute intervals of the hyaloplasmic filopodium. The growth of the hyaloplasmic filopodium (outlined by arrow heads) is about 3 microns per minute in this particular epithelial cell. The entire cell cluster migrates at approximately the same speed. The phase light vesicles (*) migrate in a saltatory manner and therefore its migration cannot be determined with a three minute time lapse interval. (3,100 X)

(a) Time at 0 minute.

(b) Time at 3 minutes.

(c) Time at 6 minutes.

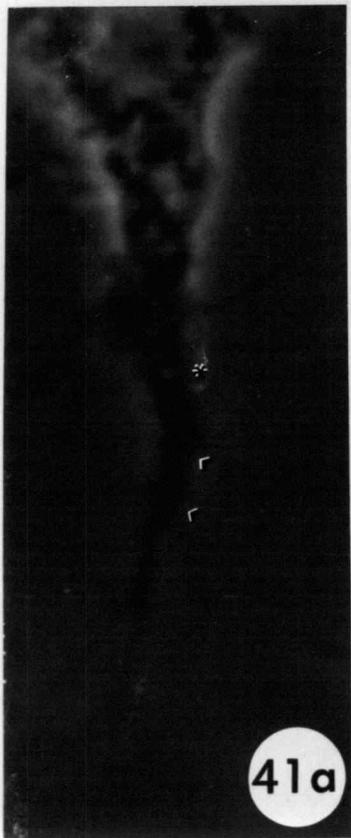
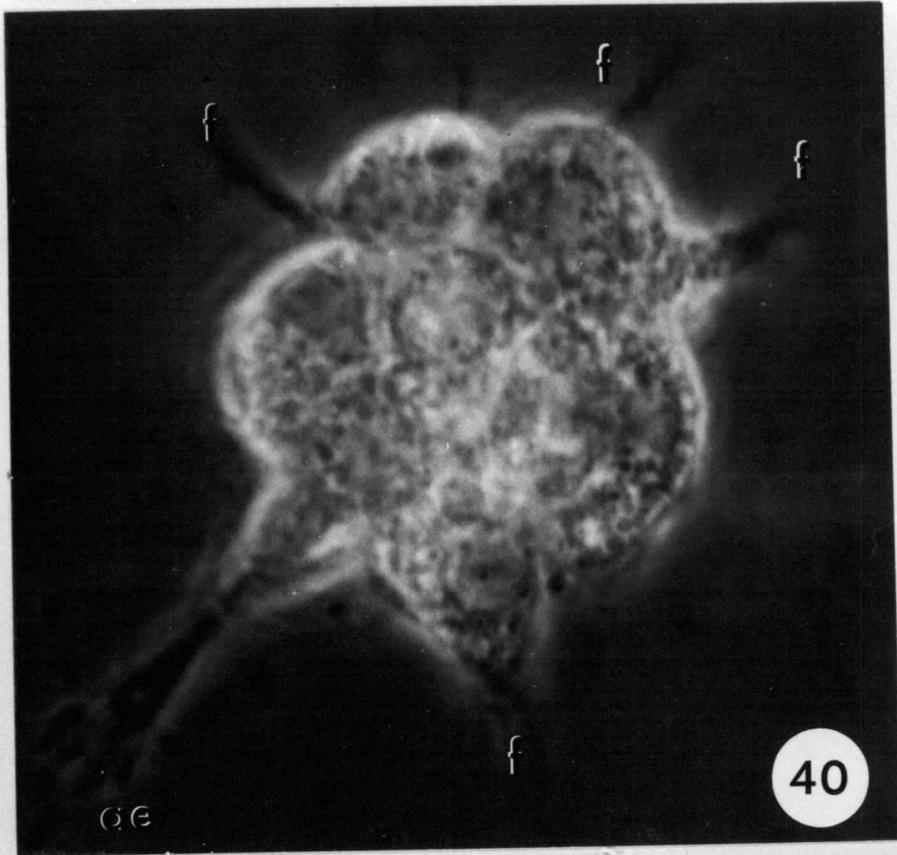


Figure 42. Time lapse sequence of a cell cord growing centrifugally from an aggregate which has been allowed to settle on a glass slide treated with a glycoprotein from the cell surface of sea urchin cells. The formation of phase-gray vesicles (*), retraction of filopodia (+), and growth of the hyaloplasmic filopodia (arrow heads) can be followed in many of the advancing cell cords. Pigment cells (P) remain spherical and do not play an active role in migration. (2,100 X)

- (a) Time at 0 minute. Arrow indicates site of cytoplasmic blebbing.
- (b) Time at 5 minutes. A cytoplasmic fragment (cf) results from blebbing.
- (c) Time at 10 minutes. Endocytosis (*) has occurred with the formation of a phase-gray vesicle.

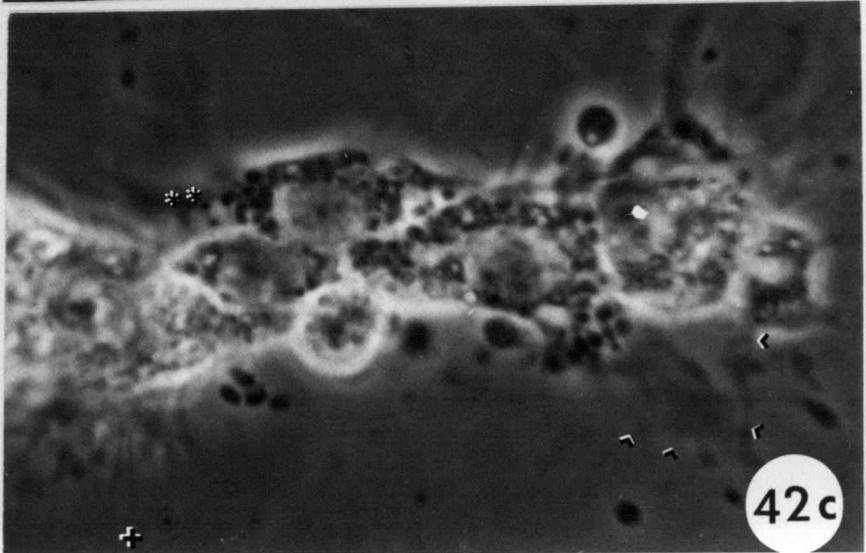
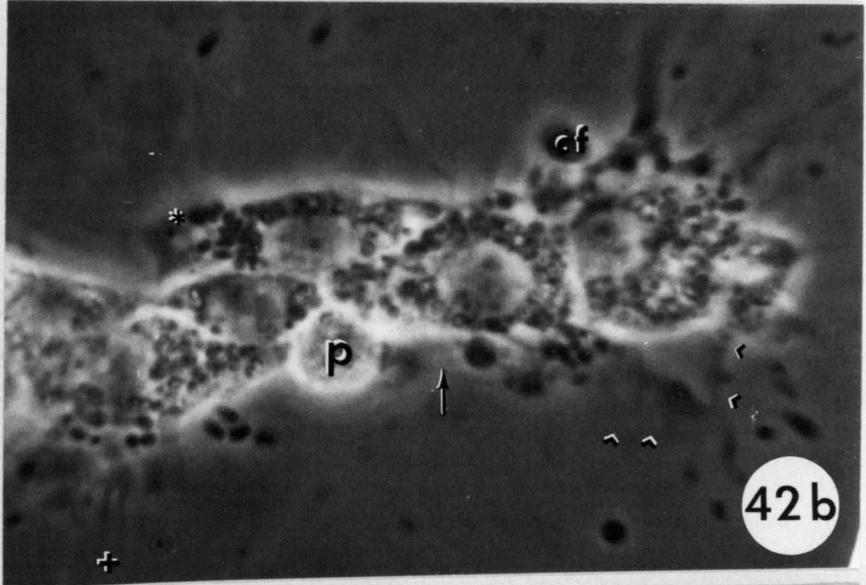
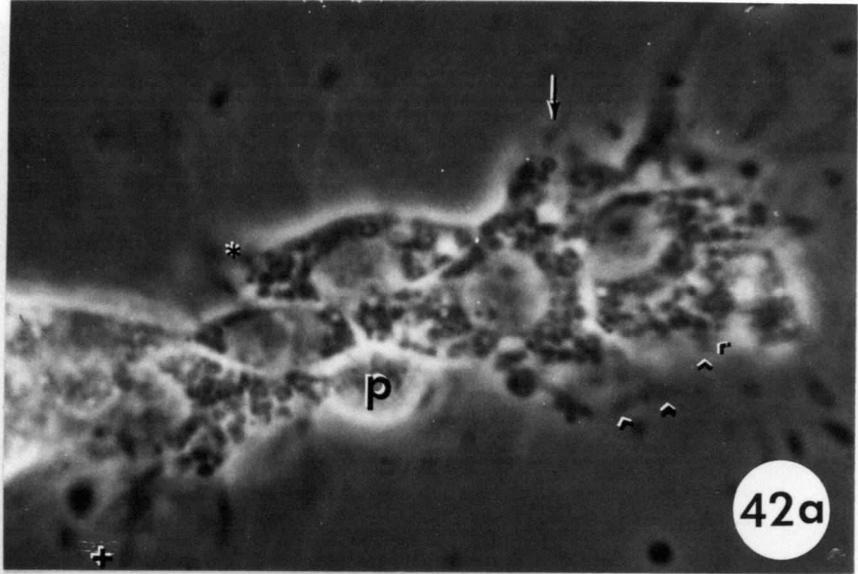
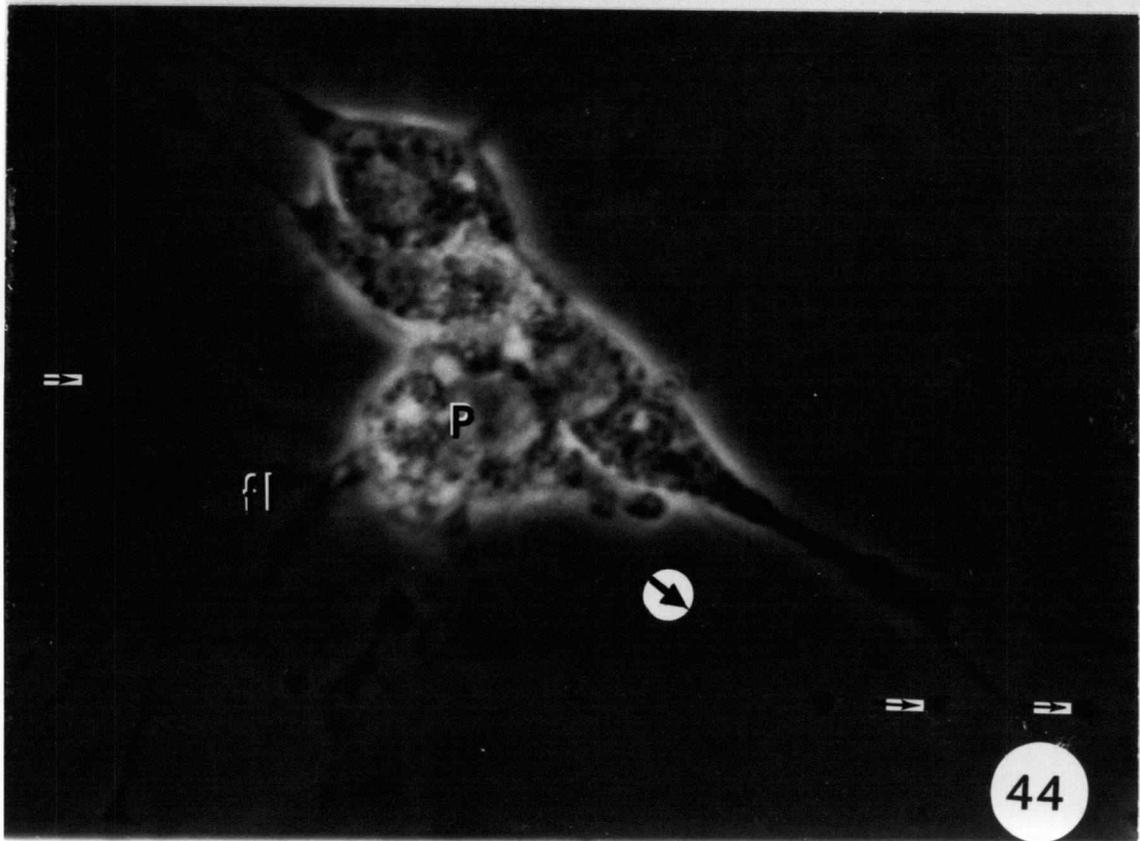
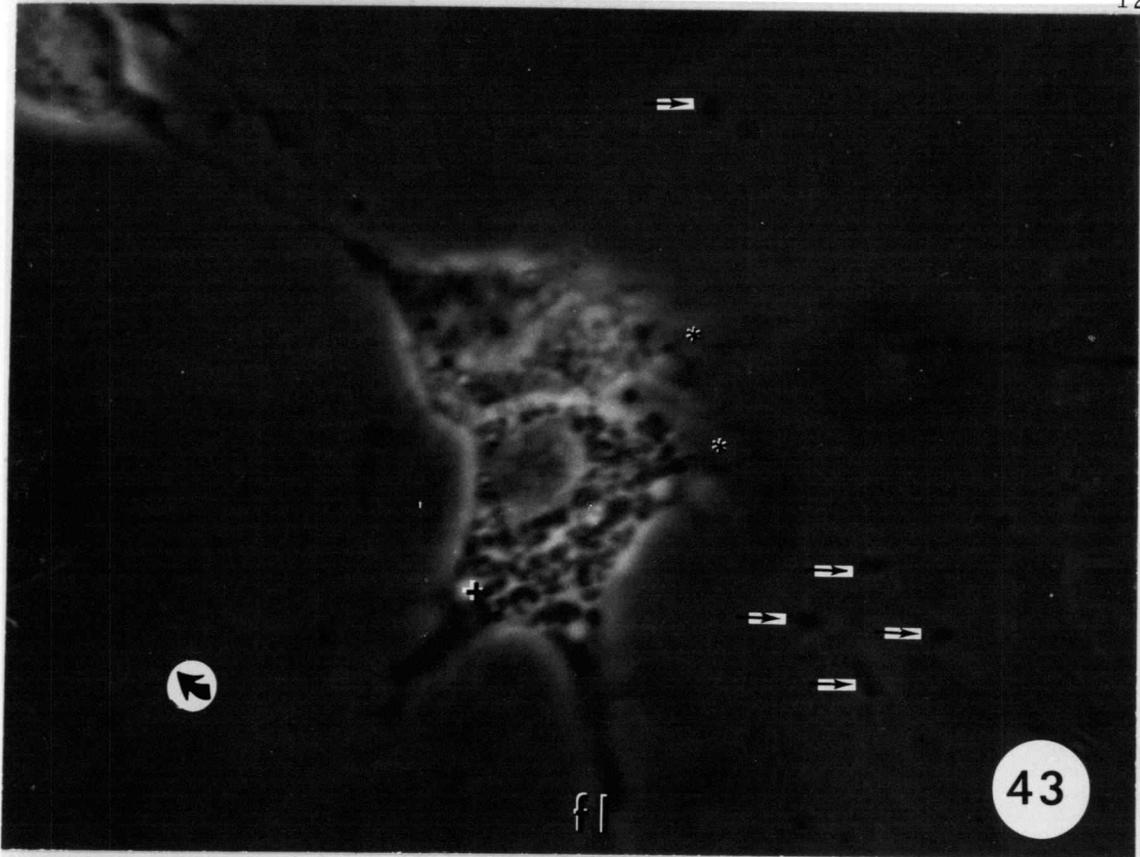


Figure 43. Cluster of two epithelial-like cells cultured on a glass slide treated with a glycoprotein suspected of enhancing adhesion. Hyaloplasmic filopodia (fl) anchor the cells to the substrate. Within the cells lysosome-like vesicles (+) seem to be evenly distributed while the phase gray vesicles (*) involved with endocytosis are located at their site of formation in the hyaloplasmic sheet. The direction of migration of the cell cord which left this cell cluster is indicated by the heavy arrow. "Foot-prints" of hyaloplasmic fragments (small arrows) can be detected on the glass slide. (2, 100 X)

Figure 44. Cluster of four cells cultured on a glass slide treated with a glycoprotein suspected of enhancing adhesion. Only limited hyaloplasmic filopodia can be detected from the non-migratory pigment cell (P). The heavy arrow indicates the direction of migration of the cell cord which left this cell cluster. "Foot-prints" of hyaloplasmic fragments (small arrows) can be detected on the glass slide (2, 100 X)



- Figure 45. The mechanical separation of cells in an aggregate one hour after the onset of reaggregation. Both epithelial cells (E) and mesenchymal cells (M) maintain their straight and angular cellular boundaries. The direction of force is indicated by the arrows. (1,550 X)
- Figure 46. The mechanical separation of cells in an aggregate three hours after the onset of reaggregation. In migrating epithelial cells (me) the firm adhesion of the ledge edge allows the lamella to be drawn out into a hyaloplasmic projection. The direction of force is indicated by the arrows. (2,000 X)
- Figure 47. The mechanical separation of cells in an aggregate five hours after the onset of reaggregation. The normally straight and angular boundaries of the epithelial cells are drawn into numerous cytoplasmic projections as the cells are separated from each other. (3,100 X)
- Figure 48. The mechanical separation of cells in a large aggregate seven hours after the onset of reaggregation. While the margins between epithelial cells are drawn into numerous cytoplasmic projections the margins between mesenchyme (m) and epithelial (e) cells form few cytoplasmic projections. (1,700 X)

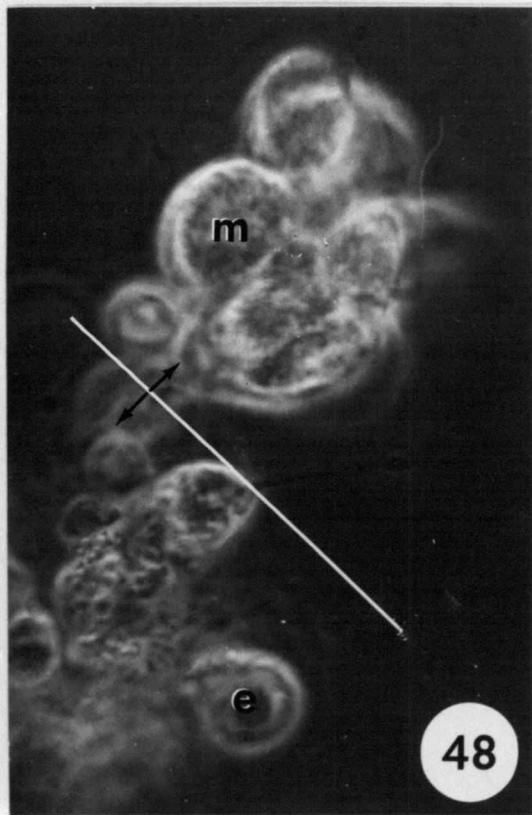
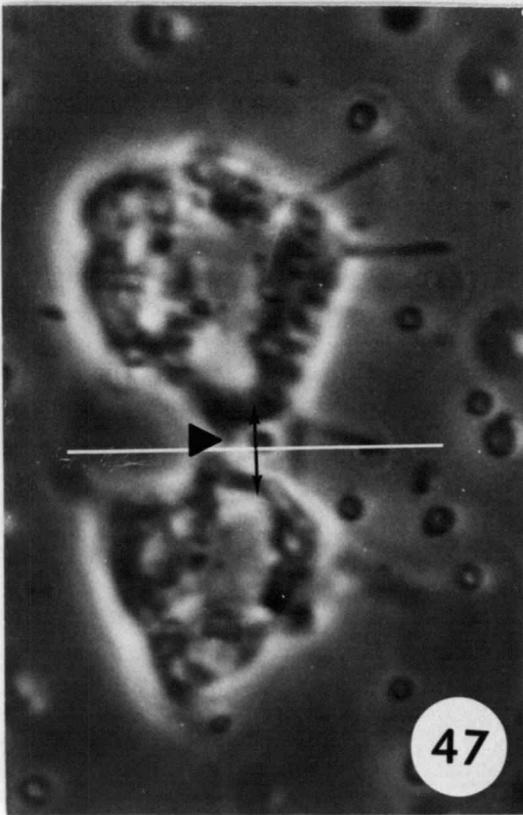
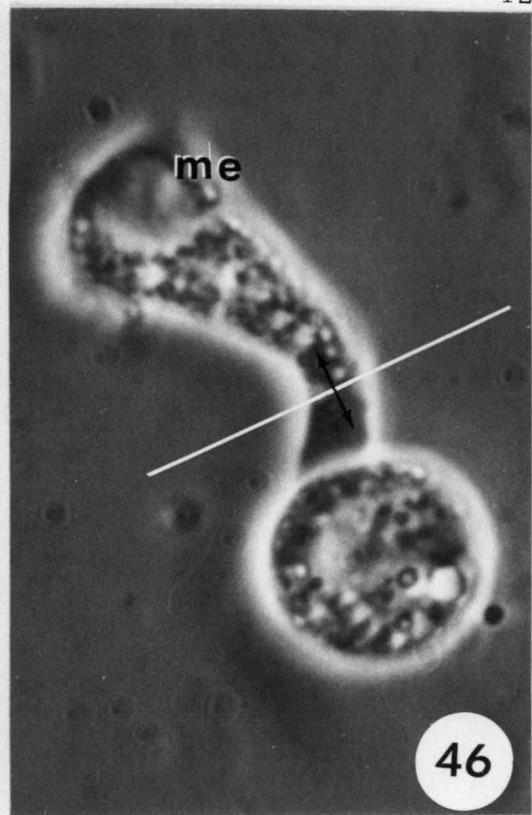


Figure 49. Cell appositions (CA) formed between two epithelial cells thirty minutes after the onset of reaggregation. The plasma membranes at the site of apposition are non-parallel and are separated from each other by an electron lucid distance of approximately 100 Å. Mitochondria (M), yolk (Y), nucleus (N), and lipids (L) are not localized within the cell at this time. (50,400 X)

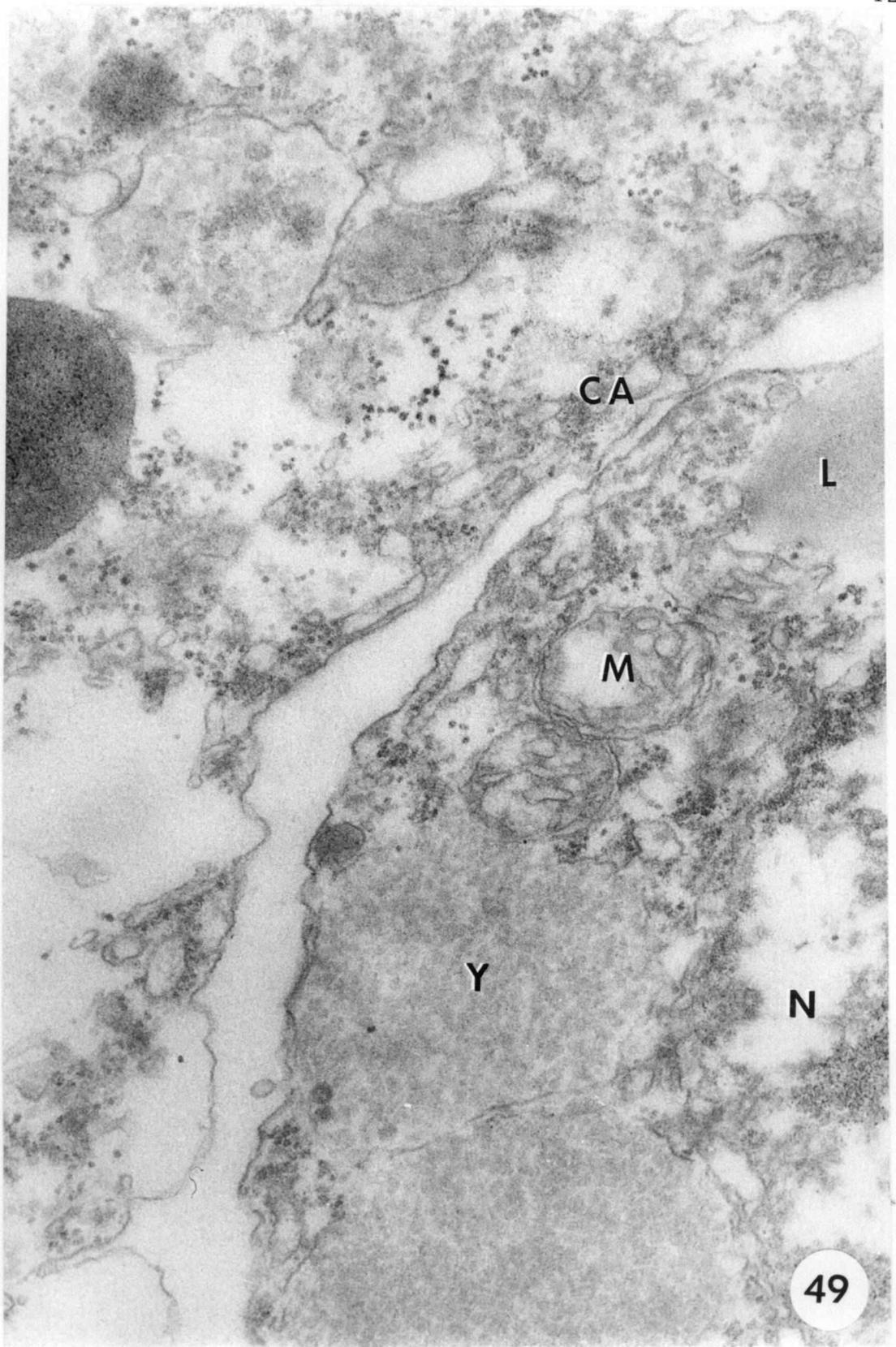


Figure 50. Cell appositions formed between a non-migratory epithelial cell (E) and a migratory epithelial cell with a well formed hyaloplasmic bleb (HB). The arrow indicates the direction of migration towards the cell free surface. The mitochondria (M), lipid (L), and yolk (Y) appear to be evenly distributed within the non-migratory cell. (22,600 X)

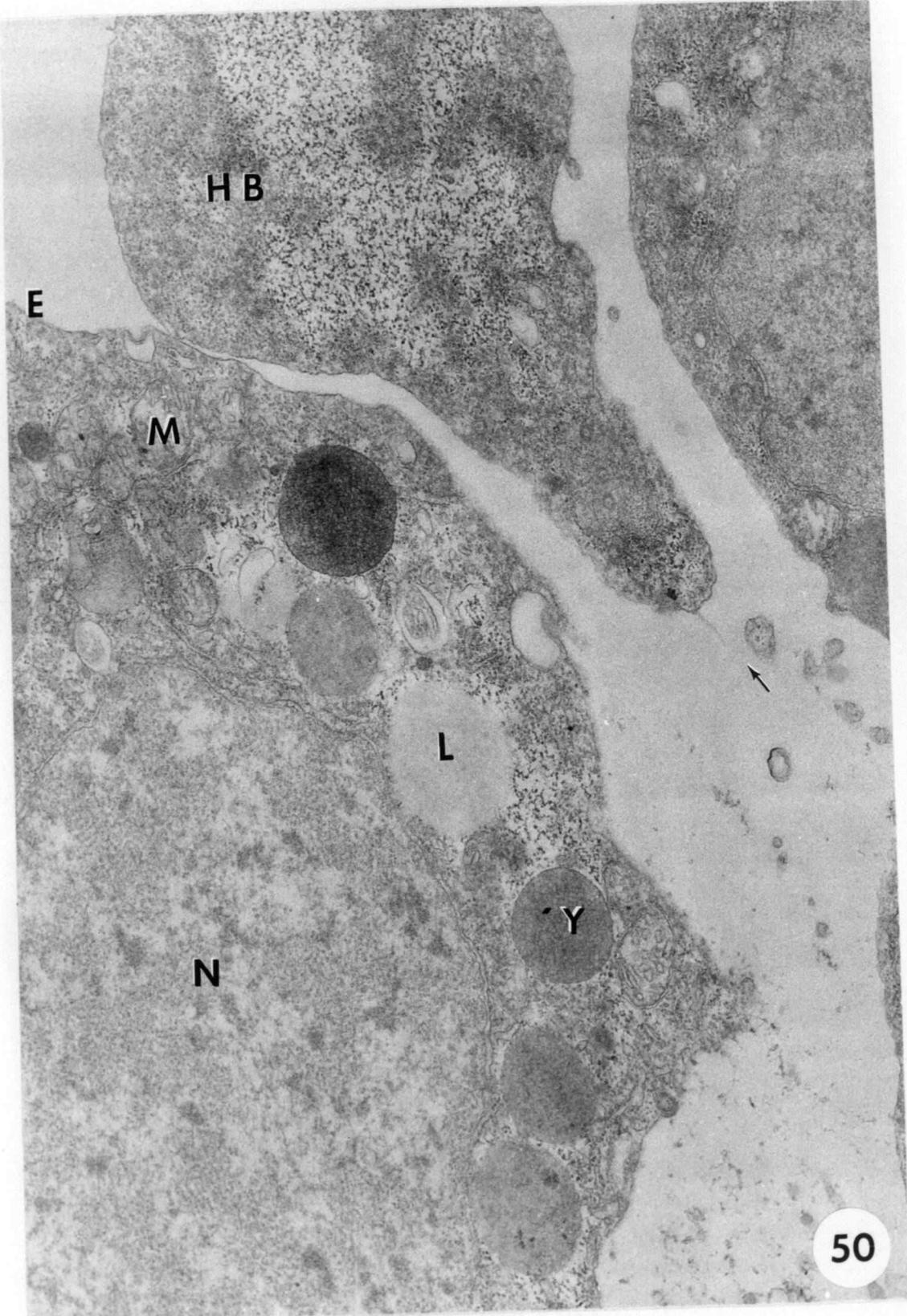


Figure 51. Six hours after the onset of reaggregation, cell contacts are formed by the overlapping lamella (La) of the leading edge (LE). While cell appositions frequently occur along the margin of the lamella only few occur along the margins of the lamellipodium (Lap). At this stage of reformation, the enlarging leading edge has begun to form the presumptive blastocoel (pB), which is distinct from the cell free surface of the epithelia (CFS). (42,500 X)

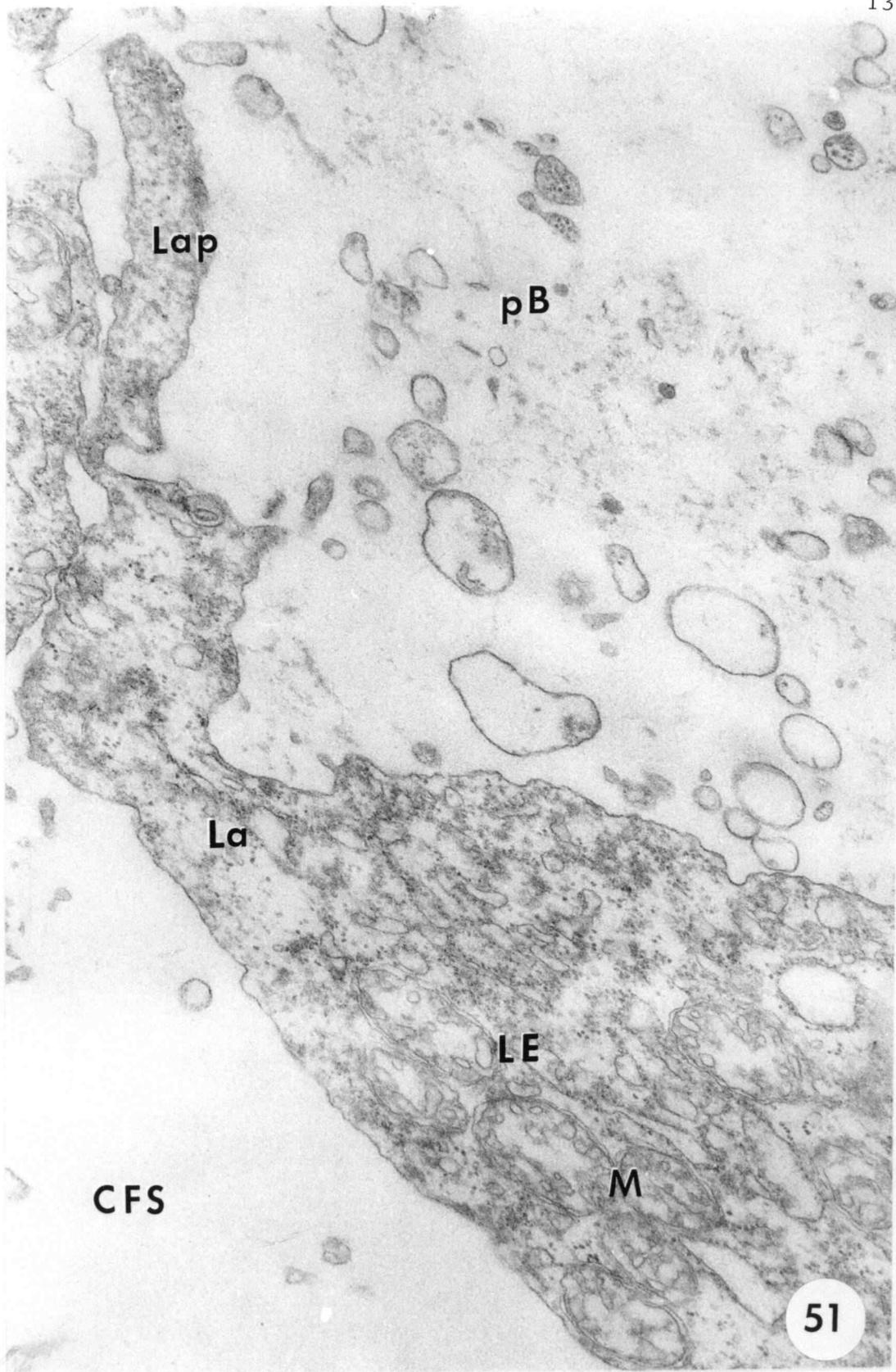


Figure 52. Cell junctions formed at the cell free surface (CFS) margins of the epithelial cells 10 hours after the onset of reaggregation. Electron dense material occurs between the apposed plasma membranes as well as the region subjacent to the junction (arrows). (55,300 X)

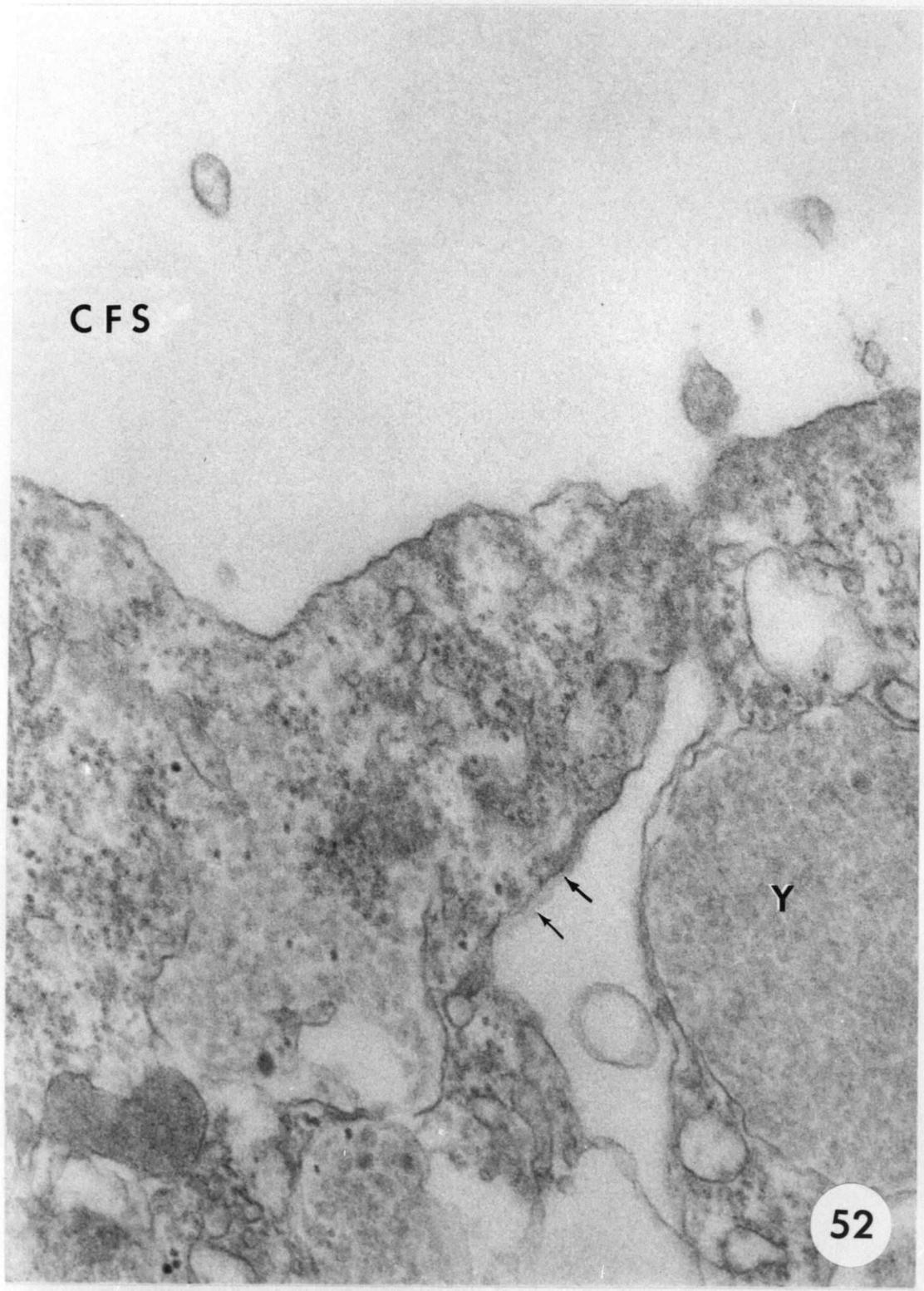


Figure 53. The distinct polar orientation of the epithelial cell is apparent 12 hours after the onset of reaggregation. The cell junction (CJ) is formed between the apposing plasma membranes at the base of a "V"-shaped (V) indentation between the two cells. Septate bar formation (arrows) is already suggested at this early stage. The lysosome-like vesicles (LV) and phagosomes (P) are localized along the blastocoel (B) margin of the epithelial cell. Yolk (Y) and mitochondria (M) appear to be evenly distributed within the cytoplasm. (33,700 X)

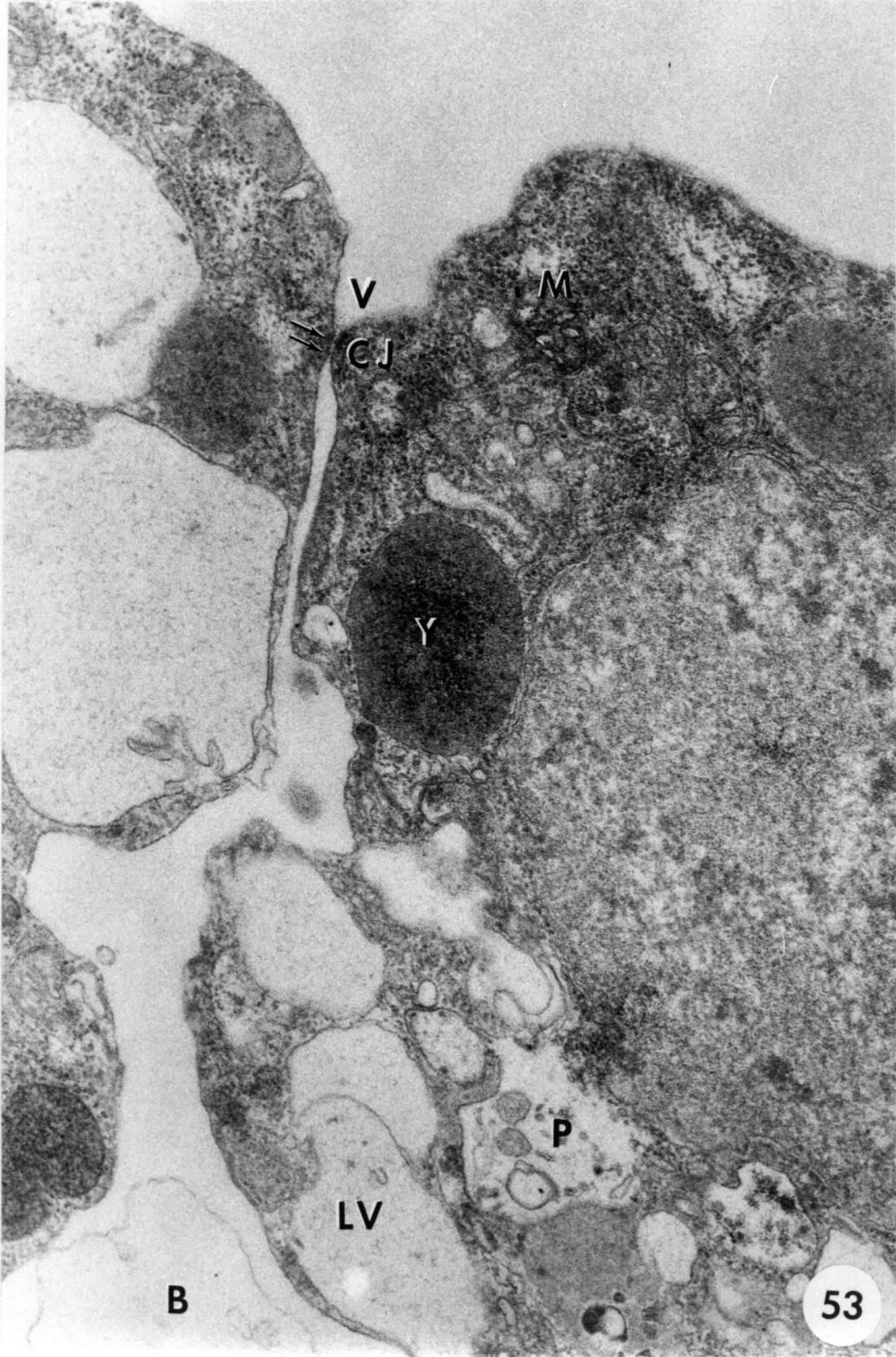


Figure 54. Cell junctions (CJ) form along the cell free surface (CFS) at the apical margin of the cell 24 hours after the onset of reaggregation. Electron dense material occurs subjacent to the area of the cell junction. Yolk (Y) and mitochondria (M) are evenly distributed in the epithelia cell. (55,300 X)

