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RUSSELL ALBERT WHITEHEAD for the Ph. D. in Zoology
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Title TIME-LAPSE AND ELECTRON MICROSCOPE STUDY OF CULTURED EPITHELIUM SUBJECTED TO CALCIUM DEPLETION WITH EDTA AND ITS SUBSEQUENT RECOVERY

Abstract approved [redacted]
(Co-major professor)

[redacted]
(Co-major professor)

Primary monolayer cultures of chick renal epithelial cells grown on plastic coverslips were reversibly dissociated with the chelating agent ethylenediaminetetraacetic acid (EDTA). The cellular responses during dissociation and reaggregation in Hank's balanced salt solution (BSS) were observed with phase optics and recorded by time-lapse cinemicrography.

The epithelial cells grow as a confluent sheet closely adherent to the plastic substrate. EDTA induces a series of responses by the cells of the monolayer. The chelation of divalent cations by EDTA induces the cells of the monolayer to relinquish mutual adhesive contacts. On newly freed surfaces veiling is initiated for a brief period and then subsides. Cellular contraction continues followed by very active surface blebbing. On continued chelation this

activity also ceases at which time the cells are completely separated, fully rounded and highly refractile.

Cultures containing fully separated cells were perfused with Hank's BSS and allowed to reassociate into a coherent monolayer. After a brief refractory period surface blebbing resumes and continues for a time giving way to a less vigorous surface expansion. The cells flatten to the substrate, expand centrifugally, and make contact with adjacent cells. This initial contact is extended over greater surface areas until a coherent sheet of cells indistinguishable from the untreated cultures has been established.

The EDTA effects are interpreted as being a result of the extraction of divalent cations while the recovery responses are interpreted as a reversal of the EDTA effects by the calcium containing medium.

The responses during separation and recovery are discussed in terms of altered sol-gel relationships, reversible cellular permeability properties and reversible changes in the cellular adhesiveness.

The sequence of events during separation and reconstitution of the confluent sheet have been studied with the electron microscope to establish changes in the ultrastructure corresponding to those observed with the light microscope. The fine structure of the

untreated tissues has been studied with special emphasis on the cellular interrelationships. With the exception of the intracellular cement all adhesive modifications are disrupted during EDTA induced separation and those that are disrupted exhibit a striking recovery during incubation in Hank's BSS. During the separation and recovery sequences microvilli are elaborated at the free surfaces of the cells, they are retracted on fully separated cells and reappear during recovery. Their possible role in cell contact is discussed.

In addition to the surface effects EDTA elicits disruption of certain intracellular components. Peripheral microtubules are destroyed during the separation sequence and reappear in the recovery cells, however, those observed in association with the centriole are not affected. The microtubules are discussed in terms of a labile cytoskeleton functioning in formation and maintenance of cell asymmetries. Polyribosomes are decreased in numbers during chelation. This is interpreted as being due to extraction of magnesium by the chelating agent and its removal permits their increase during recovery.

The mitochondria, endoplasmic reticulum, Golgi lamellae and the centriole and its derivatives are not altered by treatment.

Many of the EDTA induced responses are discussed in the

light of events occurring during normal mitosis. The surface responses and cytoplasmic effects of EDTA treatment suggests that a related phenomenon occurs during normal mitosis.

TIME-LAPSE AND ELECTRON MICROSCOPE STUDY OF CULTURED EPITHELIUM SUBJECTED TO CALCIUM DEPLETION WITH EDTA AND ITS SUBSEQUENT RECOVERY

by

RUSSELL ALBERT WHITEHEAD

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APPROVED:

[redacted]

Associate Professor of Zoology
Co-Major Professor
In Charge of Major

[redacted]

Assistant Professor of Zoology
Co-Major Professor
In Charge of Major

[redacted]

Chairman of Department of Zoology

[redacted]

Dean of Graduate School

Date thesis is presented December 8, 1966

Typed by Maryolive Maddox

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INTRODUCTION

Divalent cations, expecially calcium ions have been implicated in a wide variety of cellular functions, among which and not the least important is their participation in maintenance of cellular structure. The influence exerted by calcium and other divalent cations on cellular properties and activities has been thoroughly reviewed by Heilbrunn (1952).

Among the possible roles suggested for cellular calcium is the proposal by Dornfeld and Owczarzak (1958) that calcium ions may act as intermolecular links in maintaining the structural integrity of the interphase plasma membrane. The earlier work of Alexander, Teorell and Aborg (1939) suggested that such a calcium mediated linkage was likely. Webb and Danielli (1940) proposed that the plasma membrane may be regarded as a reservoir for calcium ions which are bound at the cell surface in a calcium proteinate complex. Direct support of this proposal was obtained by Lansing and Scott (1942) when they demonstrated an accumulation of calcium in spodograms prepared from plasma membranes.

Ringer (1890) and Herbst (1900) first demonstrated that this surface calcium may be involved in maintenance of tissue integrity. Since these early reports, it has become generally accepted that calcium ions are a major factor in the formation and maintenance of intercellular adhesion.

Zwilling (1954) introduced the use of the chelating agent ethylenediaminetetra-acetic acid (EDTA) for dissociation of embryonic chick cells. Following agitation in EDTA suspensions of viable cells were obtained which reaggregated readily when the EDTA was removed from the culture. Under physiological conditions this organic chelating agent has the ability to complex with and to bind divalent cations and thus provides a means whereby calcium may be removed from various tissue systems. Experimental removal of calcium ions and its subsequent replacement is a method that has been extensively employed to examine the role of calcium in cell adhesion as well as other surface activities and cellular properties. Owczarzak and Dornfeld (1962) using EDTA dispersive methods have provided additional evidence that calcium ions associated with the cell surface play a critical role in intercellular adhesive phenomena. Dornfeld and Owczarzak (1958) used time-lapse cinematography to record the surface responses of cultured chick heart fibroblasts perfused with EDTA. They observed the responses to treatment in

all stages of the cell cycle and noted that interphase cells responded to EDTA by withdrawal of cell processes with subsequent "surface bubbling and ectoplasmic blebbing" (page 245). Metaphase cells were not affected by treatment; however, anaphase bubbling persisted and telophase expansion and migration were prevented. The EDTA effects were completely reversible and recovered cells resumed normal configurations and activity including further mitoses. The same authors Owczarzak and Dornfeld (1962) recorded the responses of cultured epithelial sheets to 0.003 M EDTA perfusion and observed complete separation of cells with subsequent surface bubbling identical to that observed in fibroblasts. When the chelating agent was removed complete recovery followed with concomittant reconstruction of coherent epithelial sheets. The EDTA effects were interpreted as resulting from the extraction from and binding of divalent cations at the cell surface. Similar results were obtained by Robbins and Micali (1965) in which interphase HeLa cells subjected to 0.01 M EDTA incubation exhibited cytoplasmic bubbling, whereas, metaphase cells under similar conditions responded with simple isotropic expansion. They proposed that calcium depletion results in a local weakening of the cortical gel structure establishing a gelation gradient between the endoplasm and the cortical ectoplasm with subsequent cytoplasmic herniations. This concept

of cortical gel solation by calcium extraction is given credence by the observation that EDTA decreases the viscosity of the peripheral cytoplasm in mouse ascites cells as shown by displacement of peripheral lipid granules under centrifugation (Nishimura, DiPaola and Hill, 1955). Borei and Bjorklund (1953) demonstrated a similar loss of rigidity in EDTA treated sea urchin eggs, as shown by increased elongation during centrifugation. They interpreted this loss of rigidity as being due to the loosening of intermolecular bonds of the cortical structure by chelation of cations.

Most recently L. Weiss and Clement (1966) investigated the effects of EDTA mediated calcium withdrawal on cellular deformability. Among other results they noted that following calcium depletion the cell surfaces were much more readily deformed. They interpreted these results in terms of surface viscosity changes or gel-sol transformations. They further proposed that such changes could occur naturally thus permitting the cells to elaborate low radius of curvature probes with which initial cell to cell contacts may be established. On readministration of calcium containing media the surface properties of the cells reverted to those observed prior to extraction of calcium. DeHaan (1958) showed that with proper control of the concentration, timing and duration of EDTA treatment it was possible to predictably alter morphogenetic movements

in developing embryonic chick heart. These effects were interpreted as resulting from temporal inhibition of intercellular adhesion with a correlated inhibition of normal cell motility.

In studying the effects of EDTA on cell adhesion in slime mould DeHaan (1959) observed that treatment of the pseudoplasmodium caused extensive dissociation of the cells accompanied by surface bubbling similar to that discussed above. Under the influence of 0.003 M EDTA, free vegetative cells retracted their pseudopodial extensions and rounded up. Surface bubbling and bleb formation followed and normal aggregation was prevented. All effects were reversible following calcium replacement. He suggested in agreement with Dornfeld and Owczarzak (1958) that surface bleb formation is the result of a state of plasma membrane instability induced by calcium depletion. Thomason and Schofield (1961) using time-lapse cinemicrography failed to record any surface activity or cytoplasmic bubbling in mouse Ehrlich ascites tumor cells treated with 0.003 M - 0.006 M EDTA solutions. They concluded that such responses were "unspecific reactions to ill treatment and not related to the calcium concentrations of the cells or their environment" (page 492). L. Weiss (1960) concerned with the possibility of cell damage due to EDTA, measured the effects of treatment on the viability, diameter and dry mass of murine sarcoma-37 cells and

detected no variance from untreated cells in any of these parameters. Similarly Zwilling (1963) found no disproportionate death rate in nodal cells of embryonic chick after EDTA dissociation and reaggregation. Drosophila salivary glands dissociated with EDTA, however, showed extensive deformation of cell contents with marked swelling of the nucleoli (Kaufman and McDonald, 1957). Letham (1960) applied EDTA dispersive techniques to plant cells and found that its effectiveness was markedly influenced by pH, concentration and temperature but was less damaging to the cells than other macerating agents.

Investigations have been made employing the techniques of electron microscopy to study the phenomena of EDTA induced cell dispersion and of reassociation following removal of the chelating agent. Observations were made concerning changes in adhesiveness, physiology and cytoplasmic structure. Lynn, Fortney and Brown (1964) observed ballooning of mitochondrial membranes and condensation of the mitochondrial matrix following EDTA treatment. They concluded that EDTA decreased the permeability of the mitochondrial membranes and the ballooning of the outer membrane was the result of trapping of water between the two membranes following matrix dehydration. Harris and Leone (1966) also reported distention of the outer mitochondrial membranes along with matrix

condensation in OsO_4 fixed mouse liver cells and in glutaraldehyde fixed McCoy cell strain cultures. In addition they noted distention of intracristal spaces as well as general cytoplasmic coagulation. Peachey (1964), however, noted that EDTA treatment did not induce these effects in all mitochondria; only a portion displayed this response while many appeared unaffected by treatment. No effect was observed in mitochondria of EDTA treated gastric gland oxyntic cells of Rana by Sedar and Forte (1964). They did show that the electron opaque material of the desmosomes disappears permitting the separation of cells at this point. Similar effects were demonstrated in the zonula adhaerens and the tight junction (zonula occludens). Apical microvilli were retracted whereas, no effect was observed in the mitochondria, endoplasmic reticulum, nuclei, ribosomes, glycogen or zymogen granules. All effects on the components of the juctional complex were reversible on administration of calcium with especially striking recovery of the electron opaque material associated with the desmosomes. They concluded that calcium is required for the preservation of the fine structure of the junctional complex and the desmosome was suggested as a likely site for calcium bridge formation. Hays, Singer and Malamed (1965) did an electron microscope study on the effects of calcium withdrawal on the structure and function of the toad bladder.

Electron micrographs were presented showing that when calcium was withdrawn by EDTA chelation the epithelial cells detached from one another and then from the substrate tissue. Microvilli with dense fibrous cores were observed in partially and fully separated cells. An unrolling of complex plasma membrane convolutions was observed and all expressions of cell attachment were disrupted; the desmosomes appearing somewhat more persistent as separation occurred above and below this component prior to separation of the desmosome plaques. Free cells were rounded with extensive vacuole formation, some mitrochondrial swelling and nuclear distortion. Electron microscopy of rat liver perfused with EDTA revealed a decrease in mutual cell contacts accompanied by extensive vacuolization of the cytoplasm (Coman, 1954). Easty and Mutolo (1960), however, detected no separation of adjacent cells nor vacuole formation in similarly treated rat liver and suggested that the results of Coman (op. cit) were the result of artifacts caused by methacrylate embedding. In the same investigation Easty and Mutolo (op. cit) studied the affects of EDTA on the adhesiveness of Walker ascites tumor cells of the rat and observed that incubation in EDTA was no more effective in separating the cells than incubation in saline. They suggested that the role of calcium in cellular adhesiveness had been over emphasized in these tissues. By careful perfusion of rat

liver with EDTA, Leeson and Kalant (1961) were able to demonstrate progressive separation of adjacent cells, culminating in completely separated and isolated liver parenchymal cells. Their results disagreed with the findings of Coman (op. cit.) in that no alterations of cell organelles were detected including preservation of normal intact plasma membranes. These findings were also diametrically opposed to those of Easty and Mutolo (op. cit.) whose results were interpreted as being due to inadequate perfusion techniques. Pseudopodial formation and microvilli resembling those in untreated liver bile caniliculi were observed on the surface of partially and fully separated cells. Berwich and Coman (1962) found that removal of calcium with EDTA caused considerable reduction of cell to cell adhesiveness in buccal epithelial cells but treatment with EDTA had no effect on the attachment to the substrate. Neuriminidase, however, reduced the stickiness to the substrate, whereas, it had no affect on mutual cell adhesion. They suggested that mutual adhesiveness is dependent on calcium bridge formation while attachment to the substrate depends upon a substance containing mucopolysaccharides. This supported the proposal that mutual adhesiveness of cells and substrate attachment are two independent phenomena (Coman, 1961). Centrifugation methods were used by Easty, Easty, and Ambrose (1960) in a quantitative study of

adhesiveness to glass of various cell types and the effects of different agents on this adhesiveness. It was observed that while various enzymes reduced the cell-glass adhesiveness, EDTA was without effect. Although EDTA did apparently increase the distance between the cells and the substrate as shown by interference reflection microscopy it failed to dislodge them completely from glass (Curtis, 1964). Rinaldini (1959), however, stated that EDTA can detach chick embryonic heart and breast muscle cells from glass after vigorous shaking. Forte and Nauss (1963) studied the physiological effects of EDTA on the bullfrog gastric mucosa and observed among other changes an increased movement of sucrose across the mucosa after treatment. This was considered to be a result of expansion of the intercellular spaces due to cell separation. The effects were reversed when the EDTA was replaced by calcium laden media. EDTA administered at a pH below 6.0 was observed to elicit no response on the mucosa, thus providing physiological evidence of the effective range of chelation proposed by Letham (1960). Curran, Zadunaisky and Gill (1961) found similar reversible changes in frog skin subjected to EDTA treatment, as did Hays, Singer and Malamed (1965) in EDTA treated toad bladder epithelium.

In comparing the dispersive effects of several calcium chelating compounds on perfused rat liver Anderson (1953) found that

following homogenization EDTA yielded 60% isolated whole cells, a percentage that compared favorably with other agents studied. Melnick (1955) used 0.02% EDTA and obtained effective separation of viable cells from monkey kidneys, whereas, Ganapathy, Hildebrandt and Riker (1964) incubated plant callus tissue and obtained a percentage yield of single cells equal to that from other dispersing agents. Lesseps (1963) dissociated embryonic chick retina cells in a solution containing EDTA, papain and l-cysteine and allowed them to reaggregate in culture. Electron micrographs of dissociated cells showed surface undulations and microvilli while cells in reaggregation showed these surface irregularities as the initial sites of mutual cell contacts.

It has been adequately established that calcium depletion with EDTA does in fact elicit tissue dissociation accompanied by unique cell surface reactions in a variety of tissues. Its employment by investigators requiring cell dispersive methods has been widespread. The majority of studies employing light microscopy have of necessity been conducted on cells grown in culture while the greater number using electron microscopy have been done on in vivo systems of adult organisms. Thus there is little direct correlation between the responses observed with phase optics and those observed at the ultrastructural level.

The present study attempts to compensate for this discrepancy by examining a single tissue with the entire spectrum of available magnifications. The effects of EDTA on cultured embryonic chick mesonephric kidney epithelia have been investigated with phase-contrast microscopy, time-lapse cinemicrography and electron microscopy. Comparisons are made between normal untreated epithelial sheets and epithelia subjected to progressive EDTA separation and their restitution into coherent sheets of epithelia.

MATERIALS AND METHODS

Tissue culture methods

The mesonephric kidney was removed from seven to nine day old chick embryos, thoroughly minced and cultured for three days at 37.5°C. Epithelia to be fixed for electron microscopy were cultured in Maximov depression slides using plastic cover slips rather than glass to facilitate subsequent separation of the resin embedded tissue and the cover slip. Cultures which were to be treated and/or photographed by time-lapse cinemicrography were cultured in the same medium but in Rose chambers to permit their perfusion with the EDTA and recovery solutions. The growth medium consisted of 79.5% Hank's balanced salt solution (BSS), 20% bovine serum, 0.5% glutamine, 100 units of penicillin per ml, and 100 units of streptomycin per ml. Its pH was adjusted to 7.3 prior to use. All cultures were examined with phase-contrast optics after three days incubation to insure selection of vigorous colonies. Only those having coherent sheets of epithelia firmly attached to the cover slips were retained for experimental purposes.

Time-lapse cinemicrography

Cinemicrographic records were obtained of control, treatment

and recovery sequences for use as standards of comparison with cultures fixed for electron microscopy. Selected Rose chamber preparations maintained at 37.5°C were photographed at a time-lapse interval of two or four seconds. After recording the appearance and activity of control cultures, the chambers were perfused by gravity flow with 0.002 M EDTA carried in Hank's BSS adjusted to pH 7.2. Epithelia were subjected to EDTA treatment until the cells were maximally separated and rounded after which the culture chambers were perfused with normal Hank's BSS and the tissue allowed to recover. Twelve ml of fluid were flushed through the two ml chamber at each perfusion to insure complete fluid displacement.

Electron microscopy

Tissues to be fixed for electron microscopy were handled in the following manner.

A constant temperature bath was set to maintain sterile Columbia dishes filled with Hank's BSS at 37.5°C. Twenty-five Maximov cultures were opened and the plastic cover slips containing epithelial colonies were immersed in the medium for 15 minutes, a procedure which served to float off loose debris and non adhering cells as well as to allow simultaneous initiation of treatment of all cultures. The culture medium was replaced as quickly as possible with the 0.002 M

EDTA treatment solution to initiate cell separation. Cover slips with adhering tissues were removed from the treatment medium and fixed in 3% glutaraldehyde for 30 minutes at various stages or degrees of separation. The latter were predetermined with reference to the time-lapse cinemicrographic record and correlated with direct phase-contrast microscopy of an "observation" culture run simultaneously with the treatment. This sampling was followed until maximum separation was attained at which time the chelating medium was replaced on the remaining cultures by normal Hank's BSS. Fixation of re-associating cells in a progressive series was continued until complete restitution of coherent epithelial sheets was observed. Photographs were obtained of the "observation" culture at each fixation period through out the entire sequence. This procedure was repeated on three different sets of Maximov cultures.

Fixed cultures were processed according to the following procedure:

All cultures were washed for one hour in Sorenson's phosphate buffer at pH 7.3, postfixed in 2% osmium tetroxide, dehydrated in a graded ethanol series and allowed to stand over night in a 50:50 mixture of absolute ethanol and araldite. The mixture was drained off and the cover slips were placed with the adhering tissues upward in small aluminum pans and flat imbedded in the final araldite resin.

Polymerization of the resin occurred at 60°C for 48 hours. Following polymerization the cover slips were separated from the resin leaving the cells at the surface of the araldite sheet. Individual epithelial growths were selected under phase-contrast optics, cut out and glued to araldite pegs. After trimming, the blocks were sectioned with glass knives on a Porter Blum MT-1 ultramicrotome. Thin sections were picked up on formvar coated grids and doubly stained with saturated uranyl acetate and Reynold's lead citrate. Electron micrographs were obtained using either an RCA EMU 2 D or an RCA 3H electron microscope and subsequently photographically enlarged.

RESULTS

Observations with time-lapse cinemicrography

Epithelial cells cultured on plastic in a fluid medium by the method described (Figure 1) are identical in all respects to epithelia grown directly on glass or using plasma clot techniques. Their appearance is that of a confluent sheet of cells apparently migrating outward from the explant as a monolayer on the plastic substrate. Free borders of the cultures are characteristically active showing considerable veiling action accompanied by pinocytotic activity. Movement of the particulate cytoplasmic elements is especially striking and suggests an active translocation rather than random Brownian movement. Usually the mitochondria appear filamentous, however, their labile nature is evident as they are observed to move, bend and constantly change shape. Interphase nuclei are distinct as large relatively lucid spheres containing one or two prominent nucleoli. In a number of cells a rather large juxtanuclear area displays a different aspect than the rest of the cytoplasm. This area being invaded less by cytoplasmic particles has a brighter appearance than the adjacent cytoplasm.

Primary cultures of chick renal epithelia give a characteristic series of responses when treated with EDTA. The most obvious are

the release of mutual cell contacts, cellular contraction, surface bubbling and complete cell segregation. This pattern of responses can be completely reversed by removal of the chelating agent.

Time-lapse cinemicrographic records of epithelial cultures treated with 0.002 M EDTA at pH 7.3 are essentially the same as those obtained by Owczarzak and Dornfeld (1962) using 0.001-0.003M EDTA at pH 7.8. One major difference is that the lower pH extended the response to treatment over a longer period of time, whereas, the higher pH and concentration consistently resulted in complete and abrupt cell separation within six to six and one half minutes (Owczarzak, personal communication). By using the less vigorous chelating action and thereby extending the response to treatment, it is possible to record more fully the sequence of events leading to complete dissociation of the cells. Under this treatment the separation of cells is characterized by a series of striking events albeit part of one continuous process. Figure 2 shows a series of time-lapse frames selected to illustrate the major events occurring during the sequence of treatment and recovery.

The onset of responses to treatment is manifested shortly after application of the EDTA by disruption of cellular cohesion in that portion of the cells farthest removed from the substrate. The flattened regions of the cells adjacent to the substrate retain their

position and remain in intimate contact with neighboring cells. This response occurs so abruptly (four to five minutes) that it suggests a release of tension and/or a contraction rather than a response to osmotic changes. The general effect of the phenomenon is to elicit a change in contour of the cells from a uniformly flat configuration to one having delicate edges and a very thick central region containing most of the cytoplasm. All particulate material is occluded from the periphery and crowded toward the cell center. The peripheral areas thus appear very thin, homogeneous and completely free of particulate material, suggesting that in these regions little cytoplasm remains between the plasma membranes. At this point the bright juxtanuclear area mentioned is still discernible. After a few minutes the thin peripheral areas of adhering basal regions of the cells begin to separate from each other and at some of the newly freed surfaces veiling activity is initiated and becomes very pronounced. As separation continues small spaces between cells become evident and grow progressively larger as the more persistent cell contacts relinquish their adhesiveness. As the separation progresses sites of persistent attachment grow narrower but remain attached by cell processes which become more attenuated until their presence is scarcely discernible. When observed directly with phase optics and careful adjustment of the illumination these

very fine remnant fibers can be detected extending from cell to cell as well as from the cell to the substrate. Certain of these points maintain contact even after the cell bodies are displaced from one another by considerable distances.

A period of very active surface blebbing occurs shortly after the onset of basal separation. Large homogeneous rather hyalin blebs are rapidly extruded and retracted at the cell surfaces. Blebbing continues for about 30 minutes and then gradually subsides and the cells become completely rounded, static and highly refractile.

Replacement of the EDTA solution with a calcium containing medium illicits the onset of the recovery response. Shortly after the EDTA is removed the separated and rounded cells begin to extrude ectoplasmic blebs from their surfaces which are identical to those observed during calcium removal. These blebs are not completely retracted with the result that the cytoplasm moves progressively outward eventually giving the appearance of a pseudopodium the distal end of which retains blebbing activity. Pseudopodial extensions occur from all surfaces of the cells and these often merge to form larger processes which expand centrifugally. Distally the pseudopodial extensions continue to bleb and expand until contact is established with those from adjacent cells. At the onset the extensions are free of particulate materials, however, once the cells begin to

flatten the cytoplamic particles migrate out into the newly available space. After about two hours in the recovery medium the cells re-establish mutual contacts, flatten to the substrate, initiate veiling at the free surfaces and are reaggregated into a coherent sheet showing no major differences from those observed prior to EDTA treatment.

It is noteworthy that during the entire sequence of cell separation and recovery of all fields recorded with time-lapse cinemicrography not a single cell was observed to release attachment to the substrate This held true for cultures grown on glass, on plasma clot and on plastic substrates.

Electron Microscopy

For electron microscopic studies the sequence of separation and recovery was arbitrarily divided into six phases corresponding to some of the major events observed. The categories assigned were as follows:

(1) Normal or untreated

(2) Partial separation

(3) Active blebbing

(4) Complete separation

(5) Partial recovery

(6) Complete recovery

Because of the nature of the sequence and overlapping of the assigned categories several observations may apply to two or more of the groupings while others may apply better to phenomena characteristic to events not included in the categories.

Untreated cultures

In order to detect changes induced by EDTA treatment thorough electron microscopic survey has been made on control cultures. Electron microscopy of such cultures sectioned normal to the plane of growth reveals details of structure and activity not detectable under phase-contrast microscopy.

Free surface. Sections through the ruffled membranes of the peripheral cells show a complex of whorls, extensions, and other configurations depending upon the angle of sectioning of each portion of the veiling membrane (Figures 3 and 4). These processes are homogeneous, free of cytoplasmic organelles and of variable thickness. Medial to the veiling membranes, vesicular profiles are apparent and are suggestive of pinocytotic activity. Many of the peripheral cells have a large number of microvilli on their lateral free surfaces which possess electron dense fibrous cores (Figures 3, 4 and 5). The latter may frequently be observed extending

medially into the cytoplasm of the cell proper. Microvilli are also observed in the intercellular spaces formed at the point of convergence of three or four cells and are very reminiscent of those observed in bile canaliculi and other glandular ductules (Figures 6a and 7). Similar structures are also characteristic of the upper free surface of the cells although they are reduced in number and density when compared to those observed on the lateral cell surfaces (Figure 9).

Mutual contact surfaces. Contact surfaces between adjacent cells show a variety of modifications many of which have been assigned adhesive functions. Among the adhesive modifications observed are parallel unmodified contacts, complex interdigitations, mortice and tenon sites, desmosomes, electron dense areas of undetermined nature and plaques of intercellular cement.

The simple unmodified 100-200 Å spacing between cells has been accepted as fairly accurately representing the gap which exists between most cell surfaces. The interface of mutually apposed surfaces in three day primary cultures of chick renal epithelia is most commonly represented by such unmodified contacts. These interspaces are relatively electron lucid, whereas, plasma membrane differentiation is limited to rather infrequent observations of micropynocytotic vesicles budding into the cytoplasm. Plasma

membranes of such contact surfaces consistently lie parallel to one another. Figure 8 illustrates a contact surface displaying such a parallel, unmodified, electron lucid interface.

Interdigitation of cell surfaces is often very complex and suggests that this may be a very efficient mechanism for intercellular attachment. In sites of interdigitating surfaces the plasma membranes also run parallel to one another with about 100-200 Å separation and little electron opacity is exhibited in the intervening space (Figure 9).

Simple interdigitations of the mortice and tenon type, observed with less regularity, are similar in most respects to the more complex interdigitations and may differ from the latter only in a matter of the degree of complexity. They appear as simple knoblike protrusions of one cell into a complimentary concavity in an adjacent cell with the mutual interspace sometimes being filled with an amorphorus material of medium electron opacity (Figure 10).

The desmosomes are relatively large electron dense areas of local attachment composed of two dense plaques from which tonofibrils radiate into the cytoplasm of the two participating cells. Between the electron opaque plaques is an electron lucid space of approximately 200 Å. The structure of this unit is similar to those observed in many adult tissues and is illustrated in Figure 11.

Very frequently amorphous areas of increased electron density are observed subjacent to the apposed plasma membranes of cells at the margins of the cultures (Figures 3 and 7). These areas lack the ultrastructure characteristic of desmosomes although their general appearance and disposition suggests that they do function in intercellular adhesion. In keeping with the nomenclature assigned junctional complexes by Farquhar and Palade (1962) the term "zonula diffusa" is proposed for this area and will be employed where relevant. These adhesive modifications have not been observed at any sites within the cultures other than at the most peripheral attachments between adjacent cells at which site they occur consistently.

The concept exists that in a variety of tissues an intercellular cementing material may be deposited between the cells and may play a role in cellular cohesion. Figure 12 illustrates discontinuous plaques of very electron opaque material which have been deposited in the intercellular spaces. The deposition of this material does not result in an increase in the width of the intercellular space but is apparently confined within the limits of the standard 100-200 Å gap. This amorphous material may well represent cement functioning in intercellular adhesion.

Cytoplasmic organelles. Mitochondria are among the more abundant and conspicuous components of the cytoplasm. They are characteristically bounded by a double membrane system, the inner membrane being highly folded to form numerous sharply defined cristae. The mitochondrial matrix is uniquely dense and intramitochondrial granules are often present. The shape of the mitochondria varies; filamentous, branched, and donut-shaped mitochondria have been observed and many profiles are ovoid to spherical although in serial sections their filamentous nature frequently can be determined. Figures 7, 10, 12, 14, 15 and 21 illustrate the mitochondrial structure as determined at various magnifications.

In addition to the mitochondria, densely staining lipid droplets are present in varying amounts. Ancillary histochemical observations with the oil red O technique have confirmed that lipid droplets are present and increase in abundance with the age of the culture. Other histochemical observations have shown granules which give a positive acid phosphatase reaction as shown by the Gomori method. Dense variably staining and structured bodies are observed with the electron microscope. These may correspond to the acid phosphatase positive granules and thus may represent lysosomes (Figure 10).

Organization of the cytoplasmic membrane system is limited although fragments of rough tubular endoplasmic reticulum are sparsely scattered through the cytoplasm (Figures 13 and 16). The Golgi system, although infrequently observed and rather limited in extent, consists characteristically of parallel lamina of smooth surfaced membranes (Figure 18 c). In contrast to the lack of membrane associated ribosomes, free ribosomes and polysomes are profusely distributed through out the cytoplasmic ground substance. The polysomes assume a variety of configurations including aggregates of various sizes, chains of various lengths, rosettes, concentric spirals, and distinct darkly staining helical structures. The helical polysomes are approximately 350 Å in diameter, variable in length and are characterized by a gyre of 25°. These dimensions agree with measurements by several authors for similar structures in other cell types. Figures 3 and 13 illustrates the variations observed in polysome conformation.

Under the fixation procedure used, subcellular structures are observed which are rarely preserved in tissues fixed with osmium tetroxide alone. Cytoplasmic microtubules similar in general morphology to spindle tubules are well preserved by glutaraldehyde fixation and have been observed in nearly all cells in which they have been sought. In cultured chick renal epithelial

cells, microtubular elements have a characteristic diameter of 200-250 Å and an electron lucid center of about 80 Å. The walls of the tubules are approximately 80 Å thick and although Ledbetter and Porter (1964) have shown the microtubule walls to be composed of smaller subunits, no such structure was discernible in this study. There is no branching and single tubules may extend for several microns in a section. In certain cells the microtubules are highly oriented, whereas, in others the orientation is lacking. When orientation of the microtubules is observed, the alignment is generally parallel to the long axis of the cell and often associated with orientation of mitochondria and other cytoplasmic organelles (Figures 14 and 15). In sections through the cell periphery, the oriented microtubules are often observed terminating at or near the plasma membrane (Figure 15), however, physical connection to the latter has not been demonstrated. Microtubules have been observed through out the cytoplasm and are especially abundant in a region near the nucleus. In many sections it is found that this area is adjacent to an indentation in the nuclear membrane and the microtubules appear to radiate from this site (Figure 17). Connections of microtubules to cytoplasmic organelles other than to the centriole have not been established, although in a few cases such a connection to the mitochondria may be insinuated.

The cylindrical centrioles are typically paired, the members of the pair sometimes lying at right angles to one another or end to end. Figures 18a and 18b shows smaller electron dense paracentriolar bodies or centriolar satellites attached to the centriole. These are observed to be the site of attachment of cytoplasmic microtubules which radiate out in all directions from these structures. An additional expression of the centriole-microtubule relationship is shown in a sequence of micrographs depicting various stages in the development of rudimentary cilia (Figure 19). The pictures show that a ciliary vesicle is formed into which the microtubular elements of the cilium extend. The growth of the cilium apparently progresses with elongation of its microtubular elements and corresponding expansion and elongation of the ciliary vesicle. The major aspects of this phenomenon are similar to those demonstrated by Sorokin (1962) in cultured fibroblasts. The microtubules of the ciliary shaft, in contrast to the microtubules associated with the centriolar satellites, originate from the ends of the principal centriolar cylinder (Figure 19).

In addition to the cytoplasmic microtubules finer filaments similar to those associated with the desmosomes and within the cores of microvilli are observed in the cytoplasm. These filaments are frequently observed to be oriented at right angles to the

plasma membrane to which they may be attached (Figure 20). Oblique sections in which dense bundles of these filaments are observed on only one side of the cells are frequently encountered. This arrangement suggests that these fibers may lie primarily in the cytoplasm of the flattened basal part of the cell. Sections taken perpendicular to the substrate plane do in fact often show bands of fine fibers running parallel to the substrate just above the plasma membrane (Figure 21).

The cytoplasmic ground substance is a rather uniformly staining substance with intermediate electron opacity that may appear to be finely granular or interspaced with fine fibrous material.

Nucleus. The nucleus which is frequently invaginated is bounded by a double membrane which is penetrated by numerous pores (Figures 3, 22 and 23). The dimensions of the pores (500 Å) are consistent with those observed in other cell types. Sections which graze the nuclear membrane show these to advantage as dark rings with less dense centers and permit observation of the arrangement and spacing of these pores. Transverse sections of the nuclear membrane show the pores less distinctly but they can be readily detected by the presence of a material of greater opacity which lies just outside the opening in the cytoplasm (Figure 3).

The chromatin is dispersed through the nucleus in the form of granules and filaments and usually shows some condensation on the inner side of the nuclear membrane. However, on the nucleoplasm side of the membrane there are chromatin free regions directly beneath the nuclear pores. The nucleoli are very evident as large masses of densely staining granules which sometimes appear to be arranged in a continuous chord like structure (Figures 3, 6, 8, 12 and 29). The nucleolar particles have dimension similar to those observed in free cytoplasmic ribosomes.

Treated cultures

Partial separation

Figures 24, 25 and 28 show groups of epithelial cells which were briefly exposed to 0.002 M EDTA prior to fixation. Free surfaces of the cells at the periphery of the culture exhibit numerous microvilli some of which show fibrous cores that may extend into the underlying cytoplasm. The intercellular spaces are expanded and characterized by similar microvilli and larger contorted extensions. The latter are probably a consequence of release of contact between cell surface interdigitations (Figure 26). At this stage of separation the cells are clearly relinquishing contacts. Nevertheless,

the electron opaque areas of attachment at the periphery of the cultures, the sites of intercellular cementing material and the desmosomes persist (Figures 24, 25, 27, 28, 30 and 31). Cytoplasmic and nuclear components show no displacement or structural transfigurations during this phase of detachment. Partially separated cells which have been subjected to more extended calcium chelation but preceding inception of the violent blebbing response resemble in most respects those cells subjected briefly to EDTA. On further separation of the cells, a relative decrease in the number of microvilli and cell surface projections accompanied with general withdrawal of major cell processes is observed. Although the cultures do not show complete cell separation, the only adhesive modifications of the cell surface that persist are the depositions of intercellular cement. Cellular extensions which may correspond to the retraction fibers observed under phase-contrast are noted in some sections and cytoplasmic vacuolization is more extensive.

Active surface blebbing

Figures 32, 33 and 34 show cells from cultures that have been fixed during active surface blebbing. The cells are more fully separated, possess no microvilli and are somewhat rounded or lobulate in form. Cytoplasmic vacuoles are evident while other

cytoplasmic inclusions appear unaffected by treatment. No cell surface adhesive specializations are evident although certain areas show restricted sites of unmodified intercellular contact surfaces. The impression is that the cells are for the most part separated, more completely contracted and rounded-up than cells exposed to shorter treatment but they still retain some degree of flattening to the substrate. The blebbing response characteristic of this phase of treatment is especially evident in Figures 33 and 34. The protoplasmic blebs are typical of those observed in many electron micrographs but their ultrastructure differs in some respects from those observed by Robbins and Gonatas (1964) in anaphase HeLa cells. The homogeneous appearing blebs are circumscribed by a plasma membrane which is indistinguishable from those of untreated cells. The blebs are distinguished by the fact that the only cytoplasmic inclusions incorporated into them are the ribosomes and polysomes. At the base of the blebs there is clearly a zone of delicate fibrous material which may be an expression of the cortical gel region proposed by Robbins and Micali (1965). A suggestion that such a zone may encompass the entire cell is provided in Figure 36. The blebs are present in diverse sizes and shapes and this variance may be due to the fact that they are continuously extended

and retracted from the cell surface. The larger blebs are bulbous while the smaller ones frequently exhibit a more complicated palmate configuration (Figures 33 and 34).

Maximum separation

Electron micrographs of maximally separated cultures show that the microvilli and other surface modifications characteristic to untreated or partially separated cells are absent (Figures 35, 36 and 37). In nearly all cases cell attachment mechanisms are lacking. In the cases where cells fail to separate completely it is consistently observed that the intercellular cement is the mechanism that persists at points of contact (Figure 38). Desmosomes, and other adhesive specializations are absent entirely. Some of the cells at this stage exhibit blebs but their configuration is much more regular than those fixed during the period of active blebbing (Figures 35, 36, 37 and 38). At the base of the blebs a fibrous layer is evident through which the ground substance, ribosomes and polysomes have extruded forcing the membrane outward. Beneath the plasma membrane in the non-blebbing areas a zone devoid of large cytoplasmic organelles is set apart from the rest of the cytoplasm. The mitochondria, lipid granules and other inclusions of the cytoplasm are not observed in this zone.

Cultures which have been subjected to calcium depletion with EDTA until they were observed optically to be maximally separated, generally exhibit some degree of cytoplasmic vacuolization (Figures 35, 36 and 38). This is considered to be an effect of permeability changes due to calcium extraction since untreated cultures subjected to parallel fixation procedures fail to show such extensive vacuole formation. The appearance of the mitochondria at this state is usually not altered from that observed in untreated cultures (Figures 36 and 37); however, in some isolated cells the mitochondria do show intracristal swelling. This swelling is believed to be a result of cellular degeneration rather than an effect of EDTA treatment. Support for this interpretation is provided by the fact that the large majority of cells at maximum separation do not show this artifact. In contrast to the findings of Lynn, Fortney and Brown (1964) as well as those of Harris and Leone (1966), no ballooning of the mitrochondrial membranes or matrix condensation is observed even in those degenerate cells showing intracristal swelling. The configuration of the Golgi system in fully separated cells corresponds to that observed in control cultures (Figure 35). The endoplasmic reticulum also appears unaltered by treatment and is apparent as sparsely scattered rough surfaced tubular profiles (Figure 35). Free ribosomes and polysomes are distributed through the cytoplasm as in untreated cells; however, there seems to be a considable

reduction in the frequency of the helical polysomes as well as in the spiral and chain-like configurations. The few that are observed do not appear to be structurally altered by the chelation process. Cytoplasmic microtubules are considerably reduced in number and those that are observed in fully chelated cells are consistently associated with the centriole or its ciliary derivative (Figures 36, 37 and 19f). Evidence here is inconclusive but it appears that the microtubules associated with the centriolar structures have qualities that in some way render them more resistent to calcium depletion than those not having this association. No detectable change can be observed in the fine structure of the centriole or its ciliary derivatives (Figures 36, 37 and 19f). The nuclei may appear irregular in shape and the cytoplasmic organelles often appear closely packed (Figures 35, 36 and 38). The impression is given that much of the ground substance between the particulate components has been forced out into the blebs by an active contraction of the peripheral zone, or by crowding due to vacuole formation. No change has been demonstrated in the structure of the plasma membrane of either the fully rounded cell or in the membrane encompassing the cytoplasmic blebs.

Partial recovery

Epithelial cells which have been incubated for 90 minutes in Hank's BSS subsequent to full separation in EDTA exhibit characteristics intermediate between those observed in completely separated and in fully recovered cultures. Figures 39, 40, 41, 42 and 43 illustrate portions of such partially reconstructed epithelial sheets. The microvilli which were completely withdrawn at full separation are once more characteristic of the unobstructed surfaces as well as existing on surfaces approaching each other (Figure 43). Within 90 minutes after removal of the EDTA the cells reestablish sufficient union to allow formation of intercellular adhesive modifications (Figure 43). Contact sites are observed which are interpreted as desmosomes previous to complete differentiation of the fibrillar elements. In some cases indications of fibrillar structure may be detected. In addition, the surface extensions appear to be progressively interdigitating with those from adjacent cells. Typical microvilli, however, are rarely observed on surfaces which have established contacts.

At this stage of recovery almost all contacts occur at the tips of blunt surface projections (Figures 39, 40 and 41). These surface irregularities may represent microvilli which after establishing

contact, shorten and become distended due to centrifugal movement of the cytoplasm. Indirect evidence for such a proposal is provided by the observation that retention of the microvilli is characteristic only in the broader intercellular spaces formed at the junction point of three of four cells (Figure 39). Retention of microvilli is obvious where no mutual approach has been made, i.e. at the free surfaces of the cells. On the other hand progressive shortening of microvilli may be observed on the reverse side of a cells where contacts are forming with a neighboring cell surface (Figure 40). The microvilli thus appear to make contacts with opposing cell surfaces after which a zipper action may result in extension of contact surfaces. In other areas a second factor appears active in effecting increased mutual contact. Once cell contacts have been made, subsequent movements of the cell processes seem to be directed in such a manner as to promote maximum surface apposition. This behavior of reaggregating cells which results in increased contact surface has been termed "contact promotion" by Curtis (1961). Figures 44, 46 and 47 depict cells of reaggregating cultures in which their shape and relationships give the impression of active contact promotion. Intercellular adhesive specializations are apparent only in the extreme tips of several of these cells, while surface undulations have decreased considerably in the intervening intercellular spaces.

Complete recovery

Cells that have been subjected to maximum separation in EDTA and subsequently allowed to reaggregate in Hank's BSS for three hours are shown in Figures 48, 49 and 50. It is clear that the chelation and recovery sequence has not altered their final appearance from that observed in control cultures nor has the structure of any of the cytoplasmic inclusions been irreversibly affected by treatment. Electron micrographs of the contact surfaces between cells which have been reaggregated into a coherent epithelial sheet show an ultrastructure similar to that described for normal cells. Typical 100-200 Å unmodified parallel contacts are reestablished through the cultures and the recovery of the localized desmosomes is especially striking (Figure 50). In the latter the intercellular space and the intracellular tonofibril components are as identical to those observed in control cultures. Interdigitating surfaces have reestablished elaborate contacts similar to those found in untreated tissues (Figure 49), while the intercellular adhesive cement is frequently observed between the cells and is indistinguishable from that observed in control cultures (Figure 48). The electron opaque contacts (zonula diffusa) described in control cultures are again apparent in the cultures at similar peripheral sites (Figures 44 and

45). Microvilli have reformed on the lateral free surfaces of the cultures and micropinocytotic activity is evident. The cytoplasmic microtubules, which at complete separation were limited to those associated with the centrioles, are again dispersed through the cytoplasm (Figure 42). The helical polysomes and similar chain-like structures which appear to be less abundant in fully separated cells are observed with a frequency comparable to that in control cultures (Figure 50). The structure of the mitochondria as well as other cytoplasmic organelles does not differ in any way from those of untreated tissues (Figures 46, 47 and 49). Cells which were incubated in the complete growth medium for 24 hours subsequent to EDTA dispersion show no structural alteration. They are similar in all respects to untreated cells and are healthy in appearance (Figure 51).

The final impression is that colonies of epithelial cells subjected to complete calcium depletion by EDTA, as judged by their maximum separation, reaggregate into coherent sheets on recovery and are in no way permanently affected by the treatment.

DISCUSSION AND CONCLUSIONS

Time-lapse-cinemicrography

Chick renal epithelia grown on plastic coverslips present an appearance similar to those grown on glass or on plasma clot as shown by a comparison of phase-contrast photomicrographs and time-lapse cinemicrographs. The cells are able to flatten and adhere to plastic with a tenacity equal to that observed using the more conventional culture methods. The work reported by Rappaport, Poole and Rappaport (1960) and that of L. Weiss (1960, 1965) points out that the surface charge on the substrate as well as the constituents of the medium is important in determining the ability of the cells to approach and adhere to the substrate. Although no attempt has been made to determine the surface charge on the plastic coverslip, it is apparent from the successful adhesion that the required conditions are fulfilled in the culture system employed. The ruffling of the leading edge of the sheet and its accompanied pinocytosis is similar to that described by other authors (Abercrombie, 1958, 1961; Ambrose, 1961; Rose, 1964; Abercrombie and Ambrose, 1962). Much of the movement of the cytoplasmic particles does not appear to be the result of Brownian bombardment since many of the granules, e.g. mitochondria, appear to travel back and forth along

the same path in a saltatory fashion and in non-random patterns. The apparent non-random activity may well be a phenomenon requiring the expenditure of metabolic energy.

The large relatively clear juxtanuclear regions observed in several cells may represent Golgi zones similar to those described by Rose (1964). On the other hand they may represent centrioles associated with microtubular structures which may act to exclude the other cytoplasmic organelles. This possibility will be discussed more fully with reference to electron microscopic observations.

During the course of several time-lapse recordings and numerous direct phase-contrast observations no mitoses were observed suggesting that the migration of the cells from the kidney tissue and subsequent centrifugal migration of the coherent sheet is not due to increasing cell numbers. The mitotic index of cultured chick renal epithelium is known to be extremely low but the complete absence of division figures may have been due to an inhibitory effect of the streptomycin included in the culture medium. The cells do, however, move centrifugally in the absence of observed mitotic activity with concommitant expansion of the confluent epithelial sheet. Therefore, the outgrowth may instead be the result of active migration of the cells upon release of contact inhibition as proposed by Abercrombie and Ambrose (1962). The subsequent expansion of

the sheet could then occur as the individual cells increase their adhesiveness to the substrate and simultaneously decrease cell to cell contact resulting in flattening in the manner described by Gustafson and Wolpert (1963).

The response to treatment was extended over a longer duration and therefore differed in some respects from those recorded by Owczarzak and Dornfeld (1962) in which the cells separated abruptly at about six minutes treatment. The slowed down response fits well with the proposal of DeHaan (1958) that there is a direct correlation between the amount of calcium in the surrounding medium and the extent of cellular contact. Thus a rapid extraction of calcium would be expected to disrupt intercellular adhesiveness quickly while a less vigorous chelation of the calcium would result in a slower release of contacts. Letham (1960) has demonstrated that the effectiveness of EDTA as a chelating agent is influenced by pH, temperature and concentration. Its ability to complex with and to bind calcium is maximum at about pH 7.8 and decreases with a lowering of pH. Therefore, time relationships obtained using 0.002 M EDTA at pH 7.2 differed from those observed using higher concentrations and pH.

The observation that the portion of the cells farthest removed from the substrate relinquished mutual contact long before the basal

region did, suggests a difference in adhesiveness of the two regions. That adhesion to the substrate is greater than that between the mutually opposed lateral cells surfaces is evident from the degree of flattening to the coverslip. The model presented by Gustafson and Wolpert (1963) emphasizes that in order for cells in contact with one another to assume a flattened sheet the adhesion to the substrate must be in excess to that between their mutual surfaces. The early release of adhesion in the upper regions of the lateral contact surfaces results in a change in contour of the cells as predicted by the model proposed by Gustafson and Wolpert (op. cit.). They point out that cells are normally spherical unless altered in shape by external forces and that adhesion to the substrate and other cells can be the force responsible for the transition from the spherical state to the flattened conformation. In attaining this flat expanded condition, tension develops within the cell surfaces. Upon release of the lateral contacts due to calcium chelation, the tension generated within the cell surfaces by the external adhesive forces is released and the upper portions forcibly contract while the more adhesive substrate attachment persists. This indicates that the lateral adhesive mechanisms are weaker and/or may be more calcium dependent than attachment to the substrate and thereby more readily disrupted by calcium withdrawal. Differences of adhesive

mechanisms between lateral and basal attachments cannot be detected with phase contrast microscopy; however, the discussion of electron microscopy will show a number of adhesive modifications between opposed cells which are sensitive to calcium extraction.

On further chelation the adhering basal regions slowly begin to relinquish contact at which time membrane ruffling indistinguishable from that observed on the free edges of control cultures is initiated. It is generally agreed that the ruffled membrane is the primary locomotive organ in both fibroblasts and in epithelial sheets. All reports concerning its function emphasize that it is a very adhesive structure. Thus the ruffled membrane is considered to be responsible for the initial adhesion between fibroblasts and between epithelial cells in addition to being that portion of a cell having the greatest adhesiveness to the substrate, (Ambrose, 1961; Abercrombie, 1961; Abercrombie and Ambrose, 1962; Curtis, 1962).

It is difficult to explain the appearance of these veiling membranes at this time in the light of the foregoing reports. In this situation veiling activity is initiated simultaneously with release of contact due to reduced cell to substrate adhesiveness and while the cells are contracting. This apparent discrepancy might be explained if the appearance of the veiling at the newly freed surfaces

was considered as a manifestation of the cell's attempt to maintain contact and not being successful it continues to veil as long as possible. Rand quoted by Abercrombie (1961) has stated that epithelia will not tolerate a free edge. Thus the elaboration of the veiling surface between separating cells may be an attempt to eliminate this newly exposed surface by reestablishing contacts. Abercrombie (1961) has pointed out that contact inhibition selectivity inhibits the activity of the ruffled membrane whereas, release of contact inhibition allows its development. It is then probable that the release of contact occasioned by calcium withdrawal frees the cells from the stabilizing affect of contact inhibition resulting in the observed activity. The fact that this was observed at receding cell surfaces did not at first support the proposal that the ruffled membrane is the primary organ of locomotion and generally located at the leading edge of an advancing cell. However, after closer consideration the conclusion was drawn that this could indeed be the situation. The forces responsible for the rounding-up of the cells are apparently of sufficient magnitude to overcome the attempt of the ruffled membrane to move outward. This is especially probable since calcium withdrawal would conceivably reduce the adhesion to the point that any attempt at movement would be defeated by slippage.

The surface blebbing elicited by EDTA is identical to that recorded by Owczarzak and Dornfeld (1962) in similar cultured epithelia and is interpreted as being due to alteration of the sol-gel state of the interphase cells. Heilbrunn (1952) has pointed out that a gelation differential exists between the cortical ectoplasm and the endoplasm in interphase cells and most recently Robbins and Micali (1965) have reemphasized this hypothesis. Divalent cations, notably calcium ions, are known to have the capacity to gel protoplasm, whereas, their removal can be expected to result in solation of the gel state (Heilbrunn, 1952; Borei and Bjorklund, 1953; Shapiro, 1941; Nishimura, DiPaolo and Hill, 1955; and others). Upon EDTA treatment calcium ions are extracted from the cell surface (cortex) with a simultaneous increase in permeability to the aqueous medium. If a gelation differential exists in the interphase cell as proposed by Robbins and Micali (1965) such changes in permeability could conceivably result in a dilution mediated expansion of the more fluid endoplasm which is unequaled by the expansion of the more rigid cortical ectoplasm. This unequal expansion would generate an internal hydrostatic pressure resulting in herniation of the endoplasm through regions of relative cortical weakness, the surface blebs being a visible manifestation of such pressures. On prolonged calcium extraction the rigidity of the cortex is reduced

to a condition equal to that of the endoplasm so that no gelation differential persists. At this time blebbing subsides and the cells become smoothly rounded and highly refractile. On readministering of calcium containing media the cells do not immediately resume blebbing but rather do so only after a refractory period of some duration has passed. This sequence of responses suggests that under the specific conditions of the experiments, there seems to be a critical level of available calcium at which blebbing occurs. This can also be inferred by the observation that during calcium withdrawal, blebbing is not induced until a certain degree of calcium-EDTA chelation is reached. Violent blebbing ensues at this level and continues for some time and then subsides. The refractory period observed in Hank's BSS prior to resumption of surface activity suggests that at a specific level of available calcium the gelation gradient is reestablished between the endoplasm and the cortex to the extent that it results in hydrostatically induced blebbing. This level is reached both during calcium withidrawal and readminstration following destruction of the gradient with EDTA. Renewed activity continues until the calcium reaches a level required to reestablish the permeability characteristics of control cells as well as stabilize the regelling cortical ectoplasm. At this time unequal expansion of the

interior and the cortex can no longer occur and blebbing ceases. This interpretation conforms in principle with that offered by Dornfeld and Owczarzak (1958) as well as that presented by Robbins and Micali (1965).

Other environmental alterations known to affect the sol-gel system of protoplasm have been shown to illicit a similar surface response in interphase cells. Thus Rosenberg (1963) observed blebbing in mechanically deformed cells while Landau (1960, 1961), Landau and McAlear, (1961), and Goldring and Landau, (1961) showed that high hydrostatic pressure induced similar responses in a variety of cell types. Robbins and Micali (1965) demonstrated a blebbing reponse induced by low temperature. Miszurski (1949) interpreted the blebbing effect of colchicine treatment on fibroblasts as being due to reversible changes in the sol-gel equilibrium of the cortical layer of interphase cells. Cooper, Goldring and Klein (1962) gave a similar interpretation to the surface blebbing observed in L-strain cells subjected to a gas interface. Belkin and Hardy (1961) demonstrated that application of heavy metal ions could induce similar surface changes in a variety of cell types and Lewis as early as 1923 detected blebbing in tissue cultures exposed to alkaline solutions. Most of these latter experiments show that changes in the sol-gel state of interphase cells can indeed be

responsible for the blebbing action observed.

The blebs elaborated at the surface of interphase epithelial cells during calcium abstraction are morphologically indistinguishable from those widely observed on mitotic cells during anaphase. (Strangeways, 1922; Lewis, 1942; Chambers, 1938; Hughes and Swann, 1948; Hughes, 1952; Boss, 1955; Rose, 1964; Robbins and Gonatas, 1964; Lettre and Schleick, 1954; Lettre, Albrecht and Lettre, 1951). The close morphological similarity between the activity displayed by dividing cells and that induced in interphase cells by calcium extraction has prompted Dornfeld and Owczarzak (1958) to propose that a mechanism similar to that provided by EDTA chelation may be operative during normal cell division i.e. loss of surface calcium into the cell interior allows the cells to relax and round up and become more permeable to the aqueous medium. On release and return of this interior calcium to the surface a cortical-endoplasmic differential similar to that described above can be established. Blebbing is initiated and continues as long as permeability conditions allow. Robbins and Micali (1965) consider that such a mechanism is likely but propose that the "sink" for the calcium is the condensation of the chromatin rather than the developing spindle as proposed by Dornfeld and Owczarzak (1958). In either case, however, the shift of the calcium from the exterior to the interior of the cells and the reverse can initiate sol-gel changes

capable of inducing bleb formation.

Thomason and Schofield (1961) were unable to consistently observe surface blebbing in Ehrlich ascites tumor cells during EDTA treatment. They concluded that such surface activity when observed is an "unspecific reaction to ill treatment and not related to the calcium concentration of the cells or their environment" (page 492). The inability of these authors to induce such a response in these cells may be due to a number of factors. (1) Tumor cells are known to have a low calcium content and/or a low binding capacity for this ion (deLong, Coman and Zeidman, 1950; Zeidman, 1947; Coman, 1953, 1960; Abercrombie and Ambrose, 1962). (2) Ascites tumor cells are generally spherical and relatively nonadhesive, again indicating a possible low calcium content. (3) Thomason and Schofield preceded the EDTA treatment with a calcium free Ringer's solution which can illicit surface responses similar to EDTA. In previous studies these authors observed just such a phenomenon.

If all of these factors are considered in the light of the foregoing proposal concerning the mechanism whereby surface blebs may be produced, one would not expect extensive surface blebbing to occur. The evidence that ascites tumor cells have a low calcium concentration and are free nonadhesive spherical cells further

suggests that a gelation differential between the endoplasm and the cortex may not exist. This suggestion plus the fact that the cells were washed in a calcium free solution prior to the removal of calcium with EDTA indicates that any gelation differential present prior to EDTA treatment was equalized during the calcium free wash. Treating cells of known low calcium content which have been further depleted of calcium by a calcium free wash, with EDTA would be equivalent to the treatment of epithelial cells until they were fully rounded and then treating again with EDTA. No blebbing would be expected since the proposed gelation differential would have previously been destroyed if it had been present at all. It would also be equivalent to EDTA treatment of rounded metaphase cells in which the required differential between the endoplasm and cortical ectoplasm is lacking (Robbins and Micali, 1965). Thus it seems that the lack of surface responses reported by Thomason and Schofield is exactly the situation to be expected under the conditions of their investigations and indeed may lend indirect support to the proposed mechanism for surface bleb formation.

During the very violent blebbing, the static rounded phase and the blebbing observed during recovery, no cells were observed to relinquish contact with the substrate. This observation

does not lend support to the many investigations that suggest that EDTA can by itself separate cells from the culture substrate or from connective tissues in vivo. The violent surface activity, the perfusion methods used, and the fact that the cells were rounded up as well as being susceptible to being pulled off by gravity, suggest that if EDTA did indeed cause cell to substrate separation, it would have been immediately apparent. Since this did not occur the conclusion is drawn that EDTA does not affect the cell to substrate attachment to the extent that cells are released from the surface. This would imply that substrate attachment may not be calcium dependent or may require more extensive chelation to give such a reaction. Generally methods using EDTA to produce cell suspensions involve some sort of mechanical disruption in conjunction with the chelating agent. This no doubt can supplement the EDTA action so that the cells are released from the substrate as well as from one another. However, little consideration is given to the mechanical effects when such studies are reported. These results concur with those of Coman (1961) and Berwich and Coman (1962) in which it was pointed out that cell to substrate attachment and mutual cell adhesion may be two independent phenomena.

Following the refractory period, in which the cells are

apparently unresponsive to added calcium, the fully rounded cells begin to extrude cytoplasmic blebs. These protrusions are not fully retracted and their distal ends continue to expand forming pseudopodial extensions. Eventually the blebbing activity subsides in favor of a random probing action by the expanding cytoplasmic sheet. The peripheral expansion allows the cells to flatten and spread on the substrate until random (Lucey and Curtis, 1959) intercellular contacts result. These initial contacts gradually become more extensive in a zipper-like manner similar to that described by Speigel (1954) until the once isolated cells have become consolidated into a single coherent epithelial sheet. Such extensions of contact surfaces has been termed contact promotion by Curtis (1960, 1962).

The calcium contained in the recovery medium may participate in several of the phenomena observed during the recovery of the fully rounded cells. Danielli and Davson (1935) demonstrated that a decrease in permeability accompanies an increase in calcium content in protein-lipid layers. Alexander, Teorell and Aborg (1939) proposed that calcium ions caused increased molecular packing within the surface membrane resulting in the lower permeability. Similar observations concerning the affects of calcium on cell permeability have been made by innumerable investigators.

Incorporaion of calcium into the surface of the expanding cells would thus decrease the influx of the aqueous medium into the interior. Concurrent incorporation of calcium ions into the cortex would facilitate its gelation and enhance its ability to maintain the spread condition of the cells. This cortical gelation would also reestablish a stable solation-gelation differential. This differential, however, can not be expected to cause surface blebbing since the permeability decrease no longer permits adequate water influx to produce the necessary internal hydrostatic pressures. Thus the blebbing response is supressed after adequate calcium is bound at the cortex and the sol-gel characteristics of the normal interphase cell is reestablished.

The transition of the cells from the relaxed spherical condition to that in which the cells are spread and flattened to the substrate involves an obvious increase in surface area. Such an increase in surface area necessitates an equivalent increase in the plasma membrane and the subjacent cortex of the cells. Alexander, Toerell and Aborg (1939) suggest that calcium is necessary for the integrity of the cell membrane and Mazia (1940) depicts the surface of sea urchin eggs as a reservoir in which calcium ions are bound. Similarily Webb and Danielli (1940) have shown that the cell membrane may be considered as a storage area for calcium

ions. The evidence provided by these authors as well as many others suggests that calcium may play a structural role in the plasma membrane and/or the subjacent cortex. Thus calcium incorporation into the cell surface may be required to facilitate the membrane and cortex formation demand by the increasing surface area. Whether the newly formed membrane is due to a synthetic process or whether it is the result of stretching of the relaxed membrane present in the fully rounded cells is unknown. It is interesting, however, to note that Moscona and Moscona (1963) were able to inhibit aggregation of dissociated embryonic cells with agents known to inhibit protein synthesis. They interpreted this lack of reaggregation as being due to prevention of synthesis of a cell binding extracellular material but the inhibition of reaggregation could be explained equally well if the effect was considered to influence motility rather than adhesion. For the spherical dissociated cells to reaggregate they must become motile which requires changes in shape and consequently formation of additional surface membranes. In addition Moscona (1961) demonstrated that adhesion of dissociated cells to glass and their ability to reaggregate was decreased proportionately with decreased temperature and he suggested that a synthetic process was required to permit adhesion. Steinberg (1962 a), however, considered this

thermal response as being an effect on surface motility of the cells and does not agree with Moscona that inhibition of synthesis of intercellular material is the cause for nonaggregation. Thus this also may be considered as an inability of the cells to elaborate additional membrane material which would be required for the cells to flatten and adhere to the substrate. In this same light Spiegel (1954) observed that EDTA dissociated sponge cells remained spherical and isolated and did not reaggregate in static culture. Reaggregation did, however, occur when the cells were brought into mutual contact by shaking. His interpretation was that EDTA did not effect adhesion but rather prevented reaggregation by inhibiting the amoeboid movement of the rounded cells. Here the lack of motility is associated with a calcium deficiency which conceivably could prevent synthesis or organization of the pseudopodial membranes normally observed in reaggregating sponge cells.

The presence of calcium ions at the cell surface, or as an adsorbed layer on the cover slip, or incorporated in the medium itself may facilitate the adhesion of cells to the substrate. Gustafson and Wolpert (1963) have pointed out that cells are normally round unless deformed by an external force and that adhesion to the substrate or to adjacent cells is the most important spreading factor. The spreading and attachment of the cells to the substrate may be

mediated by calcium ions acting as divalent links between the cell and the substrate as proposed by Rappaport, Poole, and Rappaport (1960). Calcium may act to reduce the depth of cationic clouds surrounding the cells and the substrate thus permitting the cells to come into intimate contact with the coverslip, (L. Weiss, 1960) or it may aid in coacervate formation during elaboration of an extra cellular adhesive material (Rinaldini, 1958). The divalent cations in this sense may be functioning only to allow cell flattening during which the initial attachment may occur solely through physico-chemical phenomena between the cells and the substrate (Curtis, 1962). Calcium ions thus may play a role in the formation of the primary adhesions to the substrate but in the light of the observations made during EDTA treatment it is proposed that such a calcium mediated mechanism, whatever its nature, is subsequently replaced or supplemented by one that is less calcium dependent.

The cells expand and become flatter in a manner similar to that depicted by Taylor (1961) and due to their increasing adhesiveness to the substrate they eventually make contact with one another at which time calcium may participate in formation of lateral intercellular adhesions. The mechanism of calcium mediated adhesion at this time may be any or all of those proposed earlier as well as being involved in the formation of more permanent adhesive

modifications of the cell surfaces. Thus the role of calcium ions is reaggregation of dissociated cells is complex and may vary from site to site between cells and between cells and their substrate and may differ in importance depending upon the state of reaggregation being studied.

Electron microscopy

Epithelial adhesive mechanisms

Lateral surfaces of adjacent cells are characterized by a variety of modifications many of which have been assigned adhesive functions. Among the contact mechanisms observed in the cultures of chick renal epithelia are parallel unmodified contact surfaces, plaques of intercellular cement, desmosomes, complex interdigitations, simple mortice and tenon junctions and a contact modification which has been termed zonula diffusa. This zonula appears to be more common to cultured epithelial sheets than of intact tissues.

The unmodified surfaces normally run parallel to one another with regular spacing of about 100-200 Å. Such uniform intercellular contact interspaces are very commonly observed between cells within the monolayer cultures. Curtis (1962) has pointed out that this is probably the most common expression of

intercellular contact relationships. He suggests that the common occurrence of the 100-200 Å gap between parallel surfaces is the result of the interaction of repulsive and attractive forces acting on the opposed surfaces. This space would then represent the distance at which no net attraction or repulsion is acting on either surface.

The frequent observations of micropinocytotic vesicles invaginating into the cytoplasm from these spaces suggests that the material present within this 100-200 Å space is most likely a fluid of relatively low viscosity rather than a definite viscous cement and probably indicates open communication between intercellular spaces in a sinusoidal arrangement. Needless to say, at full separation of the cells this close juxtaposition does not exist; however, following full recovery in Hank's BSS the close proximity is reestablished. The effects of EDTA treatment and the mechanisms whereby the standard mutual contacts are reestablished will be more fully discussed.

The concept of an intercellular cement or "glue" material existing between cells and being responsible for maintaining tissue integrity has attracted the support of a large number of workers (Gray, 1926; L. Weiss, 1960; Rinaldini, 1959; Easty and Mutolo, 1960; Letham, 1960; Moscona, 1961, 1963 a,b and c). Such cements have long been considered as a mechanism of intercellular adhesion in both adult and embryonic tissues.

In many adult tissues the adjacent cells may be widely separated by an intercellular matrix. The connective tissues are well known for the conspicious intercellular material between their constituent cells. In contrast little conclusive evidence exists confirming that a substance which serves an adhesive function does exist between the cells of epithelial tissues. Edds (1958), however, holds the view that a homogeneous cementing material is found between the cells in a wide variety of tissues, adult and embryonic. Most of the scanty evidence suggesting the presence of an intercellular cement between the closely apposed membranes of epithelial cells has come from electron microscopy studies (Robertson, 1960). As already mentioned, a 100-200 Å intercellular space is commonly observed between many cell types. It has been reported to contain a substance having a density at least equal to that of the general cytoplasmic background of the cells. Although this weakly staining substance does represent an intercellular material, little can be concluded concerning its role in intercellular adhesion. Although intercellular cementing has been attributed to this material, it has been difficult to demonstrate this postulate either with the light or the electron microscopes. Mercer and Shaffer (1960) have attributed this lack of evidence to the inability of standard electron stains to adequately reveal the nature of the intercellular material.

Curtis (1962) has pointed out that the presence of a viscous material between cells or on the surface of approaching cells would tend to hinder the formation of mutual contacts by preventing the close approach of the cells. On the other hand he also suggests that such a viscous substance, if secreted into the intercellular spaces after initial adhesion has been established, would because of its nature tend to prevent subsequent separation of the cells. When cells do approach to within 100-200 Å of one another any fluid present between them would tend to prevent separation regardless of its viscosity while the strength of the adhesion would increase with the viscosity of such a material.

The fact that intercellular cements are common in adult tissues but rarely seen in embryonic tissues suggested to Abercrombie (1962) that the adhesion of embryonic cells may be the result of direct calcium linkage which is replaced by a definite protein cement in adult tissues. Easty and Mutolo (1960) also suggested that the protein cement is more important than calcium in maintaining adhesion in adult tissues.

Electron microscopy of cultured chick renal epithelial cells has revealed that a densely staining material is deposited in the intercellular spaces. It is observed in discontinuous plaques between the cells and does not appear to appreciably increase the

dimensions of the intercellular gap but rather seems to be deposited within the limits of the standard 100-200 Å space. It stains very deeply with the uranyl acetate-lead citrate method employed and the evidence presented here suggests that it definitely does play a role in cell adhesion. During the treatment sequence, those sites, in which this material is detected maintain contact longer than any of the other adhesive modifications observed. Electron micrographs frequently show cells which have not completely relinquished contacts among cells judged by phase-contrast microscopy to be fully separated. Most frequently it is observed that these persistent sites of attachment are confined to regions of apposed cells between which the dense material appears. This suggests that the material definitely does have an adhesive function. Apparently the contact of the cells at these sites is not disrupted by calcium chelation as extensively as that of other adhesive specializations. Thus this material may represent an intercellular cement which is not affected or at least is disrupted less readily by the chelation process. It may be that the cement contains a large amount of calcium thereby requiring more extensive chelation in order to disrupt it or it may consist of a material which is not calcium dependent and is therefore unaffected by calcium extraction. An additional interpretation might be that the intercellular cement is calcium dependent

during its formation but may bind calcium too tightly to allow EDTA extraction under the conditions of treatment.

The observation of a similar intercellular cement in completely reconstructed epithelial sheets does not necessarily indicate that this material is reformed after the cells are reassociated since no stage of separation or recovery was observed in which it was not present.

Since intercellular cements are more characteristic of adult tissues than embryonic tissues one might expect that older cultures would contain a more extensive distribution of this dense material. Younger cultures than those studied might be expected to lack these sites entirely.

Cells attached to one another by a mechanism that seems as tenacious as an intercellular cement would not be expected to move in relation to one another. However, as will be discussed later with reference to the desmosomes these cells are known to exhibit active movement within the cultures. Whether the cells held together by an intercellular cement are capable of movement in relation to one another or in group movement in relation to the other cells in the culture, or whether this attachment mechanism is labile and capable of being disrupted and reformed is not known. The fact that the sites of intercellular attachment seem to be

somewhat calcium independent suggest that if the latter possibility is the case a different mechanism from that proposed for the transient nature of the desmosome must be postulated.

The desmosome is a more tangible cell surface specialization functioning in maintaining contact between cells of epithelial origin. This structure has been demonstrated in a wide variety of epithelial tissues and the overwhelming opinion is that it is a site of increased adhesiveness. Its presence as a localized fibrous structure between adjacent cells prompted the classical interpretation of cytoplasmic bridges between cells. Porter (1954) first described the fine structure of the desmosome and reported that it consisted of local thickenings of the apposed cell membranes from which fibrous material radiated into the cytoplasm of the participating cells. In addition he demonstrated that no continuity existed between the adjacent cells at the desmosomes and therefore, no true intercellular bridge existed at these sites. Fawcett (1958) has emphasized the ubiquitous distribution of these structures at contact surfaces of columnar and cuboidal epithelia and has described the ultrastructure of the desmosome in terms similar to those used by Porter (1954). Overton (1962) has described the sequence of events which occur during the development of desmosomes of the chick blastoderm and most recently Kelly (1966) has published a

thorough analysis of the structure of this cellular component in developing newt epidermis. He has presented an elaborate model depicting the tonofibril-plaque-plasma membrane relationships and agrees with the concept that the desmosomes are highly organized local attachment devices which supplement the more generalized adhesive mechanisms. The increased adhesiveness of cells at these structures (nodes of Bizzozzero) has been demonstrated by P. Weiss (1958) who observed them to maintain persistent attachment while adjacent cell surfaces separated when subjected to a variety of treatments.

Epithelial tissues grown in culture assume a flat monolayer conformation and the cells within confluent sheets continuously shift about and actively change position in relation to one another (P. Weiss, 1958; Curtis, 1962; Owczarzak, unpublished). It has been pointed out by Weiss (op. cit.) that this continuous movement must rule out the possibility of stable mutual attachments between epithelial cells within such an aggregation. It would therefore, be surprising to observe desmosomes in such a dynamic system unless they are either very labile or are not in reality involved in maintaining cell to cell adhesion.

The presence of desmosomes between cultured epithelial cells has not previously been reported. The observation of

desmosomes in electron micrographs of control cultures was consequently somewhat unexpected. Desmosomes in normal untreated cultures are similar to those observed in many tissue types, adult and/or embryonic. They are characterized by dense plaques subjacent to the apposed plasma membranes from which numerous tonofibrils radiate into the cytoplasm of the participating cells. The space present between the apposed surfaces is approximately 100-200 Å.

During the treatment sequence with EDTA the desmosomes were observed to be somewhat more tenacious in their adhesion than adjacent unmodified surfaces. Separation is frequently observed adjacent to these attachments while the desmosomes themselves maintain contact. At full separation no intact desmosomes, intracellular plaques or tonofibrils are observed. Apparently the treatment by calcium chelation disrupts not only the intercellular attachment but in some way destroys the structure of the desmosomes. In contrast to these results Overton (1962) and Overton and Shoup (1964) have shown that trypsin dissociation of chick blastoderm and chick duodenal mucosa epithelial cells results in separation of the desmosomal plaques but preserves the intracellular dense material and the tonofibrils. Sedar and Forte (1964) have obtained results similar in some respects to those of Overton (1962) using EDTA

dispersive methods on the junctional complex between oxyntic cells of the gastric glands of Rana. They observed disruption of the intercellular dense material characteristic of normal desmosomes, separation of the desmosomal plaques and destruction of the tonofibrils although the amorphous plaque material did not appear altered. Hays, Singer and Malamed (1965) also showed that calcium withdrawal with EDTA disrupts the lateral desmosomes between epithelial cells of the toad bladder and have noted that variation exists in the rate of separation at sites of different contact mechanisms.

The apparent difference between the results of Sedar and Forte (op. cit.) and those presented here may be due to the fact that the intracellular amorphous plaque material may have been missed during sectioning of the fully separated cells. In addition it is possible that the differences in the tissues studied may explain the variation in results. It is also possible that the situation in the adult tissue used by Sedar and Forte (1964) is more stable or permanent than in cultured embryonic epithelia and may therefore, require more extensive chelation to fully disrupt the desmosomal components. That this may be the case is suggested by the limited cell separation observed in the EDTA treated oxyntic cells.

During the recovery sequence there is a striking restoration of the desmosomes. It appears that upon reestablishment of contact

between two cells some sort of contact stimulus is provided which initiates the formation of these specialized structures. Within 90 minutes the recovery of the desmosomes has occurred; the dense material subjacent to the apposed plasma membranes is present and the tonofibrils are again evident radiating into the cytoplasm.

It is possible that during the calcium extraction the tonofibrils are destroyed by some action such as solation or depolymerization of the fibrous material. In any case they are not apparent in the fully separated cells. On readministration of the calcium containing medium these structures become apparent when contact is established between adjacent cells. This may indicate that the calcium allows regelation, repolymerization or resynthesis of the fibrous material which according to Porter (1954) has a proteinaceous nature. The mechanism, whereby these surface specializations are induced at specific contact sites is not known.

The observations that the desmosomes are present in control epithelia, are absent at maximum spearation and are reformed when the cells reassociate, suggest that they are very labile structures and can be destroyed and reformed rapidly. The work of Petry, Overbeck and Vogel (1962) indicates that this does occur in vaginal epithelium. Indeed such a very labile nature would be a requirement for cells in vivo which have a high mitotic index since

mitotic cells relinquish all mutual contacts and round up. Similar observations have been made on cells grown in culture. Thus during division the mitotic cell must have an ability to release desmosomal contacts which are reformed by the daughter cells subsequent to completion of cleavage. Forte and Nauss (1961) have suggested that the desmosome is a likely site for calcium bridge formation while others have proposed that the dense amorphous material may be a substance containing a high concentration of calcium. In the light of these possibilities the action of EDTA becomes more interesting. It is possible that processes occurring during preparation for mitosis may temporarily reduce the surface calcium in mitotic cells with concurrent disruption of the desmosomal attachments allowing the cells to round up. Following telophase the daughter cells assume configurations characteristic of the tissue type and reestablish contacts with other cells at which time desmosomes may reform. The recovery of the normal configuration and desmosome restoration would coincide with mitotic events during which calcium ions may be released to the surface permitting their utilization in adhesion. The effects of experimental removal of calcium and those observed during preparation for mitosis suggests in agreement with Dornfeld and Owczarzak (1958) and Robbins and Micali (1965) that processes involved in mitosis may involve

mechanisms similar to the chelating action of EDTA.

The mechanism whereby, these structures act in cell adhesion is not understood and Curtis (1962) points out that it is uncertain whether desmosomes do have any function at all in cell adhesion. The overwhelming opinion, however, is that they are sites of strong intercellular adhesion but apparently they must be considered as labile structures capable of being disrupted and reformed with ease. The work presented here and that of Sedar and Forte (1964), Petry, Overbeck and Vogel (1961), Overton (1962), and Hays, Singer and Malamed (1965) all support such a proposal. The possibility that the desmosomes are stable and very adherent structures can not be excluded since it is possible that they may have been detected only in cells which are not moving with reference to one another but may be moving in relation to the rest of the cells in the culture. Thus they could be moving but still possess stable adhesions between the member of the pair.

Complex interdigitations of the apposed cells surfaces are occasionally observed in normal cultures. The configuration of these interdigitations suggests that they may serve as very efficient adhesive mechanism. Such regions could also serve an equally important function in that the complex interlocking of surfaces would facilitate exchange of metabolites from cell to cell or from cell to

interspace. This latter function has been assigned to the complex infoldings of the plasma membrane into the cytoplasm at the base of kidney tubule cells as well as in other tissues. In the cultured cells it is not likely that such a function would be required since the cells are flat and extensively exposed to the culture medium. Thus it is likely that the primary function of the interdigitation between the cultured epithelial cells is that of intercellular adhesion.

During the process of separation these interdigitated surfaces relinquish attachment and frequently result in microvilli and larger projections being played out from the cell surfaces. At full separation these projections are withdrawn into the cell surface. Following the complete reassociation of the epithelial sheet similar complex interdigitating surfaces are observed. A possible mechanism whereby such regions can form will be discussed more fully concerning the role of microvilli but will not preculde the possibility that similar regions may result from active surface movements between two apposed cell surfaces. It may also be possible that areas of interdigitation may readjust in such a way as to result in straight parallel unmodified contacts as well as acquiring local specializations such as desmosomes or intercellular cement within the limits of the interdigitated areas.

Simple interdigitations of the mortice and tenon type are

observed with less regularity than those of the more complex nature. They are nevertheless similar in some respects to the latter and may differ only in the degree of complexity. Since these surface modifications are observed infrequently and are not extensive in disposition it is not likely that they are structures having as their primary function the increase of surface area for more efficient exchange of metabolites (Fawcett, 1958). Thus their primary function is most likely that of maintaining intercellular adhesion. In a few cases the space between the cells immediately around the button like process is filled with material of medium electron opacity. This might be an intercellular cement but differs in appearance from that discussed earlier. During the sequence of separation and recovery the mortice and tenon sites disappear as would be required for full separation and are reestablished in fully recovered cells.

It has been observed that the intercellular contacts at the peripheral free borders of the monolayer colony are characterized by surface specializations unique to this region of the culture. These modifications seem to be very adhesive structures and appear as two dense amorphous zones beneath the apposed plasma membranes of the adhering cells. In this sense they resemble the amorphous plaques of desmosomes, however, no intracellular fibrils have been detected in connection with these dense thickenings. Since the

dense material is amorphous and not associated with a cytoplasmic fiber system the term zonula diffusa has been assigned these structures. Structures of similar appearance have been described in other systems and have been interpreted as incipient desmosomes prior to differentiation of the tonofibril elements. That this is not the case in the present situation is suggested by the evidence offered earlier that the desmosomes are capable of being formed very rapidly. The primary cultures employed were three days old and since the desmosomes have been observed to be capable of complete reconstruction within 90 minutes after full separation, one would expect most desmosomes present in untreated cultures to exhibit their characteristic fibrillar nature. In addition the consistent and exclusive observation of these structures at the free surfaces of the epithelia does not agree with the distribution of the desmosomes which were observed between cells through out the culture. Desmosomes having characteristic tonofibrils have frequently been observed within the same sections in which the zonula diffusa was observed and examination of electronmicrographs reveals that the only factors they have in common are the dense subsurface deposits and the obvious adhesive function. Other authors propose that they are typical desmosomes in which the tonofibrils have been disrupted by fixation. Although such artifacts may be a consequence of inadequate

fixation the evidence presented suggests that this is not likely the cause of the structures observed at the periphery of these cultures.

If the zonula diffusa does represent an adhesive mechanism characteristic of adult tissues it most likely is an expression of the terminal bar which would be expected near the free borders of kidney tubule cells. Thus they may be an in vitro expression of the terminal bars observed in vivo. This structure resembles that of the terminal bar more closely than any other attachment device although the latter is also reported to display a fibrillar nature similar to that of the desmosome (Fawcett, 1958). If it is an in vitro expression of the terminal bar one would not expect and identical appearance. Terminal bars are found as a circumferential band just below the free borders of columnar and cuboidal epithelial cells. In this situation, however, the cells are growing as a flattened monolayer, the cells resembling those of a simple squamous epithelium. Changes in conformation during a transition from the in vivo columnar shape to the flattened cells in vitro might require considerable alteration in the intercellular relationships at the site of the terminal bars. The fact that the dense bands are observed at the periphery of the culture regardless of the level of the section suggest that these may be continuous bands running just below the surface of the adhering cells. If this is the case they would resemble the terminal

bars of in vivo columnar epithelia.

The effect of calcium chelation on the zonula diffusa parallels that on the desmosomes. Early in the treatment sequence the zonula diffusa is observed to maintain contact longer than the unmodified surfaces much the same as that described for the desmosomes. This frequently results in a situation in which the surfaces of the internal cells are separating but the adhesion persists at the periphery of the cultures. On further chelation the zonula diffusa relinquishes contact and is not apparent at maximum separation. Apparently the calcium extraction destroys its adhesive nature in a manner similar to the effect on the desmosomes. It may also be that the cells simply pull apart during the treatment with the result that any adhesive modifications present would be disrupted. A significant point, however, is that the electron dense amorphous material normally observed along the inner aspect of the apposed membranes has not been observed within the cytoplasm of the fully separated cells. The possibility of missing a zonula diffusa in the form of a continuous terminal bar-like band during sectioning would be very unlikely since sections taken at nearly any level should reveal such dense areas if they exist.

The zonula diffusa as described is very conspicuous in partially recovered cultures and their adhesive function is especially

evident at this time. During recovery these structures reappear and seem to be a very significant factor in reestablishing a coherent sheet of cells. They are again observed only at the cell junctions forming at surfaces exposed to the recovery medium. These structures could also be sites of intercellular calcium bridge formation similar to that proposed by Sedar and Forte (1964) for the demosomes.

In the fully recovered cells these structures are again apparent in locations similar to those in untreated cultures.

If these sites can be considered as regions in which calcium bridges exist then their transient nature in the treatment and recovery sequence might be explained in the same manner as that of the desmosomes.

The foregoing discussion concerning the variety of structures and surface specializations which play a role in maintaining the integrity of an epithelial sheet suggests that any discussion of cell adhesion exclusively in terms of one mechanism or another is unrealistic. There is no reason to expect that all the adhesive specializations discussed would be affected equally by treatment with any one single agent, nor would it be expected that in all cases would calcium ions be involved to the same degree, if at all. Adhesion must be considered as involving different mechanisms operating at

different sites within the culture as well as showing temporal differences. This is evident from observations on the time series samples obtained during EDTA induced separation, viz. certain of the modifications maintain contact while others relinquish contact more readily. P. Weiss (1958) has emphasized that contact between cells or cell adhesion must be envisioned as a series of active spots rather than a phenomenon which occurs uniformly over the entire cell surface. The results presented here establish that at least five different mechanisms may be involved in cell adhesion within simple primary epithelial cultures and emphasize that each one must receive appropriate consideration during the course of investigations into the phenomenon of cell adhesion. Under the treatment employed here the desmosomes seem to be more adhesive than the unmodified contacts whereas the intercellular cement appears to be still more persistent than the desmosomes. If a different agent, is used to dissociate the cells, it might be very likely that one of the other contact mechanisms would be disrupted first.

Many authors maintain that EDTA separates cells from the substrate as well as from one another; our results do not bear this out. During the entire sequence of EDTA treatment and recovery no cells were observed to be released from the substrate while mutual contacts were readily disrupted. The substrate attachment

persisted even at complete cell separation and was not released through the washing and fixation procedures. In addition, cultures treated by perfusion were in an inverted position in which release of substrate contacts and the subsequent disappearance of cells would have been readily recorded by time-lapse photography. One would also expect that the flow of fluid through the chamber during perfusion, supplemented by the effect of gravity, would certainly result in observable substrate contact release if it does occur on calcium chelation. On the contrary no such release of cells from the substrate was recorded even after full separation of the cells from their neighbors.

In studying the methods employed by a number of investigators who imply that cell to substrate attachment is destroyed by calcium depletion it seems significant that in order to yield a free cell suspension mechanical forces are required in addition to calcium chelation. The EDTA dispersive methods used are usually accompanied by shaking, massaging or some other mechanical disruptive force. Thus the proposal by Coman (1961) that mutual cell adhesion to the substrate are two independent phenomena appears valid in light of the observations presented here.

Surface phenomena

Free borders of epithelia rarely present a smooth surface but instead are usually characterized by surface projections or microvilli which extend outward from the cell into the adjacent space. The number of microvilli present on the free surface of cells varys from a very few as in endothelial cells to very many as revealed in electron micrographs of the striated border of absorptive cells in the intestinal epithelia. The abundance of regularly arranged microvilli in cells known to have absorptive functions has resulted in the interpretation that these processes act primarily by increasing the effective absorptive surface area. Fawcett (1958) points out that although microvilli do certainly amplify the absorbing surface, their frequent observation on epithelia of known secretory function prevents them from being indiscriminately assigned absorptive functions. He has compiled an extensive list of epithelia which show similar processes, some being absorptive, some secretory and others apparently involved in fluid transport. He emphasizes that the significance of microvilli in secretory cells is unknown while those found in sites such as bile canaliculi of hepatic cells may somehow be concerned with fluid transport or propulsion. Dempsey (1958) also lists several additional sites that are

characterized by microvilli and in agreement with Fawcett also stresses that these processes are characteristic of secretory cells as well as those having known absorptive function. Dalton, Kahler and Lloyd (1951) demonstrated the presence of a core material within the microvilli which extended into the cytoplasm of the cells. The presence of this electron dense core material as well as its extension into the apical cytoplasm was confirmed by Fawcett (1958). Following improvement in fixation methods a similar internal structure has been observed within the microvilli of a wide variety of tissues (McNabb and Sandborn, 1964; Ito, 1965; Overton and Shoup 1964; Hays, Singer and Malamed, 1965; Taylor, 1966). Most of these workers consider the axial core as being a filamentous structure but Taylor (1966) has proposed a tubular structure similar to the microtubules observed within the cytoplasm of many cell types.

A review of the literature concerning the universality of microvilli on free epithelial surfaces has been made resulting in the formulation of an opinion that whenever epithelia possess free surfaces, microvilli are formed to varying degrees as an unspecific response to the environmental situation. This seems to hold true for secretory cells, absorptive cells and cells having neither of these as their primary function. Thus there seems to be an

additional function still to be defined, for these processes which may be common to epithelia in general. Fawcett (1958) has stated tht the present interpretations concerning the function of microvilli "are speculative and will remain so until further work is done correlating changes in the configuration of the surfaces with experimental alteration in the physiological activities or environmental conditions of the cells" (page 38).

In the present study microvilli have been demonstrated on the free surfaces of untreated cultures. They may well function in absorption and are frequently associated with micropinocytotic uptake of materials from the medium. In addition the general appearance and disposition of the microvilli in untreated cells suggests that they may also serve in intercellular adhesion in a manner similar to that proposed by Dempsey (1958). They interdigitate with one another forming a zipper-like intercellular attachment which in the previous discussion of cellular contacts was considered to be one of the major adhesive mechanisms.

During the treatment sequence described in this study, striking changes occur with regard to the number and disposition of the microvilli. Implications are that they form rapidly when ever new free surfaces are exposed. Early in treatment, when the cells are just beginning to separate, microvilli are observed in

very large numbers. This elaboration of microvilli may be an expression of the reduced viscosity of the cell surface due to calcium depletion. Recently Weiss and Clement (1966) reported that reduced viscosity due to EDTA treatment results in an increased surface deformability which permits the elaboration of numerous "low radius of curvature probes" or microvilli. On further chelation the cells separate and become smoothly rounded with the loss of these surface processes. The loss may be nothing more than an expression of the rounding process with incorporation of microvilli into the cell proper due to prevention of cell surface extensions by increased cellular turgor. On the other hand the disappearance of the microvilli may be due to solation of the axial core as a result of continued calcium extraction which prevents gelation or repolymerization of the supporting filaments. A solation of the supporting axial core has been suggested as a possible mechanism by which the microspikes of Taylor and Robbins (1963) might be obliterated from the cells surface.

When the EDTA is replaced by Hank's BSS the cells gradually flatten with a centrifugal flow of cytoplasm which ceases when contact is made between cells. Sections taken just prior to, during and shortly after contacts are reestablished show that numerous microvilli complete with filamentous cores have formed on the free

cell surfaces. Evidently at this time the turgor of the cell is reduced sufficiently to allow projections from the cell surfaces while the calcium concentration has increased sufficiently to allow gelation or repolymerization of the filamentous supporting core.

From the results obtained during the reestablishment of mutual contacts it appears that the microvilli of reaggregating epithelial cells may act in readhesion of cells in two different ways. At some sites of contact the surface projections may progressively interdigitate in a zipper-like action. This mechanism of attachment no doubt results in the final complex interdigitations of the opposed cell surfaces observed in control and fully reassociated cultures. During the reassociation of cells the microvilli may also function as low radius of curvature probes by which adjacent cells may overcome existing potential energy barriers and the contacts so formed are stabilized by the formation of calcium bridges between negatively charged groups situated on adjacent cell surfaces (Coman, 1964; Bangham, Pethica and Seaman, 1958; Berwich and Coman, 1962; Steinberg, 1958, 1962; Kuckler, Marlowe and Merchant, 1960; De Haan, 1958; Pethica, 1961; Rappaport, 1960; Gey, 1954; Lesseps, 1963; Weiss, 1966; and Taylor, 1966). The length of such calcium bridges has been calculated to be 5-10 Å (Hill, 1956; Bangham and Pethica, 1960;

Pethica, 1961; Curtis, 1962) and very close apposition of cells must be attained before this mechanism can be effective in adhesion. The spatial requirements for calcium linkage is thus in opposition to the 100-200 Å gap proposed by Curtis, (1960, 1962) as the distance at which attractive and repulsive forces become equalized. Bangham and Pethica (1960) and Pethica (1961) have calculated that cell surfaces could approach within 5-10 Å if the areas of approach have radii of curvature of the order of 0.13μ or less, a value derived from the fact that the repulsive energy is directly proportional to the radii of curvature of the two approaching surfaces. Curtis (1962) stated that "cell processes 1300 Å or less are rare" (page 109) but the results of this study as well as a review of recent literature suggest that the presence of such small projections on the free surfaces of epithelia is the rule rather than the exception. The present electron microscopic studies show that approaching cell surfaces do possess projections of sufficiently small dimensions and support the proposal by Lesseps (1963) that such processes may permit calcium bridge formation by facilitating "reduction of the effective repulsive energy between the approaching cells"(page 181). Thus the surface projections (microvilli) would seem to be able to participate in intercellular adhesion either by interdigitation or by allowing intimate approach of the cell

surfaces within the limits required for calcium bridge formation.

Sections taken at about 90 minutes recovery show numerous sites at which progressive interdigitation of adjacent cell surfaces is apparent. At other areas within the same section, intercellular contacts are apparent at the tips of blunt surface projections. These sites of contact surface irregularities are interpreted as representing microvilli which have established contact, shortened and become distended due to centrifugal movement of the cytoplasm and/or the spreading effect as the cells become more extensively associated (contact promotion). Additional support for this interpretation resides in observations of free cells which have not made contacts but which still possess microvilli on all surfaces. Likewise cells having established contact on but one side retain microvilli on all surfaces. Likewise cells having established contact on but one side retain microvilli on their free surfaces. Low power electron micrographs including several cells in contact show that the only sites retaining microvilli are those areas of junction between three or four cells in which the intercellular space is still considerably expanded.

On further reassociation, the intercellular contacts become more extensive and as shown in electron micrographs the blunt projections become progressively shorter and broader until at full

reaggregation they are rarely seen and typical contact interspaces are common. In confluent sheets microvilli are apparent only on the free surfaces and in the intercellular spaces in which close contacts have not been established. Their appearance in the latter case gives the impression that they are indeed attempting to bring about contact and if successful could result in a phenomenon of membrane interdigitation similar to the membrane knotting described by Waddington, Perry and Okada (1961) between blastomeres of Limnea.

The evidence presented here strongly suggests that microvilli do indeed participate in the establishment of intercellular contacts but does not preclude their having additional functions. The timing of their appearance as cells are just beginning to separate, their absence on full separation and their subsequent reappearance during readhesion seems to support such a proposal.

Time-lapse records obtained during the most active period of surface blebbing clearly demonstrate that this activity occurs prior to maximum rounding of the cells which are essentially separated from one another and in the process of contraction. The variable appearance of the blebs in electron micrographs depends upon the extent of expansion or retraction which exists at the time of fixation. This is expected in the light of their rapid formation

and retraction during treatment. It is not possible, however, to state specifically the position of any bleb within a differentiation cycle.

Anaphase blebbing has been demonstrated extensively with light microscopy and is similar in appearance to EDTA induced activity. In addition a variety of experimentally induced conditions have been reported to elicit a similar response; however, in only a few studies were the authors concerned with the changes in the fine structure occurring during this phenomenon. Landau and McAlear (1961) have shown electron micrographs of surface blebs on primary human anmion cells which had been subjected to and subsequently released from highly hydrostatic pressure. They interpreted the blebs observed as being similar to those in anaphase and telophase mitotic cells and stress that the entire content of the blebs is composed of membranous components of the endoplasmic reticulum encompassing a few mitochondria with a minimum incorporation of the cytoplasmic matrix. In contrast the study of the ultrastructure of mitotic cells by Robbins and Gonatas (1964) has shown that anaphase blebs contain no discernible membranous structures or cytoplasmic inclusions. They show instead that the content of the anaphase blebs consists entirely of ground substance.

The blebs observed in cells treated with EDTA resemble those of mitotic cells much more closely than do those produced by variations in pressure. This is what may be expected in the light of the proposed gelation-solation mechanism for the action of EDTA on these cells. Blebs formed on withdrawal of divalent ions do not contain any cytoplasmic structures other than ribosomes and polyribosomes. The configurations of the smaller palmate blebs is identical to the blebs demonstrated by Robbins and Gonatas in anaphase cells.

No change was discernible in the structure of the plasma membrane that could be correlated with blebbing activity in EDTA treated cells; however, a more concentrated study should be done to establish this as a fact. There is agreement, however, with the observation by Robbins and Gonatas that the plasma membrane encompassing the anaphase blebs is not structurally altered from that of non-blebbing surfaces.

The frequent observation of a delicate fibrous zone at the base of the blebs is interesting. This zone could be a manifestation of the cortical region proposed earlier. It appears as if the fibers act to prevent the passage of larger cytoplasmic organelles into the bleb, whereas, those of sufficiently small dimensions may be forced through the mesh. In some cases the presence of this

zone can be detected beneath the plasma membrane around the entire cell in which case the larger inclusions are maintained at a rather uniform distance from the cell surface. It is probable that the results obtained by Landau and McAlear (1961) are the effects of excessive pressure under which such a barrier may undergo complete solation which would allow the mitochondria and the endoplasmic reticulum to be extruded into the forming bleb. Robbins and Gonatas do not show such a zone beneath the amorphous blebs; however, the lack of structure in the anaphase blebs suggests that a discriminatory mechanism of some sort must exist to prevent extrusion of the larger cytoplasmic organelles.

The striking similarity in the ultrastructure of the EDTA induced blebs and those at normal anaphase gives added support to the proposal (page 50) that EDTA action may be similar to calcium mediated events which occur during normal cell division.

Cytoplasmic components

Numerous polyribosomes having a variety of conformations are observed in the cytoplasm of control cells. Since their presence has been associated with protein synthesis it can be concluded that these cells are engaged in a similar activity. They exist as long chains, concentric spirals, rosettes, aggregates and helical

structures having very characteristic configurations. Similar structures have been reported in a wide variety of tissues, especially those lacking extensive endoplasmic reticulum.

Helical polyribosomes have recently been observed in the cytoplasm of other cells types (Echlin, 1965; Maniloff, Morowitze and Barnett, 1965; Behnke, 1963; Waddington and Perry, 1963). Pappas (1956) reported helices in the nucleus of Amoeba proteus and Roth, Obetz and Daniels (1960) demonstrated that in the amoeba the helices were present in interphase, disappeared by late prophase and reappeared in telophase. Stevens and Prescott (1965) also reported association of these structures with the nuclear pores in amoeba. These last authors, however, do not consider them as polyribosomes but rather as some sort of packaged messenger RNA. Most recently Scharff and Robbins (1966) have described helical polyribosomes in cultured HeLa cells and like Roth, Obetz and Daniels (1960) noted that the helices as well as other ribosomal configurations disaggregated at metaphase. They pointed out that the rapid decrease in protein synthesis associated with mitosis may be due to the disappearance of the polyribosomes. They also point out that the disappearance of polyribosomes could not be due to either the lack of RNA synthesis or to increased ribounclease activity. The half life of m RNA is three to four hours, whereas, the duration of

metaphase is only 15 minutes. In addition no increase in ribonuclease activity that could account for their loss was detected during metaphase. This suggests that at the onset of metaphase the existing polyribosomes are disrupted directly by a mechanism unique to this phase of the cell cycle.

In the present electron microscopic study it has been observed that the number polyribosome configurations is significantly reduced in cells fully separated by chelation. It is conceivable that the polysome disorganization may be due to the chelating action of EDTA. Magnesium is known to be involved in the binding of single ribosomes into the polyribosome structure and its removal by EDTA might be expected to disaggregate the polyribosomes into the single ribosome particles which are very numerous in the separated cells. No quantative or statistical methods were used; however, their very low observed frequency following EDTA treatment allows little doubt that their numbers are reduced by chelation.

The observation by Roth, Obetz and Daniels (1960 and by Scharff and Robbins (1966) that polyribosomes are absent during metaphase indicates that some process requiring magnesium may be occurring at that time and could conceivably result in its extraction from the polyribosomes. Such a possibility is interesting in the light of the proposal by Dornfeld and Owczarzak (1958) that "a

mechanism similar to EDTA action is operative during normal cell division" (page 247). They suggest that the developing spindle might exhibit a chelating action and have proposed that the surface phenomena observed during mitosis are due to the release of divalent cations into the cell interior. This agrees with the theory of Heilbrunn (1952) in which calcium released from the cortex during mitosis was considered to be used in the formation of the mitotic apparatus. Robbins and Micali (1966) have offered a modification of these ideas concerning the metaphase surface activity, and proposed that the condensation of the chromation is responsible for the binding of the divalent cations.

If any or all of these propositions are correct one would not expect the extraction of divalent cations from the cell surface without their concurrent extraction from the polyribosomes embedded in the neighboring cytoplasm.

The work of Sofer, George and Iverson (1966) showed that cells in which spindle formation was blocked with colchicine still exhibited a decrease in protein synthesis at metaphase. This in conjunction with the fact that colchicine blocked cells still show the reduction in polyribosomes (Scharff and Robbins, 1966) suggests that spindle formation is not responsible for dissaggregation of the polyribosomes and therefore may not be the reason for the release of

divalent cations from the surface into the cell interior during mitosis. This does not, however, rule out the proposal by Robbins and Micali (1966) that the condensation of the chromatin may have the ability to bind divalent cations from the cell surface. Thus it is possible that the disappearance of the polyribosomes at metaphase maybe related to their reduction in EDTA treated cells in much the same way as normal surface blebbing is related to that observed during EDTA treatment.

The fact that some polyribosomes are observed in fully separated cells may be accounted for by the fact that the full effect of the chelating agent may not be expressed in the time required for complete separation of the cells. The cells were either fixed for electron microscopy or perfused with Hank's BSS when they were observed to be fully separated. If they had been incubated in the EDTA solution beyond that point it is probable that no polyribosomes would remain.

Harris and Leone (1966) have emphasized that possible deleterious effects of EDTA dispersive methods on cytoplasmic organelles and have presented a series of electron micrographs of osmium fixed mouse liver cells showing extensive mitochondrial damage. These authors have observed condensation of the mitochrondrial matrix, extensive ballooning of the outer mitochrondrial membrane,

distention of the intracristal spaces and a general degeneration of these organelles all of which were attributed to the EDTA treatment. Other authors have observed similar alteration of mitochondrial structure during EDTA treatment (Lynn, Fortney and Brown, 1964; Peachey, 1964).

The results presented here are diametrically opposed to those of the above investigators and support the observations of Sedar and Forte (1964) and Leeson and Kalant (1961) in which EDTA treatment had no effect on mitochondrial ultrastructure.

The untreated mitochondria of cultured chick renal epithelia are identical to those demonstrated in similar cells by Taylor (1966) and Gonatas and Robbins (1965). They are unique in that they possess a very dense matrix material which contrasts sharply with the numerous well defined cristae. The ultrastructural features of the mitrochondria are not altered by the sequence of EDTA treatment and recovery. Those observed during cell separation, at complete cell dispersion and subsequent to full reassociation in the recovery medium are indistinguishable from those of control cultures. In isolated cultures some cells do show intracristal expansion but the inconsistent observation of such damage suggests that these are situations in which the cells are undergoing degeneration rather than being a specific effect of EDTA treatment. Support for this interpretation is provided by the presence of identical isolated cells in

control cultures and by the fact that the frequency of observations of this artifact was not increased following maximum separation in EDTA. The ballooning of the mitochondrial membranes and the matrix condensation discussed by Lynn, Fortney and Brown (1964) and Harris and Leones (1966) was not observed even in the few cultures showing intracristal swelling of the mitochondria. Several investigators have studied the effects of EDTA dispersive methods on the viability of a variety of cell types and have obtained results showing that the percent of cell death was not increased above that of other dispersive techniques. Cells damaged to the extent demonstrated by Harris and Leone (1966) could not be expected to survive, however, chick epithelial cultures treated with EDTA until maximum separation, recovered completely when the medium was replaced by Hank's BSS. Electron micrographs of such recovered cultures showed perfectly normal mitochondria.

The degenerative changes observed by the foregoing investigators could very likely be due to post mortem changes occurring in the liver tissues prior to fixation. That this may indeed be the case is suggested by the work of Leeson and Kalant (1961) in which direct perfusion accomplished cell separation without accompanying mitochondrial alteration. Other factors could no doubt contribute to the picture presented in the degenerate tissue. Osmuim tetroxide

fixation was employed and may have exaggerated the effect. Although similar changes were reported in glutaraldehyde fixed McCoy cell strain in tissue cultures only electron micrographs of osmium fixed tissues were presented. Another factor that very well may have contributed to the degenerative process is the long duration of treatment (one to six hours) to which the tissues were subjected prior to fixation.

In recent years a growing interest has developed concerning the nature and function of cytoplasmic microtubules. Prior to the advent of glutaraldehyde fixation (Sabatini, Bensch and Barnett, 1962), microtubules were observed almost exclusively as subunits of highly organized structures such as cilia or flagella. It appears that in these specialized situations, the tubular subunits are less susceptible to disruption by osmium tetroxide fixation while microtubules unrelated to the ciliary or flagellar elements are readily destoyed. In contrast, cytoplasmic microtubules are especially well preserved with glutaraldehyde and it has become increasingly apparent that they are ubiquitous cell organelles. This ubiquity is well documented in several recent reviews in which extensive lists illustrate the diversity of the animal cell types known to possess cytoplasmic microtubules (Behnke, 1964, 1966; Anderson, Weissman and Ellis, 1966; Sandborn et. al., 1964; Slauterback, 1963; Porter,

Ledbetter and Badenhauser, 1964). In addition, microtubules of similar morphology have been demonstrated in a variety of plant cells (Ledbetter and Porter, 1963, 1964; Hepler and Newcomb, 1964; Newcomb and Bonnett, 1965). This ubiquity of microtubules might be expected since it has been demonstrated that the mitotic spindle fibers common to dividing cells are in fact tubular in nature (Harris, 1962; Kane, 1962; Roth, 1962; Robbin and Gonatas, 1964). However, the widespread observation of morphologically similar structures dispersed through the cytoplasm of interphase cells following glutaraldehyde fixation has stimulated considerable interest in microtubules not associated with cell division. As mentioned earlier the epithelial cells used in this study are exclusively interphasic cells and one of the striking features of their fine structure is the abundance of cytoplasmic microtubules.

In general morphology, the microtubules resemble those observed in other interphase cells as well as those of the mitotic spindle and the subunits comprising the shafts of cilia and flagella. Thus they appear in cross section as spherical tubules approximately 220-250 Å in diameter having a rather electron dense outer wall and a less dense inner core. In longitudinal section they present the appearance of two dense lines running parallel to one another for considerable distances separated by an electron lucid zone.

A number of precise measurements have been made and differences in diameters have been reported to exist between tubules of the mitotic spindle, those comprising ciliary and flagellar shafts and those lying in the more peripheral cytoplasm. Although these differences may exist, little significance can be assigned to size variations until measurements have been made on a wide variety of tissues under identical fixation and embedding conditions. Differences in the preparation methods could conceivably result in a range of diameters equal to the range of sizes proposed for these tubules. Slauterback (1963) has pointed out that there are two general size classes of microtubules, 120-200 Å for spindle tubules while those of the ciliary shaft and other specialized situations are about 270 Å. He proposes that this bimodal size distribution is coincident with functional specialization. This may be a valid assumption; however, the range of sizes presented in the literature would suggest that there may be a continuous spectrum of diameters presented by microtubules which may be a result of the conditions prevailing during preparation for electron microscopy or differences in cell types or cell size.

Although it seems that little emphasis can at present be placed on size variations there seems to be some correlation between the disposition of the microtubules in the cultured epithelial cells and

their functional capacities. In these interphasic cells there appears to be three expressions of the cytoplasmic microtubular system which do seem to be related to specific functional potentialities. In two of these situations an obvious association with the centriole is noted while in the third the microtubules do not seem to be related to this organelle.

It is generally known that microtubules comprise the shaft of cilia and it is believed that they form by extension of the centriolar tubules. A similar relationship is observed in the electron micrographs presented here depicting the various developmental stages of rudimentary cilia in embryonic chick renal epithelium. During the development of a cilium, the centriole, activated by some stimulus, initiates the production of a ciliary vesicle into which the developing cilium extends. The growth of the cilium progresses by elongation of its microtubular elements into this vesicle. The organization of the tubules in the ciliary shaft has been shown by several authors to be directly related to the tubular organization of the centriole cylinder (Sotelo and Trojillo-Cenoz, 1958; Sorokin, 1962; Renaud and Swift, 1964). This similarity in organization suggests that these microtubules are produced at the end of the cylinder and that the tubules of the centriole may act to determine the pattern of organization of the ciliary components. The phenomena involved in the formation of cilia described here are in accord with those

described by the above investigators, however, no evidence is available to suggest a mechanism whereby the tubules are extended. If the tubules of the centriole do act as a model for the formation of the microtubules one would expect a synthesis of tubular protein within these sites and a progressive outward extension of the completed structures.

In contrast to the organization of microtubules into the cilium shaft another system of microtubules is observed which has an entirely different type of association with the centriole. Where those of the cilium are characteristically associated with the distal end of the centriole cylinder these microtubules are associated with the centriolar satellites or paracentriolar bodies. This satellite microtubule relationship has been described by several authors (De-THE, 1964; Gonatas and Robbins, 1965; Szollozi, 1964; Slauterback, 1963). The microtubules appear to be attached to these sites and radiate out into the surrounding cytoplasm much as they would in the formation of a monaster. De-THE (1964) suggests that the synthesis of microtubular protein may occur within the satellites. Taylor (1966) suggests a similar possibility but also points out that there is no evidence precluding the possibility that microtubules could be synthesized independently in the cytoplasm and secondarily attached to these sites. This association of tubules to the satellite bodies rather than to the centriole proper is characteristic of the

spindle tubules during mitosis (Taylor, 1965) and in fact De-THE has suggested that the observation of such a relationship in interphase cells might indicate that these microtubules represent spindle tubules that persist in the resting cell. Lettre and Lettre (1958) proposed that spindle fibers might persist during interphase. Gonatas and Robbins (1965) have shown a similar relationship between the neurotubules and centriolar satellites in ganglionic cells of the chick embryo and they implied that the spindle tubules and the neurotubules are homologous structures. Taylor (1966) also reported tubules extending the length of nerve processes. He points out, however, that the temporal relationship between the mitotic events in these cells and the formation of the nerve processes precludes the possibility of their being formed from spindle tubules. The tubules observed radiating from the satellites of cultured chick renal epithelial cells are interpreted as being remnants of preexisting spindle tubules or as incipient spindle tubules prior to the onset of subsequent mitoses. Since these cells have not been observed in division it may be suggested that the tubules are a manifestation of incomplete spindle development.

In some cells the microtubules associated with the centrioles are so numerous that they form an area from which other subcellular organelles are excluded. Mention was made earlier of bright

juxtanuclear areas which were recorded with time-lapse cinemicrography and were less frequently invaded by cytoplasmic inclusions. They may represent zones heavily permeated by the cytoplasmic microtubules rather than the Golgi region which is very limited in these cells. The association of microtubules with the centriolar satellites in one case and their direct production in another from the distal end of the centriole cylinder to form a rudimentary cilium points out that their disposition is coincident with functional specialization.

A third and probably the most interesting disposition of cytoplasmic microtubules is frequently encountered in these interphase epithelial cells. In this case, the microtubules, morphologically identical to those previously discussed are found through out the more peripheral cytoplasm without obvious relationship to the centrioles. Microtubules which bear no apparent relationship to the centrioles have been demonstrated in a variety of cell types, (Taylor, 1965, 1966; Sandborn, et. al., 1964; Behnke, 1964; Beyers and Porter, 1964; Tilney and Porter, 1964) and in fact microtubules of typical morphology are observed in cells of higher plants in which centrioles have not been demonstrated (Ledbetter and Porter, 1963, 1964; Newcomb and Bonnett, 1965; Hepler and Newcomb, 1964). Therefore the microtubules observed in the marginal cytoplasm are

not necessarily dependent upon the centriole for their organization and may be expected to possess different properties from those produced at or near the centriole.

Microtubules have been observed in all cells of untreated cultures. In many cells they are not arranged in any precise order, however, in others they are very abundant and may exhibit a striking alignment parallel to the long axis of the cell or cell process and coinciding with a comparable orientation of the mitochondria and other inclusions. The cells extending to the free-borders of the epithelial sheet also show a particularly high degree of microtubular orientation. In this case it is perpendicular to the free edge of the colony and extends to the plasma membrane. In the more central cells of the colony the microtubules are less abundant and are infrequently oriented.

A variety of functions have been postulated for microtubules. The simplest and least acceptable interpretation is that these structures represent nothing more than discarded remnants of preexisting spindle tubules. Their consistent and predictable observation in interphasic epithelial cells, their abundance and their highly organized disposition suggests, however, they are not merely residual fragments of those present during mitosis but that they are an expression of a function not directly related to mitosis.

Sandborn et. al. (1964) have proposed that the cytoplasmic microtubules may form a complex network of connecting channels between other organelles and the plasma membrane. Thus a continuous microcirculatory system capable of shunting ions and macromolecules from place to place within the cell is postulated. Their parallel alignment, their straight non-branching course through the cytoplasm and the paucity of connection with other organelles suggests that this hypothesis is not applicable to the cells studied.

DuPraw (1965) also proposed that the cytoplasmic organelles are connected to one another and to the nuclear membrane by a system of microtubules. He proposes that the microtubules are contractile elements and are responsible for the saltatory movements of the cytoplasmic inclusions. Behnke (1964), however, in a study of a variety of cell types failed to observe any connection between the microtubules and other structures. He suggests that the interphase microtubules may become functional as spindle tubules either by being properly oriented or by being depolymerized into subunits in early prophase with subsequent repolymerization into functional spindle fibers. He maintains that this proposal gains credence from the observation that microtubules are not observed outside of the region of the spindle during mitosis.

Although the above proposed functions for the microtubules can not be excluded at present it is felt that overwhelming evidence

suggests that they may function as a labile cytoskeleton which is involved in the formation and maintenance of asymmetrical cell shapes. This implies that they act as an intracellular framework which may impart direction to the cytoplasmic flow characteristic of elongating and flattening cells. Thus their observation in untreated cells and especially their high degree of oreintation in those cells known to undergo most rapid form changes may indicate that they participate in the formation and maintenance of the flattened conformation and when not precisely ordered their rigid nature would still premit their serving an internal supporting function.

Porter and his associates have presented a series of reports that individually support such a proposed function. Collectively they provide convincing evidence that microtubules are a feature characteristic of cells undergoing dynamic changes in shape and following such changes they may act to support the resulting asymmetrical conformation. Ledbetter and Porter (1963) demonstrated that microtubules were aligned parallel with the cytoplasmic streaming patterns in plant cells and suggested that they function in initiation and propagation of cytoplasmic circulation. Bickle, Tilney and Porter (1966) observed microtubules disposed peripheral and parallel to the migration channels of pigment granules in melanophores and Silviera and Porter (1964) showed that microtubules

were involved in elongation of flatworm spermatozoids. Tilney and Porter (1965) have observed that the axonemes of heliozoan axopods are composed of a highly organized system of microtubules which function in the formation and support of axopodia. They point out that the microtubules are responsible for the formation and maintenance of cell form and the close association with cytoplasmic streaming again suggests that they play a role in cytoplasmic movements. Kitching and Cragg (1964) have shown similar axopodial microtubules in Actinosphaerium and point out that no contractile properties can be assigned these structures.

Probably the most convincing evidence that microtubule orientation is an expression of dynamic changes in cell shape has been presented by Byers and Porter (1964). They have shown that microtubules appear concurrently with the cell elongation accompanying the morphogenetic movements occurring during lens placode development. During the period of cell elongation microtubules are very abundant and are disposed parallel to the axis of elongation. When the cells cease to elongate and become relatively static the microtubules are rapidly lost and if further morphogenetic changes occur, involving cell elongation, these structures are once again observed aligned parallel to the long axis of the cell. This close temporal association between the appearance of

microtubules and cell elongation coupled with their disappearance when cytoplasmic movement ceases indicates that they are indeed involved in dynamic changes of cell shape. Porter and Tilney (1965) and Porter, Ledbetter and Badenhauser (1964) have pointed out that similar microtubules are consistently and predictably associated with cytoplasmic movements and therefore closely related to development and preservation of a symmetrical cell shapes.

Other investigators have observed a similar relationship between microtubules and directed cell movement and/or determination and preservation of cell shape (DeTHE, 1964; Anderson, Weissman and Ellis, 1966; Robbins and Gonatas, 1964; Clark, 1965; Fawcett and Witebsky, 1964).

The collective evidence that a microtubule-cell extension association does exist has prompted Tilney and Porter (1964) to state that "whenever an organism constructs a linear extension of itself such as in cilia or flagella, in axopods of nerve cells and even in dividing cells, microtubules are synthesized in the direction of the forming extension" (page 340). The microtubule orientation in control cultures is not characteristic of all cells but rather is limited to a small percentage of cells most of which are at the perimeter of the culture. This might indicate that these cells were expanding or putting out motile extensions at the time of fixation while those

having randomly disposed as well as fewer tubules are considered as being relatively static. This is what might be expected considering the motile nature of the cultured cells and their ability to change shape. The formation of microtubules in in vitro propagated cells is therefore likely to be a consequence of the transformation from the relatively static in vivo condition of the cells to the dynamic situation existing in culture.

Cells which have been subjected to calcium extraction with EDTA lend support to the opinion that a difference may exist between those microtubules associated with the centriole and those more widely dispersed in the cytoplasm. In fully separated cells the ciliary microtubules and those associated with the centriolar satellites remain unaltered while those participating in cytoplasmic movement or in maintaining the a symmetrical conformation are entirely absent. Thus a difference exists in that the latter are more labile to the altered conditions imposed by chelation of calcium. Taylor (1966) has pointed out that a variation exists among microtubules with respect to their susceptibility to disruptive forces. The effects expressed under EDTA treatment may be due to such differences. Such variations in the responses to calcium extraction may be extrapolated to suggest that more fundamental differences may exist in the nature and composition of the different microtubules.

The disruption of the microtubules normally distributed through the cytoplasm may be a direct consequence of calcium depletion resulting in a solation or depolymerization of their protein structure. If this possibility is correct it would be as expected since calcium withdrawal is known to result in solation of gelled proteins. Porter and Tilney (1964) have noted that the microtubules of heliozoan arms are destroyed by high hydrostatic pressure which is known to solvate gelled proteins.

If their disruption is due to calcium extraction with EDTA, the observation by Behnke (1964) that mitotic cells do not have microtubules outside the spindle region again might suggest that events occurring during mitosis may have an action similar to that of EDTA. Thus the internal shunting of calcium during condensation of the chromatin as proposed by Robbins and Micali (1965) may act as an internal chelator thus eliciting the disruption of these more labile microtubules. This would infer that a difference does indeed exist between these tubules and those of the mitotic spindle since the latter are obviously not disrupted at this time.

On the other hand the paucity of these structures in fully rounded cells may be due to the fact that the cells are spherical and static. As a result the conditions of changing cell shape and cytoplasmic movement associated with the presence of microtubules

do not exist in the peripheral cytoplasm. Mitotic cells also round up and become relatively symmetrical showing little if any cytoplasmic streaming. In this case the lack of peripheral microtubules might be readily explained without invoking the idea that they are reoriented to form the mitotic apparatus or depolymerized and reformed in the developing spindle. Therefore the lack of peripheral microtubules in rounded mitotic cells may simply be a consequence of the transformation from the dynamic situation of motile interphase cells to the spherical state of those in mitosis. The absence of peripheral microtubules in cells possessing true spindle tubules points out that differences must exist in the tubules themselves or in the conditions existing at their respective developmental sites.

When fully rounded cells are allowed to spread in Hank's BSS they regain their asymmetry and cytoplasmic streaming resumes. Electron micrographs taken during the recovery sequence show that typical microtubules are once again characteristic of the cytoplasm and are frequently oriented with the long axis of the cytoplasmic extensions. Cells which have reaggregated fully into a coherent sheet exhibit distribution and orientation of microtubules similar to that observed in control cultures. The reapplication of the calcium laden medium may facilitate their polymerization or

gelation while the cell asymmetry and cytoplasmic streaming generally associated with their appearance is reestablished.

The observation that these microtubules are present in untreated cells, are absent when the cells are fully rounded by calcium chelation and reappear rapidly throughout the cytoplasm when the recovery conditions are appropriate indicates that these are very labile structures. This concurs with the observation of Beyers and Porter (1964) that cells exhibiting cytoplasmic movement in forming cell asymmetries consistently show microtubules while similar cells which have become static and are no longer elongating lose these structures promptly. The results discussed here may be an experimentally induced situation in vitro comparable to the reversible situation described by Beyers and Porter (1964) during the morphogenetic changes characteristic of the developing lens of the embryonic chick.

These results concur with the view that microtubules, because of their abundance, disposition and apparent rigidity may serve as a labile cytoskeleton. Thus it is suggested that they play a role of obvious significance in maintaining cell to cell associations and are intimately associated with cytoplasmic movements and formation and preservation of cellular asymmetry.

In addition to the microtubules discussed, finer non-tubular filaments have been observed in the cytoplasm of these cells. The

microfilaments have dimensions similar to the tonofibrils associated with the desmosomes. Hay (1961) has pointed out that similar filaments are common to many epithelial cell types and that a supporting function is generally ascribed to them. Palay and Karlin (1959) have shown that the terminal web characteristic of certain columnar epithelia is composed of similar cytoplasmic filaments. The filaments observed in the monolayer cultures may be an in vitro modification of those expected in the terminal web of the kidney tubule cells. Biberfield (1965), however, suggests that the synthesis of analagous filaments in cultured liver cells may be an expression of dynamic changes that occur as cells adjust to the in vitro situation.

The disposition of these fibers along the lower surface of the cells and the apparant association with the cell membranes suggest they may be involved in sustaining the flattened condition of the cells. In this sense they would be acting as a supporting structure which could supplement a similar function proposed for the microtubules. In addition their constant association with the lower and peripheral cell surfaces indicates that they play a part in cell to substrate attachment. Taylor (1966) has consistently observed a corresponding disposition of filaments in cultured epithelia and has postulated a similar function. Bronsted and Carlson (1961)

have observed a fibrous cytoskeleton in the cortex of expanded basal epithelial cells of sponge gemmules. They noted that they exhibited maximum development when the cells were most expanded and also suggested that they aid in substrate attachment and maintenance of cell shape. The filaments therefore appear to be functionally similar in some respects to the larger microtubules and in fact Taylor (1966) has noted a close association between the two. The nature and significance of this association remains to be determined.

Although these filaments were not observed in fully chelated cells, and their absence could be accounted for by a solation process mediated by calcium depletion, it is felt that the evidence is not sufficient to warrant such a proposal at this time. In addition they were rarely observed in recovering cells although they would be expected. Their lack of detection in fully rounded cells and their infrequent observation in recovered cells might be due to their being lost because of the difficulty obtaining the first section from the block surface. Only the first few sections would cleave the appropriate plane required for their demonstration since the filaments are most abundant immediately adjacent to the substrate plane.

Electron micrographs taken of cells fixed at full separation

frequently show the cytoplasm to be filled with large vacuoles while the nucleus is often highly distorted. Hays, Singer and Malamed (1965) and Lesson and Kalant (1961) have observed similar vacuole formation and nuclear distortion in other tissues subjected to EDTA dispersive methods. It is assumed that the vacuoles are a direct result of the increased permeability elicited by calcium extraction with EDTA. During their formation the numerous large vacuoles may contribute to the internal pressure and thereby facilitate the extrusion of ground substance during the blebbing activity. In addition the distorted shape of the nucleus in the fully rounded cells may in part be a consequence of pressures created by these enlarging vacuoles.

Cells which have recovered in Hank's BSS show a decrease in cytoplasmic vacuolization with the nucleus showing a corresponding recovery to the normal spherical condition. The addition of the calcium containing medium permits the cells to reestablish permeability properties similar to those of control cultures and thus normal cytoplasm and nuclear configurations are observed in recovered cells.

In addition to the variety of surface structures and intracellular components shown to be reversibly affected by calcium depletion, a number of organelles are unaltered during treatment.

Neither phase-contrast nor electron microscopy has revealed structural changes in the cell membranes, mitochondria, fragmentary endoplasmic reticulum or the limited Golgi lamellae. Although the nucleus is distorted in fully rounded cells and reverts to the spherical condition in recovered cells no consistent alterations were observed in the structure of the nuclear membrane during any phase of the sequence. The nucleolus maintains its characteristic structure through the sequence of calcium abstraction and readministration. Thus certain specific cellular components are in no way altered by EDTA treatment while those profound changes in ultrastructure as well as those observed with phase-contrast microscopy are completely reversible subject to incubation in the calcium containing recovery medium. A number of the effects of EDTA on the cellular fine structure have been correlated with those observed with phase-contrast microscopy. In addition many of the effects calcium withdrawal are manifested at the ultrastructure level and at best can only be indirectly correlated with those observed with the light microscope.

SUMMARY

A time-lapse cinemicrographic and electron microscopy study has been made on primary cultures of chick renal epithelium subjected to calcium depletion with EDTA and subsequent recovery in Hank's BSS.

The time-lapse record shows that the chelation process elicits a series of responses by the cells of a coherent monolayer of tissue. They relinquish mutual contacts, initiate veiling on the newly freed surfaces, exhibit surface blebbing and eventually become fully rounded and highly refractile.

The release of cell contacts is attributed to the removal of divalent ions from intercellular adhesive mechanisms while the veiling of the freed surfaces is interpreted as an attempt by the cells to form or maintain contacts. The blebbing phenomenon and final rounding of the cells are interpreted in terms of sol-gel transformations and alterations in the membrane permeability properties, both being elicited by the removal of cellular calcium.

On readministration of Hank's BSS to fully separated cells the blebbing activity resumes. The cells spread and flatten to the substrate eventually reaggregating into a confluent sheet indistinguishable from control cultures. The calcium contained in the

recovery medium apparently plays a role in the reattachment to the substrate as well as in the membrane formation required during the transition from the spherical condition to the flattened state of the fully recovered cells. In addition the calcium may facilitate extension of intercellular contact surfaces. The effects of calcium chelation with EDTA as observed with the light microscope are therefore completely reversible resulting in intact sheets of epithelial cells.

Electron microscopy has been done on untreated epithelial cultures and has revealed details of ultrastructure and intercellular relationships not discernible by phase-contrast microscopy. The fine structure of the untreated cultures has been described as has that of cultures fixed at progressive stages in the sequence of dissociation and reaggregation.

Electron micrographs depicting surfaces of mutual contact show a variety of adhesive modifications. The desmosomes, surface interdigitations, mortice and tenon sites and the zonula diffusa, all are disrupted by the chelation process while the sites of intercellular cement are relatively resistant to the effects of calcium depletion. The desmosomes are completely disrupted upon calcium removal with EDTA but show a complete and rapid recovery in Hank's BSS. The ability to be disrupted and reformed readily coupled with the

known motility of the cultured cells has prompted the proposal that these structures have a labile nature. The loss and reappearance of the intracellular structure of the desmosomes are interpreted as being directly mediated by the depletion and reapplication of calcium required for their structural integrity.

Dense thickenings are observed beneath the plasma membranes exclusively at contact sites at the periphery of the culture. The zonula diffusa is disrupted by calcium depletion and reappears on reassociation of the cells. It is interpreted as a labile adhesive structure being calcium dependent and may represent *in vitro* modifications of the terminal bar seen *in vivo*.

Interdigitations of apposed surfaces relinquish contacts under the effect of EDTA mediated separation as do the mortice and tenon type of contacts and both are reformed upon recovery in Hank's BSS.

A dense amorphous intercellular material has been shown in discontinuous plaques between cells of untreated cultures. This material is interpreted as an intercellular cement which persists between cells at maximum treatment with EDTA. It is proposed that this cement is relatively less susceptible to calcium depletion and therefore less calcium dependent than the other adhesive mechanisms.

The free surfaces of untreated cells are characterized by

submicroscopic microvilli having electron dense cores. During treatment with EDTA microvilli are at first elaborated at newly freed surfaces following which they are retracted. At full separation they are completely absent, however, they reappear in large numbers during recovery in Hank's BSS. They may function in reestablishing the coherent sheet of cells either by acting as low radius of curvature probes enabling intimate calcium bridge formation or by interdigitating with those from other cells. In the latter case they could form the complex infoldings observed between fully recovered cells. Their elaboration during early treatment is discussed in terms of surface viscosity changes occasioned by calcium removal. Their complete disappearance on further chelation and their reformation in the recovery medium may represent disruption of the supporting cores by calcium extraction with subsequent reformation in Hank's BSS.

The ultrastructure of the surface blebs induced by EDTA is described and resembles very closely those described during mitosis. The blebs observed with electron microscopy are correlated with those observed with time-lapse cinemicrography.

Various polyribosome configurations are observed in untreated cultures but their number is considerably reduced in fully sepatated cells. It is postulated that the EDTA extracts magnesium

required for the binding of ribosomes into polyribosomes and is discussed in the light of similar disruption occurring in normal metaphase cells. In fully reassociated cells they are as numerous as in untreated cells.

Three expressions of a microtubule system have been described in untreated cells. Those associated with the centriole in rudimentary cilia or associated with the centriolar satellites are not disrupted by EDTA treatment. The microtubules showing no association to the centriole, however, are absent in fully chelated cells but reappear during recovery in Hank's BSS. These latter microtubules are frequently oriented with the long axis of a cell process in both untreated and recovering cells. It is postulated that they comprise a labile cytoskeleton which may function in the formation and maintenance of cell asymmetries and is associated with directional cytoplasmic movement. It is also proposed that a difference in susceptibility to treatment and therefore more subtle differences may exist between the variously disposed microtubules.

A layer of non-tubular microfilaments is observed at the flattened base of untreated cells. They run parallel to the substrate plane and it is proposed that they aid in maintenance of the flattened condition and/or attachment to the substrate.

The cytoplasm is highly vacuolated and the nucleus is

considerably distorted in fully chelated cells. The formation of the vacuoles is assumed to be a result of permeability changes elicited by removal of surface calcium while the nuclear distortion may be a consequence of vacuole crowding. These effects on the nucleus and cytoplasm are reversed during incubation in Hank's BSS.

The ultrastructure of the mitochondria, the Golgi lamellae, and the fragmentary endoplasmic reticulum are described and do not appear to be altered by the chelation process.

The profound surface changes and those occuring in the cytoplasmic structures are discussed in the light of the proposed sol-gel transformations, ion flux, and permeability changes occurring during normal mitosis. All changes are completely reversible when calcium is supplied in the medium.

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APPENDIX

Figure 1. A phase-contrast photomicrograph illustrating a typical growth of chick renal epithelial cells cultured on a plastic coverslip in a Maximov depression slide. (560 x)

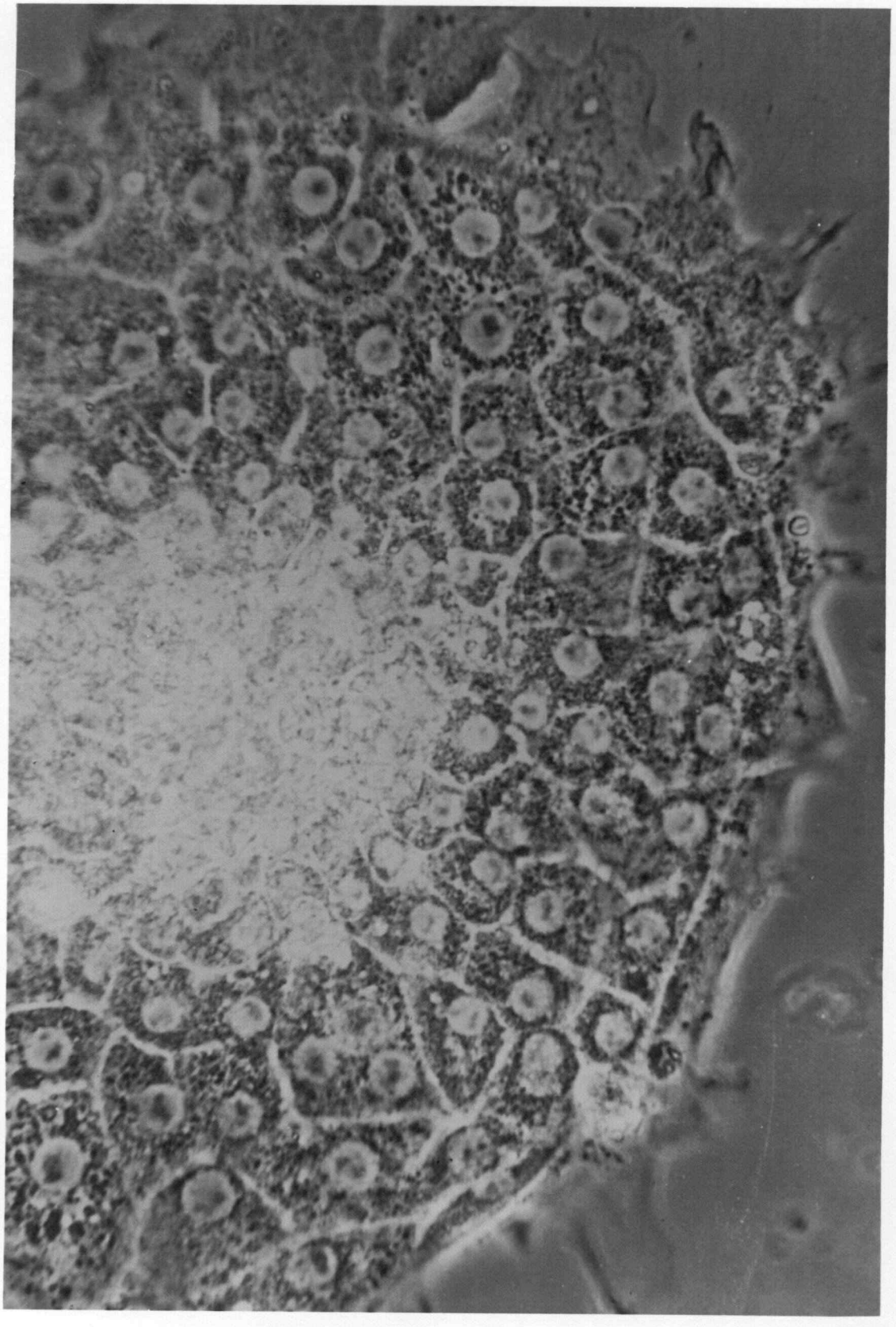


Figure 2. A sequence of frames abstracted from a time-lapse cinemicrographic film showing part of an epithelial culture during separation in and recovery from EDTA treatment. (850 x).

(a) Untreated Culture

(b-e) Response to treatment with 0.002 M EDTA

(b) Five minutes forty-eight seconds: partial separation and cellular contraction of the uppermost surfaces. Veiling is apparent between some of the cells.

(c) Ninteen minutes and twenty seconds: note further rounding of cells and retraction of the basal regions.

(d) Twenty-six minutes and forty-six seconds: surface blebbing initiated with further cellular contraction.

(e) Sixty-one minutes and twelve seconds: cessation of blebbing, further retraction and rounding of most cells. The response of the cell in the lower right appears to be slower than the others.

(f-h) Recovery response in Hank's BSS.

(f) The same field after being perfused with Hank's BSS and incubated for 14 minutes and 28 seconds. Note the resumption of the blebbing response.

(g) Thirty-two minutes twenty seconds: continued blebbing and centrifugal expansion of the cells.

(h) One hundred and forty-two minutes forty seconds: note the near complete recovery of some cells. The cells have flattened to the substrate, expanded and established extensive contacts.

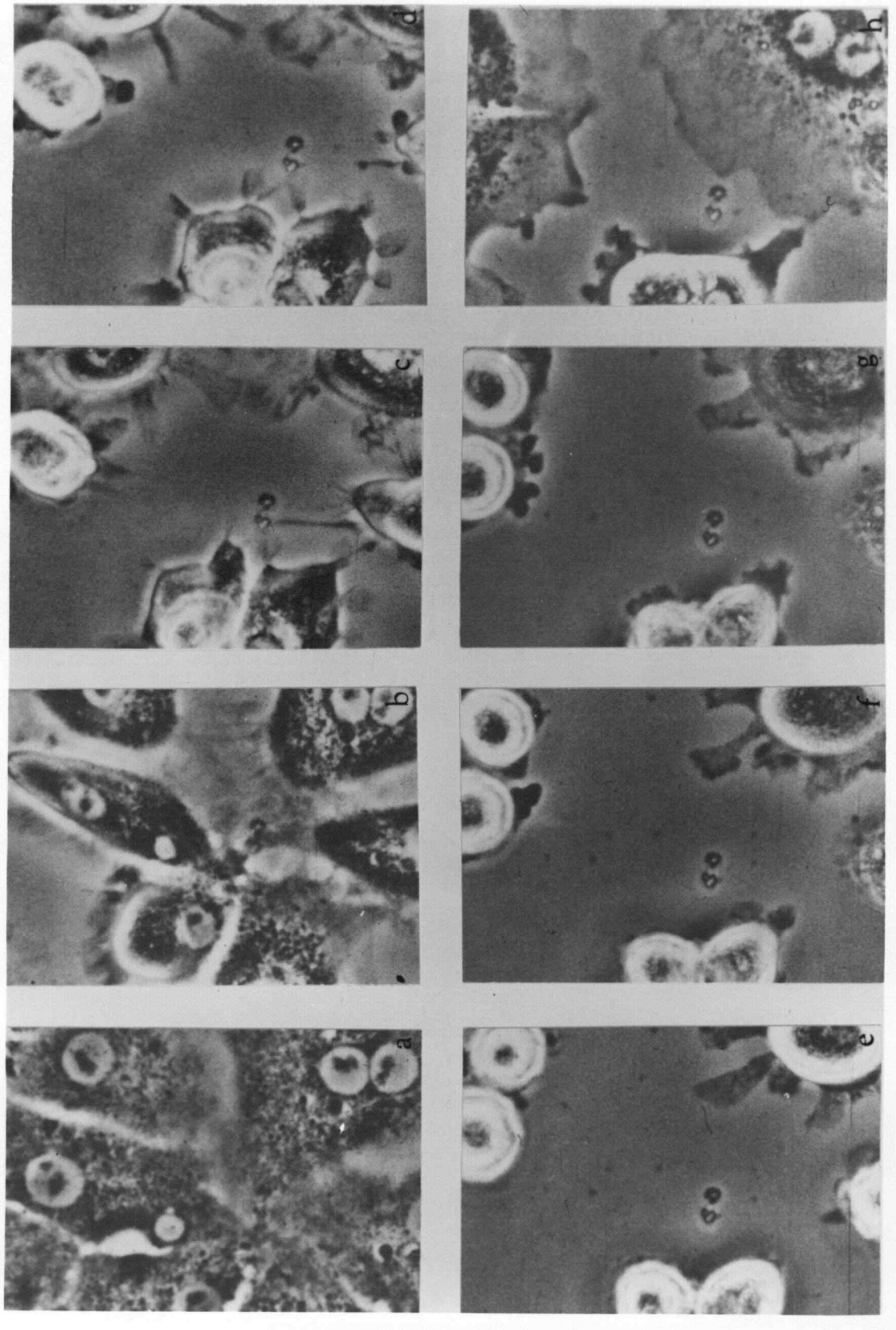

a
b
c
d
e
f
g
h

Figure 3. Two cells from the periphery of an untreated culture. Note the surface ruffled membrane (Rm) and surface microvilli (Mv). The zonula diffusa is clearly shown at the peripheral contact between the two cells (arrow). Also note the abundance of helical polyribosomes (h), scattered microtubules (t) and the opaque regions outside the nuclear pores (arrows). The nucleolus (nu) appears as a continuous cord. Nucleus (n). (10600 x)

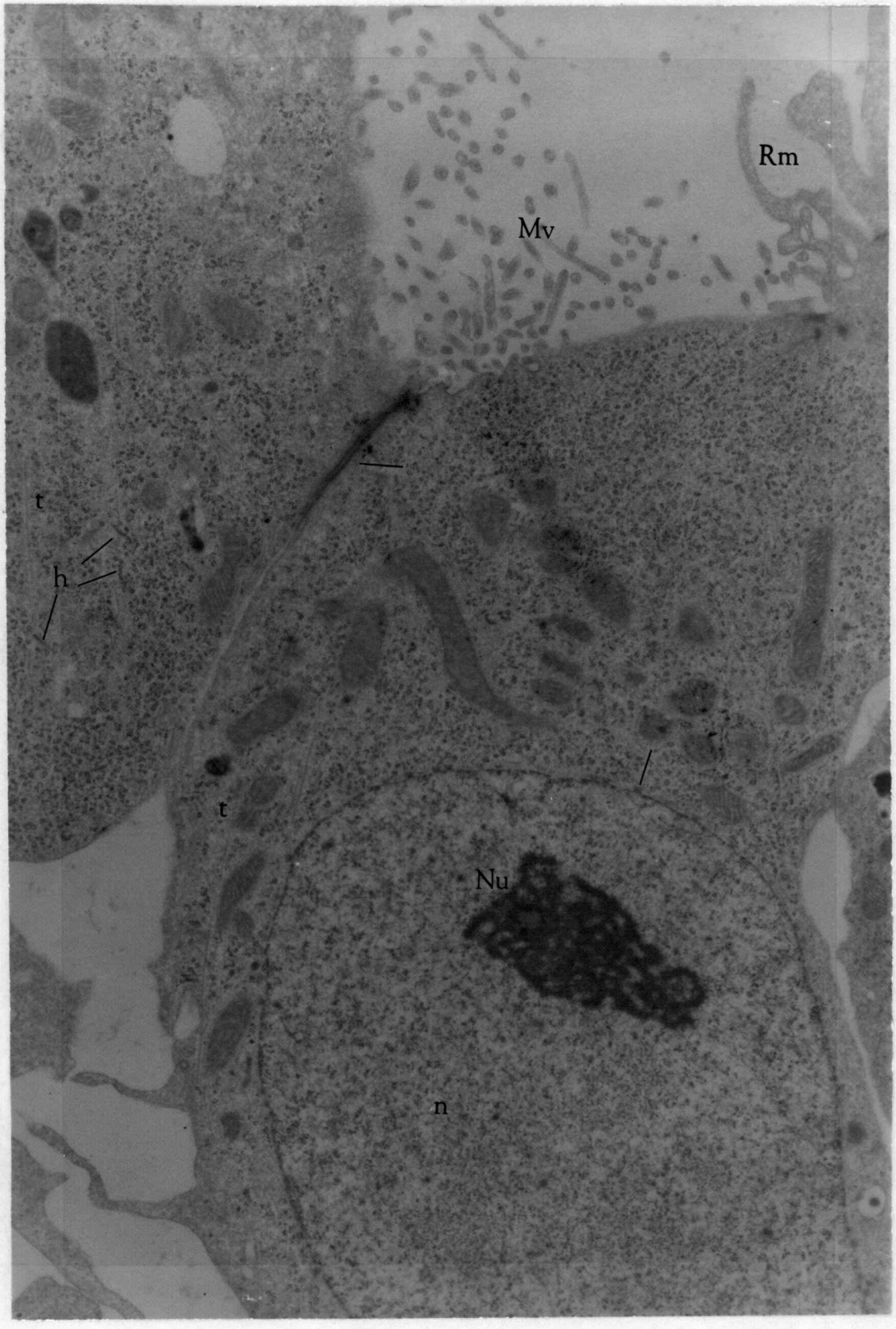

Rm
Mv
t
h
t
Nu
n

Figure 4. Untreated cells showing the surface ruffled membrane (Rm). Note its association with numerous vesicles (v). Microvilli (Mv) are also shown on the free surface. Nucleus (n). (4500 x)

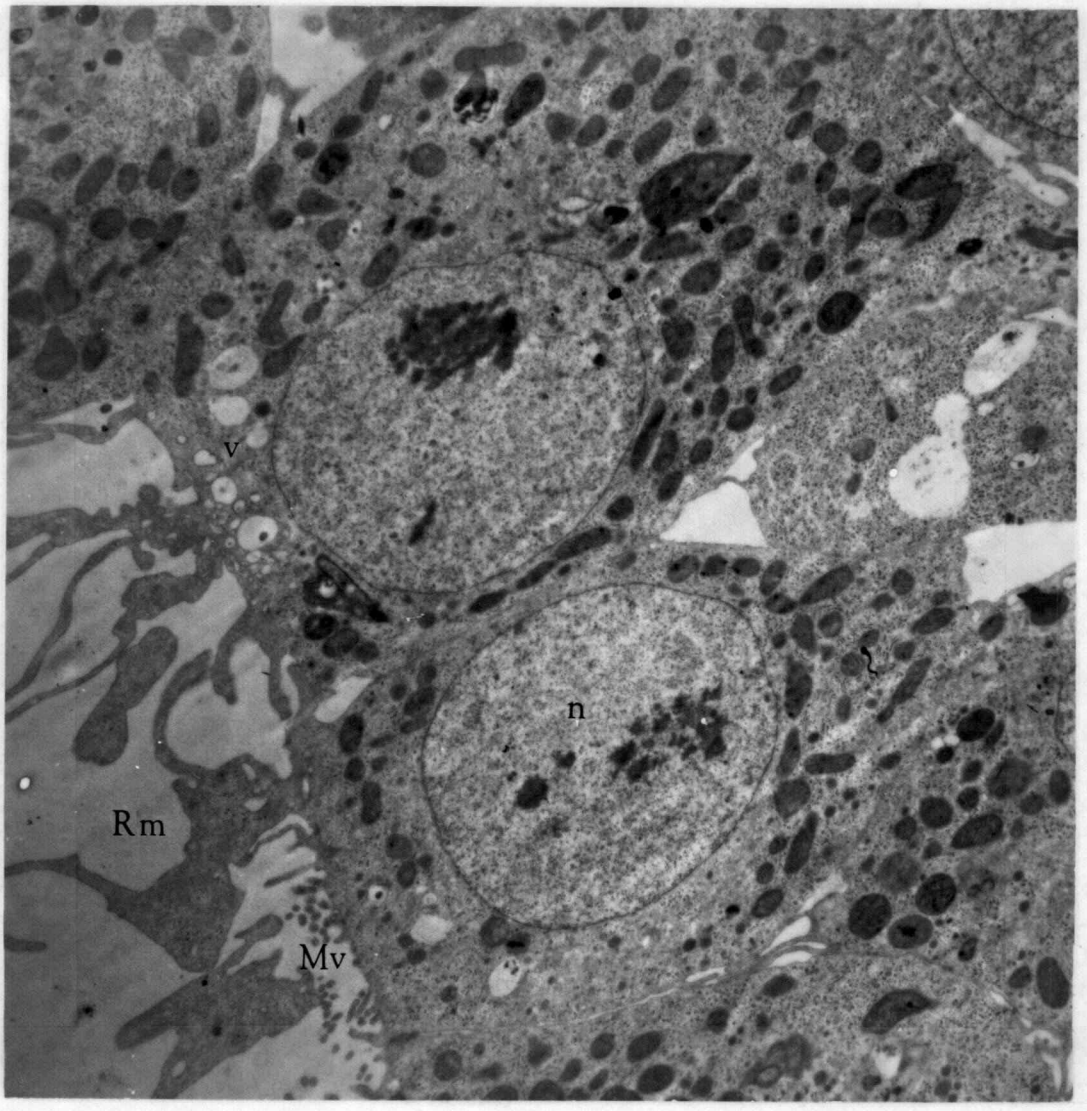

v
n
Rm
Mv

Figure 5. Surface microvilli (Mv) and their association with micropinocytotic vesicles (v). (60,000 x)

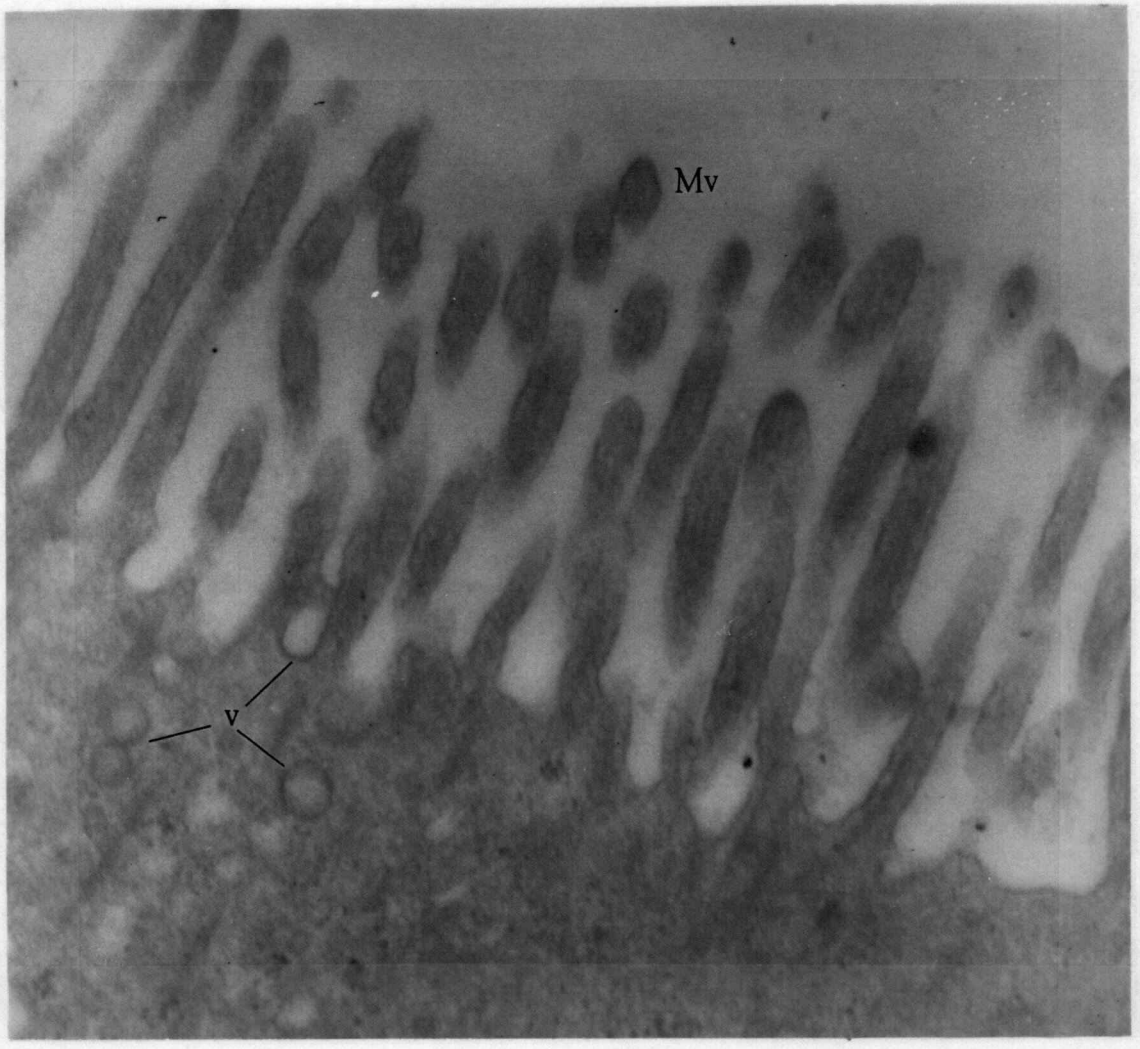

Mv
v

Figure 6. (a) Microvilli (Mv) occupying an intercellular space. Micropinocytotic vesicle (v). (4500 x)

(b) The centriole (c) and the surrounding organelle free zone. Nucleus (n), Nucleolus (Nu). (4500 x)

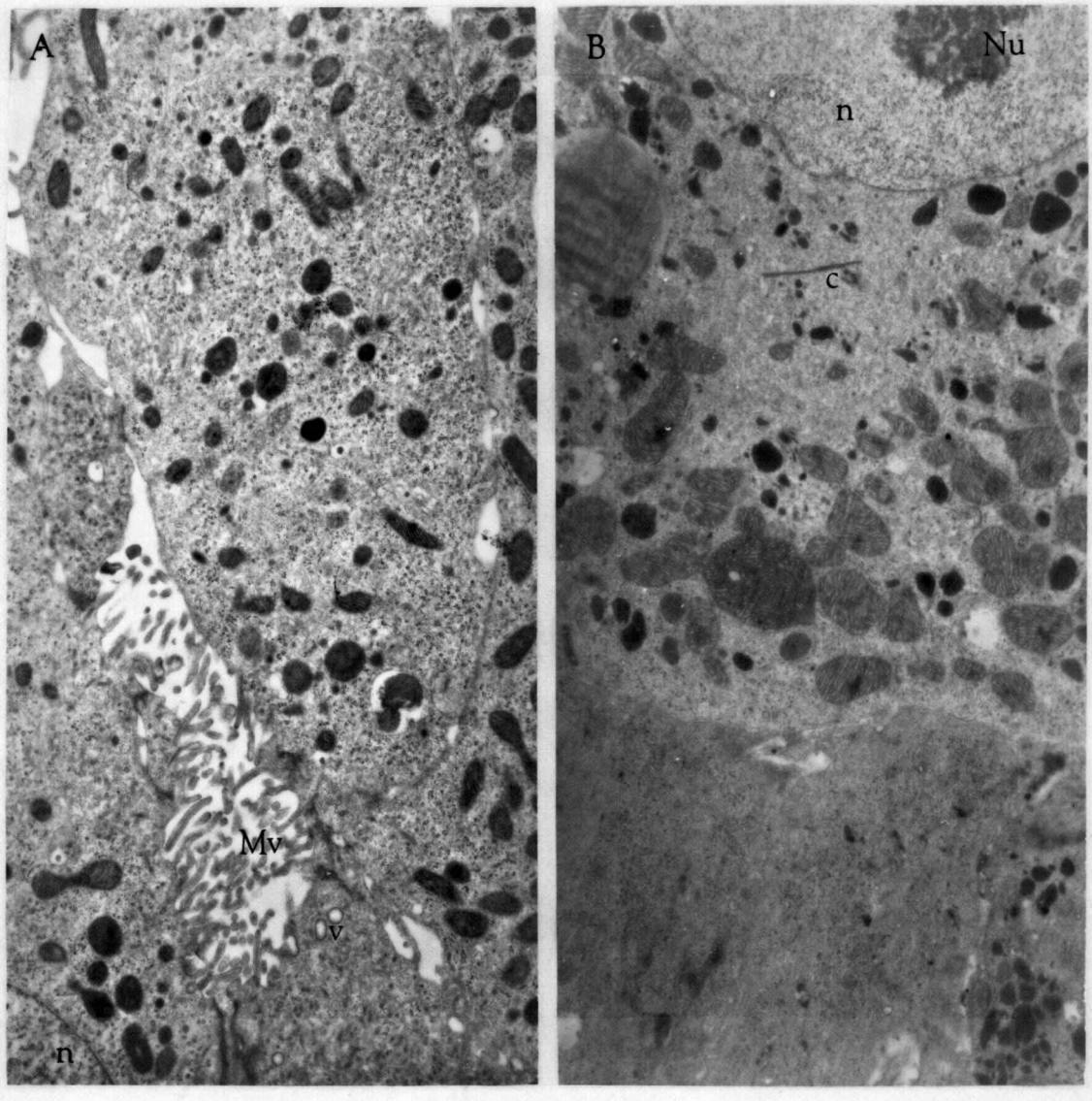

A
Mv
v
n
B
Nu
n
c

Figure 7. The junction point of three untreated cells showing the microvilli (Mv) in the intercellular space and the dense zonula diffusa where the cells are in contact (arrows). Nuclei (n), Vacuoles (V), Mitochondria (M), lipid droplets (l). (18, 000 x)

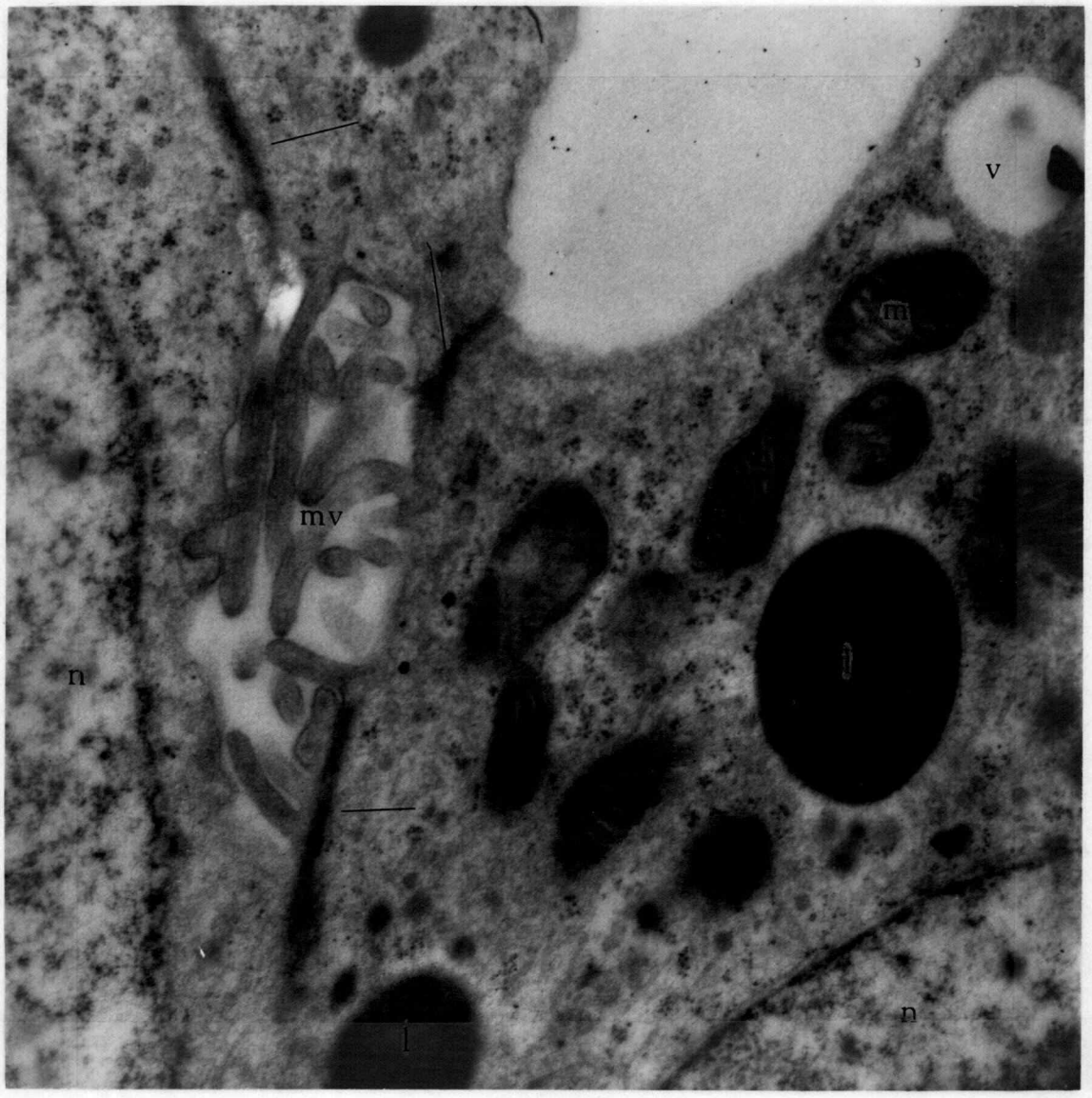

v
m
mv
n
n

Figure 8. A typical unmodified contact surface between two untreated cells. Note the granular nature of the nucleolus (nu). Nucleus (n). (12,000 x)

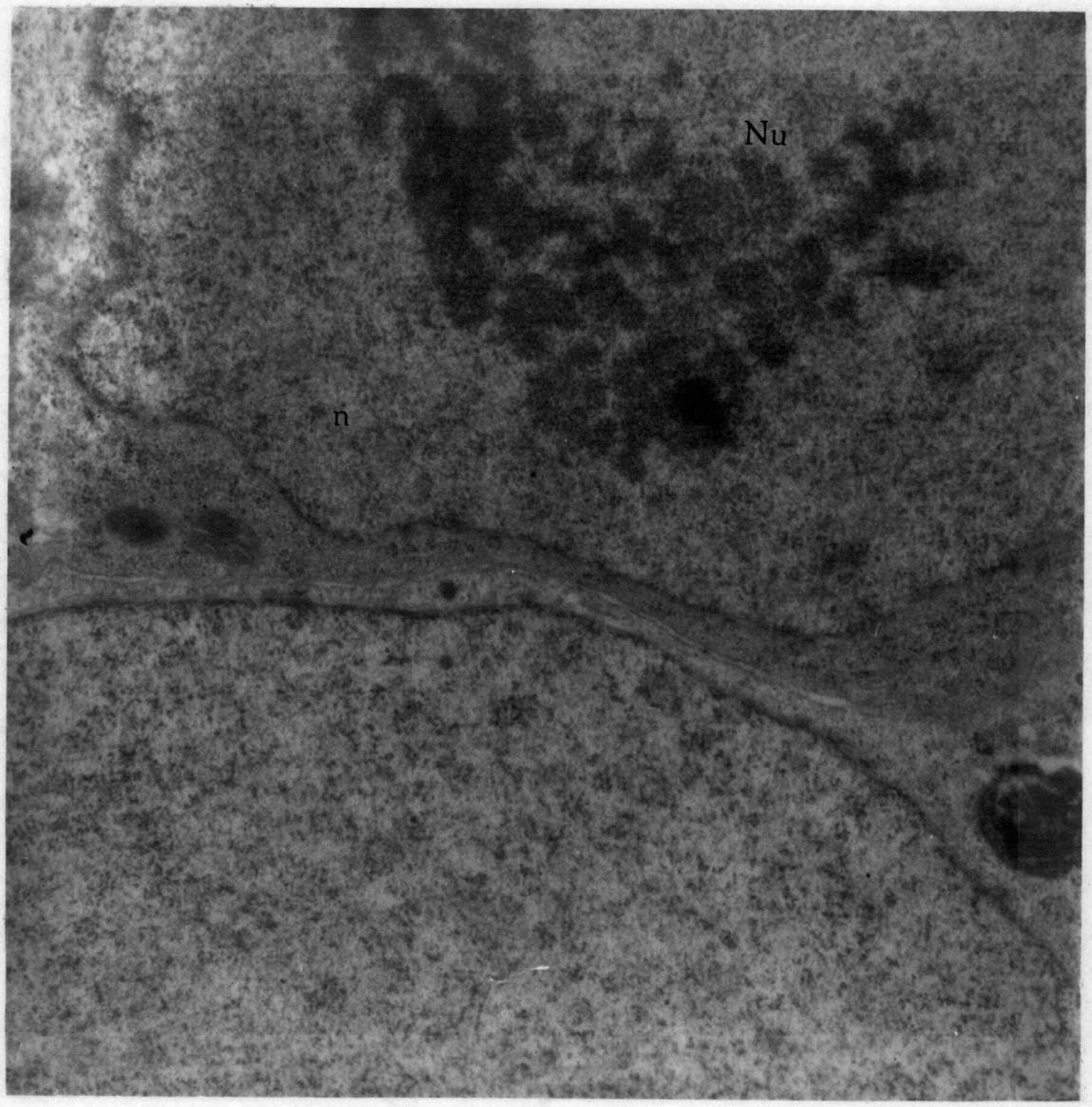

Nu
n

Figure 9. A section perpendicular to the plane of growth of an untreated culture. Extensive surface interdigitation (i) is shown between the cells. Microtubules (t) are shown exclusively in cross section. Microvilli (Mv); Nucleus (n). (18,000 x)

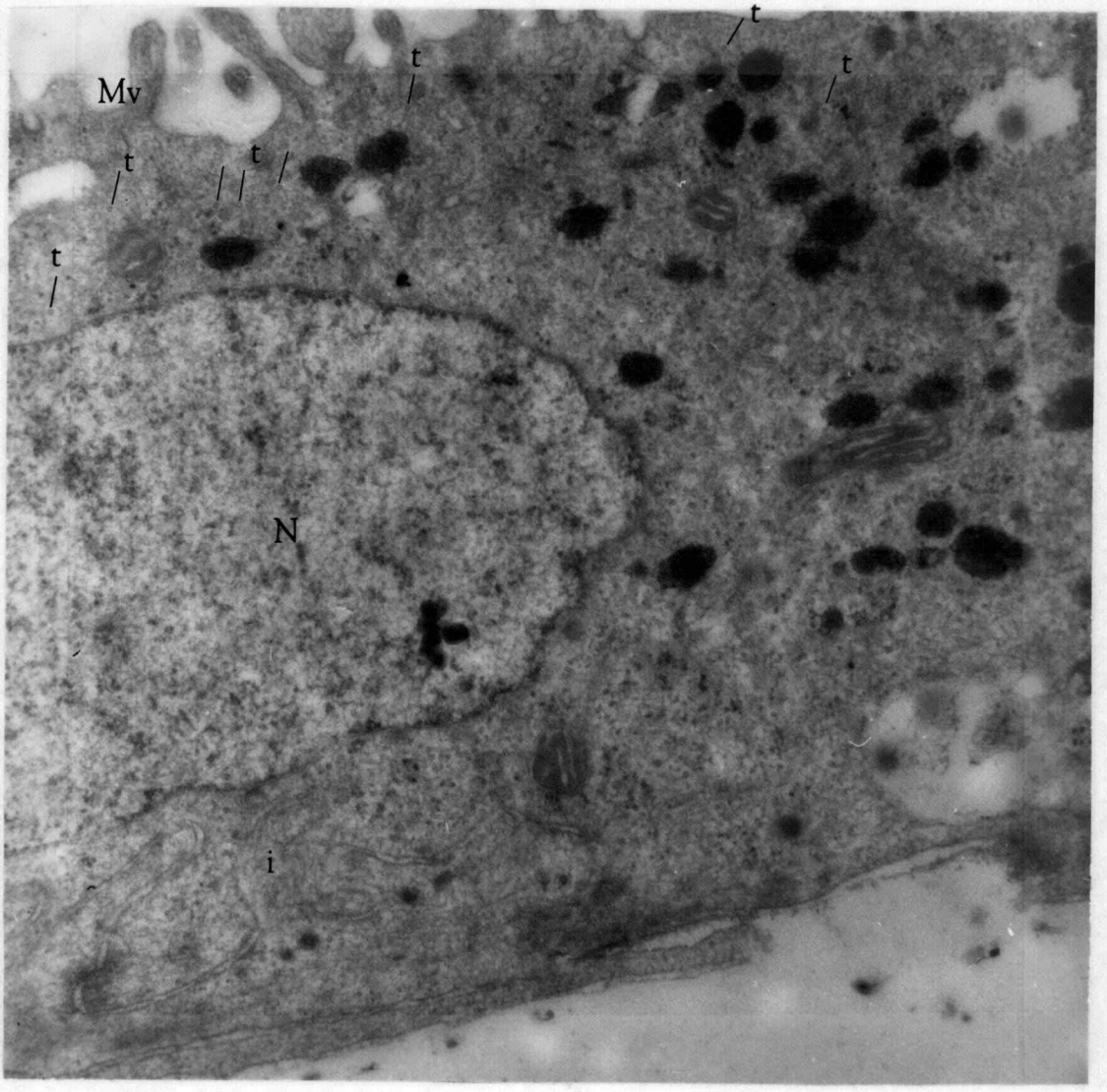

Mv
t
t
t
t
t
t
N
i

Figure 10. A contact surface between two untreated cells showing the mortice and tenon type contact (arrow). Nucleus (n); Lipid droplet (l). (9000 x)

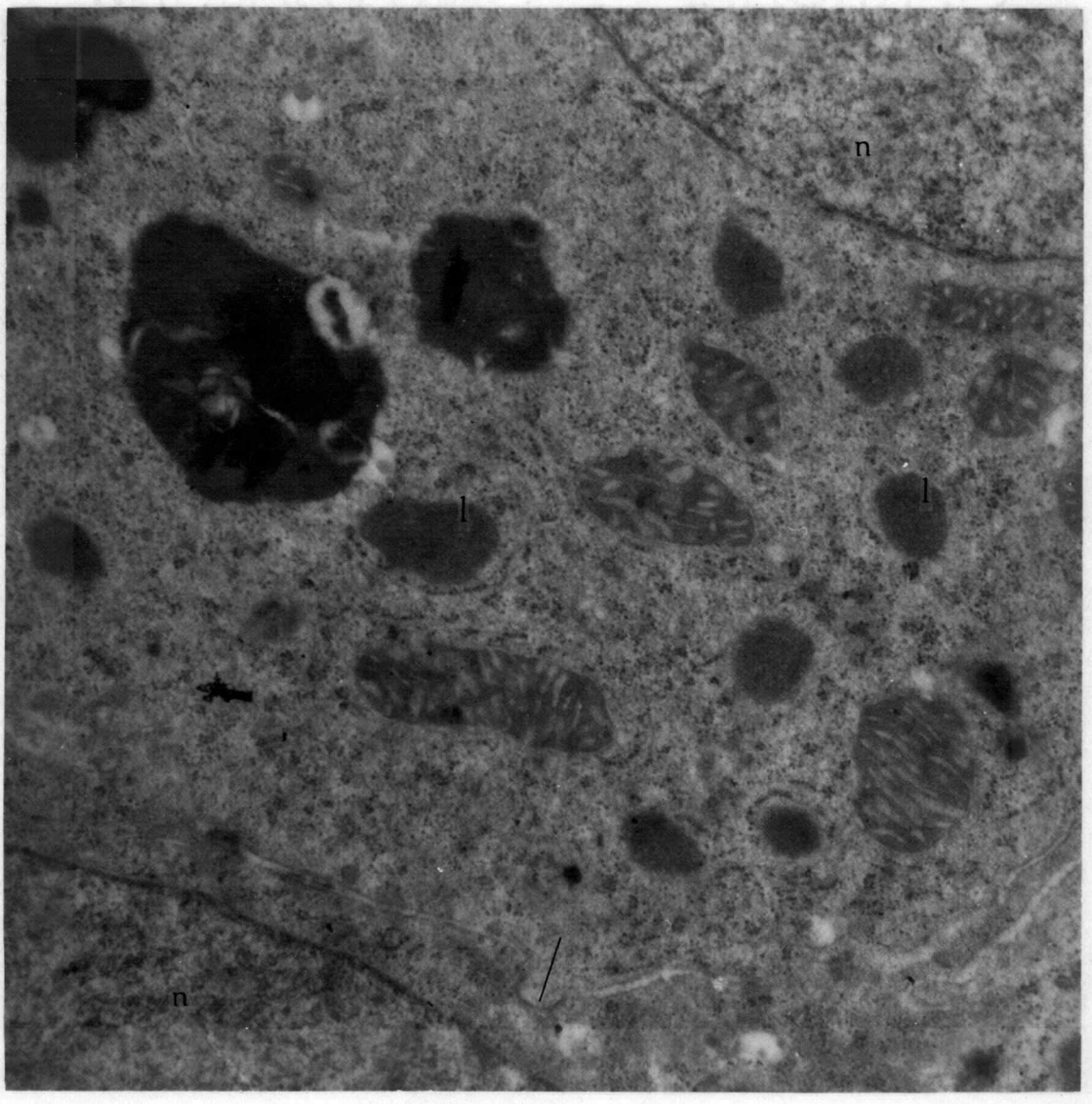

n
l
l
n

Figure 11. An untreated culture showing a desmosomal contact site (d) with extensive tonofibril elements (f). (60, 000 x)

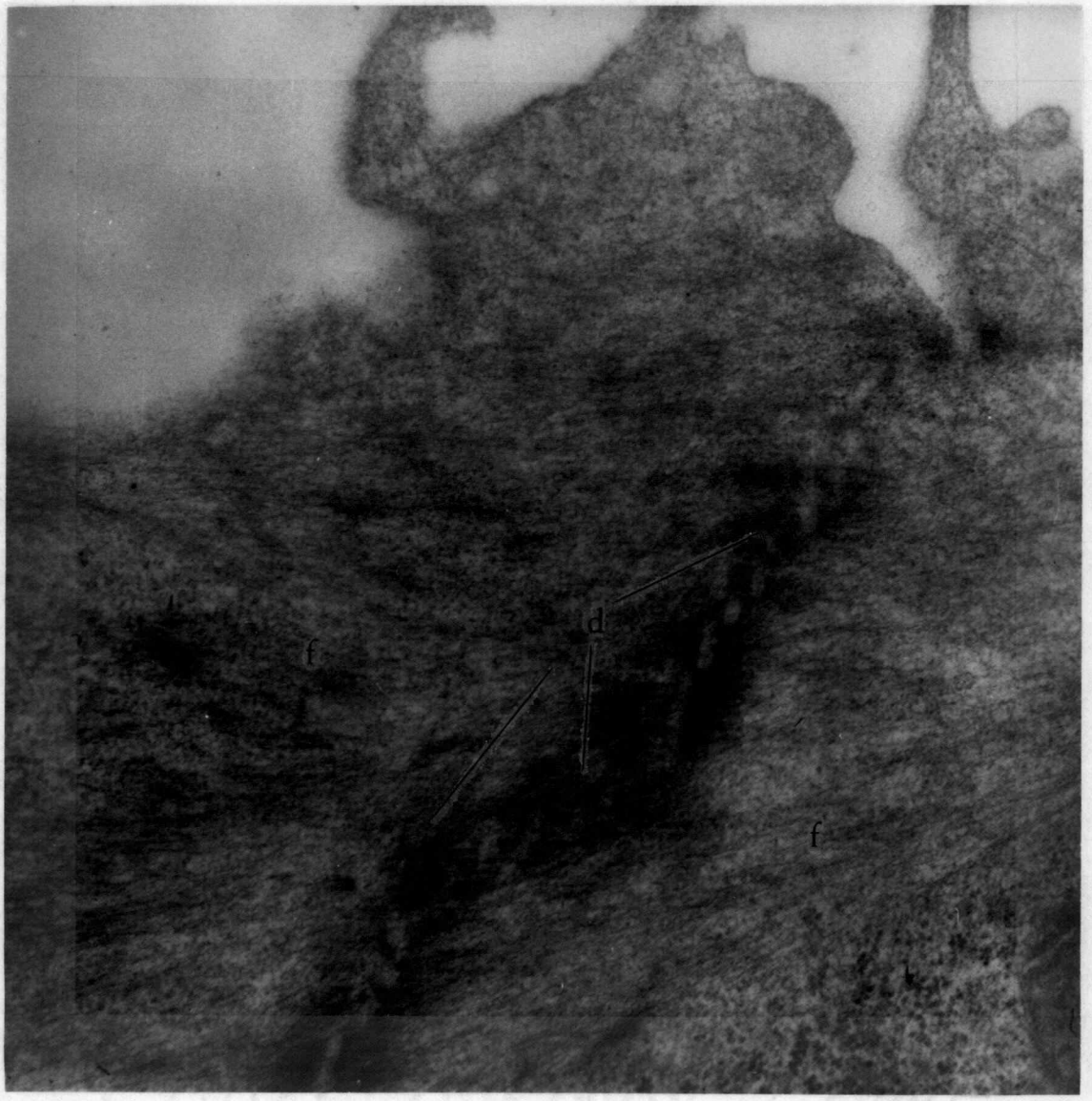

d
f
f

Figure 12. Portions of two untreated cells between which lie depositions of dense intercellular material (arrows) Micropinocytotic vesicles (v) are shown beneath the plasma membrane. Microtubules (t); Nucleus (n); Nucleolus (nu). (9000 x)

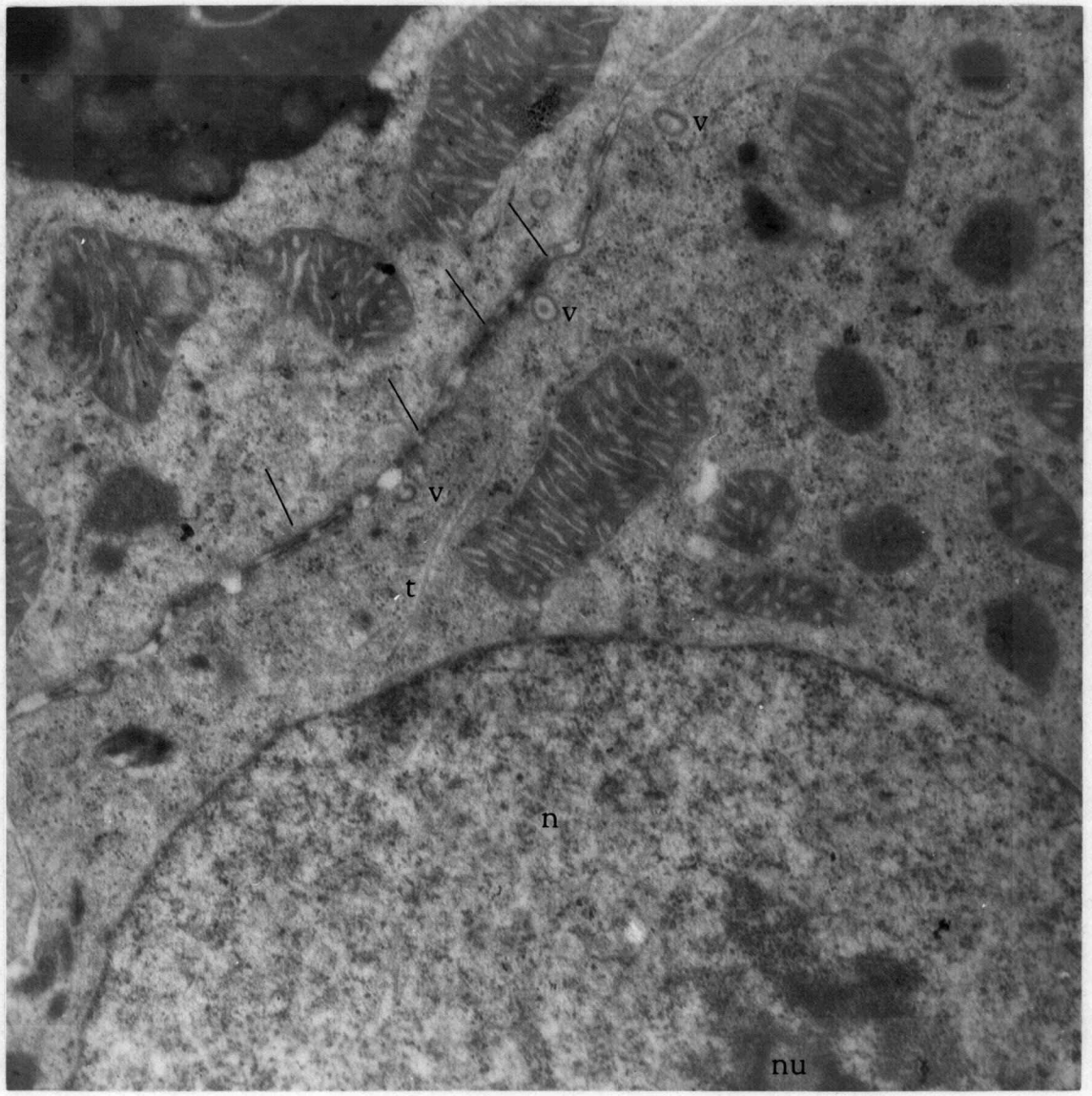

v
v
v
t
n
nu

Figure 13. A portion of the cytoplasm of a cell from a control culture showing fragments of endoplasmic reticulum (er), mitochondria (m) and a microtubule (t). Microvilli (Mv) are present on the surface and a variety of polyribosome configurations are illustrated (arrows). (16, 500 x)

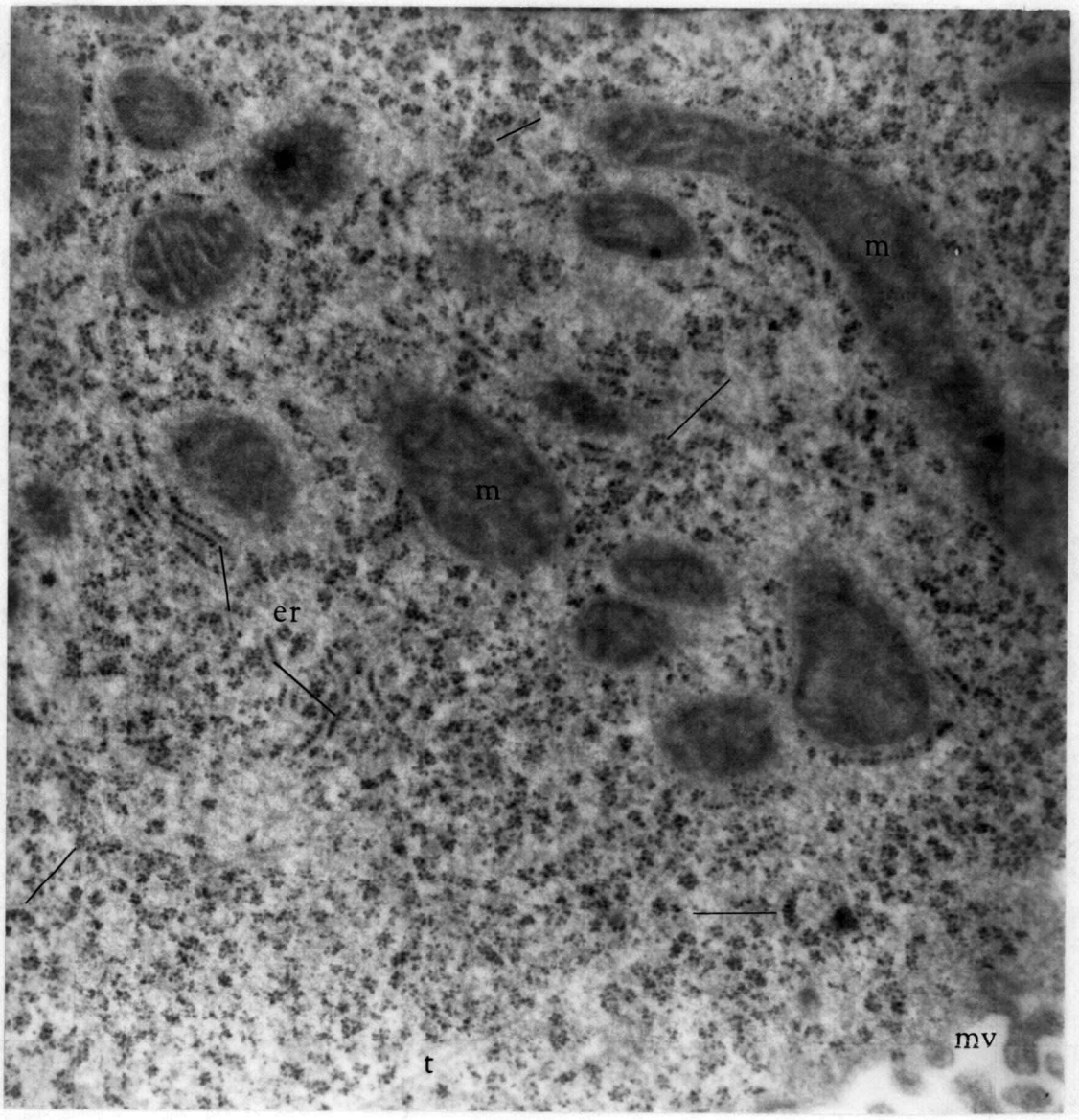

m
m
er
t
mv

Figure 14. Portions of three untreated cells. Note the orientation of the microtubules (t) and the mitochondria (m) is parallel to the long axis of the center cell. A network of delicate filaments (f) occupies the lower left corner of the figure. The arrow indicates a site that may be a contact between the microtubules and a mitochondrion. Lipid droplet (l). (9000 x)

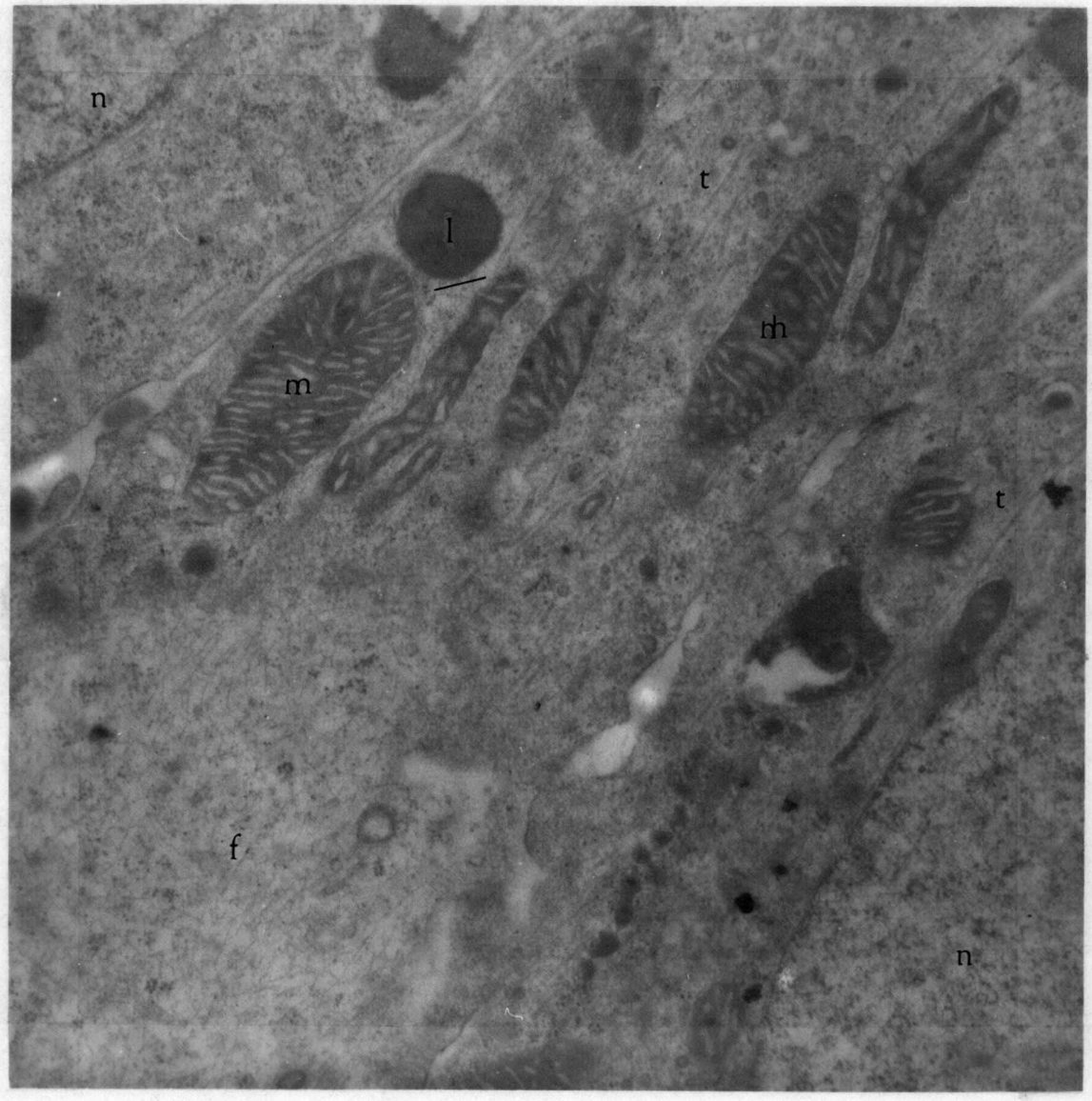

n
t
l
m
m
t
f
n

Figure 15. Orientation of microtubules (t). Note their relation to the edge of the cell, the longitudinal axis of the cell process and the orientation of the mitochondria (m). Lipid droplets (l). (16,500 x)

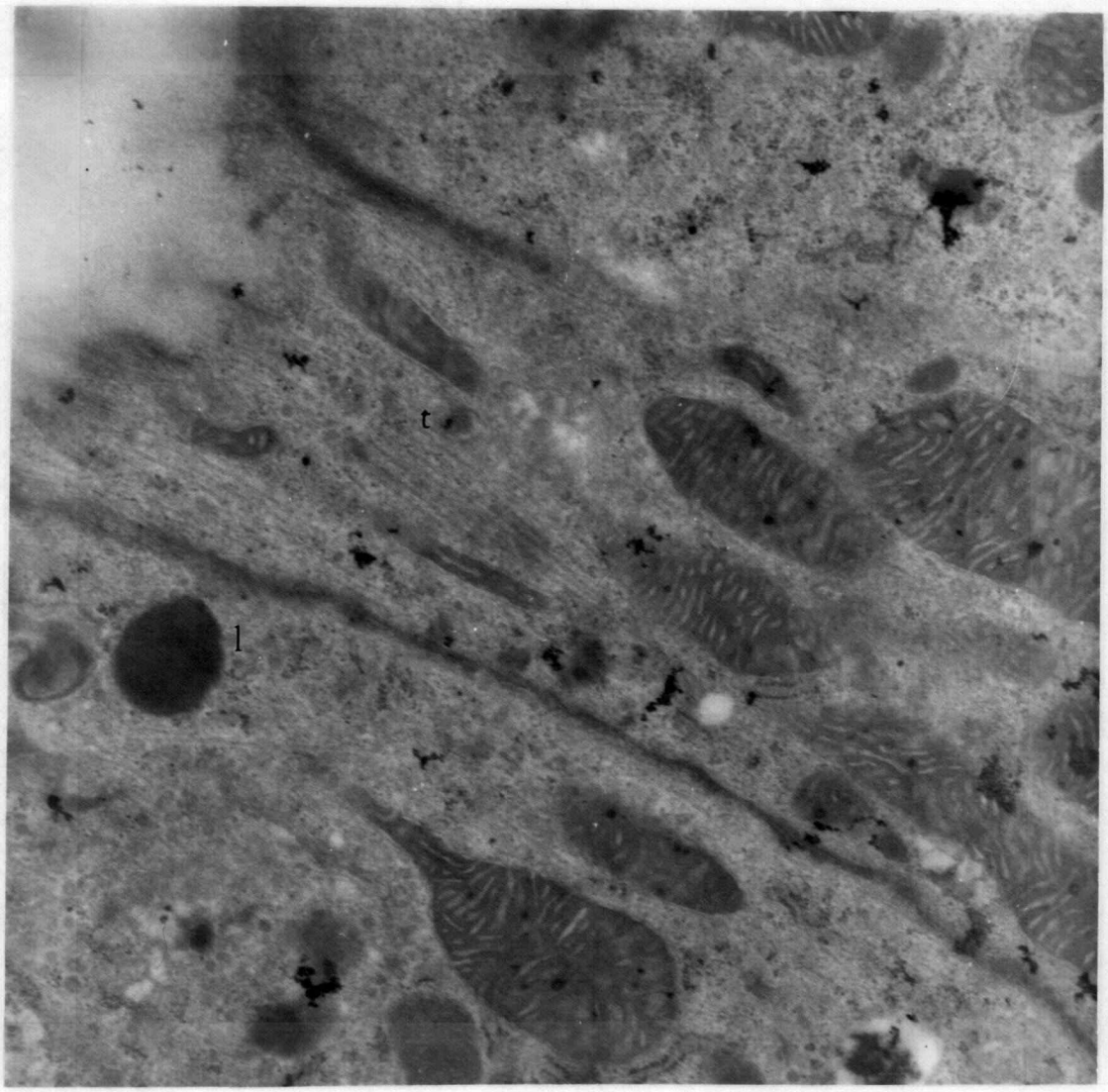

t
l

Figure 16. An array of microtubules (t) frequently observed in the juxtanuclear area. Nucleus (n); Nucleolus (nu); Endoplasmic reticulum (er). (9000 x)

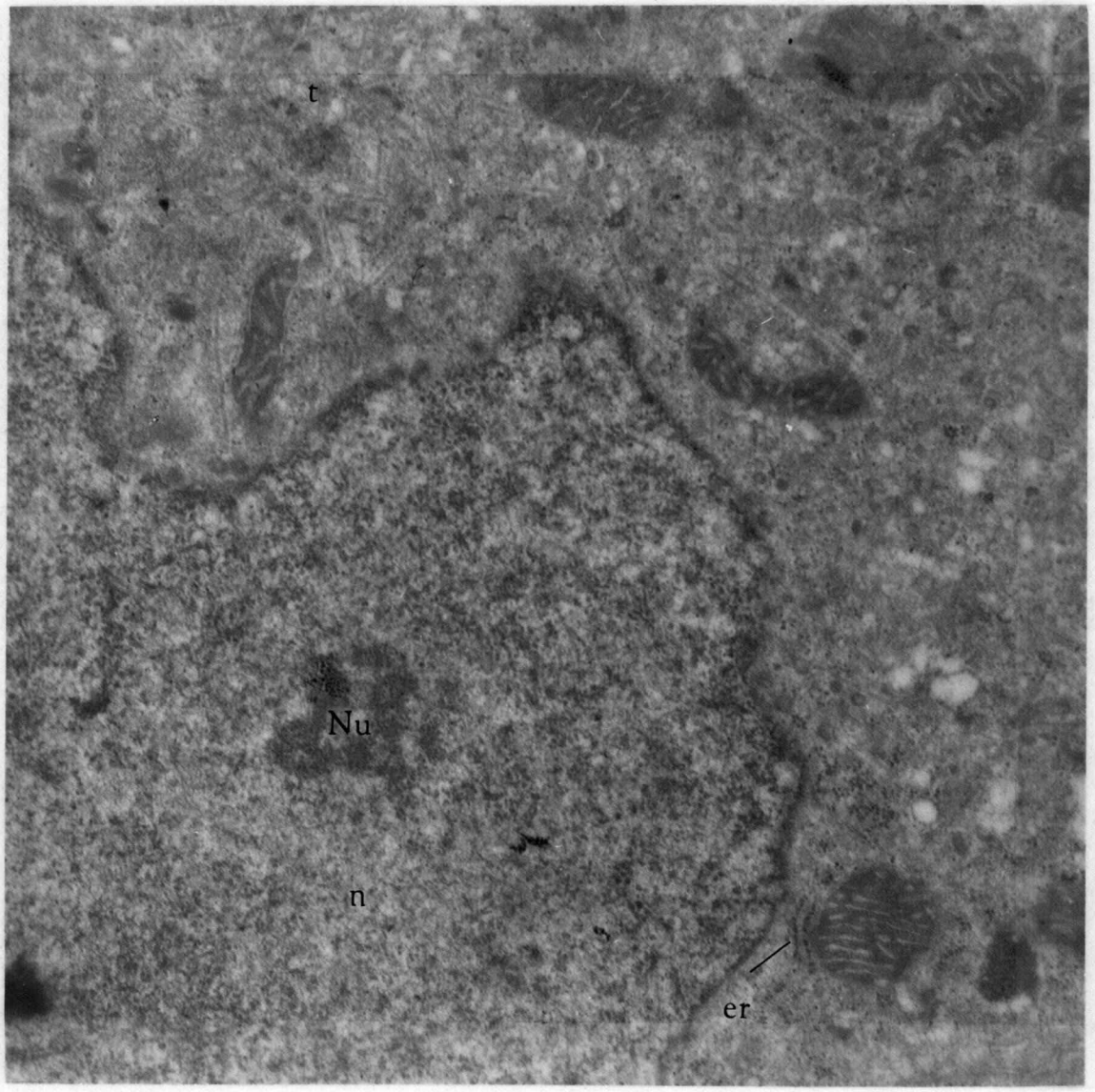

t
Nu
n
er

Figure 17. A portion of an untreated cell showing the association of the centriole (c) with the indented nuclear area. Microtubules (t) radiate from a dense body (arrow) which may be a paracentriolar satellite. Nucleus (n); Nucleolus (nu); Mitochondrion (m); Endoplasmic reticulum (er). (16, 500 x)

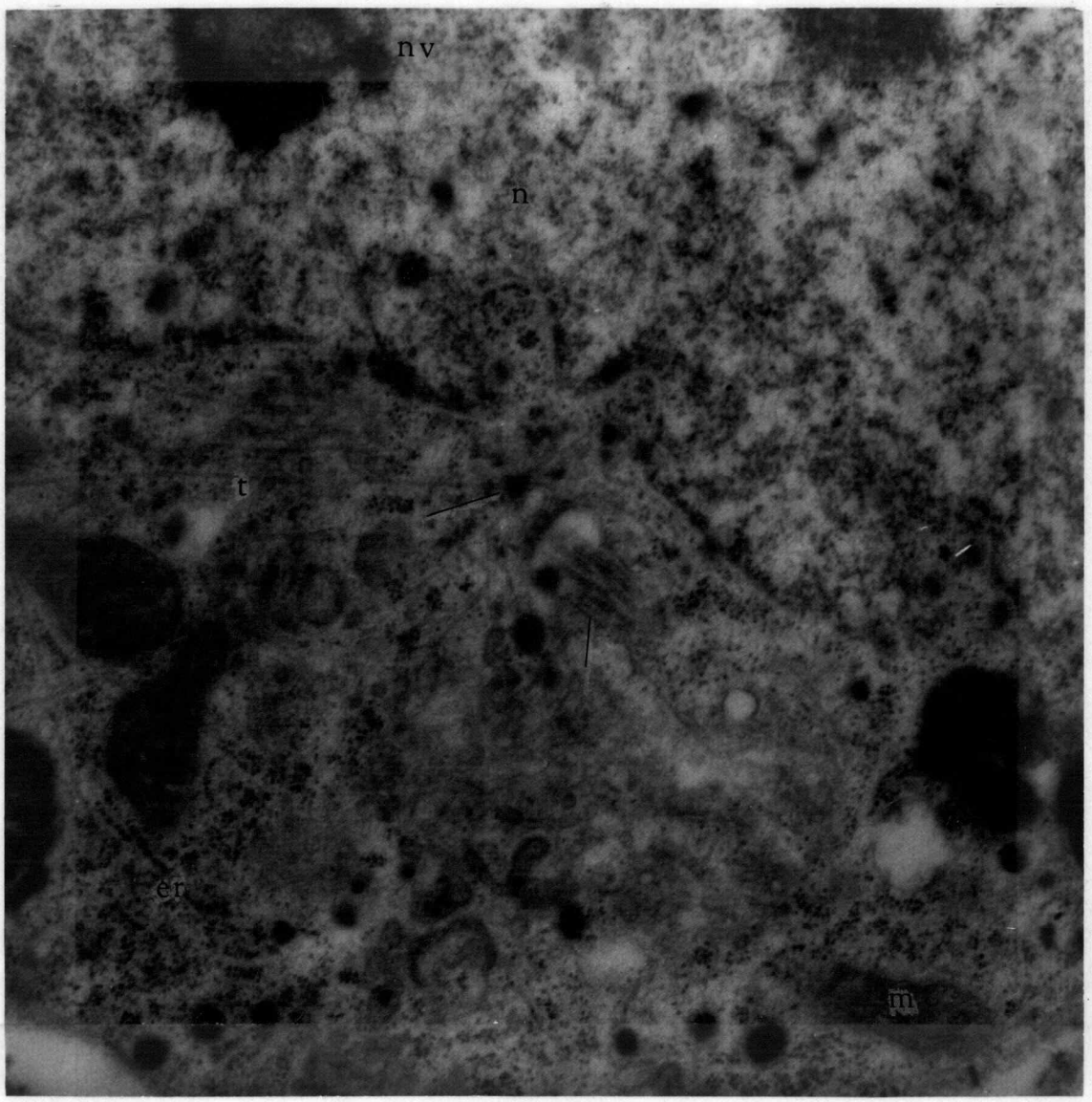

nv
n
t
er
m

Figure 18. (a). The relationship between the paracentriolar bodies and the centriole cylinder. Microtubules (t) are shown radiating from these bodies. Partial separation. (60, 000 x)

(b) Lower magnification of the same centriole as in (a). Note the relatively clear region around the centriole. (18, 000 x)

(c) Section of an untreated cell showing the limited Golgi zone (g). Centriole (c). Partial separation. (18, 000 x)

(d) The edge of a cell sectioned through the basal layer of filaments (f). (4500 x)

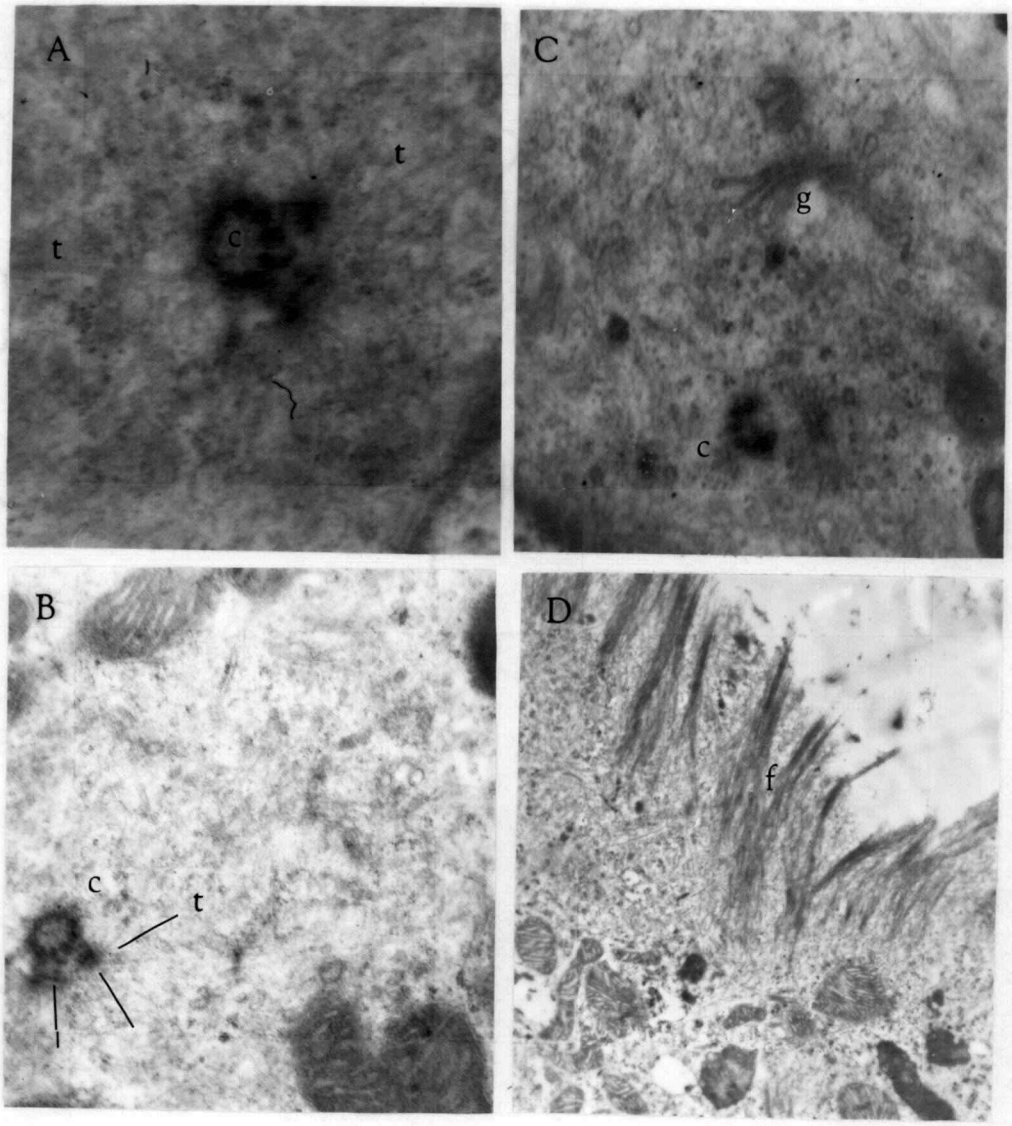

A
t
t
c
C
g
c
B
c
t
D
f

Figure 19. Various expressions of the rudimentary cilia observed in cultured chick renal epithelium.

(a) A small ciliary vesicle (cv) is present at the end of the one member of the pair of centrioles (c). Note the dense body (arrow), the paracentriolar body from which microtubules (t) radiate. Fully recovered cell. (40, 000 x)

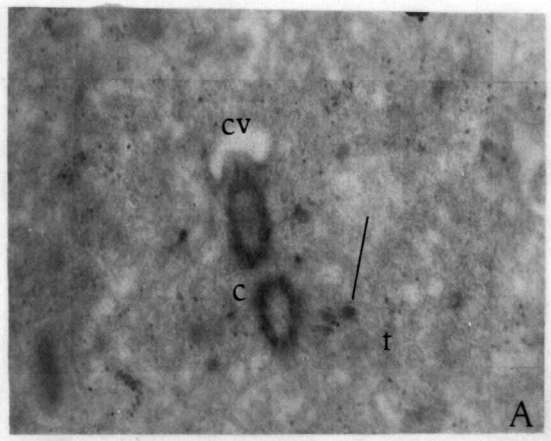
(b) Higher magnification showing a similar ciliary vesicle (cv). Centriole (c) (50, 000 x)

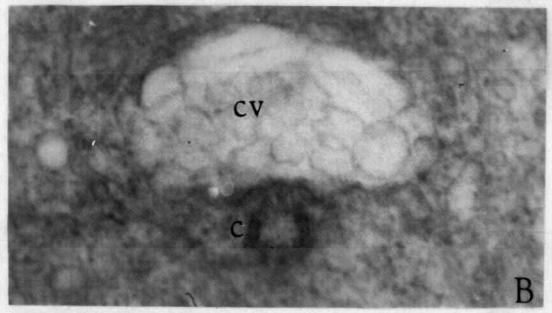
(c) A small cilium extending into the ciliary vesicle (cv) and attached to the centriole (c) from which radiate numerous microtubules (t). Nucleus (n) (12, 000 x)

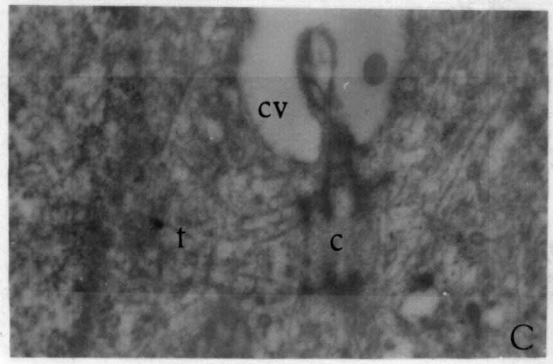
(d) Similar cilium-ciliary vesicle-centriole relationship. Note the formation of ciliary microtubules (t) from one end of the centriole (c) and the radiating microtubules from the other. (50, 000 x)

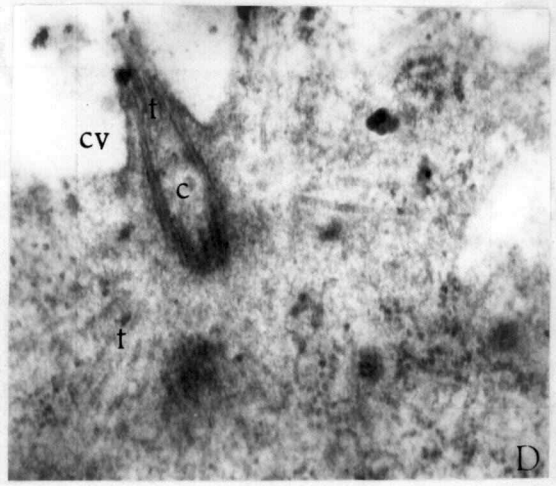
(e) Both centrioles (c) are present. The ciliary tubules (t) appear continuous with those of the centriole and extend into the relatively well developed cilium. Ciliary vesicle (cv). Untreated culture. (40, 000 x)

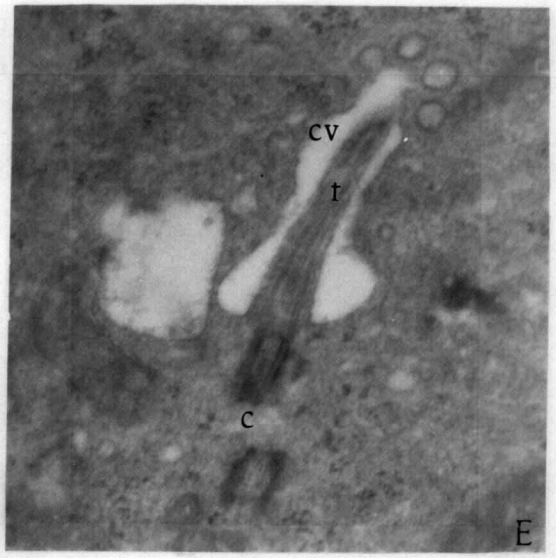
(f) A higher magnification showing the relationship between the centrioles (c), the ciliary microtubules (t), the ciliary vesicle (cv) and the cytoplasmic microtubules (t). Maximum separation. (100, 000 x)


cv
c
t
A


cv
c
B


cv
t
c
C


t
cv
c
t
D


cv
t
c
E

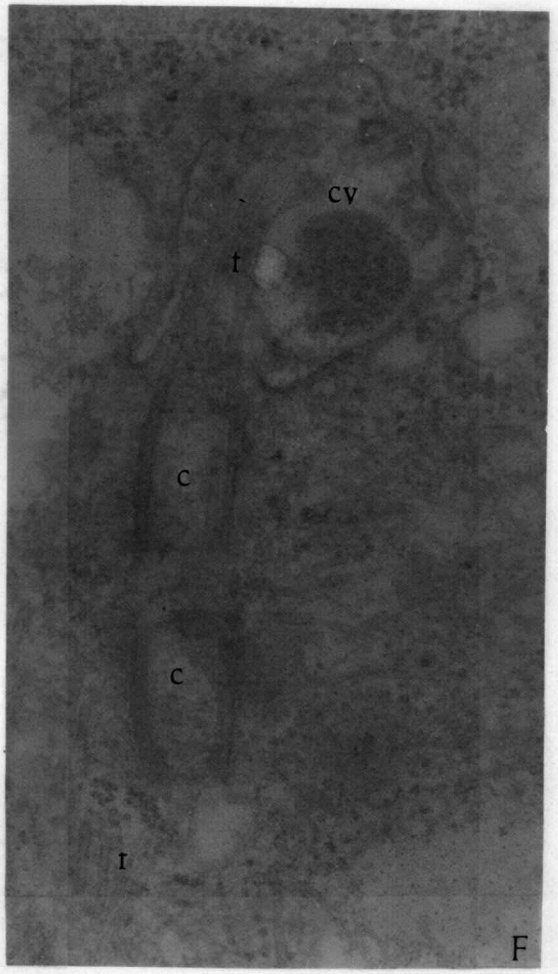

cv
t
c
c
t
F

Figure 20. Untreated cells sectioned close to the substrate showing the orientation of the basal filaments (f). Mitochondria (m); Lipid droplet (l); Microtubule (t). (12,000 x)

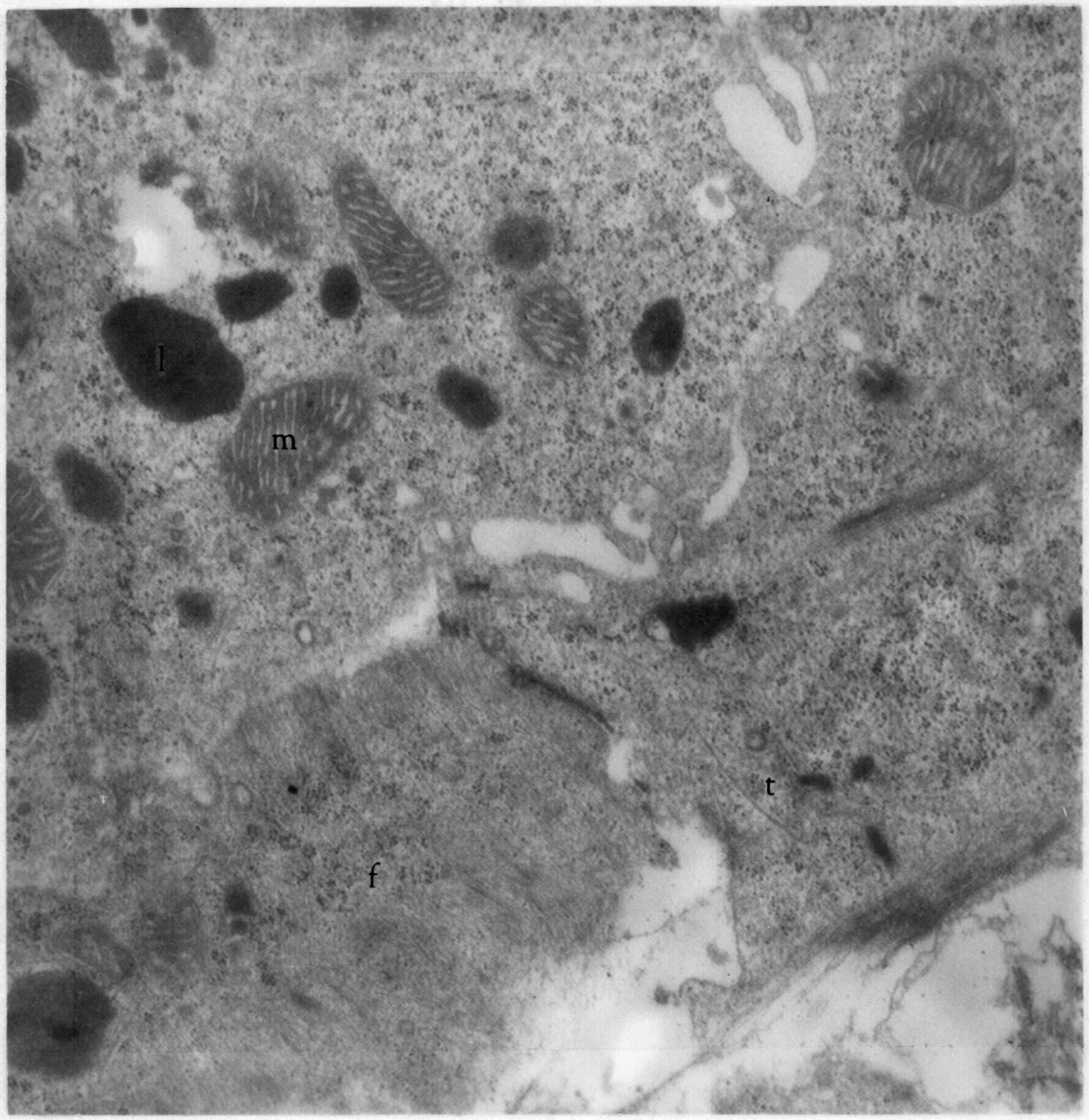

l
m
t
f

Figure 21. All sections taken perpendicular to the plane of growth. The lower edge of the cells represents the surface in contact with the substrate.

(a) The lower portion of the cells show transverse sections of numerous microfilaments (f). Note the dense amorphous material at the lower surface of the cells (arrow). Microvillus (Mv); Mitochondria (m); Lipid droplet (l); Nucleus (n). (18, 000 x)

(b) The basal filaments (f) are sectioned longitudinally while microtubules (t) are shown in cross section. Mitochondria (m); Lipid droplet (l). (18, 000 x)

(c) The filaments (f) are again cut in cross section as are the microtubules (t). (18, 000 x)

(d) The extremely flattened periphery at regions of contact between two cells. Note the extension of the basal filaments (f) and the deposit of dense amorphous subcellular material (arrows). (18, 000 x)

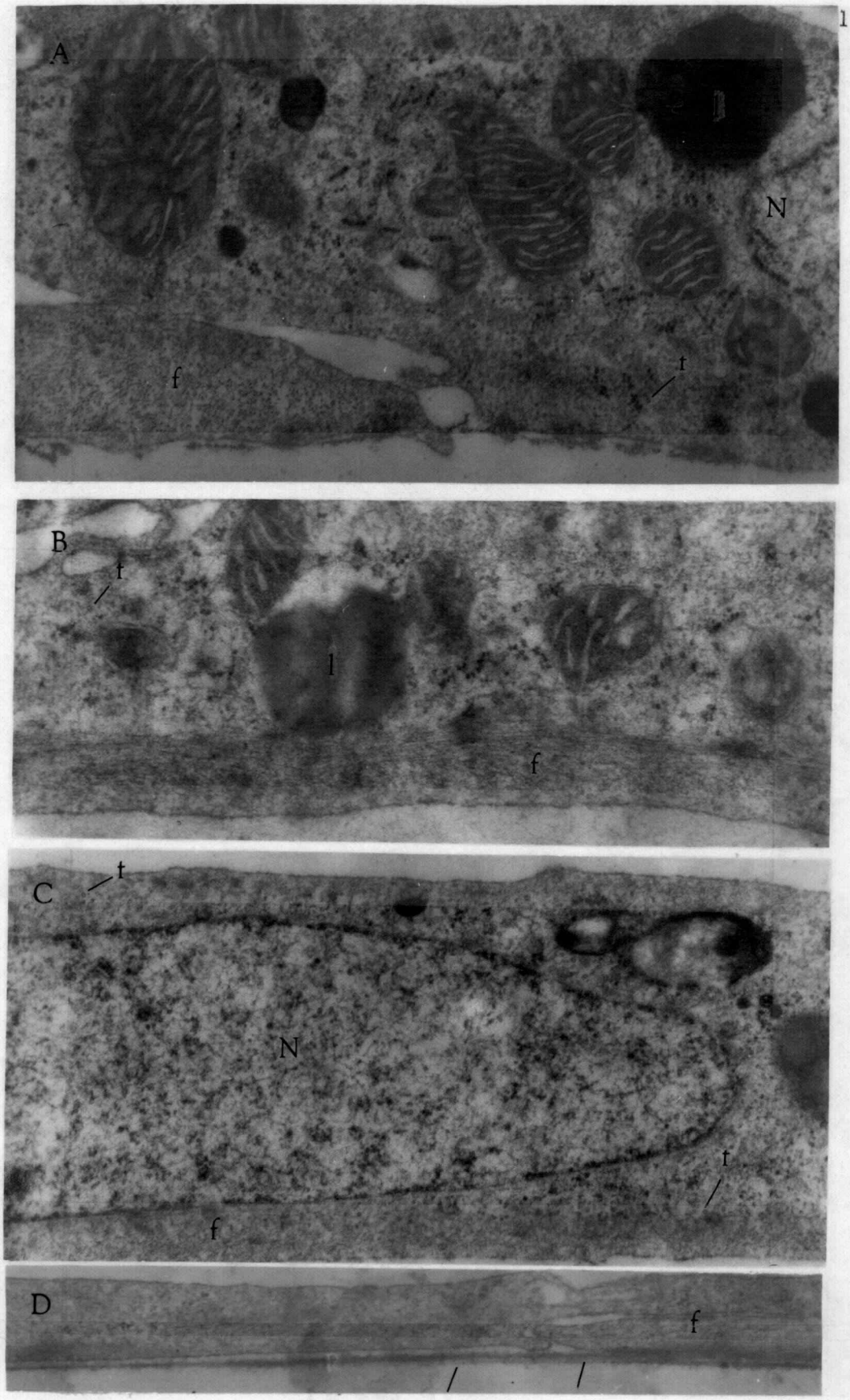

A
N
f
t
B
t
l
f
C
t
N
t
f
D
f

Figure 22. A grazing section of the nuclear membrane of an untreated cell showing the nuclear pores (p), some with opaque central cores. Extremely long chain-like polyribosomes are evident (arrows). Microtubules (t). (24, 000 x)

/

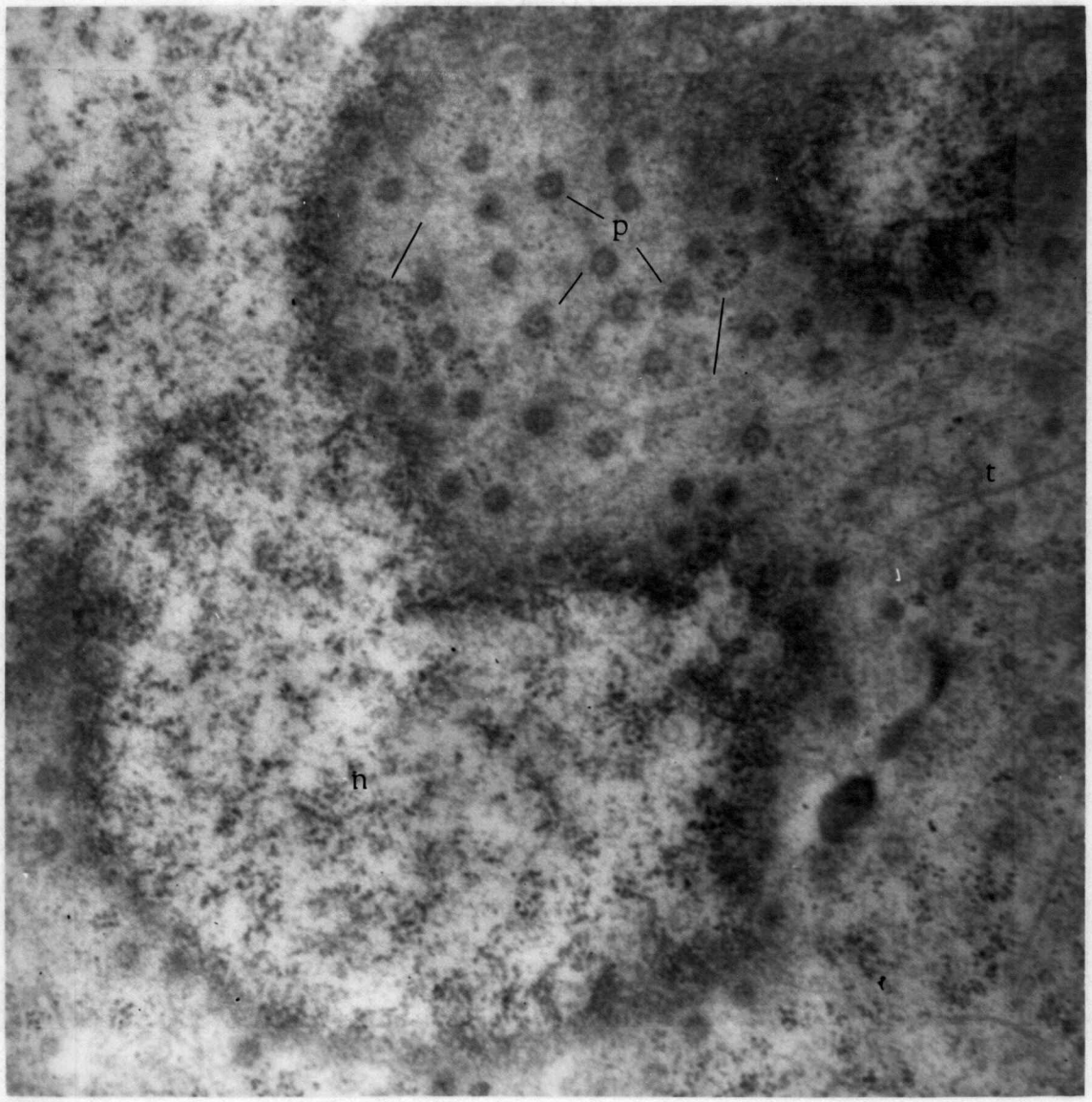

p
t
h
t

Figure 23. A portion of a cell just after application of EDTA. Numerous microvilli (mv) are present and microtubules (t) are abundant between the nucleus (n) and the cell menbrane. Nuclear pores (p). (12, 000 x)

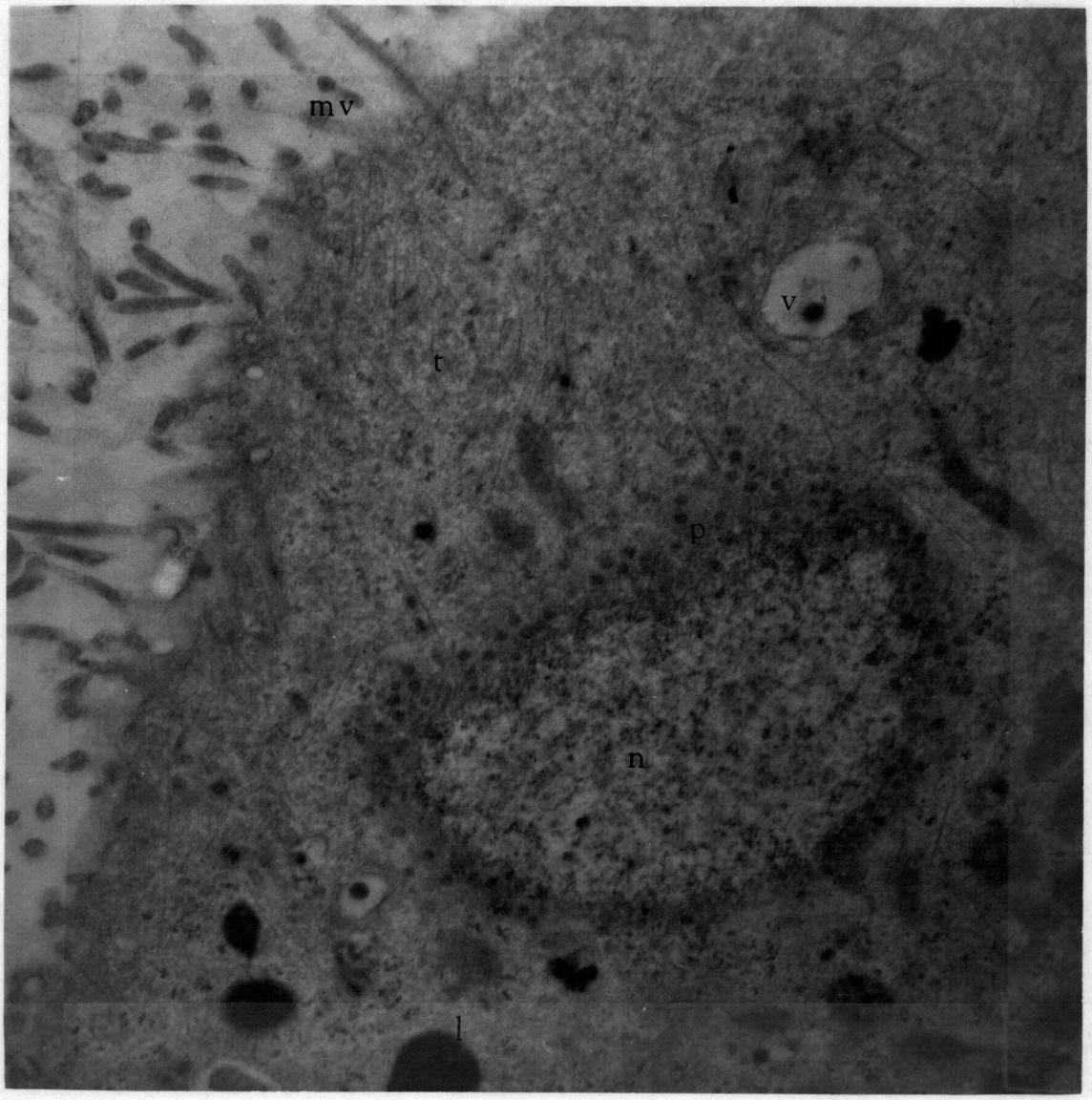

mv
v
t
p
n
l

Figure 24. Peripheral cells of a culture very briefly exposed to EDTA. The peripheral location of the zonula diffusa is shown (arrows). Many microvilli (mv) are shown in cross section in which dense cores may be seen. Nucleus (n). (4000 x)

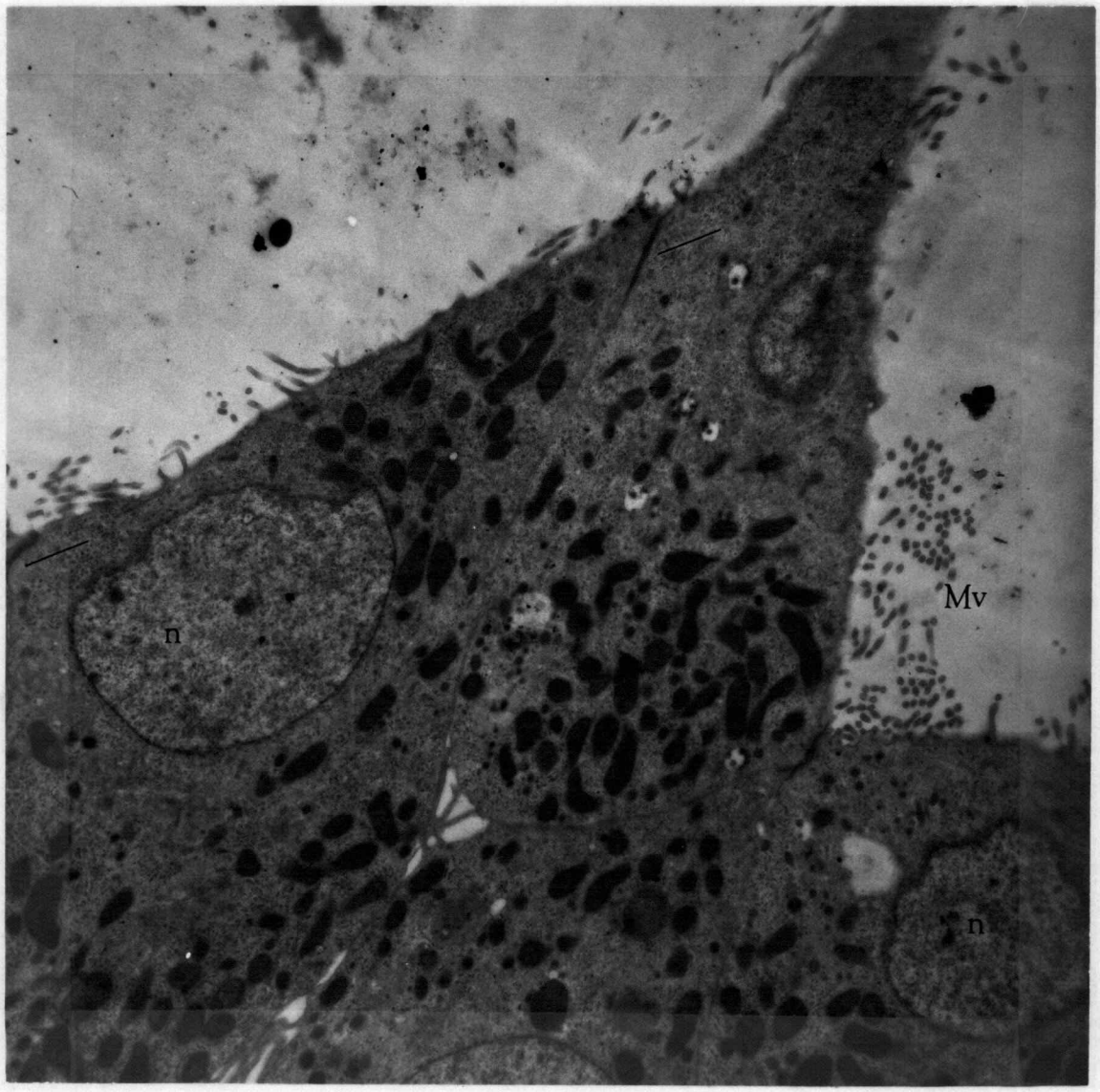

n
Mv
n

Figure 25. Periphery of a culture briefly treated with EDTA. Note the surface microvilli (mv) and the interdigitating processes. The (arrow) depicts a zonula diffusa. Nucleus (n); Nucleolus (nu); Lipid droplet (l). (4000 x)

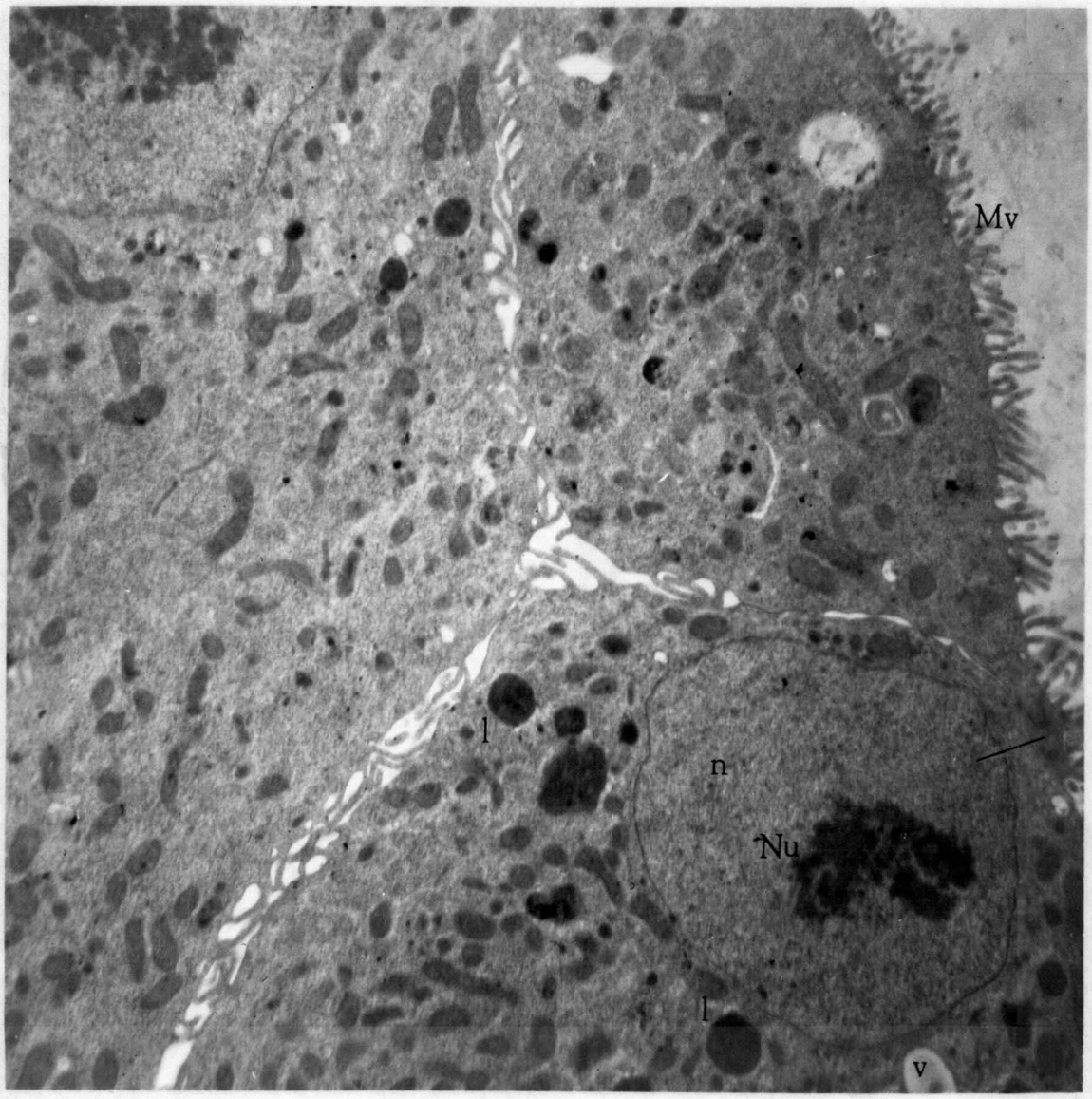

Mv
l
n
Nu
l
v

Figure 26. An intercellular space between two cells subjected to brief EDTA treatment. Note the long interdigitating processes. Microtubules (t); Helical polyribosome (h). (30,000 x)

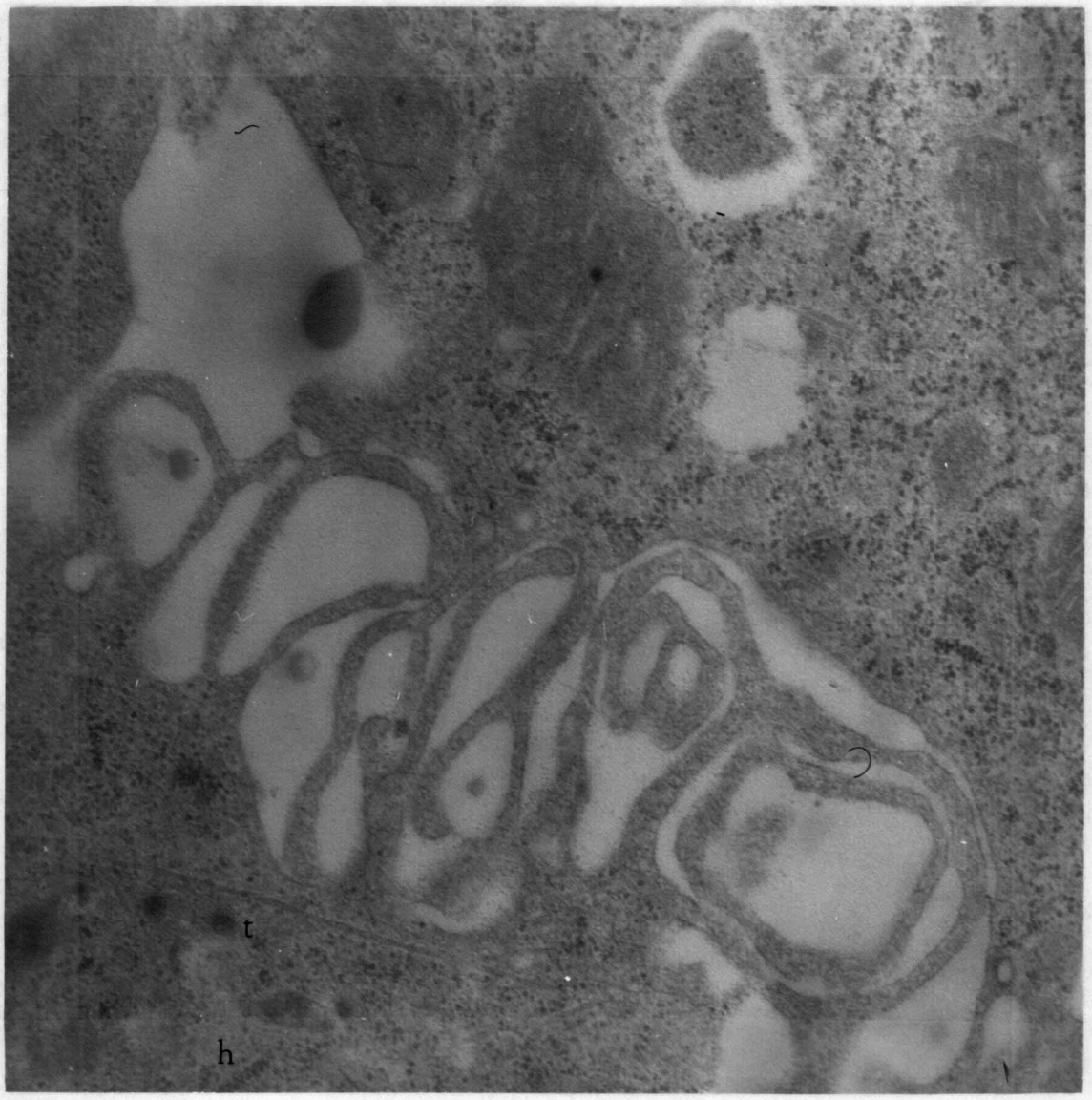

t
h

Figure 27. A desmosome (d) between two cells subjected briefly to EDTA. Note the separation lateral to the attachment plaque. Tonofibrils (f); Microtubules (t). (60, 000 x)

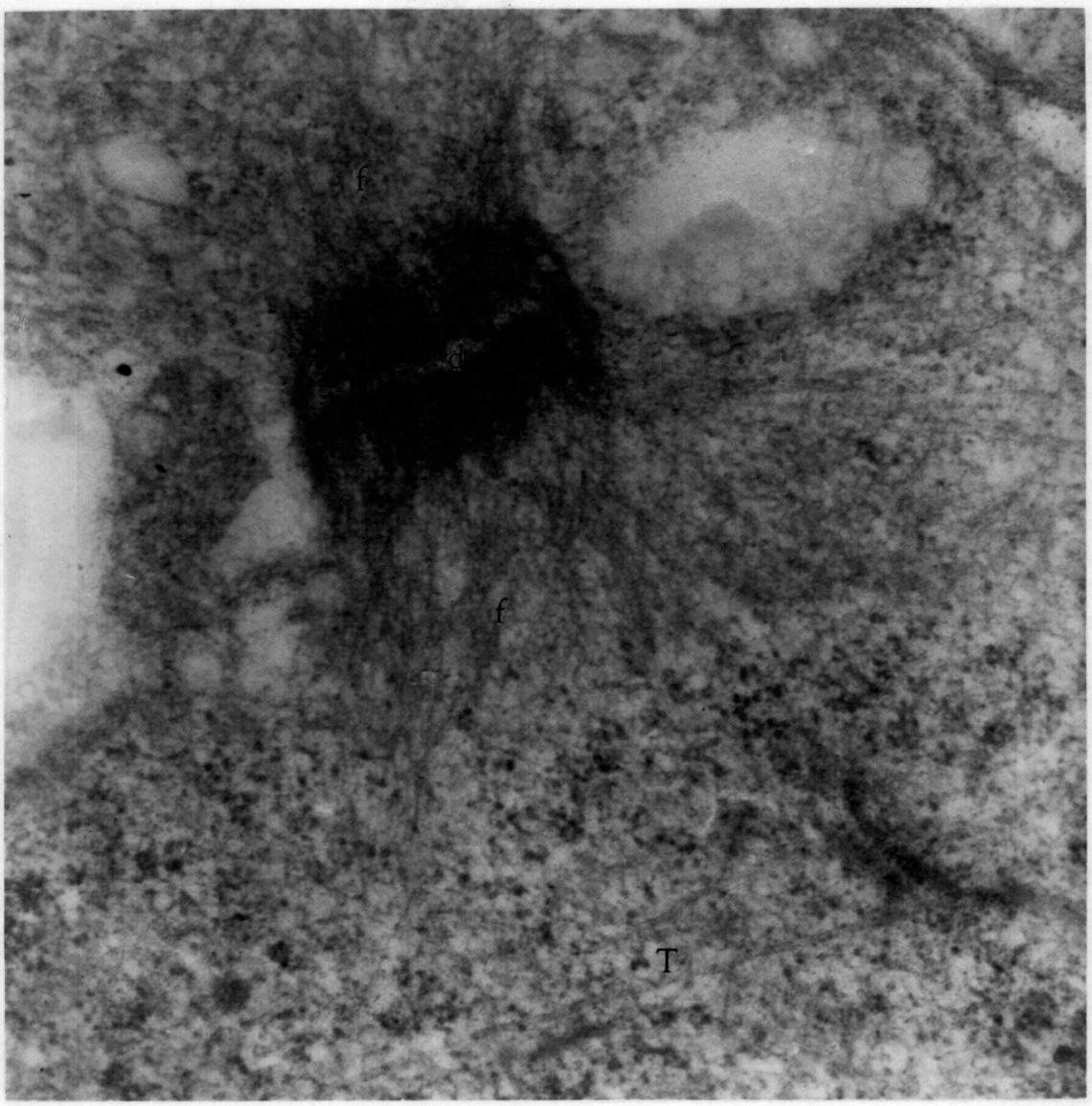

f
f
f
T

Figure 28. Several cells from a culture subjected to partial separation with EDTA. Microvilli (mv) are very apparent on the free surfaces as well as in the intercellular spaces. Nucleus (n). (3000 x)

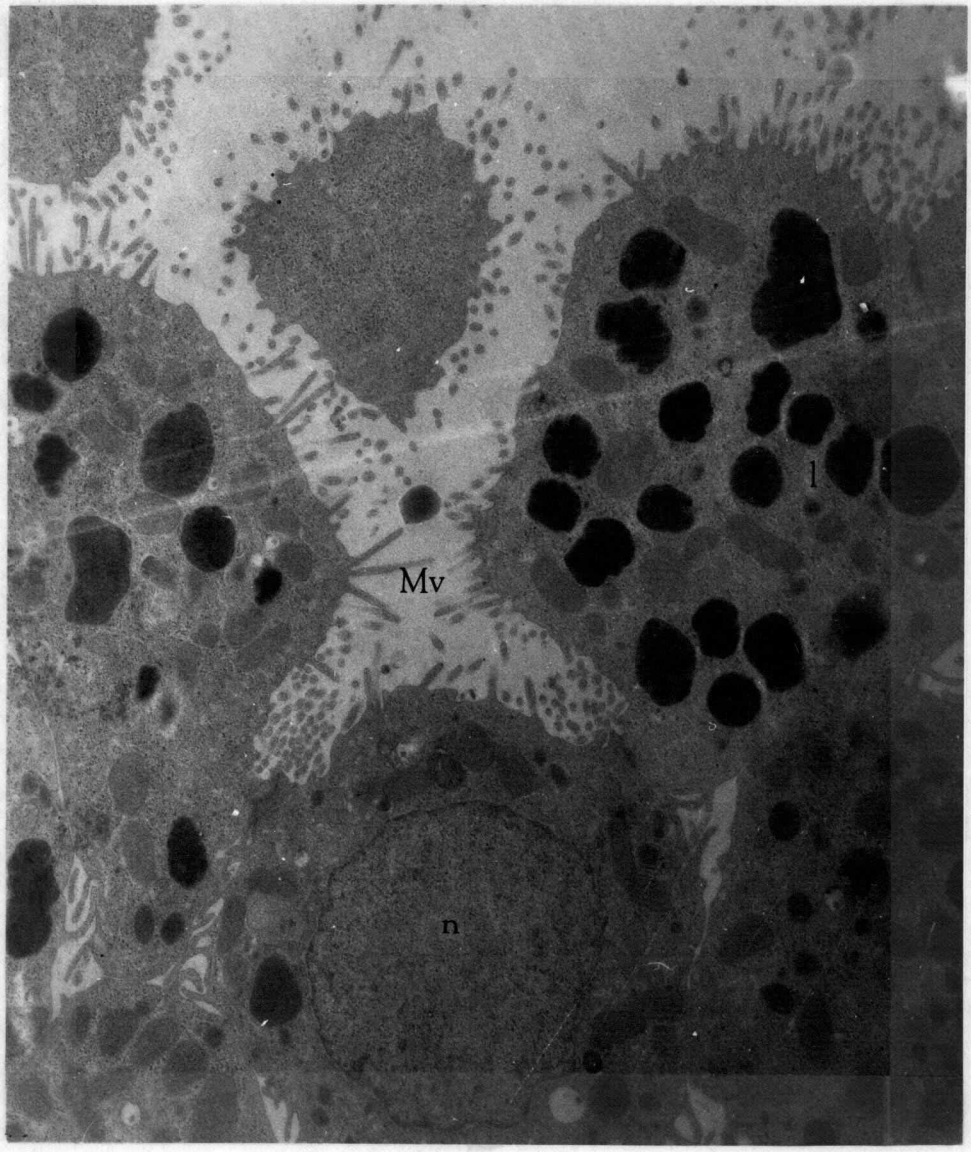

l
Mv
n

Figure 29. A portion of a cell from a culture subjected to partial separation. The nucleus (n) is beginning to show an irregular margin and the granular nature of the nucleolus (nu) is evident. A part of a cilium (ci) is shown extending into the ciliary vesicle (cv). (12,000 x)

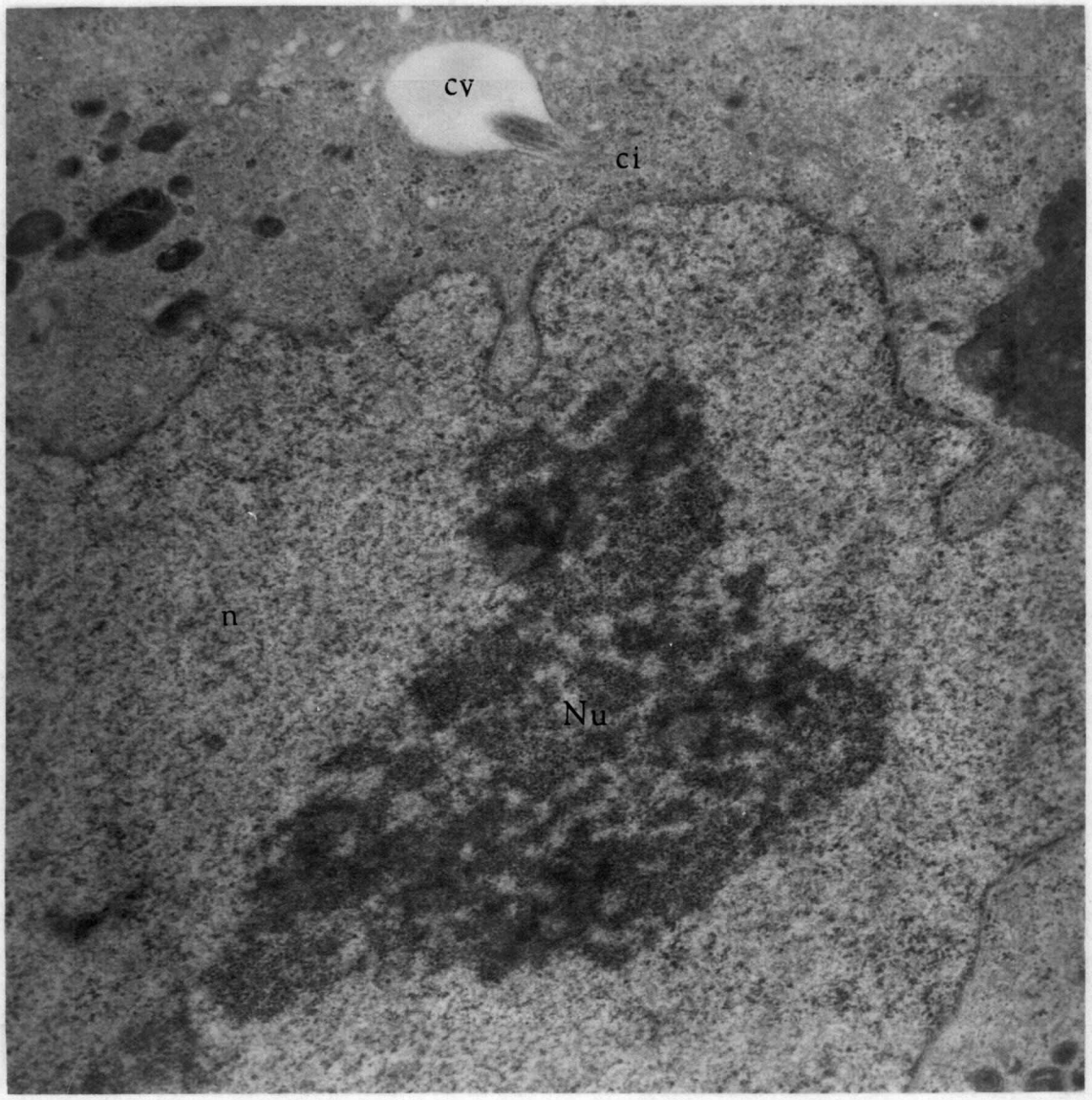

cv
ci
n
Nu

Figure 30. Portions of three cells which have been partially separated with EDTA. Microvilli (mv) are apparent in the intercellular spaces. The desmosome (d) has not relinquished contact while the mortice and tenon structure (arrow) apparently is doing so. Microtubules (t). (18, 000 x)

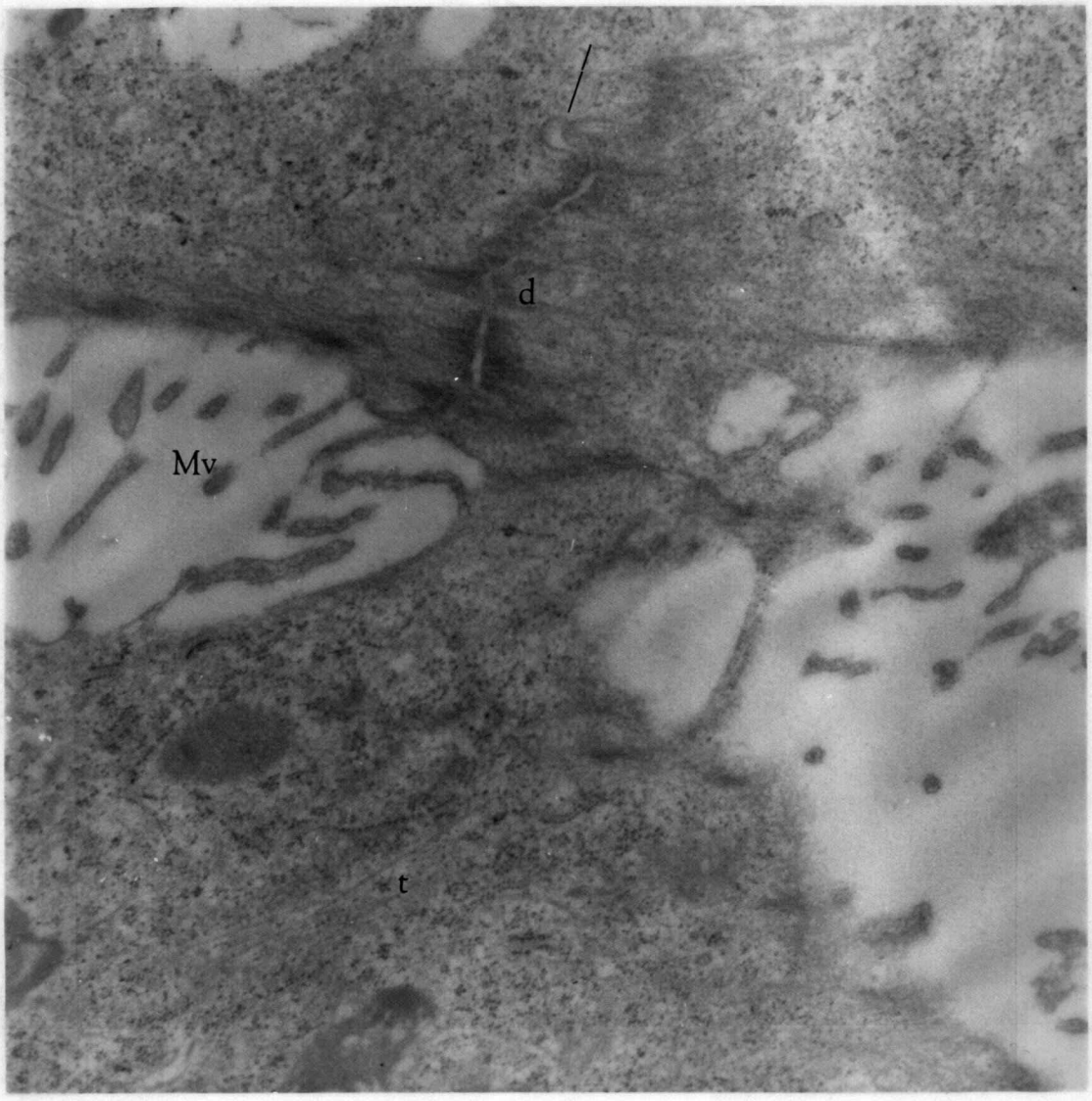

d
Mv
t

Figure 31. Portions of two cells subjected to partial separation. Microvilli (mv) are apparent on the surfaces. The very dense band may represent a zonula diffusa sectioned close to the surface. Two microtubules (t) show a precise parallel alignment. (18,000 x)

MV
t

Figure 32. Cells fixed during the active blebbing stage. Note that the cells are not fully rounded but lobulate in shape. Filaments (f); Nucleus (n); Nucleolus (nu); Mitochondria (m). (4000 x)

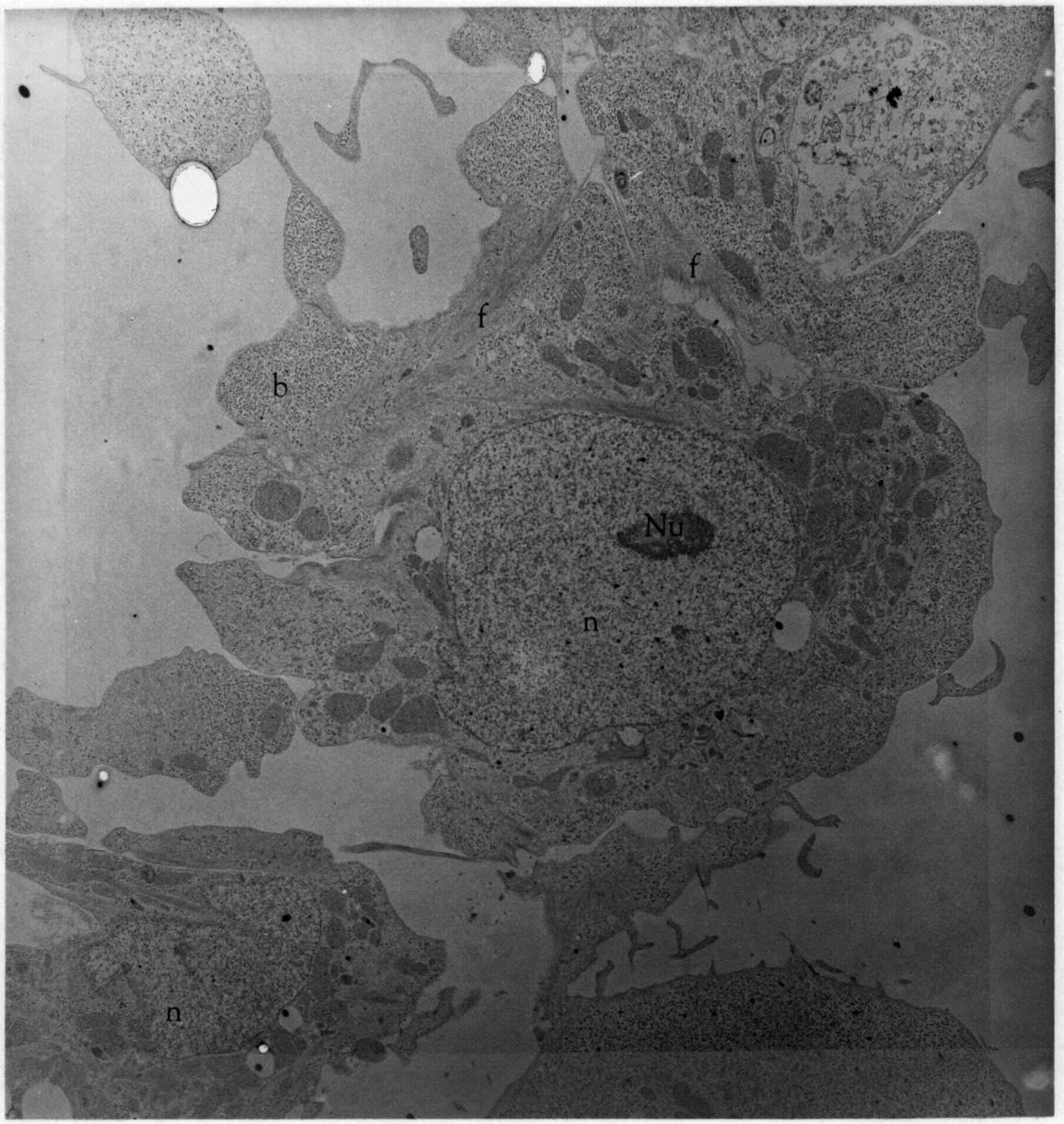

f
f
b
Nu
n
n

Figure 33. The edge of a cell fixed during the active blebbing stage. Note the variation in size and shape of the blebs (b) and their homogeneous nature. Filaments (f); Mitochondria (m). (4000 x)

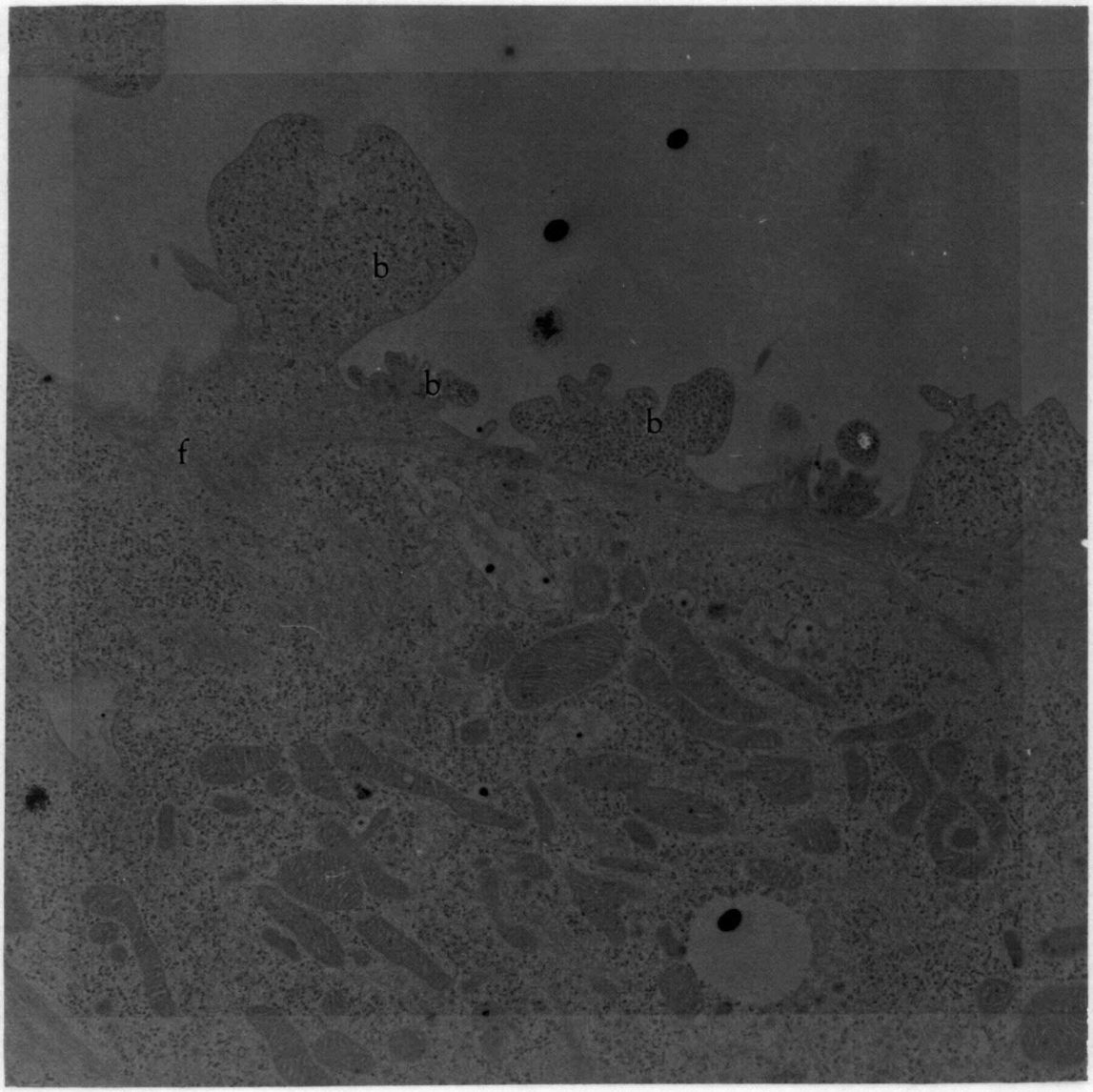

b
b
b
f

Figure 34. Higher magnification of the blebbing surface depicted in Figure 33. Note that the only constitutuents in the blebs are the ribosomes and polyribosomes. Filaments (f); Microtubules (t).

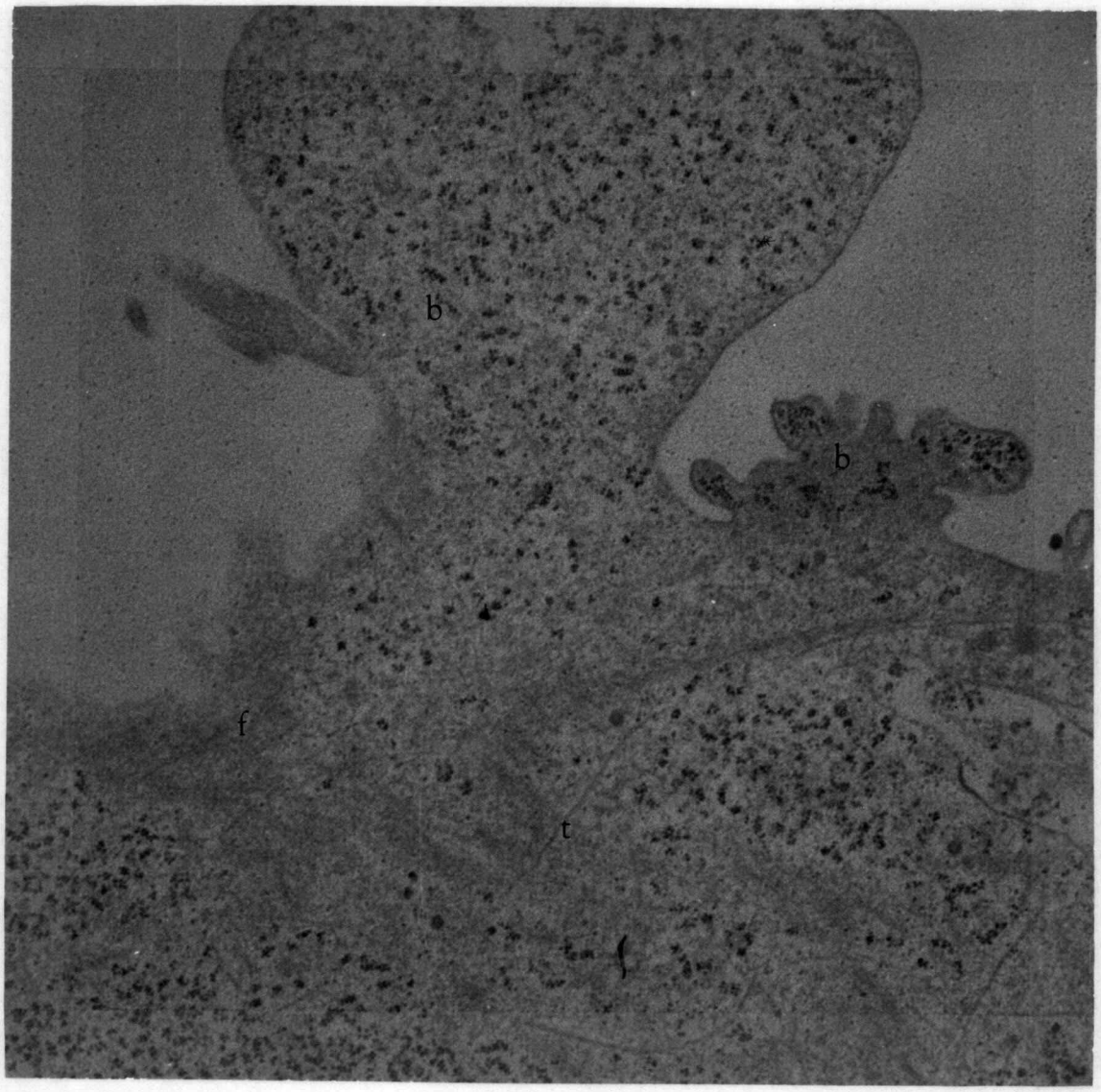

b
b
f
t

Figure 35. An isolated cell at maximum treatment with EDTA. The Golgi lamellae (g), endoplasmic reticulum (er) and nucleolus (nu) appear unaltered. The cytoplasm is vacuolated (v) and the nucleus (n) is compressed. Bleb (b). (5500 x)

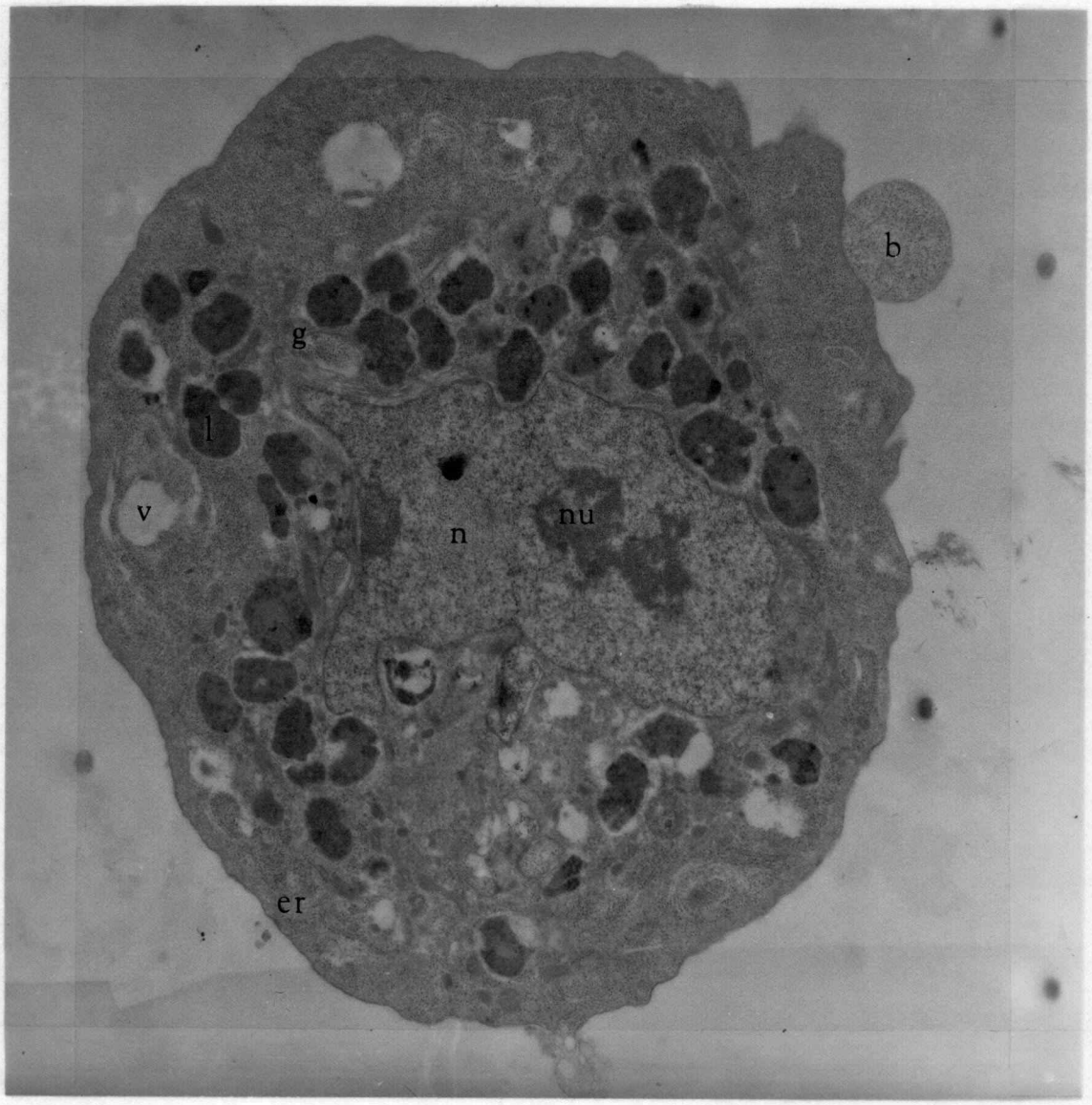

b
g
l
v
n
nu
er

Figure 36. An isolated cell at maximum separation. Note the large homogeneous bleb (b) and the delicate fibrous material running along its base (arrows). The cytoplasm contains numerous vacuoles (v) and the nucleus (n) has an irregular shape. Note the unaltered appearance of the mitochondria (m), the centriole (c) and the microtubules (t) associated with it. (19,700 x)

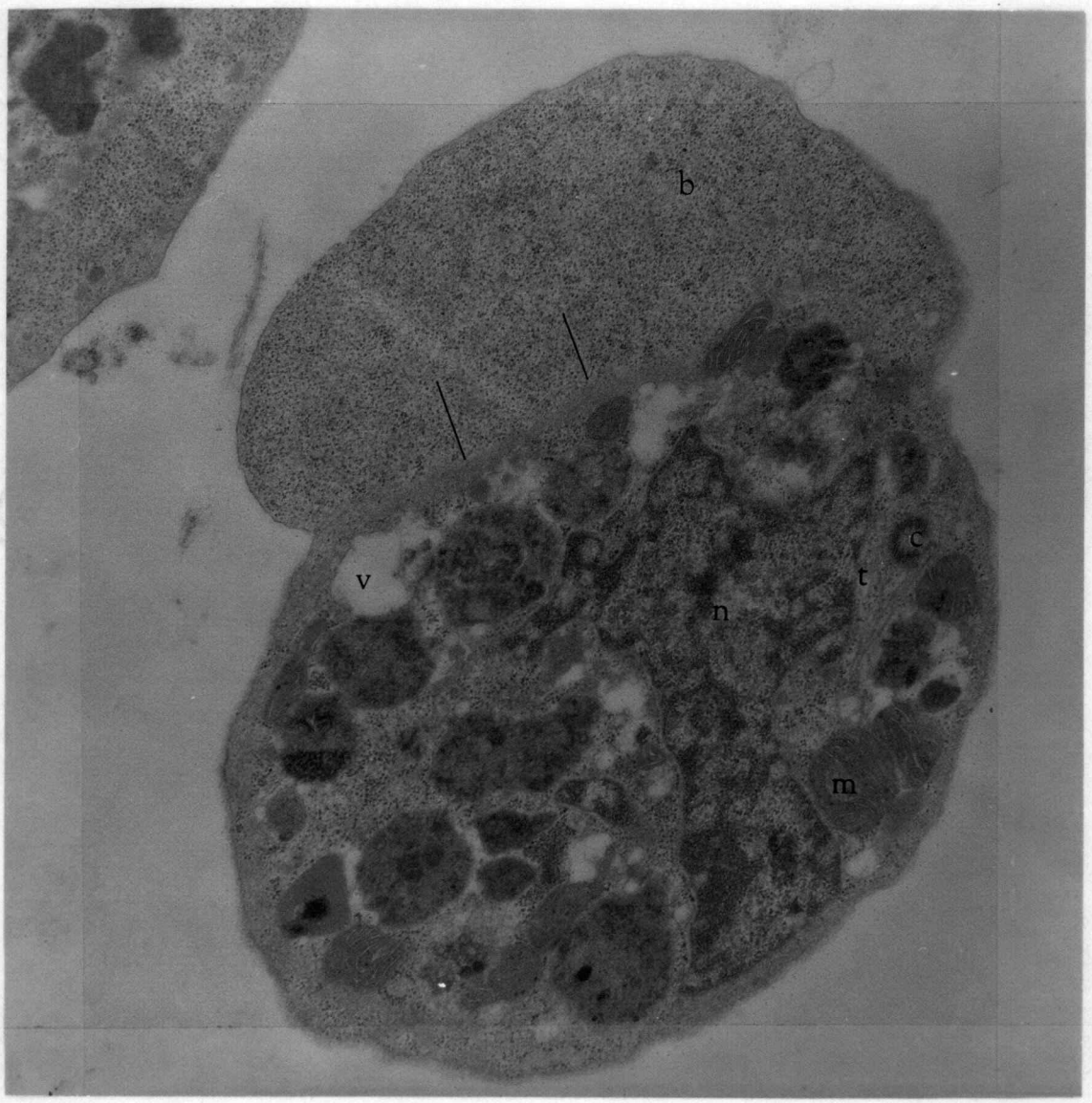

b
v
n
t
c
m

Figure 37. Higher magnification showing a portion of the same cell as in Figure 36. The tubular nature of the centriole (c) is apparent as are the microtubules (t) which appear to originate from a site outside the centriole cylinder (arrow). Mitochondria (m). (57,500 x)

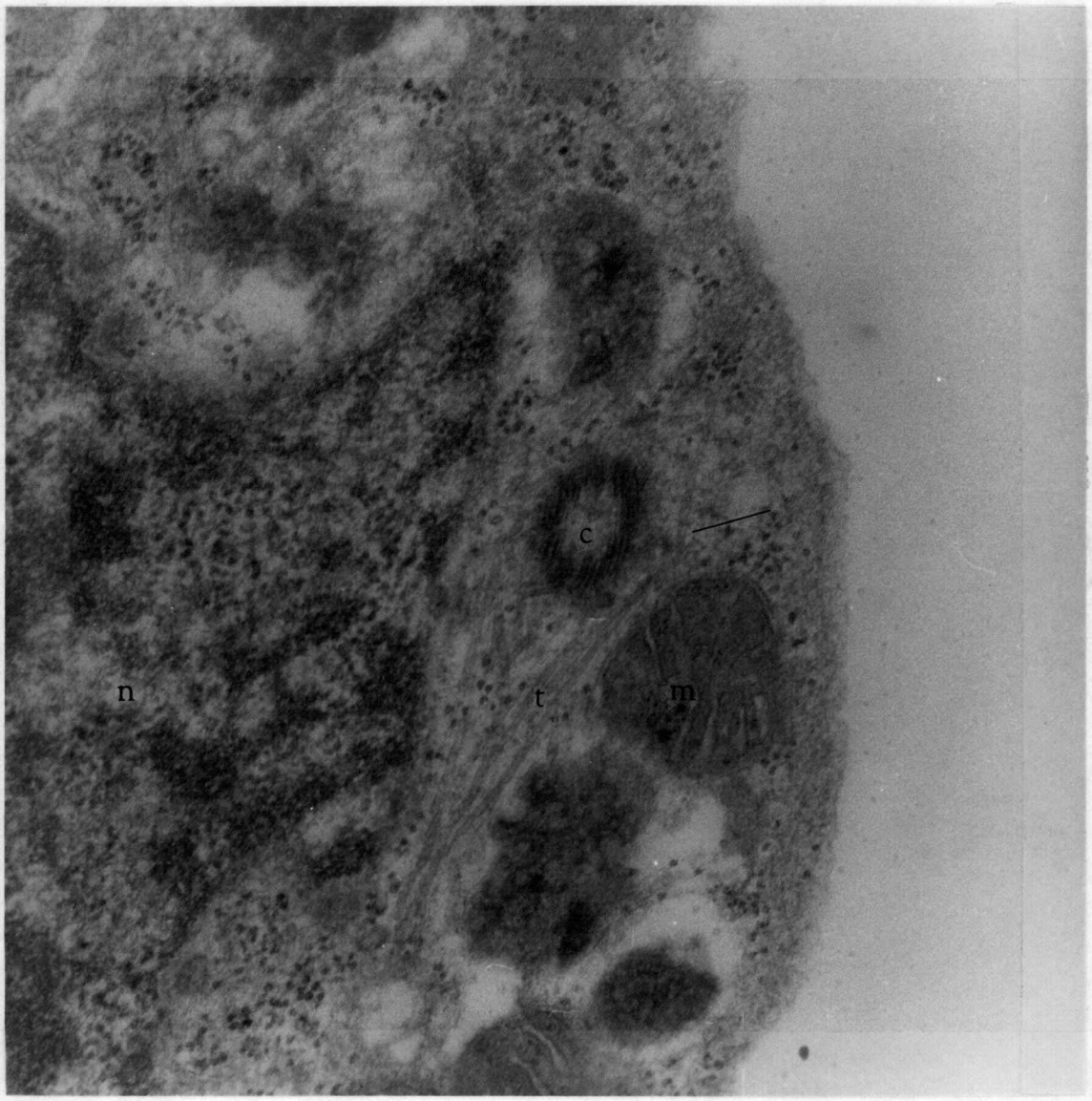
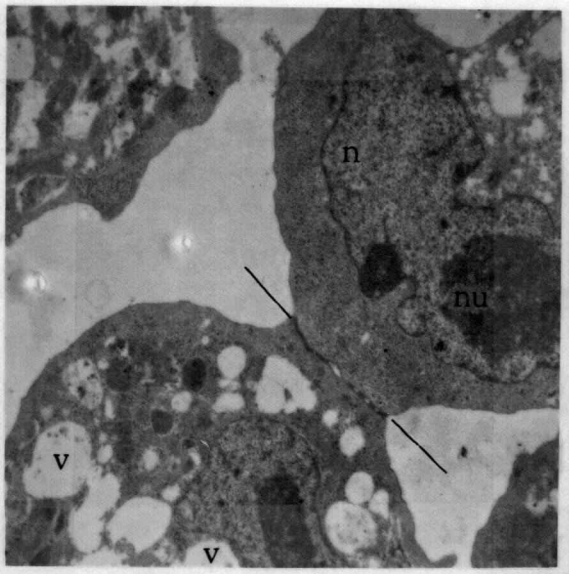
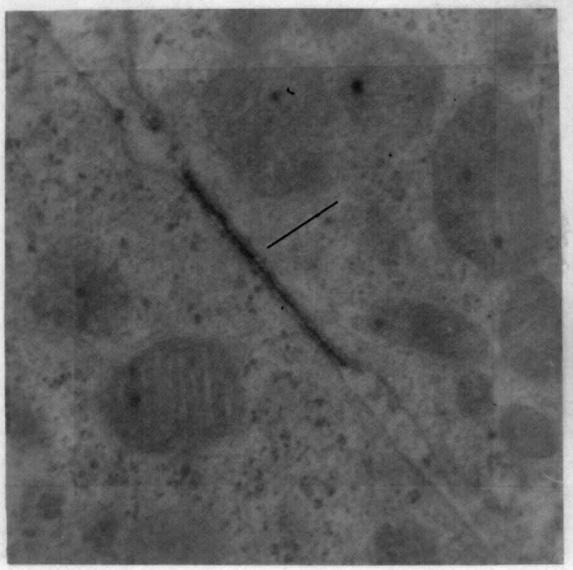

c
n
t
m

Figure 38. Three electron micrographs at varying magnifications depicting the persistent attachment at sites of intercellular cement (arrows). All cases represent cultures that have been maximally separated in EDTA.

(a) (4500 x)
(b) (15,000 x)
(c) (57,500 x)


n
nu
v
v



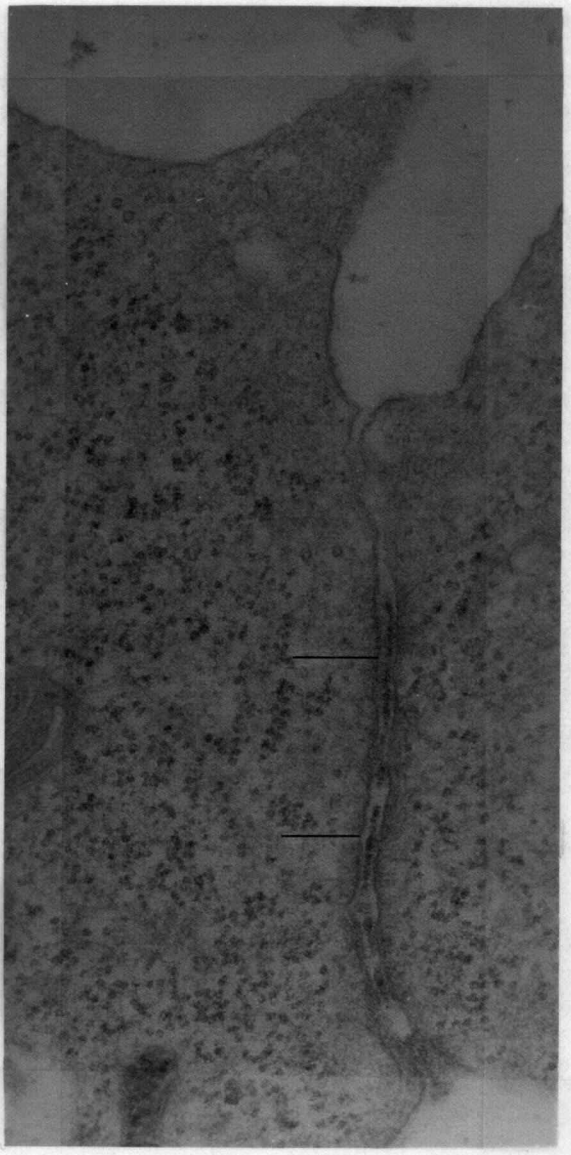


Figure 39. Low power electron micrograph showing several cells after 90 minutes in the recovery medium. Microvilli (mv) are apparent only in the expanded intercellular spaces while numerous contacts between cells occur at the tips of blunt surface projections (arrows). Nucleus (n); Nucleolus (nu); Mitochondria (m). (6000 x)

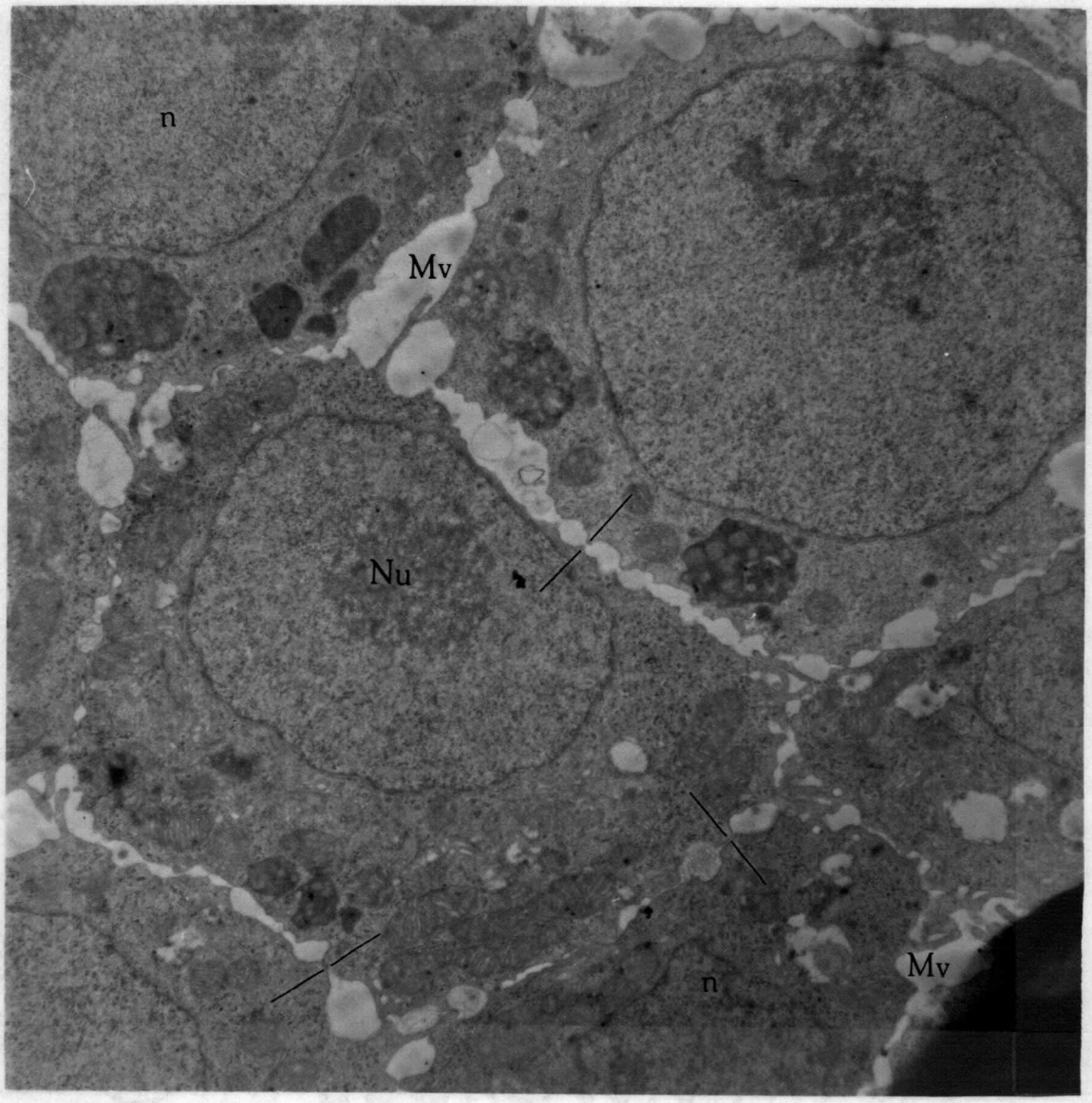

n
Mv
Nu
n
Mv

Figure 40. Portions of cells which have been incubated in Hank's BSS for 90 minutes following full separation in EDTA. Microvilli (mv) are very numerous on the non-contact surfaces while contacts between the cells occur at the tips of blunt surface projections (arrows). Nucleus (n); Centriole (c); Ciliary vesicle (cv). (9000 x)

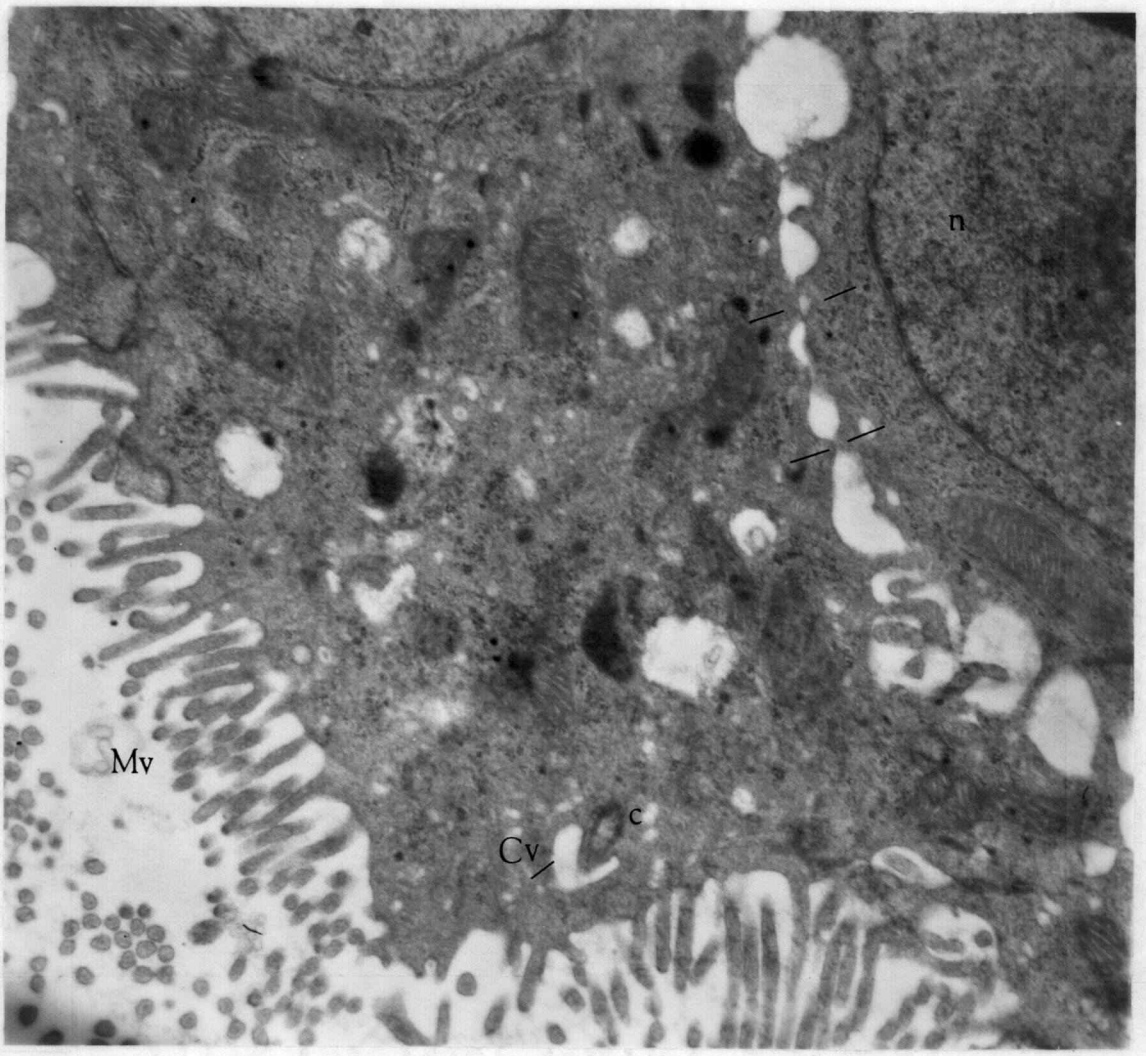

n
Mv
Cv
c

Figure 41. Cells which have been incubated in the recovery medium for 90 minutes. Contacts occur at blunt surface projections and incipient desmosomes may be present (arrows). The limited Golgi zone appears normal (g). Nucleus (n); Vacuole (v). (12, 000 x)

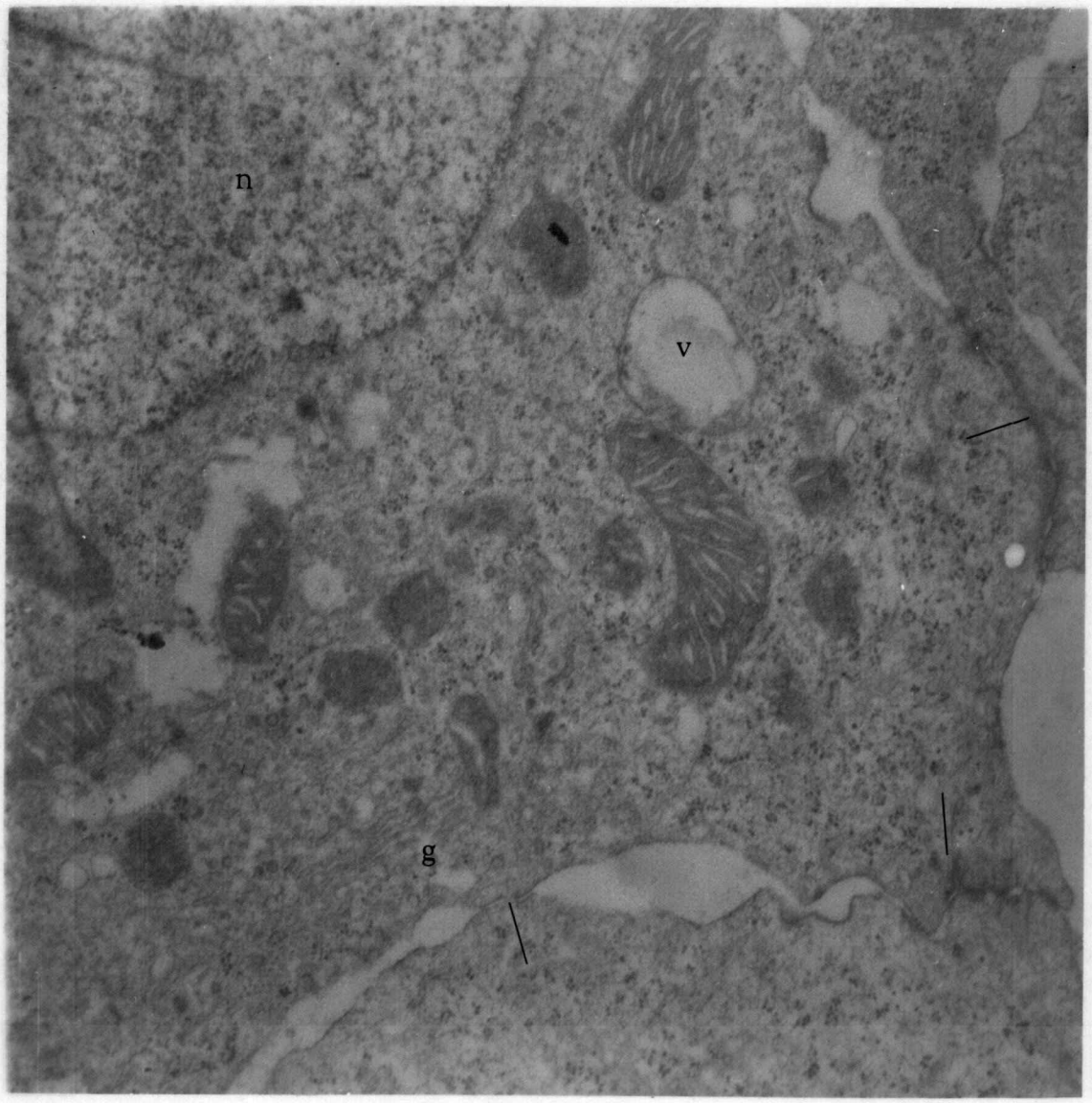

n
v
g

Figure 42. An electron micrograph obtained from a section through a cytoplasmic extension during recovery from EDTA treatment. Note the corresponding orientation of the filaments (f), mitochondria (m) and the microtubules (t). The cell center is to the upper right while the apex of the extension is to the lower left. (12,000 x)

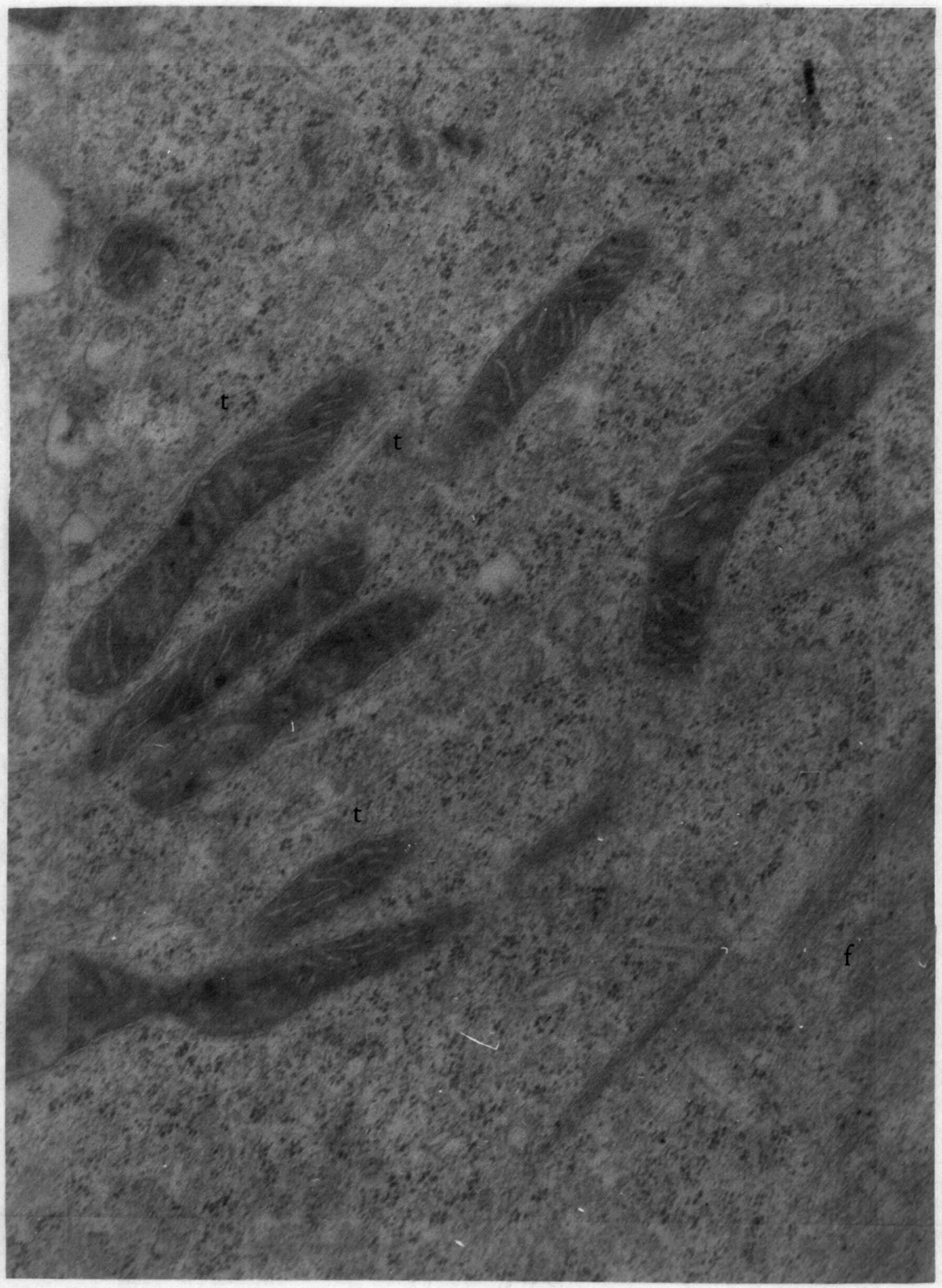

t
t
t
f

Figure 43. A montage showing cells a 90 minutes recovery in Hank's BSS. Surface microvilli (mv) are numerous and show electron dense cores. At the lower right surface processes appear to be progressively interdigitating in a zipper-like fashion. Dense thickenings (arrows) between the upper two cells may represent incipient desmosomes, as some fibril differentiation is present. Microtubules (t); Mitochondria (m); Nucleus (n); Nucleolus (nu). (12,000 x)

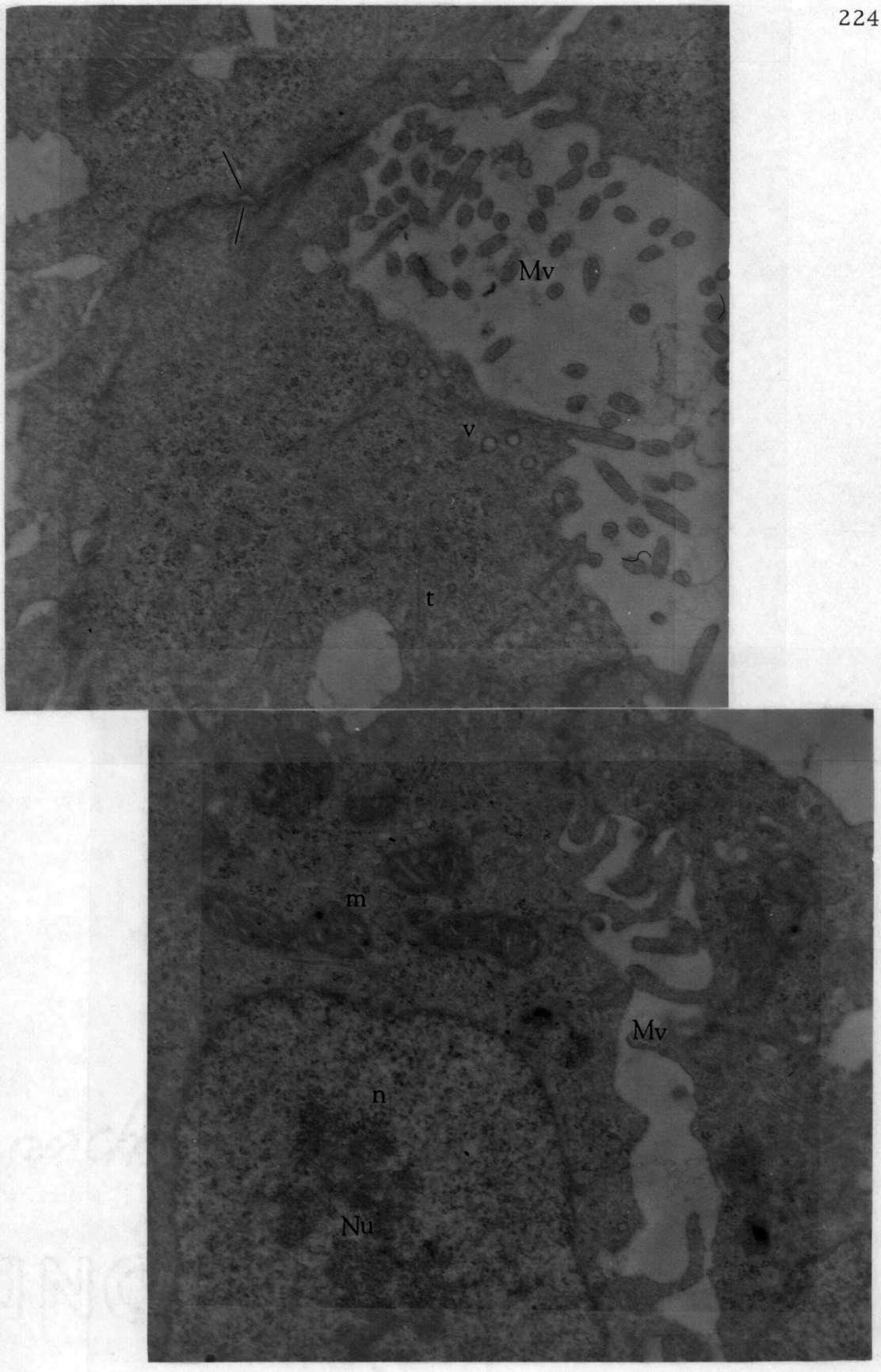

Mv
v
t
m
Mv
n
Nu

Figure 44. Montage depicting cells after 120 minutes recovery in Hank's BSS. Microvilli (mv) are present on the exposed surface but are absent on the contact surfaces. The conformation of the cells suggests that extension of contact has occurred (contact promotion). A zonula diffusa has formed between the two lower cells (arrows). (9000 x)

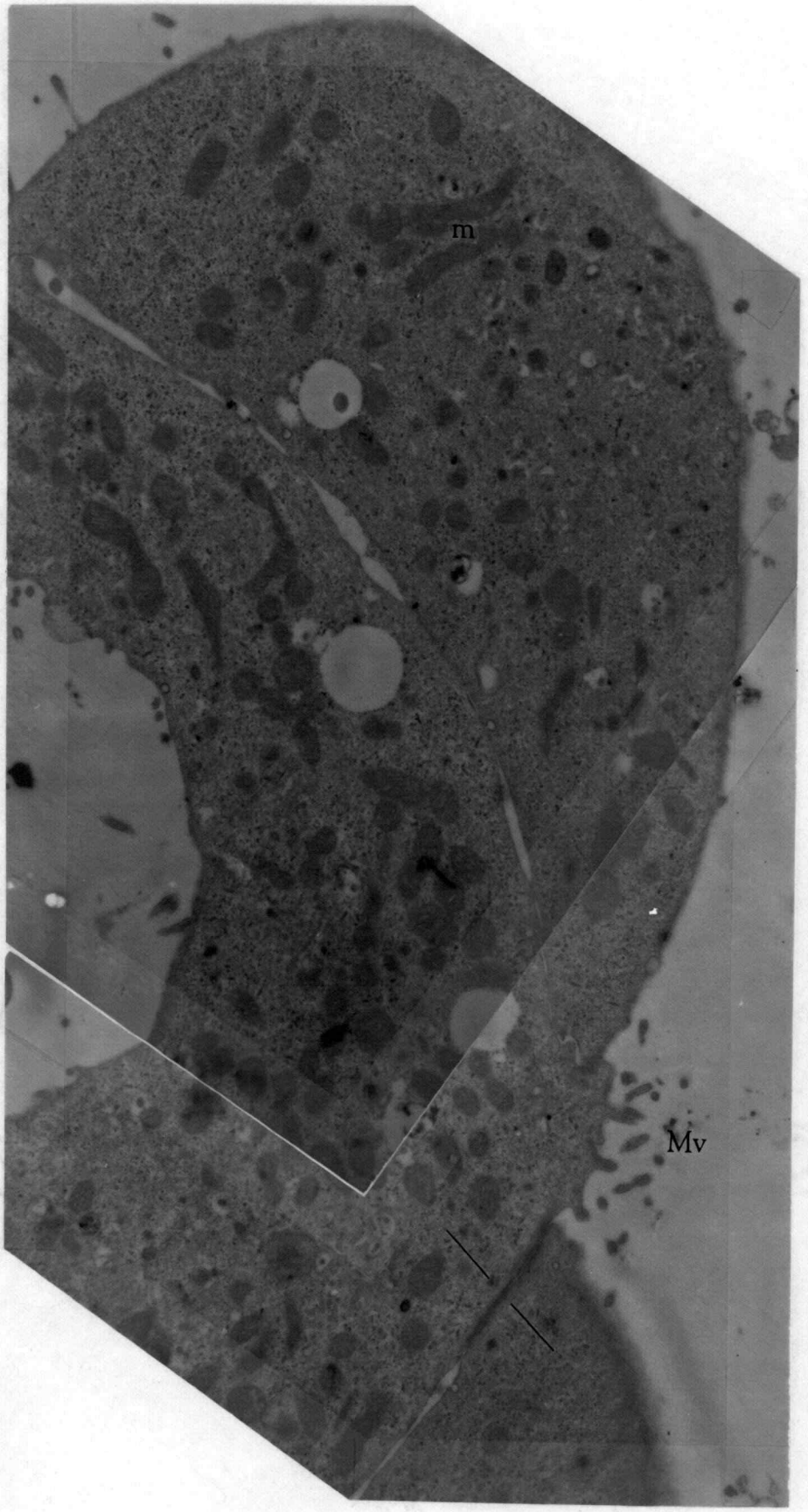
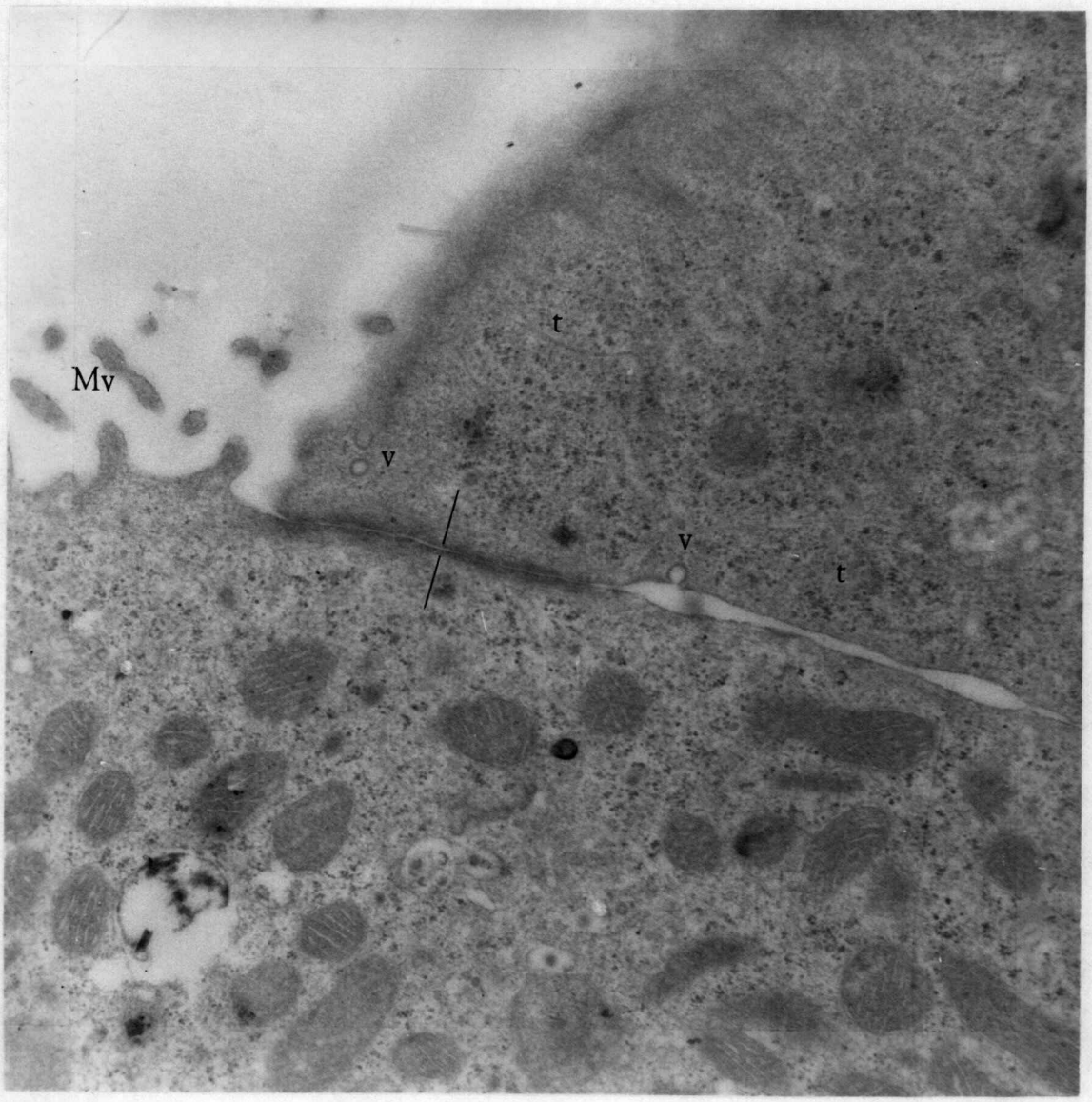
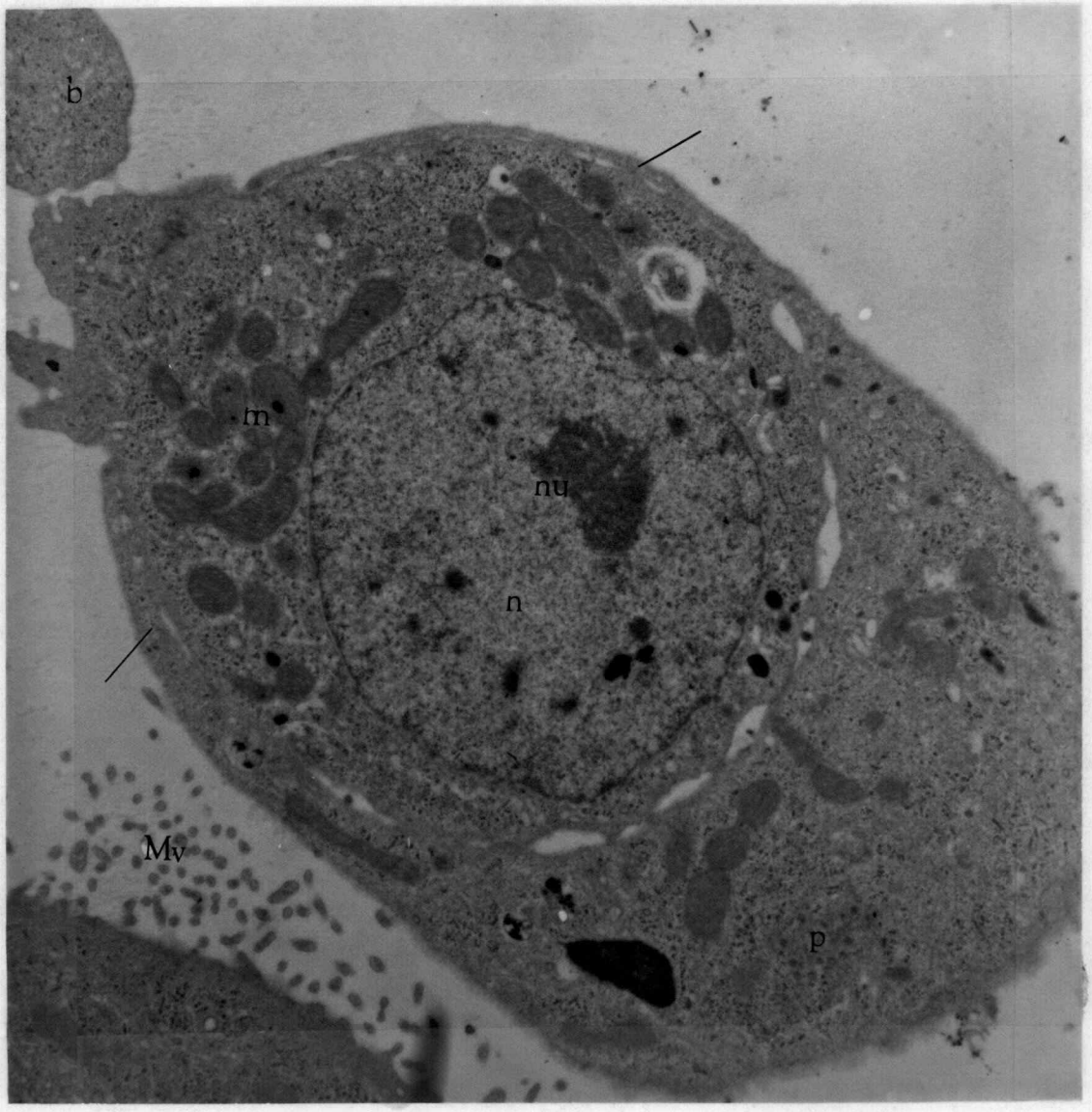

m
Mv

Figure 45. A higher magnification showing the zonula diffusa illustrated in Figure 44 (arrows). Microvilli (mv); Micropinocytotis vesicles (v); Microtubules (t). (18,000 x)


Mv
v
t
v
t

Figures 46 and 47. Sample sections of two cells having reestablished extensive contact subsequent to full EDTA treatment. Note that one cell has nearly surrounded the other (arrows). Such a situation may be taken as evidence for contact promotion. Microvilli (mv) are present on free surfaces but broad projections only are observed at contact regions. The bleb (b) in the upper left supports the proposal that blebbing activity occurs in regions of relative surface weakness since support may be provided by the outer cell at all points except that which shows the bleb. Nucleus (n); Nucleolus (nu); Mitochondria (m); Nuclear pores (p). (9000 x).


b
m
nu
n
Mv
p

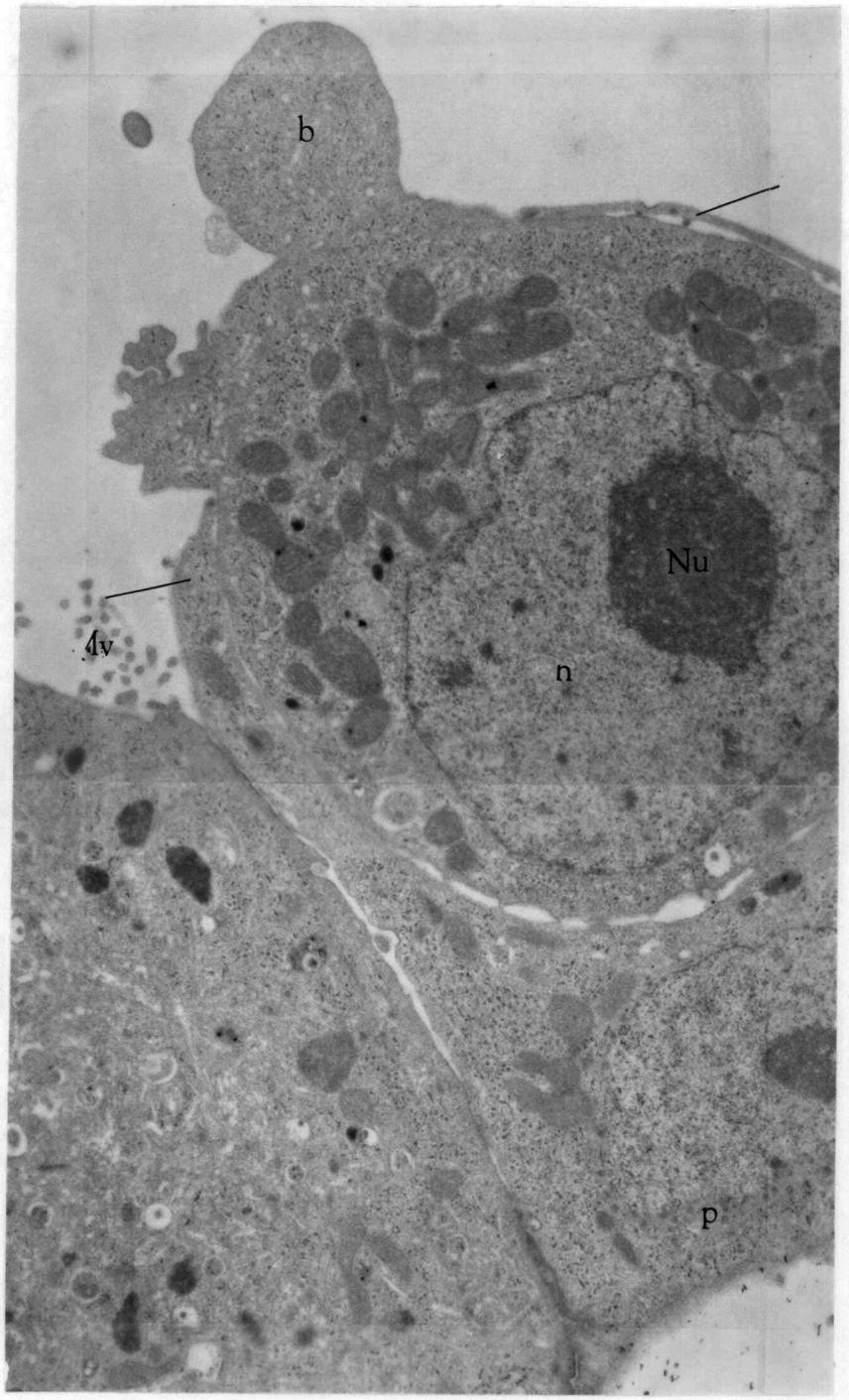

b
Nu
lv
n
p

Figure 48. Contact surface between two cells following separation and recovery. Intercellular material is present (arrows). Nucleus (n); Nucleolus (nu); Mitochondria (m); Micropinocytotic vesicles (v); Microtubules (t). (12,000 x)

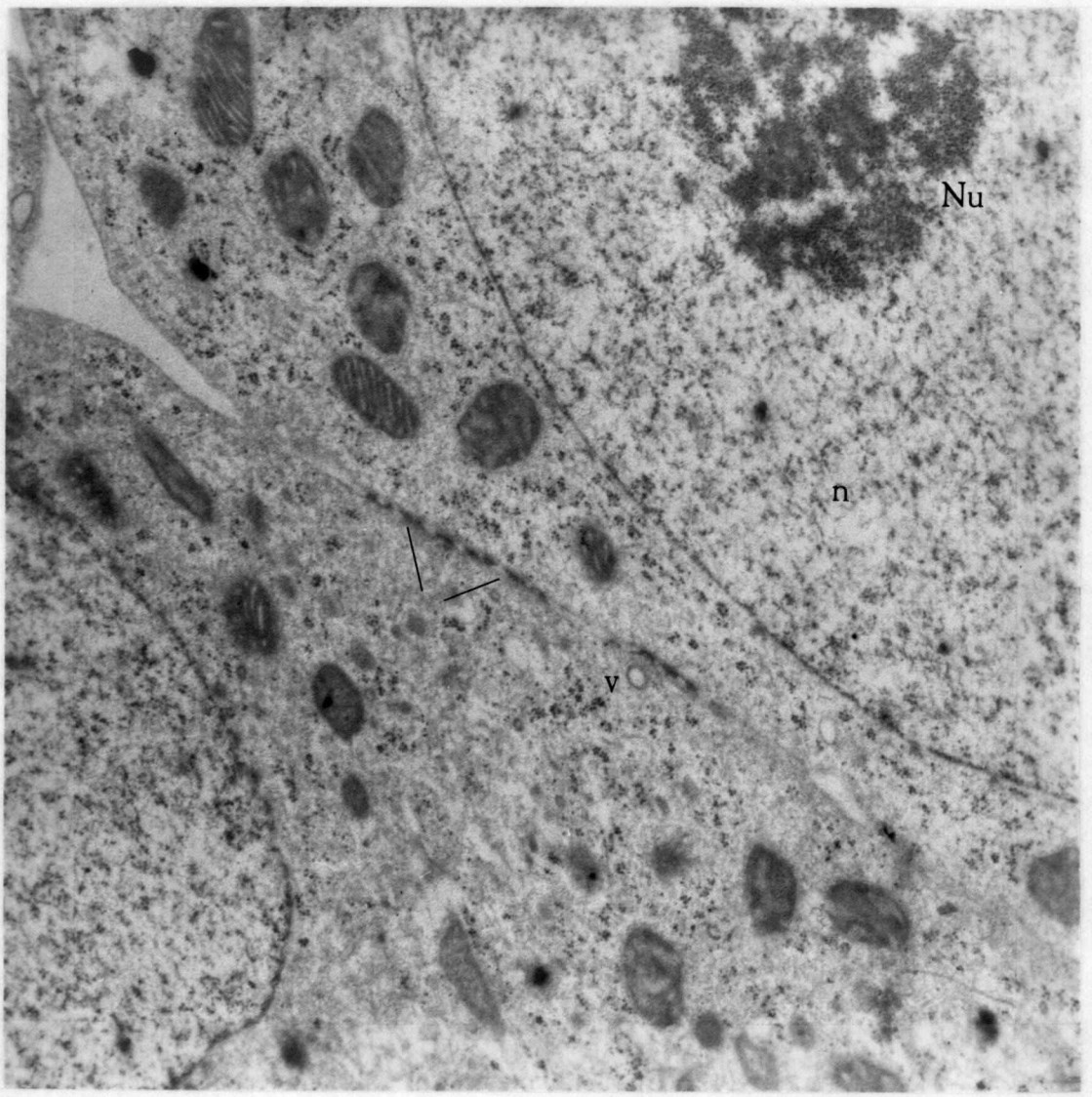

Nu
n
v

Figure 49. A contact surface between two cells following recovery in Hank's BSS. Complex surface interdigitation has occurred (arrows). Micropinocytotic vesicles (v). (24,000 x)

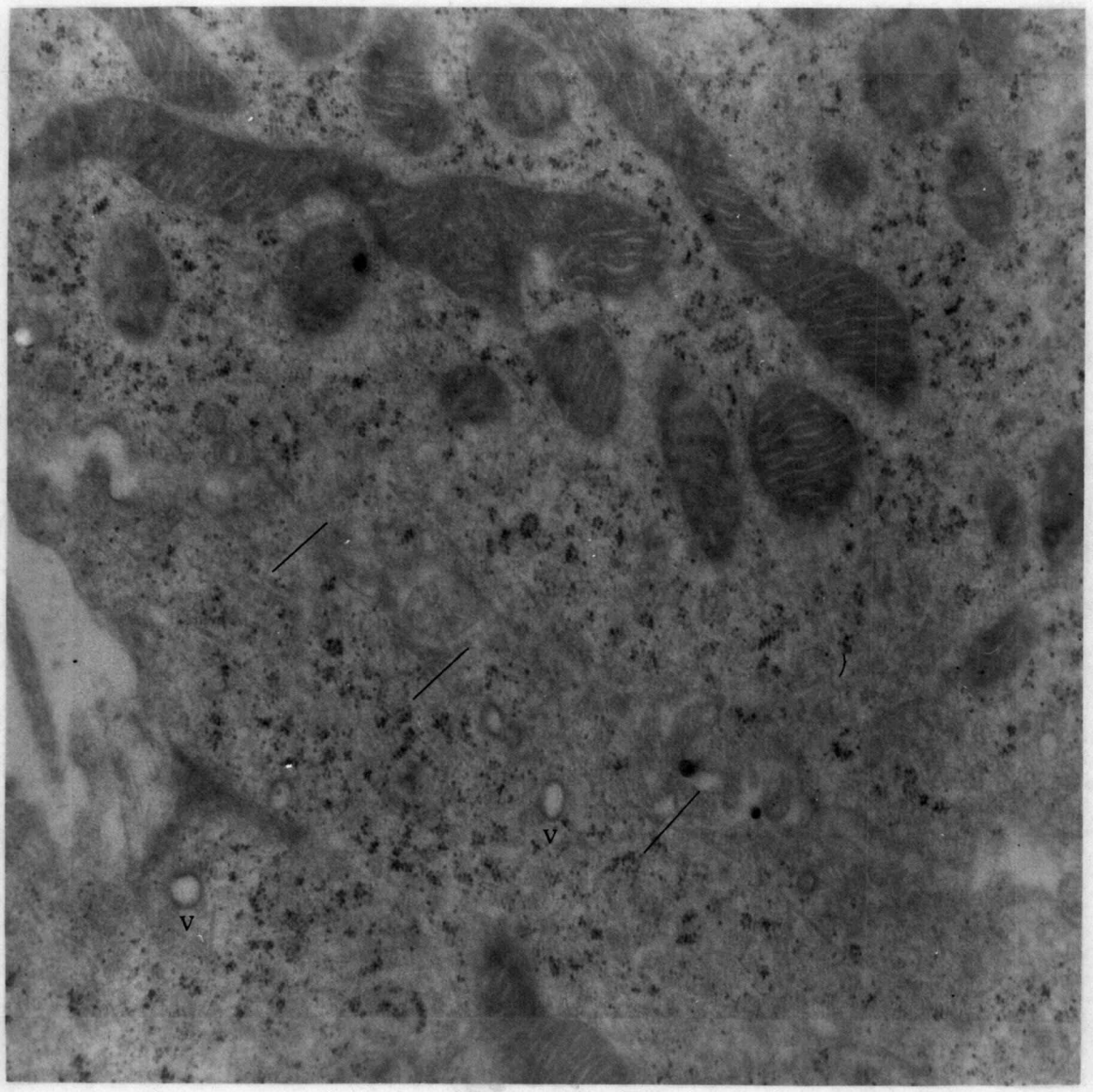

v
v

Figure 50. Desmosomal contacts between cells that have been incubated in the recovery medium for three hours. The structure of the desmosome (d) is complete including extensive tonofibrils (f). Helical polyribosomes (h); Intercellular cement (arrow). (31, 500 x)

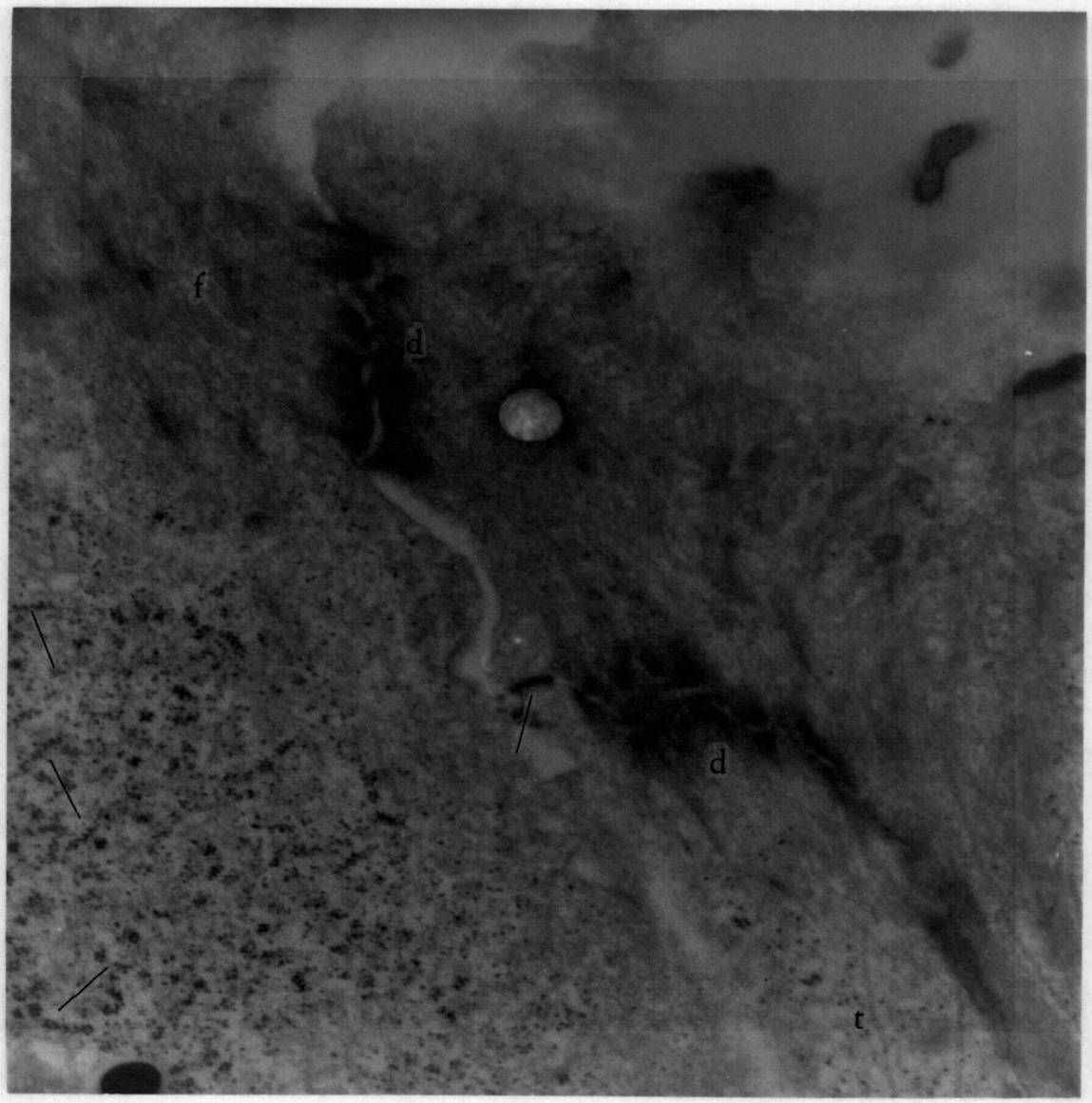
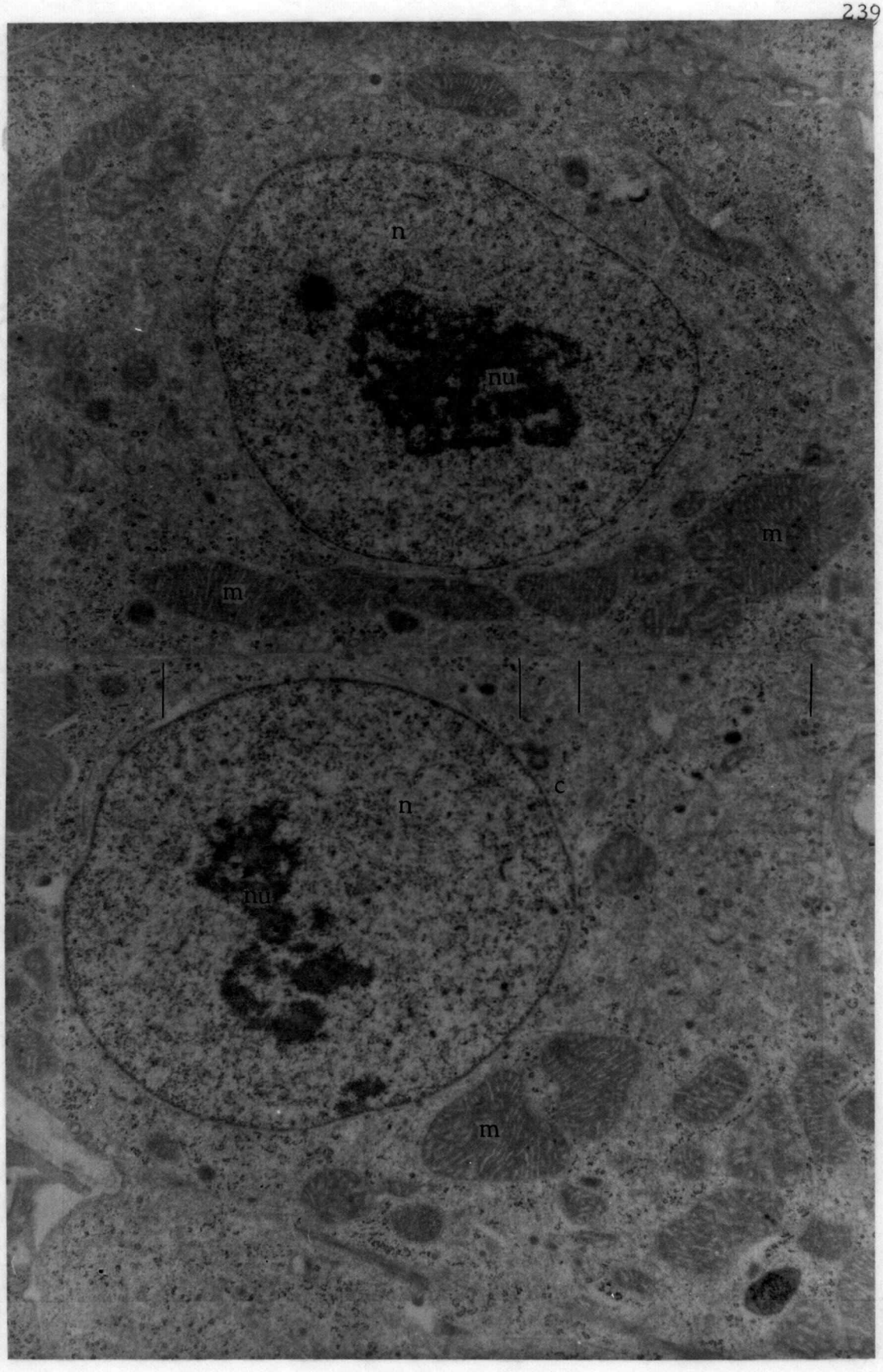

f
d
d
t

Figure 51. Cells from a culture that has been incubated in Hank's BSS for 24 hours subsequent to maximum EDTA treatment. A typical close contact has been reestablished between the cells (arrow) and interdigitation of the surfaces is shown. The nucleus (n) and the nucleolus (nu) appear normal as do the mitochondria (m). Centriole (c). (10, 600 x)


n
nu
m
m
n
c
nu
m