CELL BEHAVIOUR IN CHICK BLASTODERM EXPANSION

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DEDICATION

TO MY FATHER.

DECLARATION

I hereby declare that this thesis is my own composition and that, except where otherwise stated, the experimental work was performed by me alone.

None of the material in this thesis has been submitted for any other degree.

Abdulgader O. A. El-Gadi

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SUMMARY

The behaviour of the leading edge cells during the early stages of epiboly (stage 3 to 16, Hamburger and Hamilton, 1951) has been investigated in this study using light, transmission and scanning electron microscopy.

The results showed a consistency in the distribution of junctions between these cells throughout these stages. However, there is a significant increase in both the width of the leading edge and the number of its cells around the second day of incubation (stage 11-13). The significance of this increase is discussed in relation to the reported increase in the rate of expansion of the blastoderm at around that time (Downie, 1976).

As the leading edge migrates centrifugally over the vitelline membrane, it requires the addition of new cells to cover an increasingly wider area. This study provides evidence that cells recruited into the edge are the deep layer cells which are found on the basal lamina of the peripheral part of the blastoderm and not the ectodermal cells as is generally thought.

The opposite task faces the leading edge as it passes the equator of the egg and occupies a smaller area of vitelline membrane, that is how to accommodate the large number of edge cells into a smaller space? The current study provides the first account of the unique strategy that the chick blastoderm uses to achieve this task. Some of the leading edge cells become stationary at points around the circumference of the edge. This allows just enough cells to continue their movement to cover the rest of the vitelline membrane and in turn the whole yolk. As more cells are added to the original stationary cells and become stationary themselves, long stationary streaks develop, giving the blastoderm edge a crescent-shaped appearance. The difference in the end of epiboly between the chick blastoderm and other embryos with similar systems is discussed in this study.

The study also reports for the first time that cell death occurs in the leading edge cells during the late stages of development. This occurs in the oldest stationary cells then progresses centrifugally to the younger ones.

As the stationary edge cells die, they are replaced by the nearby ectodermal cells which attach strongly to the vitelline membrane - an unusual behaviour for ectodermal cells which never attach to this membrane during the earlier stages of epiboly.

The time and position of appearance of stationary points was found to be unrelated to the stage of development . The length of the stationary streaks and their complexity was found also to be different from one blastoderm to another even in blastoderms which have been incubated for the same length of time. Another peculiar finding of this study is that the extent of blastoderm expansion and the time it seals the vitelline membrane is also different from one embryo to another at any particular time of development.

Indirect immunofluorescent staining of pieces of blastoderm edges cultured on glass confirmed previous reports that the shape of the epithelial cells does not depend on the integrity of an intact microtubule system and that microfilaments are the essential elements in maintaining the shape of the leading edge cells.

Immunofluorescent staining for fibronectin distribution in the blastoderm edge showed it to be present in the basal lamina, and within a few attached edge cells during the first 2 days of expansion, but not between edge cells, or at the edge cell-vitelline membrane interface. However, extracellular fibronectin was detected in the stationary streaks after 3 days. These results differ from previous reports, and reasons for these differences are discussed.

Experiments to study the behaviour of blastoderm cells lacking an edge on the inner surface of the vitelline membrane revealed that the blastoderm

has the ability to form a regenerated edge which moves rapidly on the substratum. Cultures of blastoderm pieces with or without the leading edge on the outer surface of the vitelline membrane showed that these cells were unable to expand on this substratum. The reasons behind this behaviour are discussed.

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CHAPTER ONE

INTRODUCTION

The scope of this introduction is to throw some light on the general properties of epithelia which relate to their ability to migrate, and then to give special consideration to a particular epithelium, the chick blastoderm.

1.1- PROPERTIES OF EPITHELIAL SHEETS RELEVANT TO EPITHELIAL SPREADING:

1.1.1- ORGANISATION OF EPITHELIA DURING MOVEMENT:

Conflicting reports exist about whether, during the spreading of an epithelial sheet on a substratum, the only cells that attach to the substratum are the marginal "edge" cells while the rest of the sheet is pulled along passively, or whether the submarginal "non-edge" cells are actively involved in the process.

Vaughan and Trinkaus (1966) studied the *in vitro* movement of epithelial cells and reported that the only cells which adhere to the substratum are those located at the free edge of the explant and therefore are the only ones which are capable of active movement. They suggest that the movement of these marginal cells leads to the movement of the whole sheet. The non-edge cells on the other hand do not seem to attach to the substratum. However, if the attachment of these non-edge cells with other neighbouring cells are broken and so they themselves become edge cells, they attach to and actively move on the substratum.

The results of Vaughan and Trinkaus (1966) were confirmed by DiPasquale (1975) who reported that *in vitro* experiments on several epithelial cell types showed that although some submarginal cells sometimes attach to the

substratum, there is no evidence to support their involvement in the movement of the sheet.

Several other models have been put forward concerning the role of both marginal and submarginal cells in expansion of an epithelial sheet.

The first of these models is the "rolling and sliding" movement proposed by Krawczyk (1971) for epidermal cells in mice and supported by the findings of Gibbins (1978), Pang *et. al.* (1978), and Repesh and Oberpriller (1980). In this model the marginal cells adhere to the substratum and the submarginal cells move over them and in turn attach to the substratum. The submarginal cells have been shown to develop hemidesmosomes with the substratum (Krawczyk and Wilgran, 1973). This means that the cells which are in contact with the substratum remain stationary while the cells on top of them roll over and make new attachment sites with the substratum. More evidence for this model was provided by Ortonne *et. al.* (1981).

The second model is based on the work of Radice (1980 a,b) on wound healing in *Xenopus* larvae, where he used time lapse cinematography and transmission electron microscopy to study the *in situ* and *in vitro* migration of epithelial cells into the wound area. He reported that both experiments showed that the submarginal cells possess leading lamellae which make close contact with the substratum, a feature of actively moving cells.

In this model the marginal cells of the epithelial sheet adhere to the substratum and move over it, and the submarginal cells will immediately occupy the space left by them i.e. both marginal and submarginal cells retain their position in relation to each other. Support for this model comes from the work of Udoh and Derby (1982) who reported piling up of cells as movement ceases during wound healing in tadpole tail epidermis. The model has also been supported by Takeuchi (1983) in his work on corneal epithelium indicating that submarginal cells elongate at right angles to the direction of the marginal cells.

Radice (1980 a&b) reported that the movement of submarginal cells is much slower in *in vitro* experiments than in *in situ*, and suggested that this could be due to the effect of tension generated by the marginal cells which move in the wider space needed to be covered *in vitro* while their movement *in situ* is confined to a smaller space and so they are under less tension.

1.1.2- CELL REARRANGEMENT IN EPITHELIAL SHEETS:

The short distance movement of cells within an epithelium, so that individual cells indulge in a process of changing their immediate neighbours, is given the term "cell rearrangement".

This process is thought to be the most common process by which an epithelial sheet can alter its dimensions (Fristrom, 1988). As far back as 1944, Waddington reported that the elongation of the notochord in amphibians can only be brought about by one of two mechanisms; either that cells change shape to reflect the elongation and narrowing of the tissue mass,or that they undergo rearrangement and eventually return to their original shape.

The idea of cell rearrangement had not been recognised as an important morphogenetic mechanism for more than 30 years until it was revived by the work of Fristrom (1976) and Fristrom and Fristrom (1975) on the elongation of the imaginal leg discs in *Drosophila*. They argue that, given the fact that cell division, cell death, and movement of cells in or out of the plane of the epithelium are constant, the elongation of the discs can only occur as a result of either the elongation of the cells themselves or by cellular rearrangement. They reported that there is no evidence for any elongation of cells themselves and that cells in the discs rearrange themselves so that the number of cells in the long axis increases while the number of cells around the circumference decreases in proportion to that increase.

At about the same time Jacobson and Gordon (1976) reported that cell rearrangement is responsible for giving the proper shape to the cells of the

neural plate in the newt embryo.

Evidence for epithelial cell rearrangement comes from a time lapse cinematographic study on gastrulation in *Xenopus* conducted by Keller (1978). He showed that cells in the upper layer of the involuting marginal zone exchange neighbours at the same time as the mass of cells becomes longer and narrower.

More evidence for epithelial cell rearrangement comes from the work of Kageyama (1982) on the embryo of the Medaka, *Oryzias latipes*, using silver staining methods. He argued that the extension of the marginal zone of the enveloping layer during the course of epiboly in this embryo is a result of rearrangement of the component cells of that zone.

Further direct evidence for cell rearrangement was demonstrated more recently by Keller and Trinkaus (1987) who used time lapse cinematography and silver staining to study the process of epiboly in another teleost embryo (*Fundulus*). As the enveloping layer passes the equator of the yolk and moves towards the vegetal pole, cells which used to occupy the marginal positions of this layer translocate to occupy submarginal positions. This results in the reduction required for the circumference of the layer to be accommodated into the narrower space available. At the same time submarginal cells undergo cell rearrangement by reducing the boundaries between them and shifting to more submarginal positions.

In this way, cell rearrangement occurs in *Fundulus* without disrupting the integrity of the permeability barrier provided by the apical tight junctional seal of this epithelium. This barrier needs to be impermeable throughout for the normal course of development to proceed (Bennett and Trinkaus, 1970).

Cell rearrangement also occurs during the secondary invagination of the archenteron of the sea urchin gastrula (Ettensohn, 1985a; Hardin and Cheng, 1986), and at the neurula stage of *Xenopus* where it leads to the lengthening

and narrowing of the notochord (Keller *et. al.*, 1985).

Cell rearrangement can also occur within an epithelium without changing the shape of that epithelium, as has been recently shown by Nardi and Adams (1986). They reported that cell rearrangement is involved in the patterning of the moth wing. They have shown that the cells of the scale primordia, which are distributed randomly at first, align themselves into regular transverse rows with a few rows of unspecialised epithelial cells. Scales then start to grow from these aligned rows in the proximal-distal direction.

1.1.2.1- Types of cell rearrangement:

According to the source of force needed for cell rearrangement to take place there are two types ; passive and active cell rearrangements.

A) Passive cell rearrangement:

Generally speaking, passive cell rearrangement, as in the cases described above for amphibian and fish embryos,is brought about by the tendency of the cells in an epithelium to return to their original shape after the distortion of that shape by an external force exerted on that epithelium by another tissue. For example, in the epiboly of *Fundulus* the external force exerted on the epithelial cells of the enveloping layer is generated by the syncytial layer which underlies the enveloping layer and spreads over the yolk ahead of it. In amphibian embryos cell rearrangement is triggered by the active movement and arrangement of mesenchymal cells of the deep cell layer during its extension (Keller, 1986).

Honda *et. al.* (1982) studied wound healing in the epithelia of the cat cornea using time lapse cinematography. He showed that cells at the margin of the wound first elongate to cover the centre of the wound. Having done that, they then rearrange themselves by shortening their cell boundaries while maintaining contact with adjacent cells until the apices of four cells intersect and return to their original shape. In this case the force needed for this passive

rearrangement has resulted from the migration of the marginal cells to cover the wound.

B) Active cell rearrangement:

This type of cell rearrangement tends to be a general feature of invertebrate epithelia. Here the driving force of the process is generated within the epithelial cells themselves.

Active cell rearrangement have been demonstrated by Fristrom and Fristrom (1975), and Fristrom (1976) during the elongation of the imaginal leg discs of the *Drosophila*, and by Ettensohn (1985a) in the archenteron of the sea urchin. In both cases cells rearrange themselves so that their number increases in the long axis of the plane of the tissue but decreases around the circumference.

1.1.3- THE CYTOSKELETON IN MOVING EPITHELIAL CELLS:

Epithelial cell movement and stability are achieved by the interaction of the cytoplasmic polymer systems represented by microfilaments, microtubules, and intermediate filaments, along with adhesions to the substratum and neighbouring cells.

1.1.3.1 **Microfilaments:**

Microfilaments are actin-based structures which form a network around the apical and basal surfaces of epithelial cells. The ring of microfilaments associated with the *zonula adherens* is the most prominent region of this network. It is this ring of microfilaments which brings about the contractions of the epithelium which is then transmitted to neighbouring cells. Such contractions lead to invagination of the sheet when it occurs at the apical surface of the cells, while contractions at the basal surface lead to their evagination. Both processes cease when the tissue is treated with cytochalasin B which disturbs these actin-based structures (Owaribe *et. al.*, 1981).

Jacobson *et. al.* (1986) propose in their "cortical tractor" model for epithelial folding that cortical microfilaments found at the apices of epithelial cells accumulate there as a result of cortical flow of adhesive structures apicalwards. They suggest that if the rate of recycling of these adhesive structures is slower than their insertion at the basal surface, a pile-up of these structures occur at the apical surface of the cells leading to the prevention of detachment of the cells.

Microfilaments are also involved in the elongation of epithelial sheets as in the case of the nematode *C.elegans* (Priess and Hirsh, 1986). Here the actin filamentous bands which are distributed transversely beneath the apical side of each epithelial cell contract and lead to the anterior-posterior elongation of the hypodermal cells with corresponding narrowing of the circumferentially located cells. The force which results from this elongation leads to shape change of the embryo from spherical to worm-shaped.

Owaribe *et. al.* (1981) used chick glycerinated retinal pigmented epithelial cells developed *in vivo* and *in vitro* to study the effect of the contraction of the circumferential microfilaments at the apical surface of individual cells on the shape of the whole epithelium. They reported that when the monolayer epithelium is transferred to an Mg-ATP solution each cell starts to contract and as a result the whole epithelium becomes divided into small cell groups. Further contractions in the cells of each group led to the lifting of the in turn detaches from the substratum.

Owaribe and Masuda (1982) suggested that the microfilaments responsible for such contractions at the apices of the pigmented epithelial cells are those which are loosely packed with random polarity, and are associated with *zonula adherens* (see below), as the ones which were found to be associated with the same structure in isolated brush borders from the intestinal

epithelium (Rodewald *et. al.*, 1976). Microfilaments are also involved in the cell rearrangement which is responsible, as shown above, for the elongation of the imaginal leg discs of *Drosophila* (Fristrom, 1976).

In addition, microfilaments are important elements in the protrusive activities of epithelial cells. These protrusive activities play an integral part in the locomotion of single cells (Trinkaus, 1984). The basal surfaces of epithelial cells exhibit these activities (Fristrom, 1988). Bereiter-Hahn *et. al.* (1981) proposed a model for locomotion of isolated *Xenopus* epidermal cells involving microfilaments at the cortex of these cells. They suggest that when an area of the cell body forms a lamella the cortical filaments contract and pull the cell membrane which faces the culture medium towards the coverslips on which these cells have been grown. As a result of these contraction a flow of cytoplasm into the lamella occurs and, as other fibrils at the base of the cells contract, the whole cell body moves in the direction of the lamella.

Chernoff and Overton (1979) suggested that microfilaments are responsible for the migration of the edge cells of the chick blastoderm, since treatment of these cells with cytochalasin B led to the retraction of the edge cells and to their detachment from their substratum, the vitelline membrane.

1.1.3.2- **Microtubules:**

Two classes of microtubules coexist in cells: a stable class and another which polymerises and depolymerises rapidly (Shulze and Kirschner, 1987). These cytoskeletal elements have been associated with stabilisation of cell shape and the shape of some cellular organelles. For example the distribution of microtubules parallel to the long axis of columnar epithelial cells may functions to stabilise the elongated shape of these cells (Byers and Porter, 1964; Burnside, 1973). They also play an important role in cell division maintaining Golgi structure and the distribution of the cytoplasmic components of cells (Simons and Fuller, 1985), and in cytoplasmic flow in some cells.

Microtubules are known to play an important role in maintaining the polarised morphology of fibroblasts (Vasiliev and Gelfand, 1976). Treatment of cultured fibroblasts with microtubule-destroying drugs such as colcemid, colchicine, and nocodazole leads to the loss of the polarised morphology of these cells and inhibits their ability to spread on substrata (Vasiliev *et. al.*, 1970; Gail and Boone, 1971; Goldman, 1971). Although this seems to be a general role in fibroblasts, exceptions have been reported. Gelfand *et. al.* (1985) reported that small fragments of fibroblastic cells are unaffected by such treatment while larger intact fragments are.

The dependence of epithelial cell morphology and spreading on their microtubule systems is, however, more controversial. Domnina *et. al.* (1985) reported that some epithelioid cell lines exhibit the same response to microtubule-disrupting drugs as fibroblasts. However, several reports (DiPasquale, 1975 on chick embryo gut epithelial cells; Chernoff and Overton, 1979 on chick blastoderm edge) argue that the maintenance of the morphology of epithelial cells in these different embryonic tissues does not depend on microtubules.

More recently, Middleton *et. al.* (1988) studied the effect of two microtubule destroying drugs, colcemid and nocodazole, on the shape of cultured embryonic chick heart fibroblasts and two epithelial cell types; corneal epithelial cells and epidermal epithelial cells. They reported that the drugs inhibited both the elongation and spreading of fibroblasts but did not significantly affect such processes in the two epithelial cell types, although the microtubules of the latter cells had clearly been destroyed by the drugs. As to the reason behind such differences between the effect of these drugs on the polarisation of fibroblasts and epithelial cells, Middleton and co-workers put forward two possible answers; a) it may be due to the way the microtubules in these two cell types interact with their relevant intermediate filaments which are chemically different from each other. Fibroblasts contain vimentin filaments which are

known to break down when microtubules are disrupted (Franke *et. al.*, 1978; Wang and Goldman, 1978; Henderson and Weber, 1981), while intermediate filaments in epithelial cells are made of cytokeratin filaments which do not generally break down when microtubules are destroyed (Osborn *et. al.*, 1977; Henderson and Weber, 1981; Franke *et. al.*, 1978). Alternatively b) it may be due to the fact that these epithelial cells are smaller in size than fibroblasts and so they are not dependent on microtubules to maintain their morphology. This explanation is already partially supported by the work of Gelfand *et. al.* (1985) who found that, even between fibroblasts themselves, the effect of these microtubule-disrupting drugs depends on the size of the fibroblastic fragments.

The role of microtubules in the orientation of cells during their migration is another a controversial issue. Gotlieb *et. al.* (1981) who studied the relation between the location of microtubule organising centres (MTOCs) and the direction of migration of cultured endothelial cells, reported that in the majority of migratory cells of this type MTOCs are located in front of the nuclei towards the direction of movement of the sheet while they are randomly distributed in non-migratory cells. They claimed that MTOCs, their associated microtubules, and centrioles play a role in establishing the direction of cell migration. Trinkaus (1984) suggested that this role of microtubules is a passive one. He explains that once one lamella has dominated over the others it will orientate the migrating cell towards its direction, generating tension which could help the alignment of microtubules in that particular direction.

Evidence for the independence of directional epithelial cell movement on microtubules comes from the work of Euteneuer and Schliwa (1984) on fish epidermal keratocytes. They reported that individual cytoplasmic fragments which lack any microtubules, centrioles or nucleus are capable of assuming the same directional locomotion as intact cells. They concluded that the force for movement of epidermal cells is generated and regulated locally, in the lamellae.

1.1.3.3- **Intermediate Filaments**

Intermediate filaments are stable structure forming a major component of differentiated epithelial cells. Their role in these cells is thought to be mechanical; they may be involved in maintaining the shape of the cells and in giving them the ability to stretch. As stated above, these intermediate filaments are composed of cytokeratins in epithelial cells while those of fibroblasts are vimentin-containing filaments. The role of these filaments in early embryonic development has yet to be demonstrated.

1.1.4- EPITHELIAL INTERCELLULAR JUNCTIONS:

An important feature characteristic of epithelial sheets is the presence of two circumferentially arranged sets of junctions below the apical ends of the cells. These junctions are the *zonula adherens* and *zonula occludens* (occluding junctions).

1.1.4.1- ***Zonula adherens*:**

The function of the *zonula adherens* is to adhere adjacent cells together. They are associated with a dense mat of circumferentially distributed actin filaments just beneath the plasma membrane of the cells (Farquhar and Palade, 1963; Staehelin, 1974).

1.1.4.2- ***Zonula occludens*:**

In vertebrate epithelial cells occluding junctions are represented by tight junctions. They are located at the apical side of the cells above the *zonula adherens*. They function as permeability barriers by preventing the diffusion of small molecules via the intercellular spaces. In these junctions, the outer plasma membranes of the cells look inseparable (Staehelin, 1974).

Although tight junctions are sometimes absent from some epithelia such as the epithelium lining the blood capillaries (Fawcett, 1966), they are especially well developed in epithelia which separate two very different media with

respect to their osmotic pressure such as in the case of the epithelium of the enveloping layer of *Fundulus* which separates the embryo from the highly saline surrounding medium. Here they are distributed in such a way as to form an unleaky barrier, while at the same time permitting cell rearrangement (Keller and Trinkaus, 1987).

The other two types of junctions that are common between epithelial cells are *macula adherens* (desmosomes) and gap junctions.

1.1.4.3- **Desmosomes:**

Desmosomes are adhesive in nature (Kolega, 1986; Overton, 1973), and because of this adhesive nature they seem to influence the positioning of cells within the tissue i.e. they are involved in the process of sorting out of cells (Trelstad *et. al.*, 1967; Overton, 1977). In her experiments on mixed aggregates of cells, Overton (1977) found that cells which are capable of forming the most desmosomes sort out internally while those which are less capable of forming desmosomes are displaced to the periphery.

Desmosomes usually develop between cells in an epithelium under mechanical stress (Lackie, 1986). They are anchored to the cytoplasm with tonofilaments, a type of intermediate filament, which have no contractile proteins and which originate in the cytoplasm and loop in and out of the desmosomes. In this way these filaments provide the desmosomes with their extremely adhesive properties which makes them resist any mechanical stress exerted on the cells which they adhere together (Trinkaus, 1984).

1.1.4.4- **Gap junctions:**

Gap junctions are widely distributed in epithelia. They couple cells chemically and electrically (Gilula, 1980) and have been implicated in intercellular communication (Gilula *et. al.*, 1972; Goodenough and Revel, 1970; Revel *et.al.*, 1973) probably by means of cell-to-cell transfer of metabolites

(Gilula *et. al.*,1972; Pederson *et. al.*,1980). They are also thought to be involved in the co-ordination of cellular activities such as cell movement in cell sheets (Andries *et. al.*,1985). Evidence for the importance of gap junctions in cell communication comes from the work of Warner *et. al.* (1984). They reported that disruption of these junctions in *Xenopus* by microinjection of antibodies to gap junction proteins has resulted in abnormal embryonic development.

1.1.5- CELL SUBSTRATUM INTERACTION:

One of the distinctive characteristics of epithelial cells cultured *in vitro* is that they migrate as a cohesive sheet. Several studies have dealt with the interaction between the epithelial cells and the substrata on which they move, and it is generally accepted that cells which make close contacts with the substratum are highly motile when compared with those which make focal contacts,also known as dense plaques. Radice (1980b) used interference reflection microscopy (IRM) to study both *in vitro* and *in situ* locomotion and cell-substratum interaction in *Xenopus* epidermal cells. He reported that as the cells move, most of their leading lamellae and some parts of the cell body show broad grey regions which represent areas of close contact between these cells and their substratum.

Radice (1980b) also noted that within these grey areas are some darker spots which represent areas of focal contacts. Cells with focal contacts are found to be very few at the edge area and move slower than the cells without them. He also noted that the pattern of close contact changes rapidly as the cells move and that areas of focal contacts do not move relative to the substratum but persist for several minutes within the larger area of close contact, before fading.

It has been shown that regions of focal contacts appear under IRM as dark spots because they are the regions where the separation between the cell body bundles of actin filaments or stress fibres (Burridge, 1981 and Burridge *et. al.*,

1987). Cells with fewer stress fibres migrate more rapidly (Couchman and Rees, 1979 for fibroblasts, and Radice, 1978 for epidermal cells).

Couchman and Rees (1979) and Couchman *et. al.* (1982) also noticed that for fibroblasts grown in culture, as time progresses, both stress fibres and focal contact develop and this results in a decrease in the rate of cell movement.

The appearance of stress fibres is thought to be a result of an increase in surface fibronectin; cells with less surface fibronectin, such as transformed cells, lack both stress fibres and focal contacts. However, if fibronectin is added to these transformed cells they regain their normal morphology and develop both structures (Ali *et. al.*, 1977; Burridge *et. al.*, 1987; Willingham *et. al.*, 1977).

Kolega *et. al.* (1982) have studied cell-substratum contact in amphibian leukocytes, amphibian epidermal cells, chick heart fibroblasts, and canine kidney epithelial cells using both IRM technique and time-lapse cinemicrography and confirmed the findings of the previous investigators in that, basically, close contacts are associated with rapid cell movement while focal contacts are associated with reduced cellular movement and maintenance of the spread cell shape.

1.2- THE CHICK BLASTODERM AS A SYSTEM TO STUDY EPITHELIAL CELL BEHAVIOUR:

The blastoderm of avian embryos expands to encompass the extremely large mass of the egg yolk in a process called epiboly. Because of the wide area that is needed to be covered by that expansion, the process is indeed the most extensive type of epithelial spreading that is found during the morphogenesis of any vertebrate embryo.

In the freshly laid hen's egg the blastoderm is a small membrane circular sheet of tissue just 3mm in diameter lying on the yolk mass. The blastoderm and the yolk mass are enclosed by a membrane. After about 10 hours of

incubation, the blastoderm margin attaches to the overlying vitelline membrane and starts to expand centrifugally. It reaches the equator of the egg by the third day of incubation and during the fourth day it encompasses the whole yolk (Bellairs,1963).

The blastoderm during the first day of incubation (stage 4-6 of Hamburger and Hamilton, 1951) is divided according to its appearance into two concentric zones, a central translucent zone called the *area pellucida* and a denser outer zone called the *area opaca*. The embryo forms from the central part of the *area pellucida* while the lateral parts of this area along with the *area opaca* form the extraembryionic tissue. The blastoderm is originally composed of two layers, an upper layer or the ectoderm and a lower layer, the hypoblast, which is more or less equivalent to the endoderm. It is thought that the hypoblast develops as a result of ejection of its cells from the upper layer due to differences in the surface properties, with the hypoblast cells being less adhesive (Zalik and Sanders 1974, Eyal Giladi *et. al.* 1975 and Bellairs 1986). After the appearance of the primitive streak, cells pass through it into the hypoblast and form a central area, the definitive endoderm (Bellairs, 1953). The latter displaces the hypoblast to the border of the *area pellucida* (England and Wakely, 1986).

The *area opaca* is divided according to the morphology of its cells into three zones; the inner zone, the syncytial zone and the "margin of overgrowth" (Bellairs, 1963; Bellairs *et. al.*, 1969). It is this latter area which I will be concentrating on throughout this literature review.

The cells of the "margin of overgrowth" are called the "leading edge cells" (Downie and Pegrum, 1971) and are the only cells of the blastoderm which attach to and actively move on the inner surface of the vitelline membrane. These cells migrate centrifugally across the vitelline membrane leading to the expansion of the whole blastoderm (New, 1959; Bellairs, 1963; Bellairs *et. al.*, 1969).

New (1959) attempted to answer the question why the leading edge cells of

the blastoderm are the only cells that have the ability to attach to the vitelline membrane and move over it. He put forward two possible explanations: a) that the cells of the leading edge may be intrinsically different, with regard to their surface structure, from the remaining cells of the blastoderm OR b) that they may be intrinsically the same but their marginal position may enable them to behave differently. His further experiments on the blastoderm made him favour the first explanation. His explanation was further supported by other workers (Bellairs and New, 1962; Bellairs, 1963; Bellairs *et. al.*, 1969).

The ultrastructure of the leading edge cells has been studied by Downie and Pegrum (1971). They reported that these cells have an abundance of microtubules, bundles of cortical filaments, and oriented lamellae which attach to the vitelline membrane via attachment plaques. These are all important features of actively moving cells (Baker and Schroeder, 1967).

The expansion of the blastoderm to cover the whole yolk involves an increase in its area of over 200 fold. This increase must involve a great deal of growth. Schlesinger (1952) conducted a series of cauterisation experiments on the blastoderm of the chick embryo. He argued that growth occurs actively from the edge area and not by cell proliferation in any other part of the blastoderm. His experiments however included many artifacts which are known to affect the spreading of the edge cells, such as damaging parts of the vitelline membrane which are crucial for edge cell movement. Downie (1976) studied cell proliferation in the blastoderm of the chick embryo and reported that cell proliferation has never been observed among the edge cells but only in the rest of the blastoderm. These findings have been recently supported by and Monnet-Tschudi and Kucera (1988) who used autoradiography to study the same problem.

Downie (1976) also reported, however, that the rate of proliferation is not the same throughout the blastoderm but differs from one area to another and from one stage of development to another; at the start of expansion all non-

edge cells proliferate at a rapid rate but as expansion proceeds the proliferation becomes restricted to certain areas. For example, at stage 17-18 (H.H.), cell proliferation is low at two points, near the *area vasculosa* and near the leading edge, and is high in the area which lies between the two low points. As expansion proceeds further cell proliferation in the blastoderm becomes restricted to a narrow area around the *area vasculosa*.

1.2.1- TENSION IN THE BLASTODERM:

New (1959) noted that during the centrifugal active migration of the edge cells over the vitelline membrane the rest of the blastoderm came under tension. This mechanical tension is thought to be important in preventing the non-edge cells of the blastoderm from piling up and in their maintaining the sheet-like arrangement. New argues that this tension is essential for the normal expansion of the blastoderm.

The generation of tension in the blastoderm by the edge cells was also supported by Bellairs (1963) and later by Bellairs *et. al.* (1967) who stressed its importance for the normal development of the embryo, indicating that experimentally decreased tension leads to cell death in the blastoderm and to poor differentiation.

Downie (1976) also implicated the edge cells with the generation of tension in the blastoderm and stated that this tension is a result of an imbalance between the rate of the centrifugal movement of the edge cells and the rate of proliferation of the non-edge cells i.e. the growth of the blastoderm. He also measured the amount of tension in the blastoderm at different stages of development by noting the retraction that these blastoderms undergo after the detachment of the edge cells from the vitelline membrane. He reported that tension is at its highest level at the start of the expansion when the rate of cell proliferation (growth) in the blastoderm is low, then it drops dramatically at later stages when the rate of growth catches up with the rate of edge migration

and so releases the tension on the blastoderm.

Kucera and Monnet-Tschudi (1987) confirmed the presence of tension in the blastoderm and reported that increasing the tension experimentally leads to abnormal embryonic development. They, however, did not agree with New (1959), Bellairs (1963), and Downie (1976) on the origin of the tension. They claim that real time cine analysis showed that tension is not generated by the movement of edge cells but by the contraction of the non-edge cells of the intermediate and/or the peripheral zones of the *area opaca*.

Kucera and Monnet-Tschudi (1987) also claim that tension is not equally distributed throughout the blastoderm but differs from an area under maximum strain represented by the posterior part of the primitive streak to an area under less stress represented by the *area pellucida*. They claim that the tension has no effect on the leading edge cells themselves.

1.2.2- FIBRONECTIN AND CHICK DEVELOPMENT:

Fibronectin appears in the chick embryo shortly before gastrulation in the ectodermal extracellular matrix. It seems to play an essential role in cell migration during morphogenesis. In fact, gastrulation (Boucaut *et. al.*, 1984 in amphibian embryos; Duband and Thiery, 1984 in chick embryo), neural crest migration in chick embryos (Bronner-Fraser, 1981; Duband and Thiery, 1982; Newgreen and Thiery 1980; Ravasio *et. al.*, 1983) and movement of mesoblasts (Harrisson *et. al.*,1984) are all related to the presence and distribution of fibronectin in the embryo. For example, Ravasio *et. al.* (1983) and Boucaut *et. al.* (1984) reported that *in vivo* and *in vitro* studies using antibodies to fibronectin suggest that migration of embryonic cells require the guidance of fibronectin in the spatially organised extracellular matrix, and Duband and Thiery (1982) showed that the distribution of fibronectin plays a crucial role in guiding the migrating neural crest cells to their target sites.

High concentration of fibronectin have been found in arrays of fibrils along

the border between the *area pellucida* and *area opaca* of the gastrulating chick embryo (Critchley *et. al.*, 1979 and England, 1982). Contradictory reports exist as to the function of this band.. It has been suggested that it is involved in guiding the migration of mesoblast cells and the posterior movement of primordial germ cells (Critchley *et. al.*, 1979, Wakely & England, 1979 and England, 1980,1982). However, Andries *et. al.* (1985) reported that there are only few cells on this band and that these cells are spherical and have no lamellae, suggesting that the band is not a favourable substratum for the spreading of cells while, on the other hand, cells on the basal lamina outside this band show the features of actively moving cells. They suggested that this band might serve as a barrier that stabilises the partition between the embryonic and extraembryonic tissue and also stabilises the polarity of the early blastoderm.

Monnet-Tschudi *et. al.* (1985) studied the distribution of fibronectin in the chick blastoderm and reported that it is associated with the basal lamina of the ectodermal part of the blastoderm and is also found all around the actively moving edge cells and the mesodermal cells.

Kucera and Monnet-Tschudi (1987) examined the interaction between fibronectin and the ectodermal cells of the blastoderm. They used antibodies to fibronectin to see the effect that the disruption of its normal distribution has on the blastoderm. They reported retraction of the *area opaca* and distension of the *area pellucida*. They also reported that fibronectin fibrils are distributed in a radial pattern in those areas which contract radially i.e. intermediate and peripheral zones of the *area opaca*. They concluded that the mechanical activities of the ectodermal cells may play an important role in the *in situ* assembly of extracellular matrix components. These findings are similar to those of Singer (1979) who indicated that fibronectin rich fibres are oriented with respect to bundles of intracellular microfilaments of fibroblasts cultured *in vitro*.

More recently Lash *et. al.* (1990) demonstrated that fibronectin is present

between the cells of the chick blastoderm edge acting as a substratum, in both cell-cell and cell-substratum adhesion and movement. They suggested that the leading edge cells themselves secrete fibronectin during their migration and utilise it as a substratum for their migration.

1.2.3- CONCLUDING REMARKS ON CHICK BLASTODERM:

The chick blastoderm provides a very fertile field for the study of epithelial cell behaviour. It represents a unique system of epithelial interaction and behaviour since, firstly it is composed of an epithelial sheet of cells in which only the marginal edge cells are in contact with the substratum, the vitelline membrane, at any time; secondly these edge cells are themselves different from any other actively moving marginal epithelial cells in that they are multilayered which suggests that there must be an excellent degree of co-ordination between them in order to be able to expand in such an elegant way to cover the whole of the egg yolk; and thirdly, since there is no cell proliferation among edge cells it is interesting to know how such a small band of cells which used to encircle a blastodisc only a few millimetres wide could possibly encircle a blastoderm which is 20 times as wide when the blastoderm reaches the equator of the egg.

Finally the later stages of epiboly in the chick embryo have been totally ignored by scientists and it would be very worthwhile to compare the system here with the one which has been extensively investigated in the teleosts *Fundulus* and *Oryzias*.

1.3- AIMS AND OBJECTIVES OF THE CURRENT RESEARCH:

This project is intended to focus on the following points:

1- Detailed comparison of the organisation of the leading edge cells at 15 hours, 1 day, 1.5 day, 2, 2.5, 3, and 3.5-4 days of incubation and the possible source of cell recruitment into the leading edge.

2- Morphometric analysis of the leading edge at the above stages of development and its relation to the of expansion of the blastoderm.

3- The behaviour of edge cells at the end of epiboly.

4- Immunofluorescent staining of the blastoderm to determine the orientation of both microtubules and microfilaments in both edge and non-edge cells, and the effect of inhibitors of these cytoskeletal elements on both the shape and movement of these cells.

5- The distribution of fibronectin in the chick blastoderm.

6- The behaviour of non-edge cells cultured *in vitro* on the inner and outer surface of the vitelline membrane.

CHAPTER TWO

MATERIAL AND METHODS

2.1- Light microscopy:

Fertile white leghorn eggs were incubated at 37.5 °C and in a humidified atmosphere. When they reached the required stage of development they were opened and their blastoderms dissected out in Tyrode's balanced salt solution and as much as possible of the adhering yolk was washed off the vitelline membrane, taking care not to disturb the leading edge cells or detaching them from the membrane.

The vitelline membranes with the attached leading edge cells were mounted on glass rings to keep the blastoderm as flat as possible before being fixed for two hours in Karnovsky's half strength fixative (Karnovsky, 1965) prepared as follows:

- Dissolve 10 gm. Paraformaldehyde in 125 ml. of distilled water by heating to 60-70 °C on a magnetic stirrer.
- Add a few drops of 1N sodium hydroxide (NaOH) solution to the mixture until the mixture becomes colourless (usually 2-4 drops of NaOH were sufficient).
- Cool then add 25 ml. of 25% glutaraldehyde solution (TAAB England).
- Make up to a total volume of 250 ml. with 0.1 M cacodylate buffer (pH 7.4), then add 0.125 gm. anhydrous calcium chloride.

The 0.1M cacodylate buffer used above was prepared by dissolving 4.28 gm. of sodium cacodylate in 100 ml. distilled water and 2.7 ml. of 0.2 N HCl then added. The solution was then made up to a total volume of 200 ml. with distilled water.

After 2 hours in the fixative, the specimens were washed four times in the buffer over a period of about an hour, and post-fixed for 1.5 hour in 1% (W/V) osmium tetroxide in cacodylate buffer at 4 °C. The specimens were then washed three times in cacodylate buffer for about an hour. They were then processed for embedding in Araldite resin in the following way:

- Dehydrate in 30% alcohol (ethanol)	10 min.
- Dehydrate in 50% alcohol	10 min.
- Dehydrate in 70% alcohol	10 min.
- Dehydrate in 90% alcohol	10 min.
- Dehydrate in absolute alcohol	2X10 min.
- Dehydrate in dried absolute alcohol	10 min.
- Dehydrate in absolute alcohol : propylene oxide (3:1)	10 min.
- Dehydrate in absolute alcohol : propylene oxide (1:1)	10 min.
- Dehydrate in absolute alcohol : propylene oxide (1:3)	10 min.
- Dehydrate in propylene oxide	2X10 min.

- Two changes in pure propylene oxide for 10 min. each.

The specimens were transferred to open glass vials containing a mixture of equal volumes of Araldite resin and propylene oxide and left overnight on a rotator to allow the evaporation of the propylene oxide and its substitution with the resin. The specimens were then transferred to aluminium plates containing pure resin in an oven set at 60 °C and left for 36-48 hours to allow the polymerisation of the resin.

Semi-thin sections (1µm thick) for light microscopy were cut with glass knives on a Reichert OMU3 ultramicrotome and mounted on standard glass slides. They were then stained with 1% toluidine blue in 1% borax (aluminium borate), mounted with DPX and examined under a Wild M20 light microscope.

Photographs were taken on a Wild M20 photomicroscope using Kodak Panatomic-X films (ASA 32).

2.2- Transmission electron microscopy (TEM):

Ultrathin sections of areas of interest to be viewed under the transmission electron microscope (TEM) were cut on the ultramicrotome and collected on slot grids which had been coated with a film of 1% formvar in chloroform. They were then stained with uranyl acetate and lead citrate (Reynolds,1965) for 5 minutes each and examined under a Zeiss 902 TEM.

2.3- Scanning electron microscopy (SEM):

Specimens intended for examination under the Scanning electron microscope (SEM) were transferred after the last change in dried absolute alcohol in section 2.1 into a critical point drying unit, mounted on aluminium stubs using double sided sellotape, and sputter coated for 6 minutes with gold/palladium. They were then examined under a Philips 500 SEM.

2.4- Blastoderm cell extraction using Triton X-100:

Some of the blastoderms at different stages studied have been extracted to improve detection of different junctions between their cells (Andries and Vakaet, 1985b).

Blastoderms intended for extraction were washed as usual in Tyrode's buffer then treated for 5 minutes in a solution of 0.5% (V/V) Triton X-100 in a PIPES buffer made up of 10 mM PIPES, 0.3M sucrose, 5.5 mM magnesium chloride, and 1 mM EGTA. The final pH of this buffer was 6.8. The extracted blastoderms were then rinsed in the PIPES buffer and fixed for 30 minutes at room temperature with 1% (V/V) glutaraldehyde in PIPES buffer. After fixation, specimens were washed in 0.1 M cacodylate buffer and treated for 20 minutes with 0.2% (W/V) tannic acid in cacodylate buffer. They were then post-fixed in 0.2% (W/V) osmium tetroxide in 0.1 M cacodylate buffer for 20 minutes, and stained *en bloc* with 0.1% (W/V) uranyl acetate in water for 5 minutes. The specimens were then rinsed in water, dehydrated and processed

for embedding in resin as in section 2.1 . Semi-thin sections for light microscopy and ultrathin sections for transmission electron microscopy were then cut.

2.5 Immunofluorescence:

2.5.1 Staining for microtubules and microfilaments in the blastoderm:

2.5.1.1 Staining for microtubules and microfilaments in whole mounts:

We intended at the beginning of this experiment to look at the distribution of microtubules and microfilaments in both the leading edge cells and the other non-edge cells of the blastoderm while they were in their natural environment i.e. with the leading edge cells still attached to the vitelline membrane. However, despite using different fixatives such as methanol, formaldehyde and glutaraldehyde, and following different dehydration procedures such as clearing the preparation with xylene before staining with anti-tubulin or anti-actin antibodies, I have not been able to visualise these structures due to the very bad resolution which was a result of the opaqueness of the vitelline membrane. Therefore, the results of these staining methods were very poor and I examined the distribution of microtubules and microfilaments in cultured edge cells.

2.5.1.2 Staining for microtubules and microfilaments in cultured edge cells :

Immunofluorescence staining was performed on cells that had been grown *in vitro* on glass coverslips. The following procedure was followed under sterile conditions:

After incubation for 1 - 2 days, the eggs were opened in sterile Tyrode's solution at room temperature and the blastoderm removed and any adhering yolk washed off. Small fragments of the blastoderm edge with the attached non-edge cells were then excised and each fragment was cultured using a small drop of culture medium on a coverslip in a petri dish. The culture medium used consisted of 90 ml. Ham's nutrient mixture F-12 (Gibco, UK)

supplemented with 10 ml. fetal calf serum, 2 ml. penicillin and 2 ml. streptomycin. The position of the edge cells was marked by scoring the coverslip with a diamond pencil. The cultures were given about 1 hour to attach to the substrate before adding more medium. The cultures were incubated for between 6 and 48 hours. The cultures were then processed for staining according to the method of Brinkley *et. al.* (1980) as follows:

1- Discard excess medium then rinse in phosphate buffer saline (PBS).

2- Immediately fix for 5 minutes in absolute methanol at -20 ^{o}C.

3- Fix for 2 minutes in absolute acetone at -20 ^{o}C

4- Air dry the coverslips then rinse in PBS.

5- Incubate for 30 minutes at room temperature in the primary polyclonal antibody, anti-tubulin or anti-actin (ICN ImmunoBiologicals, UK) (both raised in rabbit). The primary antibodies were made up to the dilution of 1:30 with PBS.

6- Wash coverslip 5 times in PBS over a period of 25 minutes.

7- Incubate in the dark for 30 minutes at room temperature in fluorescein isothyocyanate (FITC) labelled anti-rabbit IgG (ICN ImmunoBiologicals, UK), made up to a concentration of 1:10 with PBS.

8- Wash as in step 6.

9- Mount on glass slides using a mixture of PBS and glycerol (1:9 V/V) that has been brought up to pH 9 with 1N NaOH. This alkaline medium was used to minimise the fading of fluorescence from the cultures when they were exposed to ultraviolet light.

10- Seal the edges of the coverslips with nail polish and store in light-tight boxes at -20 ^{o}C, only if immediate viewing was impossible.

The stained sections were examined with a Leitz Ortholux II microscope using incident light fluorescence with an HB050 light-pressure mercury lamp.

Photographs were taken on Kodak ektachrome film (ASA 400), using 2X KP490 (exciting), KT 510 (dichromic mirror), and K 515 (suppressing) filters.

2.5.1.3 Controls:

These were dealt with in the same way as above except that they were incubated in PBS instead of the primary antibody.

2.5.2 The *in vitro* effect of microtubule and microfilament inhibitors on the shape and expansion of the blastoderm

Here the cultured blastoderm cells were incubated in a medium containing a microtubule inhibitor, Colchicine (Sigma, St. Louis, MO), at a concentration of 0.1 μg/ml, or microfilament-destroying drug, cytochalasin D (Sigma, St. Louis, MO), at a concentration of 5 μg/ml for a certain period of time (details of which will be included in the results). They were then processed for immuno-fluorescence staining for microtubules and microfilaments as described in section 2.5.1.

2.5.2.1 Controls:

Cultures intended as controls were incubated without the inhibitors and then processed for staining as in section 2.5.1.2. Controls for the specificity of the staining were processed as in section 2.5.1.3.

2.6 The distribution of fibronectin in the blastoderm of the early chick embryo:

To study the distribution of fibronectin during the expansion of the blastoderm, the eggs were opened after 1-3 days incubation and pieces of the blastoderm, which include the attached edge cells and small areas of the ectodermal cells near to it, were fixed for 4-5 hours in a mixture of 96% ethanol and 1% acetic acid (99:1) (Harrisson *et. al*, 1984), washed in water, and processed for embedding in wax as follows:

- Dehydrate in 95% alcohol 10 min.
- Dehydrate in absolute alcohol 10 min.
- Clear specimens in xylene 5-10 min.
- Transfer into a mixture of equal volumes of xylene and paraffin wax for 20

minutes.

- Transfer to pure wax I for 20 minutes.
- Transfer to pure wax II for 20 minutes.
- Embed in pure wax.

6 um thick sections were then cut on a Beck microtome and mounted on glass slides which had been previously coated with albumen (10-15 sections per slide). After the sections had completely adhered to the slides and dried, they were cleared in Histoclear and hydrated in descending grades of ethanol. After the last change in alcohol they were rinsed in water then prepared for staining as follows:

- Wash thoroughly for 15-20 minutes in PBS (pH 7.2).
- Incubate for 30 minutes at 37 ^{o}C in mouse anti-fibronectin antibody raised in rabbit. The antibody was diluted to 1:60 with PBS.
- Rinse and then wash for 5 min. in PBS.
- Incubate for 30 minutes, in the dark, in FITC labelled anti-rabbit IgG (H+L) raised in goat (1:200 with PBS).
- Rinse and wash in PBS 5 min.
- Mount with coverslips using glycerol/PBS mixture (9:1 V/V).

The preparations were then viewed and photographed as in section 2.5.1 .

Following the publication of a study conducted by Lash *et. al.* (1990) on the distribution of fibronectin in the leading edge of the chick embryo, which reported results different from the ones I obtained following the above method, I tried the method they used (Ostrovsky *et. al.*, 1983).

2.7 **Cultures of non-edge blastoderm pieces on the inner surface of the vitelline membrane**:

Eggs were incubated for 1-2 days in order to get chick embryos at stage 4 - 13 (Hamburger and Hamilton, 1951). The blastoderms along with vitelline membrane were removed and washed in Tyrode's solution and the attached

yolk washed off the vitelline surface of the blastoderm. The leading edge which attached to the vitelline membrane was cut from the blastoderm, then small strips (3 mm2) were cut from the area of the remaining blastoderm adjacent to the original place of the edge i.e. pieces were cut from the periphery of the *area vitellina externa*. These pieces were then explanted on the inner surface of vitelline membranes which had been set up on glass rings according to the method of New (1955), using thin albumen as a culture medium. The side of the blastoderm which was in contact with the vitelline membrane, i.e. whether it was the ectodermal or endodermal side, was always noted down.

Time-lapse photography was performed on some of the explants at a rate of a frame every 3 minutes using a Wild M20 microscope with Bolex cine camera and a time lapse apparatus made in the University of Glasgow. After a period of 18-24 hours of filming the explant was fixed for 2 hours in Karnovsky's half strength fixative and processed for examination under light and transmission electron microscopy as described in section 2.1. and 2.2.

2.8 Silver nitrate staining of the blastoderm cells:

To demarcate the boundary of the leading edge and ectodermal cells of the blastoderm at the end of epiboly the method of Kageyama (1980) was applied as follows:

- Blastoderms at the desired stage of development were removed from the egg and cleared of as much as possible of the attached yolk.
- They were then fixed for 3-10 min. in Stockard's fixative (5 parts formalin, 4 parts glacial acetic acid, 6 parts glycerine and 85 parts distilled water) at room temperature.
- Rinsed twice with distilled water.
- Placed in a solution of 0.5% silver nitrate for 1 to several minutes until the boundaries became conspicuous under the dissecting microscope.
- Quickly rinsed in distilled water to remove the excess of silver nitrate and mounted on glass slides.

CHAPTER THREE

ORGANISATION OF EDGE CELLS AT STAGES BETWEEN 15 HOURS AND 2.5 DAYS OF DEVELOPMENT

3.1 INTRODUCTION:

The expansion of the chick blastoderm to cover the yolk sphere of the egg has been under serious investigation for nearly 40 years. One of the early studies done on this subject was that of Schlesinger (1952) who, believing at that time that the blastoderm uses the yolk as a substrate for its expansion, assumed that the blastoderm depends for its expansion only on the edge cells and that the rest of the blastoderm does not contribute to the process. He reached his conclusion by relying on a series of injury experiments. He reported that if the blastoderm was injured distal to the advancing edge it would continue to expand until the edge reaches the place of injury: then it stops. If, however, the injury was made anywhere in the blastoderm behind the edge, the latter would continue advancing without regard to the injured areas. In his opinion the edge area was so important because of the formation of new cells round what he called free nuclei lying outside the blastoderm. Schlesinger's experiments however did not consider an important factor which was later found to play an integral part in blastoderm expansion. This factor is the vitelline membrane.

Shortly after Schlesinger, New (1955 and 1959) demonstrated that for the blastoderm to expand it needs to attach to the overlying vitelline membrane and not to the yolk as previously thought. New (1955) utilised this fact to develop a novel method for culturing the chick embryo on this membrane using glass rings for support. This method, which is known as the New Culture

method, is widely used for direct observations of many aspects of chick development.

In his study, New (1959) showed that the blastoderm attaches to the vitelline membrane only by its edge area, for when this edge is detached from the membrane the expansion of the whole blastoderm comes to a halt. He also reported that it is the properties of the cells of the edge rather than their position in the blastoderm which give them the ability to attach to and move over the vitelline membrane. This finding was later supported by the work of Bellairs and New (1962), Bellairs (1963) and Bellairs *et. al.* (1969). New (1959) also indicated that the edge cells must not only attach to the vitelline membrane to advance towards the equator of the egg but also that they must do so in a precise polarity with respect to that membrane; for when the blastoderm was cultured on the vitelline membrane upside-down, so that the edge cells which were originally in contact with the membrane were now facing away from it, the edge area curled under the blastoderm to bring those edge cells once again into their original position, then spread backwards resulting in the transformation of the inverted blastoderm into a hollow vesicle.

New (1959) also stressed the importance of the vitelline membrane in blastoderm expansion indicating that the blastoderm would expand only if its edge is in contact with the inner surface of the vitelline membrane. He compared the expansion of the blastoderm on normal and fixed vitelline membrane and found that blastoderms expand normally on the untreated membrane while formalin or alcohol fixed membranes led to weak expansion. New (1959) also reported that blastoderms cultured on the outer surface of the vitelline membrane did not expand.

New's (1959) findings could explain the failure of several attempts to achieve blastoderm expansion on plasma (Waddington, 1932) and agar clots (Spratt, 1947; Spratt and Haas, 1960). They also give a completely different interpretation of the findings of Schlesinger (1952), on the basis that when he

made the injury in front of the blastoderm edge he in fact had torn the vitelline membrane at that area so that the blastoderm continued expanding until its edge cells ran out of vitelline membrane and so the expansion stopped. When the injury was made behind the edge it did not affect the vitelline membrane in front of the edge and therefore it continued movement.

Bellairs *et. al.* (1963) examined the nature of the vitelline membrane and reported that it is composed of two surfaces; an inner surface facing the yolk and an outer surface facing the albumen. These two surfaces were found to be both structurally and chemically different, with the inner surface being the one arranged into a meshwork of fibrils which sustains cell attachment and movement. Bellairs and her co-workers were however unable to identify the composition of the vitelline membrane proteins but in any case these proteins were thought not to include collagen; the composition of the vitelline membrane is still unknown and obviously needs more careful examination with today's advanced protein analysis technology.

The emphasis in the years that followed was switched to general organisation, fine structure and behaviour of the edge cells, which enable them to carry out the task of pulling the whole of the blastoderm to cover the yolk sphere.

Downie and Pegrum (1971) studied the fine structure of the leading edge cells after about one day of incubation. They reported that these cells have oriented lamellae which underlap and overlap each other, and which attach to the vitelline membrane by attachment plaques and that these cells possess bundles of cortical microfilaments and abundant microtubules; all of these are features of actively moving cells.

Chernoff and Overton (1979) provided another study on the cells of the leading edge of 1 to 1.5 day chick embryos with respect to their attachment sites and the effect of cold treatment. They reported similar findings as far as the general shape and organisation of these cells are concerned. In addition

they found that cold treatment of the edge cells caused their lamellae and the projections on their basal surface, those in contact with the vitelline membrane, to become more prominent , and that the vitelline surfaces of these cells were resistant to cold treatment as they remained flat throughout treatment.

More recently, Andries *et. al.* (1985) made an extensive study on the distribution of junctions among the edge cells of chick embryos which had been incubated for 15 hours in an attempt to get some information about the mechanism of locomotion of these cells. They reported the presence of gap junctions and well developed desmosomes between the proximal cells of the edge and incipient desmosomes and some tight junctions between the ventral cells of the mid-part as well as the distal area i.e. those not in contact with the vitelline membrane. More developed desmosomes as well as networks of tight junctions were observed between those cells which are in contact with the vitelline membrane. They concluded from the above distribution of junctions that the cells of the edge retain their position relative to each other and to the vitelline membrane, and they proposed that the movement of edge cells follows the model of epithelial cell movement suggested by Radice (1980a) in which it is suggested that as marginal cells move forward the cells behind them occupy their position without intermingling of marginal and sub-marginal cells.

Andries and Vakaet (1985 a & b) made similar studies to the one just described and claimed that intercellular communication occurs in the leading edge through gap junctions between its cells. They implicated the presence of these gap junctions with synchronisation of movement between the different cells of the edge to increase their rate of migration and to maintain this unique morphological unit. They also reported that the discontinuous presence of tight junctions between these cells indicates that these junctions do not function as a barrier to prevent material from passing across the cells.

It is clear from the several papers cited above that the leading edge of the chick blastoderm presents an interesting field for studying cell movement and

behaviour in an epithelial sheet. However, all the previous work described above has been done on blastoderms that have just attached to the vitelline membrane or are at the very early stages of expansion. Therefore, this current study has been designed to look at the organisation of the edge cells from the early stages when they have just attached to the vitelline membrane to the stage when they pass the equator of the yolk. The much later stages as blastoderm expansion nears completion will be described in a later chapter of this thesis.

I have used light microscopy as well as scanning and transmission electron microscopy to investigate whether the organisation of the edge cells remains the same throughout the different stages of development or whether there are any differences in such organisation which could be related to the variations in the rate at which the edge cells move on the vitelline membrane as reported by Downie (1976) who found that the expansion of the blastoderm at the early stages of development is slow (200 um/hr. *in vivo* and 169um/hr. *in vitro*) but increases from the second to the third day of development (555 um/hr. *in vivo* and 285-384 um/ hr. *in vitro*) before becoming slow again after that stage (292 um/hr).

To achieve this, eggs were opened after 15 hours, 1 day, 1.5 day, 2 and 2.5 days of incubation, to get blastoderms at stages 3, 6, 8-9, 11-13, and 15-16 (Hamburger and Hamilton, 1951) respectively. These blastoderms were fixed and processed for study under light microscopy, transmission and scanning electron microscopy (TEM and SEM) as described in chapter 2. Some of these blastoderms were treated with Triton X-100 to enable me to study more closely the type and distribution of junctions between the edge as well as non-edge cells, and in addition to look for the orientation and distribution of cytoskeletal elements in these cells. This Triton X-100 treatment is described in chapter 2.

This chapter will also include morphometric analysis of the width of the edge area, i.e. the area covered by these cells on the vitelline membrane, at the different stages studied. The average height of the leading edge, the number of

its cells and the length of its leading lamellae at each stage will also be presented as part of the morphometric analysis.

3.2 Methods:

Before proceeding to describe the organisation of edge cells it is important to define some of the terms which will be used in the description. The diagram in Figure 3.1 shows that the leading edge of the blastoderm can be divided into three areas according to the position of the cells of each area relative to the ectodermal part of the blastoderm. These areas are: a) a proximal area which include those cells in contact with or very near the ectoderm; b) a distal area where cells possess free lamellae which they extend on the vitelline membrane and c) a mid-part which includes all cells lying between the proximal and distal areas. This division of the edge area is, therefore, not based on equal thirds of the areas described.

Other terms used throughout this thesis are shown also in Fig.3.1. These include: a) The ectodermal part of the blastoderm in which the cells are elevated over the vitelline membrane and do not make contact with it, and b) the deeper layer cells which are those non-edge non-ectodermal cells located on the basal lamina of the ectodermal cells. The cells of the leading edge itself are divided according to their position on the vitelline membrane into upper layer cells facing the yolk and lower layer cells nearer to the vitelline membrane.

The width of the blastoderm at a certain stage of development means that length of the vitelline membrane which the edge cells occupy at that stage of development. To measure the width, three blastoderms at each stage of development were fixed, sectioned and stained with toluidine blue as described in chapter 2. Ten sections chosen at random from each of these blastoderms were used to measure the width of the edge, with a fine eyepiece graticule and X40 objective, from the most proximal cell to the tip of the distalmost cell.

The mean width and any deviation from that mean were then calculated (Table 1).

As a comparative measure of the number of cells in the edge, I counted in any one section the number of nuclei containing a nucleolus. This measure was chosen as it seemed possible that cell size might vary considerably in different stages of the edge. Counting only sections that passed through a fixed point of relatively constant size seemed to me the best way of getting a comparison of cell numbers in such a structure. This, of course, does not give an **absolute** measure of cell numbers but does allow a comparison between stages to be made. Again, three blastoderms from each stage of development were used and the number of nuclei containing a nucleolus were counted, using X100 oil-immersion lens, from ten randomly chosen sections per blastoderm at each stage. The mean and standard deviation for each stage is calculated and one way analysis of variance (ANOVA) was used to test for any statistically significant difference between the different stages.

I also attempted to measure the lengths of the leading lamellae of edge cells, to check if these change with stage. It was necessary here, again, to devise a common convention for lamella measurement. The proximal end of a lamella was denoted by the first intracellular organelle visible in the cytoplasm of the cell (lamellae are free of organelles resolvable with the light microscope). The distal tip was the furthest extent visible using a X100 oil-immersion lens. Because of the difficulty of resolving cell boundaries within the edge, only the distalmost edge cells were measured in this way. The mean length of lamellae was then calculated from 3 blastoderms at each stage of development and 10 sections per blastoderm were randomly chosen for the measurements.

I also attempted to measure the height of the edge to check if it changes from one stage of development to another. To do this it was necessary to measure first the mean area of sections of the edge at each stage, then divide that by the mean width of that edge at that stage. To achieve this, Camera

Camera Lucida drawings of samples of the area of leading edge attached to the vitelline membrane at the different stages studied were made. These were then enlarged using a photocopying machine and the mean area and the standard deviation were calculated for each stage using the programme "Cherry Digitiser" on the BBC microcomputer which measures the area of any structure by tracing its outlines. Again, ten randomly chosen sections per blastoderm were drawn and three blastoderms for each stage were used.

The appearance of the edge cells will be presented here stage by stage. These descriptions will include a summary of the morphometric data which will be presented in detail at the end of the results section.

3.3 Description of stages:

3.3.1 Stage 3 (15 hours) blastoderm edge:

The area of the vitelline membrane covered by the leading edge at this stage i.e. the width of the edge, was found to be $104 \pm 9.5 \mu m$ and the mean height of the edge area is $25 \mu m$. Light microscopy revealed that the edge is multilayered i.e. 3 to 4 cells deep. The cells possess a large number of yolk vacuoles as is the case in all cells of the blastoderm at this early stage of development (Fig.3.2 b). These yolk vacuoles make it very difficult to study these cells under the light microscope. One feature of the edge cells which distinguish them from non-edge cells, in addition to their relative position, is their prominent nuclei. A few lacunae i.e. small intercellular spaces, have also been observed between the edge cells at this stage (see Fig.3.2b).

The number of edge cells at this early stage is 2.9 ± 0.7 cells. Lamellae from the proximal cells underlie the cell bodies of cells lying distal to them and those of the latter cells underlie the cells located still more distally, and so on. These lamellae are all oriented in the direction of movement of the edge, except that some lamellae from the proximal cells nearest to the ectoderm are pointing centripetally i.e. opposite to the direction of movement. Scanning electron microscopy of the edge area showed that the cell bodies are swollen due to the

presence in the edge cells of the large number of yolk vacuoles described above (Fig. 3.2a). The cells of the distal area of the edge and some of the cells immediately behind them send out thin lamellae which overlap and underlap each other (Fig. 3.3a). The length of the distal lamellae was 30 $\pm$ 3.4 μm. Some of these lamellae end in very thin filopodia which come in contact with the lamellae of the neighbouring cells.

Transmission electron microscopy of the edge cells at this stage showed that those at the proximal area are connected together by several desmosomes as well as points of close contact, small areas of tight junctions (Fig. 3.3b). Desmosomes are mainly found between the cell bodies of the proximal cells while areas of close contact are usually associated with the underlapping and overlapping lamellae. Desmosomes become less frequent between the cells of the mid-part (Fig. 3.4a) and are completely absent between the distalmost cells and their lamellae (Fig. 3.4b). Areas of close cell contact are, however, still frequently seen between the cells of the latter two areas of the edge. The most proximal cell of the edge has a centripetally oriented lamella as well as a centrifugally oriented one (Fig. 3.5a).

TEM also revealed that the edge cells at the different levels are rich in cortical microfilaments and microtubules which are oriented towards the direction of movement (Fig. 3.5a&b). A few of the cells which are in contact with the vitelline membrane send a number of microvillous projections which protrude into the membrane (Fig. 3.6a). I have not been able to distinguish any difference in the distribution of these projections between the different areas of the edge at this stage.

The ectodermal cells immediately proximal to the edge are 9 - 11 um in height and 7 -8 um in width. They contain many yolk inclusions but these are considerably smaller than those of the edge cells. The ectodermal cells, whether they are located near the edge or away from it, display typical junctional complexes between them. These junctions consist of tight junctions

connecting the apical surface of the cells followed by a number of small desmosomes which end in fine tonofilaments (Fig. 3.6b). Areas of interdigitation of the adjacent cellular membranes are also common. Many microvilli protrude from the apical surface towards the vitelline membrane but do not come into contact with it at any time (Fig. 3.7a). On the other hand, the basal surface of these cells, i.e. the surface which faces the egg yolk, is covered with a continuous basal lamina which underlies all ectodermal cells up to the beginning of the leading edge (Fig. 3.7b). On this basal lamina, another type of cell located distal to the *area vitellina interna* is seen. These are the deep layer cells which are found to be distributed both individually (Fig. 3.8a) and in groups (Fig. 3.8b). They possess prominent nuclei which are similar to those of the leading edge cells.

3.3.2 Stage 6 (24 hours) blastoderm edge:

The leading edge is again multilayered and is now 123 $\pm$ 5.7 μm wide, a small increase on stage 3. The number of edge cells at this stage is 3.2 $\pm$ 0.5 cells. These cells appear more compact than those at the previous stage due to the disappearance of the large yolk vacuoles and their replacement with much smaller ones (Fig. 3.9). As a result of such transformation the mean height of the edge is reduced to 15.5 μm. As is the case of edge cells at all stages, the nuclei of these cells are prominent and can be very easily distinguished from the non-edge cells of the blastoderm.

The leading lamellae of distalmost cells are flattened and overlap and underlap each other, ending in thin cytoplasmic processes which make contact with the vitelline membrane (Fig. 3.10a). The length of these lamellae is 23 $\pm$ 3 μm i.e. shorter than at stage 3. Again, no specialised junctions have been detected between these lamellae. Desmosomes have the same distribution between the cells as described in the previous stage (Fig. 3.10b), being abundant between the proximal and less between the cells of the mid-part of the edge. Again, a few of the cells which are in contact with the vitelline

membrane send a number of microvillous projections which protrude deeply into the membrane (Fig. 3.11a). Microfilaments are clearly seen in these projections. Again, I have not noticed any changes in the distribution of these projections between the different areas of the edge. The one thing that is noticeable as far as these projections are concerned is that they are larger and penetrate more deeply into the vitelline membrane than at the earlier stage.

The ectodermal cells of the blastoderm at this stage have decreased slightly in height to 6 um but increased in width to 12 μm. They display the same junctional complex described for the earlier stage, namely tight junctions and desmosomes: in addition, areas of interdigitations between the membranes of the these cells are also present (Fig. 3.11b).

The deep layer cells which cover the basal lamina of the ectodermal cells can now mostly found distributed as individuals. They possess thin leading lamellae which spread over the basal lamina oriented towards the direction of the leading edge (Fig. 3.12a). Some of these deep layer cells are in contact with the edge (Fig. 3.12b).

3.3.3 Stage 8 (39 hours) blastoderm edge :

The edge area (Fig. 13) occupies a width of 123 ± 7 μm, about the same as stage 6, and its mean height is 12 um. The number of cells in this edge is 3.8 ± 0.6. The leading lamellae of the distal cells are shorter than those of the earlier stages, being 20.5 ± 3 μm. The general distribution of junctions between the cells of the different areas of the leading edge is similar to that described for the previous stages. A number of microvillous projections into the vitelline membrane are sent here also from the mid-part and distal leading edge cells which are in direct contact with the substratum. These projections can be rarely seen extending from the proximal cells into the vitelline membrane. This, however, does not mean the total absence of these projections in the proximal area of the edge but that they are noticeably less frequent than in the

other two areas.

Microtubules are abundant in the edge cells and are generally oriented parallel to the direction of movement.

The ectodermal cells are arranged as a very thin sheet with a mean height of 4 μm and a mean width of 14 μm. The deeper layer cells are found on the basal lamina of the ectodermal cells (Fig.3.14a) and the most distal of them are actually in direct contact with the proximal edge cells (Fig. 3.14b).

3.3.4 Stage 11-13 (48 hours) leading edge:

The width of the leading edge has now increased to 153 ± 8 μm, a slight but statistically significant increase on the earlier stage width, and its mean height has increased to 22.5 μm. The number of cells has also increased to 5.5 ± 0.7. Large yolk-filled granules are a common feature of edge cells at this stage. Lacunae i.e. large intercellular spaces which vary in size are another feature of the edge cells. These lacunae along with the yolk granules give the edge cells their vacuolated appearance (Fig. 3.15). The distal-most cells of the edge area attach to the vitelline membrane with their thin fan-like lamellae. The length of these lamellae is 13.6 ± 3 μm. These lamellae communicate with each other via very thin filopodia (Fig. 3.16a). Here again, some of the proximal edge cells which are in contact with the ectodermal cells possess centripetally oriented lamellae.

At the TEM level the proximal-most cells of the edge possess many microtubules, the majority of which are orientated towards the direction of movement some but are orientated at right angles to this (Fig. 3.16b). In some instances bundles of microfilaments were seen extending throughout the length of some of the cells which are separated from the vitelline membrane by only thin underlapping lamellae produced by other cells located proximal to them (Fig. 3.17a). On the upper surface of some proximal edge cells, some darkly stained lamellae extending from some deeper layer cells were frequently seen

(Fig. 3.17b). The distribution of junctions between the edge cells is again similar to that described for the previous stages except that, as the edge has increased in width, the desmosomes in the mid-part of the edge have become restricted to those cells nearer to the proximal area of the edge while they are less frequent between the cells located more peripherally.

The ectodermal cells have, by this stage, decreased further in height to 3 μm while increasing in width to 18 μm. These cells are again connected by apical tight junctions followed by a number of desmosomes (Fig. 3.18a) and in many instances this is followed by interdigitations of their cell membranes. Some microtubules and microfilaments are present at the apical surface of these ectodermal cells (Fig. 3.18b). Deep layer cells have the same distribution as in the previous stage.

3.3.5 Stage 15-16 (60-63 hours) leading edge:

The edge cells have by this stage transformed their original organisation from multilayer to bilayer, giving the edge area the shape of a thin sheet of cells under both light and scanning electron microscopes (Fig. 3.19). There is a significant increase in the width of the edge to 216 ± 13 μm, while there is a slight decrease in its mean height to 18.5 μm. The number of cells forming the edge has increased to 7.2 ± 1.0 cells.

The distalmost cells behave as usual by sending broad fan-like lamellae to attach to the vitelline membrane (Fig. 3.20a). These lamellae are considerably shorter than those of the very early stages; being only 13.6 ± 3.4 μm. Many thin cytoplasmic processes originating at these lamellae make contact with neighbouring cells and the vitelline membrane (Fig. 3.20b).

The ectodermal part of the blastoderm has by this stage thinned down as a result of a further decrease in the height of its cells now measuring 2 μm. At the same time, the width of the ectodermal cells has increased to 22 μm. Desmosomes are again found mainly between the cells of the proximal area of

the leading edge. Again, deep layer cells are distributed on the basal lamina of the ectodermal cells.

The stages which follow will be dealt with in a later chapter as the edge cells at the late stages of epiboly differ in both their organisation and behaviour from the stages already described.

3.4 Morphometric analysis of the changes in the leading edge at the stages of development studied above:

The purpose of this analysis is to determine if there is a significant difference in the width and height of the leading edge area and in the relative number of cells and length of leading lamellae between the different stages of development, and how these differences could relate to the reported changes in the rate of expansion at different stages (Downie 1976).

3.4.1 The width of the edge:

The results showed that the leading edge covers a relatively narrow area on the vitelline membrane at stage 3 ($104 \pm 9.5\mu m$). There is then a statistically significant increase in this width at stage 6 to $123 \pm 6\mu m$ ($P < 0.001$). There is no significant increase in the width between stages 6 and 8 ($P > 0.75$). However, the increase in edge width is highly significant between stages 8 and 11 ($P < 0.001$) and stages 11 and 16 ($P<0.001$) (Table 3.1 & Fig. 3.21a).

3.4.2 The height of the edge:

The height of the edge follows a slightly different pattern from that described for the width. The mean edge height at stage 3 is $25\mu m$. It then drops sharply to $15.5\mu m$ and $12\mu m$ at stages 6 and 8, respectively. The edge then increases in height reaching a mean height of $22.5\mu m$ at stage 11. It then drops back to 18.4 um at stage 16 (Table 3.1 & Fig. 3.22a).

3.4.3 The number and size of the cells:

The relative number of cells at each stage of development is in direct relation to the width of the edge. There is no significant difference between the number of cells at stage 3, 6 and 8 where it was found to be 2.9 $\pm$ 0.7, 3.2 $\pm$ 0.5 and 3.8 $\pm$ 0.6 cells, respectively. The number of cells was found to increase significantly after stage 8 when it reaches 5.5 $\pm$ 0.7 cells at stage 11 and 7.2 $\pm$ 1.0 cells at stage 16 ($P < 0.001$) (Table 3.1 & Fig. 3.21b).

The size of cells, on the other hand, follows exactly the same pattern as the height of the edge described in the above section; it is high (896 μm2) at stage 3, dropping to 593 μm2 at stage 6 then to 407 μm2 at stage 8. It increases again to 620 μm2 at stage 11 before dropping back to 567 μm2 at stage 16 (Table 3.1 & Fig. 3.22b).

3.4.4 The length of the leading lamellae :

There is a continuous decrease in the length of the lamellae from stage 3 through to stage 16 (Fig.3.23).

Table 3.1: **Measurements of the leading edge and its cells at different stages of development.**

STAGE OF DEVELOPMENT	AREA (μm2) ±SD	WIDTH (μm) ±SD	HEIGHT μm	NO.of cells ±SD	CELL SIZE (μm2)	Lamella Length (μm)±SD
3	2599±352	104 ± 10	25.0	2.9 ± 0.7	896	30 ± 3
6	1916±350	123 ± 6	16.5	3.2 ± 0.5	598	23 ± 3
8	1534±228	123 ± 7	12.2	3.8 ± 0.6	403	20.5 ± 3
11	3434±520	153 ± 8	22.0	5.5 ± 0.7	624	13.6 ± 3
16	4008±667	216 ± 13	18.5	7.2 ± 1.0	556	13.6 ± 5

SD= Standard deviations from the mean.

Cell size was obtained by dividing the area of the edge by the number of cells.

No standard deviations are shown for cell size and the height of the edge because figures were obtained by division.

Fig.3.1- A diagram showing the terms used in describing the blastoderm edge where (D) are the distal edge cells, (DL) are the deep layer cells, (E) is the ectodermal part of the blastoderm, (L) the leading lamella, (MP) is the mid-part cells of the edge, (P) are the proximal cells of the edge, (W) is the width of the leading edge and (Vm) is the vitelline membrane which is composed of inner (I) and outer (O) surfaces. Abbreviations are not necessarily the same on other figures.

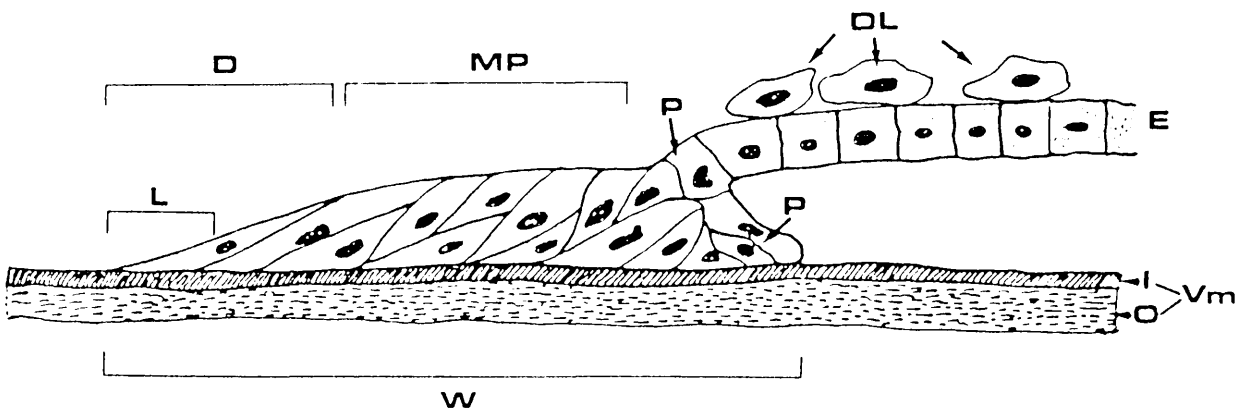

Fig.3.1

Fig.3.2 a- Scanning electron micrograph (SEM) showing the appearance of stage 3 (15) hours leading edge. Broad leading lamellae (L) are spread from the distal cells of the leading edge (Le) on the inner surface of the vitelline membrane (V). Bar = 10 µm.

Fig.3.2 b- Light micrograph of the leading edge of a stage 3 blastoderm. Notice the large yolk vacuoles (Y) in these cells at this stage. Large intercellular spaces or lacunae (lc) are noticeable. The ectoderm (E) does not come into contact with the vitelline membrane (V). Arrowhead shows the leading lamella of a distal cell. Bar= 50 µm.

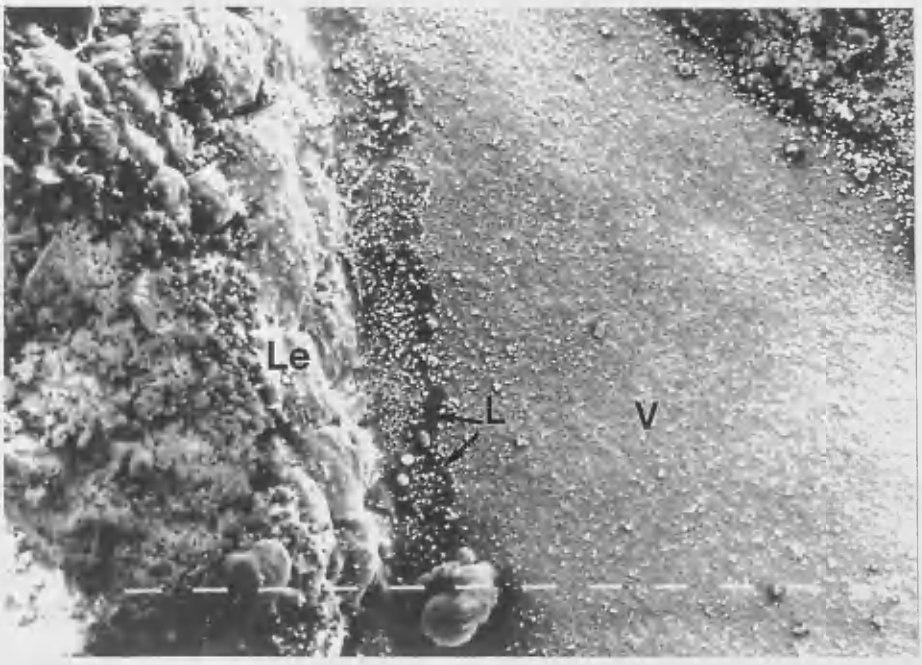

Fig.3.2a

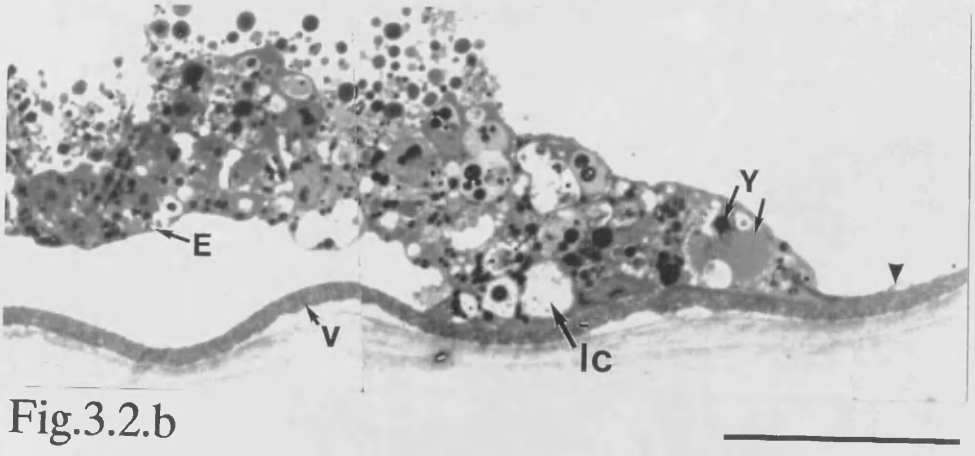

Fig.3.2.b

Fig.3.3 a- SEM showing the leading lamellae of stage 3 edge cells. Each lamella underlaps the cells in front of it. Some of these lamellae end in thin filopodia (arrows) which come in contact with the neighbouring cells. V= vitelline membrane. Bar= 1 μm.

Fig.3.3 b- Transmission electron micrograph (TEM) of the proximal edge cells of stage 3 blastoderm showing that these cells are connected together by some desmosomes (D) and close points of contact (arrowheads). Bar= 1.3 μm. X 24.5K.

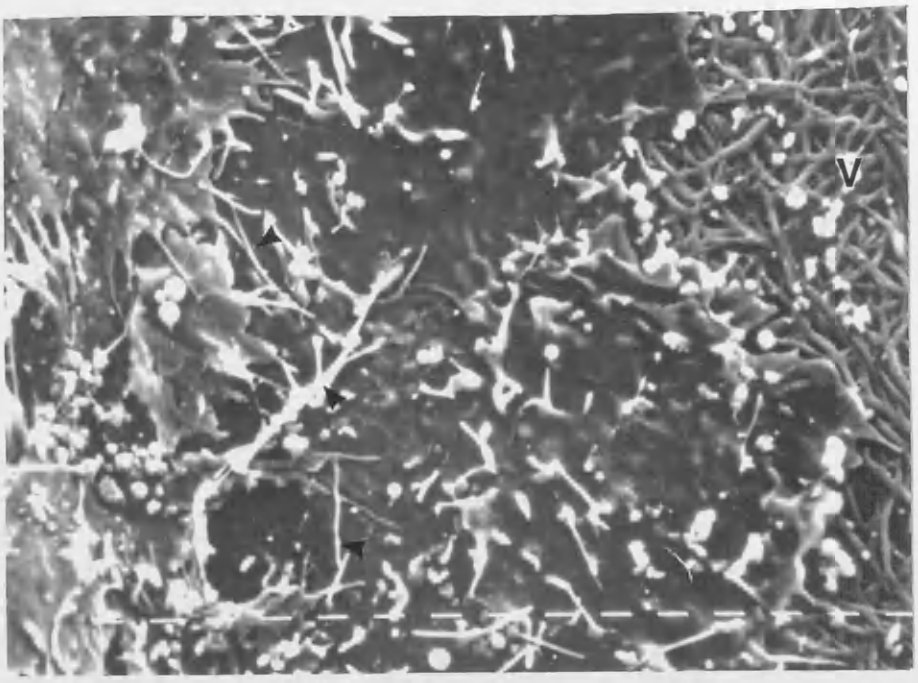

Fig.3.3a

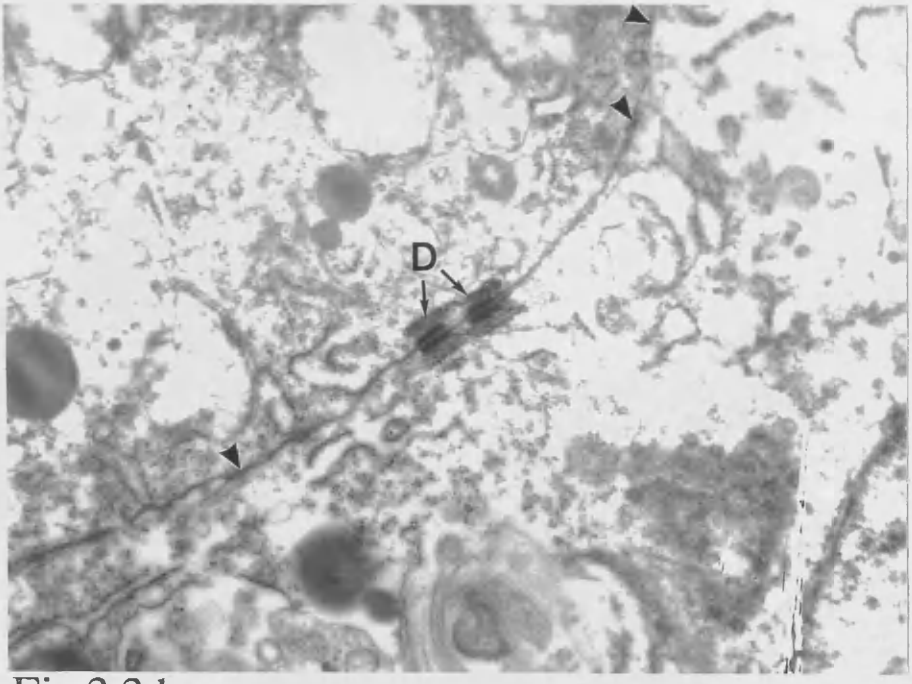

Fig.3.3 b

Fig.3.4 a- TEM of the midpart of the leading edge at stage 3. Desmosomes (D) are less prominent between the cells of this area. Cells are also connected by points of close contact (arrowheads). Bar= 0.87 μm. X 41K.

Fig.3.4 b- TEM showing the absence of desmosomes between the distal cells of the leading edge at stage 3. L= underlapping lamella; V= Vitelline membrane. Bar= 1.24 μm. X 23K.

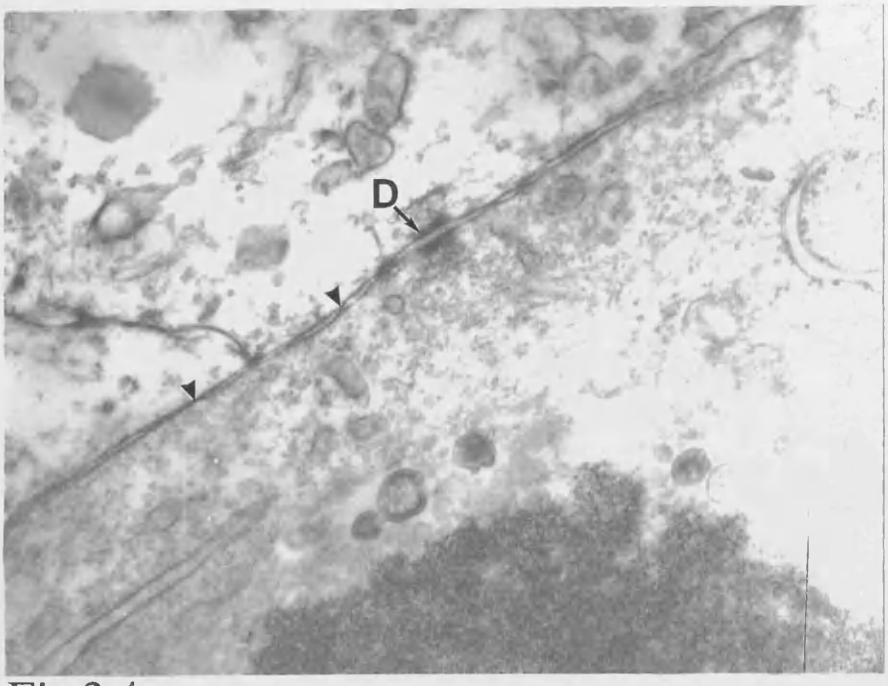

Fig.3.4 a

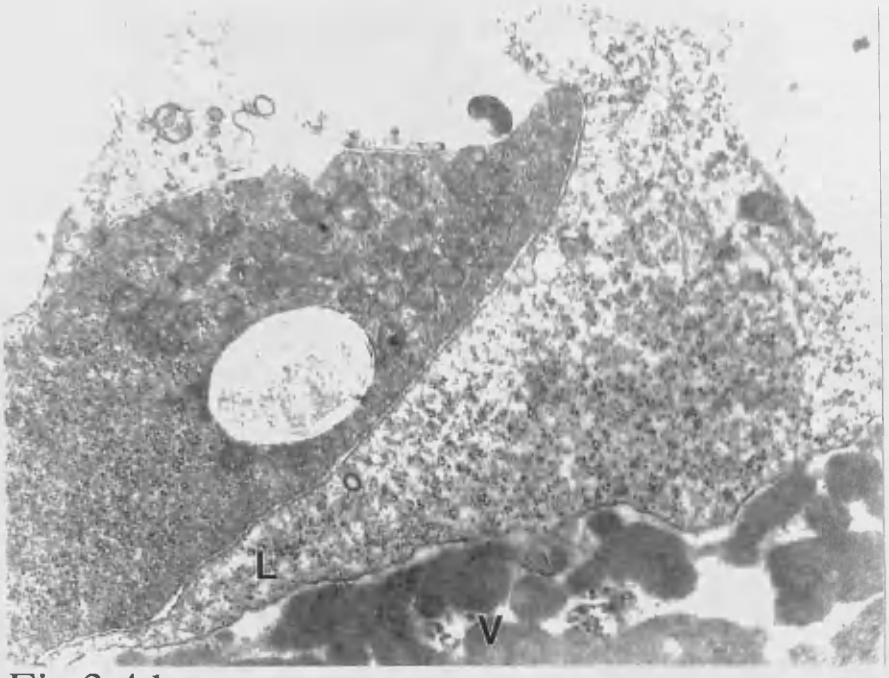

Fig.3.4 b

Fig.3.5 a- TEM showing a centripetally oriented lamella (CL) of the most proximal cell of stage 3 leading edge. This cell also possesses a centrifugally oriented lamella. Cortical microfilaments (cf) are also visible. V= vitelline membrane. Bar= 2.3 μm. X 15.6K.

Fig.3.5 b- TEM showing that the leading edge cells possess microtubules (arrowheads) orientated towards the direction of movement (solid arrow). Bar= 0.46 μm. X 36.7K.

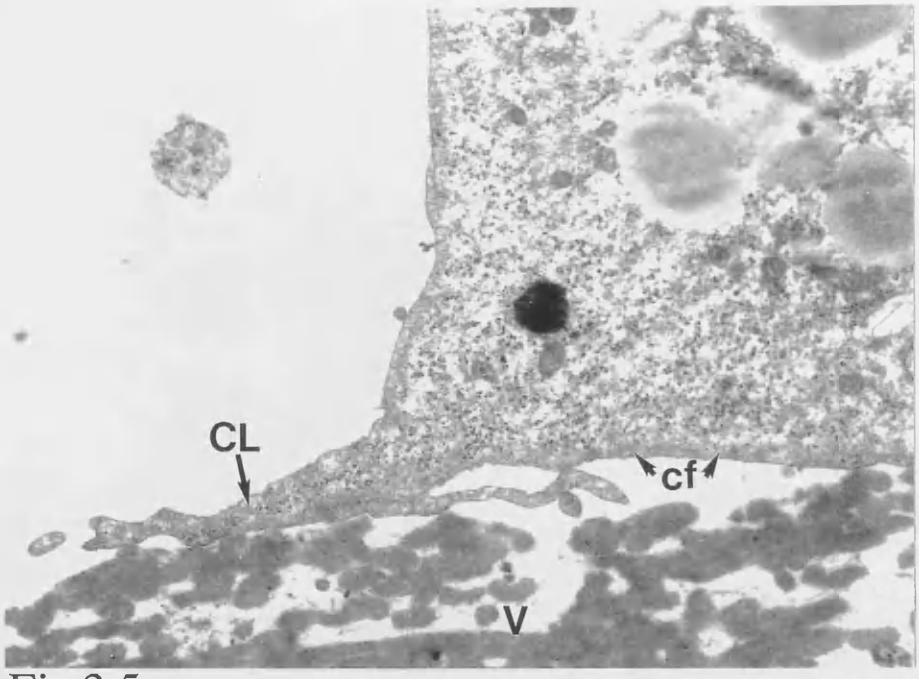

Fig.3.5 a

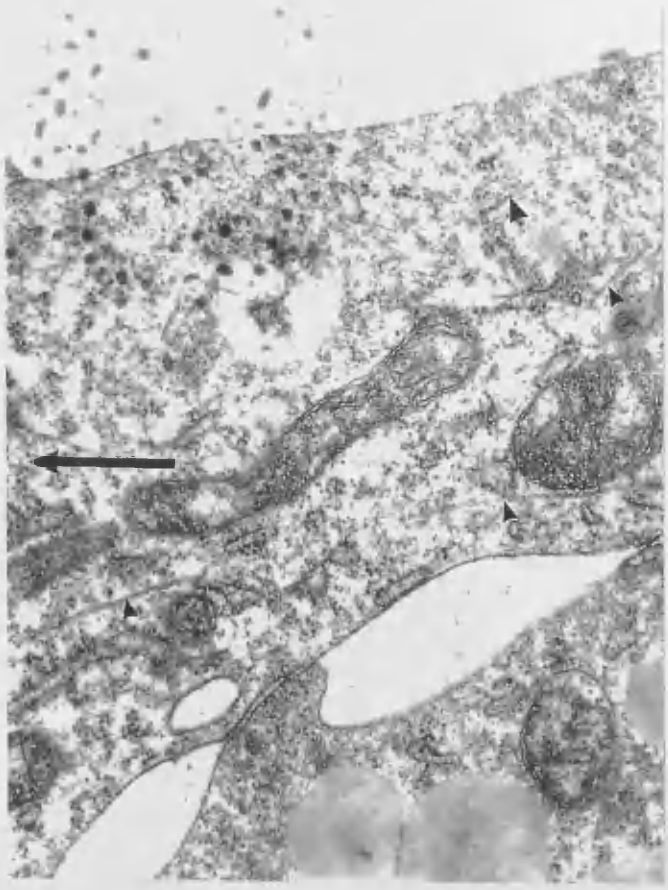

Fig.3.5 b

Fig.3.6 a- TEM showing a microvillous projection (MP) protruding from the basal surface of a leading edge cell into the vitelline membrane (V) of stage 3 blastoderm. Bar= 0.83 μm. X 39K.

Fig 3.6b TEM of ectodermal cells of stage 3 blastoderm. The cells are connected at their apical surface by a junctional complex consisting of tight junction (TJ) followed by desmosomes (D) and interdigitations of the lateral plasma membranes (I). Bar= 0.83 μm. X 39K.

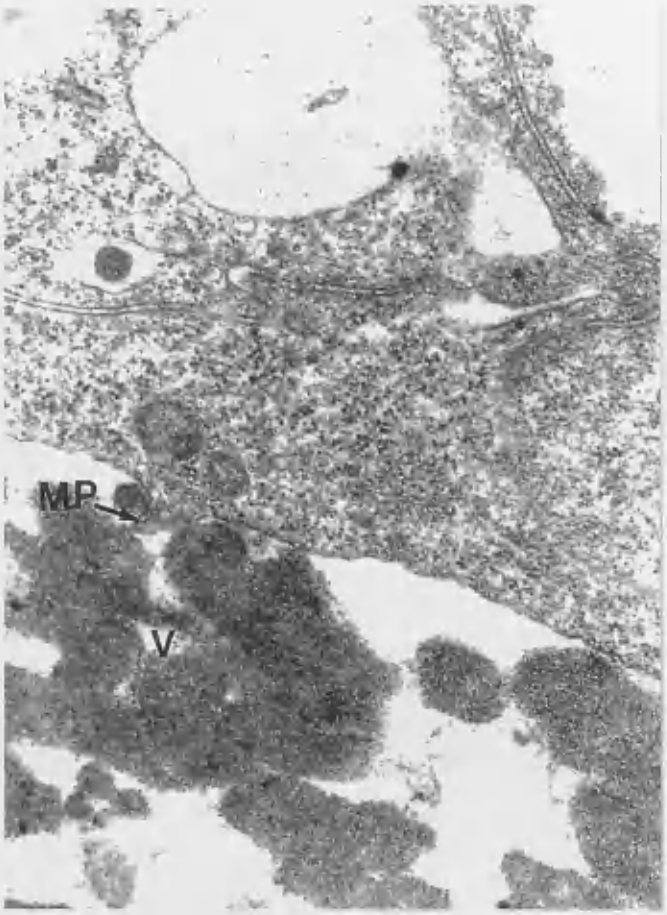

Fig.3.6 a

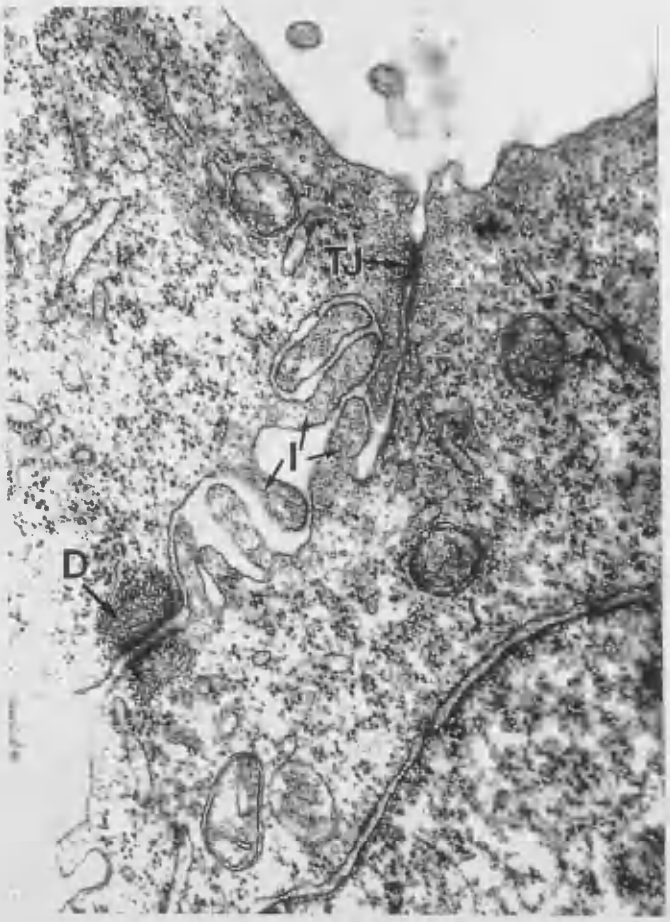

Fig.3.6 b

Fig.3.7 a- SEM showing the apical surface of the ectodermal cells of stage 3 blastoderm. Microvilli mark the boundaries between the cells and are also scattered over the apical surface. Bar= 1 μm.

Fig.3.7 b- TEM showing the basal surface of the ectodermal cells (E). A basal lamina (BL) covers this surface of the cells and extends to the periphery of the proximal edge cells. D= deep layer cell. Bar= 0.32 μm. X 102.85K.

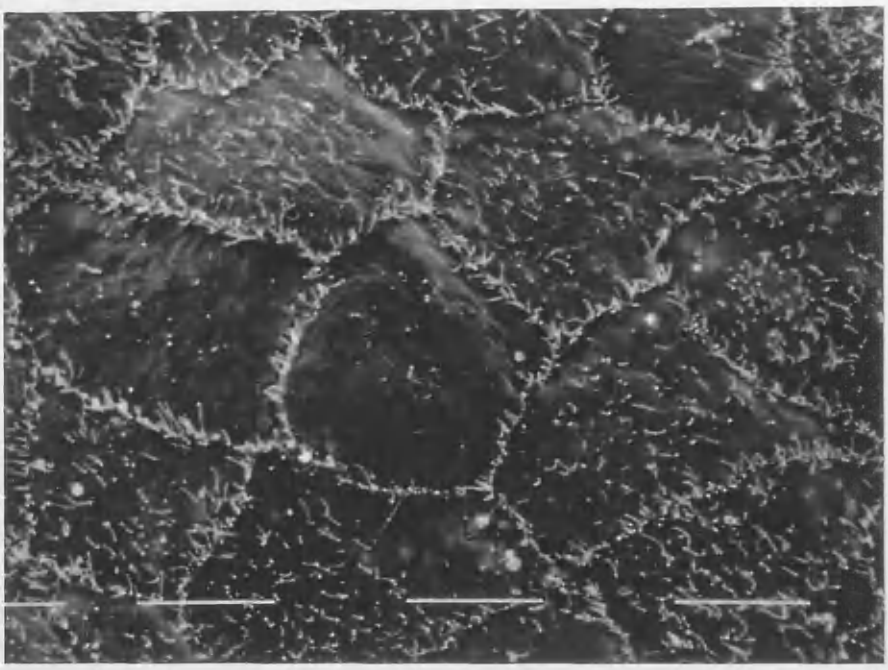

Fig.3.7 a

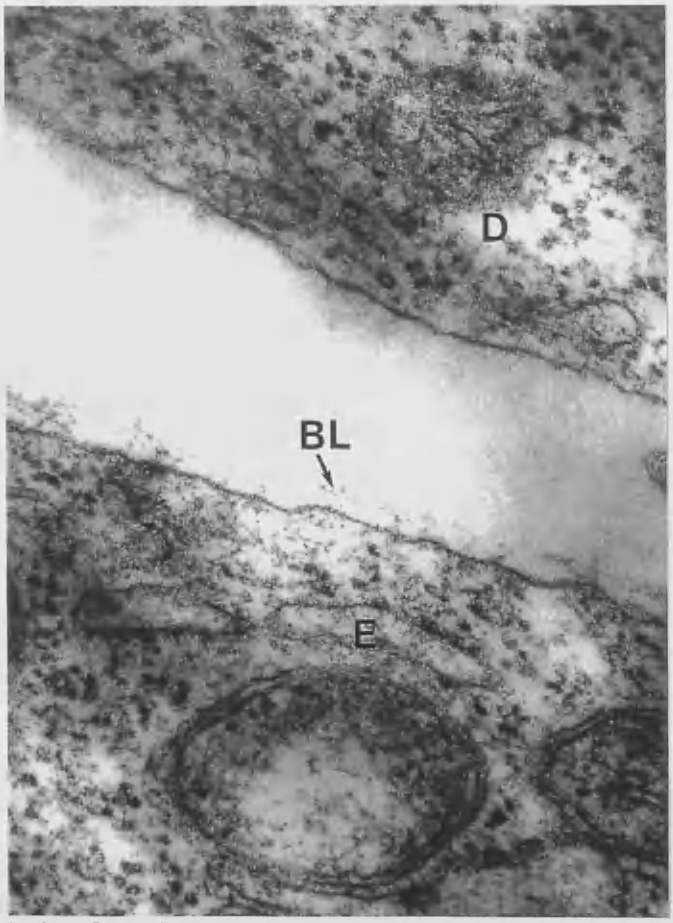

Fig.3.7 b

Fig.3.8 (a&b)-TEMs showing deep layer cells (D) distributed as individuals (a) and in groups (b) on the basal lamina of the ectodermal cells (E). Bar= 5.3μm. X 10K.

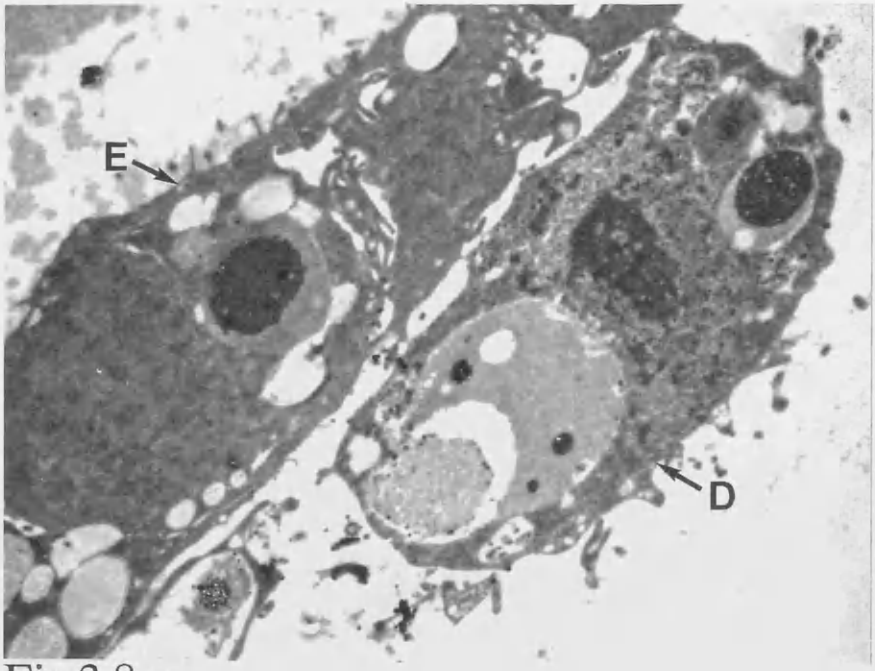

Fig.3.8 a

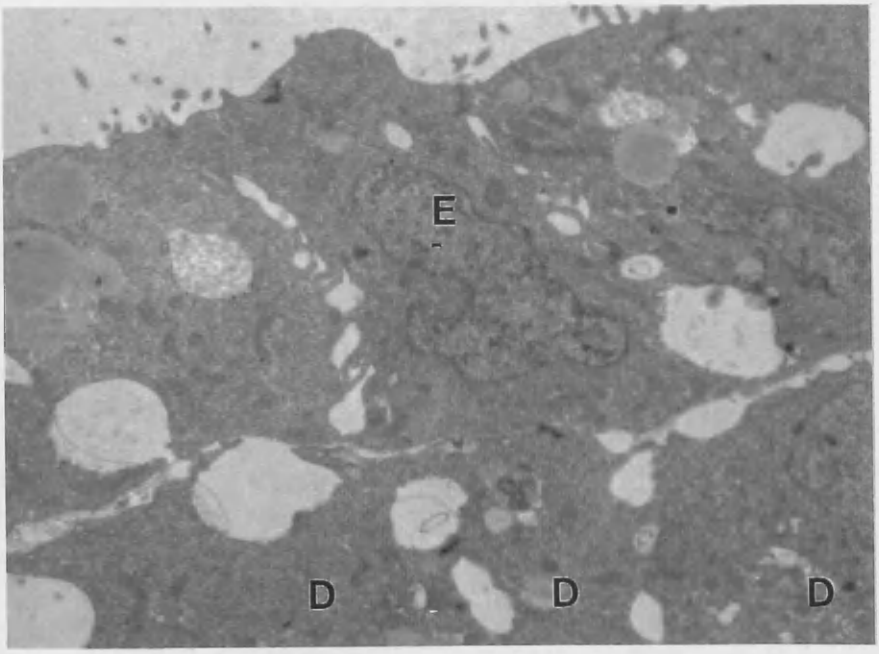

Fig.3.8 b

3.9 Light (b) and SEM (a) of the leading edge (Le) at stage 6. The cells have a more compact appearance than those of the previous stage. E= ectoderm; V= vitelline membrane. Bar in (a) = 10 μm and in (b) = 50 μm.

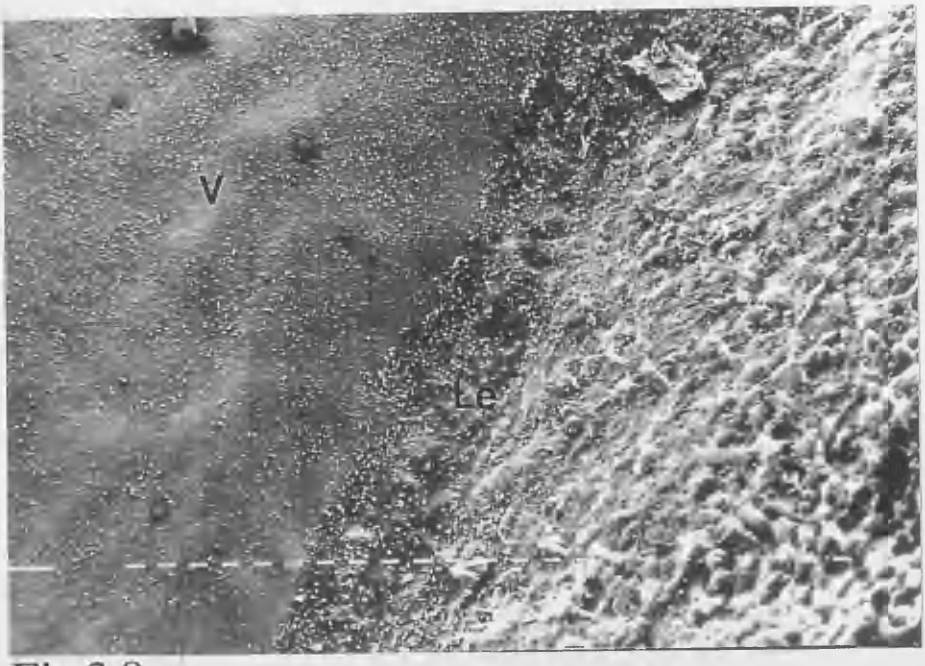

Fig.3.9 a

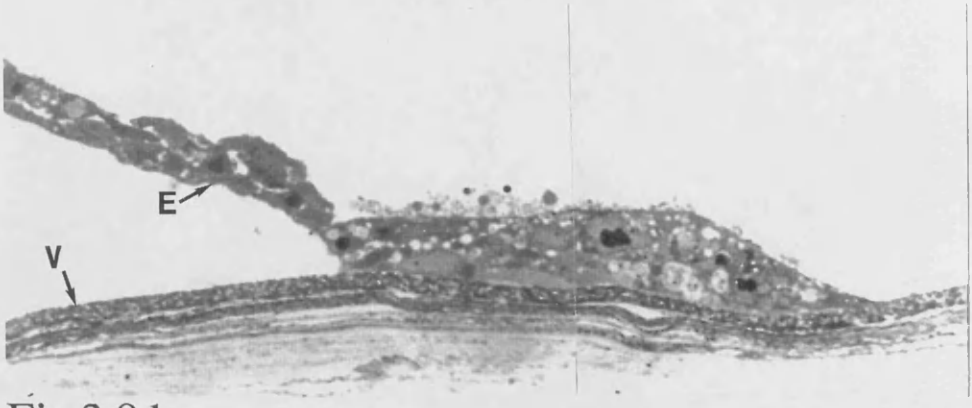

Fig.3.9 b

Fig.3.10 a- SEM of the distal cells of stage 6 leading edge showing the lamellae (L) which underlap each other. The free lamellae end in thin processes (arrowheads) which make contact with the vitelline membrane (V). Bar= 1 μm.

Fig.3.10 b- TEM showing the presence of desmosomes (D) and points of close contact (arrowheads) between the proximal cells of stage 6 leading edge. Desmosomes are less pronounced between the midpart cells and are completely absent at the distal part of the edge. Bar= 1.1 μm. X 18.5k.

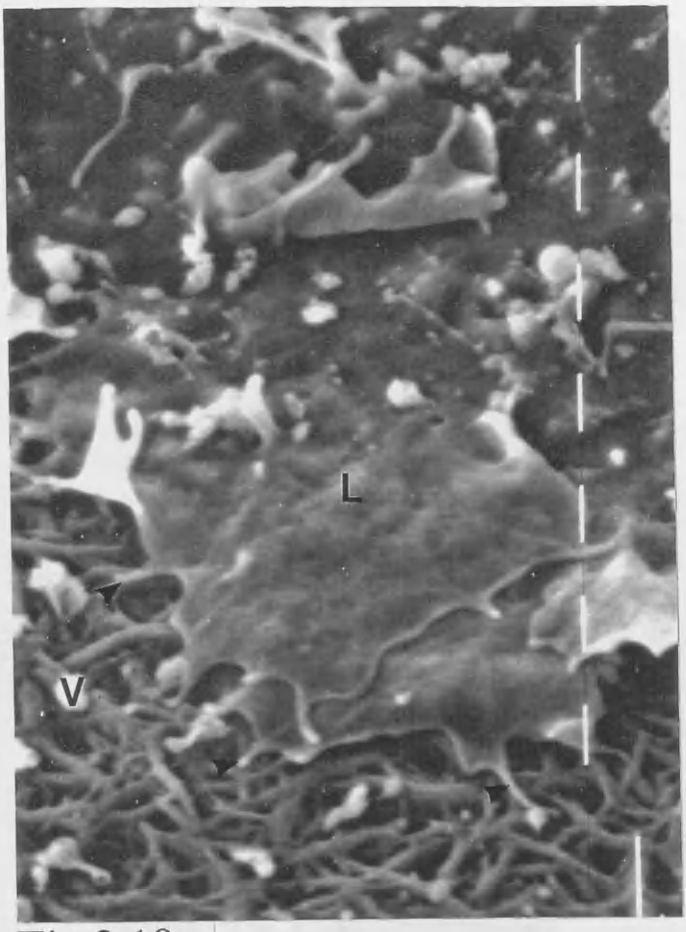

Fig.3.10 a

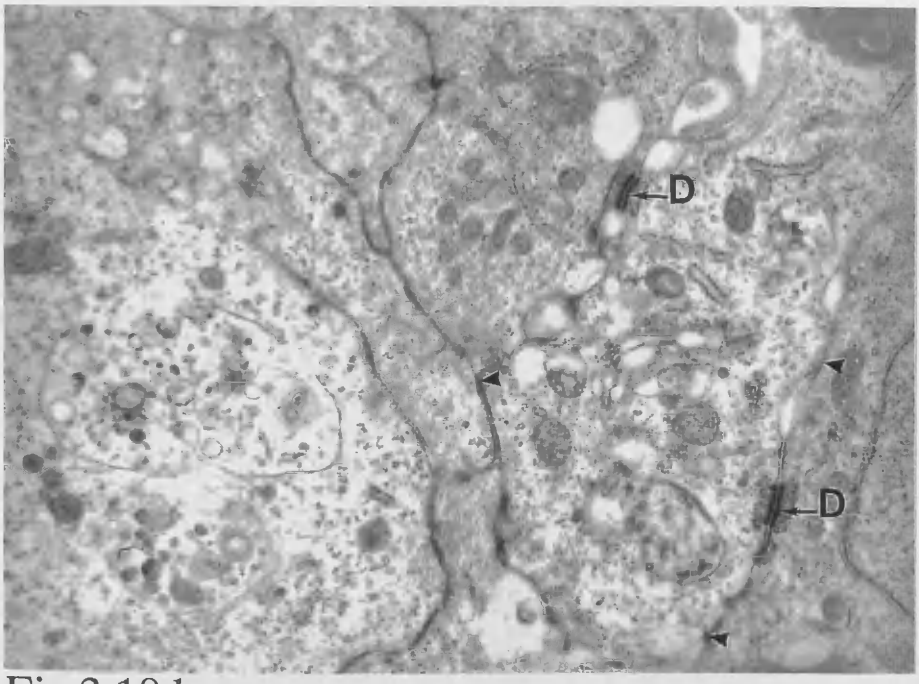

Fig.3.10 b

Fig.3.11 a- TEM showing a microvillous projection (MP) protruding into the vitelline membrane (V) from the cells in contact with it, in a stage 6 leading edge. Bar= 0.46 µm. X 41K.

Fig.3.11 b- TEM showing the persistence of the same junctional complex between the ectodermal cells at stage 6. TJ= Tight junction; D= Desmosome; I= interdigitations of the cellular membranes. Bar= 0.73 µm. X 24.5K.

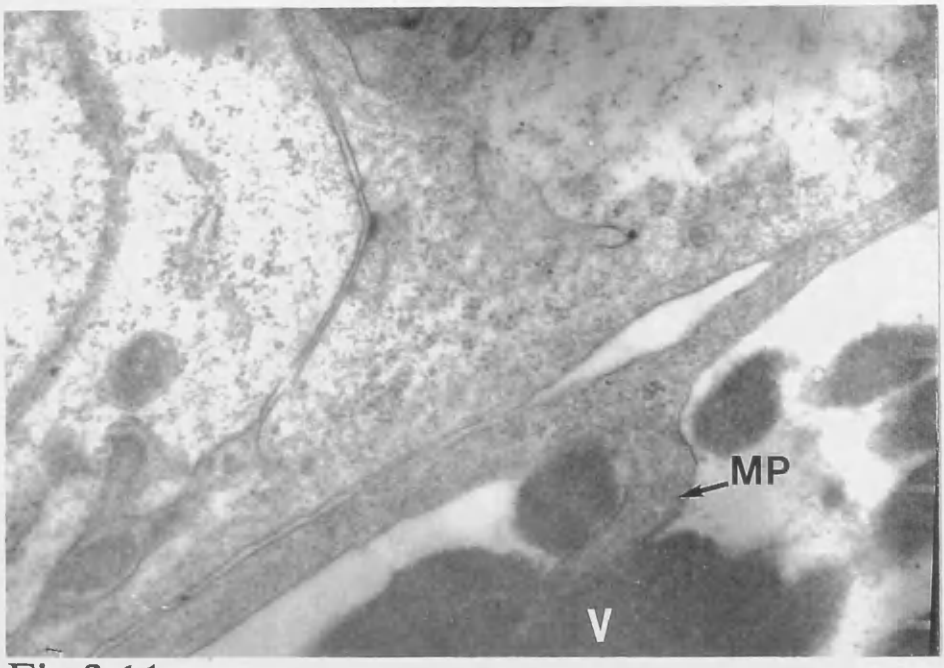

Fig.3.11 a

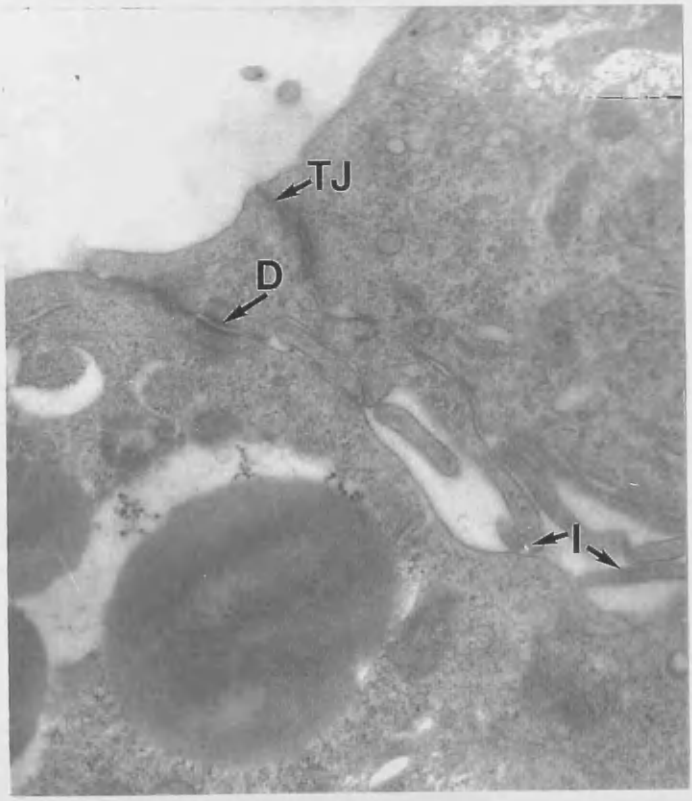

Fig.3.11 b

Fig.3.12 a- Light micrograph of stage 6 blastoderm showing some deep layer cells (arrowheads) on the basal surface of the ectodermal cells (E). Area marked by square is shown in Fig.3.12 b. Bar= 50 µm.

Fig.3.12 b- TEM of the area marked in Fig. 3.12a showing a darkly stained deep layer cell (D) in contact with the ventral surface of the proximal edge cells (PLe). Bar= 3.8 µm. X 9.4K.

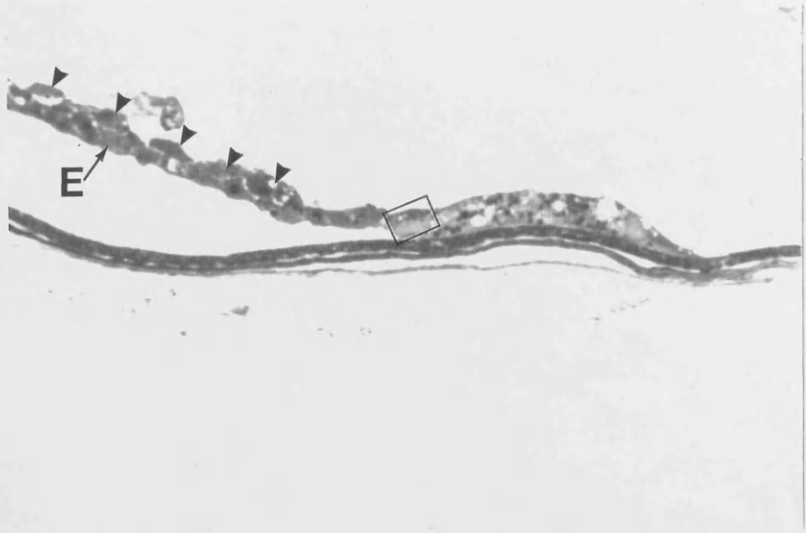

Fig.3.12 a

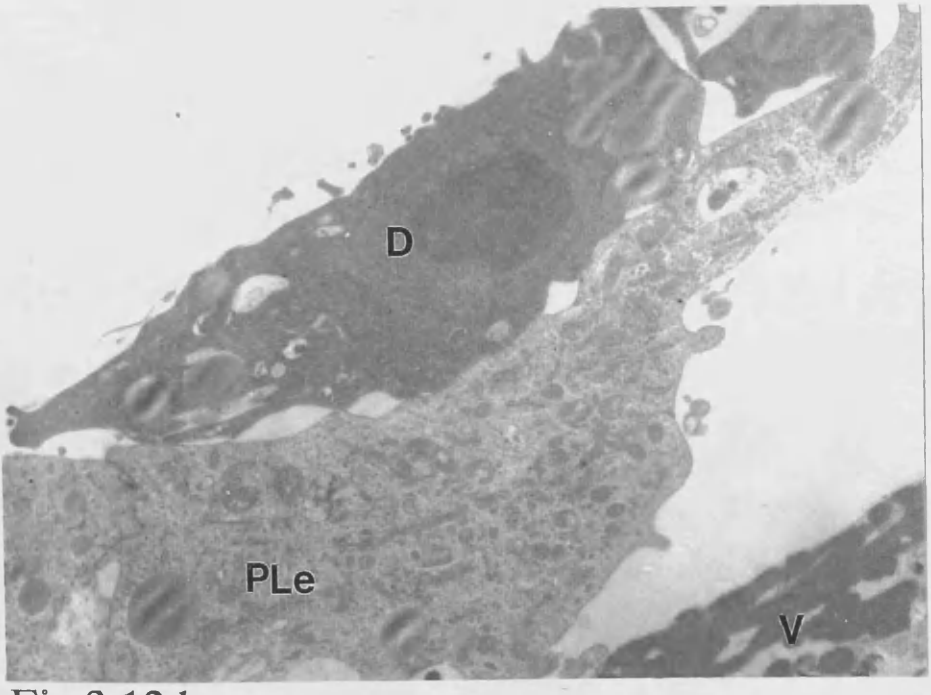

Fig.3.12 b

Fig.3.13 a- SEM showing the general appearance of the leading edge (Le) at stage 8. V= Vitelline membrane. Bar= 10 μm.

Fig.3.13 b- Light micrograph of the leading edge of stage 8 blasto-derm. Deep layer cells (arrowheads) are found on the basal side of the ectoderm (E). One of them (arrow) is in contact with the leading edge. V= Vitelline membrane. Bar= 50 μm.

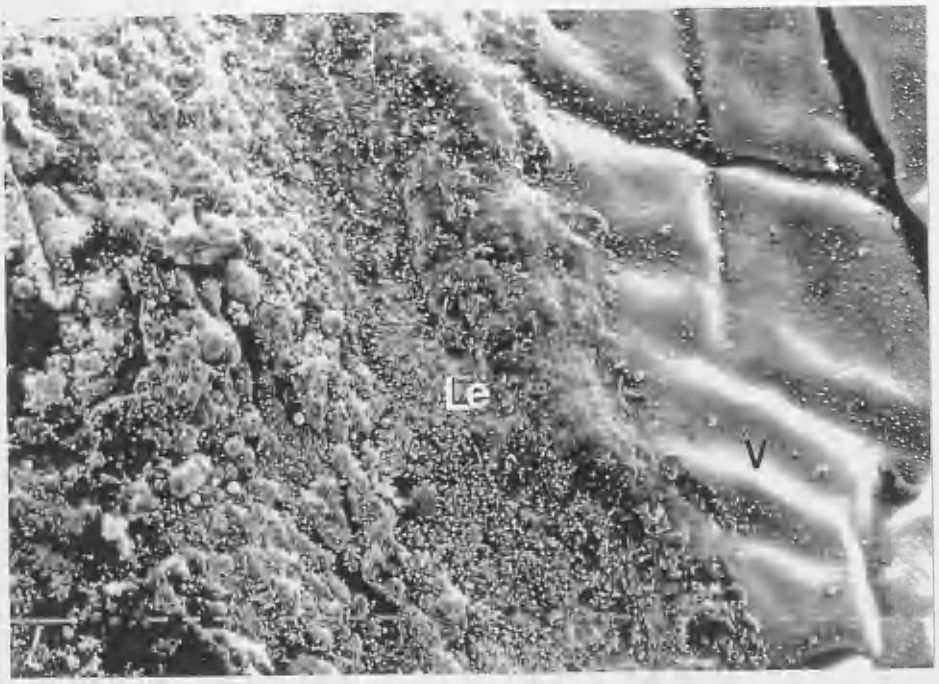

Fig.3.13 a

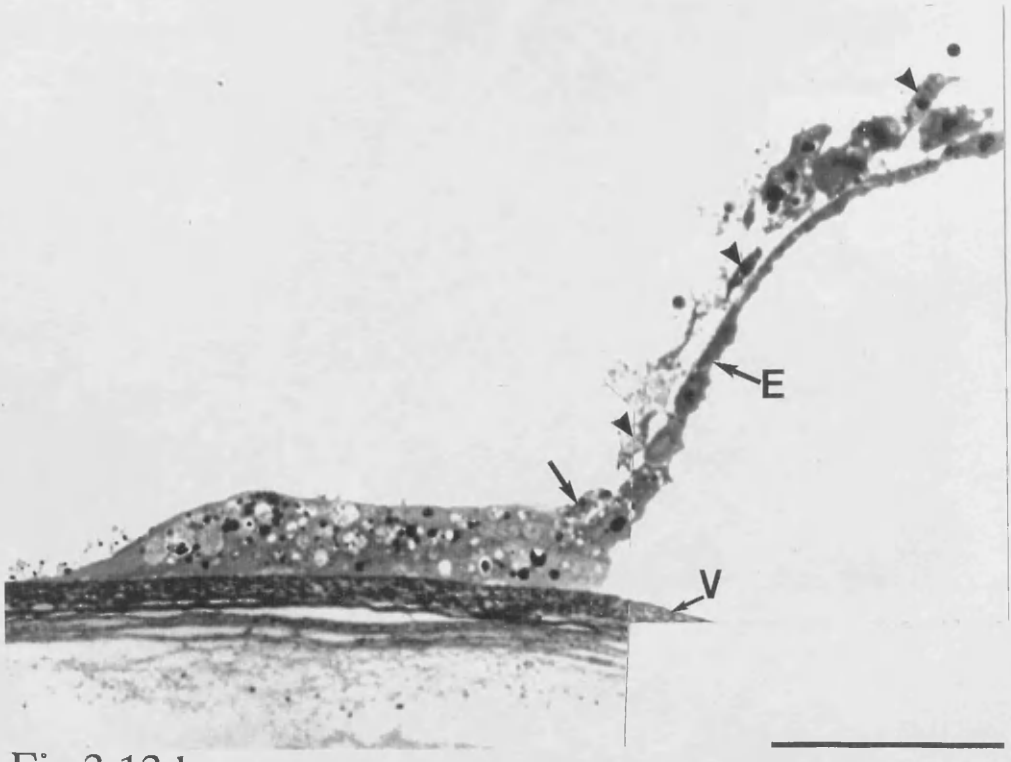

Fig.3.13 b

Fig.3.14 a- Light micrograph of another stage 8 blastoderm edge showing a stream of deep layer cells (arrowheads) near the leading edge. Le= leading edge; E= ectoderm; V= vitelline membrane. Bar= 50 µm.

Fig.3.14 b- TEM showing one of the deep layer cells (D) in the vicinity of the leading edge with its leading lamella (L) making direct contact with the proximal cells of the edge (PLe). Bar= 5.2 µm. X 6.67K.

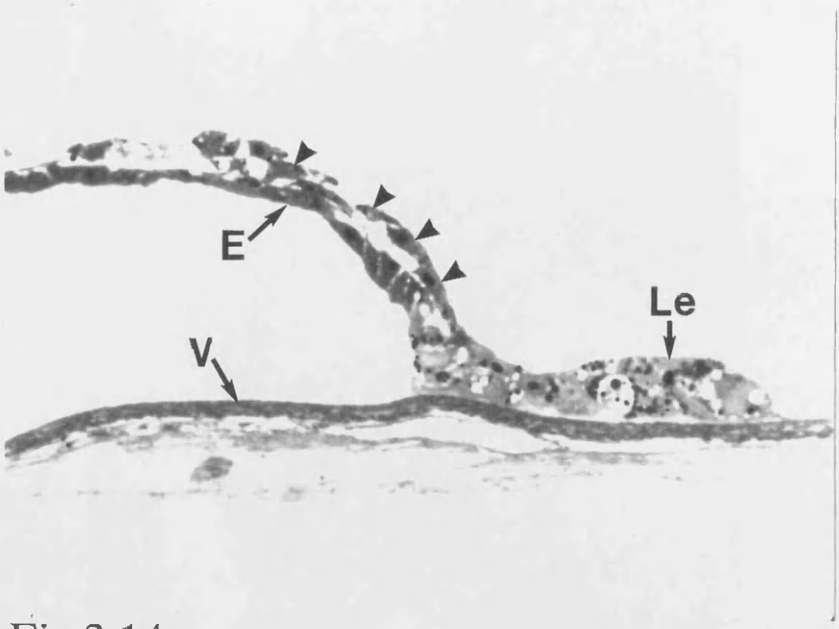

Fig.3.14 a

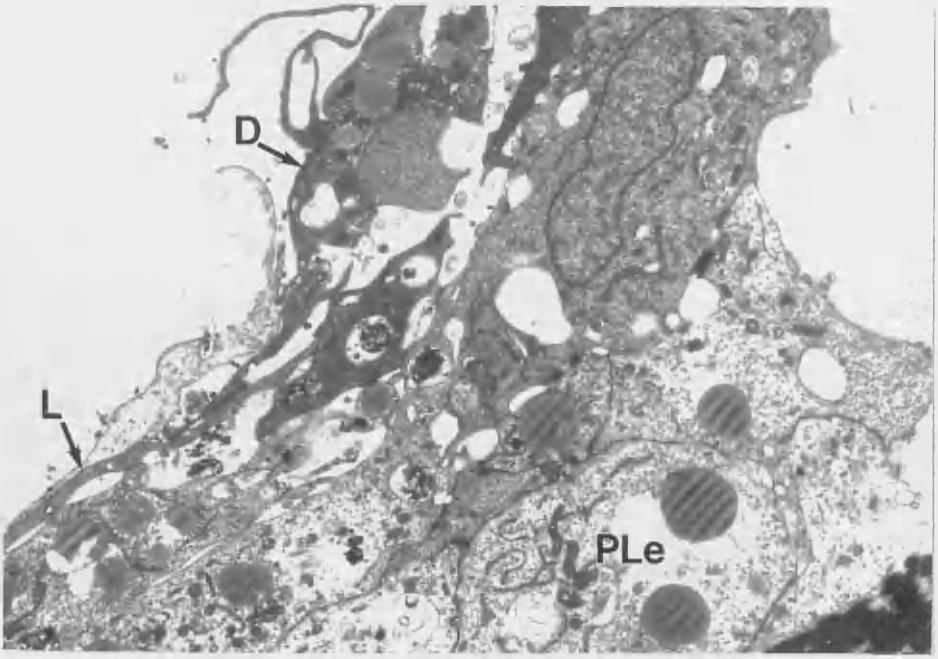

Fig.3.14 b

Fig.3.15 a- SEM showing the general appearance of the leading edge (Le) at stage 11. Bar= 10 µm.

Fig.3.15 b- Light micrograph of the leading edge of a stage 11 blastoderm showing the vacuolated appearance of the cells. E= Ectoderm; V= Vitelline membrane. Bar= 50 µm.

Fig.3.15 a

Fig.3.15 b

Fig.3.16 a- SEM of stage 11 blastoderm showing the fan-like leading lamellae (L) which make contact with each other via thin filopodia (arrowheads). Bar= 10 μm.

Fig.3.16 b- TEM of stage 11 leading edge showing that microtubules (T) seem predominantly orientated towards the direction of movement. In some cases some microtubules are found to be oriented at right angle to that direction. Cortical microfilaments (F) are also present. Bar= 0.46 μm X 41K.

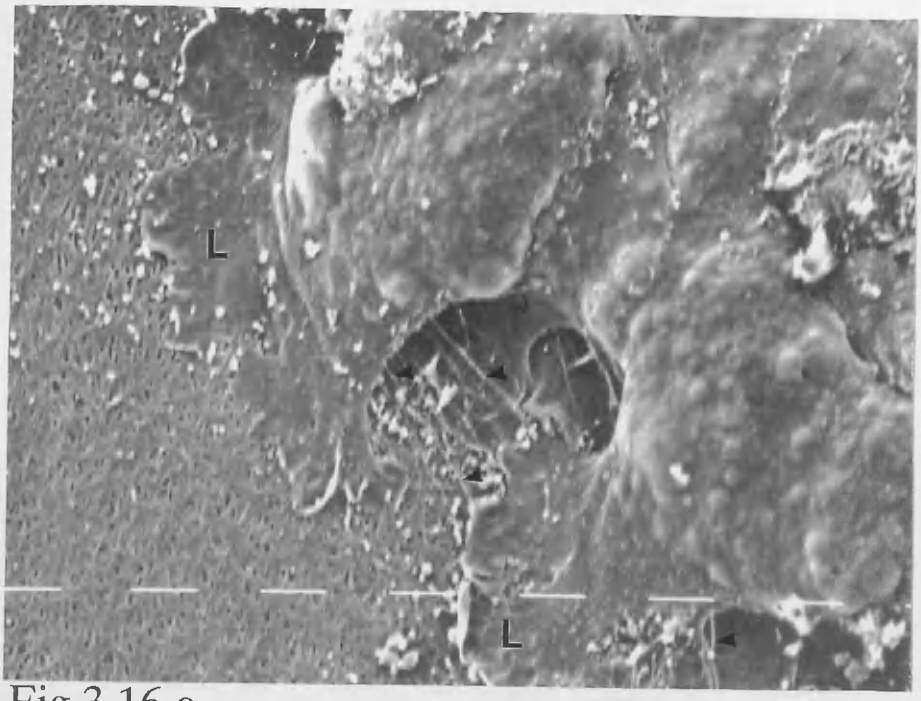

Fig.3.16 a

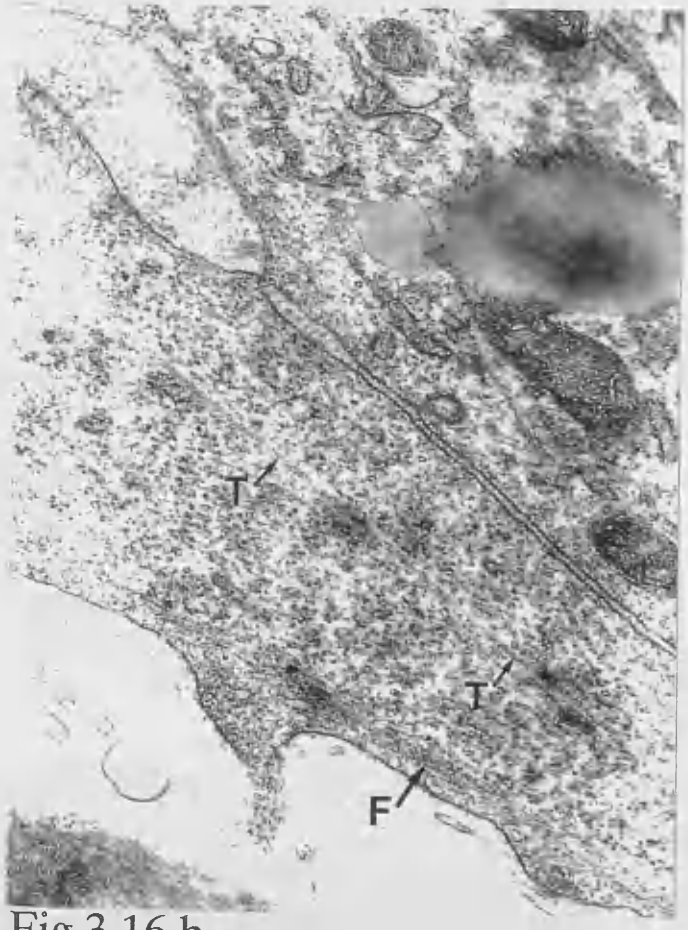

Fig.3.16 b

Fig.3.17 a- TEM of stage 11 blastoderm edge cells showing a bundle of microfilaments (F) extending along the basal surface of one of the proximal edge cells which is separated from the vitelline membrane (V) by a leading lamella (L) from a more proximal edge cell. Bar= 11.2 μm. X 8.8K.

Fig.3.17 b- TEM of stage 11 proximal edge cells showing a darkly stained lamella (L) extending on the upper surface of the cells. Notice the series of desmosomes (D) between these cells. Bar= 5.7 μm. X 8.96K.

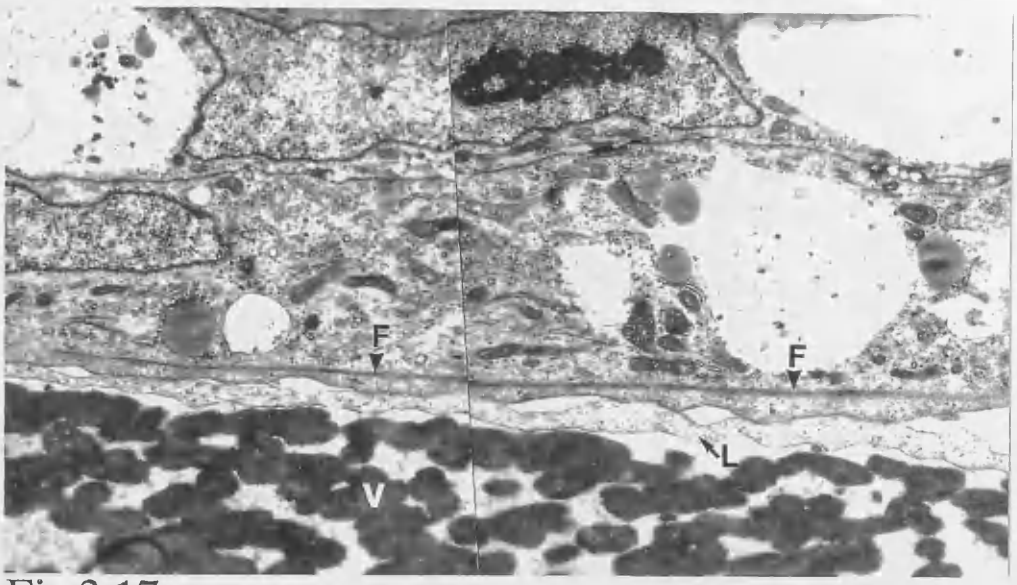

Fig.3.17 a

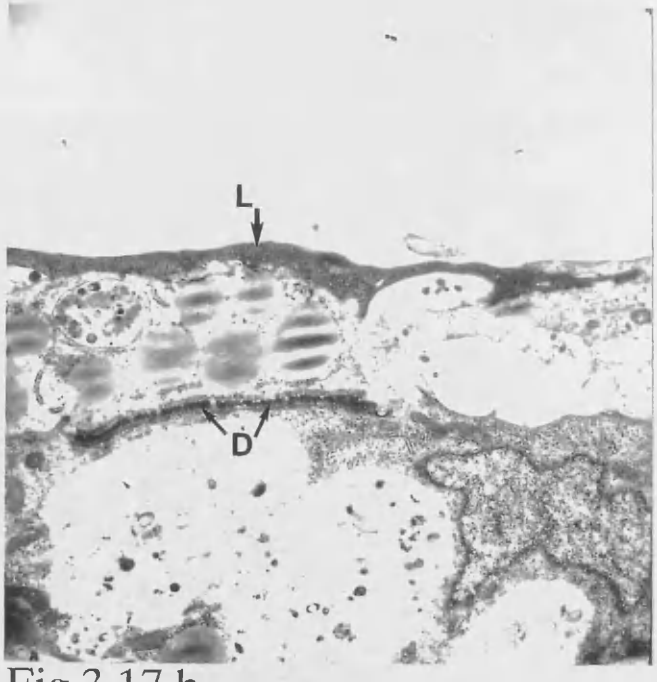

Fig.3.17 b

Fig.3.18 a- TEM of the ectoderm of stage 11 blastoderm. Here again, these cells are connected by an apical tight junction (TJ) followed by a number of desmosomes (D) and interdigitation of their membranes (arrowheads). A basal lamina (BL) covers the basal side of these cells. Bar= 3.57 μm. X 12.4K.

Fig.3.18 b- TEM of an ectodermal cell as above showing the orientation of microtubules (arrows). Microfilaments (F) extend into the microvilli which protrude from the apical surface. Bar= 0.29 μm. X 63.4K.

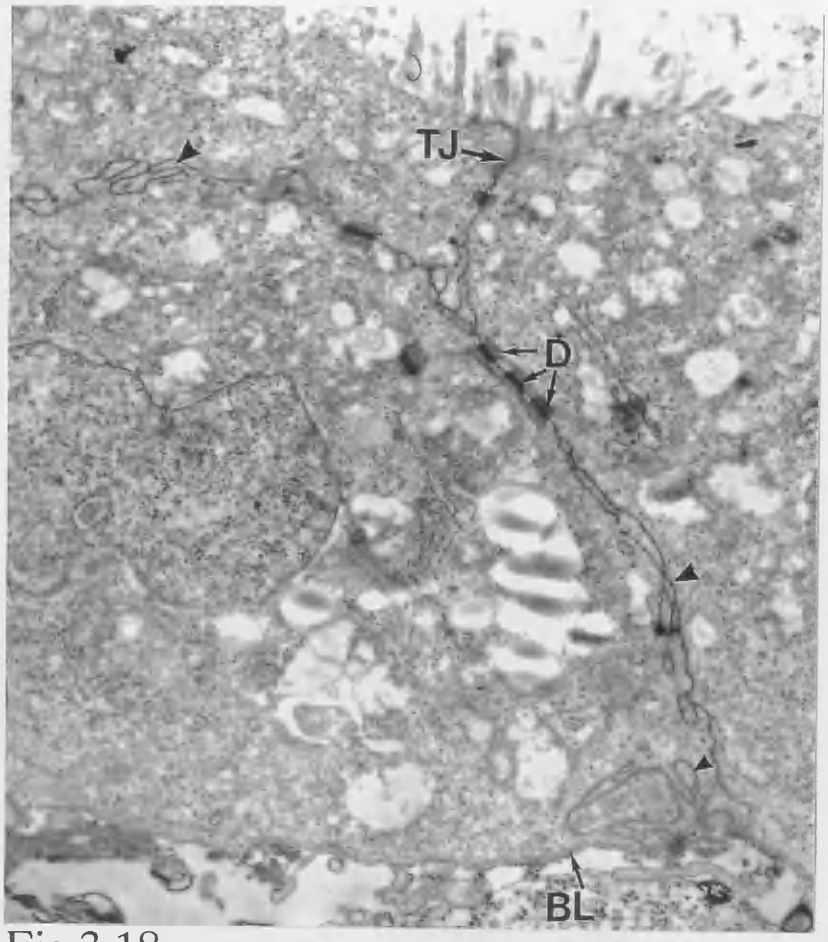

Fig.3.18 a

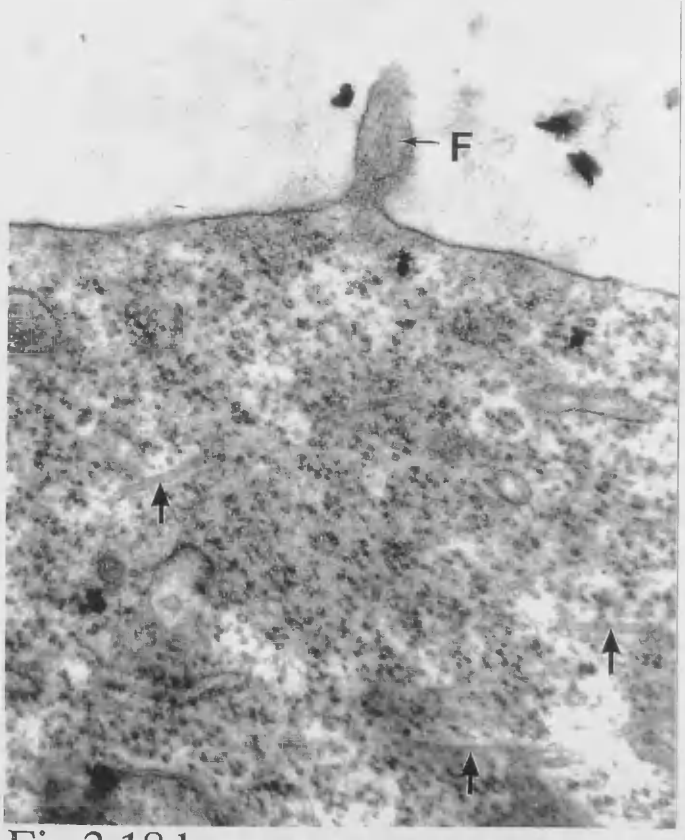

Fig.3.18 b

Fig.3.19 a- SEM of a stage 16 blastoderm edge (Le). V= Vitelline membrane. Bar= 10 µm.

Fig.3.19 b- Light micrograph of the leading edge of a stage 16 blastoderm. The cells of the edge at this stage have a bilayer instead of a multilayer organisation. E= Ectoderm; V= Vitelline membrane. Bar= 50 µm.

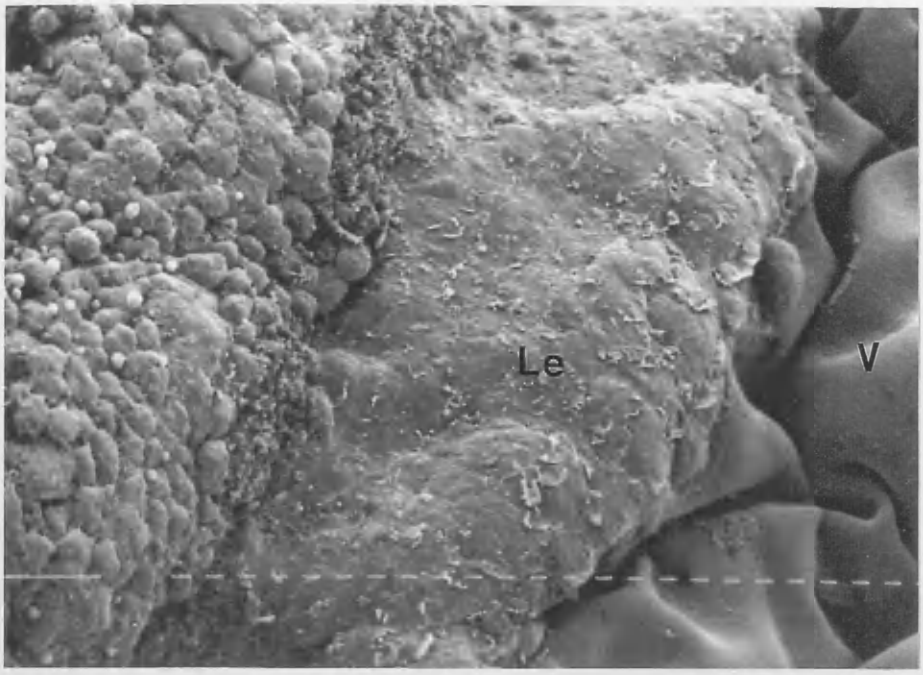

Fig.3.19 a

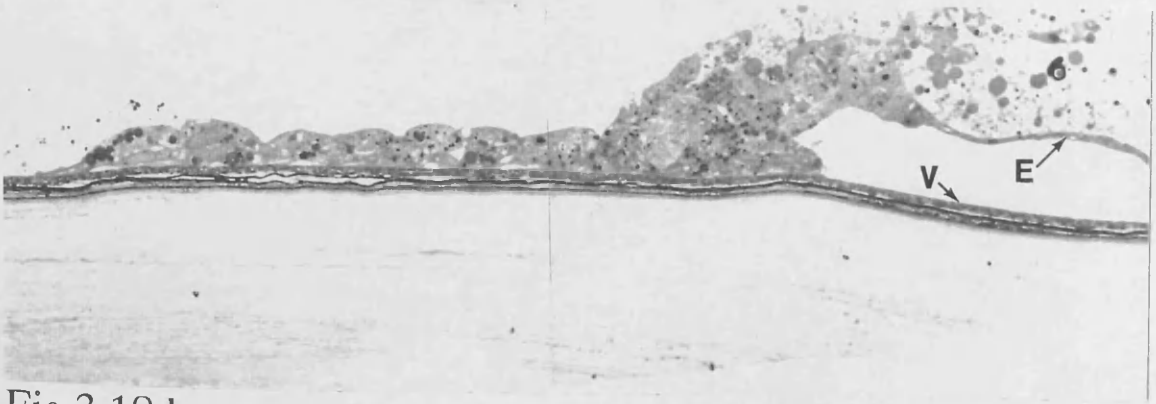

Fig.3.19 b

Fig.3.20 a- High magnification of an area in Fig.3.19a showing the overlapping of the leading lamellae (L). Bar= 10 µm.

Fig.3.20 b- SEM showing the short leading lamellae (L) of stage 16 distal edge cells extending thin cytoplasmic processes (arrowheads) to make contact with the vitelline membrane (V) and the neighbouring cells. Bar= 10 µm.

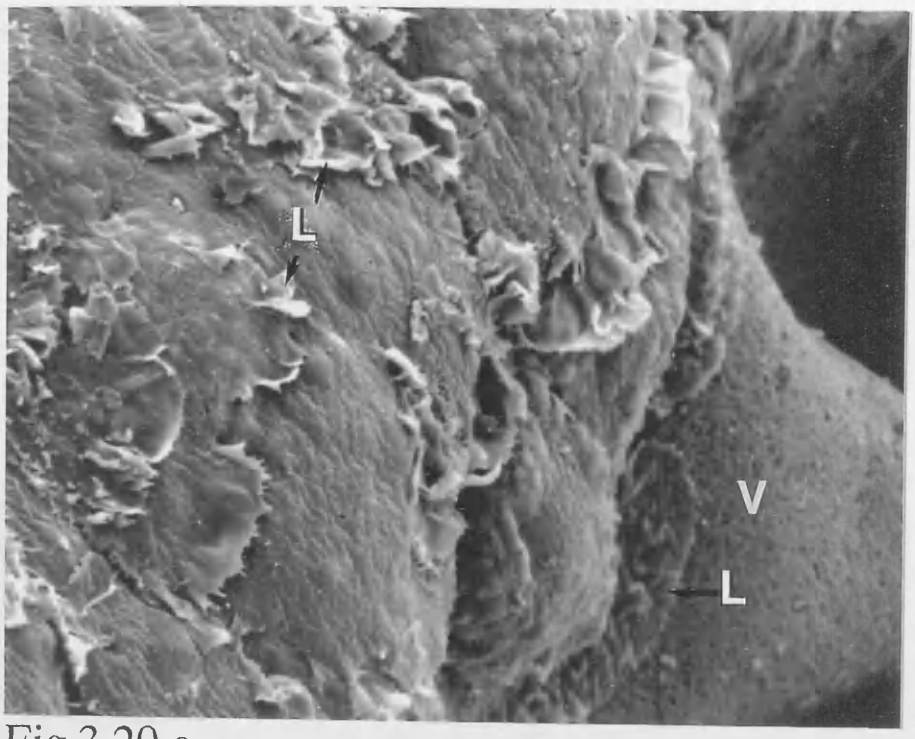

Fig.3.20 a

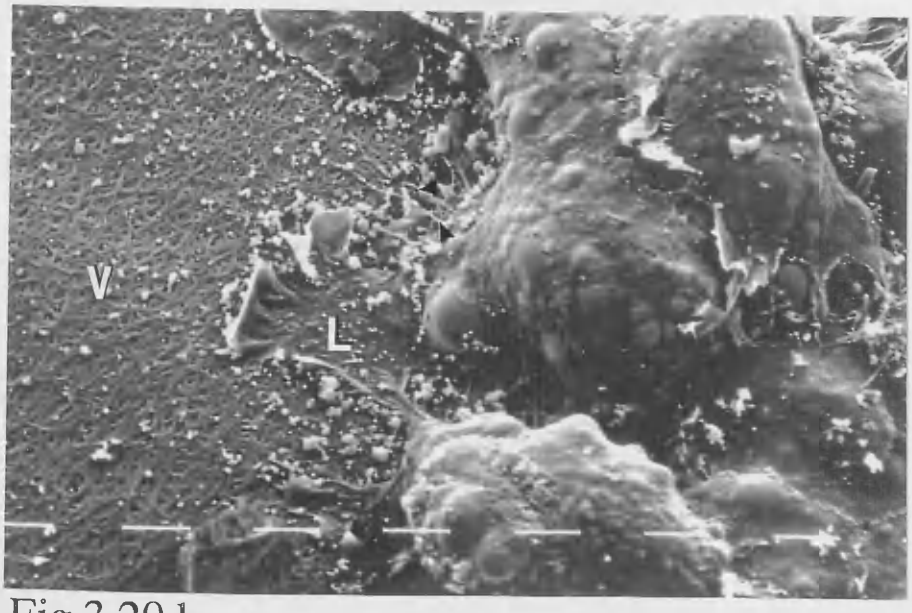

Fig.3.20 b

Fig.3.21 a- A graph showing the relationship between the stage of development and the width of the leading edge. There is a statistically significant increase in this parameter by stage 11 and 16 (2 and 2.5 days of incubation, respectively).

Fig.3.21 b- A graph showing the relationship between the stage of development and the number of cells in the leading edge. As in the above parameter, there is a statistically significant increase in the number of edge cells at stage 11 and 16.

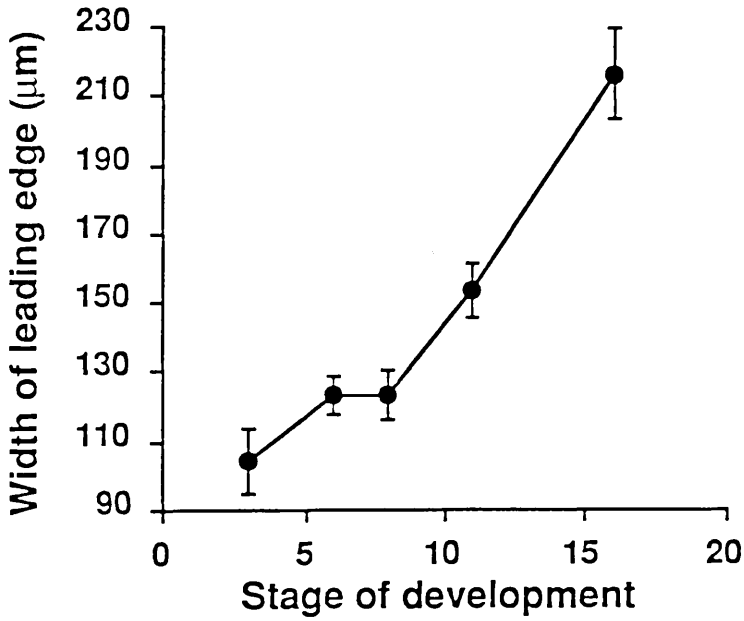

Fig.3.21 a

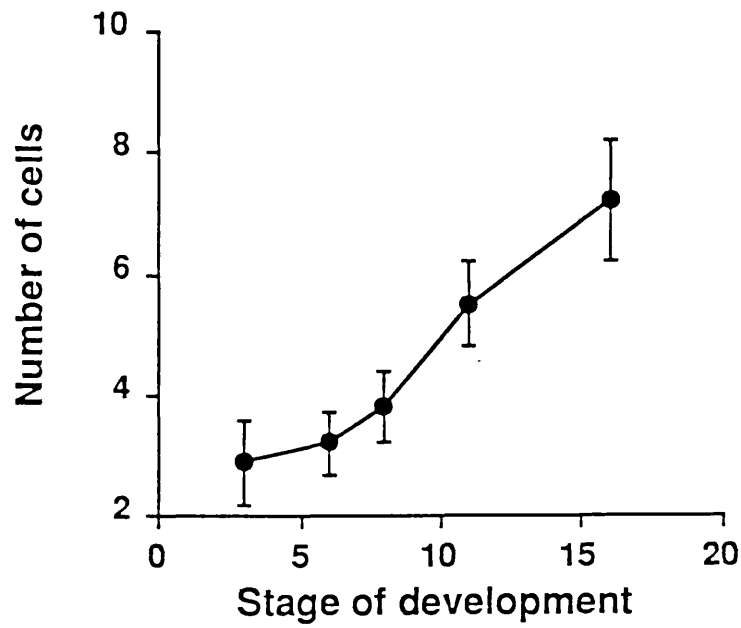

Fig.3.21 b

Fig.3.22 a- A graph showing changes in the height of the leading edge as expansion proceeds. There is no consistent pattern in this parameter.

Fig.3.22 b- A graph showing that also the size of the leading edge cells do not follow a consistent pattern.

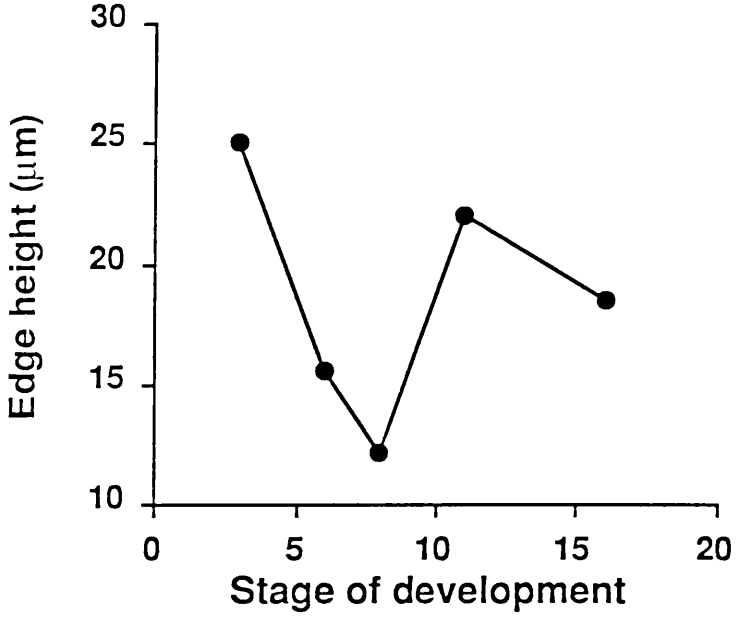

Fig.3.22 a

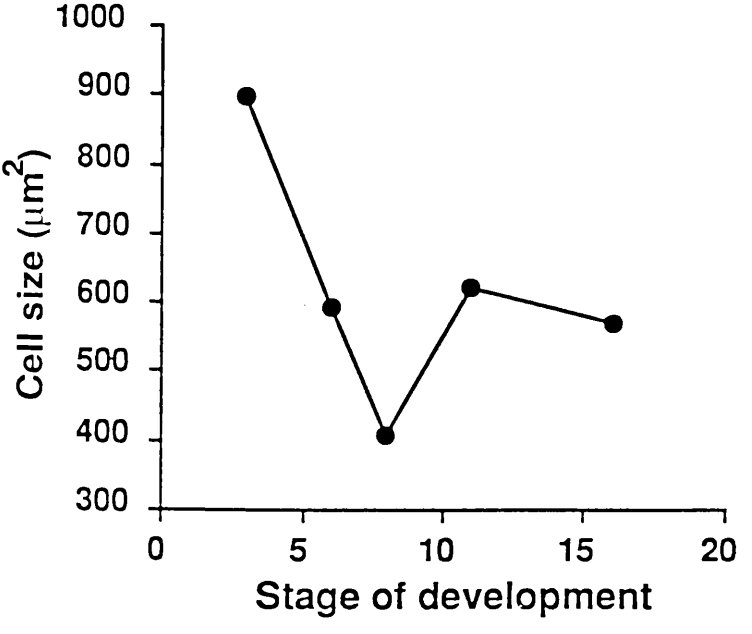

Fig.3.22 b

Fig.3.23- A graph showing the continuous decrease in the length of the distalmost leading lamellae from stage 3 through to 16.

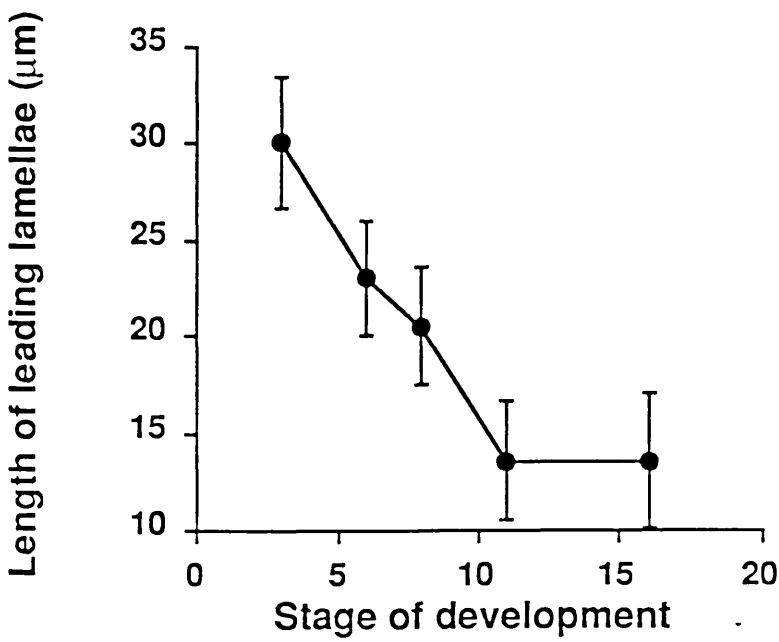
Length of leading lamellae (μm)
35
30
25
20
15
10
0
5
10
15
20
Stage of development

Fig.3.23

3.5 DISCUSSION

Only very few studies have been done on the shape and behaviour of the leading edge of the early chick blastoderm, and all these studies have concentrated on the very early stages of development, mostly around the first day of incubation (Downie and Pegrum, 1971; Downie, 1971; Chernoff and Overton 1979; Andries and Vakaet, 1985a,b and Andries *et. al.*, 1985). As such, these studies have not given the full series of events which take place during the development of this very important structure on which the life of the developing embryo depends.

The work covered in this chapter was designed to follow the development of the edge to see if there are any noteworthy changes in the shape and behaviour of its cells from the stage of their early attachment to the vitelline membrane to the stage at which they have surrounded 1/2 - 2/3 of the yolk sphere. The final stages in surrounding the yolk sphere will be described in detail in chapter 5.

This study also presents the first account of morphometric changes which occur in the leading edge from one stage of development to another and relates these changes to the reported variations in the rate of movement of the edge (Downie, 1976).

3.5.1: Yolk content of edge cells and nearby ectoderm:

It has been reported (Bellairs and New 1962) that the cells of chick embryos up to one day old are rich in yolk granules and suggested that these are the source of nourishment for the developing embryo before the onset of blood circulation. My observations are in total agreement with Bellairs' findings. Yolk inclusions were found in the edge as well as in the ectodermal cells of the blastoderm at stage 3 (H&H). Since the edge cells are the crucial element in the spreading of the whole blastoderm, it was not unexpected to find

that these cells are filled with large yolk inclusions which would be used as the source of energy needed for such a demanding activity.

At stage 6 (after about one day of incubation) I found that the edge cells contained much less yolk,in the form of small granules, presumably as a result of its usage during the early stages.

I also found that the edge cells which are in direct contact with the yolk have an irregular surface from which villi protrude. These villi may be involved in the phagocytic activity reported by Bellairs and New (1962). This phagocytic activity could account for the reappearance of the relatively large vacuoles which are one of the features of edge cells at stage 11. The amount of yolk in the edge cells at the stages that follow was found to be noticeably less than that at stage 11. This does not mean that phagocytosis is not taking place at these stages but perhaps that the yolk which has been uptaken by the cells is being used more rapidly due to the increasing demand for energy as both the rate of movement of the edge and the area that it covers increase.

The ectodermal cells of the blastoderm at the early stages of development are also active. They show a high degree of proliferation and growth to increase the diameter of the blastoderm as the edge cells continue their centrifugal migration (Downie, 1976). To achieve this, the ectodermal cells need a substantial energy source and, hence, the presence in them of the yolk.

3.5.2 Edge cell lamellae and contacts with the vitelline membrane:

a) Lamellae, contacts and microfilaments

Downie (1971), Downie and Pegrum (1971) and Chernoff and Overton (1979) reported that, at least at the stages that they studied, the leading edge cells of the blastoderm possess leading lamellae which are generally oriented towards the direction of movement of the edge and are underlapping and overlapping each other. The results of the present study suggest that this is the

case not only for the early stages that the above authors described but also for the later stages of development, even after changes in the general organisation of the edge cells (see later).

Although most of the work concerning cell locomotion has been carried out on fibroblasts moving on artificial substrata, there seems to be a general belief that the main locomotory motor in both fibroblasts and epithelial cells is their leading lamellae (Abercrombie *et. al.* 1970 a&b, 1971 & 1977, Harris 1973, Middleton, 1973, Radice, 1978 and 1980a, Trinkaus, 1984 and Lackie, 1986).

Abercrombie *et. al.* (1970 a&b) have shown that the leading lamellae of fibroblasts protrude and withdraw in a random succession with longer time being spent in protruding than withdrawal, resulting in a net advance of the cell. The mechanism by which these leading lamellae are formed is complicated and are thought to involve both the loosely organised meshwork of microfilaments and hydrostatic pressure. Evidence for this comes from the observations that both cytochalasin which inhibits actin polymerisation (Spooner *et. al.*, 1971 and Cooper *et. al.*, 1987) and sorbitol (Trinkaus, 1984) prevented the formation of the leading edge.

An interesting question which arises here is what determines the formation of such lamellae at one end of a cell rather than another. Briefly, Harris (1973) and Erickson and Trinkaus (1976) suggested that unknown factors could cause weakening of the cortical cytoskeleton at one end of the cell. As a result, the plasma membrane cannot resist the pressure and bulges, forming the protrusion, or lamella. Oster and Perelson (1987) suggested that after the protrusion is produced, actin polymerises to fill the gap.

As mentioned above, the meshwork of microfilaments is loosely organised in the leading lamellae of actively moving cells. However, in slow moving cells the microfilaments are organised in bundles, or stress fibres, which

terminate at focal contacts with the substratum (Spooner *et. al.*, 1971 and Trinkaus, 1984). Light and transmission electron microscopy of the leading edge cells of the blastoderm have all the characteristics of actively moving cells. They possess leading lamellae which are oriented towards the direction of blastoderm expansion and are rich in cortical microfilaments. Some lamellae of the edge cells produce feet-like microvillous projections into the vitelline membrane. The significance of these are discussed below.

As far as cell-substratum contacts are concerned, it is known that fibroblasts and epithelial cells moving on plain substrata make two types of contact when examined under interference - reflection microscopy (Curtis, 1964 and Izzard and Lochner, 1976). These are focal and close contacts. Focal contacts are points of strong adhesion between the cells and their substratum and are associated with microfilament bundles and thus are thought to play some role in cell motility (Heath and Dunn, 1978 and Wehland *et. al.*, 1979). However, these focal contacts are found to be more abundant in less motile cells (Couchman and Rees, 1979; Keller *et. al.*, 1979 and Kolega *et. al.*, 1982). Even when these contacts are found in moving cells, they are usually located at the ends of retraction fibres or at the tail of the cells and are frequently broken from the substratum and left behind (Chen, 1981).

Close contacts, on the other hand, are points of weaker adhesion to the substratum and are found in the more actively moving cells. While focal contacts appear as dark streaks aligned parallel to the direction of movement, close contacts appear as brighter grey areas under the interference reflection microscope. Chen (1981) found that close contacts increase in number as the protrusive activity at the leading edge of the cells increases. Similarly, Radice (1980), using *Xenopus* tadpole tail epithelium, and Heath (1982), using chick corneal epithelium, found that the main contact between the substratum and these actively moving cells is made through close contacts with only very few focal contacts. Ireland and Stern (1982) reported that close contacts are found between the substratum and newly cultured hypoblast cells and that, as the

hypoblast cells become older and less active they develop focal contacts associated with stress fibres. Unlike focal contacts, close contacts are not associated with bundles of microfilaments.

At the stages of blastoderm leading edge development examined above, bundles of microfilaments are rarely seen in the edge cells which are in contact with the vitelline membrane and, when found, they are mainly restricted to some proximal cells, probably an indication that the cells at this part of the blastoderm edge may be moving less actively than the cells at the other two parts.

b) Length of the leading lamellae:

Measurements of the length of the leading lamellae in distal cells revealed that these lamellae decrease in length with the advance of development. Their length was found to be 31 $\pm$ 4.4 μm at stage 3, dropping to 23 $\pm$ 5 μm at stage 6, 20.5 $\pm$ 3 μm at stage 8, 13.6 $\pm$ 2.6 μm at stage 11 and 13.6 $\pm$ 5.3 μm at stage 16. These figures represent 28%, 19%, 16%, 9% and 6% of the total width of the edge at stages 3, 6, 8, 11 and 16 respectively. The significance of this decrease in the length of lamellae is not clear and I am not aware of any work predicting or showing that changes in lamella size could be important in locomotion. It is possible that a series of cells, acting in a co-ordinated way may move faster if there are a relatively large number of cells with short lamellae than if there are fewer cells with larger lamellae.

Microvillous projections and the vitelline membrane

Most of the work on epithelial spreading has been carried out on plain and solid substrata and not on a fibrillar matrix such as the vitelline membrane. I am not aware of previous work on epithelial cells, apart from edge cells, reporting epithelial cells projecting into gaps in the mesh. However, there is a clear similarity here between the projections produced by edge cells and some other cell types. For example, Haston *et. al.* (1982) found that lymphocytes

attach to and migrate on 3-dimensional matrices by a non-adhesive mechanism which is the extension and expansion of pseudopodia through gaps in the matrix. They also showed that lymphocytes attached to and moved on filters with pore sizes large enough to allow for the penetration of such pseudopodia, while they failed to attach to filters with much smaller pore sizes. They suggested that these pseudopodia act as non-adhesive anchorage points for locomotion. Similarly, Brown (1982), working on neutrophil leukocytes, found that these cells do not adhere to collagen-coated glass yet they move actively in a fibrillar matrix of collagen. Lackie (1986) supported the idea that these projections anchor the cells to their substratum and suggested that their presence is enough to allow cell movement without the need for further adhesion to the substrata, as that could only result in cell immobilisation.

The basal cells of the chick blastoderm edge i.e. those in contact with the vitelline membrane were found to send microvillous projections into the membrane. The extension of these projections into the vitelline membrane may here also be important for the movement of these cells as they may act as mechanical anchorages which allow the forward movement of cells and thus the whole edge. In addition, these anchorage points seem likely to play an important role in resisting the increasing tension on these cells as the blastoderm expands, and thus preventing the detachment of the edge cells from the vitelline membrane.

Evidence for the above conclusions comes from the observation that although these projections are found at all stages of development there are however differences in their size and depth, between stage 3 and the stages that follow; they are more prominent and penetrate deeper into the vitelline membrane during the later stages of development (stage 6 upwards) when both the rate at which the edge moves and tension in the blastoderm increase.

Another point worth mentioning about these projections is that they are mostly sent by the cells of the distal area and mid-part of the edge while they

are less frequent in the proximal area. The significance which this difference in the distribution of such projections may bear on edge movement is not completely clear but it could be related to the degree by which the different parts of the edge contribute to its movement.

It is not clear whether these projections are specific to the edge cells or to the vitelline membrane i.e. would other kinds of cells on this substrate also send projections into the vitelline membrane gaps? Although Chernoff and Overton (1977) cultured embryonic chick skin epithelial cells on the vitelline membrane, they did not mention if such cells extended any projections into the membrane. It is also not clear whether these anchorage points are of the non-adhesive kind found by Haston *et. al.* (1982) and Brown (1982).

3.4.3 Changes in the dimensions of ectodermal cells:

The ectodermal cells were found to change their height as well as width with the progress of development. The height of these cells at stage 3 was 9-11 μm. This then continues to decrease steadily until it reaches 2-3 μm at stage 16.

The width of these cells follow an opposite pattern to the height. These cells are very narrow (8 μm) at stage 3. There is then a steady increase in their width until it reaches about 22 μm at stage 16.

This decrease in height and increase in width of the ectodermal cells supports the suggestion that the blastoderm is under continuous mechanical stress due to the tension which is generated by the centrifugal migration of the edge cells (New, 1959; Bellairs, 1963; Bellairs *et. al.*, 1967 and Downie, 1976). As the tension increases, the ectodermal cells stretch to prevent the breakdown of the blastoderm. This stretching is partly responsible for the noticed increase in their width and decrease in their height; the other cause for such changes could be active shape changes by the cells, since these cells were found to retain their organisation even after being excised from the rest of the blastoderm i.e. when they are relaxed from the tension (Downie, 1976).

3.5.4 Intercellular contacts within the edge:

3.5.4.1 Desmosomes:

Desmosomes or zonula adherens (Farquhar and Palade, 1963) form on the plasma membrane of adjacent epithelial cells and play important roles in cell adhesion and maintaining the structural and functional interaction of these cells (Overton, 1973; Cowin *et. al.*, 1985; Kolega, 1986 and Pasdar and Nelson, 1989). In epithelial tissue, desmosomes usually run proximal to the tight junction at the apical end of the adjacent cells and are thought to strengthen cell attachment at that area (Steinberg *et. al.*, 1987). These desmosomes have also been implicated in maintaining tissue cohesion during mechanical stretching (McNutt *et. al.*, 1971; Lackie, 1986; and Boyer *et. al.*, 1989). It has been suggested (Staehelin, 1974) that desmosomes serve as "button-like connectors", through their attached tonofilaments, between the cytoskeletal elements of individual cells in a tissue, thus distributing the mechanical stress on one cell to the whole tissue, preventing excessive deformation of cell membranes under such stress.

Downie and Pegrum (1971) studied the ultrastructure of the leading edge cells of the chick embryo after one day of incubation and reported the presence of contact specialisations between these cells but not in the shape of desmosomes. The results from the current study showed that throughout the stages of development studied desmosomes were a common feature between the proximal cells as well as between some of the cells of the mid-part of the edge. As the cells of these two areas are the nearest to the ectodermal part of the blastoderm, which is under tension caused by the centrifugal movement of the edge, it is feasible that these desmosomes develop between these cells to resist the mounting shearing force caused by such tension and to maintain the stability of the edge as a whole.

After the second day of incubation (stage 11 upwards), desmosomes were restricted to the proximal edge cells and their immediate neighbours from the

mid-part of the edge. The remaining mid-part cells were connected by small areas of tight junctions. This change in junction pattern at the late stages may be an indication that the cells of this area are less adherent to each other than at the earlier stages. The significance of this will be discussed, in the next chapter, in relation to recruitment of cells into the edge.

3.5.4.2 Tight junctions:

Tight junctions or zonula occludens (Farquhar and Palade, 1963) are located at the apical end of the cells of the vertebrate epithelial sheet and form a selective permeability barrier to prevent the passage of material from one cell to another and at the same time seal the intercellular space from the external environment (Keller and Trinkaus, 1987 and Steinberg *et. al.*, 1987). Hull and Staehelin (1976) reported that these junctions are induced by increasing tension on cells of the digestive tract of *Xenopus*. They are also thought to play a role in the maintenance of cellular polarity (Schneeberger and Lynch, 1984).

In the present work points of close contact or tight junctions were found between the cells of all areas of the edge. It seems, here also, that the increasing tension in the blastoderm may lead to the development of these junctions between the cells of the edge to resist such tension. However, while these tight junctions may be sufficient to overcome tension in the peripheral areas of the edge, they may be incapable of doing so on their own in the more proximal areas without the assistance of more powerful junctions in the form of desmosomes.

Andries *et. al.* (1985), basing their TEM and freeze fracture observations on quail and chick blastoderms, reported that tight junctions between edge cells are distributed in a discontinuous fashion and suggested that they are leaky and may not be functioning as a permeability barrier to prevent the passage of material from one cell to another, but may play an important role in cell cohesion (Revel *et. al.*, 1973; Staehelin, 1974 and Stolinski *et. al.*, 1981).The

results presented in this chapter are in agreement with their finding, not only at the early stages that they studied but also at the later stages of development.

The presence of tight junctions between epithelial cells does not necessarily prevent the individual cells from shifting their position relative to each other. Yee (1972), working on junctions between hepatocytes during the regeneration of rat liver, noticed an increase in the number of these tight junctions when the cells were proliferating or actively moving over each other. Also, Keller and Trinkaus (1987) reported that the presence of tight junctions between the cells of the enveloping layer of the *Fundulus* blastoderm did not prevent cellular rearrangement in this layer. Our finding that tight junctions are distributed between the cells of the leading edge of the blastoderm may indicate that even though these junctions prevent the breakdown of the edge under tension, they meanwhile allow for the cell reorganisation which was found to take place around the second day of incubation, when the multilayered edge becomes a bilayer.

3.5.4.3 Gap junctions:

Gap junctions (Revel and Karnovsky, 1967) exist between opposed cell membranes of neighbouring cells. They are believed to mediate and regulate the transfer of metabolites from one cell to another and to play an important role in intercellular communication (Goodenough and Revel, 1970; Revel *et. al.*, 1973, Pederson *et. al.*, 1980; Gilula, 1980; Finbow and Pitts, 1981 and Caveney,1985). Destruction of gap junction function by antibodies to their proteins inhibited cellular communication in both amphibian and mouse embryos (Warner *et. al.*,1984 and Warner, 1987 respectively). It has also been suggested (Gabbiani *et. al.*, 1978) that these junctions play an important role in synchronisation of movement of individual cells during wound healing in rat skin.

Despite the mounting evidence of the importance of gap junctions in development, their presence does not seem to be always required for

intercellular communication, for it has been reported (Goodall and Johnson,1984) that gap junctions were absent between cells of preimplantation mouse embryos. It is also important to mention that although these junctions are implicated in the synchronisation of movement of epithelial cells, Middleton (1982) has shown that these junctions were absent between actively moving retinal pigmented epithelial cells even three hours after collision between individual cells.

Andries *et. al.* (1985) reported the presence of very small areas of gap junctions between the cells of the mid-part as well as the proximal area of the leading edge but not between the ectodermal cells and related the presence of such junctions to differences in cell shape and cytoskeletal organisation between the two cell types. In similar studies, Andries and Vakaet (1985 a&b) showed the presence of gap junctions between the cells of the proximal area of the edge and suggested that these junctions might be involved in synchronisation of movement of these cells to achieve a rapid rate of migration.

Despite the careful examination of many serial sections of edge cells at all stages of development and in spite of treating these cells with Triton-X 100 I have not come across any gap junctions at any time. The cause of this disagreement between the findings of the above authors and mine as far as the presence of gap junctions is concerned is not clear. What is not clear also is the difference in the location of gap junctions in the edge between the two papers described above: in the first Andries *et. al.* (1985) found that these junctions are present among proximal as well as mid-part cells while in the second Andries and Vakaet (1985 a&b) reported their presence mainly between the proximal cells.

The presence or absence of gap junctions between edge cells needs more careful examination perhaps by microinjection of fluorescent dye to study coupling between these cells (Kam and Pitts, 1989), a technique that I considered but was not able to attempt through lack of facilities and time.

Application of dye microinjection to study coupling between epithelial cells would be useful in the study of blastoderm expansion and in other examples of epithelial movement.

3.5.5 Other changes in the chick blastoderm edge:

3.5.5.1 Changes in width of the edge and the relative number of cells:

If we look first at changes in the width of the edge and the relative number of cells occupying that width we can see the timing of these changes correlates well with the changes in the rate of expansion of the blastoderm; at stage 3 (15 hours of incubation) the edge cells have just attached to the vitelline membrane and started their centrifugal migration. The rate of this migration was reported to be 200 μm/hr. *in vivo* and 169 μm/ hr. *in vitro* (Downie, 1976). In addition, the blastoderm that the edge is pulling is relatively very small and it is therefore not necessary for the edge at this stage to have a large number of cells covering a wide area of the vitelline membrane.

At the stages that follow a steady increase in both the width and relative number of cells of the edge was observed and although there is no significant difference in width between the stage 6 and 8 leading edge, a significant increase in the relative number of cells was recorded. A further highly significant difference in both the width of the edge and the number of its cells occurs at stages 11 and 16. This coincides with the time at which the edge is moving at its fastest rate (555 μm/hr. *in vivo* and 285-384 μm/ hr. *in vitro*, Downie, 1976). It seems, therefore, that the increase in the width of the leading edge as well as the relative number of its cells is a tactic developed by the chick blastoderm to overcome the increasing tension which results from the pulling of an increasingly larger blastoderm and to achieve a faster rate of migration to cover as much as possible of the yolk sphere in the shortest time possible. I will return to this point when discussing models of epithelial movement in chapter 8.

3.5.5.2 Changes in edge height and cell size:

If we now turn our attention to the results of measuring the size of edge cells and the height of the edge as a whole, we can see that both factors seem here also to serve the common purpose of creating a powerful motile unit as development progresses.

The exceptionally large size of edge cells and, therefore, the height of the edge at stage 3 are not an indication that these cells are highly locomotive, but are due to the large yolk inclusions within the cells. These large yolk inclusions could, in fact, be one of the reasons for the slow movement of the edge at stage; they make the cells bulky and heavy which in turn could make their movement a rather difficult one.

There is a drop in both the size of edge cells and the mean height of the edge by stage 6 and 8, presumably as a result of the usage of the yolk. At stage 11, or just before it, a highly significant increase occurs in both the size of the cells and the height of the edge. As discussed above, a significant increase in the number of cells was found at this stage. It is possible that in addition to this increase, the edge cells undergo a phase of rapid growth. This growth could be one of the factors which contribute to the rapid centrifugal migration of the edge cells at this stage.

One peculiar and rather unexpected finding in the results was the statistically significant drop in both the size of cells and height of the edge at stage 16, although it is known that the rate of blastoderm expansion is greatest at this stage. The drop in height of the edge at this stage may be a result of the decrease in the height of its individual cells as they cover more area on the vitelline membrane, as proved by the significant increase in the width of the edge at this stage. However, it was not possible to predict exactly the cause of the drop in the size of the edge cells at this stage. One possibility is that less yolk inclusions in these cells could lead to changes in cell size. The other possibility is that recruitment of smaller cells into the edge (see chapter 4) could

lead to a drop in the average size of the cells at this stage.

3.5.6 Deep layer cells:

Deep layer cells were found on the basal lamina of the ectodermal part of the blastoderm at all stages of development studied above. I will discuss the significance of their presence for blastoderm expansion in the next chapter.

CHAPTER FOUR

CELL RECRUITMENT INTO THE LEADING EDGE

4.1. INTRODUCTION:

There are two intriguing questions which can be asked about epiboly in the early chick embryo. The first is how could the blastoderm, which is about 4 mm in diameter at the start of incubation, encompass a yolk sphere which is about 4 cm in diameter. To answer this question, Downie (1976) showed that there is a considerable increase in the original volume of the blastoderm. By measuring the rate of proliferation of the blastoderm cells at different stages of development he found that during the early stages of epiboly the epiblast cells, except those of the leading edge, proliferate at a high rate. This proliferation is found to be accompanied by growth of the daughter cells, resulting in the required increase in the volume of the blastoderm. Later in development cell proliferation becomes restricted to a small area adjacent to the leading edge.

The next very interesting question is how the small band of leading edge cells which encircles the very small blastodisc at the start of incubation can at the same time encircle the blastoderm when it is about to pass the equator of the yolk after 2 to 2.5 days of incubation. There are four possible answers to this question. The first straightforward possibility would be that the edge cells increase their number by cell division. This, however, was ruled out since, as indicated above, Downie (1976), using mitotic inhibitors to measure cell proliferation, found that there is no cell division among the cells of the leading edge.

The second possibility is that the original edge cells increase greatly in size. However, work in the previous chapter shows that although edge cells do change size, there is a considerable increase in their number as expansion proceeds.

The third possibility is that cells are being recruited into the edge from the nearby ectoderm i.e. that which is in direct contact with the edge. The fourth possibility is that cells are recruited into the edge from a source other than the ectoderm.

The work described in this chapter was designed to investigate the source of the increasing number of cells in the leading edge, from ectoderm, other sources, or both.

4.2 Results:

4.2.1 Are the distal ectodermal cells incorporated into the edge?

a) Evidence from ultrastructure:

Transmission electron microscopy was used here to establish whether differences in the pattern of cell junctions exist between the ectodermal cells far from the leading edge and those immediately adjacent to it. In other words, to see if the junctions between ectodermal cells in contact with the edge become weaker to allow the translocation of cells from the ectoderm to the edge.

Examination of ultrathin sections of the ectodermal cells which are located far from the edge revealed that the pattern of junctions throughout the stages of development studied, stage 3 to 16 (Hamburger and Hamilton, 1951), is the same. The cells are always connected by typical junctional complexes made of apical tight junctions followed by a number of desmosomes and interdigitations of the plasma membranes of adjacent cells (Figs 4.1a).

When sections of the leading edge and their nearby ectoderm was examined under TEM, no change was found as far as the pattern of junctions between ectodermal cells is concerned. The junctions between the two most distal ectodermal cells is identical to the junctions described above and tight junctions and desmosomes persist even between the distalmost ectodermal cell and its neighbouring proximal edge cell (Fig.4.2.1b).

b) Evidence from edge cell collision experiments:

I carried out experiments designed to see if the confrontation of two leading edges will allow some of the ectodermal cells near the two edges to get into contact with the vitelline membrane, thus indicating that, in the presence of the leading edge, the nearby ectodermal cells will have the ability to attach to the vitelline membrane i.e. that they share at least one of the important properties of leading edge cells. In these experiments, two pieces of blastoderm, each containing a leading edge and part of the ectoderm, were cultured on the vitelline membrane. The leading edge of each blastoderm piece was positioned so that it would migrate towards the other and eventually collide with it.

The logic behind this experiment was that once the edges have collided, they cannot move further, and addition of cells from the deep layer cannot make the combined edges any wider in extent. However, addition of ectodermal cells to the combined edges could make the double edge unit wider, so that the longer the period after collision, the wider the "edge" should be, indicating that ectodermal cells have been recruited into the edge.

The cultures were fixed after 2, 6, and 24 hours from the time of collision. They were then processed in the usual way and 1 um-thick sections were examined under the light microscope. Results from such experiments showed that even six hours after the collision of the two edges the ectodermal part of the blastoderm is still elevated from the vitelline membrane and none of the ectodermal cells lying immediately near the edge have attached to the vitelline membrane or intermingled with the edge cells (Fig.4.2.a&b). The edge cells of the two edges intermingle with each other and some prominent lamellae from each of the two pieces of blastoderm are now oriented centripetally i.e. in an opposite direction to the movement of that particular edge before its collision with the other edge.

No widening of the edge occurred even after 24 hours of incubation (Fig.4.2c). This is however not a consistent result since, in some many cases, the organisation of the whole cultured edges collapses and the cultured pieces show a behaviour similar to that which results from the culturing of pieces of the blastoderm on the inner surface of the vitelline membrane (see chapter 6).

These results strongly suggest that the ectodermal cells are not recruited in the leading edge.

4.2.2: Are non-ectodermal cells recruited into the blastoderm edge?

The only obvious alternative source of new edge cells is the population of distal deep layer cells found near the edge, and described briefly in chapter 3.

a) Evidence from ultrastructure:

The deep layer cells at stage 3 are difficult to distinguish under the light microscope because of the amount of yolk attached to the blastoderm, but under the transmission electron microscope they can be found in the vicinity of the leading edge lying on the basal lamina both in groups and as individual cells. From stage 6 onwards, these cells can be seen distributed mostly as individuals.

Under the light microscope, the deep layer cells stain darkly with Toluidine blue and they are rather small. This distinguishes them from the attached cells of the leading edge, which are paler staining and larger. In many sections through blastoderms at stage 6 through to stage 16 the deep layer cells get into direct contact with the proximal part of the leading edge. In fact, some ventral cells of the leading edge, both in proximal and mid parts of the edge, have an appearance and staining characters identical to the deep layer cells (See below).

The deep layer cells extend leading lamellae towards the ventral surface of the leading edge cells. For example, the deep layer cells of stage 3

blastoderms possess leading lamellae which come in contact with the surface of the proximal area of the edge (Fig.4.3a). Similarly, at stage 6, darkly stained deep layer cells are found both on the basal surface of the distalmost ectodermal cells and on the ventral surface of some cells of the proximal edge area (Fig.4.3 b&c and Fig. 4.4). These deep layer cells seem to use the ventral surface of the edge cells as their substrate on which they extend their centrifugally oriented lamellae. These lamellae make close contact with the underlying edge cell membrane. At these points of contact, there is a condensation of electron-dense material under the surface of the cell membranes of the lamella and the edge cell (Fig.4.5).

The deep layer cells at stage 8 appear similar to those at the earlier stages; they extend leading lamellae which make points of close contact with the ventral surface of proximal edge cells {Fig.4.6 (TEM) and Fig.4.a&b (LM)}.

The deep layer cells continue to be present at the later stages of development and even at stage 11 a few of these cells can still be seen extending their darkly stained lamellae on the surface of the leading edge cells (see Fig.3.17b). In fact, deep layer cells continue to be added into the edge area even at the very late stages of epiboly (See chapter 5).

b) Evidence from edge regeneration experiments:

The results of these experiments, which point to deep layer cells as the source of new edge cells, will be presented in chapter 6.

4.2.3) Filming of edge cell migration:

The purpose of this part of the present work was to investigate whether recruitment of new cells into the leading edge could be observed directly, by filming leading edges at early stages of epiboly using time-lapse cinemicrography.

Method:

Stage 3 blastoderms were removed from the yolk and as much of the attached yolk as possible was washed off, without disturbing the attachment of the edge to the vitelline membrane. The vitelline membrane, with the attached edge, was then flattened on a coverslip and transferred to filming a chamber. This consists of a coverslip that has been sealed with vaseline to a culture dish in which the centre is removed. A few drops of culture medium (HAM F-12) were spread over the coverslip and the vitelline membrane was placed so that it was sandwiched between the two coverslips. The chamber was then sealed and edge movement was filmed using a Wild M20 microscope, with X 20 objective, with a Bolex cine camera and time-lapse apparatus.

Because a high magnification was used in order to see the nuclei of the edge cells, the advancing cells covered the field of filming in a short time and needed to be brought back to the beginning of the field at least 3-4 times during the short time of filming (2-3 hours).

The films were developed and the movement of the nuclei of the edge cells was traced.

Results:

Despite the careful cleaning of yolk of the blastoderm edge, it was not possible to examine the movement of all the cells in the edge. Therefore, only the movement of the distal cells was traced by focusing on the advance of their nuclei. Fig.4.8 illustrates the movement of these cells over a period of about 3 hours. It shows that during the migration of the edge some cells appear between the originally traced cells and continue their migration with them. For example, in Fig.4.8a 2 cells appear after 1.5 hours of filming and continue their movement between the rest of the distal cells. Also Fig.4.8b shows another 2 cells behaving similarly . There is an increase of 10% in the distance between the two most peripheral cells of the edge at the end of filming in Fig 4.8a and a 17% increase in this distance in Fig 4.8b.

On the other hand, the results showed that in some cases cells which were traced at the beginning of filming went out of focus and it was not possible to tell for sure their position: some of these, however, reappeared after a short time and could be followed again .

These results do not give conclusive evidence for the recruitment of cells into the leading edge since the new cells which appear suddenly in the films may have been originally there but out of focus at the beginning of filming. However, these results do not rule out the possibility that these cells are actually newly recruited cells which intercalate themselves between the distal cells of the leading edge thus providing the edge with extra cells to migrate into a wider area over the vitelline membrane. The fact that, as expansion proceeds, leading edge cells move apart from one another (by 10-17% in about 3 hours) further suggests that cell intercalation at the leading edge must occur.

Fig.4.1 a- TEM showing typical junctional complex between the ectodermal cells of the chick blastoderm. This is composed of an apical tight junction (TJ), some desmosomes (D) and interdigitations of the cellular membranes (arrowheads) Microvilli (mv) mark the apical surface of the ectodermal cells while basal lamina (BL) marks their basal one. Bar= 1.7 μm. X 10.3K.

Fig.4.1 b- TEM showing the persistence of junctional complex between the ectodermal cells (E) even at the periphery of the leading edge (Le). Bar= 1.78 μm. X 9.5K.

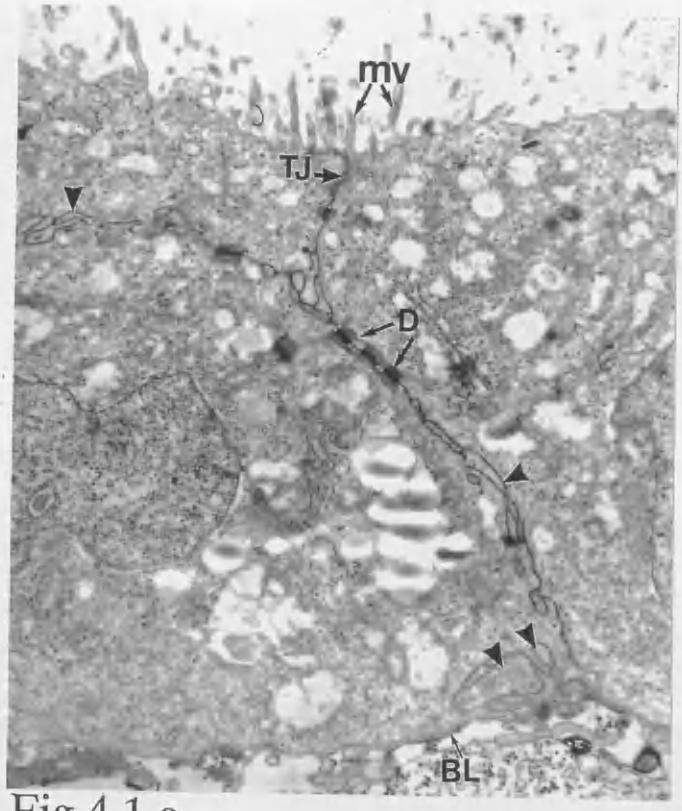

Fig.4.1 a

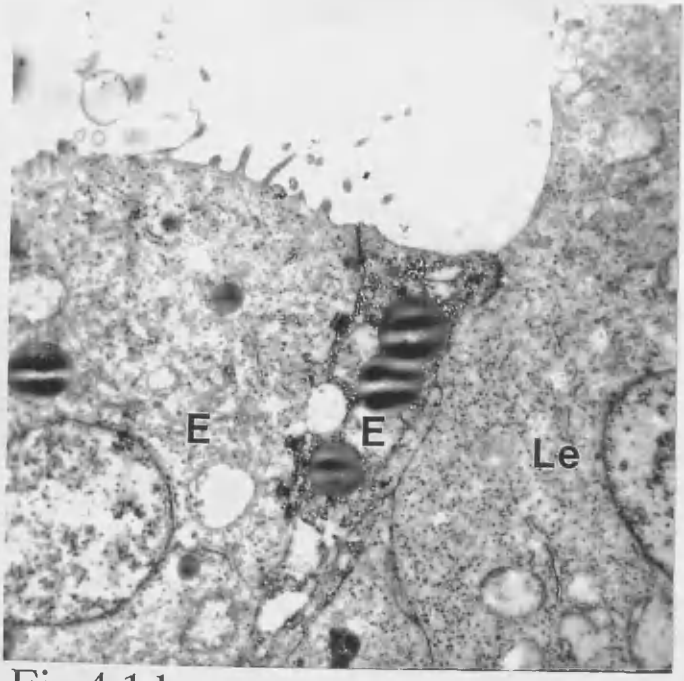

Fig.4.1 b

Fig.4.2- Light micrograph of the area of collision between 2 blastoderm edges cultured on the vitelline membrane (V) for 2 hours (a), 6 hours (b) and 24 hours (c). The ectoderm (E) does not come into contact with the vitelline membrane even after 24 hours, although it starts to show signs of disorganisation. C= area of collision between the 2 edges; D= deep layer cells. Bar= 50 μm.

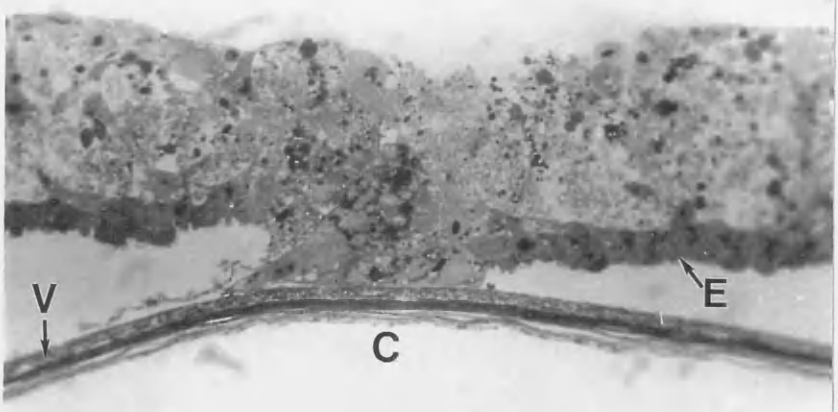

Fig.4.2 a

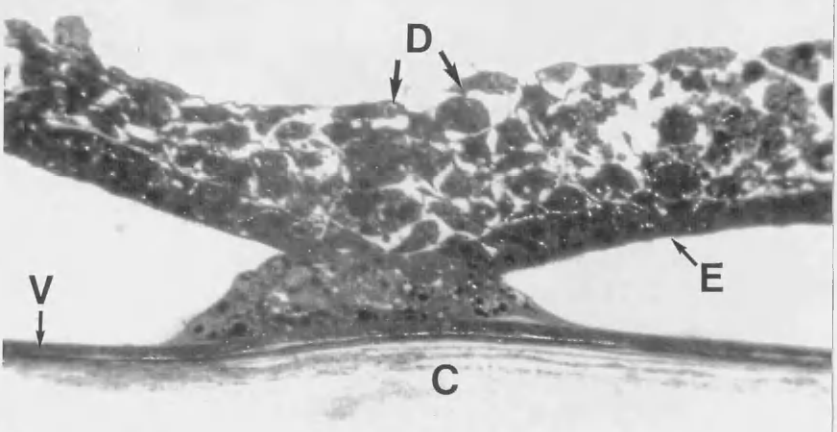

Fig.4.2 b

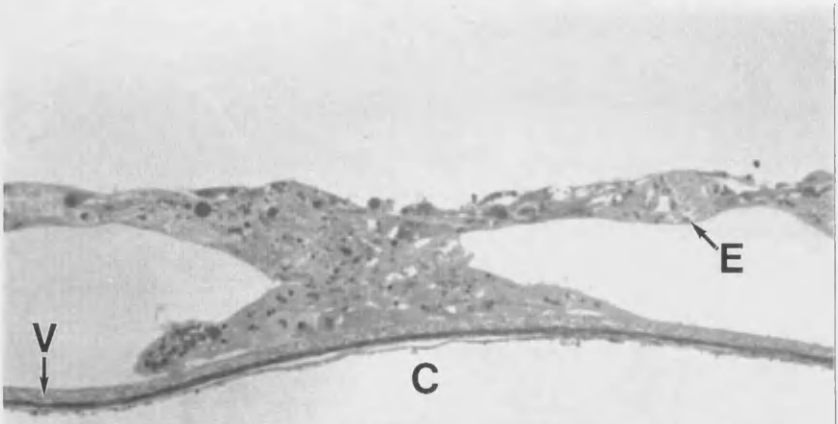

Fig.4.2c

Fig.4.3 a- TEM showing the presence of a darkly stained deep layer cell (D) on the proximal part of a stage 3 leading edge (PLe). Bar= 2.8 μm. X 5.76K.

Fig.4.3 b- Light micrograph of a stage 6 blastoderm edge. Deep layer cells (arrowheads) are on the basal surface of the ectoderm (E) and possibly one of them (marked by a large arrowhead) is making contact with the leading edge cell. Bar= 25 μm.

Fig.4.3. C- As (b) showing one of the deep layer cells (arrowhead) making contact with the leading edge. Bar= 25 μm.

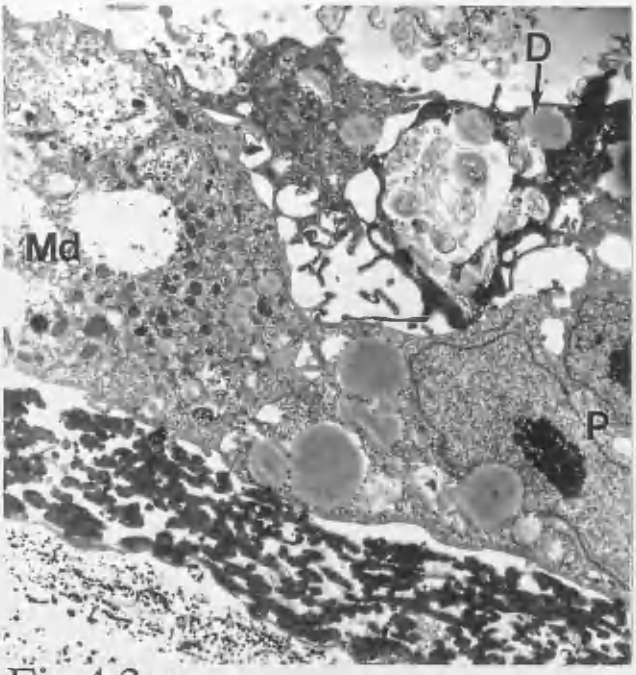

Fig.4.3 a

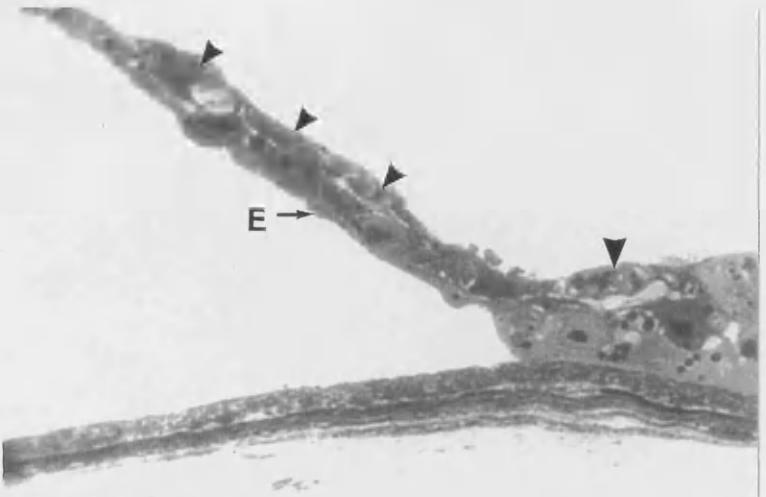

Fig.4.3 b

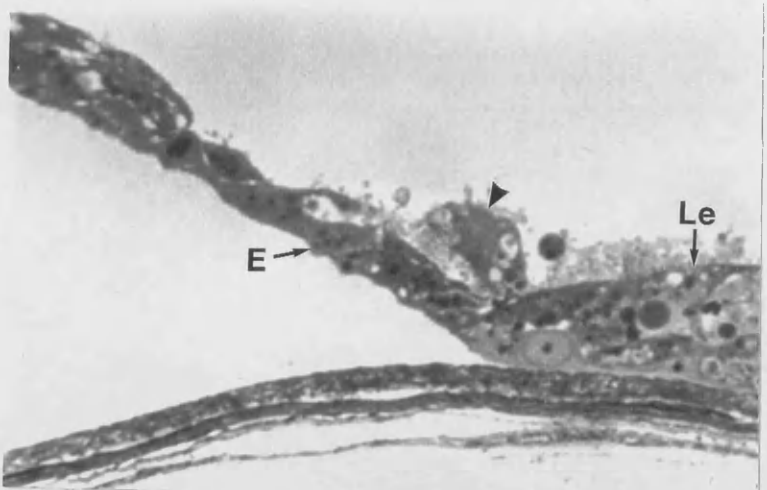

Fig.4.3 c

Fig.4.4 a- Light micrograph of a stage 6 blastoderm showing some deep layer cells (arrowheads) on the basal surface of the ectoderm. The cell marked by a square is enlarged in (b). Bar= 50 μm.

Fig.4.4 b- TEM showing the darkly stained deep layer cell (D) marked in (a) lying on the upper surface of a proximal cell (PL) of stage 6 blastoderm edge. Bar= 2 μm. X 9.2K.

Fig.4.4 c- Light micrograph of a stage 8 blastoderm edge showing two deep layer cells (arrowheads) in the vicinity of the edge (arrows) while the third (large arrowhead) is in direct contact with the leading edge (see Fig.4.5). Bar= 25 μm.

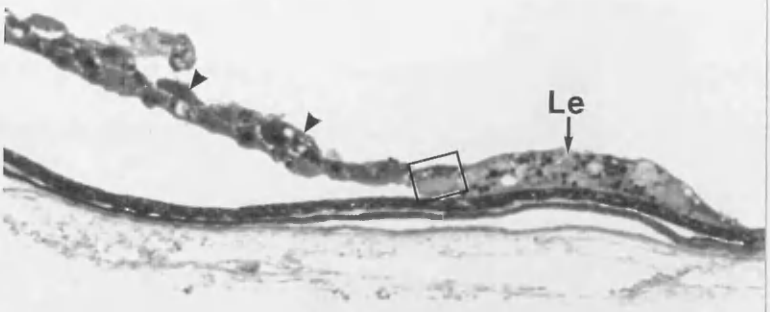

Fig.4.4 a

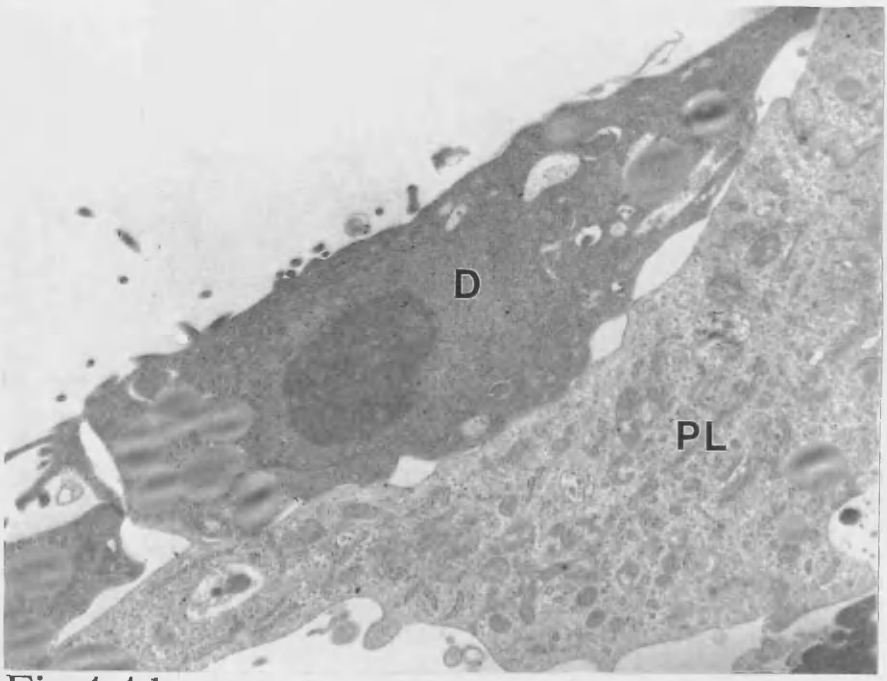

Fig.4.4 b

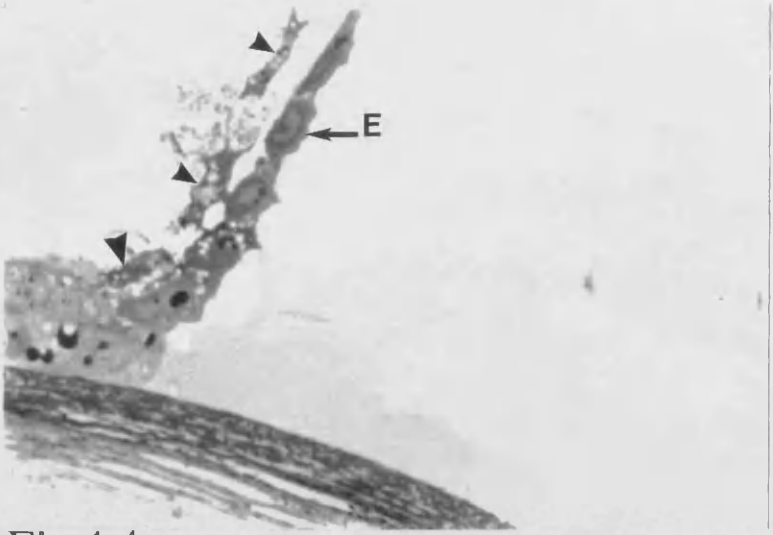

Fig.4.4 c

Fig.4.5 a- Low magnification TEM of the cell marked by a large arrowhead in Fig.4.4c. Here, the cell (D) sends leading lamellae (L) which makes contact with the cells of the edge. Another deep layer cell is on the nearby ectoderm (E). Bar= 2.8 μm. X 5.4K.

Fig.4.5 b- TEM showing the lamellae (L) from the deep layer in (a) at point of contact with the leading edge cell (Le). There is a condensation of electron dense material at the point of contact. Bar= 0.36 μm. X 43.4K.

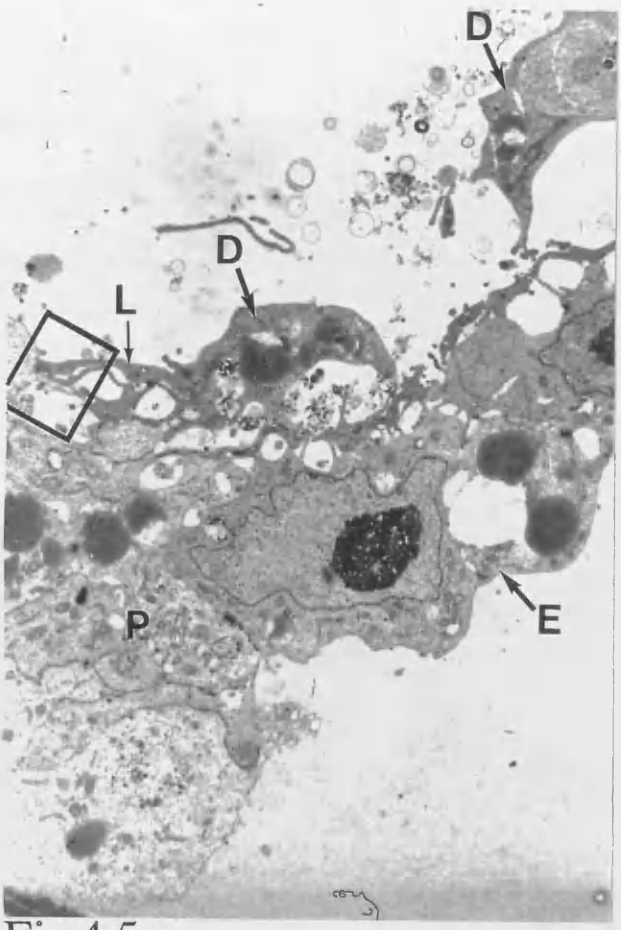

Fig.4.5 a

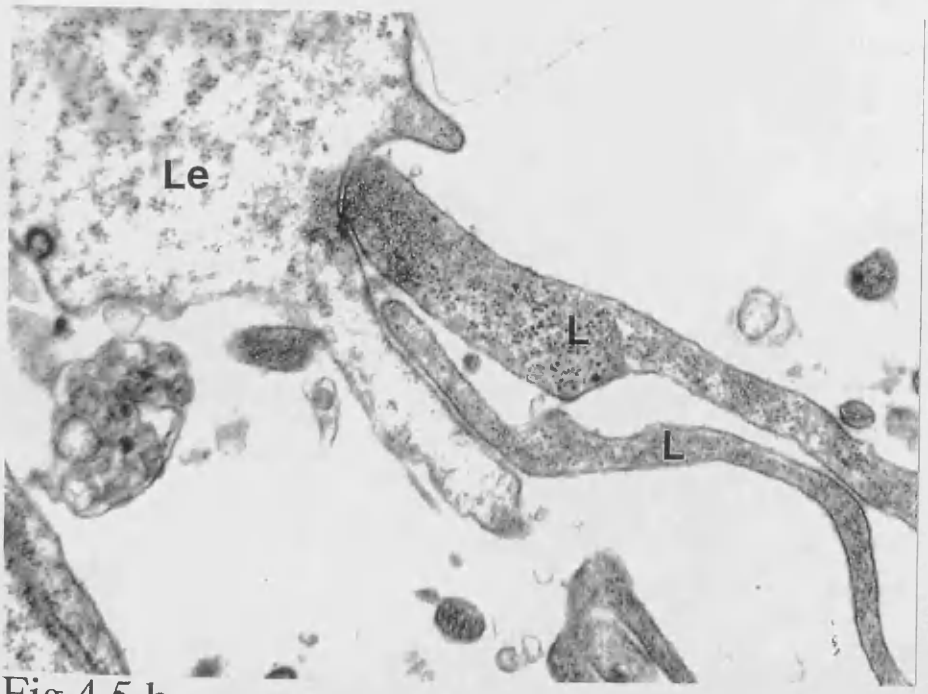

Fig.4.5 b

Fig.4.6 a- TEM of stage 8 blastoderm edge (Le) showing again the presence of a deep layer cell (D) making contact through its leading lamellae (L) with some leading edge cells (Le). Bar= 2.8 μm. X 6.67K.

Fig.4.6 b- TEM of the leading lamella (L) of the cells in (a) showing points of close contact (arrowheads) between it and the upper surface of one of the leading edge cells (Le). Bar= 1.21 μm. X 15.6K.

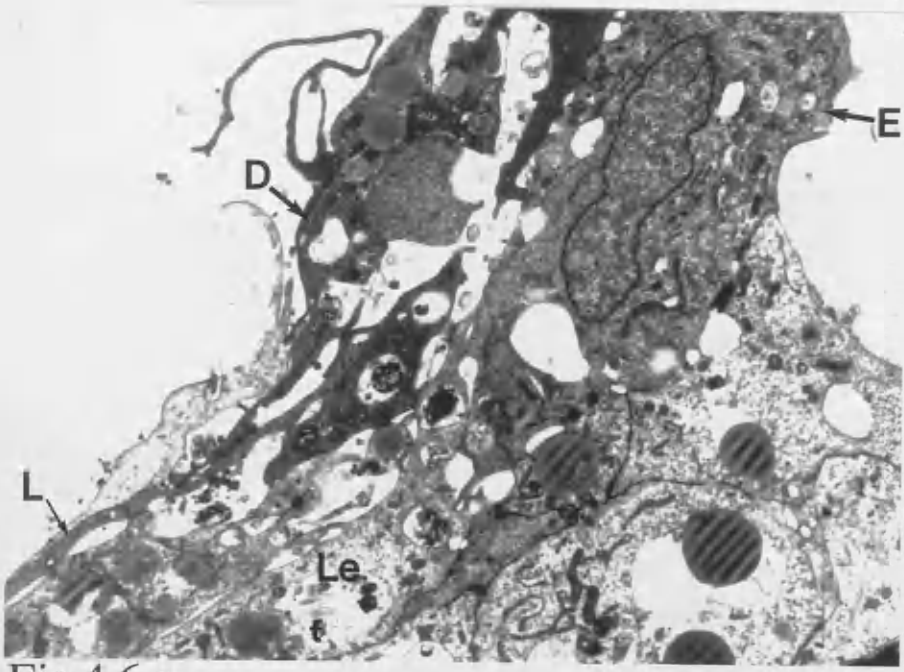

Fig.4.6 a

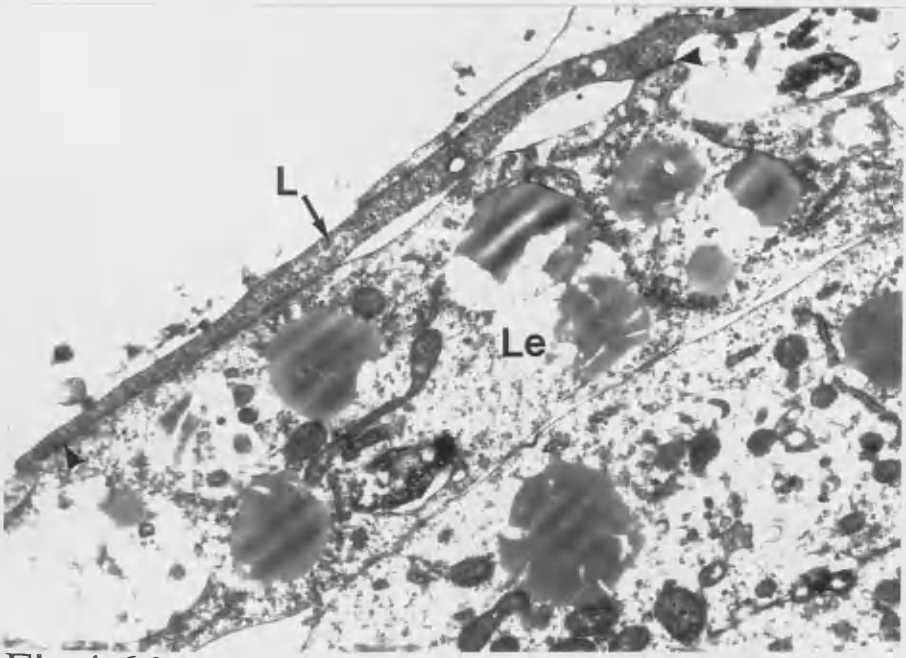

Fig.4.6 b

Fig.4.7 a- Light micrograph of a stage-8 blastoderm at the leading edge site (Le). Notice the stream of deep layer cells (D) on the ectoderm (E). Bar= 50 μm.

Fig.4.7 b- Light micrograph of a stage blastoderm showing a deep layer cell (arrow) making contact with the leading edge while others (arrow-heads) are nearby. Bar= 25 μm.

Fig.4.7 c- Light micrograph of another stage 8 blastoderm showing a darkly stained deep layer cell (arrow) spreading its lamellae on the leading edge. Bar= 25 μm.

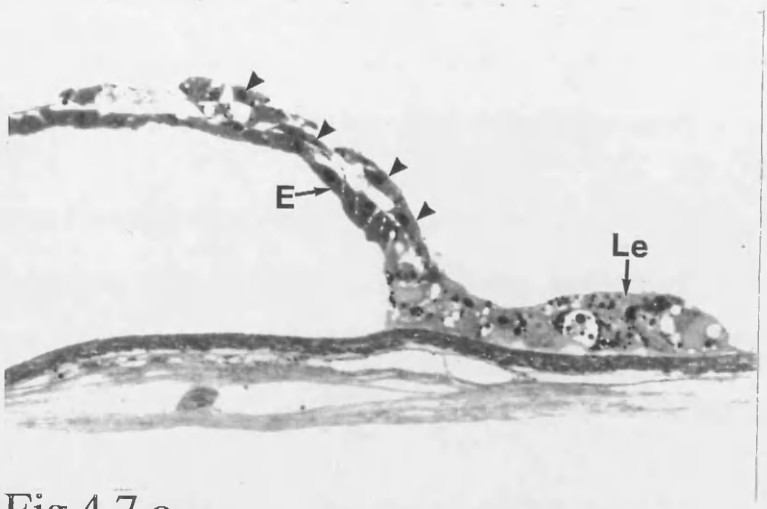

Fig.4.7 a

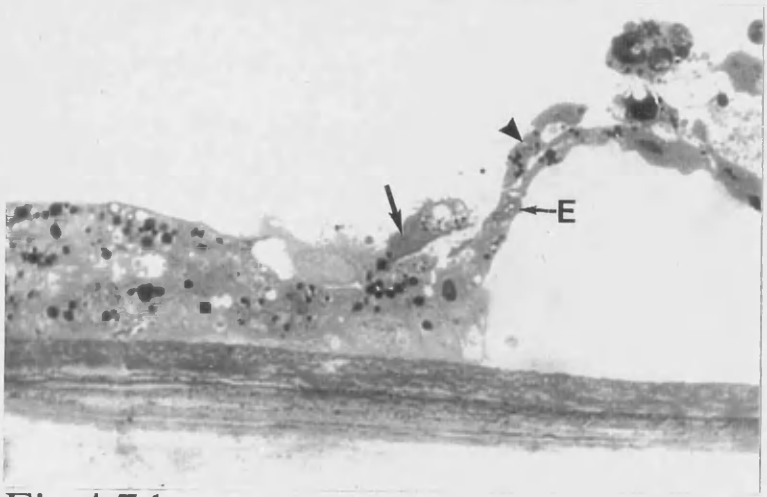

Fig.4.7 b

Fig.4.7 c

Fig.4.8- Tracing of the movements of distal edge cells followed by time -lapse cinemicrography. Each point represents the mid point of the nucleus of a distal edge cell at intervals of 10 minutes. Bar= 50 μm.

a) Notice the appearance of 2 new cells (asters) as expansion progresses. Two other cells (arrows) became out of focus and were not traced. Over 2 hours, the mean distance apart of the most proximal cells increases by 10%.

b) In this other group of edge cells, again there is an appearance of new cells (asters) between the initially traced cells. The cells marked by arrows disappeared after about an hour and were back in focus after half an hour. Over 3 hours, the mean distance apart of most proximal cells increases by 17%.

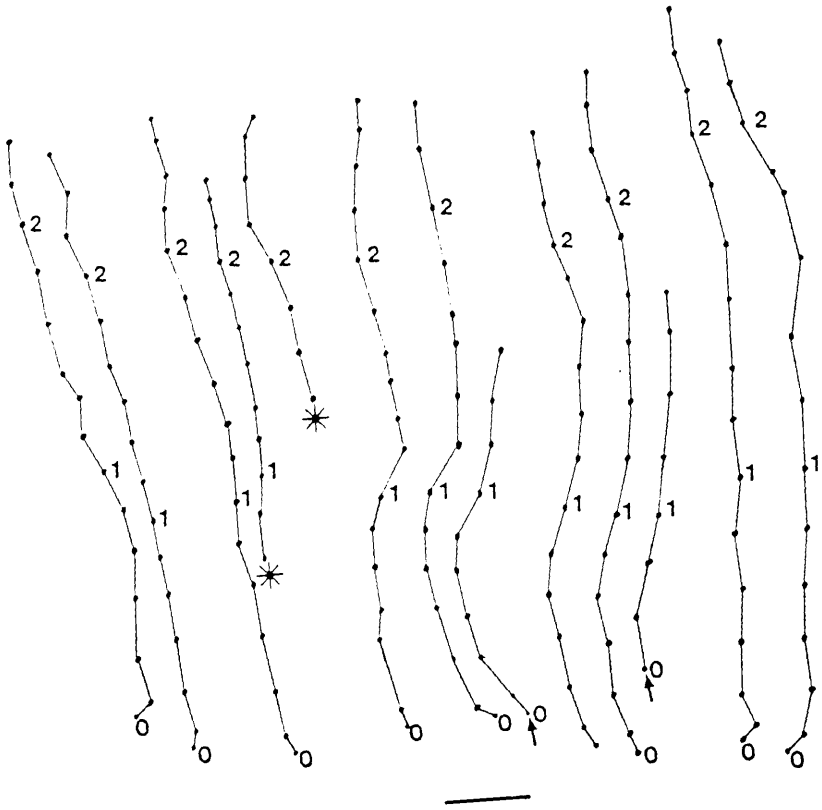

Fig.4.8 a

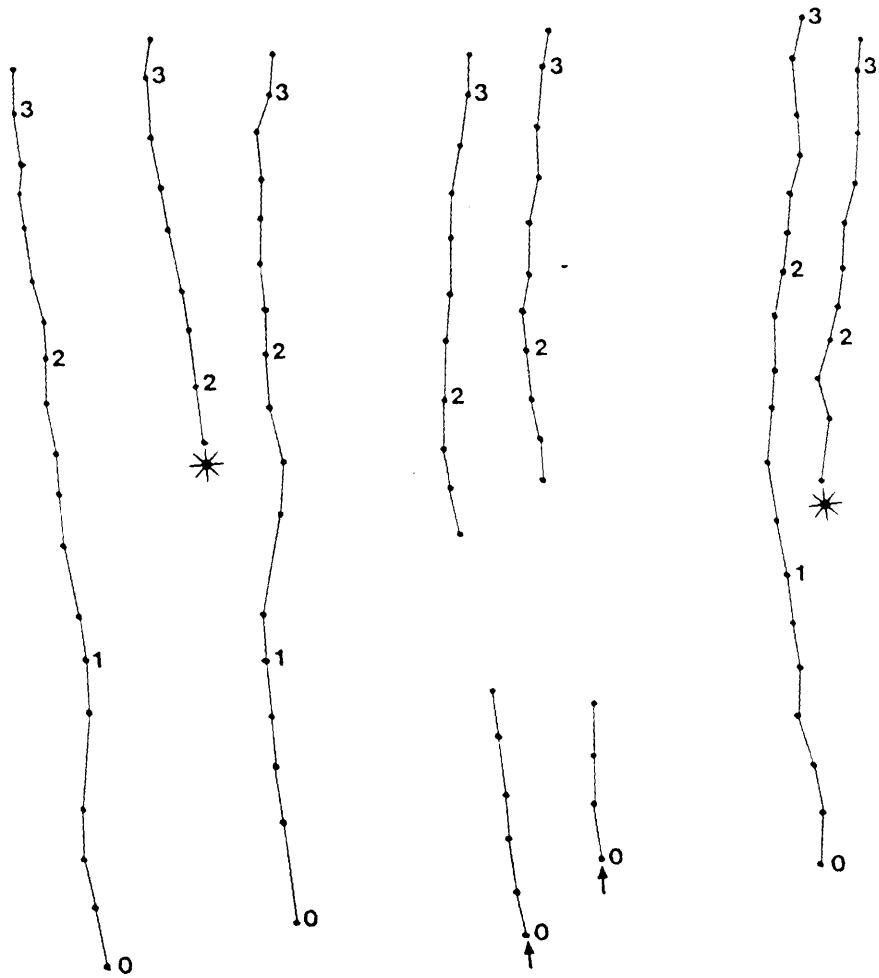

Fig.4.8 b

DISCUSSION

Chapter 3 showed that there is a steady increase in the number of edge cells as expansion of the blastoderm proceeds. Previous work on the blastoderm edge has found no evidence that the edge cells undergo cell division to increase their number as they cover an increasingly larger area of the vitelline membrane (Downie, 1976).

The idea that the most distal ectodermal cells may be incorporated into the edge seems logical but several observations argue against that possibility. First, although a large number of microvilli extend from the apical surface of the ectodermal cells into the sub-blastodermic space, these microvilli never touch the vitelline membrane and I found no evidence of the ectodermal cells, even the most distal ones, sending projections down to the vitelline membrane. Secondly, as described in chapter 3, there are ultrastructural differences between edge and ectodermal cells which make it unlikely that the latter could become normal edge cells.

Thirdly, if the ectodermal cells were to be incorporated into the edge, one would expect a change in the type of junctions which connect the ectodermal cells, probably to weaker connections nearer to the edge . However, I found no differences in the distribution and type of junctions between the ectodermal cells located far from the edge and those in contact with it. Junctional complexes which consist of apical tight junctions, desmosomes and interdigitations of the cellular membranes persist between all the ectoderm cells and even between the distalmost ectodermal cell and its neighbouring proximal edge cell.

Another point suggesting that recruitment of the ectodermal cells into the edge is unlikely is the fact that the whole blastoderm is continuously under tension as it expands over the yolk (New, 1959; Bellairs 1963; Downie, 1967

and Kucera and Monnet-Tschudi, 1987). Any disturbances in the junctions which strongly adhere the ectodermal cells to each other could lead to the breakdown of the blastoderm, a situation which never occurs in normal development.

Finally, the collision experiments described above have indicated that even when the blastoderm is relaxed the ectoderm does not make contact with the vitelline membrane but remains elevated from it. Furthermore, even under these experimental conditions, I have not been able to find any extensions of lamellae from the distalmost ectodermal cells on to the vitelline membrane.

The present work presents an alternative and highly possible source for the cells needed to increase the numbers in the blastoderm edge. This source is the deep layer cells which are present on the basal lamina of the ectoderm proximal to the leading edge. I have both ultrastructural and experimental evidence for deep layer cell incorporation into the edge.

Light microscopy revealed that the deep layer cells are present at all stages on the basal surface of the ectodermal cells and that there are always some of them in contact with the leading edge. It was actually possible to see that not only were some of these cells in contact with the edge, but some were actually incorporated into the edge and lay on the ventral surface of the edge area as shown in Fig.4.7.

Transmission electron microscopy shows that the deep layer cells extend long centrifugally oriented and darkly stained leading lamellae on the ventral surface of the proximal cells where they make points of close contact associated with condensations of electron dense material under the opposing cell membranes. The contact areas are never extensive but are always in the form of points rather than large areas.

It may be that the less pronounced presence of desmosomes between the mid-part cells of the blastoderm edge, especially between those at its ventral

surface, makes it possible for the edge cells at this part of the edge to shift position with each other and, in the process, some of the deep layer cells may incorporate themselves into the edge, leading to the observed increase in number of edge cells.

CHAPTER FIVE

END OF EPIBOLY

5.1 Introduction:

After passing the equator of the yolk sphere, the advancing blastoderm edge starts making its way towards the other end of the yolk, in an attempt to cover the rest of the sphere. As it does so it faces a major problem. This is: how could the large number of edge cells which surround a region of maximum circumference around the equator of the vitelline membrane be accommodated into a much smaller region as the expansion continues towards the other end of the egg? Related to this problem is the task of accommodating the very large surface area of the ectodermal part of the blastoderm in the increasingly smaller space.

This chapter investigates how the chick blastoderm goes about solving the above problem by looking at chick blastoderms older than 2.5 days of incubation i.e. after the stage at which blastoderm expansion passes the equator. The only previous investigations of a similar phenomenon concern the end of epiboly in teleost eggs. The strategies used by teleost and avian blastoderms will be compared in the Discussion to this chapter.

5.2 Methods:

Three to thirteen day old blastoderms were removed from the egg and the loosely attached yolk cleaned off and the embryos fixed in the usual way (Chapter 2). Some of these blastoderms were processed for SEM examination and others for embedding in Araldite resin. When possible (see below) 1 um thick toluidine blue stained sections were examined under the light microscope, and areas of interest were trimmed and processed for TEM examination. In addition, some of these blastoderms were treated with Triton-X 100 (Chapter 2

) in order to examine junctions between the cells of different areas of the blastoderm at these late stages of development.

I have also attempted using the silver nitrate staining method (Chapter 2) to outline cell boundaries of edge and non-edge cells in order to see if there are any changes in cell shape as the blastoderm nears closure.

5.3 Results:

5.3.1 Macroscopic observations: The formation of folds:

The following macroscopic description of the events that take place during the end of chick epiboly is based on Camera Lucida drawings of blastoderms at each stage of development.

5.3.1.1 Stage 18-24 (3 to 4 day old) blastoderms:

After the leading edge has passed the equator of the yolk a unique set of events starts to take place in the blastoderm edge. It starts with the appearance of several points at which the leading edge cells stop their centrifugal movement. These points appear to be distributed around the edge in no specific pattern and their position differs from one blastoderm to another at the same stage of development (Fig.5.1 a&b).

While edge cells stop movement at these fixed points, the other edge cells between the two points continue their migration. As a result, the blastoderm edge changes from a smooth circle around the yolk above or at the level of the equator to crescent-shaped areas as it passes below it. This is apparent at around 3.5 days of incubation, but can also be seen shortly before then and even as early as the 3rd day of incubation as, sometimes, the edge reaches the equator very early, giving an opportunity for the early appearance of the stationary points. It seems to me that these stationary points first appear soon after the edge passes the equator, irrespective of Hamburger and Hamilton (1951) stage of development or of the incubation time i.e. embryos differ in the

stage/time at which their edges pass the equator, but passing the equator is when the stationary areas occur.

As the moving edge advances further, more edge cells neighbouring the stationary points are added to stationary regions and by the 4th day of incubation the whole of the blastoderm edge becomes transformed into a series of U or V-shaped folds where the side lines are streaks of stationary cells while the bottom curve is the actively moving part (Fig.5.1&2). The length of some of these streaks of stationary cells can be in excess of 1 cm and their average width is 0.2 mm (Fig.5.2a).

As a result of the continuous pulling of the blastoderm by the advancing edge cells, the ectoderm to which the first (oldest) stationary cells are attaching folds over them creating a cylinder-like structure at the most proximal area of the stationary streak. One such fold has been reconstructed from Camera Lucida drawings of serial sections and is shown in Fig.5.3.

I attempted to do some quantitative analysis on the width and length of the folds at this stage of development,as well as on later stages, but was faced with the problem that these folds are so variable that it was impossible to draw a clear picture which would be representative of them at any particular stage. In other words, after the edge passes the equator of the egg, one would find that while some of the folds are at a very advanced stage, others, in the same blastoderm, are just starting to appear, leading to the formation of sub-folds and giving some of the folds a W-shaped appearance (Figs.5.1&2). Due to this diversity in the length and complexity of these folds and to the differences in the area of the vitelline membrane still to be covered by the blastoderms within the same stage of development, I show in the figures for each stage of development drawings taken from two different blastoderms.

5.3.1.2 Stage 27-31 (5-7 day old) blastoderms:

The general appearance of blastoderms is similar to those at the earlier

stages with respect to the complexity and diversity of the shape of the folds and the extent of the area covered by the blastoderm (Figs.5.4,5.5 and 5.6). Just to add to the variability of the system, I found that the area covered by the blastoderms at these stages is less in some cases than that covered by some 4 day old blastoderms (Compare Fig.5.2a with Fig.5.4, Fig.5.5 and Fig.5.6).

5.3.1.3 Stage 34-39 (8-13 day old) blastoderms:

Again, there is no specific pattern in the shape or complexity of the folds and the area covered at these late stages. The latter is variable between blastoderms of different as well as of the same stages of development. For example, while the 8 day old blastoderm shown in Fig.5.7a has covered all but a small area of the vitelline membrane, the other 8 day blastoderm shown in Fig.5.7b has still to cover an area comparable to that to be covered by the 7 day old blastoderm shown in Fig.5.6b and even larger than that which is to be covered by the 4 day blastoderm shown in Fig.5.2a.

The first time I came across a total closure of the blastoderm was in the 10 day blastoderm shown in Fig.5.9b where the leading edge cells from all directions meet and seal the remaining area. However, many 10 day blastoderms such as that shown in Fig.5.9a were still nowhere near total closure. This situation also applies to 11 and 12 day old blastoderms, where some have completely encompassed the yolk while others still have some area left to cover (Fig.5.10 and Fig.5.11).

One observation worth mentioning here is that by the 12th day of incubation the blood vessels of the expanding *area vasculosa* have reached the proximal edges of the folds i.e. the points where the first stationary cells appeared. Eventually, by the 13th day of incubation, the *area vasculosa* surrounds the whole of the yolk, leading to the completion of the yolk sac. At these very late stages there are no traces of the stationary folds or of the leading edge.

5.3.2 Microscopic observations:

I have divided this section into three broad stage groups in which the appearance and behaviour of the different blastoderm cells in each group seem to be similar, and also on the basis of whether or not it was feasible to examine the blastoderms of each group under light, transmission and/or scanning electron microscopes. The first group includes 3-4 day old blastoderms which were examined under all these methods. The second group includes blastoderms which were 5-7 day old and which were examined under light and scanning, but not transmission electron microscopes. The third group includes 8-12 day blastoderms which were mainly examined under the scanning electron microscope and occasionally under the light microscope. The factors involved in restricting the examination to one microscope or another will be discussed later.

5.3.2.1 Stage 18-24 (3 to 4 day old) blastoderms:

Silver nitrate staining of 3 and 4 day old blastoderms showed that the cells of those parts of the blastoderm which form the ectodermal layer between each two stationary streaks, i.e. those in the U or V-shaped folds of the blastoderm, are elongated with their long axis parallel to the direction of blastoderm expansion (Fig.5.12a and Fig.5.13a (SEM)). On the other hand, the ectodermal cells which occupy areas located some distance from the stationary streaks have a polygonal shape (Fig. 5.12b). In some long stationary streaks, the ectodermal sheet which is attached to the cells which have been stationary the longest fold over them forming a cylinder-like structure (Fig.5.3 & Fig. 5.13b).

Some 4 day old blastoderms were detached from their vitelline membrane in order to study the dorsal surface of the stationary cells and its relation to the membrane. Scanning electron microscopy revealed that the stationary cells form a wavy rather than a smooth sheet on the vitelline

membrane (Fig.5.14a). The areas of the vitelline membrane to which stationary cells attach was found to be wavy or wrinkled in the same way (Fig. 5.14b).

Closer examination of the dorsal surface of the stationary cells under the scanning electron microscope showed that the cells which are in contact with the vitelline membrane possess small projections which make contact with the vitelline membrane. They also have long lateral processes which make contact with neighbouring stationary cells (Fig. 5.15a) and the vitelline membrane. The areas on the vitelline membrane to which the stationary cells attach seem distorted and gaps are apparent in the fibrous meshwork which makes up the inner surface of the membrane. This contrasts with the smooth appearance of the membrane to which no stationary cells are attached and which are used only by the advancing edge cells (Fig. 5.15b).

Sections cut at right angles to the areas of stationary streaks showed that they are 2-3 cells deep and attach from both sides to the thin sheet of ectoderm (Fig. 5.16a). Transmission electron microscopy revealed that the stationary cells are connected to each other only by desmosomes (Fig. 5.16b). No other specialised junctions have been detected. The dorsal surface of these cells was found to send feet-like microvillous projections into the vitelline membrane (Fig. 5.17a). These probably correspond to the large number of projections which are seen above under the scanning electron microscope.

Dense plaques (Fig. 5.17b) have been detected on the dorsal surface of some of the stationary cells which are in direct contact with the substratum. These could well be sites for focal contact between these cells and the vitelline membrane, a situation which is rarely encountered in the actively moving cells at the earlier stages of development i.e. before these cells become stationary.

The moving cells of the edge, on the other hand, were found to transform their original organisation at these stages to being mostly one-cell deep, with the leading lamella of each cell underlapping the cell located distal to it (Fig. 5.18). The width of the leading edge could not be measured accurately at these

later stages of development due to the continuity of the moving edge cells with the stationary streaks at many points along the circumference, but I can safely say that it is in excess of 800 μm. The width is considerably larger in older blastoderms.

The distalmost cells attach to the vitelline membrane via broad leading lamellae. Some of these lamellae end in thin cytoplasmic processes which make contact with both the vitelline membrane and other neighbouring lamellae (Fig. 5.19a).

Occasionally, some of the distalmost cells detach from the main sheet of the edge both as individuals and as groups and disperse on the available space of the vitelline membrane (Fig.5.18a and 5.19b). This, however, was found in only a few cases and is not the general behaviour of the edge cells since in most of the blastoderms that I examined the monolayer of edge cells forms a continuous sheet without any detachment of its distalmost cells.

Transmission electron microscopy showed that the proximal cells of the edge maintain the presence of desmosomes between them (Fig.5.20a). However, desmosomes are almost completely absent between the monolayer of cells of the moving edge (Fig. 5.20b).

Light and transmission electron microscopy revealed the appearance for the first time of cell death among stationary cells. The appearance of cell death does not occur simultaneously but in stages. It appears first among the oldest stationary cells i.e. those which were the first to stop moving (Fig. 5.21a) before spreading distally to the younger stationary cells i.e. those which stopped later (Fig. 5.21b).

The intercellular spaces between the dying stationary edge cells are invaded by long lamellae from the nearby ectodermal cells (Fig. 5.22a).

One observation which is worth mentioning at this stage is that the

ectodermal part of the blastoderm proximal to the stationary edge zones is indirectly adherent to the vitelline membrane. This close association between the ectoderm and the vitelline membrane is not due to the direct attachment of the ectodermal cells to the substratum but is merely due to the unusual presence of albumen between the two structures (Fig. 5.22b). This will be discussed later.

5.3.2.2 Stage 27-31 (5 to 7 day old) blastoderms:

The only major difference between the leading edge cells of this and the previous stage is the extent of cell death. The oldest stationary cells have by this stage completely disappeared and the space they left behind is occupied by the long ectodermal lamellae (Fig. 5.23a). These lamellae not only extend through the space left by the edge cells and occupy it, but also come into direct contact with the vitelline membrane (Fig. 5.23b). This behaviour of ectodermal cells is in contrast with their behaviour during the early stages of epiboly.

Cell death is apparent at this stage even among the cells which lay more distally along the stationary streak i.e. those which are located nearer to the moving part of the original leading edge.

It is worth mentioning here also that the blastoderm is very sticky to the vitelline membrane due to subblastodermic albumen. This albumen was a result of the rupture of the vitelline membrane in the areas proximal to the embryo at about the 4th day of incubation (Romanoff, 1960). The thickness of this albumen, as well as of that which surrounds the outer surface of the vitelline membrane makes it very difficult to get thin sections for examination under the transmission electron microscope, and even those cut for light microscopy show shattering marks as the glass knives go through the tissue blocks. However, I tried separating some of the attached albumen from the outer surface of the vitelline membrane before fixing the specimens but in most cases that led to the breaking of the membrane and even in the few successful cases where no damage to the vitelline membrane occurred it was not possible

to cut thin sections for TEM because of the persistence of the subblastodermic albumen which solidifies after fixation, preventing consistent infiltration of Araldite into the specimens.

5.3.2.3 Stage 34-39 (8 to13 day old) blastoderms:

As I pointed out in the above section, the albumen, either sub-blastodermic or that which surrounds the vitelline membrane, makes it difficult to section the blastoderms at these late stages of development. However, in the very few cases where the surrounding albumen was partially removed, it was possible to notice that the space left by the original stationary leading edge cells has been occupied by the ectodermal cells which contain abnormally large yolk inclusions. For example, by stage 36 (10 days) the ectodermal cells are in contact with the vitelline membrane and spread onto it, resulting in a great degree of deformation of the membrane, in the shape of wrinkling at the site of attachment (Fig. 5.24a) Such deformations of the vitelline membrane are much less pronounced at areas where no attachment is taking place i.e. at those areas which lay between the original stationary streaks (Fig. 5.24b). The effects of traction that the strong attachment of the ectodermal cells generate as they adhere to the vitelline membrane is evident under the scanning electron microscope (Fig. 5.24c).

Similar deformation of the vitelline membrane occurs in the areas of attachment of the proximal moving edge cells to the membrane. This may be an indication that the expansion of the leading edge cells to cover the rest of the vitelline membrane is carried out by the more distally located edge cells.

One advantage of the thick albumen surrounding the vitelline membrane is that it makes it easy to separate the two layers of the vitelline membrane. By carefully removing the solidified albumen it removes with it the outer layer of the vitelline membrane. This made it possible to examine at least small areas of the outer face of the inner layer of the membrane under the scanning

electron microscope and to notice that the invading ectodermal cells anchor themselves to this surface by microvillous projections similar to those sent previously by the leading edge cells and the stationary cells (Fig. 5.25a).

The expansion of the edge area seems also to depend on the continuous addition of deep layer cells into the edge area as can be deduced from the association of an increasing number of these cells with the leading edge. For example, in the 8 day old blastoderm shown in Fig. 5.7, the deep layer cells seem to leave the basal surface of the ectodermal cells and move over the proximal cells of the edge to be incorporated into the edge (Fig. 5.25b). The deep layer cells persist even after the closure of the remaining area over the vitelline membrane. For example, in the 11 day old blastoderm which is shown in Fig. 5.10b, the deep layer cells seem to form a second layer on top of the monolayer of leading edge cells (Fig. 5.26). I have only been able to see this arrangement under the scanning electron microscope since all attempts made to remove the attached albumen from this area led to disturbances such as the tearing of the vitelline membrane and the separation and dissociation of the edge cells from it.

5.4 Experimental study on the effect of substratum on the behaviour of stationary cells and whether these cells are programmed to undergo cell death at a certain stage of development:

I took advantage of the fact that the stationary cells are highly adhesive to the substratum and that it is possible to get pure cultures of these cells simply by mechanically separating the attached ectodermal sheet from the stationary streaks then separating these streaks from the vitelline membrane. 4 day old stationary streaks were separated in this way then cultured on a fresh vitelline membrane using the New Culture method (New, 1953) while other streaks were cultured on glass coverslips in small petri dishes. Ham's F12 culture medium (Gibco, UK) + 10% serum was used in both types of cultures. The cultures were maintained for 3 to 5 days.

It was hoped to throw light on the following points:

A) Since the stationary cells were removed from older vitelline membranes and cultured on fresh ones, would that trigger these cells to regain the ability to spread on this new substratum? If that is the case, then it can be assumed that it is the older vitelline membranes, which may have somehow lost some of their ability to support cell movement at certain points, which causes these cells to stop moving. If that is not the case, i.e. if they behaved similarly on both old and fresh vitelline membrane, would they spread on glass indicating that they are specifically programmed to stop moving on their normal substratum?

B) The stationary cells,*in vivo*, enter the cycle of cell death at the 4th day of development and disappear completely from the stationary streaks by the 6th day. Culturing these 4 day old cells for 3 to 5 days *in vitro* would show whether these cells are destined to die at that period irrespective of the conditions they are in, or that their death can be altered by some external factors such as the presence or absence of the attached ectoderm and the vitelline membrane.

C)This experiment would also show whether the release of these cells from the tension that they are subjected to by the rest of the moving edge cells would change their behaviour and/or boost their survival.

5.4.1 Results:

The results are divided into two groups according to the substratum used to culture the stationary cells:

5.4.1.1 a) The behaviour of stationary cells on the vitelline membrane:

All of the explants of stationary cells cultured on vitelline membranes attached to them but the cells remained in the streak and were not able to spread even when kept for 5 days. The reason behind this lack of spreading seems to be the fact they that they undergo cell death. Semithin (1 µm) sections of streaks cultured for 3 days showed that cell death among these cells

is very extensive and one can barely recognise any living stationary cell within the streaks (Fig. 5.27). There was no difference in the behaviour of the stationary cells between those cultured on fresh vitelline membranes and old ones.

5.4.1.2 b) The behaviour of the stationary cells on glass:

The behaviour of the stationary cells which were cultured on glass coverslips was found to be completely different from above. The stationary cells attached to the coverslip and started to spread on it as early as the first day of culture. They spread from all sides of the explant forming a monolayer which, by the end of the 3rd day, extended a considerable distance away from the site of the explant (Fig. 5.28a). The stationary cells not only attached to and spread on their new substratum but they also looked very healthy with no sign of cell death even after 5 days in culture (Fig. 5.28b).

Fig. 5.1- Camera lucida drawings of two 3-day old blastoderms after passing the equator of the egg. Notice the appearance of stationary points as well as stationary streaks with no specific pattern. The area of the vitelline membrane yet to be covered by the blastoderm is 439 mm2 in (a) and 534 mm2 in (b). Bar= 1 cm.

Strippled areas here and in all of the following camera lucida drawings represent the position of the moving edge cells.

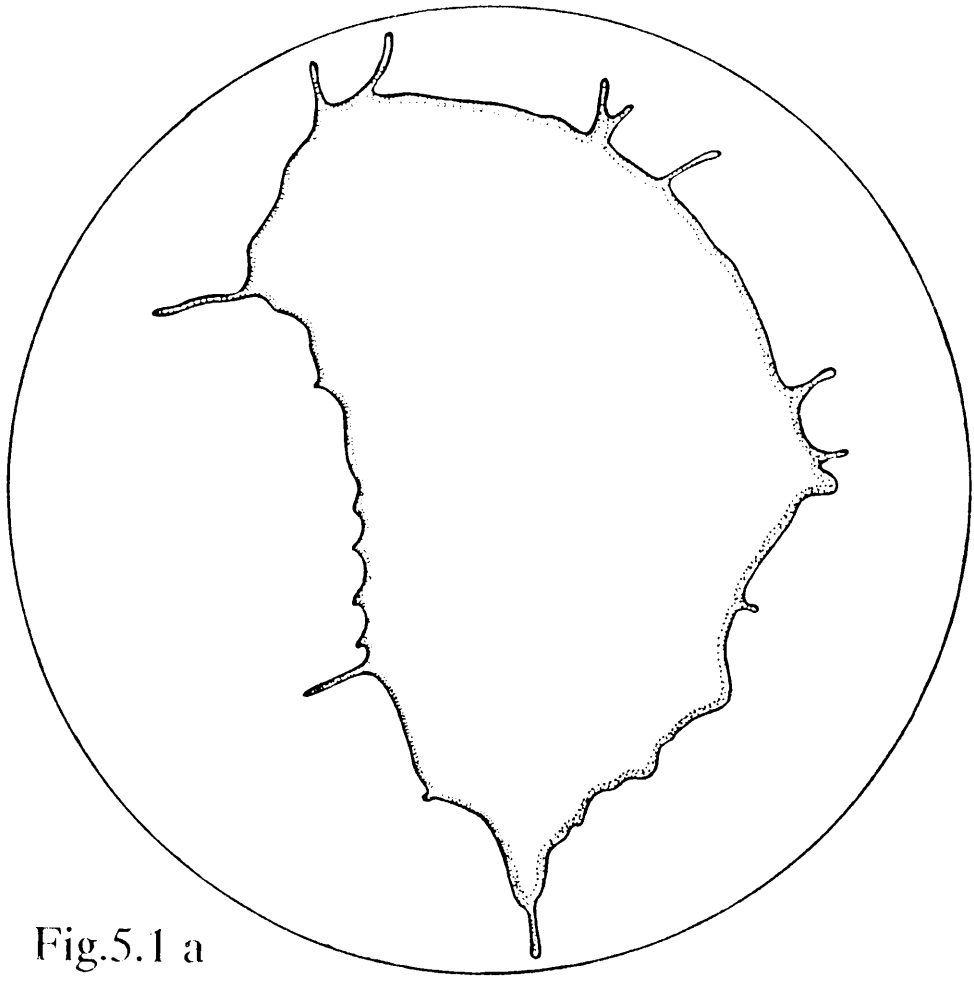

Fig.5.1 a

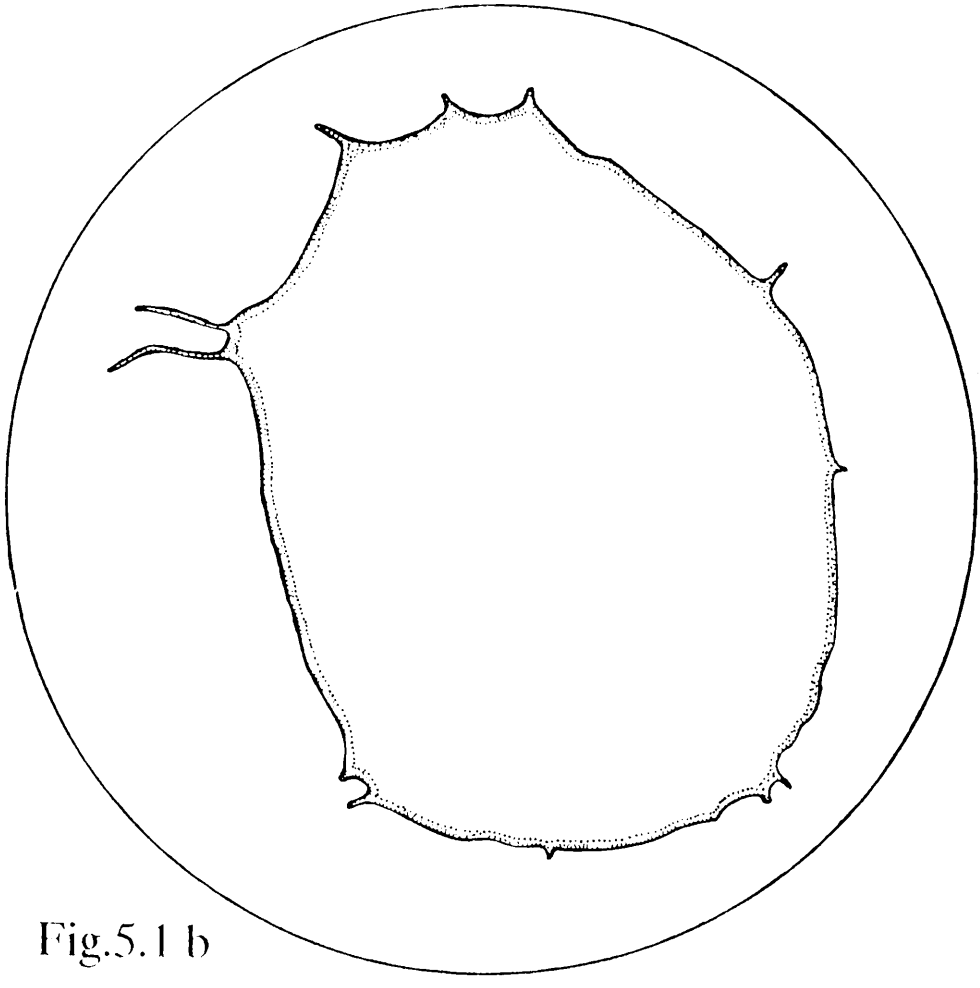

Fig.5.1 b

Fig. 5.2- Camera lucida drawings of two 4-day old blastoderms. The length and complexity of the stationary streaks differ from one blastoderm to the other as well as within the same blastoderm. There is also a great difference in the extent of expansion between the two blastoderms. Area to be covered in (a) is 34 mm2 and in (b) 272 mm2. Bar= 1 cm.

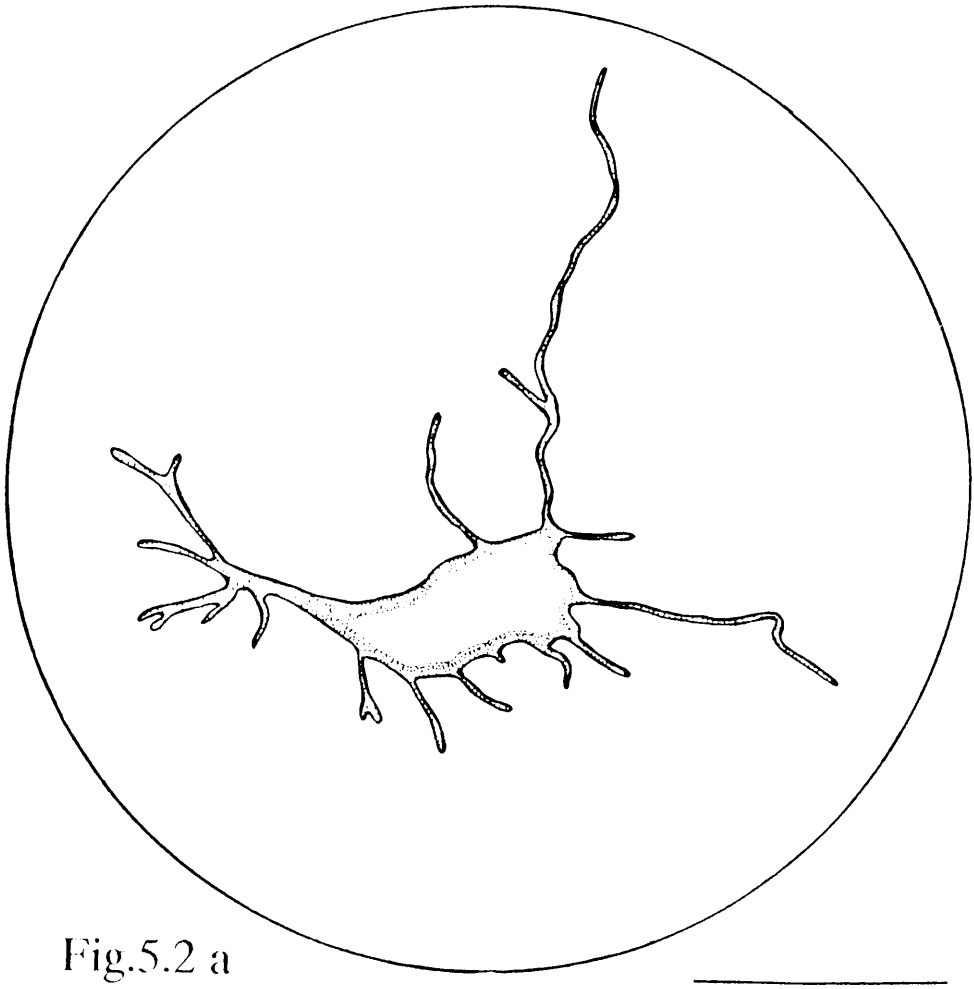

Fig.5.2 a

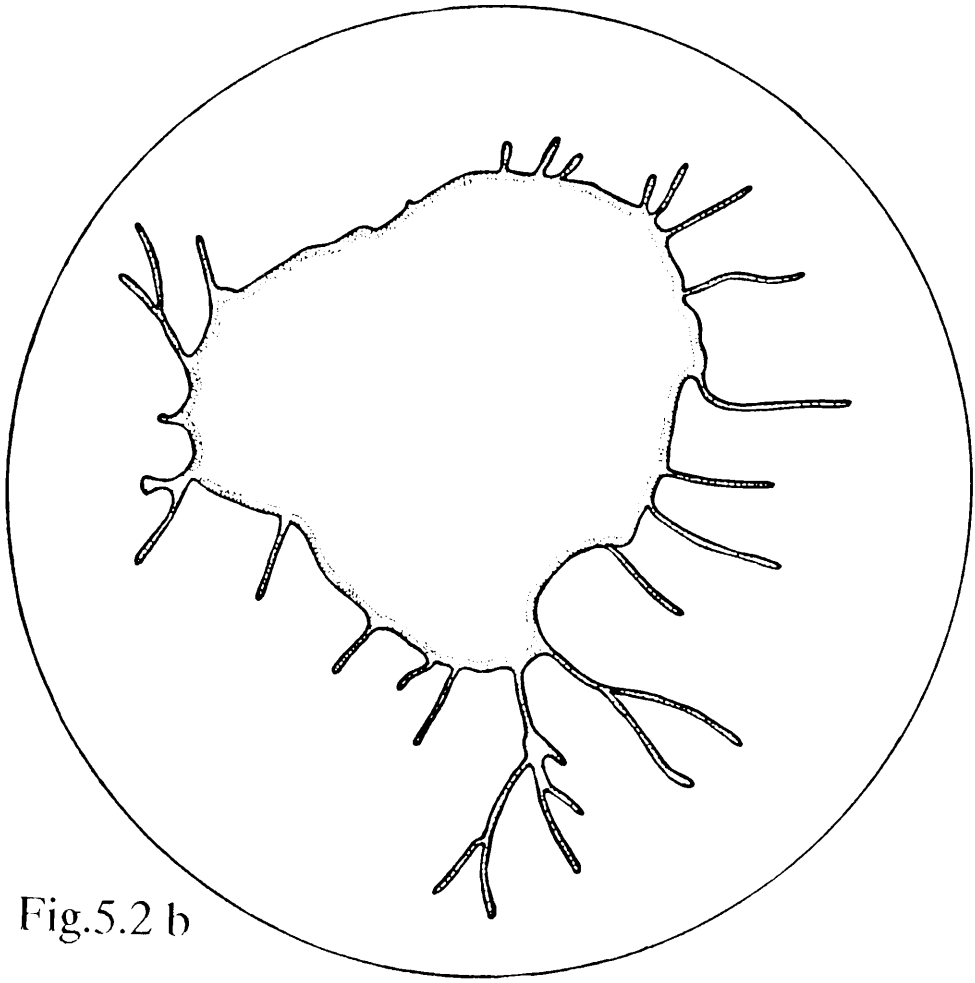

Fig.5.2 b

Fig. 5.3- Camera lucida re-construction of a 4 day stationary streak drawn from frontal sections cut through it. In this case the oldest stationary cells were covered by an ectodermal sheet as it is centrifugally pulled by the actively moving edge cells. E= ectoderm; LE= leading edge; S= stationary streak; V= vitelline membrane.

Fig.5.3

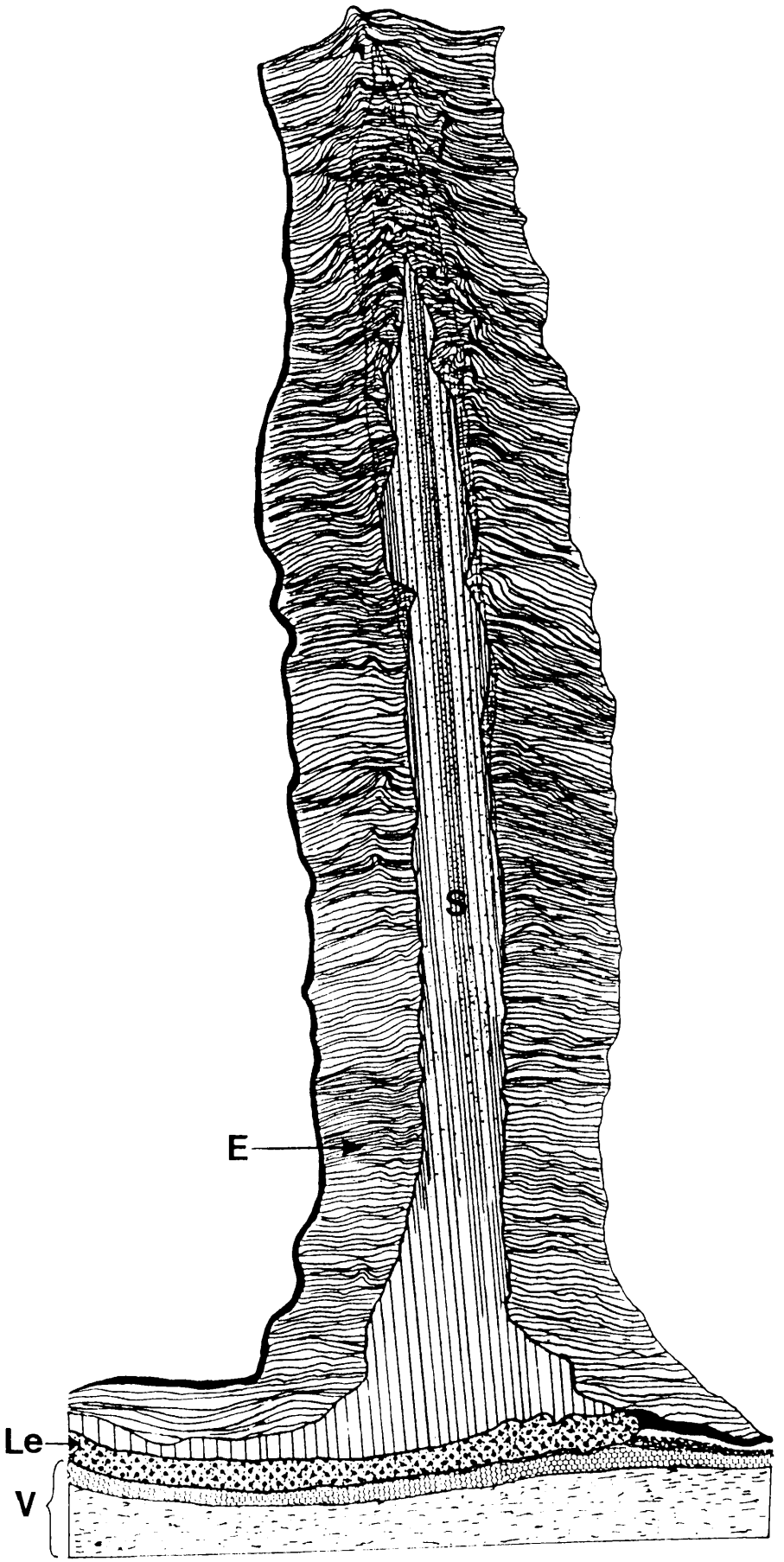

Fig.5.4- Camera lucida drawings of two 5-day old blastoderms showing again a visible difference in the length and complexity of the stationary streaks. Area to be covered in (a) 138 mm2 and in (b) 156 mm2. Bar=1 cm.

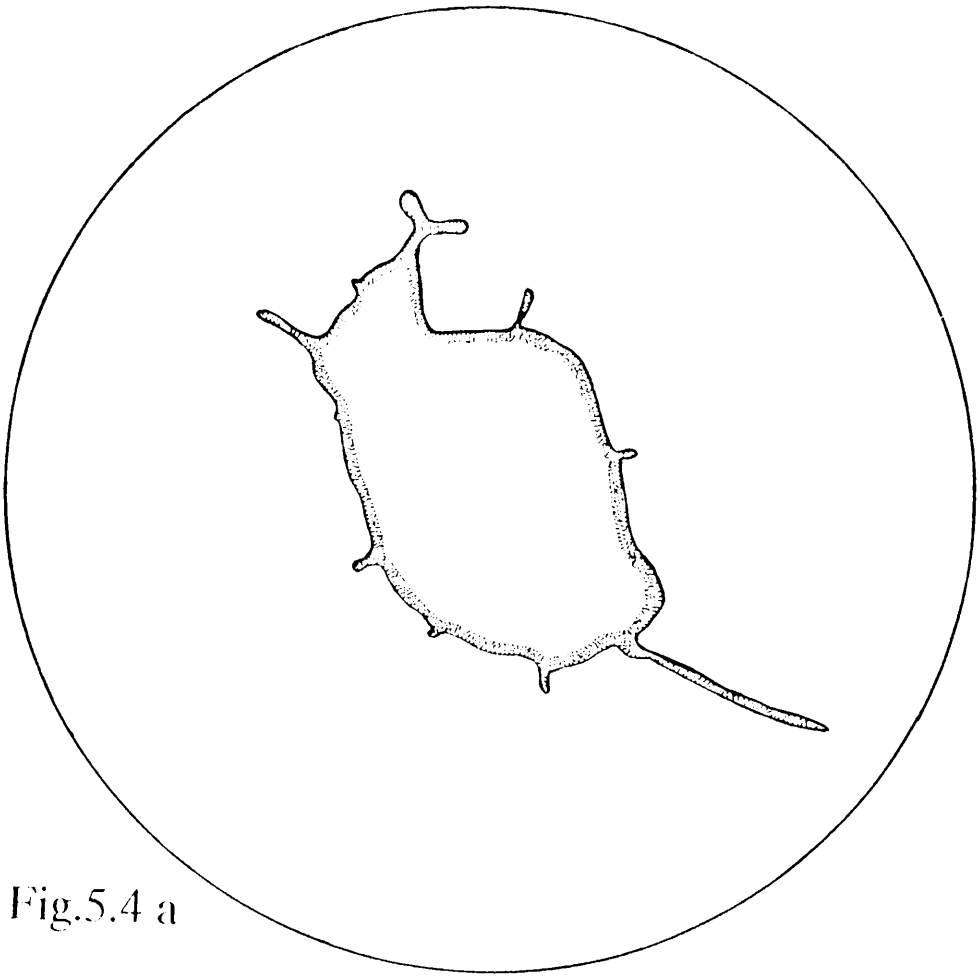

Fig.5.4 a

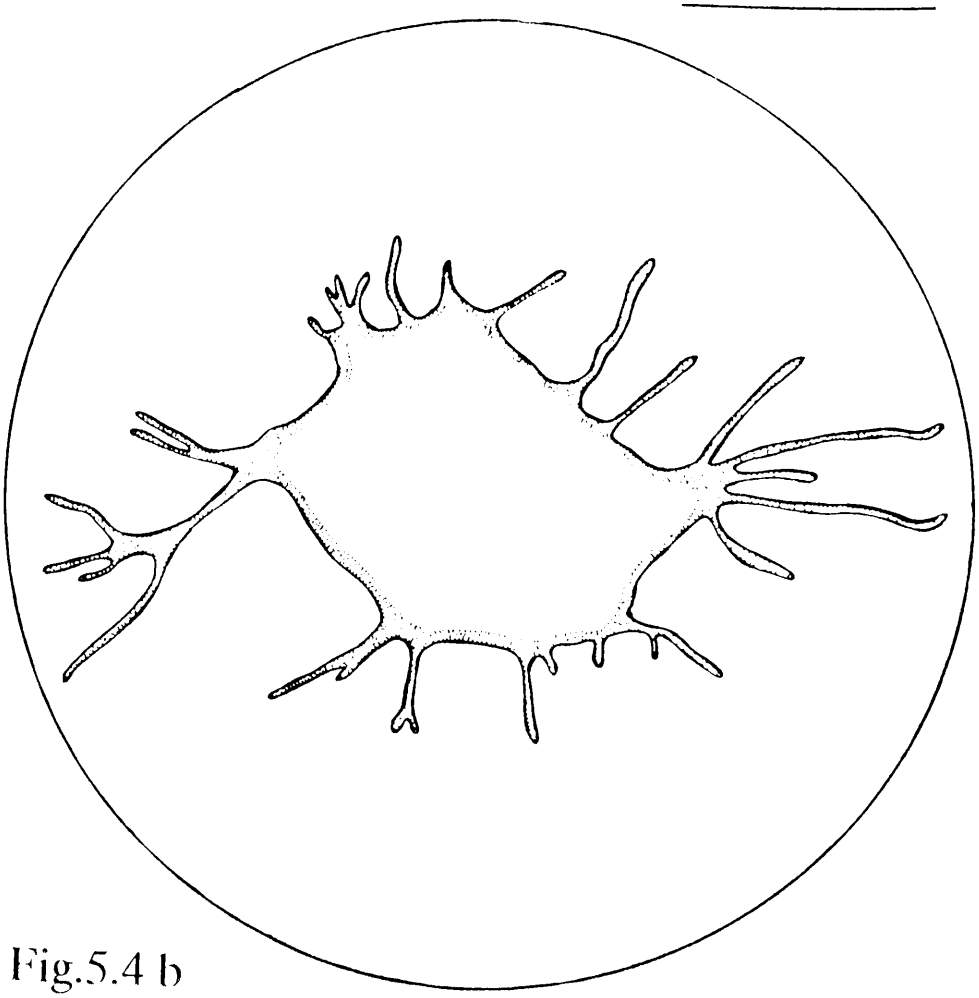

Fig.5.4 b

Fig.5.5- Camera lucida drawings of two 6-day blastoderms. Although areas still to be covered in (a) and (b) are approximately equal (183 and 185 mm2, approximately), stationary streaks show the usual differences. Bar= 1cm.

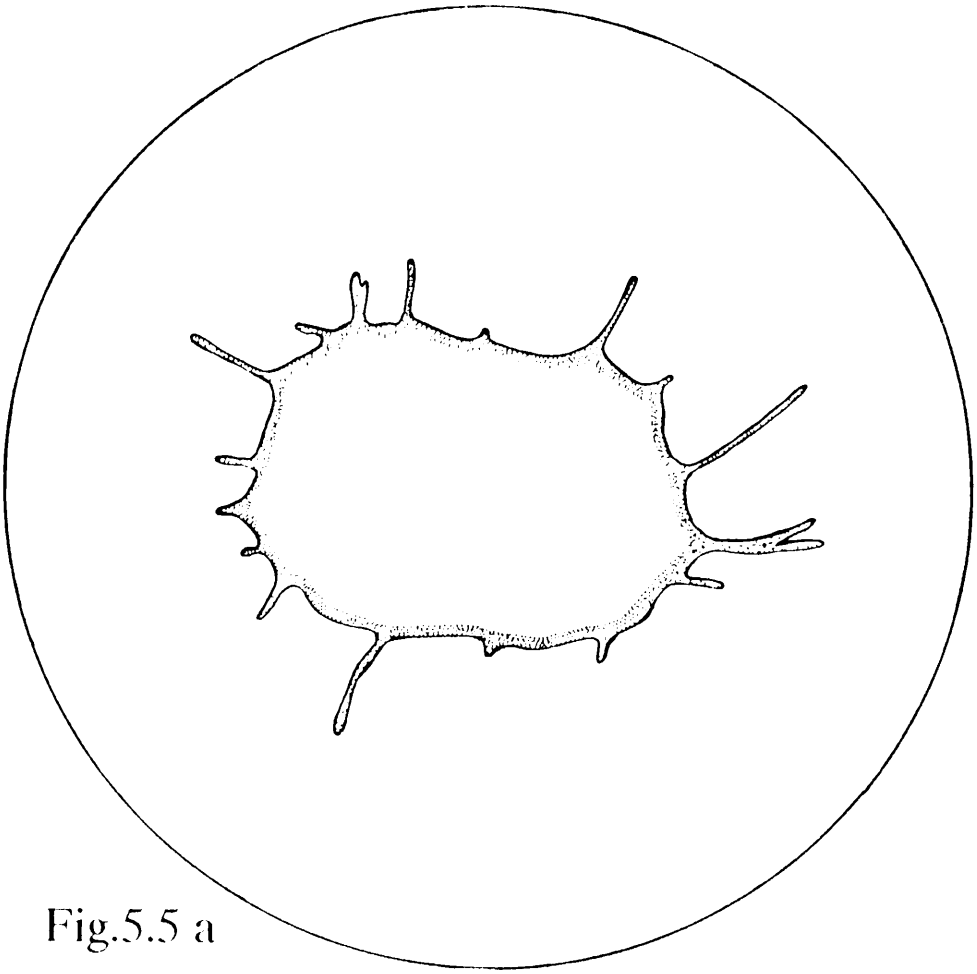

Fig.5.5 a

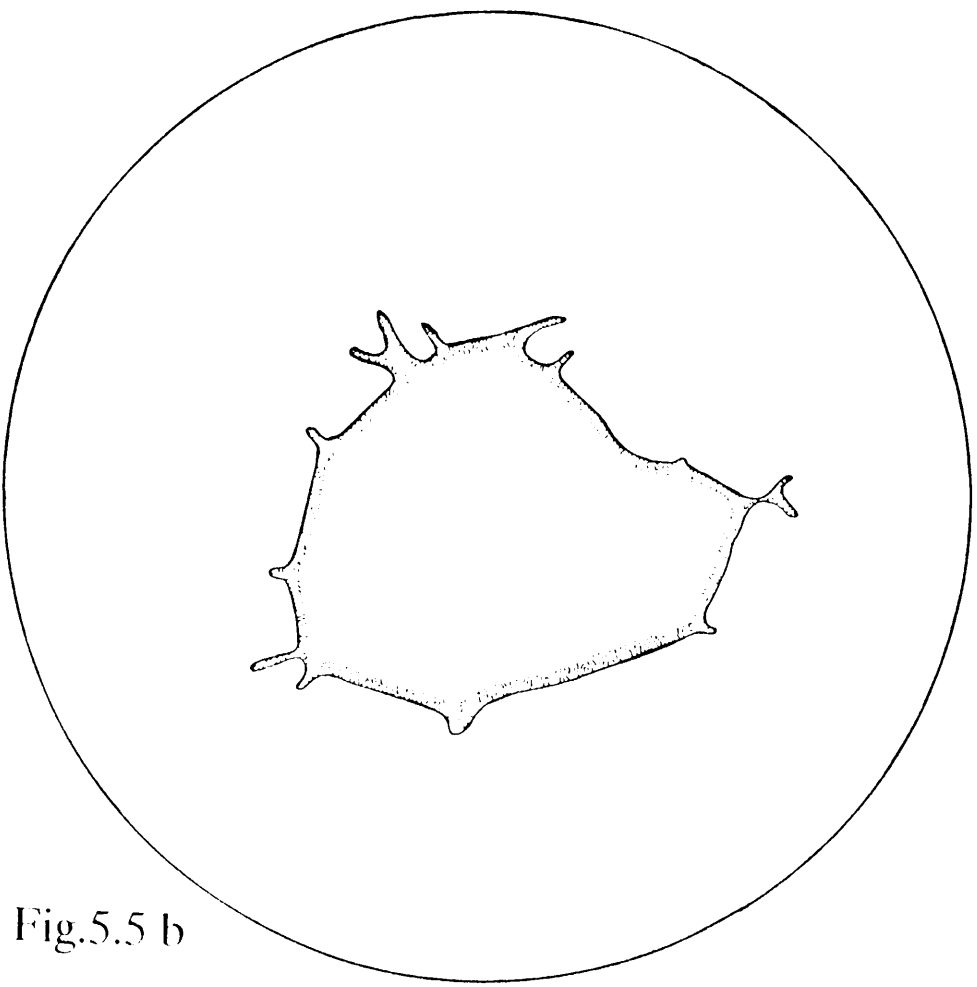

Fig.5.5 b

Fig.5.6- Camera lucida drawings of two 7-day blastoderms. Area still to be covered in (a) is 97 mm2 and in (b) 105 mm2. Bar= 1 cm.

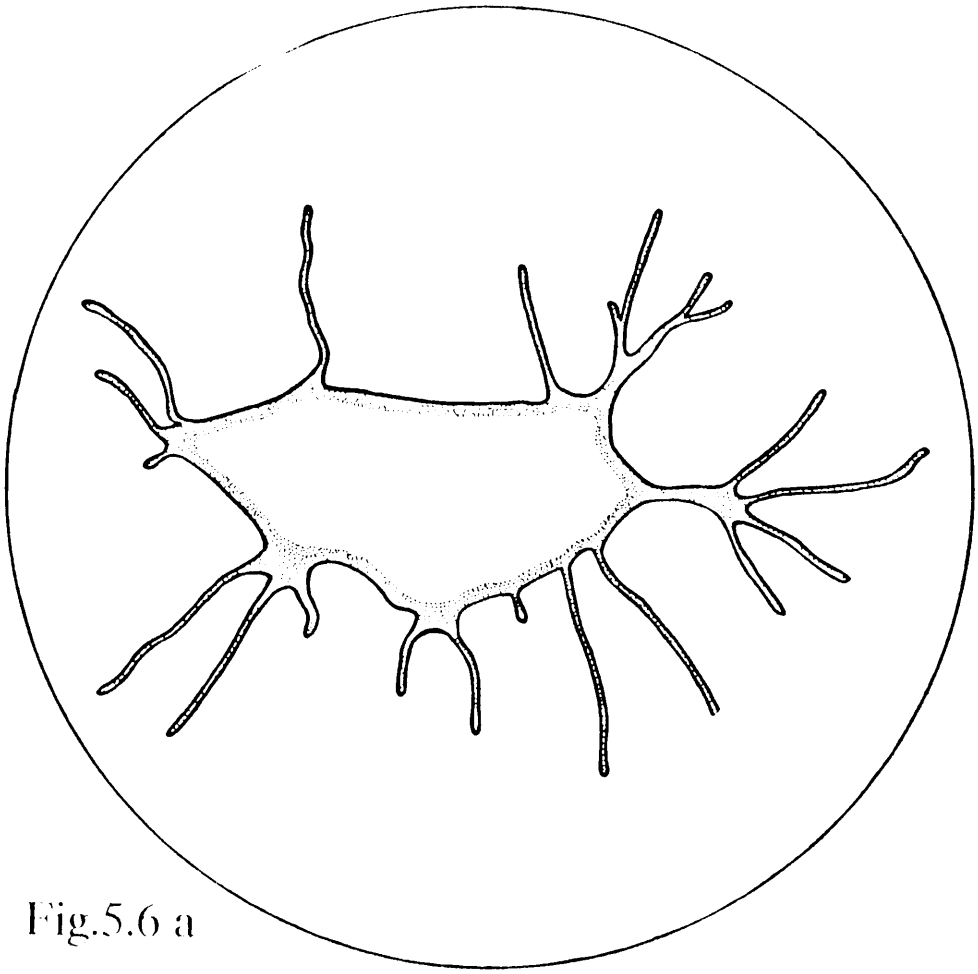

Fig.5.6 a

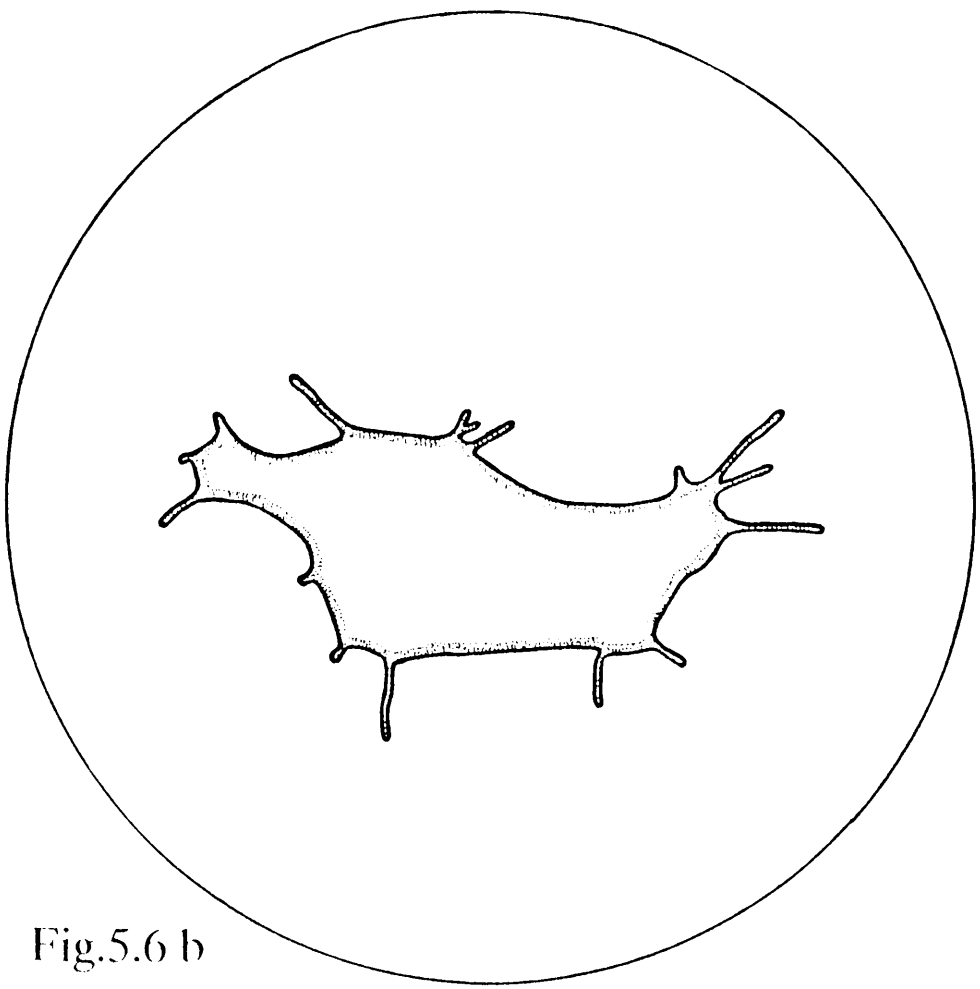

Fig.5.6 b

Fig.5.7- Camera lucida drawings of two 8-day blastoderms. Notice that while the blastoderm in (a) has covered all but a small area of the vitelline membrane (0.6 mm2), that in (b) is still to cover an area of 102 mm2. Bar= 1 cm.

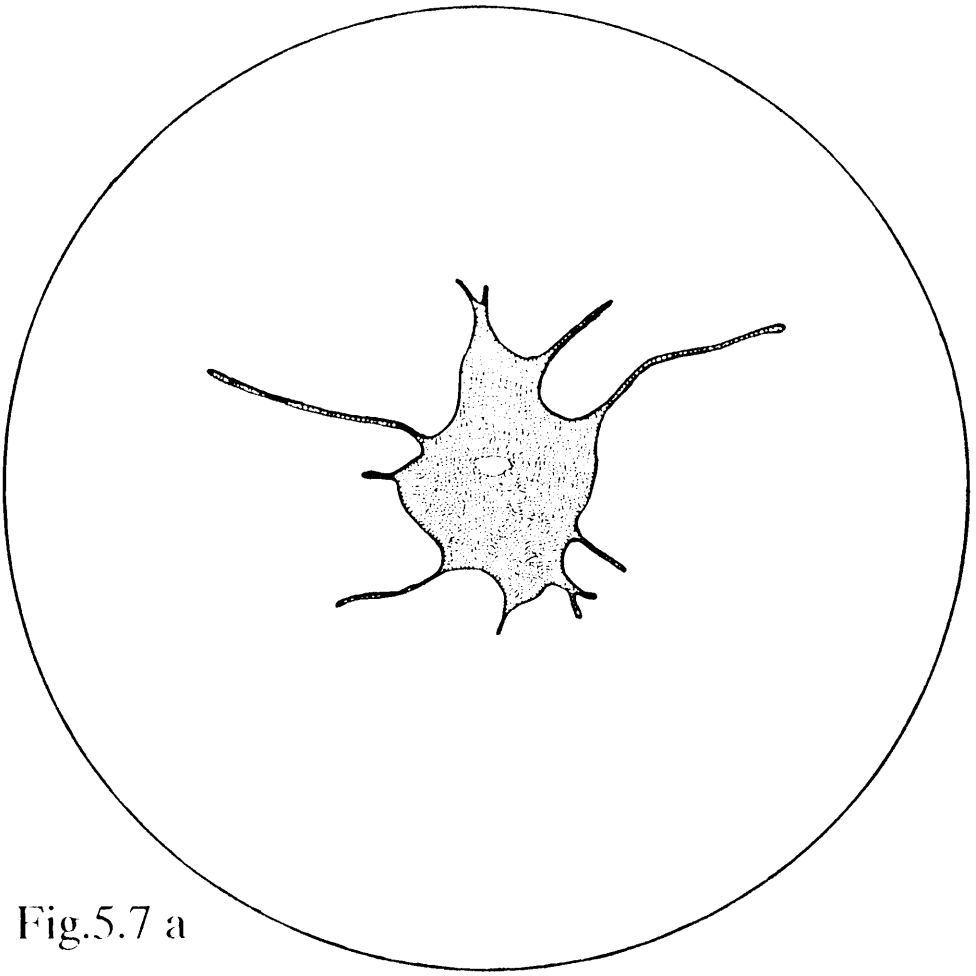

Fig.5.7 a

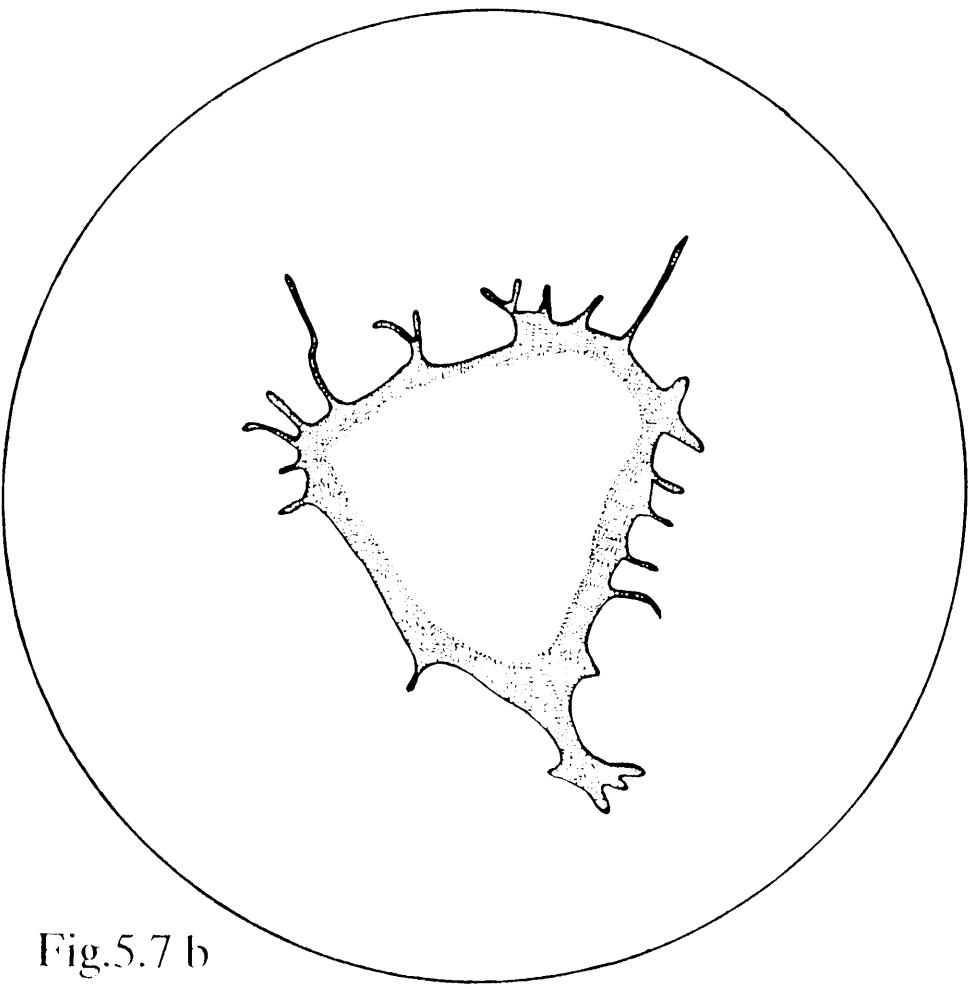

Fig.5.7 b

Fig.5.8- Camera lucida drawings of two 9-day blastoderms. Here again there is a difference between the two blastoderms in the area they have yet to cover (36 mm2 in a and 2.5 mm2 in b). Bar= 1 cm.

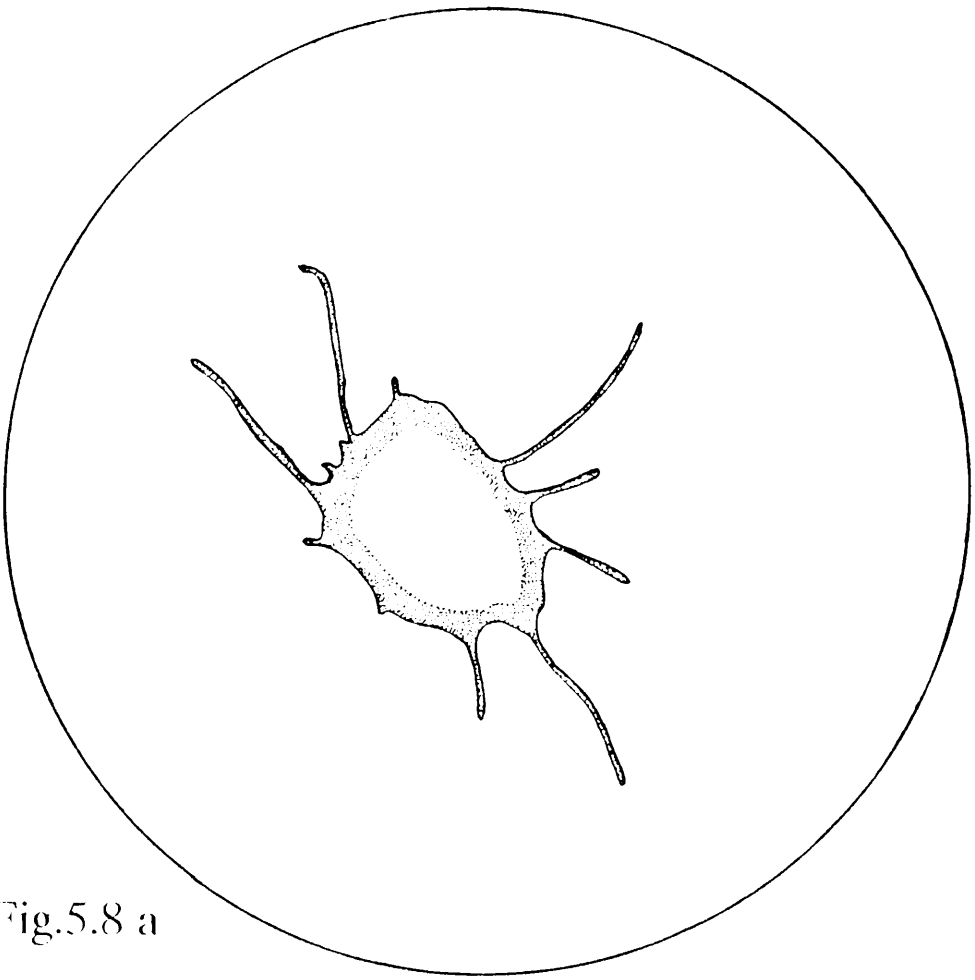

Fig.5.8 a

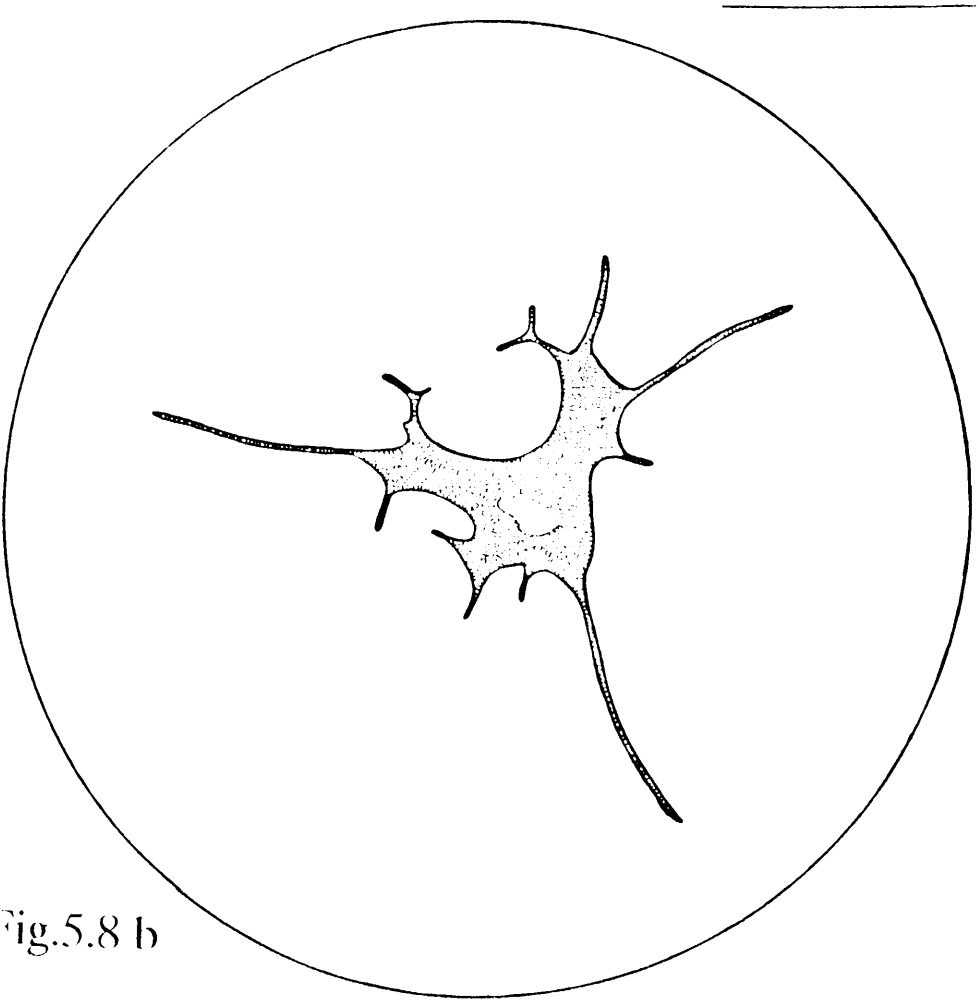

Fig.5.8 b

Fig.5.9- Camera lucida drawings of two 10-day blastoderms. This is the earliest time in which a total closure of the vitelline membrane can be seen (b). The blastoderm in (a) has still to cover an area of 101 mm2. The blastoderm in (b) has achieved total closure. Bar= 1 cm.

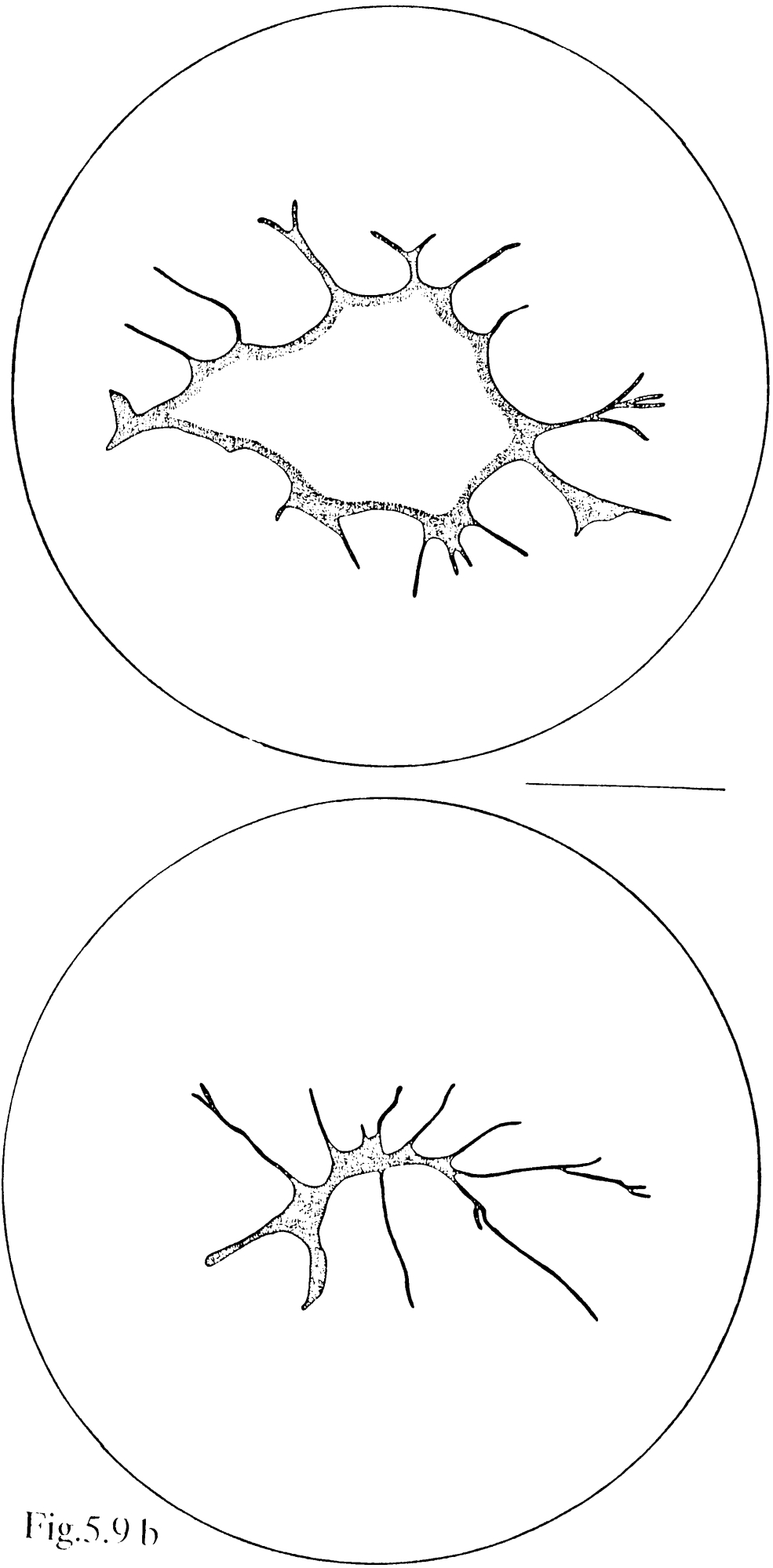

Fig.5.9 b

Fig.5.10- Camera lucida drawings of two 11-day blastoderms. While the blastoderm shown in (b) has sealed the vitelline membrane, the one in (a) is still to cover an area of 43 mm2. This area is larger than that found in the 4-day blastoderm shown in figure 2a. Bar= 1 cm.

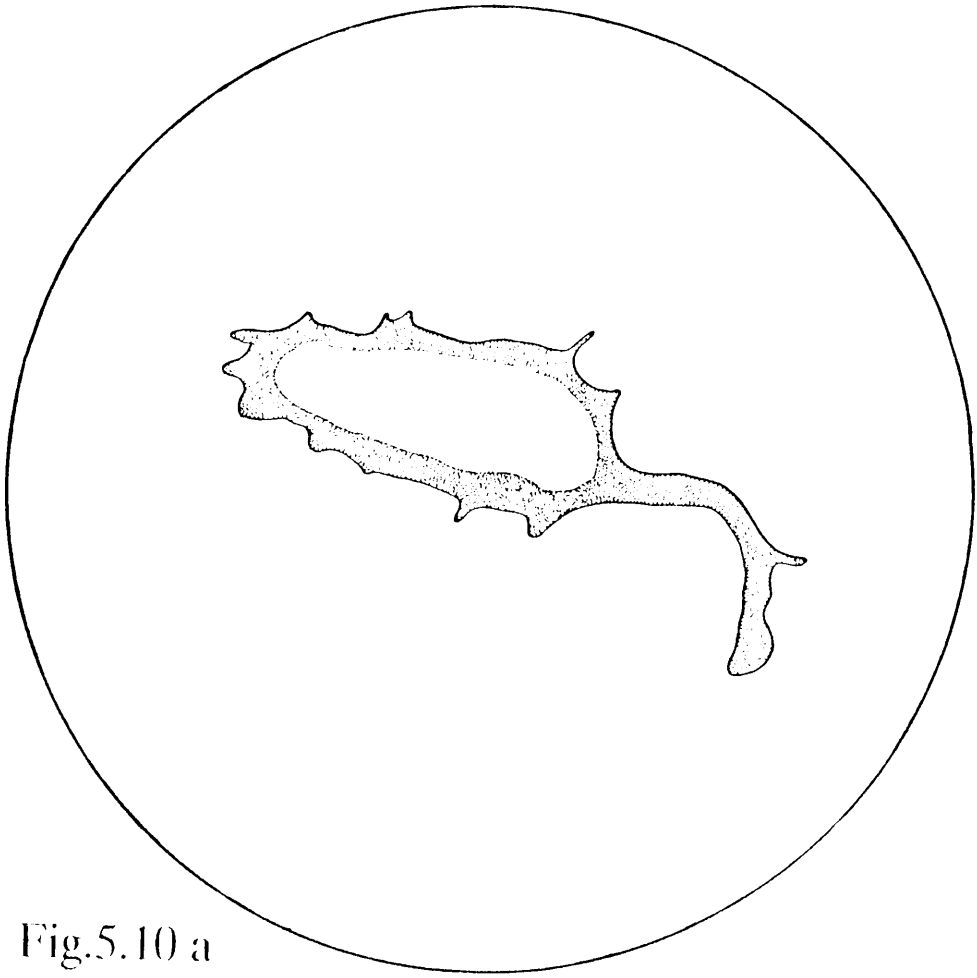

Fig.5.10 a

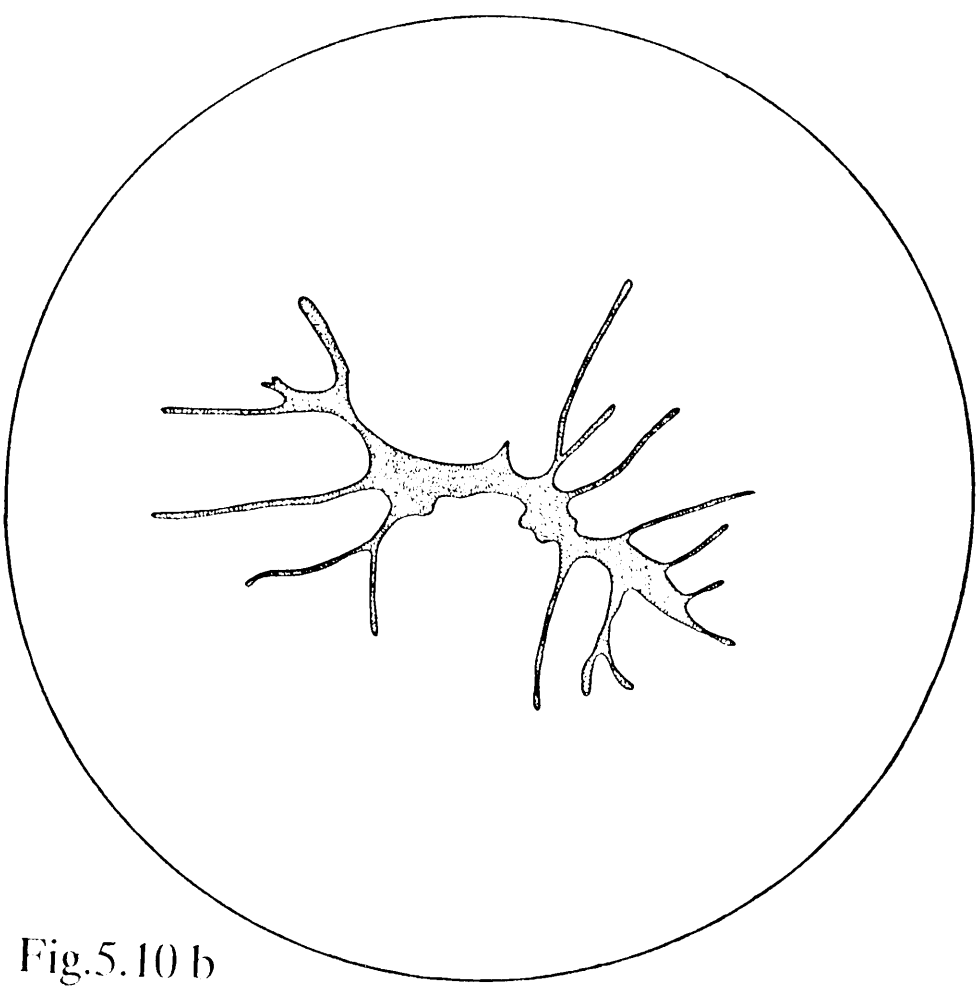

Fig.5.10 b

Fig.5.11- Camera lucida drawings of two 12-day blastoderms. Area to be covered in (a) is 0.4 mm2. Bar= 1 cm.

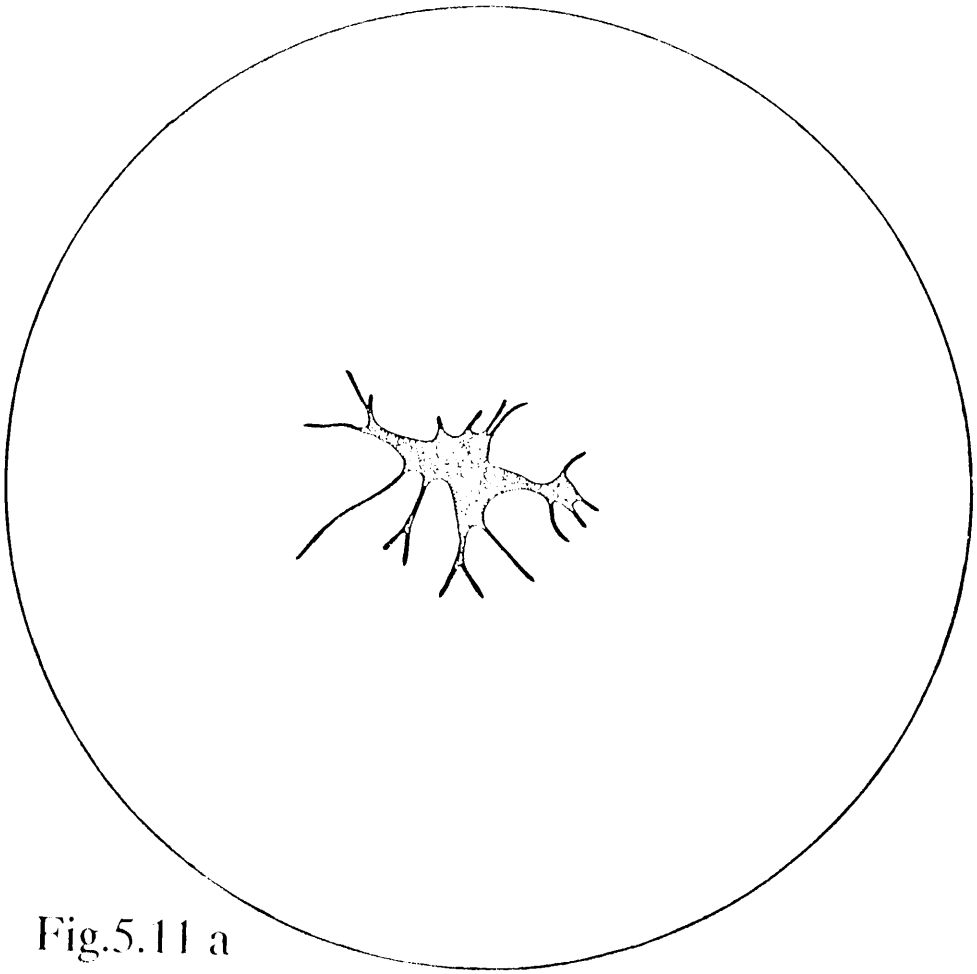

Fig.5.11 a

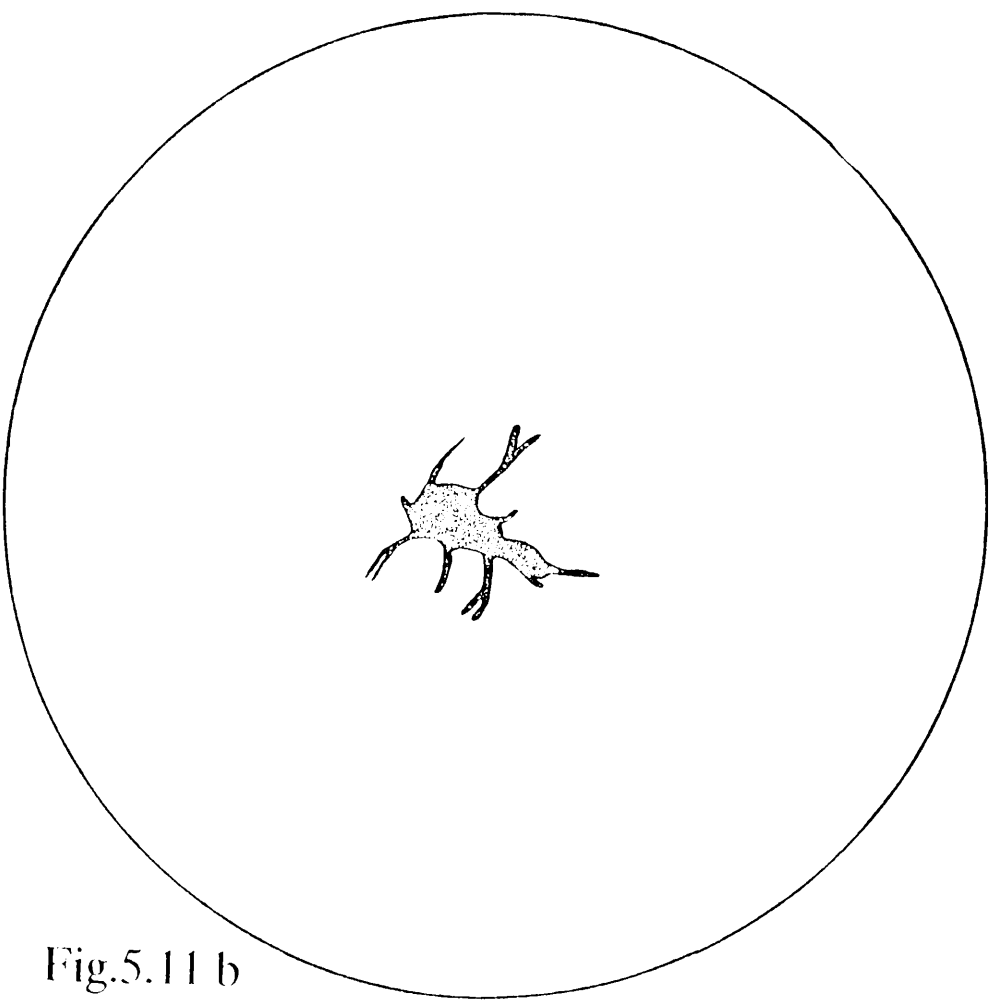

Fig.5.11 b

Fig.5.12 a- Silver nitrate staining of a 3-day old blastoderm at one of the stationary streaks (S). The ectodermal cells (E) adjacent to the streak are elongated with their long axis parallel to streak. Bar= 100 μm.

Fig.5.12 b- Silver nitrate staining of the same blastoderm as in (b) but at an area far from any stationary streak. The ectodermal cells (E) show no particular orientation and maintain their polygonal appearance. Bar= 50 μm.

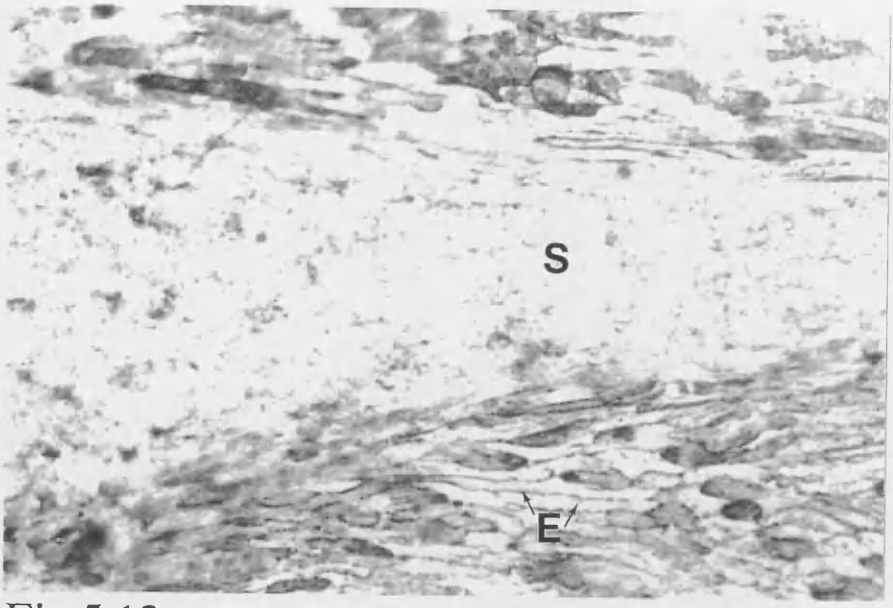

Fig.5.12 a

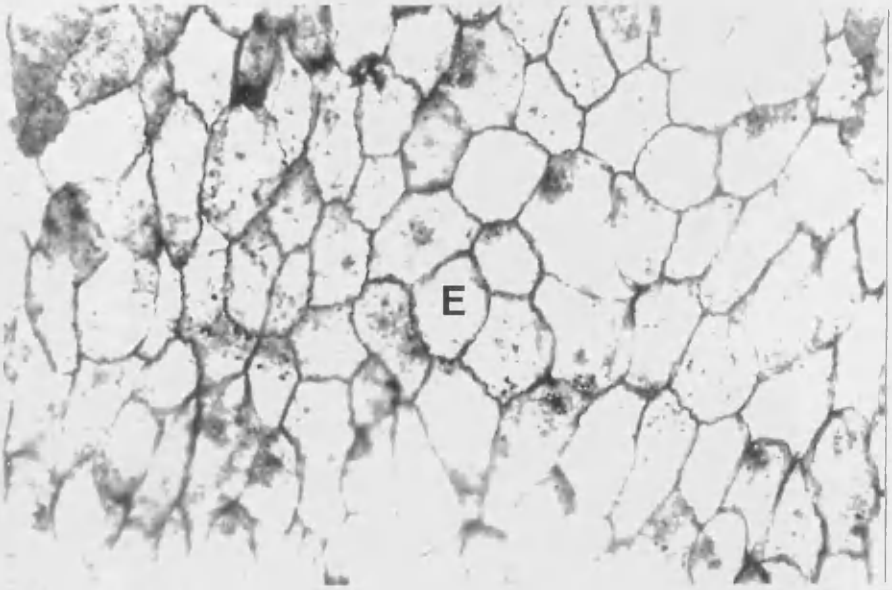

Fig.5.12 b

Fig.5.13a- Scanning electron micrograph (SEM) of an area similar to the one shown in Fig.5.12a showing the orientation of the ectodermal cells (E) near a stationary streak (S). Bar= 100 µm.

Fig.5.13 b- Silver nitrate stained stationary streak showing a cylinder-like structure formed by the folding of the ectoderm (E) over the oldest stationary cells (EF). This is similar to the one shown in Fig. 5.3. S= stationary streak. Bar= 100 µm.

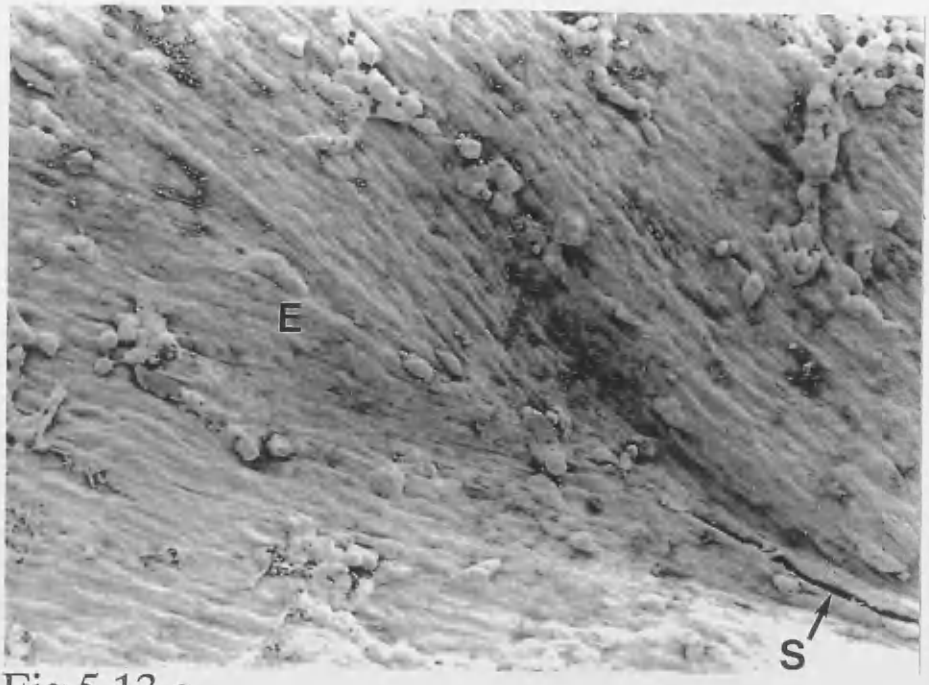

Fig.5.13 a

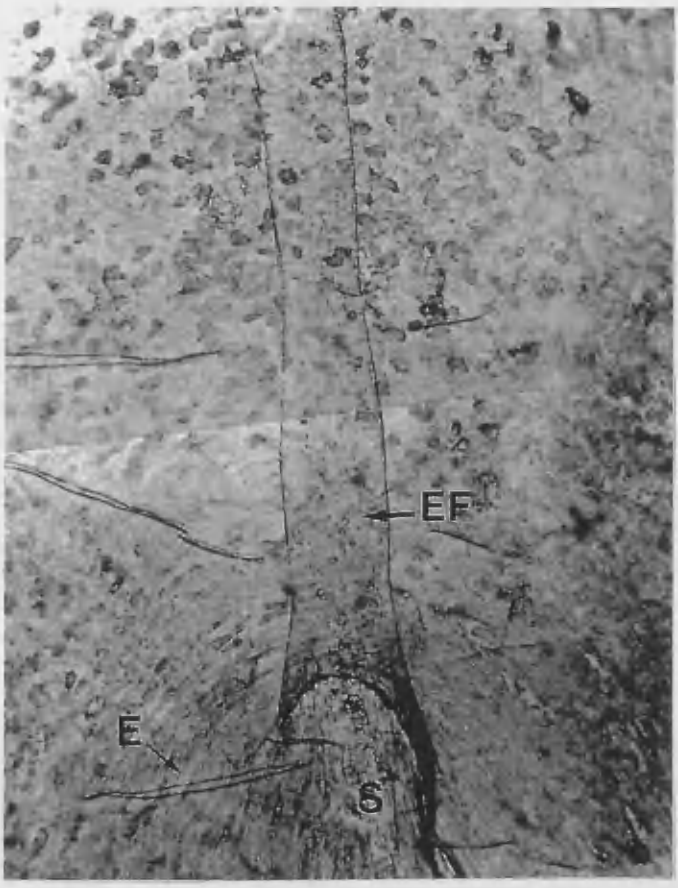

Fig.5.13 b

Fig.5.14 a- SEM of the basal surface of two stationary streaks (S) separated from the substratum, the vitelline membrane. This surface has a wavy appearance. E= ectoderm; arrows show direction of blastoderm expansion. Bar= 100 μm.

Fig.5.14 b- SEM of the inner surface of the vitelline membrane to which stationary cells used to attach. Notice the wrinkling of the membrane (asters) in a way which corresponds to the wavy appearance of the stationary streaks. The surrounding areas which have been used only by actively moving edge cells show no such wrinkling. Bar= 100 μm.

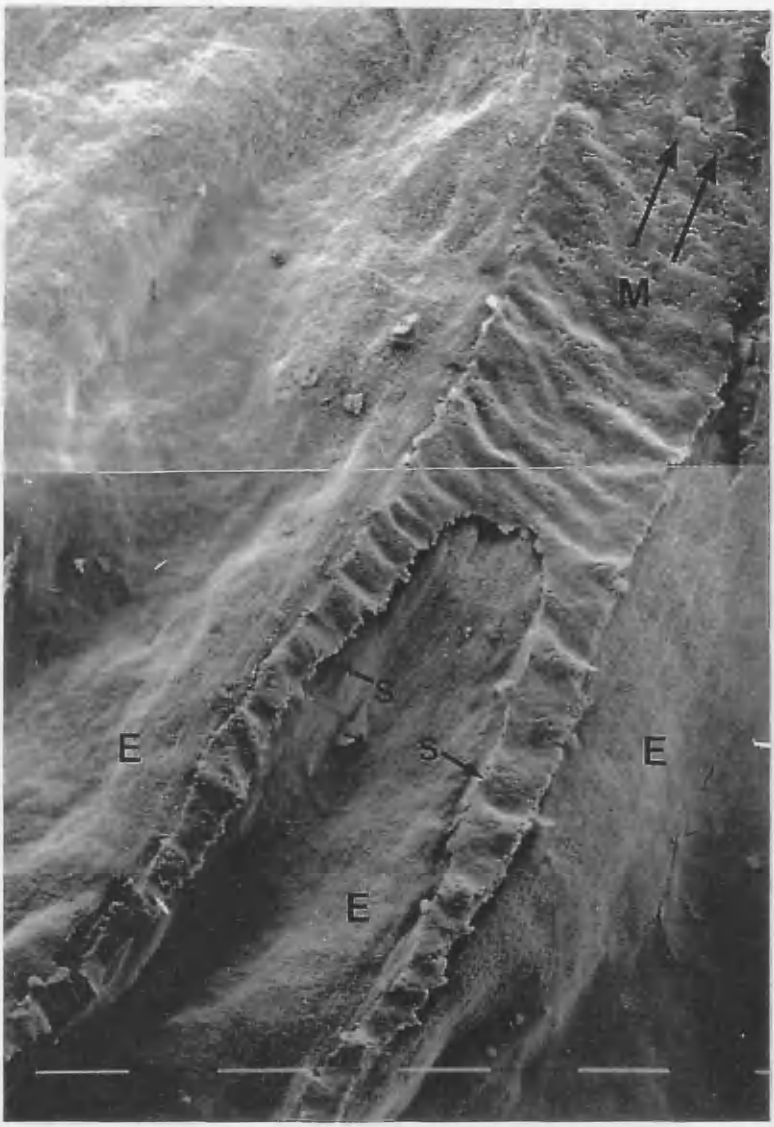

Fig.5.14 a

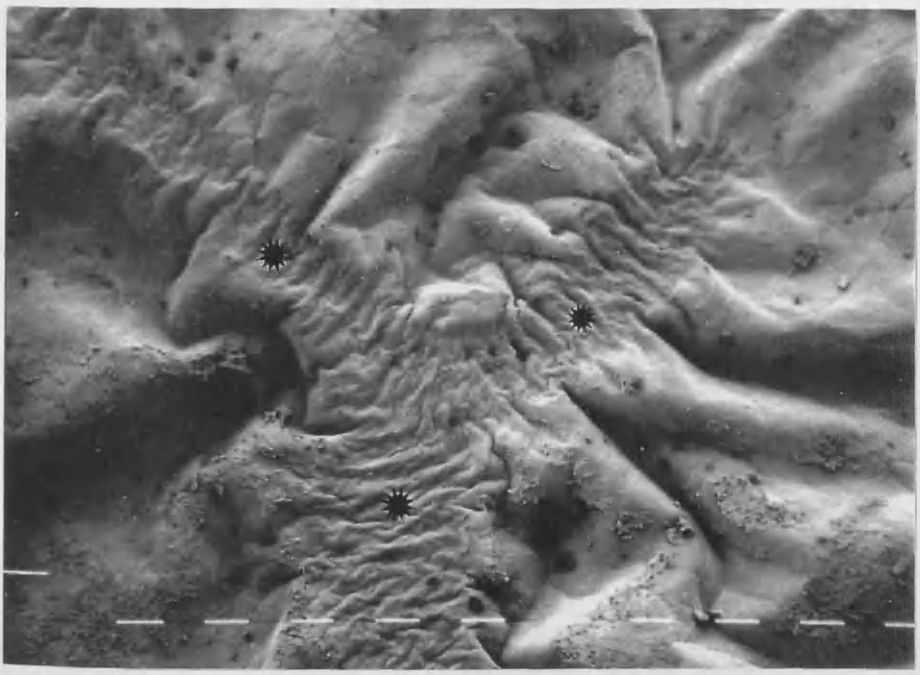
Fig.5.14 b

Fig.15 a- SEM showing the basal surface of some stationary cells (S). A few microvillous projections (arrowheads) are visible on this surface. These cells make contact with each other via thin cellular processes (arrows). Bar= 1 µm.

Fig.5.15 b- SEM showing some deformation in the fibres of the inner surface of the vitelline membrane caused by the attachment of the stationary cells (S). The left hand side of the micrograph (M) show less deformation as no stationary cells have attached to it. Bar= 10 µm.

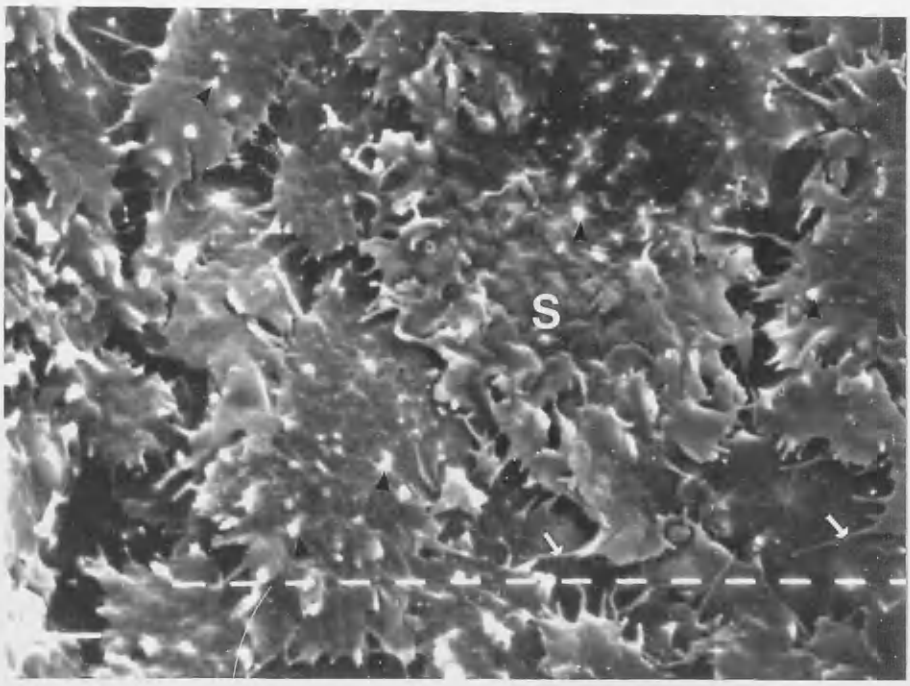

Fig.5.15 a

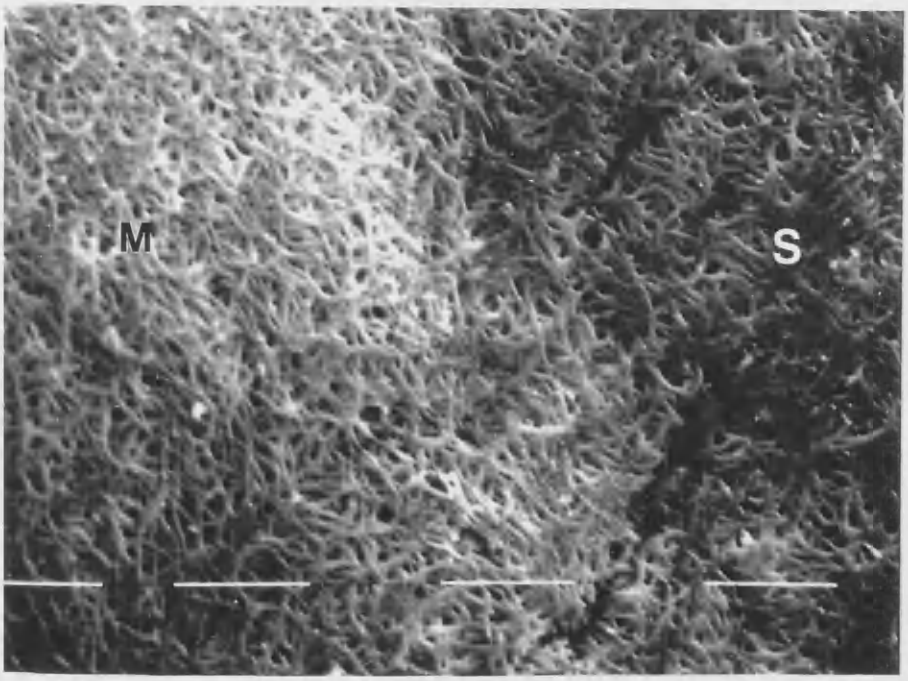

Fig.5.15 b

Fig.5.16 a- Light micrograph of one of the stationary streaks of a 3-day old blastoderm. E= ectoderm; SC= stationary cells; V= vitelline membrane. Bar= 50 μm.

Fig.5.16 b- Transmission electron micrograph (TEM) showing desmosomes (D) connecting adjacent stationary cells (Sc). Bar= 0.97 μm. X32 K.

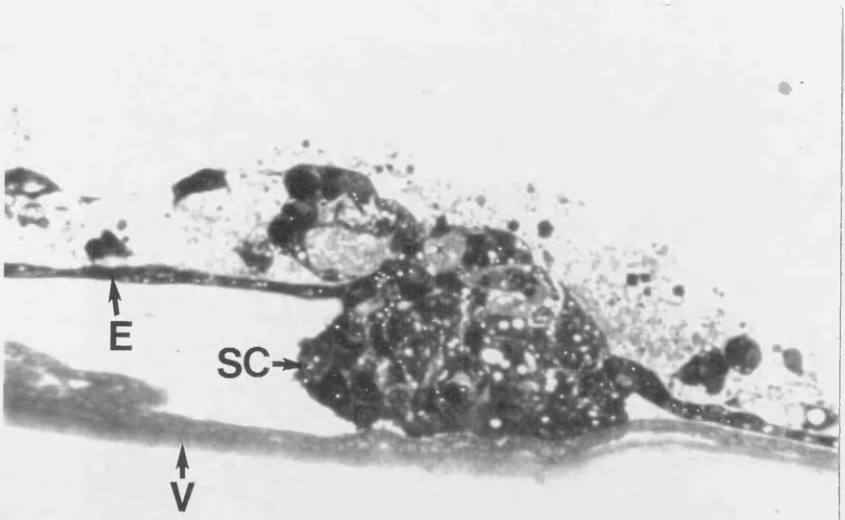

Fig.5.16 a

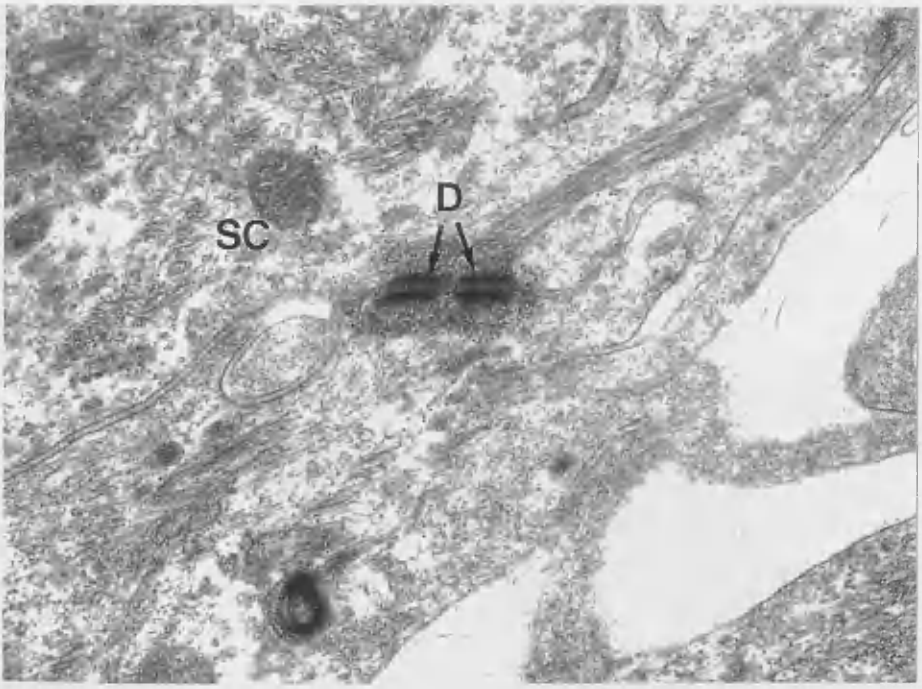

Fig.5.16 b

Fig.5.17 a- TEM of 3-day old blastoderm showing microvillous projections (MP) protruding from the basal surface of the stationary cells and penetrating deeply into the vitelline membrane surface (V). Notice that microfilaments (arrowheads) extend into these projections. Bar= 0.73 µm. X 23.5K.

Fig.5.17 b- TEM of a 3-day old stationary streak cell (SC) showing a dense plaque (DP) in point of contact with the vitelline membrane. Bar= 0.17 µm. X 100K.

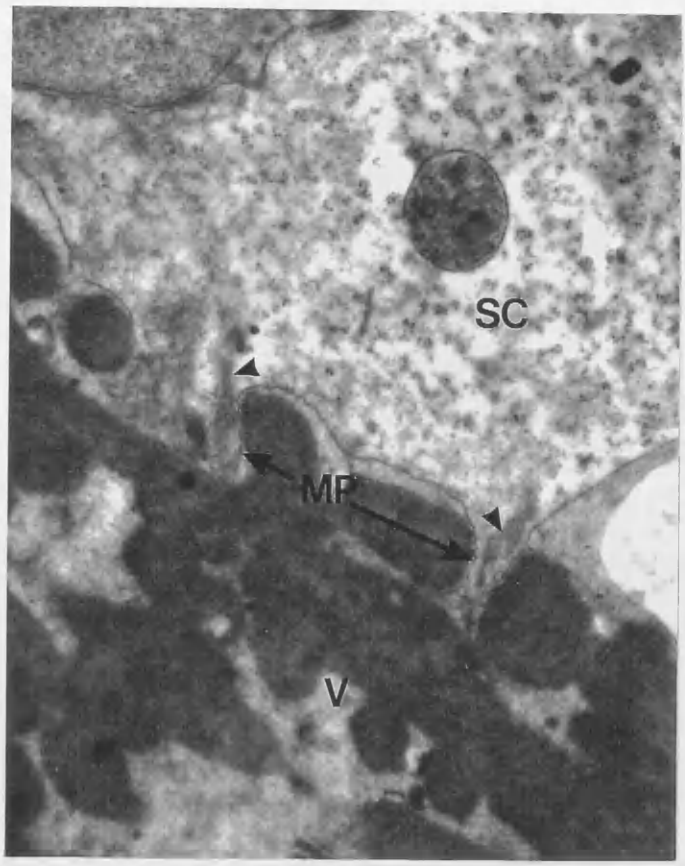

Fig.5.17 a

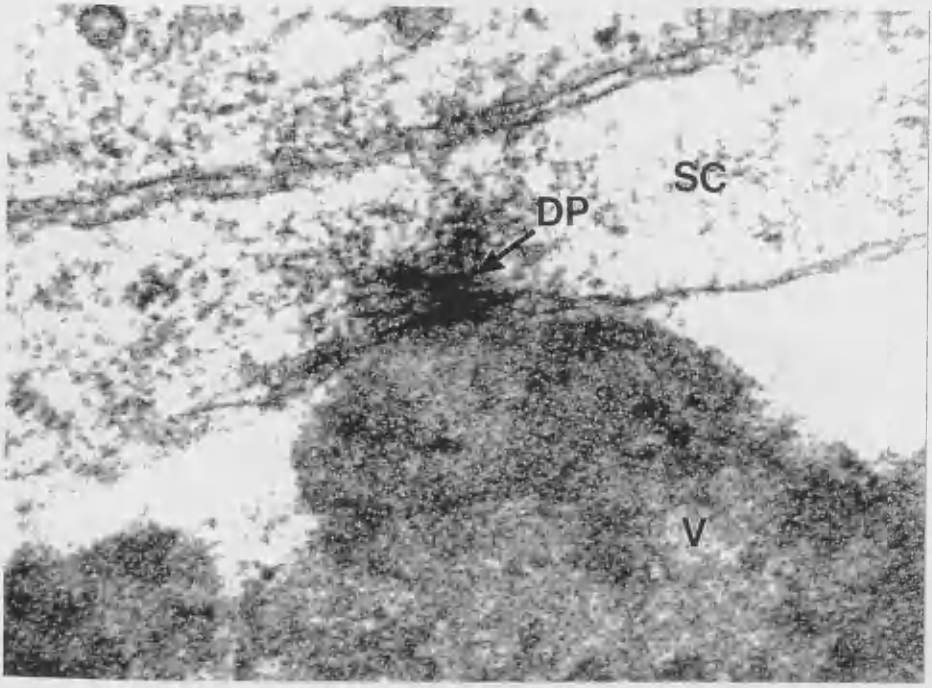

Fig.5.17 b

Fig.5.18 a- Light micrograph of a stage 18 leading edge. The edge cells have transformed from a multilayer to a monolayer (M). Bar= 50 μm.

Fig.5.18 b- A different part of the same specimen as (a) showing that in some areas the distal-most cells (d) detach from the main sheet of edge cells and migrate in singles and in groups. Bar= 50 μm.

Fig.5.18 c- SEM of stage 18 blastoderm showing the actively moving edge cells (M) forming a monolayer on the vitelline membrane (V). Part of a stationary streak (S) can be seen. Bar= 100 μm.

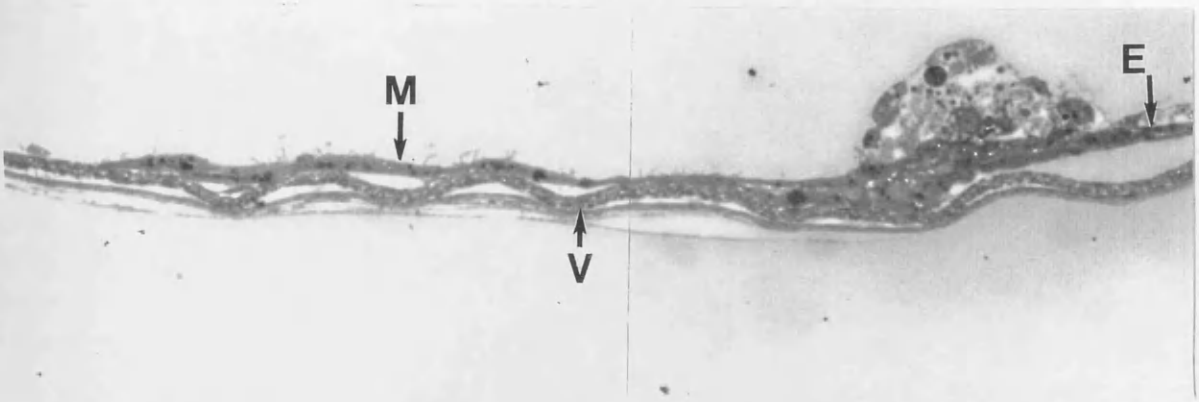

Fig.5.18 a

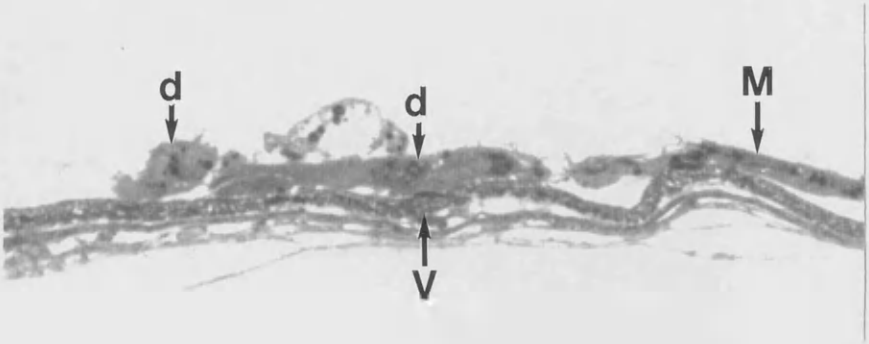

Fig.5.18 b

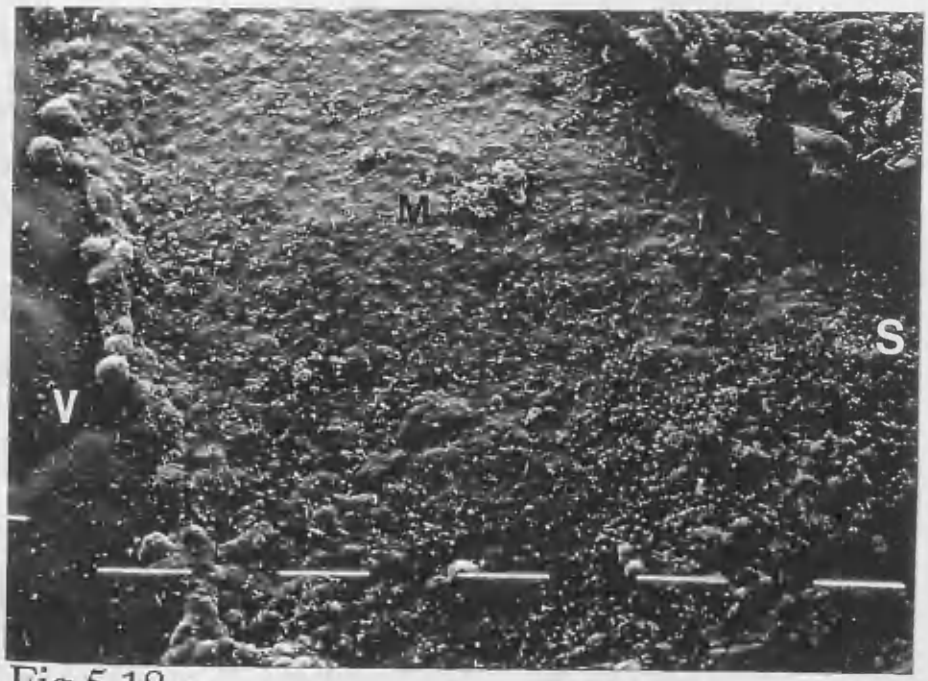

Fig.5.18 c

Fig.5.19 a- SEM of the distal leading edge cells (Le) of the blastoderm shown in fig. 5.18 c. The cells extend thin filopodia (arrowheads) to the neighbouring cells as well as to the vitelline membrane. Bar= 10 μm.

Fig.5.19 b- SEM of a 3-day old blastoderm showing the detachment of some of the distal-most cells (D) from the monolayer sheet of leading edge cells (M). Notice the crescent-shaped ectoderm (E) near the stationary streak (S). V= vitelline membrane. Bar= 100 μm.

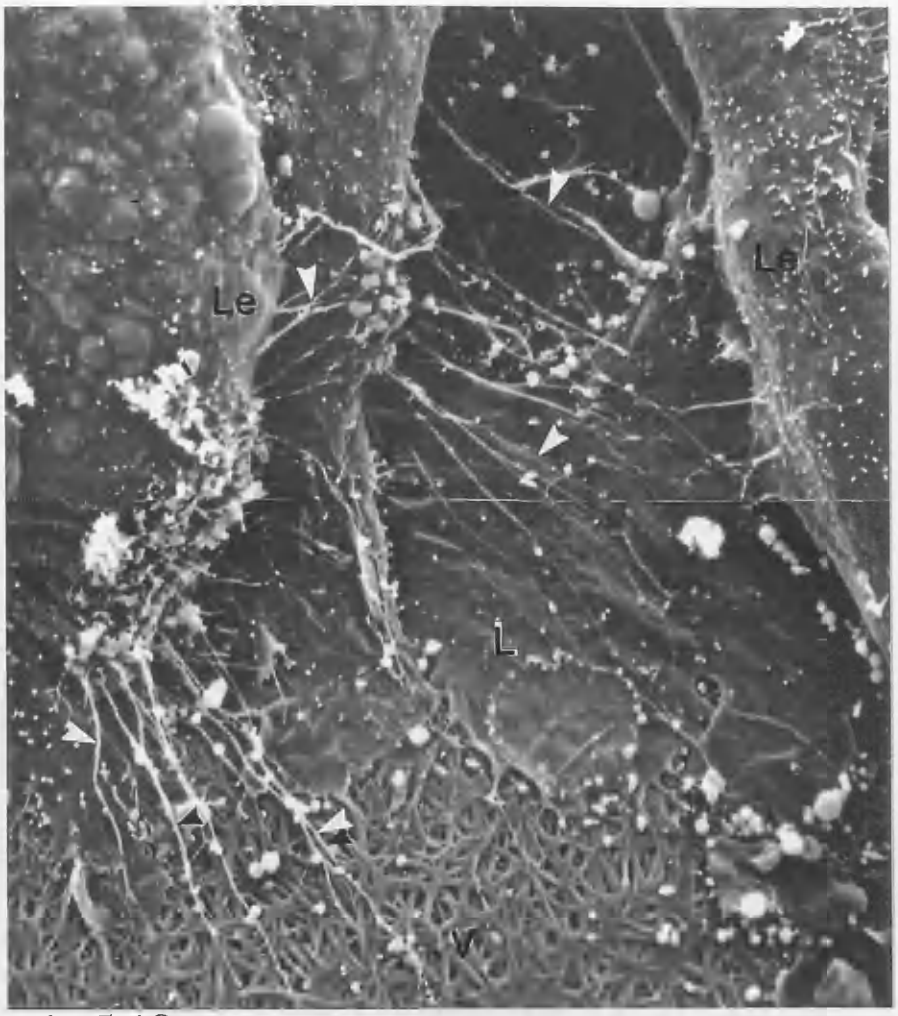

Fig.5.19 a

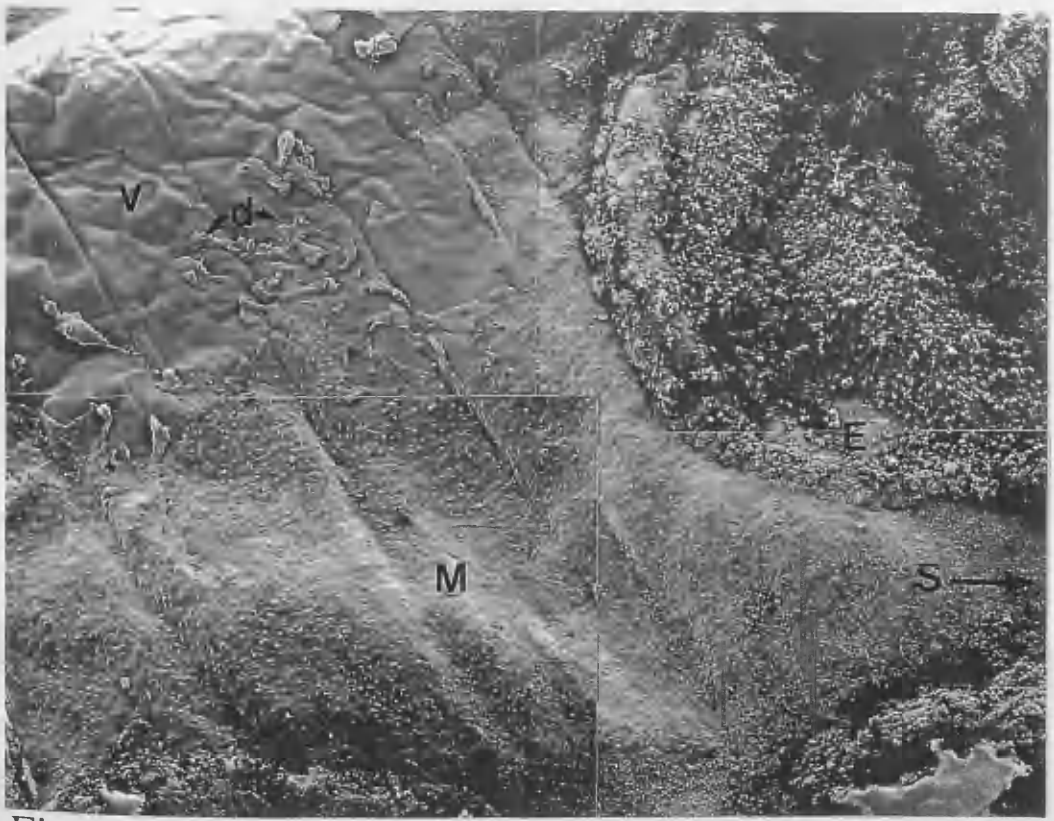

Fig.5.19 b

Fig.5.20 (a&b)- TEM showing the presence of desmosomes (arrow-heads) between the proximal edge cells (P) of a stage 18 blastoderm (a). No specialised junctions were detected between the cells of the monolayer (M) of the edge (b). Bar in a & b= 2.8 μm. X 6.5K.

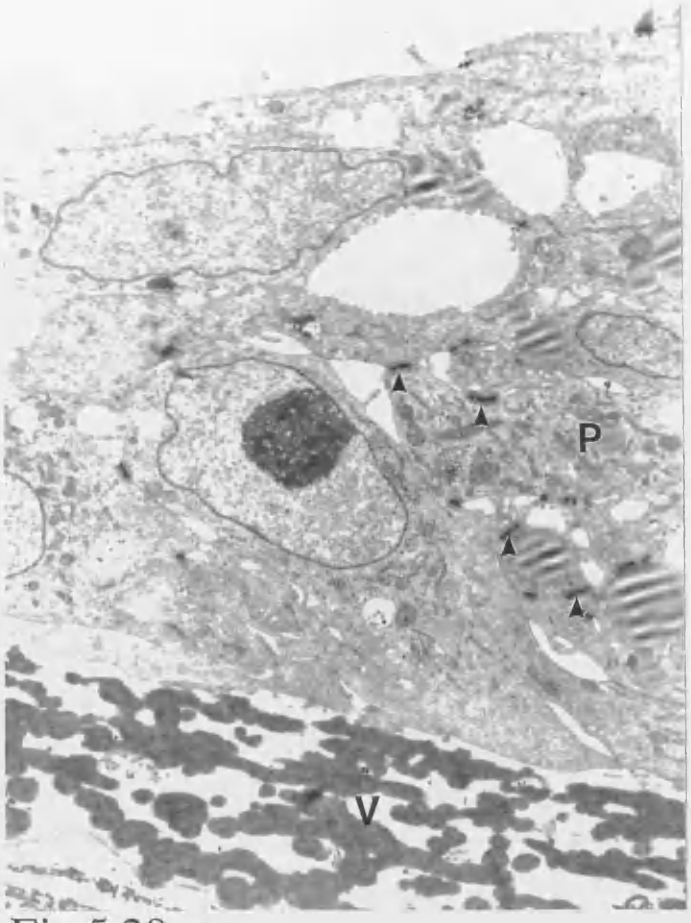

Fig.5.20 a

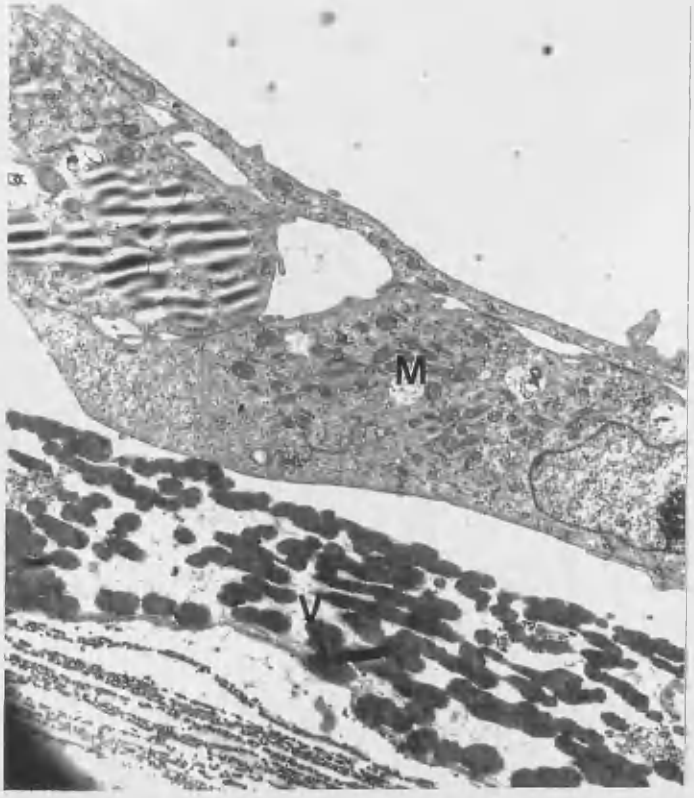

Fig.5.20 b

Fig.5.21 a- A Light micrograph (frontal section) through the oldest stationary cells (S) a 4-day blastoderm. Notice the presence of cell death (arrows) in these cells. Bar= 50 µm.

Fig.5.21 b- A Light micrograph (frontal section) through stationary cells (S) which became stationary later than the ones shown above. Cell death (arrows) can also be detected in these cells. Bar= 50 µm.

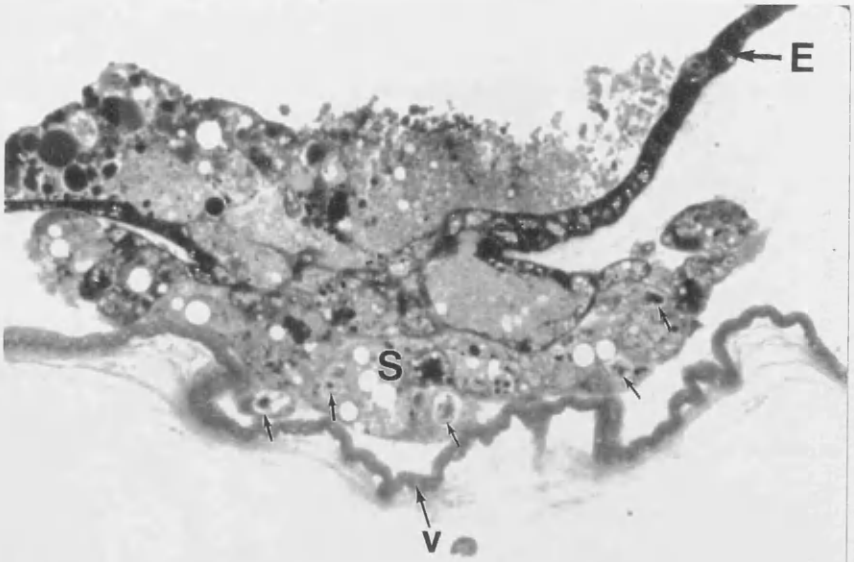

Fig.5.21a

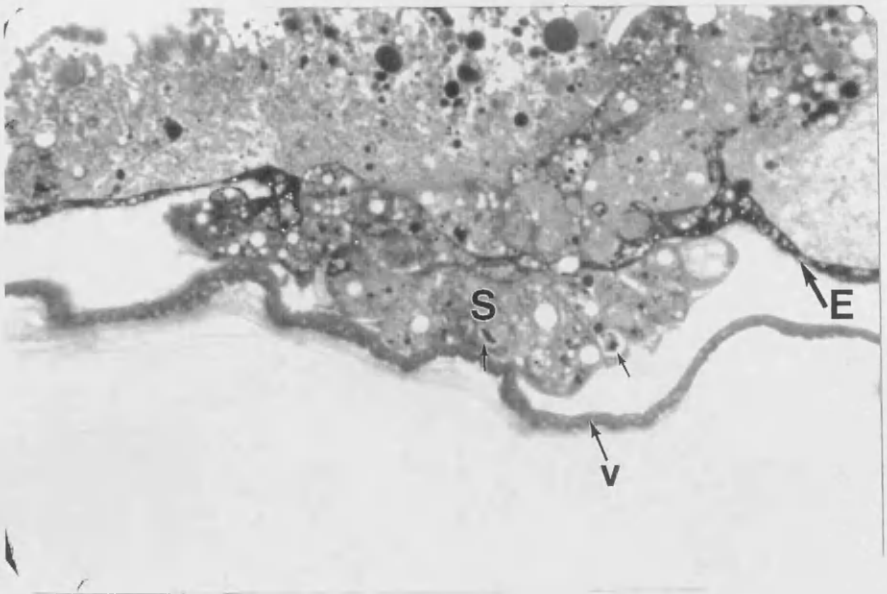

Fig.5.21b

Fig.5.22 a- TEM showing lamellae (L) sent by the ectodermal cells to the spaces between the dying stationary cells (DS). Bar= 1.7 µm X 8.55K.

Fig.5.22 b- Light micrograph of a 4-day old blastoderm. Subblastodermic albumen (A) is present between the ectodermal sheet (E) and the vitelline membrane (V). Bar= 50 µm.

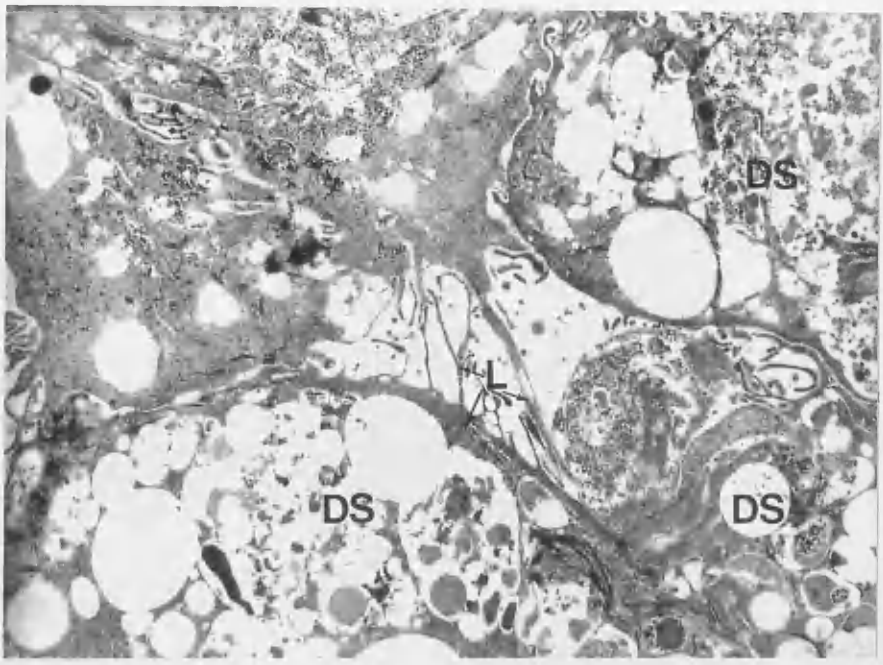

Fig.5.22a

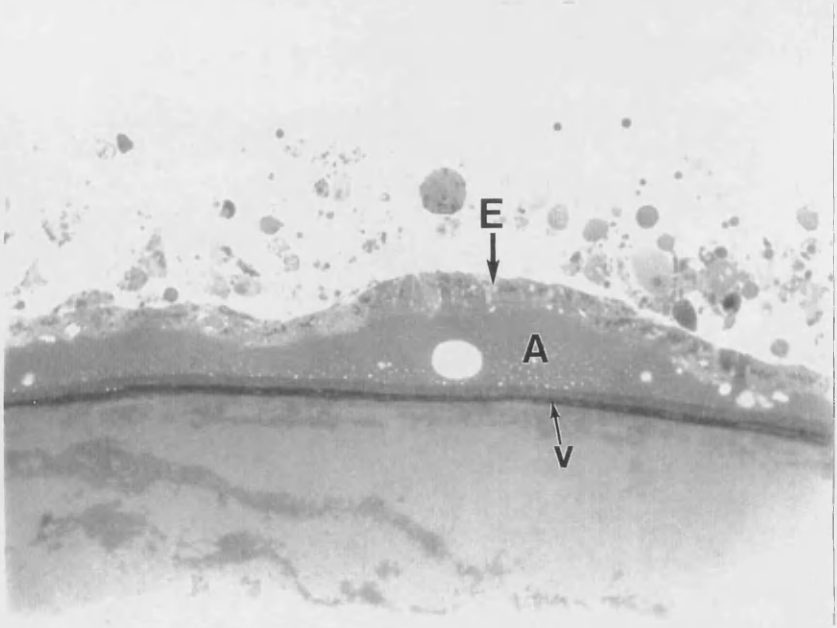

Fig.5.22b

Fig.5.23 a- Light micrograph (frontal section) through a stationary streak of a 6-day old blastoderm. The stationary cells have completely disappeared from the streak and their space (Sp) is occupied by some ectodermal lamellae. E= Ectoderm; V= vitelline membrane (V). Bar= 50 µm.

Fig.5.23 b- A magnified part of (a) showing that the space left after the death of the stationary cells is occupied by lamellae (L) of ectodermal cells. Some ectodermal cells (E) begin to attach to the vitelline membrane (V) in place of the stationary cells. Bar= 50 µm.

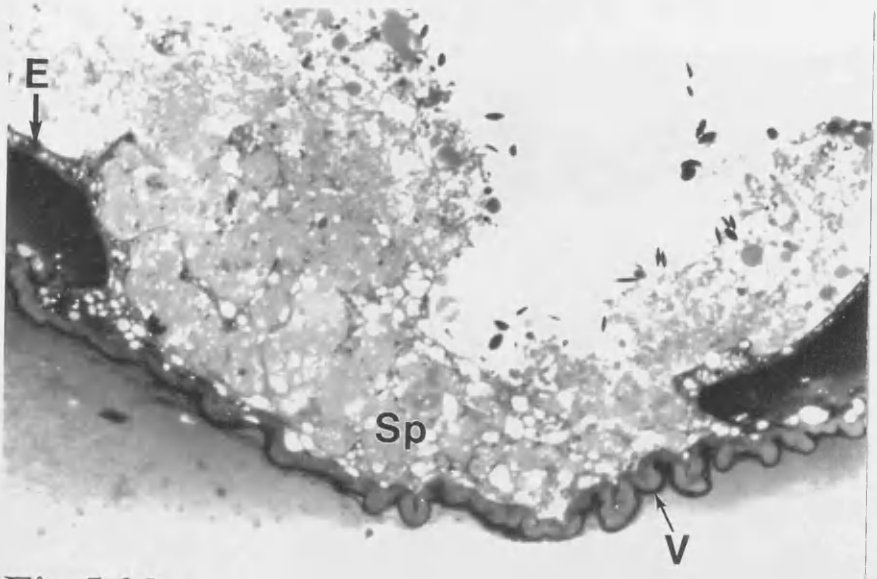

Fig.5.23 a

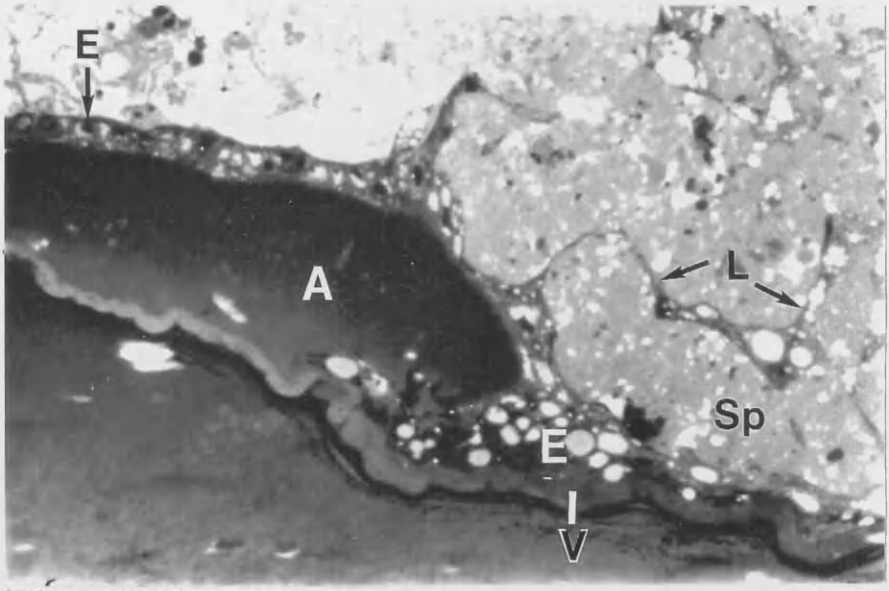

Fig.5.23 b

Fig.5.24 a- Light micrograph (frontal section) through a stationary streak of a 10-day old blastoderm. The ectodermal cells (EC) have completely occupied the space left by the original stationary edge cells. Notice the presence of large yolk droplets in these ectodermal cells. Notice also the wrinkling of the vitelline membrane(V) at areas of attachment. Bar= 100 μm.

Fig.5.24 b- Similar section as in (a) showing that the vitelline membrane (V) is greatly wrinkled at points of attachment of ectodermal cells (EC) but smooth in areas where no such attachment is present (arrowheads). Bar= 50 μm.

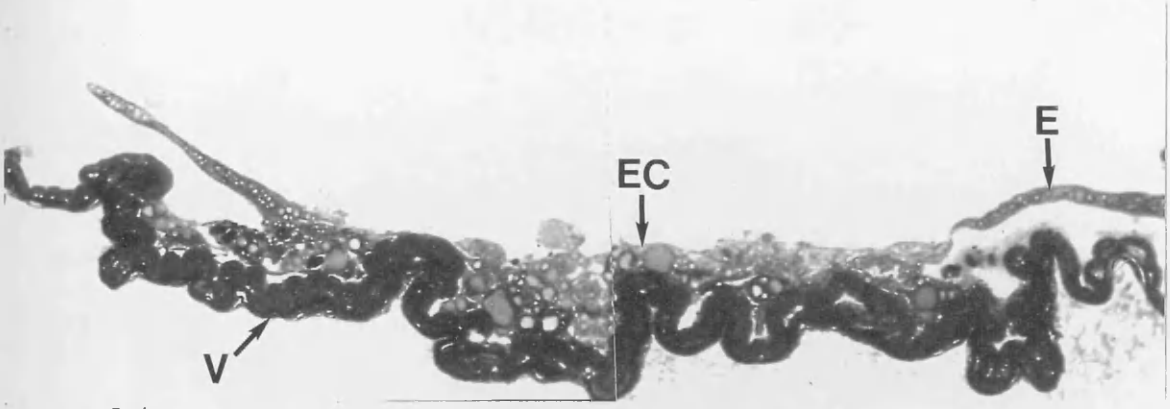

Fig.5.24 a

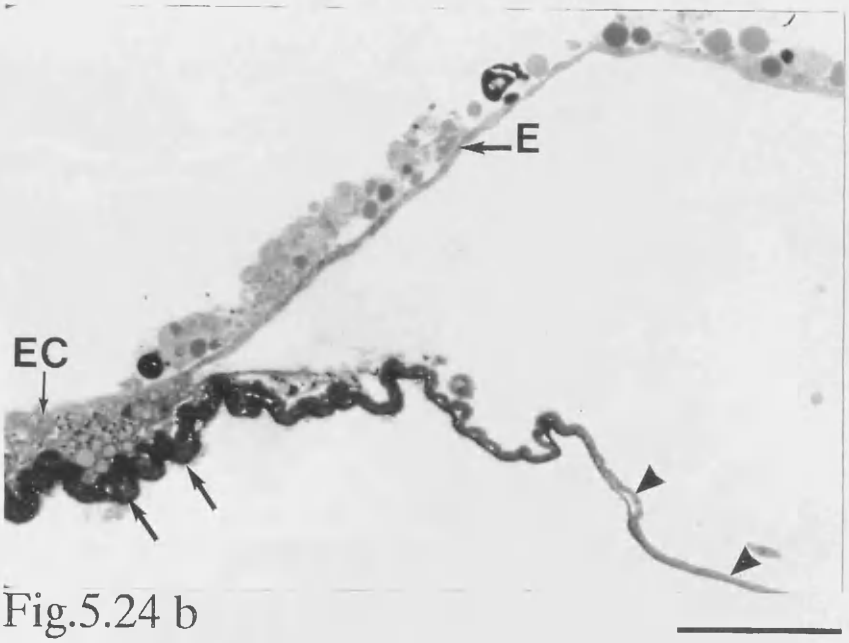

Fig.5.24 b

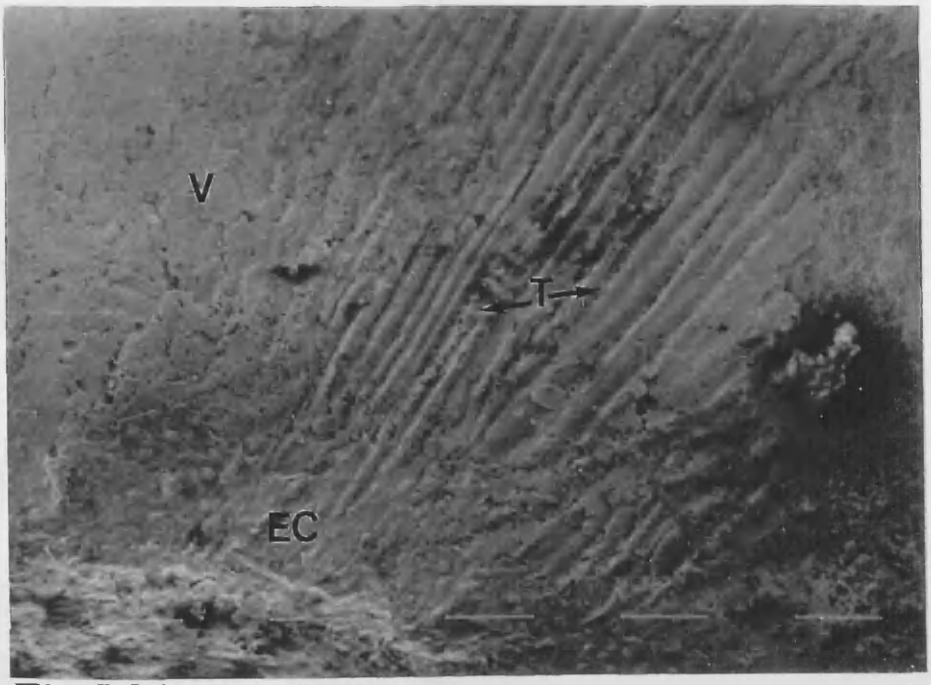

Fig.5.24 c

Fig.5.25 a- SEM showing microvillous projections (arrowheads) protruding from the ectodermal cells (EC) into the inner surface of the vitelline membrane. Here, the outer surface of the membrane has been peeled off along with the attached albumen in order to see these structures. Bar= 1 μm.

Fig.5.25 b- SEM of an 8 day-old blastoderm showing the continuous presence of deep layer cells (D) in areas between the ectoderm (E) and the leading edge cells (Le). Bar= 60 μm.

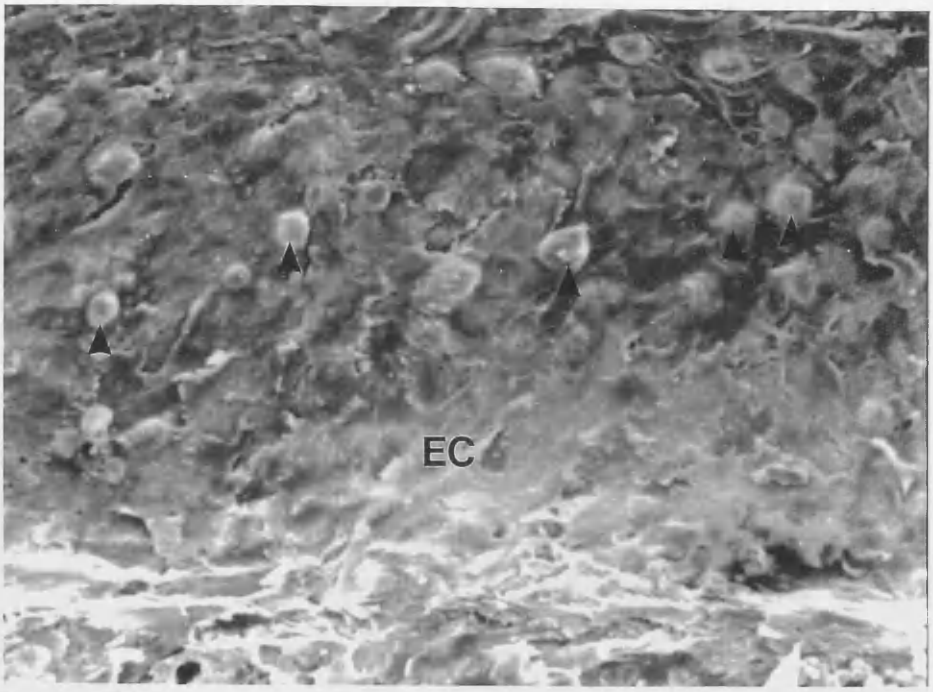

Fig.5.25 a

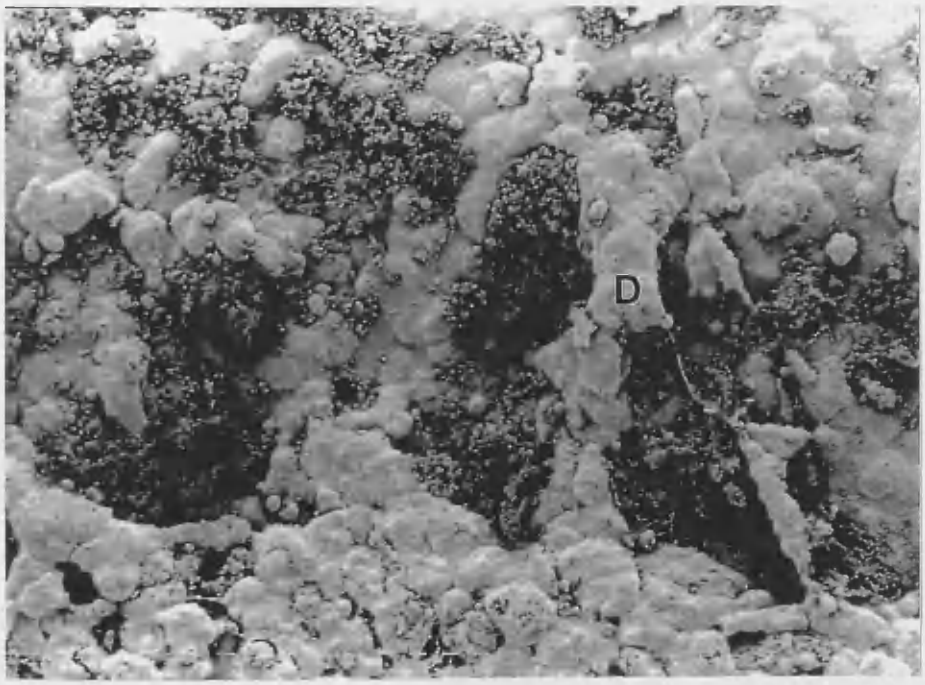

Fig.5.25 b

Fig.5.26 a- SEM of an 11 day- old blastoderm in which the leading edge has sealed the whole of the vitelline membrane. Notice that the deep layer cells (D) form a second layer over the leading edge cells. The marked area is enlarged below. Bar= 200 µm.

Fig.5.26 b- High maginfication of the area marked in (a). Deep layer cells (D) apparently continue to leave the basal surface of the ectoderm (E) and are recruited into the leading edge area. Bar= 20 µm.

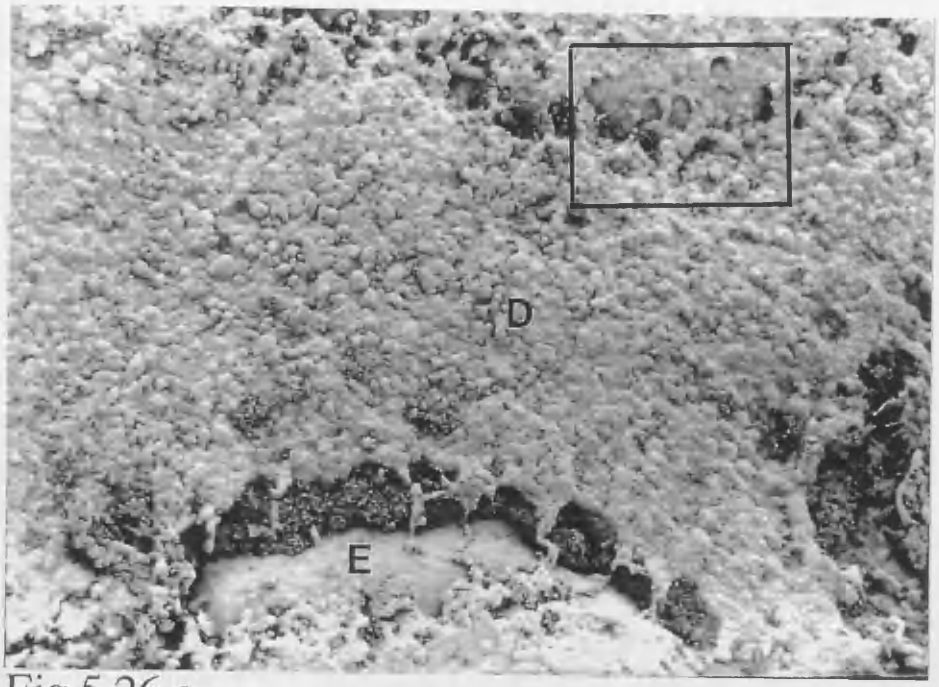

Fig.5.26 a

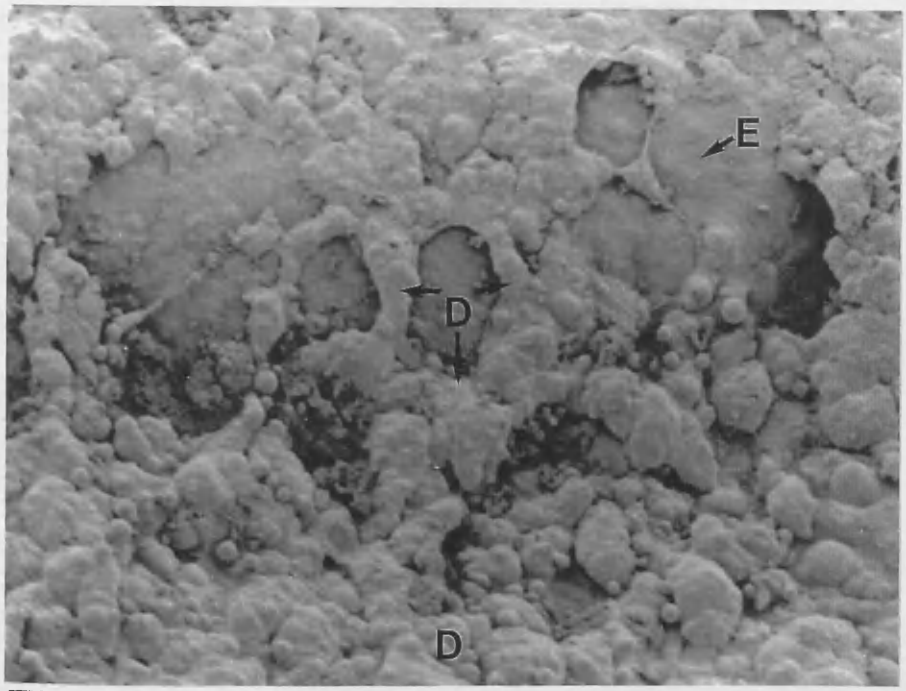

Fig.5.26 b

Fig.5.27 a- Light micrograph of stationary streak (S) cultured for 3 days on the vitelline membrane. There is no apparent migration of cells from the explant site (compare with fig.28a). Bar= 50 µm.

Fig.5.27 b- TS through a sattionary streak cultured for 3 days on the inner surface of the vitelline membrane (V). The majority of the stationary cells (SC) are dead or showing signs of cell death. Bar= 50 µm.

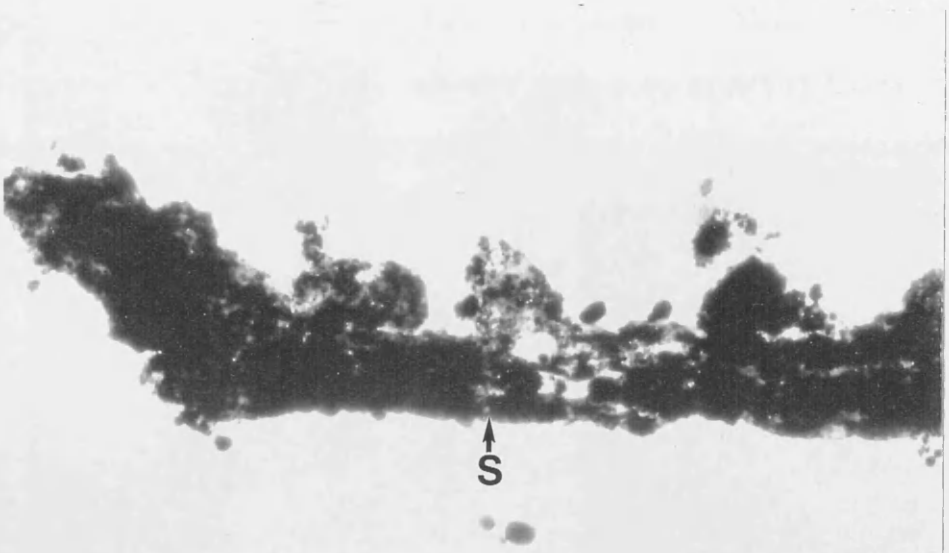

Fig.5.27 a

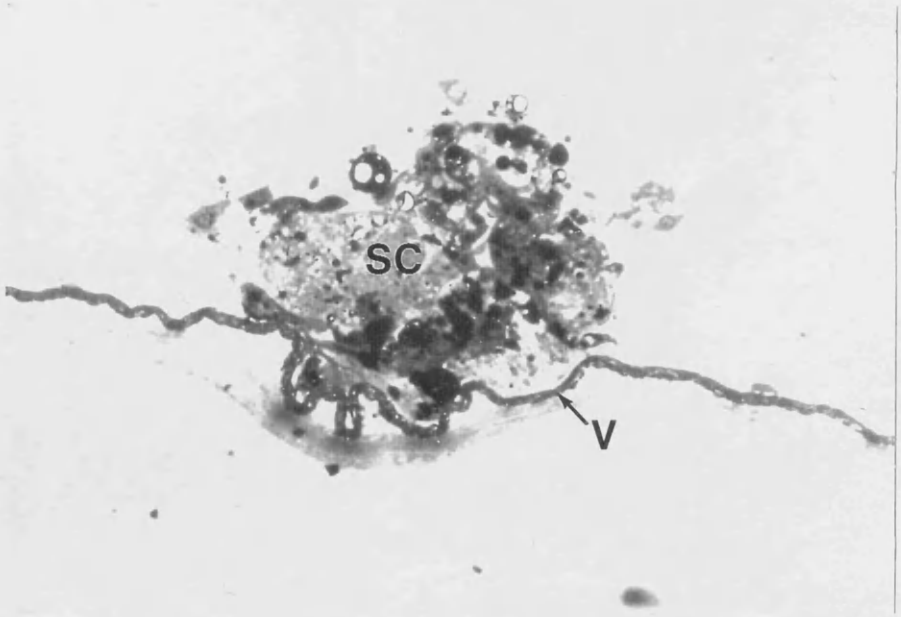

Fig.5.27 b

Fig.5.28 Light micrograph of some stationary cells cultured for 3 (a) and 5 (b) days on a glass substratum. Notice the extent of the migration from the site of the explant (EX) and their healthy appearance. Bar= 100 μm.

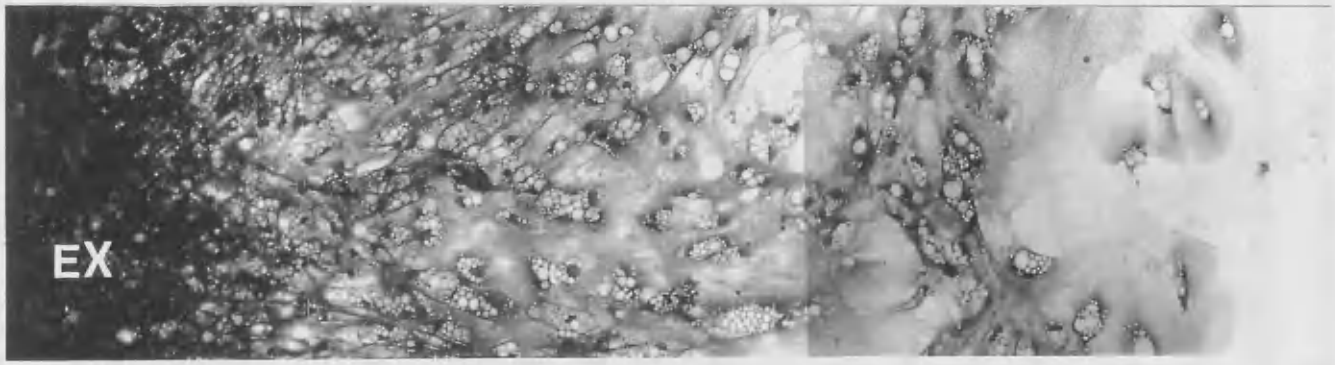

Fig.5.28 a

Fig.5.28 b

5.4 DISCUSSION

The chick blastoderm conforms to the spherical geometry of the egg and, therefore, it has to achieve two rather contrasting tasks. The first task is that it must increase the area of its ectoderm layer and its rim by which it attaches to the substratum, the vitelline membrane. Downie (1976) has shown that the area of the blastoderm increases due to division and growth of its ectodermal cells, and the previous two chapters of this thesis showed that there is a steady increase in the number of edge cells as a result of the recruitment of new cells, the deep layer cells, into the edge.

The second task is for the blastoderm to somehow accommodate the large number of its leading edge cells in the smaller space available on the vitelline membrane . In other words, the blastoderm has to decrease its circumference continuously in order to be able to expand from areas of larger circumference around the equator of the egg into areas of smaller circumference in the lower egg hemisphere. This chapter describes the way the chick blastoderm goes about achieving this task. However, before discussing the strategy adapted by the chick blastoderm to accomplish this we must first look at how other embryos tackle the same problem.

The most extensively studied embryos in this context are those of two teleosts, *Oryzias latipes* and *Fundulus heteroclitus*. In the first system Kageyama (1980 and 1982) found, using silver staining to delineate cell boundaries, that after passing the equator of the egg, the enveloping layer of the blastoderm decreases the number of its marginal cells in order to adjust to the changes in the geometry of the egg. He suggested that this decrease in marginal cells is due to cell rearrangement as well as changes in the shape of these cells.

In the second system, it was found that here also the marginal cells of the

enveloping layer of *Fundulus* decrease in number by both cell rearrangement and shape changing. Silver staining and time-lapse cinematography used by Keller and Trinkaus (1987) disclosed that after the enveloping layer had passed the equator of the egg its marginal cells translocate from their original marginal positions to submarginal ones. This results in the recorded decrease in the number of these cells from 165 around the equator to 25 as the blastoderm encompasses the yolk. At the same time, the submarginal cells undergo change in shape by tapering and narrowing. By this continuous cell rearrangement and cell-shape changes the enveloping layer of this embryo moves into an increasingly narrower space until it closes at the vegetal pole and, thus, encompasses the whole of the yolk sphere.

More recently, Weliky and Oster (1990), working on epiboly in the *Fundulus*, supported the findings of Keller and Trinkaus (1987) and put forward a computer model which simulates the events that take place during the process. Their model is based on the assumption that the cells of the enveloping layer, whether marginal or submarginal, are under two mechanical forces, first what they called an "azimuthal" stress which tends to elongate the cells in the direction of expansion i.e. perpendicular to the marginal boundary, and second a circumferential, or medio-lateral stress which tends to stretch the cells horizontally i.e. parallel to the marginal boundary. They suggest that when cells move from regions of larger to smaller circumference in the lower hemisphere of the egg during the late stages of epiboly they come under an increasingly large azimuthal stress while the medio-lateral stress on them is relaxed. As a result of this imbalance between the two forces the submarginal cells elongate so that their long axis becomes perpendicular to the marginal boundary and parallel to the direction of expansion. With further elongation, pairs of these submarginal cells separate from each other, leading to the translocation of these cells and, thus, the rearrangement of cells observed by Keller and Trinkaus (1987) in the living embryos. Similarly, the marginal cells of the enveloping layer elongate and eventually retract from the margin to

submarginal positions.

The process of epiboly in the chick embryo differs fundamentally from that of the teleost embryos described above in that while in the latter the enveloping layer expands as a single cohesive sheet over the yolk syncytial layer and so any rearrangement must occur within that sheet, the blastoderm of the chick embryo expands as a result of the migration of the leading edge cells which form a separate unique unit which differs from the rest of the blastoderm in many aspects (Downie, 1976 and New, 1959). The arrangement of this edge in relation to the rest of the blastoderm makes it impossible for its cells say to retract from it and translocate to the nearby non-edge part of the blastoderm, as is the case during epiboly in the teleost embryo. Therefore, the end of epiboly in the chick blastoderm must involve a different strategy.

The events that take place during the late stages of epiboly of the chick blastoderm have never previously been studied in any detail and so this chapter describes for the first time the unique and novel strategy that this system adopts in order to achieve the required reduction in the number of its leading edge cells as it expands to encompass the whole yolk.

After the chick blastoderm edge passes the equator of the egg, around the 3rd day of development (stage 18), some of its edge cells become stationary at certain points on the vitelline membrane. The position of these points does not only differ between embryos at different stages of development but also between embryos of the same stage. There therefore seems to be no precisely determined pattern of stationary points, in time or position.

At the same time as these points appear the leading edge cells, except for those which become stationary, continue their centrifugal movement and as development proceeds some of the moving cells which lay near the stationary points become stationary themselves. It seem that they are prevented from further movement by the pulling force exerted on them by the older stationary

cells with which they are associated. TEM revealed that the stationary cells are connected to each other via the powerful tension resisting junctions, the desmosomes. The development of these desmosomes between the stationary cells seems to serve a number of purposes. Firstly, they enable the first stationary cells to resist any force which could separate them from each other, such as that which is created by the rest of the actively moving edge cells. Secondly, they strongly connect the older cells to the most recently added stationary cells thus leading to the formation of the stationary streaks. Thirdly, they may be involved in maintaining the elongated shape of the stationary cells.

Scanning and transmission electron microscopic examination of the dorsal surface of the stationary cells revealed that this surface is rich in microvillous projections which penetrate into the meshwork of the inner surface of the vitelline membrane. As discussed in chapter 3, these projections may play an important role in anchoring the edge cells to their substrate.

The strong association between the stationary edge cells and the vitelline membrane at the later stages of epiboly can also be due to the dense plaques which are evident in the areas where these cells make direct contact with the membrane. These dense plaques are known to be areas of strong association between cells and their substratum and are more prominent in those cells which show little or no motility (Couchman and Rees, 1979; Kolega *et. al.*, 1982; Burridge, 1986 and Burridge *et. al.*, 1987).

In my opinion, the combination of the presence of both microvillous projections from the stationary cells and the areas of dense plaques between these cells and the substratum could serve to anchor the stationary cells strongly to the substratum in order to resist the pressure to move forward which is applied on them by the forward movement of the remaining edge cells. It might be that the wrinkling of the areas on the vitelline membrane to which these cells attach is evidence for such a resistance. This is in contrast to the situation observed in the moving edge during the earlier stages of development

when microvillous projections are not as numerous as in the stationary cells and dense plaques are hardly seen at all. Even when the latter are present, they occur between the dorsal surface of the moving edge cells and the underlapping lamella of another cell and not between the cells and the surface of the vitelline membrane.

The adhesion of the stationary cells to the vitelline membrane is so strong that if the blastoderm is mechanically pulled from the vitelline membrane the ectodermal part breaks very easily from the streaks of stationary cells which remain attached to the substratum. This is in contrast with the fact that the leading edge cells of the early stages of development can be very easily detached from the vitelline membrane, without causing any separation from their neighbouring ectodermal cells.

The formation of the stationary streaks leads in turn to the transformation of the smooth circumference of the blastoderm into U-shaped areas made of advancing edge cells at the bottom and stationary cells in the streaks. The number of folds and their complexity differ from one embryo to another even at the same stage of development. Also the extent of expansion of the blastoderm and, therefore, the area they need to cover are so variable that some blastoderms at say the 4th day of development were found to have expanded even more than in older ones. The reasons behind these variations are not known and merit further investigation.

The cells of the ectodermal sheet in the folded areas of the blastoderm, i.e. those which lie between two stationary streaks, change their original polygonal shape and elongate so that their long axis becomes parallel to the direction of migration of the moving parts of the edge. This change in shape is due again to two mechanical forces, a force pulling them created by the moving edge cells and another holding them in place created by their attachment to the stationary edge cells.

The elongation of the ectodermal cells reduces the proportion of the

edge circumference that each occupies and, in turn, allows more of the blastoderm to squeeze into the increasingly smaller areas which it needs to cover as epiboly nears its final stages. Whether or not this change in shape is associated with cell rearrangement similar to that described by Keller and Trinkaus(1987) and Weliky and Oster (1990) for *Fundulus* is not clear and has not been investigated in the present study due to the difficulties encountered during any attempt to get direct *in ovo* observations.

By this unique strategy the chick blastoderm achieves two objectives. First, by confining some of its marginal edge cells to stationary streaks, it allows just enough of the edge cells to continue migrating into the available area on the vitelline membrane, which gets smaller and smaller as expansion proceeds. Secondly, the transformation of the circumference of the blastoderm into the observed folds , along with the shape change in the ectodermal cells reduces the total area of the ectodermal part of the blastoderm, allowing more of this sheet to expand to cover more of the lower hemisphere of the egg.

By the third to fourth day of incubation the vitelline membrane ruptures at the animal pole (over the embryo) and slips towards the vegetal pole (Jensen, 1969). This rupturing could account for the albumen which is present at the later stages of epiboly between the ectodermal sheet of the blastoderm and the vitelline membrane. This albumen as well as that under the intact part of the vitelline membrane becomes more solid as development progresses and this could provide some support for the very thin sheet of ectoderm and prevents its breakage as well as supporting the vitelline membrane which show a decrease in its mechanical strength as it becomes older (Jensen, 1969).

Cell death in the leading edge cells:

The stationary cells, having participated actively in the early process of blastoderm expansion and then in the required decrease in the circumference of the blastoderm, enter a cycle of cell death. Cell death does not occur

simultaneously throughout the streaks of stationary cells but in stages. The oldest stationary cells which are the furthest from the moving edge and which were the first to stop their centrifugal movement are found to be the first to start dying at around the 4th day of incubation. Cell death then progresses distally to the younger stationary cells.

As the stationary cells start the cycle of cell death intercellular spaces start to appear between them. These spaces were found to be invaded by long lamellae sent by the nearby ectodermal cells. By the 6th day of incubation all the stationary edge cells, at a certain area, have died and only the lamellae of the adjacent ectodermal cells are found in their place.

The mechanism of removal of the dead stationary cells is not known but there are two possibilities; (a) the stationary cells could be added to the yolk then absorbed by the rest of the blastoderm or alternatively, (b) they could be phagocytosed by the nearby ectodermal cells. The second possibility seems the more likely since the lamellae sent by the ectodermal cells to invade the intercellular spaces surround the dying stationary cells from all sides and they could well be in the process of engulfing these cells. Also, after the stationary cells have been removed no more of the ectodermal lamellae can be seen; instead, the ectodermal cells themselves come into contact with the vitelline membrane, and cells from both sides of the original streak fuse together to occupy the space left by the stationary cells. As they do so, one feature becomes evident, that is the large yolk inclusions which these cells contain, and which is generally not a characteristic of the ectodermal cells except during the very early stages of development (Stage 3). It can be easily noticed that the yolk inclusions in these ectodermal cells are larger as well as more in number than the rest of the ectodermal cells which are further away from the areas of the stationary cells. This gives more support to the possibility that the ectodermal cells in question may well have gained their yolky appearance as a result of their phagocytosis of the dead stationary cells.

The presence of thick albumen at these areas made it impossible to examine, under the transmission electron microscope, the kind of contacts which the invading ectodermal cells have with the vitelline membrane. However, after removing the albumen along with the outer surface of the vitelline membrane it became apparent that these cells possess microvillous projections similar to those of the original stationary cells which they send into the meshwork of the inner surface of the vitelline membrane. The attachment of the ectodermal cells to their substratum is increasingly strong as can be judged from the very deep wrinkling of the vitelline membrane at the sites of attachment and the traction that these cells create in the areas of the membrane adjacent to the these sites.

The results from the experiment in section 5.4 which was designed to study the behaviour of the stationary cells on fresh vitelline membrane and glass revealed that although these cells attached to the vitelline membrane they were unable to spread on it and entered the cycle of cell death as they did *in ovo*. However, they behaved differently on glass, where they spread and stayed healthy even after 5 days in culture. By this time they would have been 9 days old *in ovo*, and they would have been completely removed from the vitelline membrane and their space would have been occupied by the adjacent ectodermal cells. These results suggest that these cells are committed to die at a certain age as long as they are in contact with the vitelline membrane, no matter whether this membrane is fresh or has been incubated for 4 days and whether they are under stress from the other blastoderm cells or not. Their survival and spreading on the glass substratum suggests that it is the strong attachment of the stationary cells to the vitelline membrane and, subsequently, their inability to move that may be the crucial factor involved in triggering these cells to enter the cycle of cell death. It would be well worth testing the behaviour of these cells on a variety of extra-cellular matrix coated substrates to test whether or not specific molecules trigger the death response in these cells.

The closure of the blastoderm by the monolayer of edge cells and the role of deep layer cells in the process:

As the process of the formation of the stationary streaks continues and the subsequent transformation of the blastoderm edge into folds continues, the moving edge cells continue their centrifugal movement to cover the rest of the vitelline membrane. As they do so they reorganise themselves into a monolayer with the leading lamellae of each cell underlapping the cell in front of it. As mentioned in the results, the width of the leading edge could not be measured accurately at these later stages of development due to the continuity of the moving edge cells with the stationary streaks at many points along the circumference, but I can safely say that it is in excess of 800 um. The width is considerably larger in blastoderms which are 7 days old or more.

The transformation of the leading edge into a monolayer could be due to the events that take place in the rest of the blastoderm. During the later stages of epiboly, the blastoderm becomes resistant to the forward pulling created by the leading edge due to the strong attachment of the stationary cells, and later the ectodermal cells, to the vitelline membrane in the stationary streaks. This resistance puts the leading edge under an increasing tension which, if not dealt with, could detach edge cells from the vitelline membrane. It seems that the leading edge solves this problem by reorganising its cells into a monolayer so that those edge cells which were not in contact with the vitelline membrane become attached to it giving the leading edge more grip on the substratum and thereby preventing any tension from detaching it from the latter.

The reorganisation of the edge cells into a monolayer may be also the only way by which the blastoderm achieves the final encompassing of the yolk. In all the blastoderms which I examined and which had completely encompassed the yolk sphere I have not come across one blastoderm in which its ectodermal parts from all directions actually meet and seal together. It may be that with the ectodermal cells invading more and more of the spaces which

were left by the dead stationary cells, and therefore with the increase in the exceptionally strong adhesion of these cells to the vitelline membrane, there comes a time when the expansion of the blastoderm either completely stops or its rate of expansion becomes considerably reduced. In fact this rate has been reported to start dropping around the 3rd day of incubation (Downie,1976) and the results here show that the final stages of expansion are very slow indeed, but very variable from embryo to embryo.

I would also suggest that the monolayering of the leading edge cells at the end of epiboly is partly a sign of slowing down and increasing disorganisation of the edge cells. As more edge cells attach, it is difficult for them all to orientate in the same direction, therefore coherent expansion in one direction becomes more difficult. A reflection of this is the breaking away of some cells from the edge, which is possible because of the lack of desmosomes at the distal parts of the edge. This also occurs in cultures when explants are attached to a surface all over, and coherent expansion is therefore not easy for the sheet of cells.

The role of the deep layer cells during the late stages of epiboly:

The deep layer cells seem to serve two functions during the expansion of the chick blastoderm. First, as discussed in the previous chapter, they recruit themselves into the edge area to provide the latter with the cells required to cover the increasing circumference of the blastoderm. Secondly, they seem to play an important role during the later stages of epiboly especially around the time of closure of the blastoderm. They are found to be incorporated into the edge area even during these stages but this time their function is most likely to be providing the monolayer of edge cells with extra cells to cover the space available on the vitelline membrane. Even at the stages when the monolayer of edge cells has sealed the blastoderm, they seem to form an other layer on top of the edge cells to give support to this area of the blastoderm at these stages. In the absence of any TEM examination it was impossible to study the kind of association made between these cells and the edge cells underneath.

CHAPTER SIX

6.1 Cultures of blastoderm explants on the inner surface of the vitelline membrane

6.1.1 INTRODUCTION:

The set of experiments described in this chapter was designed to test whether in the absence of a normal leading edge, pieces of the blastoderm which do not normally attach to the vitelline membrane during blastoderm expansion have the ability to do so when cultured on the inner surface of the vitelline membrane. It was also hoped that the behaviour of both the ectodermal and deep layer cells, which constitute such pieces, could throw some light on the degree to which either of these two cell types, or both, contribute to the movement of the normal edge *in vivo*. In other words, I hoped to present evidence based on the behaviour of these two cell types for the source of cell recruitment into the edge as it migrates to cover an increasingly wider area over the vitelline membrane.

6.2 Methods:

The methods of culturing these pieces of the blastoderm and analysis of the results have been described in chapter 2.7. Basically, small (3 mm2) explants of extraembryonic tissue were cut from just proximal to the edge of stage 4-13 (H&H) chick embryos, and cultured on vitelline membranes, using the method of New (1955) with thin albumen as medium. Explants were cultured either ectodermal side up, or down, and the pattern of explant outgrowth was followed by time lapse filming and Camera Lucida drawings at intervals. Cultures were also fixed at intervals, for semi-thin and ultra-thin sectioning.

6.1.3 RESULTS:

6.1.3.1 Macroscopic observations:

Very soon after the start of culturing on the vitelline membrane, the explants showed a reduction in their size, before they attached to the vitelline membrane and began to expand in size.

After 2 hour incubation time, two areas became visible in the explant. These areas were 1) a large dark area which occupies most of the explant and 2) a lighter narrow area surrounding the previous one (Fig.6.1b).

The lighter peripheral area expanded in all directions on the vitelline membrane giving the area a much lighter appearance (Fig.6.1c). Expansion started after 2 hours of incubation, and the rate of expansion was approximately equal all round the explant, and at approximately the same rate for several hours. The average speed at which these cells moved on the vitelline membrane was found to be 71 $\pm$ 14 μm/hr (Table 6.1). Camera Lucida drawings showing the direction and evenness of expansion are shown in Figure 6.2.

After six to ten hours of incubation the cells of the central dark area had condensed together more tightly, resulting in a darker appearance to this central area.

Once the condensed central area had formed, it started to shift its position from one side of the explant to another. After 16-24 hours of incubation much of the explant formed a condensed mass of cells which moved very rapidly in one direction, leaving behind a trail of cells attached to the vitelline membrane (Fig.6.1d). The average speed of this mass of cells was 217 $\pm$ 60 μm/hr (Table 6.1). I give this rapidly moving mass of cells the name "Regenerated Edge" to distinguish it from the similar but not identical normal blastoderm edge. The behaviour of explants was basically the same whether

cultured ectodermal side up or down i.e. in both kinds of explants a regenerated edge formed.

6.1.3.2 MICROSCOPIC OBSERVATIONS:

1um sections cut through explants that had been incubated for 2, 6-10, and 16-24 hours were stained with Toluidine blue and examined under the light microscope. Also, ultra-thin sections were examined under the TEM. The description of and distinction between the two cell types i.e. deep layer cells and ectodermal cells is based on the differences of appearance between the two cell types under light and transmission electron microscope when seen in sections from intact blastoderms. These include: a) the yolky appearance of the deep layer cells when compared to the more compact ectodermal cells and b) the difference in staining of the two cell types; the ectodermal cells are much darkly stained than the other when examined under both the light and transmission electron microscope.

6.1.3.2.1 **After 2 hours of incubation :**

The lighter area which forms the periphery of each explant, both when the ectodermal side of the explant was cultured uppermost and lowermost, was found to be made of deep layer cells. When the explant was cultured so that the ectodermal side was uppermost, all the deep layer cells were in direct contact with the vitelline membrane to which they readily attached and started their centrifugal movement (Fig.6.3a). Each of these cells possessed a leading lamella which underlapped the cell in front of it (Fig.6.3b). In addition, the deep layer cells sent a few microvillous projections into the meshwork of the inner surface of the vitelline membrane (Fig.6.4a). They also possessed cortical microfilaments and microtubules orientated towards the direction of movement (Fig.6.4b). No specialised junctions were detected between these cells or their lamellae and there was no sign of any dense plaques between the cells and the vitelline membrane.

The ectodermal cells on the other hand did not attach in any way to the vitelline membrane. Instead, they remained on top of the deep layer cells and retained all the characteristics of the ectodermal cells of the intact blastoderm, namely the presence of the tight junction at their apical side followed by a number of desmosomes and interdigitations of their lateral cellular membranes. Basal lamina separated these cells from the underlying deep layer cells (Fig.6.5a). As mentioned above, in explants where the ectoderm was uppermost, all the deep layer cells were in contact with the vitelline membrane, with no attached edge and unattached area proximal to the edge as in explants where the ectoderm was cultured facing the vitelline membrane (See below).

When the explants were cultured so that the ectodermal side was in direct contact with the vitelline membrane, the deep layer cells moved from the top of the ectoderm around the edge of the explant and came into contact with the vitelline membrane. In the meantime, the ectodermal part of the explant became elevated away from the vitelline membrane and even the peripheral ectodermal cells did not make contact with it (Fig.6.5b). The arrangement of deep layer cells and ectodermal cells in this case was similar to that when the ectoderm was uppermost with respect to their contact with the vitelline membrane and distribution of junctions.

6.1.3.2.2 **After 6-10 hours of incubation:**

Both ectodermal and deep layer cells retained their positions in relation to each other and to the vitelline membrane as described for the 2 hour explants. The two cell types were still separated from each other by a well defined basal lamina. Up to this stage the ectodermal part of the explant, which forms the dark central mass, did not come into contact with the vitelline membrane, whether the ectoderm was originally cultured up or down. On the other hand the deep layer cells, in both explant types, continued their spreading on the vitelline membrane, covering a distance of up to 3 to 6 mm from the edge of the ectoderm (Fig.6.6a). These cells were 3-4 cells deep as they first

attached to the vitelline membrane around the peripheries of the ectodermal part, but as they moved away from the explant site the outgrowth decreased in thickness until it formed a monolayer at the distal-most parts of the explant (Fig.6.6b). All outgrowth cells were attached to the vitelline membrane.

6.1.3.2.3 **After 16-22 hours of incubation:**

This was the time at which the "regenerated edge" started to move rapidly on the vitelline membrane, leaving behind a trail of cells which detached from the main mass (Fig.6.7a) . Scanning electron microscopy revealed that the advancing marginal cells of the edge possessed broad leading lamellae which are similar to those of the normal blastoderm edge (Fig.6.7b).

Semi-thin sections cut through the "regenerated edge" showed that the marginal cells of the edge have all the features of the deep layer cells namely their yolky appearance and pale staining. These cells were always located in front of any advancing edge and were followed by the darkly stained ectodermal cells (Fig.6.8a). Unlike their organisation into a monolayer during their slow movement during the early stages of explants, deep layer cells form a multilayer unit with the ectodermal cells in this "regenerated edge" stage. TEM of the deep layer cells and their lamellae showed that there were similarities between them and the distal cells of the normal leading edge. Such similarities included the lack of any specialised junctions between them, the presence of cortical microfilaments and microtubules and the presence of microvillous projections which they extended into the vitelline membrane (Fig.6.8b).

By this stage, the basal lamina separating ectodermal and deep layer cells had disappeared, and ectodermal cells did make contact with the vitelline membrane irrespective of their original position in the explant. They were oriented with their long axis in the direction of movement (See Fig.6.8a).

Despite the loss of the basal lamina the ectodermal cells and deep layer cells were not completely intermingled with each other; the former remained as

a unit connected by some desmosomes and tight junctions (Fig.6.9a). Some of these ectodermal cells extended microvillous projections into the vitelline membrane and possessed microtubules as well as cortical microfilaments (Fig.6.9b).

6.2 Cultures of the blastoderm on the outer surface of the vitelline membrane

6.2.1 INTRODUCTION:

The outer surface of the vitelline membrane is composed of several layers of fibrous materials (Bellairs *et. al.*, 1963). The outer and inner surfaces of the vitelline membrane are known to be different both morphologically and chemically (Bellairs *et. al.*, 1963; Bellairs *et. al.*, 1969, Jensen 1969 and Kido and Doi, 1987). New (1959) studied the behaviour of edge cells when cultured on the outer surface of the vitelline membrane. He reported briefly that this surface of the vitelline membrane did not support the movement of the cultured edge cells. Also, Chernoff and Overton (1977) reported, in a similar study, using the scanning electron microscope, that the cultured area of the edge adheres strongly to the substratum with no detectable movement except for the occasional breaking away of some cells from the periphery of the explants. They suggested that the difference between the degree of adhesion of the edge to the outer and inner surfaces of the vitelline membrane is due to the difference between the chemical composition of the two surfaces, which could account for differences in the texture of the two.

The present section of this chapter investigates in more detail the nature of contact of the extraembryonic cells with this surface of the vitelline membrane using light and transmission electron microscopes. I will concentrate

in the first part of this section on the behaviour of pieces of the blastoderm lacking the leading edge when cultured on the vitelline membrane, in order to compare it with the behaviour of the similar tissue when cultured on the inner surface of the membrane, as described in section 6.1 of this chapter. I will then describe briefly the behaviour on the same substratum of similar pieces of the blastoderm possessing the normal leading edge cells.

6.2.2 Methods:

The experiment was the same as described in section 6.1.1 and 2.7 except that the vitelline membrane was set up so that its outer surface was now the one on which the explants were placed. In addition to culturing blastoderm pieces lacking the edge, pieces containing the edge were also cultured on this surface of the membrane.

6.2.3 Results:

6.2.3.1 Cultures of blastoderm pieces lacking a leading edge on the outer surface of the vitelline membrane:

6.2.3.1.1 Macroscopic observations:

The general situation was similar to that described in 6.1.2.1 at the early stages of the explants, with the appearance of the slightly dark ectodermal area and the lighter area of deep layer cells surrounding it from all sides, irrespective of whether the ectodermal side of explants was placed up or down. However, while in cultures of similar explants on the inner surface of the vitelline membrane the deep layer cells started to move centrifugally at a considerable rate after about 6 hours of incubation, explants on the outer surface of the vitelline membrane showed no detectable movement at this time or even later. Also, while a rapidly moving edge started to appear after no later than 20 hours in the first set of experiments, such an edge did not form in the second set of experiments even when the explants were cultured for more than 40 hours. I cannot say for sure whether initial shrinkage of these explants

occurred in this set of experiments or not.

6.2.3.1.2 Microscopic observations:

6.2.3.1.2.1 After 2 hours:

The behaviour of blastoderm pieces cultured on the outer surface of the vitelline membrane was similar whether the ectodermal side was cultured up or down. 1 um sections through 2 hour explants revealed that the lighter periphery of the explant was made of deep layer cells while the central darker area was ectodermal (Fig.6.10a).

The deep layer cells and ectodermal sheet had the same organisation as those cultured for the same period of time on the inner surface of the vitelline membrane (See 6.1.3.2.1) with only one exception related to cells in contact with the substratum. While the deep layer cells in 2 hour old explants cultured on the inner surface of the vitelline membrane spread smoothly on that substratum with only the occasional extension of microvillous projections into it, the surface of the deep layer cells in contact with the present substratum was irregular and branched at some areas into long lamellae (Fig.6.10b) which penetrated deeply in between the layers of this surface of the membrane.

6.2.3.1.2.2 After 10-16 hours:

By ten hours, the deep layer cells, instead of migrating centrifugally as those described in section 6.1.3.2.2 for cultures on the inner vitelline membrane surface, were found between the layers of the outer surface of the vitelline membrane with no such outwards migration. The surface of the vitelline membrane to which the cells attached was wrinkled and many of its layers were broken into small islands or clumps of fibrillar meshwork which were then surrounded by the branching lamellae of the cultured cells (Fig.6.10c and Fig.6.11a). These cells as well as their lamellae possessed highly ordered arrays of microfilaments (Fig.6.11b) which ended at points on the plasma membrane

juxtaposed to the substratum (Fig.6.12). Desmosomes and points of close membrane opposition were common between these cells.

The ectodermal part of these explants, whether cultured upper-most or lower, was still separated from the deep layer cells by a continuous basal lamina.

6.2.3.1.2.3 After 16 hours and more:

While explants on the inner surface of the vitelline membrane formed a rapidly migrating "regenerated edge" shortly after this stage, explants on the outer surface of the membrane showed no sign of any active migration of their cells although there was a total collapse of their ectodermal-deep layer cell organisation, after the disappearance of the basal lamina (Fig.6.13a). Even when the culturing time was extended to 2 days, no such regeneration of the rapidly moving edge occurred (Fig.6.13b). The cells of these explants caused further wrinkling and breaking down of further layers of the substratum, as more cells penetrated deeply into it. I was not able to detect any breaking away of individual cells from the periphery of the explants.

TEM revealed that, here again, bundles of microfilaments which ended in attachment points with the substratum were a familiar sight among the cells in contact with the substratum (Fig.6.13c and Fig.6.14).

6.2.3.2 Cultures of pieces of blastoderm containing a leading edge on the outer surface of the vitelline membrane:

The pattern of behaviour of these cultures was essentially the same as that described above for pieces cultured without their leading edge. In the first 2-6 hours of incubation the leading edge attached to the outer surface of the vitelline membrane, but instead of remaining flat on the substratum they formed a clump and lost their normal proximal-distal organisation (Fig.6.15a). As the edge cells attached to this substratum they exerted tractional force on it as can be judged from the wrinkling of this surface of the membrane at the

points of attachment.

TEM of edge cells at this stage showed that they behaved similarly to deep layer cells cultured for 2 hours on the outer surface of the vitelline membrane in that the lamellae which made contact with the outer layers of the substratum had very irregular shapes and branched into smaller lamellae (Fig.6.15b). The tip of one such lamella penetrating one layer of the substratum is shown in Fig.6.16a).

The sheet of ectodermal cells behaved as in normal situations in that it remained elevated from the substratum. The deep layer cells at the side opposite to the leading edge behaved in a similar way to those of explants lacking the edge described in 6.2.3.1.2.1.

After 10-17 hours of incubation, the organisation of the explants into ectoderm and deep layer cells had collapsed and both cell types came into contact with the outer layers of the vitelline membrane. Meanwhile, the leading edge cells could no longer be distinguished from the rest of the explant cells (Fig.6.16b). The cells in contact with the outer surface of the vitelline membrane behaved exactly the same as those lacking the edge in that their lamellae penetrated between the different layers of the substratum and broke them down to small clumps of fibrous material which were collected between the cells (Fig.6.16c). Here also, bundles of microfilaments ending at the cell surface in contact with the substratum were abundant in these cells (Fig.6.17a).

No change was observed in the behaviour of these explants even when the culture time was extended to 2 days (Fig.6.17b). A monolayer of cells only a few cells wide could be seen around the periphery of these explants but no detachment of individual cells from the explants was detected.

Table 6.1 **The rate of expansion of cells from blastoderm pieces cultured on the inner surface of the vitelline membrane.**

	Rate of expansion before "regenerated edge"	Rate of expansion after "regenerated edge"
1	60 μm/hr.	210 μm/hr.
2	60 μm/hr.	160 μm/hr.
3	72 μm/hr.	140 μm/hr.
4	73 μm/hr.	267 μm/hr.
5	100 μm/hr.	300 μm/hr.
6	65 μm/hr.	227 μm/hr.
MEAN ± SD	71 ± 14 μm/hr.	217 ± 60 μm/hr.

Fig.6.1- Time-lapse frames showing the progress of 2 pieces of blastoderm explants cultured on the inner surface of the vitelline membrane (a) at start of culture, (b) after 2 hours , (c) after 8 hours and (D) after 22 hours with the appearance of the regenerated edge. The centre in all frames, which appears light in these negative copies of the time-lapse film, is the ectodermal part of the explant. Bar= 500 μm.

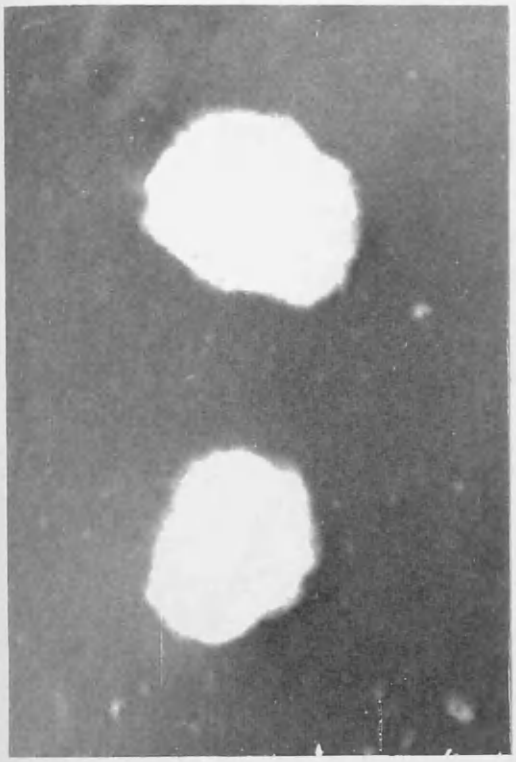

Fig.6.1 a

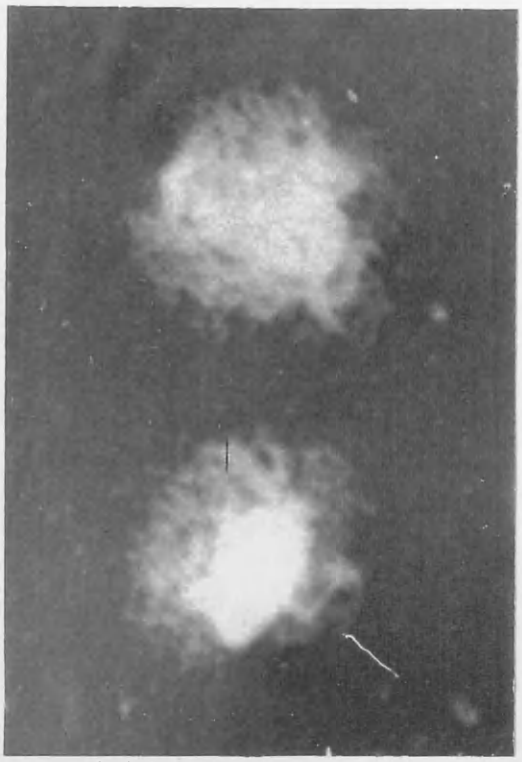

Fig.6.1 b

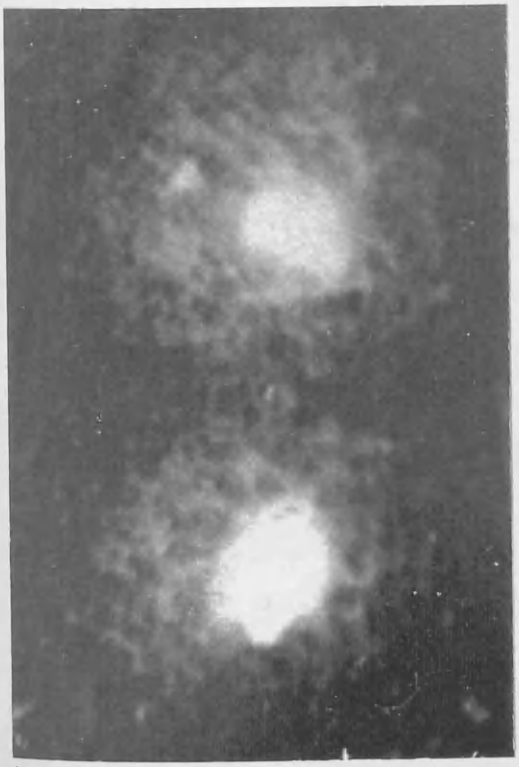

Fig.6.1 c

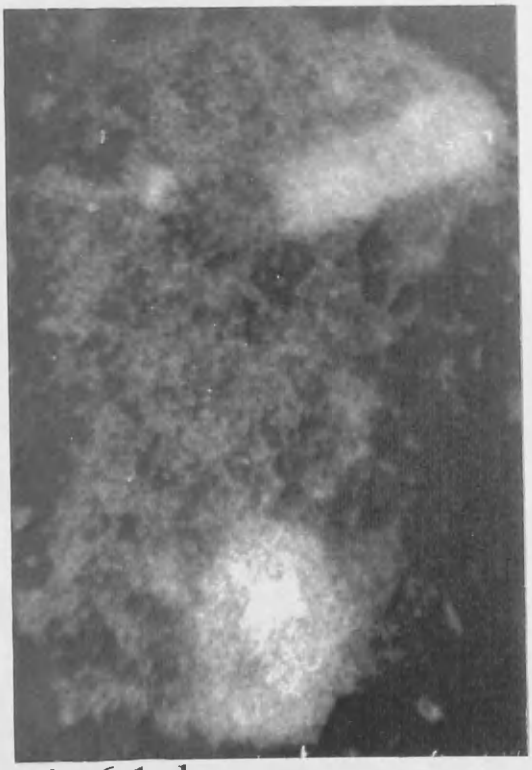

Fig.6.1 d

Fig.6.2- Camera lucida tracing of a blastoderm explant showing the evenness and extent of expansion on the vitelline membrane. Numbers indicate hours from the start of the experiment. Bar= 100 μm.

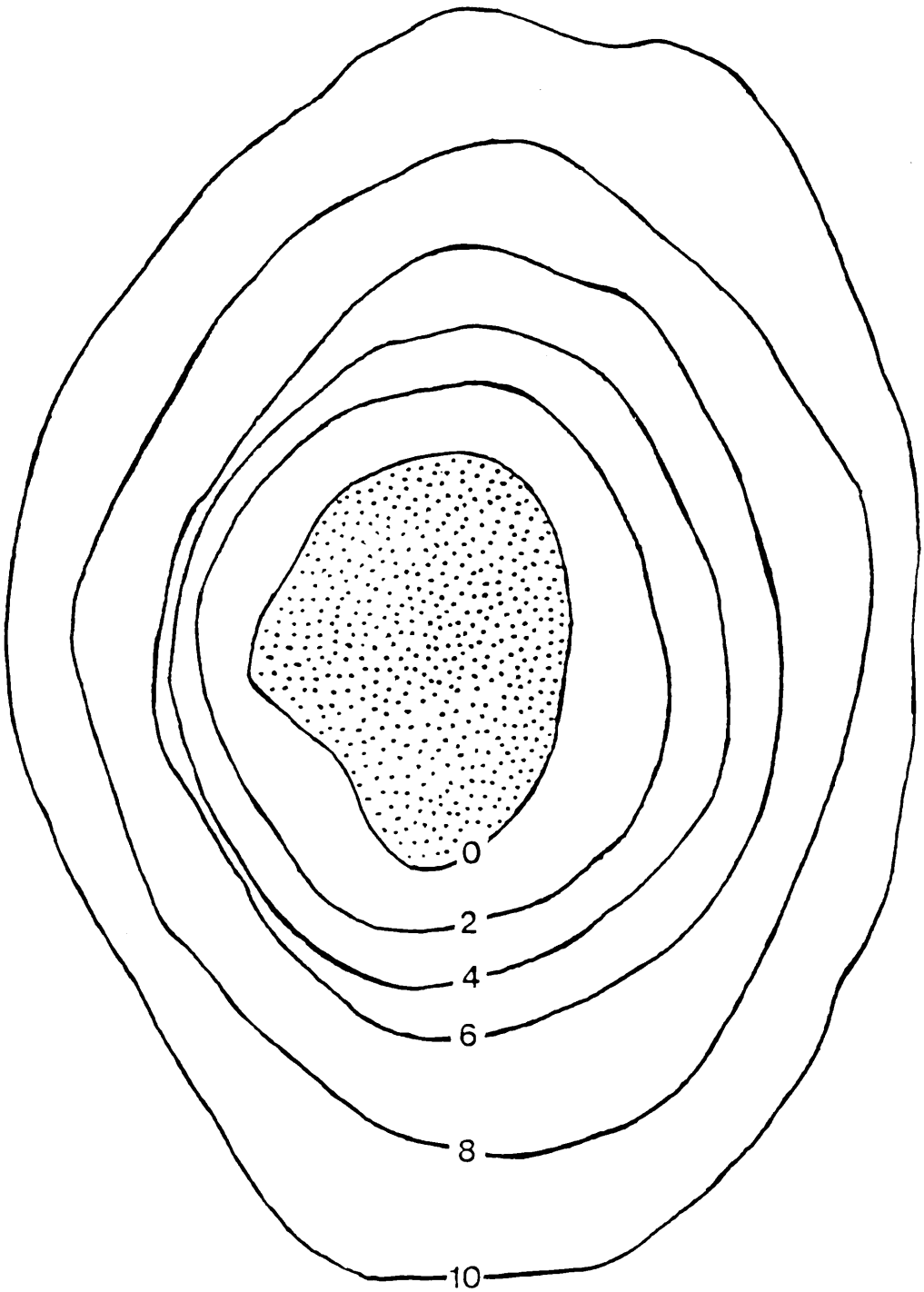

Fig.6.2

Fig.6.3 a- T.S through a piece of blastoderm cultured for 2 hours on the vitelline membrane with its ectodermal side (E) uppermost. The deep layer cells (D) started their centrifugal expansion while the ectoderm does not come into contact with the vitelline membrane (V). Bar= 50 µm.

Fig.6.3 b- TEM showing the leading lamellae (L) of deep layer cells which underlap each other over the inner surface of the vitelline membrane. Y= intracellular yolk. Bar= 1.21 µm. X 15.2k.

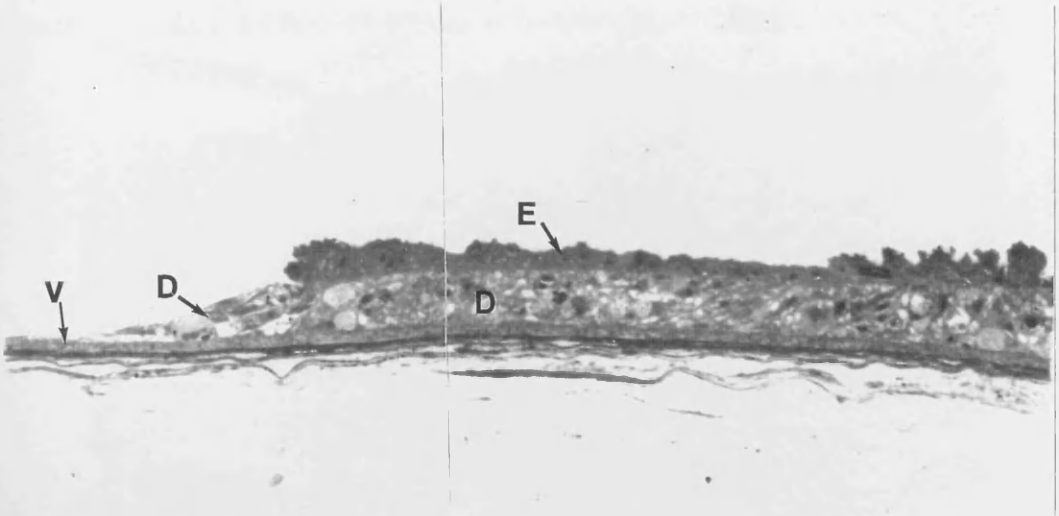

Fig.6.3 a

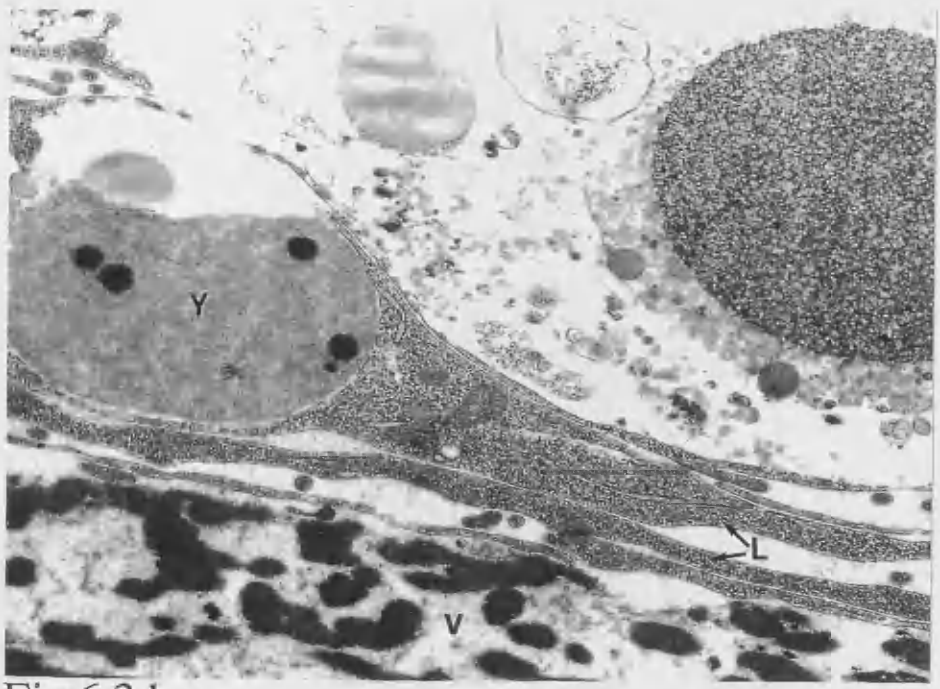

Fig.6.3 b

Fig.6.4 a- TEM of a 2-hour explant showing a microvillous projection (MP) penetrating the inner surface of the vitelline membrane (V). Bar= 0.73 μm. X 25.8k

Fig.6.4 b- TEM of a 2-hour explant showing the presence of micro-filaments (F) and microtubules (arrows) in the leading lamellae of deep layer cells. Bar= 0.73 μm. X 27K.

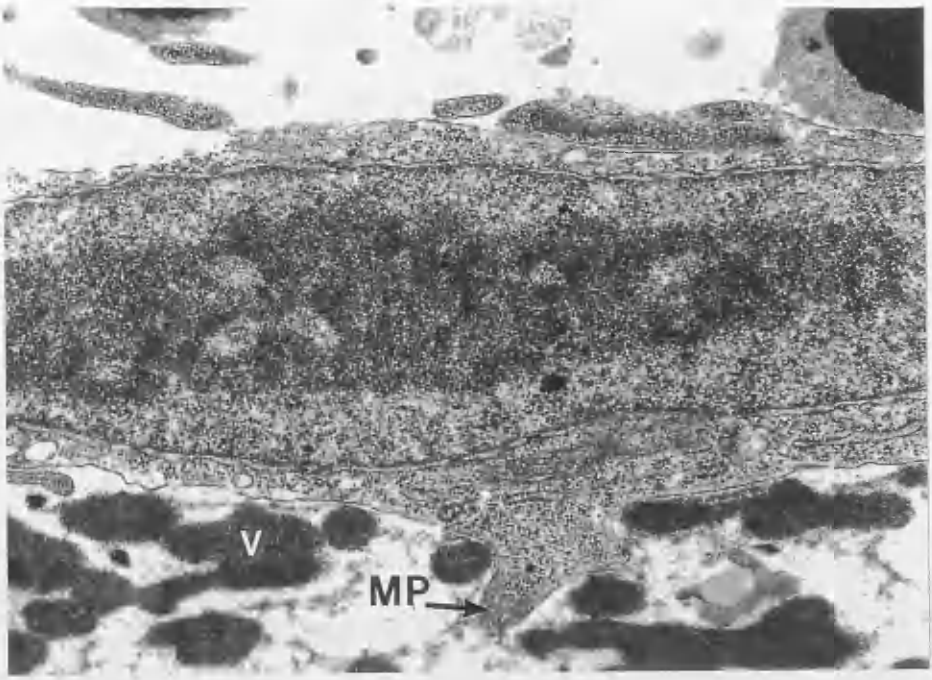

Fig.6.4 a

Fig.6.4 b

Fig.6.5 a- TEM showing the persistence of the basal lamina (BL) in a 2-hour explant. It separates the ectodermal part (E) from the deep layer cells below (D). Bar= 2.5 μm. X 7.2K.

Fig.6.5 b- Light micrograph of a 2-hour explant cultured with its ectoderm (E) facing the vitelline membrane (V). The ectoderm is elevated from the substratum while the deep layer cells (D) spread readily over it. Bar= 50 μm.

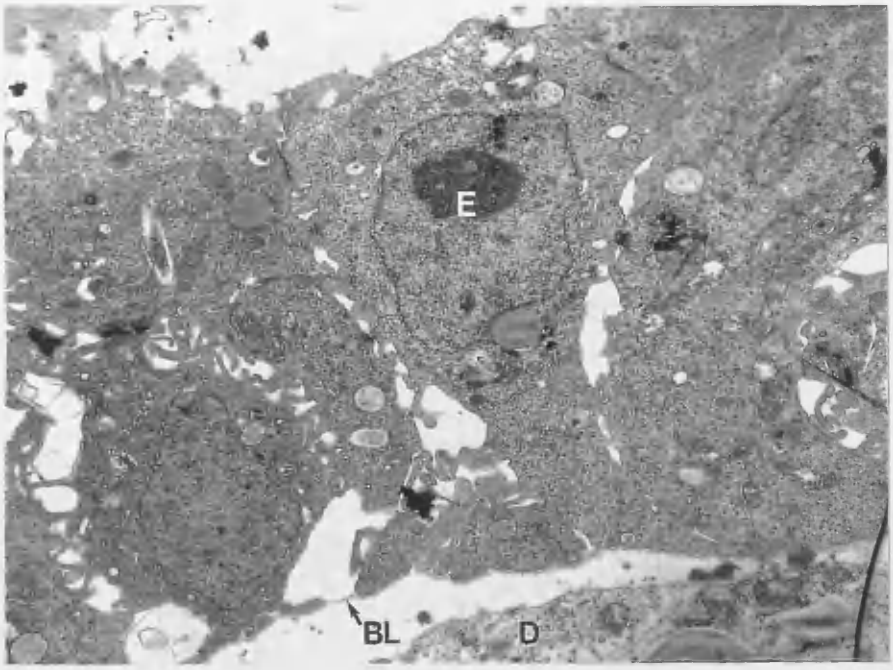

Fig.6.5 a

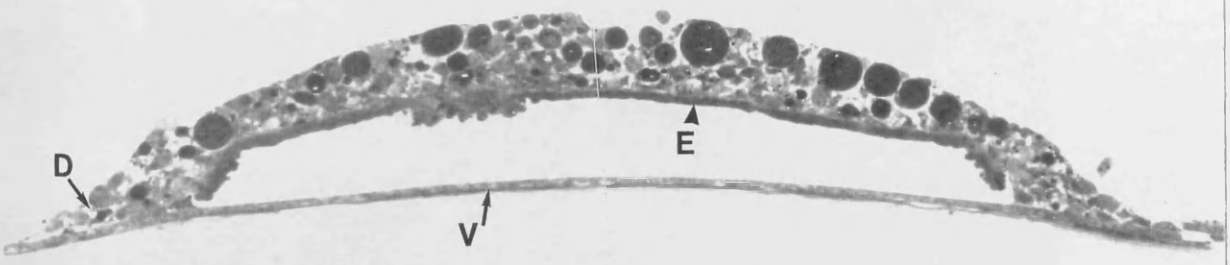

Fig.6.5 b

Fig.6.6 a- Dorsal view of an 8-hour explant showing further expansion of deep layer cells (D) on the vitelline membrane. The ectoderm (E) forms a darkly stained mass in the centre of the explant. Bar= 50 μm.

Fig.6.6 b- SEM of an explant as above showing the peripheral deep layer cells which form a monolayer (M) on the vitelline membrane (V). Bar= 10 μm.

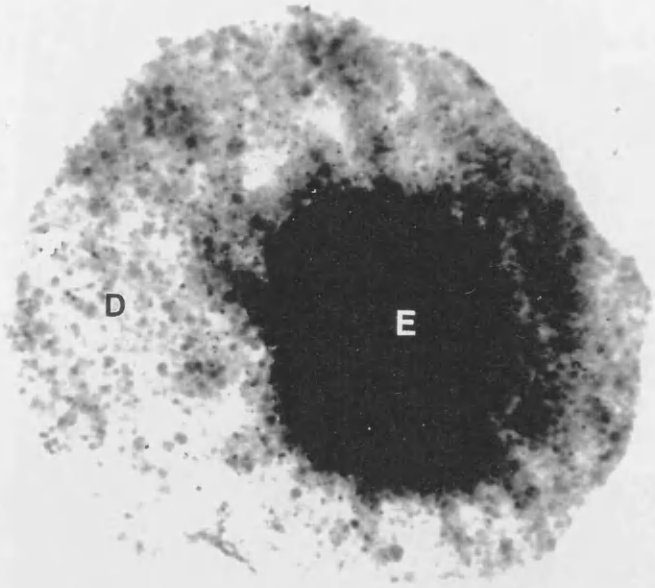

Fig.6.6 a

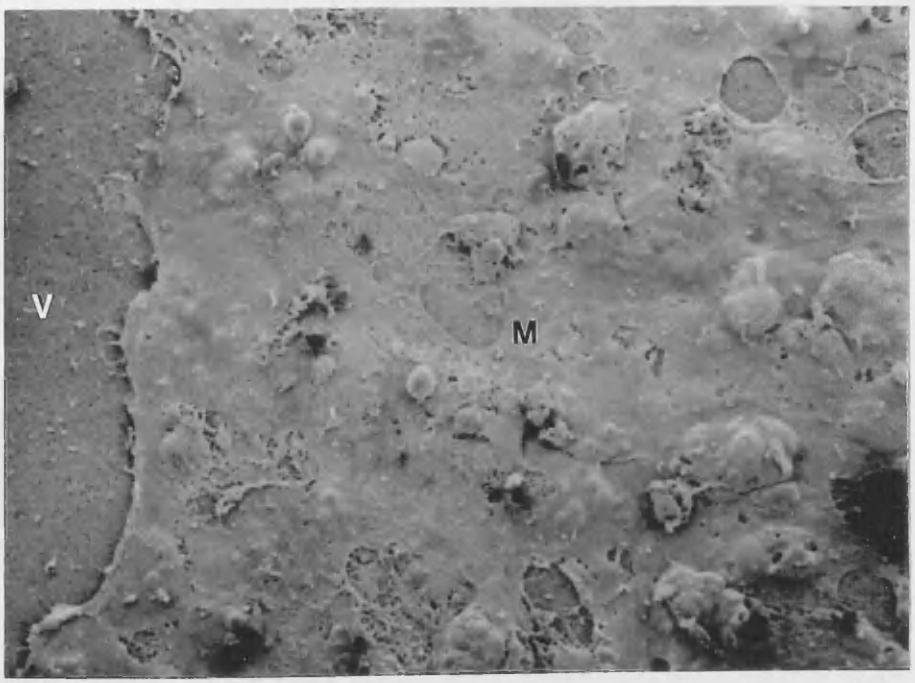

Fig.6.6 b

Fig.6.7 a- SEM of a 22-hour explant showing the formation of the regenerated edge which moves rapidly in one direction (arrow) leaving behind a trail of cells. Bar= 100 µm.

Fig.6.7 b- SEM showing the leading lamellae (L) which the peripheral cells of the regenerated edge spread on the vitelline membrane (V). Bar= 10 µm.

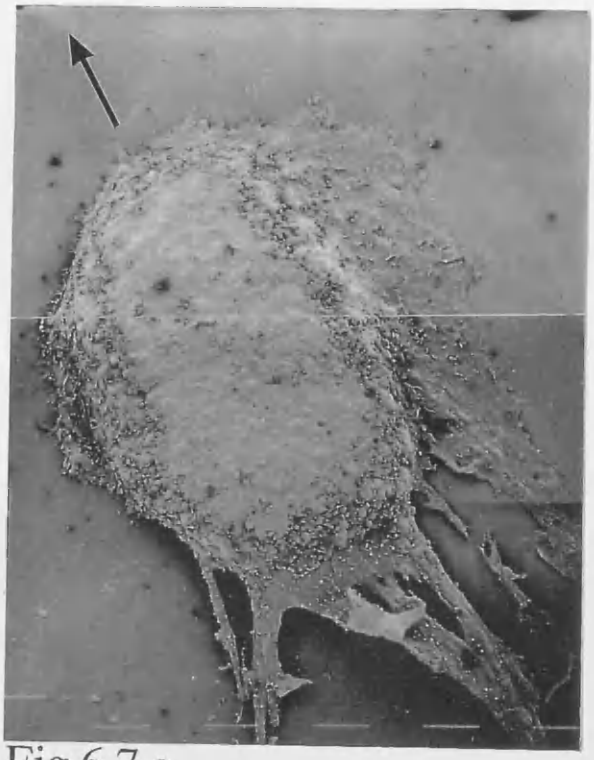

Fig.6.7 a

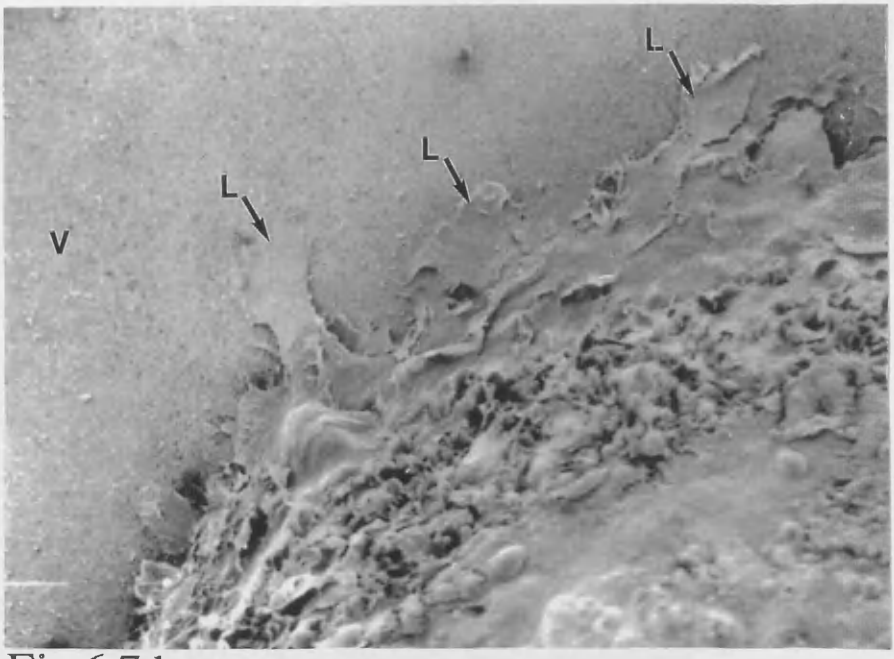

Fig.6.7 b

Fig.6.8 a- Light micrograph of a regenerated edge. Deep layer cells (D) are in front of the edge while the ectodermal cells (E) form its centre. The ectodermal cells are oriented with their long axis in the direction of movement (arrow). Bar= 50 µm.

Fig.6.8 b- TEM showing a microvillous projection from a deep layer cell (MP) penetrating the inner surface of the vitelline membrane (V). Bar= 0.29 µm. X 63.4k.

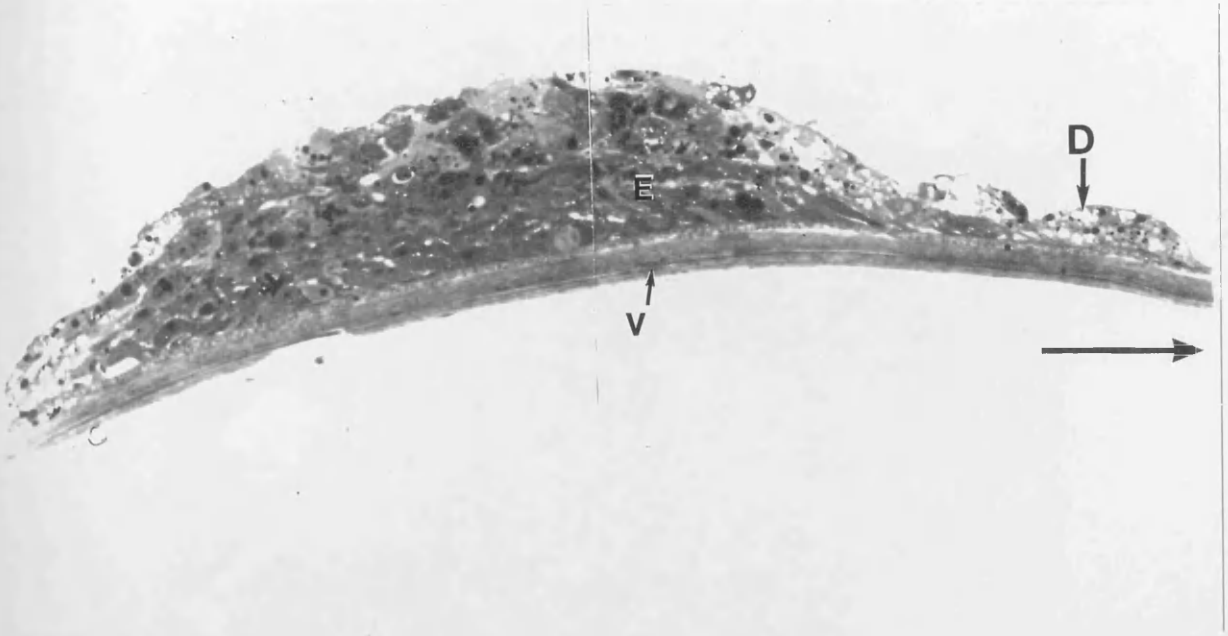

Fig.6.8 a

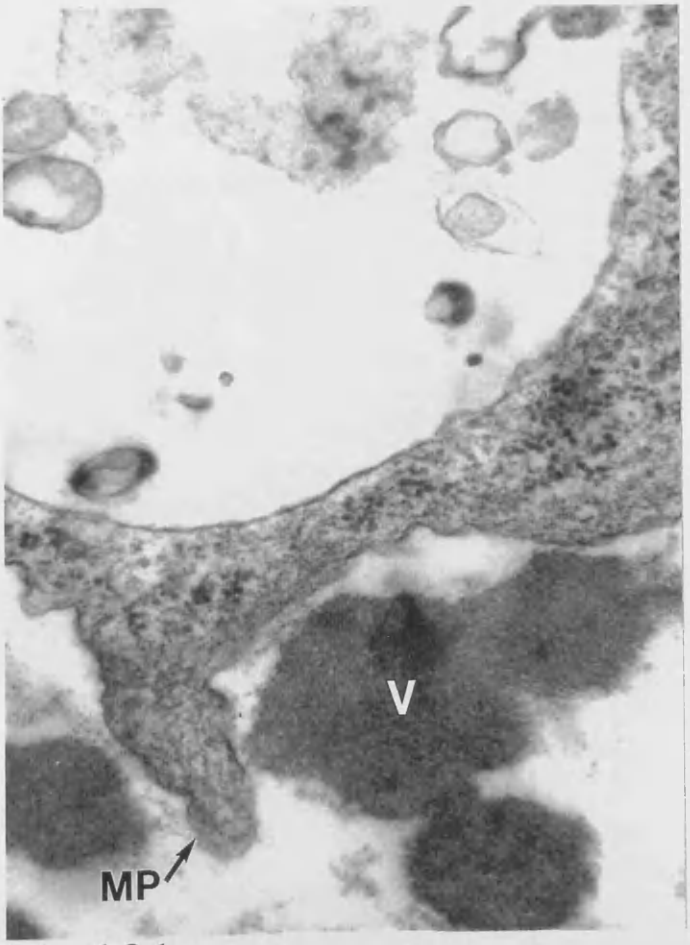

Fig.6.8 b

Fig.6.9 a- TEM showing the occasional presence of desmosomes (D) between the ectodermal cells. Points of close contact (arrowheads) are also present. Bar= 1.21 µm. X 15.6k.

Fig.6.9 b- TEM showing the cortical distribution of microfilaments (F) in the ectodermal cells. Microtubules (arrowhead) are also present and are orientated parallel to the direction of movement. Bar= 0.46 µm. X 45.4k.

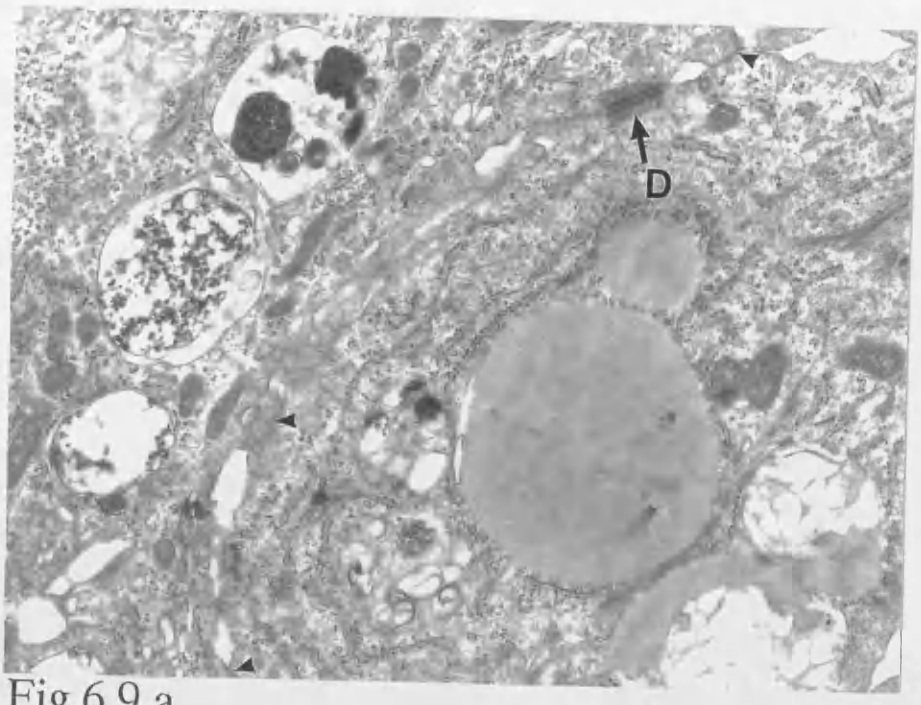

Fig.6.9 a

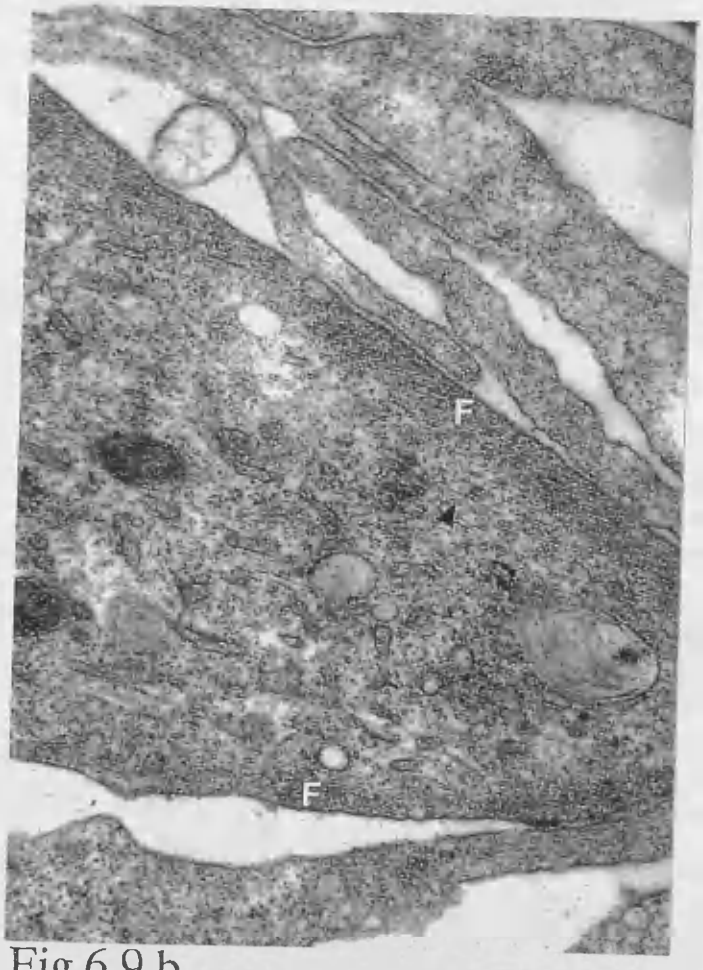

Fig.6.9 b

Fig.6.10 a- Light micrograph of a piece of blastoderm lacking the leading edge which has been cultured for 2 hours on the outer surface of the vitelline membrane (V). Only deep layer cells (D) attach to the substratum. E= ectoderm. Bar= 50 μm.

Fig.6.10 b- TEM showing the irregular appearance of the leading lamellae (L) of the deep layer cells after being cultured for 2 hours on the outer surface of the vitelline membrane (V). Bar= 2.8 μm. X 3.2k.

Fig.6.10 c- Light micrograph of a 10-hour explant showing that cells which make contact with the vitelline membrane (V) collect some of the layers of the outer surface between them. Notice that the peripheral cells of the ectoderm made contact with the substratum while the rest of the ectoderm did not. Bar= 25 μm.

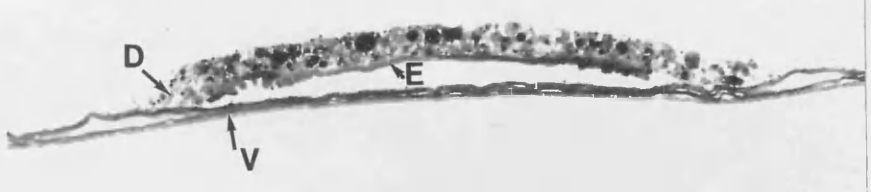

Fig.6.10 a

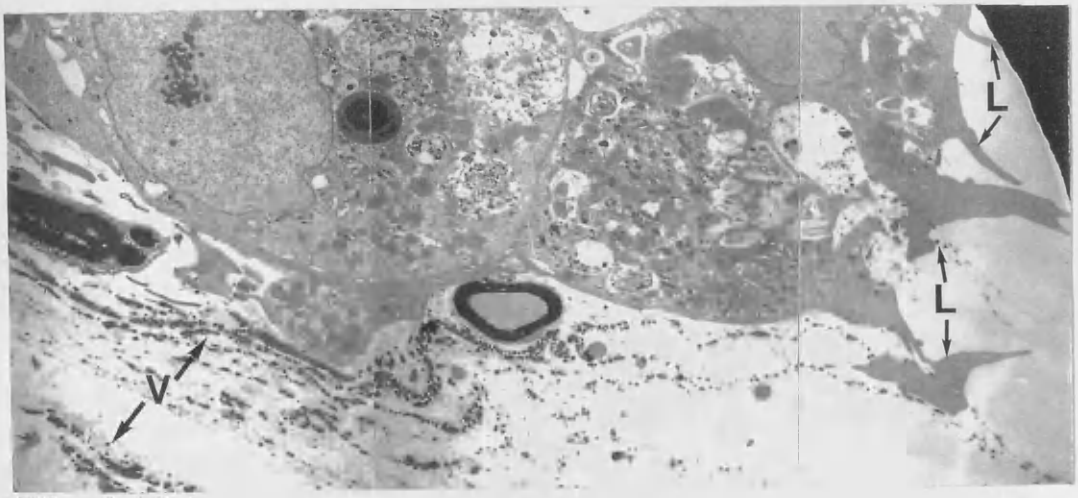

Fig.6.10 b

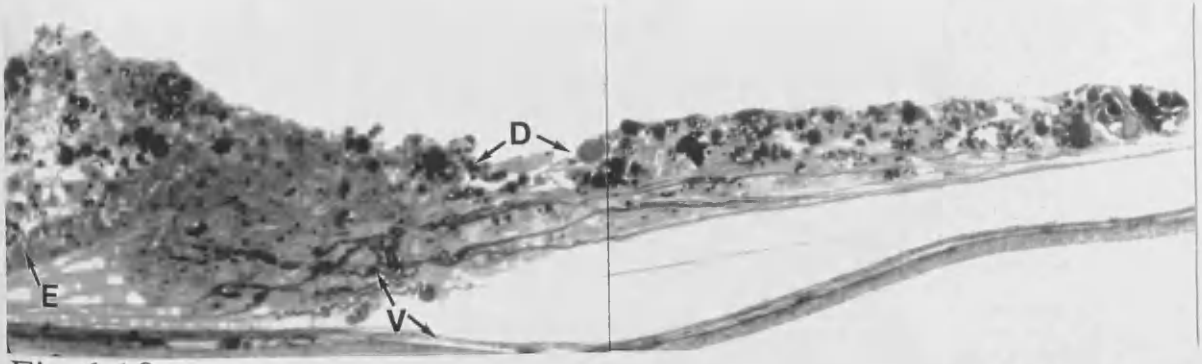

Fig.6.10 c

Fig.6.11 a- TEM showing the breaking down of the layers of the vitelline membrane outer surface (V) into clumps which are surrounded by the branching lamellae (L) of the cultured cells. The large and small areas marked in the left hand side of this are enlarged in Fig. 6.12a and 6.12b, respectively. Bar= 2.8 µm. X 6.9K.

Fig.6.11 b- TEM showing the presence of microfilament bundles (arrows) in cells in contact with the substratum. Cells are connected by desmosomes (D) Bar= 1 µm. X 19K.

Fig.6.11 a

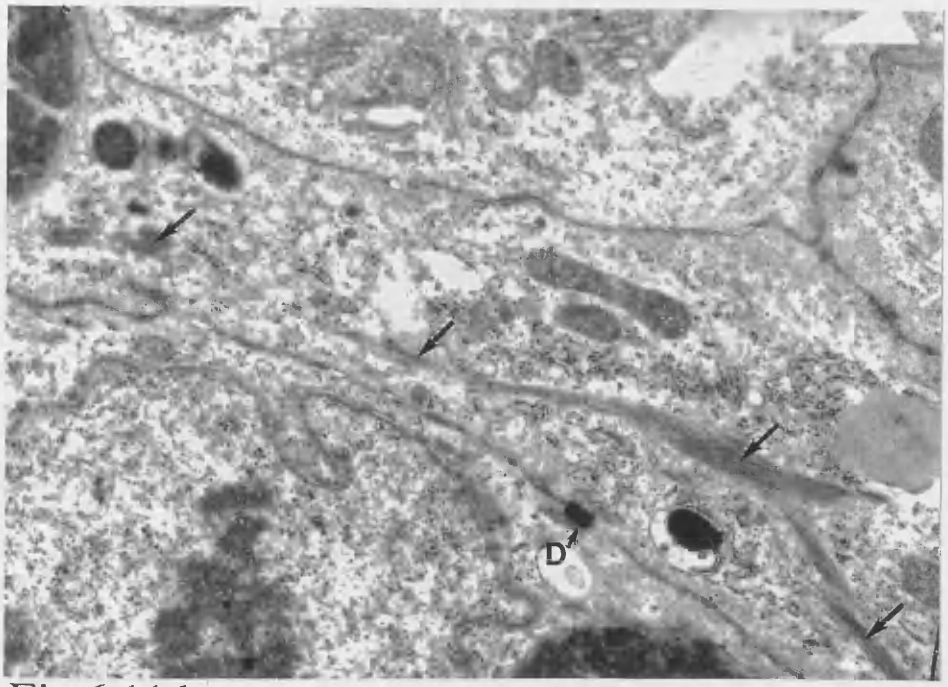

Fig.6.11 b

Fig.6.12 a- High magnification of the large area marked in Fig.6.11a showing a bundle of microfilaments (F) ending at points on the cell membrane juxtaposed to the substratum, the vitelline membrane (V). Bar= 1 μm. X 19K.

Fig.6.12 b- High magnification of the smaller area marked Fig.6.11a showing microfilaments (F) ending at the cell surface in contact with the vitelline membrane outer surface (V). Bar= 0.46 μm. X 33.5K.

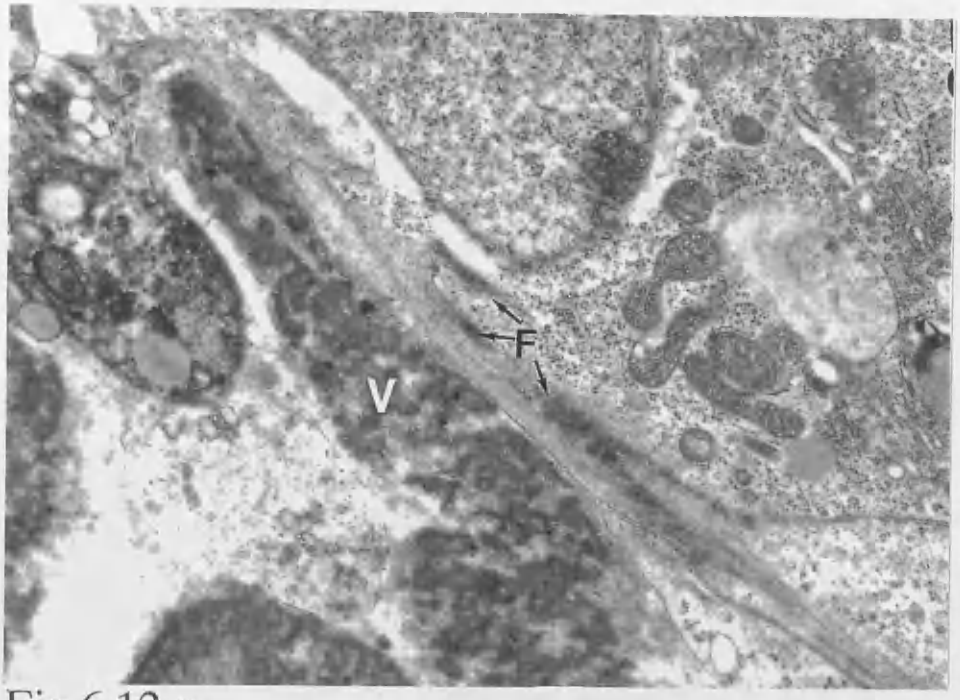

Fig.6.12 a

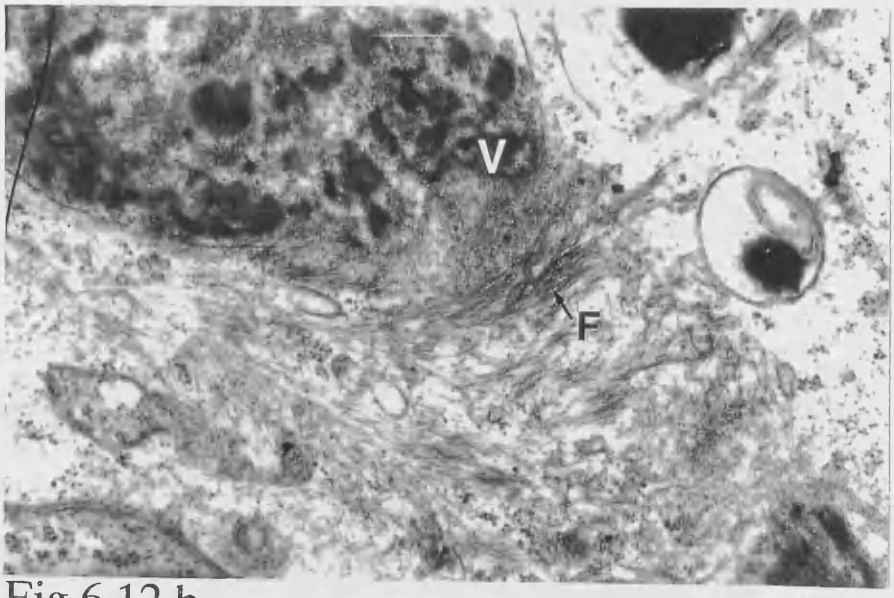

Fig.6.12 b

Fig.6.13 a- Light micrograph of an explant cultured for 17 hours on the outer surface of the vitelline membrane (V). Notice the collapse of the original organisation of the explant. Bar= 100 µm.

Fig.6.13 b- Light micrograph of an explant cultured for 45 hours on the outer surface of the vitelline membrane (V). Notice the wrinkling of the substratum at the site of explant attachment. Bar= 100 µm.

Fig.6.13 c- TEM showing a bundle of microfilaments (F) in one of the cells in contact with the substratum. From a 45-hour explant. Bar= 0.67 µm. X 28.5 K.

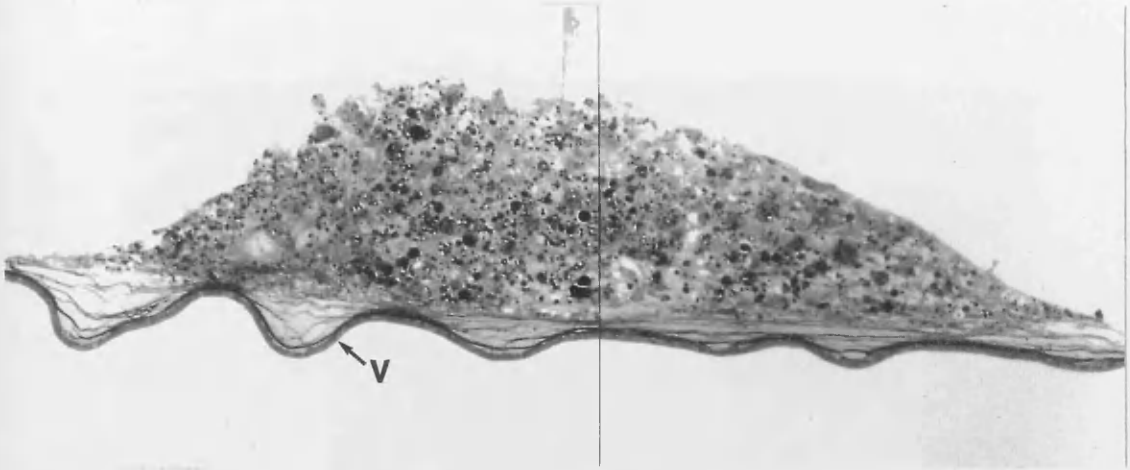

Fig.6.13 a

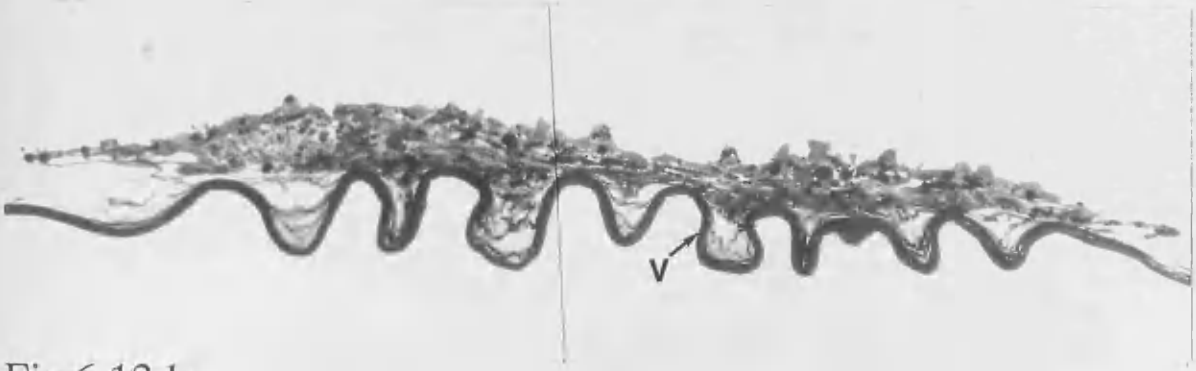

Fig.6.13 b

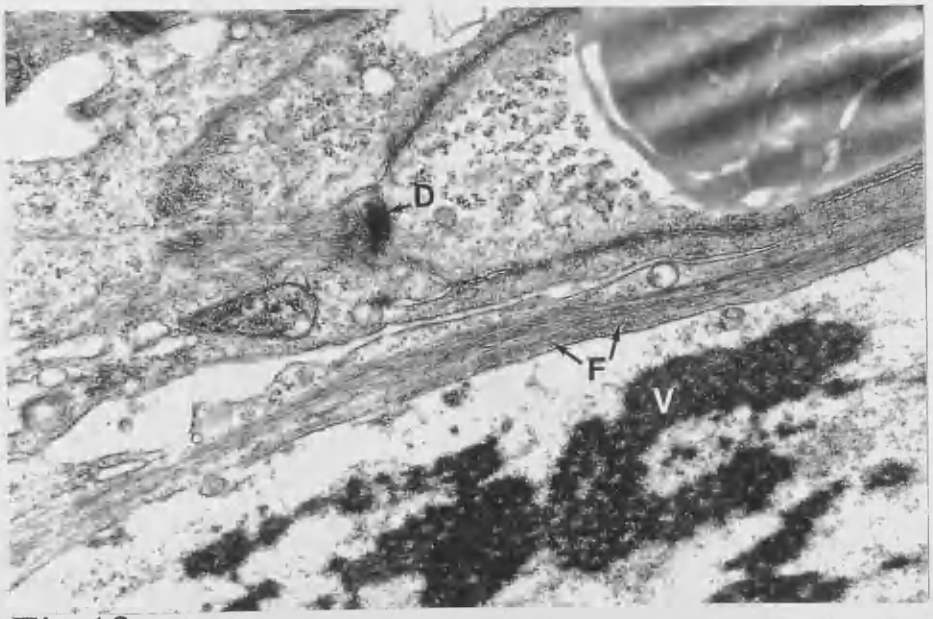

Fig.13. c

Fig.6.14 (a&b)- TEM showing the ending of microfilament bundles (F) on the surface of the cells at points of contact with the substratum (V). Bar in (a)= 0.29 μm. (X 5.4k) and in (b)= 0.29 μm. (X 52.5k).

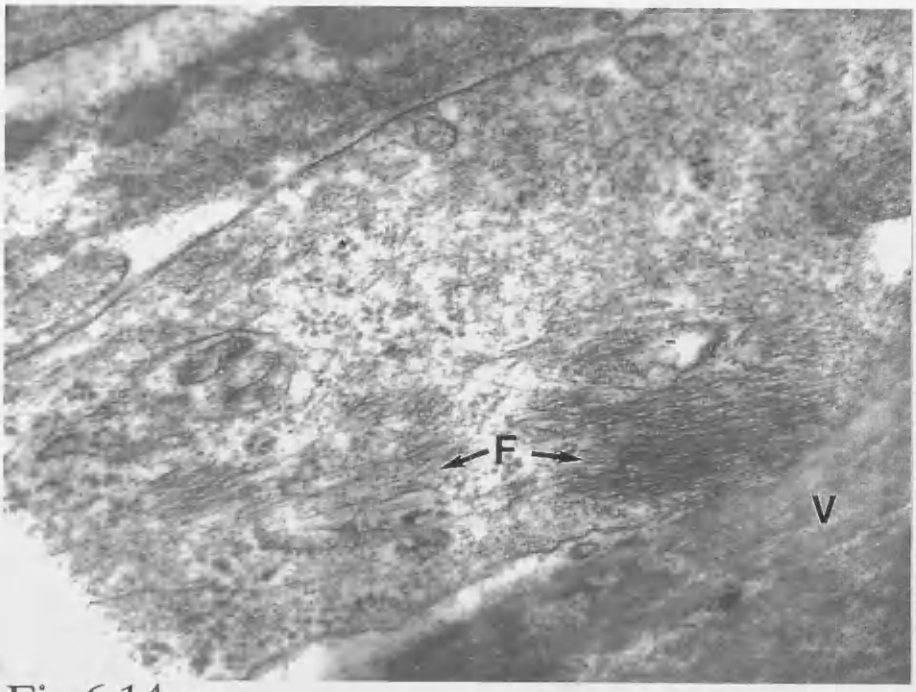

Fig.6.14 a

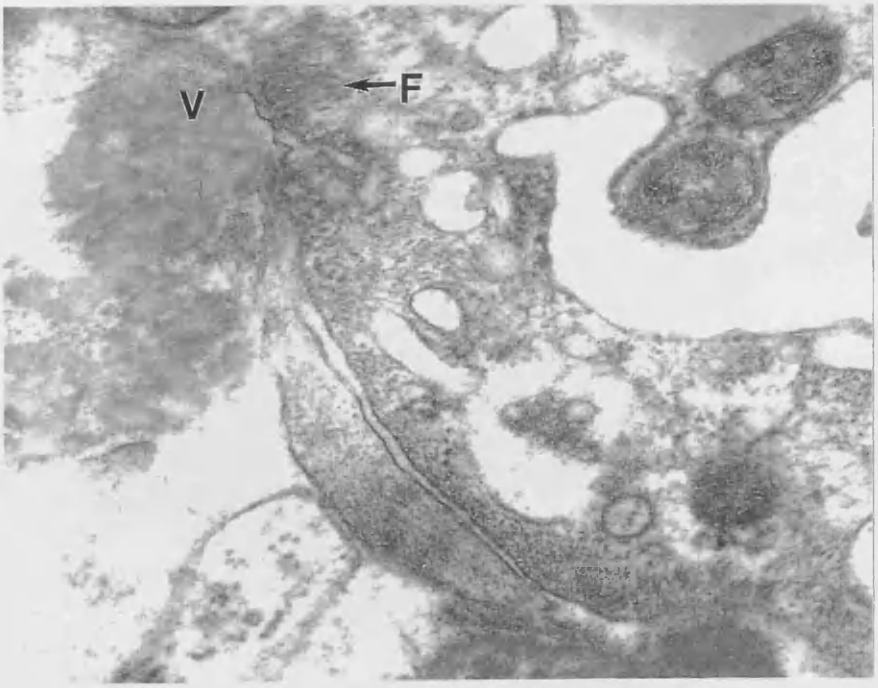

Fig.6.14 b

Fig.6.15 a- Light micrograph of a blastoderm explant containing the leading edge cultured for 2 hours on the outer surface of the vitelline membrane (V). Notice that the leading edge cells (Le) had lost their usual proximal distal organisation and are reduced into a clump of cells. The ectoderm (E) behaves as usual in that it does not come into contact with the vitelline membrane. Bar= 50 µm.

Fig.6.15 b- TEM of the area marked in (a) showing the penetration of the irregular lamellae of the edge cells (L) into the layers of the vitelline membrane and their branching between the layers. Bar= 2 µm. X 7.7k.

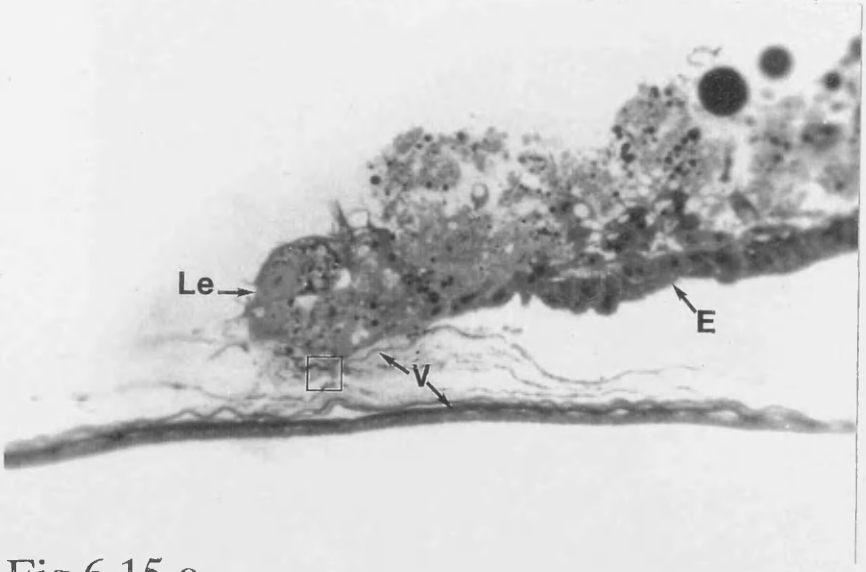

Fig.6.15 a

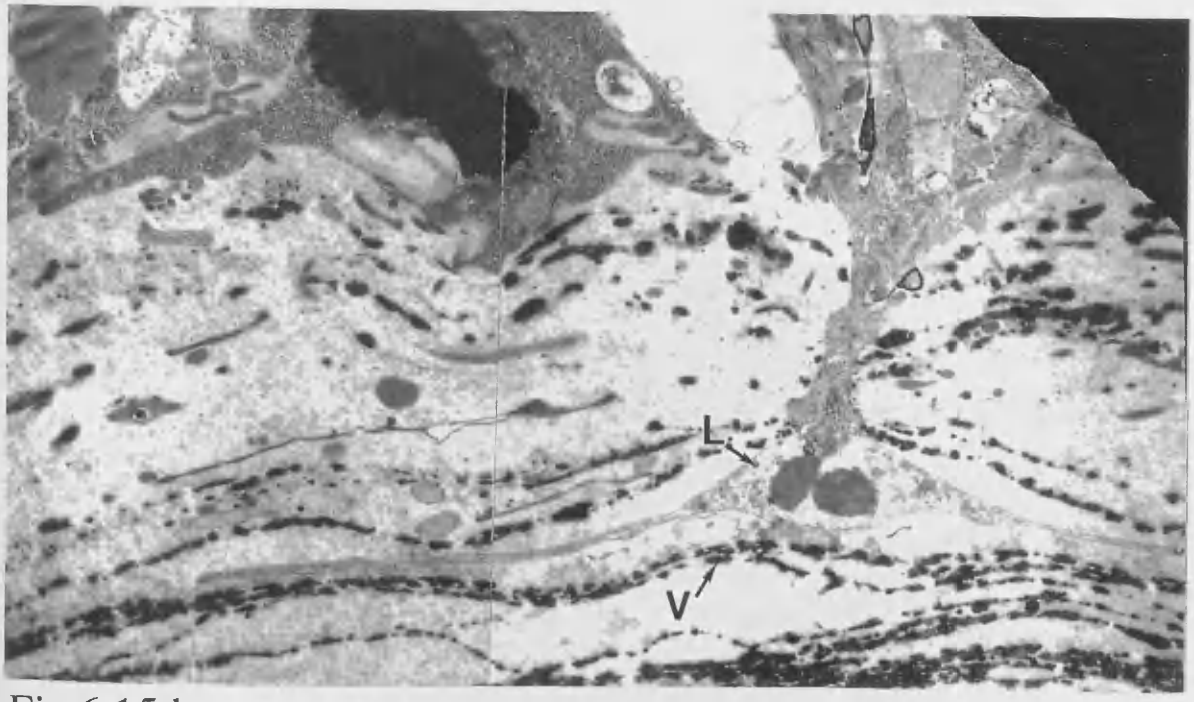

Fig.6.15 b

Fig.6.16 a- TEM showing the tip of one of the lamellae of a leading edge cell (L) penetrating a layer of the outer surface of the vitelline membrane (V). Bar= 0.46 μm. X 33.5K.

Fig.6.16 b- Light micrograph of a 17-hour blastoderm explant originally containing a leading edge cultured on the outer surface of the vitelline membrane. The original organisation of the explant has collapsed. Bar= 100 μm.

Fig.6.16 c- TEM showing the breaking down of the layers of the outer surface of the vitelline membrane (V) as the cultured cells penetrate it (arrowhead). Marked area is enlarged in Fig.17a. bar= 2 μm. X 9.25K.

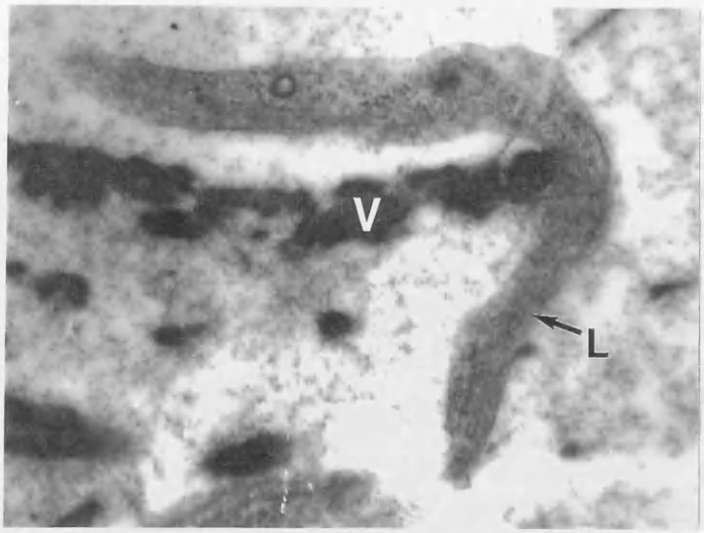

Fig.6.16 a

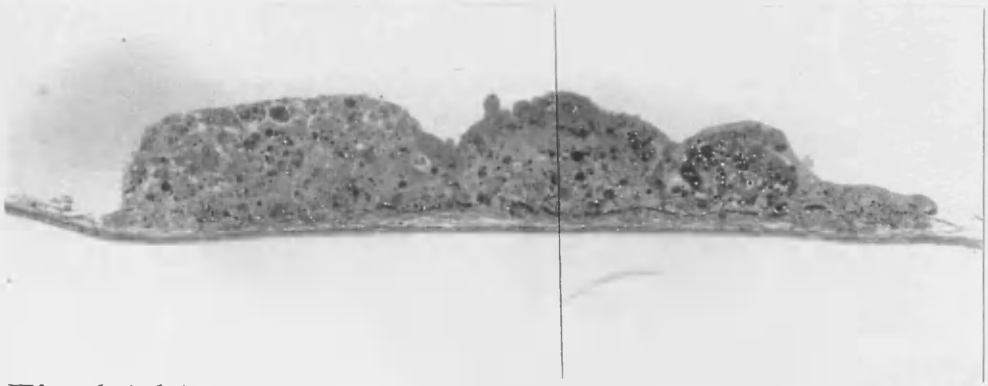

Fig.6.16 b

Fig.6.16 c

Fig.6.17 a- TEM showing the ending of microfilament bundles (F) at the cell surface which makes contact with the substratum (V). Bar= 0.67 µm. X 27K.

Fig.17 b- Light micrograph of a part of a 45-hour explant showing that even when a few cells leave the explant site, they do not seem to break away as individual cells but are forming a monolayer (M). Bar= 100 µm.

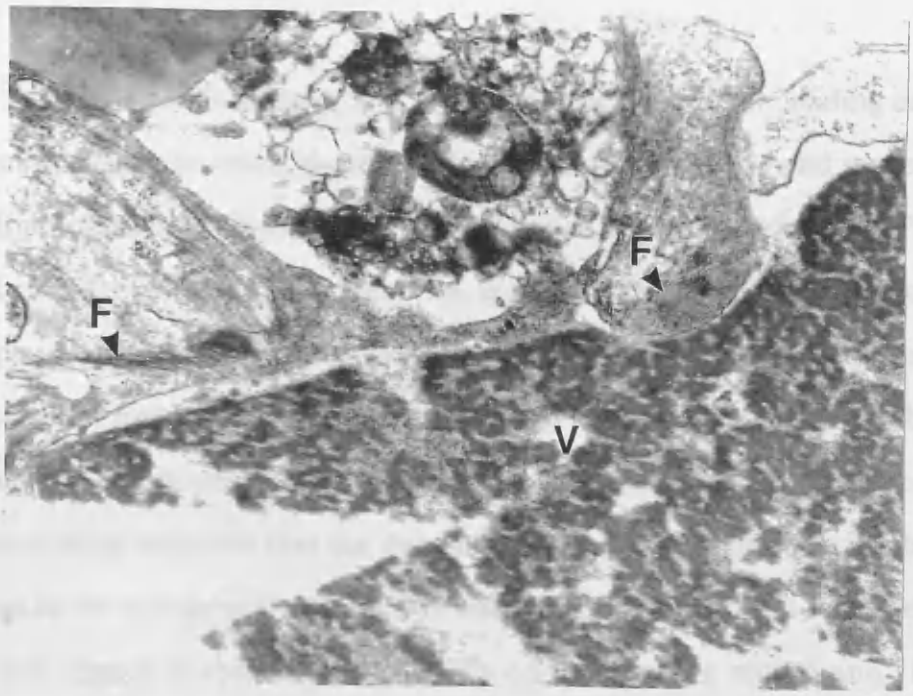

Fig.6.17 a

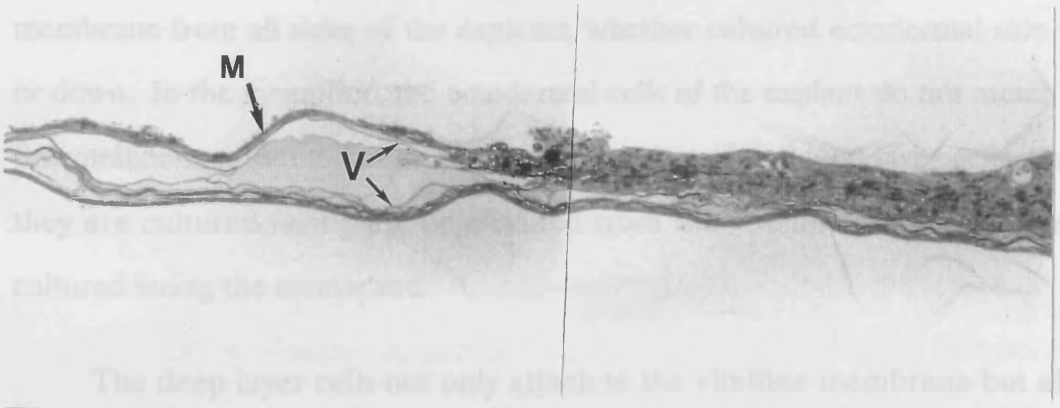

Fig.6.17 b

6.3 DISCUSSION

As discussed in chapter 3 of this thesis, the cells of the leading edge are the only cells of the chick blastoderm that normally attach to and move on the vitelline membrane (Bellairs, 1963; Bellairs *et. al.*, 1969; Downie & Pegrum, 1971 and Downie, 1976). Here we report that, given enough time and adequate conditions, pieces of the blastoderm cultured on the inner surface of the vitelline membrane without their normal leading edge have the ability to attach to and move on that substratum. Also, chapter 4 provides some ultrastructural evidence that the deep layer cells which are present on the basal lamina of the ectodermal cells are the most likely source of cells being recruited into the edge as it spreads centrifugally on the vitelline membrane. Results obtained by observing the behaviour of the cells of the explants described above give some experimental support to the findings presented in chapter 4.

As early as 2 hours after the explants have settled on the inner surface of the vitelline membrane the deep layer cells start to attach to the vitelline membrane from all sides of the explants, whether cultured ectodermal side up or down. In the meantime, the ectodermal cells of the explant do not attach to the vitelline membrane but instead remain on top of the deep layer cells when they are cultured facing up, or elevated from the vitelline membrane when cultured facing the membrane.

The deep layer cells not only attach to the vitelline membrane but also move centrifugally over it. As they do so they bear all the characteristics of the leading edge cells. These include: (A) the leading lamellae of these cells are arranged so that each lamella underlaps the cell positioned in front of it, (B) The presence of cortical microfilaments under the dorsal and ventral surfaces of the cells, (C) Microtubules are abundant and are oriented towards the direction of movement, (D) The presence in the cultured deep layer cells of

microvillous projections which penetrate into the inner surface of the vitelline membrane and which are thought to give the cells a grip on the substratum and to allow their forward movement.

Further evidence that the deep layer cells are the more likely source of cells recruited into the normal leading edge comes from the fact that when the blastoderm explants were placed so that the deep layer cells are in contact with the vitelline membrane they immediately attach to it and start their movement. However, when the explants were placed so that the ectoderm is in direct contact with the vitelline membrane it does not attach to it but instead elevates itself from it as deep layer cells leave the basal side of the ectoderm and attach to the vitelline membrane. This suggests that the deep layer cells are originally adaptable to movement on the vitelline membrane while the ectodermal cells are normally not.

The ectodermal cells in both types of explants i.e. when the ectodermal side was up or down do not make contact with the vitelline membrane until they lose their basal lamina. This occurs at a time when the deep layer cells have already moved a considerable distance from the site of the explant. As long as the ectodermal cells maintained their polarity, with microvilli and tight junctions at the apical side and basal lamina at the basal side, they were unable to attach to the vitelline membrane. Ultrastructural examination of normal blastoderms showed that the polarity of the ectodermal cells is maintained throughout the early stages of blastoderm expansion. This , therefore, gives more support for the conclusion presented in chapter 4 that the ectodermal cells of intact blastoderms, in their normal polarised form, are a very unlikely source of cell recruitment into the leading edge.

Although the ectodermal cells eventually lose their basal lamina and attach to the vitelline membrane, they do not intermingle completely with the rest of the deep layer cells but instead they sort themselves out at the centre of the "regenerated edge" by maintaining some desmosomes and tight junctions

between them. Despite these junctions the ectodermal cells align themselves in the direction of movement and seem to contribute to the movement of the whole "regenerated edge". This late behaviour of ectodermal cells i.e. their loss of basal lamina and attachment to the vitelline membrane seems to relate to the attachment of these cells to the vitelline membrane during the late stages of blastoderm closure. In both cases, these cells are able to attach to the vitelline membrane only after the loss of their basal lamina as well as the strong attachments between them, in the form of apical tight junctions, desmosomes and interdigitations of their lateral plasma membranes. However, while ectodermal cells attach very strongly to the vitelline membrane at the late stages of epiboly, causing distortion of this membrane, they behave differently in the regenerated edge stage of the explants reported above. The only difference between these cells in the two systems is the presence (in the "regenerated edge" stage) or absence (in the late stages of epiboly) of the deep layer cells. It seems that the presence of the latter cells is crucial for the ability of ectodermal cells to move on the vitelline membrane. In fact, preliminary experiments (not included in this thesis) showed that if a sheet of pure ectodermal cells was cultured on the inner surface of the vitelline membrane, it failed to show any active movement even after a prolonged time in culture, but when deep layer cells are included, as in these explants (6.1), the ectodermal cells were able to contribute to the formation of the rapidly moving "regenerated edge".

A final point on the behaviour of blastoderm pieces on the inner surface of the vitelline membrane is the change in the rate of expansion of cells of these explants before and after the formation of the "regenerated edge". Before discussing this, however, it would be useful to compare the organisation of the normal edge cells at the early stages of epiboly with that at the later stages and how that relates to the rate of blastoderm expansion at these two stages. One would notice that at the early stages of epiboly (before the edge reaches the equator of the egg) the edge has a multilayered organisation and the rate of

expansion is very rapid. However, at the late stages (after the edge has passed the equator) the rate of expansion drops sharply, as can be deduced from the period needed for the edge to reach the other end of the egg. This slowing down in the rate of expansion occurs at the time when the edge cells become reorganised into a monolayer. It seems, therefore, that the density and multilayering of the edge are essential for its rapid rate of expansion. I believe that the change in the rate of movement of cells before and after the formation of the regenerated edge follows the same pattern; the monolayer of deep layer cells migrates relatively slowly when compared to the rapid movement of the more efficient multilayered regenerated edge.

The results obtained from the set of experiments described in section 6.2 of this chapter demonstrate for the first time a detailed description of the behaviour of chick blastoderm cells cultured on the outer surface of the vitelline membrane, based on light and transmission electron microscopy.

The deep layer cells of the blastoderm pieces, cultured with their ectoderm up or down on the outer surface of the vitelline membrane, show once again their consistent behaviour discussed above namely by being always the first to attach to the vitelline membrane. However, when it comes to their next step, which is their centrifugal expansion, they are prevented from doing so.

Although the molecular differences between the two surfaces of the vitelline membrane have not been completely elucidated, Cook *et. al.* (1985) reported the exclusive localisation of an endogenous lectin in the outer layer of the membrane. They suggested that this lectin may be involved in the transport of solutes across the vitelline membrane as well as in providing bactericidal properties to the egg. They also suggested that since it is absent in the inner layer of the membrane, it has no role to play during blastoderm expansion. Lash *et. al.* (1990) suggested that the presence of this lectin in the outer layer of the vitelline membrane may have played an important role in preventing the

migration of blastoderms on this surface of the vitelline membrane, as outlined in the experiments of New (1956) and Chernoff and Overton (1977).

The results reported in the current work, although they do not contradict the previous reports, suggest another more likely cause for the lack of movement of extraembryonic cells when cultured on the outer surface of the vitelline membrane. This is the physical properties of this surface of the membrane.

It is well documented that while the inner surface of the membrane, on which edge cells normally move, is made of a meshwork of fibres 0.2-0.6 um in diameter , the outer surface of the vitelline membrane is made of several layers of a meshwork of fibres only 15 nm in diameter. I believe that while the inner surface of the vitelline membrane provides a suitable substratum for the extraembryonic cells to crawl on, the outer surface of this membrane is too fluid to withstand the crawling movement of these cells and in turn its layers of fine fibres break down under the tractional force of the cultured cells. As one layer of this surface breaks down, another layer becomes available for the cells to attach to and attempt to move on, and the cycle is repeated again and again. Therefore, the centrifugal movement of the blastoderm cells, either edge or non-edge cells, is prevented by the lack of a suitable substratum.

Harris and co-workers (1980 & 1981) showed that highly motile cells, such as leukocytes do not deform their silicone rubber substratum, while the less active fibroblasts cause extensive wrinkling of the rubber indicating that the cells are under tension. Burridge (1981) suggested that this tension is generated by stress fibres which end in strong adhesion points with the substratum. Light and TEM revealed that extra-embryonic cells cultured on the outer surface of the vitelline membrane cause not only the wrinkling of the substratum but also the collapse of the fibres which constitute this substratum. It seems very likely that these cells behave like fibroblasts cultured on silicone rubber in that they adhere strongly to the substratum by strong adhesion points

and that they are under tension. The latter suggestion is based on the fact that highly ordered arrays of microfilaments, which correspond to stress fibres in stationary fibroblasts, are very familiar in the cytoplasm of these cells. Here, however, tension is generated in response to a too fluid substratum which does not provide enough resistance to the tractional force exerted by the cells. The latter, therefore, cannot move forward but instead distort the substratum.

As a confirmation for the above suggestion that the bundles of microfilaments described above are a result of an unusually strong association between the cultured extra-embryonic cells and their above substratum, when such cells are cultured on the inner surface of the vitelline membrane (see 6.1.2) they display no such bundles of microfilaments and cause no apparent wrinkling to this substratum, indicative of less attachment of these cells to that substratum. Indeed, if we look back at the behaviour of cells during the early stages of chick epiboly (3.3) we notice no such bundles in the edge cells; however, during the late stages of epiboly we find that there are dense plaques between the stationary edge cells and their substratum and that bundles of microfilaments are apparent in the microvillous projections which penetrate into the vitelline membrane's inner surface. We also notice that the vitelline membrane is wrinkled at areas of stationary cell attachment. Such wrinkling of the inner surface of the vitelline membrane has also been observed during the stage at which ectodermal cells occupy the space of the dead edge cells and I expect these cells to possess similar bundles of microfilaments.

This work on the behaviour of extra-embryonic cells on the outer surface of the vitelline membrane, although it does not question the previous suggestions as to the cause of the absence of active movement of explants on this substratum, concludes that the lack of such movement seems most likely to be because a combination of two interrelated factors; a) the lack of enough resistance of substratum to the tractional force exerted by the attached cells which in turn results in the lack of enough purchase for the cells to move and b)

the cells stick more strongly to the substratum in the "hope" that they may move, however, the stronger the attachment becomes the more stationary the cells become. The different layers of the outer surface of the vitelline membrane break down as a result of the continuous tractional force of the cultured cells.

CHAPTER SEVEN

Immunofluorescent staining for Microtubules, Microfilaments and Fibronectin in the chick blastoderm, and effects of colchicine and cytochalasin D on blastoderm cell shape

7.1 Microtubules:

7.1.1 INTRODUCTION:

The dependency of chick epiboly on an intact system of microtubules in its leading edge cells has been subject to several investigations. However, the results of such investigations do not provide general agreement on this subject, just as there has been no agreement as to the importance of microtubules in maintaining the integrity of the epithelial cells and their ability to move in a particular direction (Vasliev *et. al.*, 1970; Euteneur and Schliwa, 1984 and Wong and Gottlieb, 1988). Downie (1975) treated early chick blastoderms in New culture with the microtubule-disturbing drug colchicine and reported that such treatment led to the arrest of blastoderm expansion. He suggested that such arrest of blastoderm expansion was a result of the effect of the drug on the epiblast, ectodermal, cells rather than the cells of the leading edge since migration of the latter cells will continue in the presence of colchicine when the centre of the blastoderm is excised. The drug is thought to interfere with the flattening of the ectodermal cells, thus preventing the whole sheet from expanding.

Chernoff and Overton (1979) made a similar study on mainly dissociated chick blastoderm cells from stage 1 or stage 7-9 (H&H) embryos *in vitro* using colcemid and cold treatment. Their results supported the findings of Downie (1975) in suggesting that microtubules are of little importance in the movement of chick blastoderm edge cells.

Mareel *et. al.* (1984) made another study on the importance of the microtubule system on blastoderm expansion but their results contradict both those of Downie (1975) and Chernoff and Overton (1979). They reported that treatment of intact blastoderms as well as fragments of the blastoderm edge with the microtubule inhibitors taxol and Nocodazole led to a rapid arrest of their expansion, suggesting that microtubules play an important role in the migration of the blastoderm edge and, therefore, blastoderm expansion. Contrary to the finding of Downie (1975) that blastoderm expansion resumes if the centre of colchicine-treated blastoderms is excised, Mareel and co-workers claimed that, in their treated blastoderms, edge migration is halted even if the centre of the blastoderm is excised, indicative of the importance of microtubules in the ability of edge cells to migrate.

The original aim of the current work was to study microtubule distribution in normal and colchicine-treated intact blastoderms attached to their substratum, the vitelline membrane, using indirect immunofluorescent staining with special reference to the distribution of microtubules in the leading edge cells. However, this aim was hampered by the simple fact that microtubule distribution was very difficult to study in intact edges in the presence of the vitelline membrane which becomes opaque during tissue processing (Fig.7.1). The aim was therefore changed to studying the same problem but on a glass substratum. In addition, only on the effect of the drug on the shape of the edge cells was studied and not their migration, since the leading edge cells do not migrate as well on a glass substratum as they normally do on the vitelline membrane. The effect of the drug on normal shape of the deep layer cells was also investigated.

7.1.2 Methods:

The detailed methods followed are outlined in 2.5.1 & 2.5.2. Basically, 1-2 day old blastoderms were removed from the vitelline membrane and pieces of blastoderm including an edge or lacking it were cultured on glass coverslips.

These were left to attach to the substratum for 2 to 48 hours then treated with colchicine (0.1 μg/ml) for different times to study the effect of the drug on the shape of edge and deep layer cells. In some instances, the drug was added to the culture medium before attachment to see if that interferes with the ability of edge and deep layer cells to attach to their substratum. Control and experimental cultures were fixed and stained for microtubule distribution by indirect immunofluorescence.

7.1.3 **RESULTS:**

7.1.3.1 The normal distribution of microtubules *in vitro*:

Edge cells grown on a glass substratum for 2-48 hours spread broad leading lamellae which are very rich in microtubules. Individual microtubules extend from the nucleus to the tip of the spreading lamellae. In the pioneer edge cells i.e. those with a free lamella, the microtubules are orientated parallel to the direction of spreading. Cells which are located immediately behind these cells show that microtubules are orientated predominantly towards the direction of expansion. However, more proximally located cells show no particular distribution of microtubules (Fig.7.2a).

The deep layer cells which attach to the glass substratum at the side of the culture opposite to the leading edge cells show a similar distribution of microtubules as for the edge cells i.e. they extend parallel to the direction of movement from around the nucleus to the tip of the leading lamellae (Fig.7.2b).

It was not possible for me to study in any detail the organisation of microtubules in the ectodermal cells which constitute the central part of the cultures. The main reason for this was that the deep layer cells overlap the ectodermal cells in this area obscuring microtubule distribution in both layers. I found it impossible to get rid of deep layer cells from the surface of the ectoderm without disturbing the attached marginal cells, even after fixation.

It is worth mentioning here that the microtubule organising centre in the majority of cells with free lamellae was located in front of the nucleus i.e. between the nucleus and the free leading lamella (Fig.7.3a).

7.1.3.2 The effect of colchicine on the shape of edge and deep layer cells *in vitro*:

Treatment of cultures with colchicine resulted in the destruction of microtubule systems in both edge cells and deep layer cells. However, in both cell types, colchicine treatment did not affect the general shape of the cells or their lamellae which still showed typical spreading on glass (Fig.7.3b). Even when colchicine was added to the culture medium before the blastoderm explant had attached to the substrate, the cultures attached readily to the substratum and spread their lamellae onto it in the usual way. This was true both for explants with an intact normal edge, and for those lacking it.

7.2Microfilaments:

7.2.1 Introduction:

The only previous work done on the distribution of microfilaments in chick extraembryonic cells is that of Chernoff and Overton (1979). That work did not look specifically for these structures in edge cells and the effect of their destruction on these cells, but was rather concerned with these structures in the whole blastoderm and in dissociated extraembryonic cells.

My work was designed to look for microfilament distribution in edge cells and the effect that destruction of these structures had on these cells. However, the presence of the vitelline membrane made it impossible to achieve this task and, as in the case of microtubule distribution, the aim of the work had to be changed to using a glass substratum to look only on the effect of microfilament destruction on the shape of cultured edge and deep layer cells.

7.2.2 Methods:

The method used was basically the same as for microtubules (7.1.2).

Cytochalasin D, a microfilament inhibitor, was added to the culture medium either before the attachment of the blastoderm pieces, soon after, or much later, then control and experimental cultures were fixed and stained for actin distribution using indirect immunofluorescence.

7.2.3 Results:

7.2.3.1 The distribution of microfilaments in normal leading edge and deep layer cells *in vitro*:

The orientation of microfilaments in cultured edge and non-edge cells was hard to determine even under the highest resolution available with our UV microscope. It has been shown however that, at least in dissociated edge cells, microfilaments are distributed all around the edge of the cells and in thin filopodia extending from the leading lamellae (Chernoff and Overton, 1979).

At several points in the cultured cells, there was a high level of immunofluorescent staining for microfilaments. These points seemed to be foci for dense arrays of microfilaments near the cell- substratum attachment sites (Fig.7.4).

7.2.3.2 **Effects of cytochalasin D on the shape of the leading edge and deep layer cells:**

Unlike colchicine, cytochalasin D had wide effects on the cell shape of cultured blastoderm pieces. These effects were related to the time at which these pieces were exposed to the drug. If the drug was added to the culture medium before the blastoderm explants were introduced to the culture dish, explants failed to attach to the substratum, even with an extended culture time. If the pieces concerned were allowed to attach to the glass substratum for a short time (2-4 hours) before adding the drug, the cells detached from the substratum within 5-10 minutes. When the drug was added to the medium after the blastoderm pieces had been in culture for 24-48 hours the cells, edge and non-edge ones, did not detach from the glass substratum but showed

dramatic effects on cell shape (Fig.7.5). The outlines of cells could no longer be recognised and the lamellae of the cells collapsed except for some filopodia containing bundles of microfilaments which extended from around the nucleus and ended in points of high immunofluorescent staining where the cells attached to the glass substratum.

7.3 Fibronectin:

7.3.1 Introduction:

Very few studies have been designed to examine the distribution of fibronectin in the early chick blastoderm and its relation to blastoderm expansion. Monnet-Tschudi *et. al.* (1985) reported that the distribution and intensity of fibronectin in the basal lamina of the ectodermal cells decreased progressively from the centre of the blastoderm towards the leading edge in stage 4-6 blastoderms. As far as the distribution of fibronectin in the leading edge was concerned, they reported that it forms a network of coarse elements oriented parallel to the edge and that fibronectin immunoreactivity was associated both with the dorsal and ventral surfaces of the edge cells. They also conducted an immuno-electron microscopy investigation using Protein A bound to colloidal gold and reported internalised staining contained in intra-cytoplasmic vesicles in the ectodermal cells. Similar staining was occasionally seen in edge cells. They reported that deep layer cells have the same distribution of fibronectin as the leading edge cells i.e. around and occasionally inside the deep layer cells.

Lash *et. al.* (1990) reported similar light microscopy results to those of Monnet-Tschudi *et. al.* (1985) except that they claimed fibronectin to be absent from the distal tips of the edge cells. Absence of fibronectin staining from the distal tip is not, however, obvious from the only figure (Fig. 7) they show to support this claim. Indeed, Fig. 7 shows abundant fibronectin staining around the lateral sides of nearby ectodermal cells, indicated as not showing such

staining in some previous studies (Mayer *et. al.*, 1981 and Harrisson *et. al., 1984*).

More recently Raddatz *et. al.* (1991) studied the distribution of fibronectin before the attachment of edge cells to the vitelline membrane in stage X-XIII (Eyal-Giladi and Kochav, 1976) embryos. They reported that the stationary loose cells of the edge showed no staining for fibronectin. They suggested that the appearance of fibronectin during the stages at which the blastoderm is expanding (Monnet-Tschudi *et. al.*, 1985 and Lash *et. al.*, 1990) may be necessary for the transformation of the originally stationary and loose edge cells to an organised unit of actively migrating cells.

7.3.2 Methods:

1-3 day blastoderms were examined for the distribution of fibronectin using the methods of Harrisson *et. al.* (1984) and Ostrovsky *et. al.* (1983).

7.3.3 Results:

7.3.3.1 The distribution of fibronectin in the chick blastoderm *area opaca* before the edge has passed the equator of the egg:

Initially I used the method of Harrisson *et. al.* (1984) to study the distribution of fibronectin in wax embedded blastoderms. The results of this method revealed that fibronectin is found mainly in the basal lamina to which the basal side of the ectodermal cells is attached. Fibronectin activity is present in this basal lamina from the centre of the blastoderm to the periphery of the leading edge (Fig.7.6a). Contrary to the findings of Monnet-Tschudi *et. al.* (1985), I was not able to detect in any of the blastoderms I studied any fibronectin staining at either the dorsal or ventral surfaces of the leading edge cells. However, staining for fibronectin was sometimes found to be localised in a few points inside some of the edge cells (Fig.7.6b). This staining may correspond to the internalised fibronectin reported by Monnet-Tschudi and his colleagues, though I have no proof of this.

Following the publication of Lash *et. al*'s (1990) paper which reported similar but not identical findings to those of Monnet-Tschudi and co-workers, I looked again at fibronectin distribution using this time the method of Ostrovsky *et. al.* (1983) which was used by Lash's team and which involves looking at the distribution of fibronectin in araldite-embedded material. The results I obtained are similar to my original results and are at variance with Lash *et. al's* . Fibronectin staining is restricted to the basal lamina of the ectodermal part of the blastoderm and to a few spots inside the edge cells (Fig.7.7). Due to the thinness of sections prepared by this method (only 1 um thick), the staining is not as strong as in sections stained according to the method of Harrisson *et. al.* (1984).

7.3.3.2 **Fibronectin distribution at the later stages of epiboly:**

Sections cut through the stationary streaks of late blastoderms (3 day or older) revealed that staining for fibronectin is similar to that of the earlier stages except for the addition of localised staining on some areas of the inner surface of the vitelline membrane where the stationary edge cells are attached. Such staining is absent in similar areas of the vitelline membrane during the early stages of epiboly and, during the later stages, in areas of the vitelline membrane where no stationary cells are attached (Fig.7.8).

Fig.7.1 a- Immunofluorescent micrograph showing the difficulty in visualising microtubules in the leading edge cells (LE) in the presence of the vitelline membrane. E= ectoderm. Bar= 50 µm.

Fig.7.1 b- Immunofluorescent micrograph of the ectodermal part of the above specimen (E), showing the same problem as above. E= ectoderm; arrowheads= mitotic figures. Bar= 50 µm.

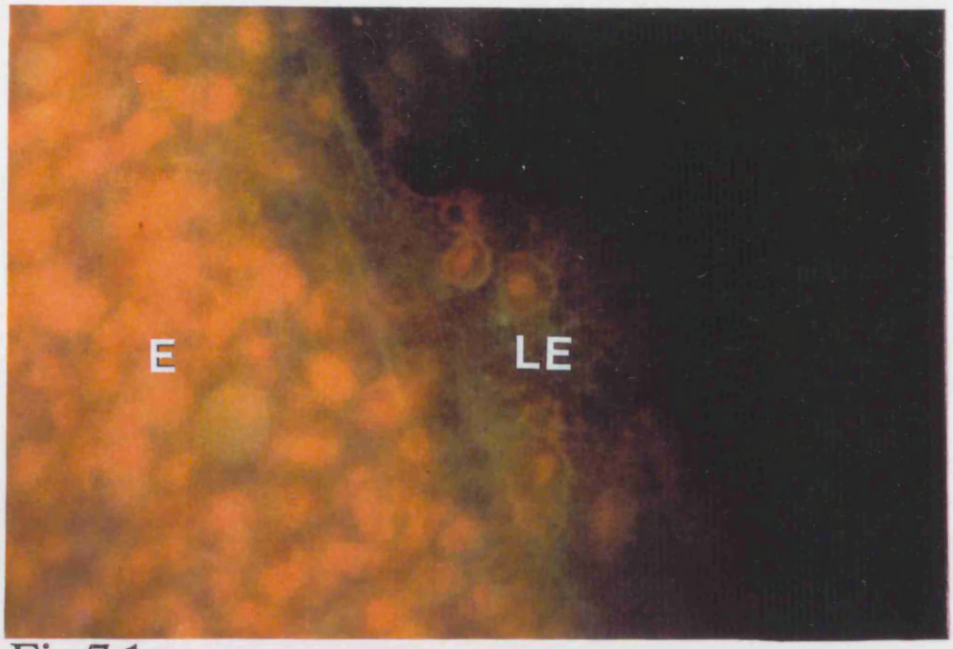

Fig.7.1 a

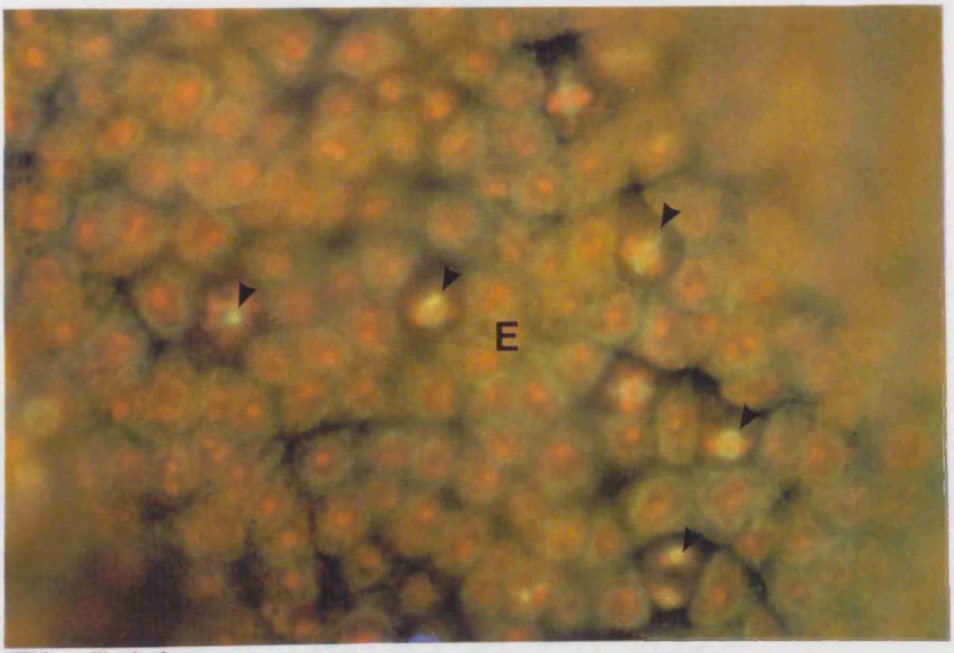

Fig.7.1 b

Fig.7.2 a- Immunofluorescent micrograph showing the distribution of microtubules in cultured leading edge cells. Microtubules extend from the perinuclear area to the tip of the lamellae (L). In the distalmost cells and the submarginal cells (sm) immediately behind them microtubules are orientated towards the direction of movement. Microtubules in the rest of the edge cells, i.e. those which are away from the margin, do not show any particular orientation. N= nucleus. Bar= 50μm.

Fig.7.2 b- Immunofluorescent micrograph showing the distribution of micro-tubules in deep layer cells which attached to the substratum (glass) at the opposite side of the culture to the leading edge. The deep layer cells show similar microtubule distribution as the leading edge cells. L= leading lamellae. Bar= 50μm.

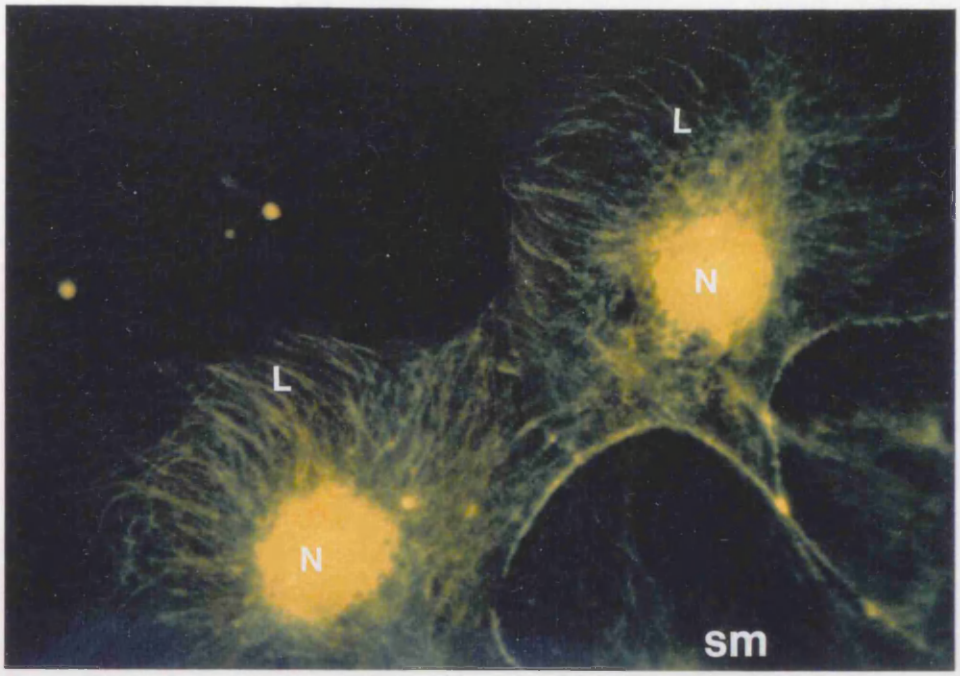

Fig.7.2a

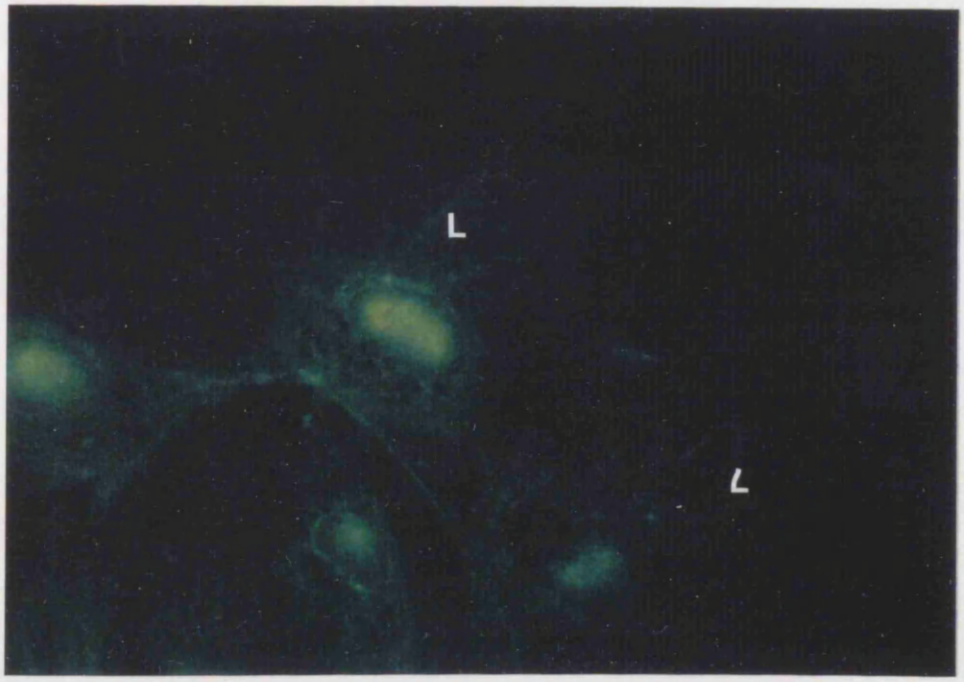

Fig.7.2b

Fig.7.3 a- A high magnification immunofluorescent micrograph of leading edge cell showing microtubule organising centre (arrow) located between the nucleus (N) and the free leading lamella (L). Bar= 25 μm.

Fig.7.3 b- Immunofluorescent micrograph showing that colchicine caused the destruction of microtubules in cultured leading edge cells but did not prevent the leading lamellae of these cells from spreading onto the substratum. L= leading lamellae; N= nucleus. Bar= 50 μm.

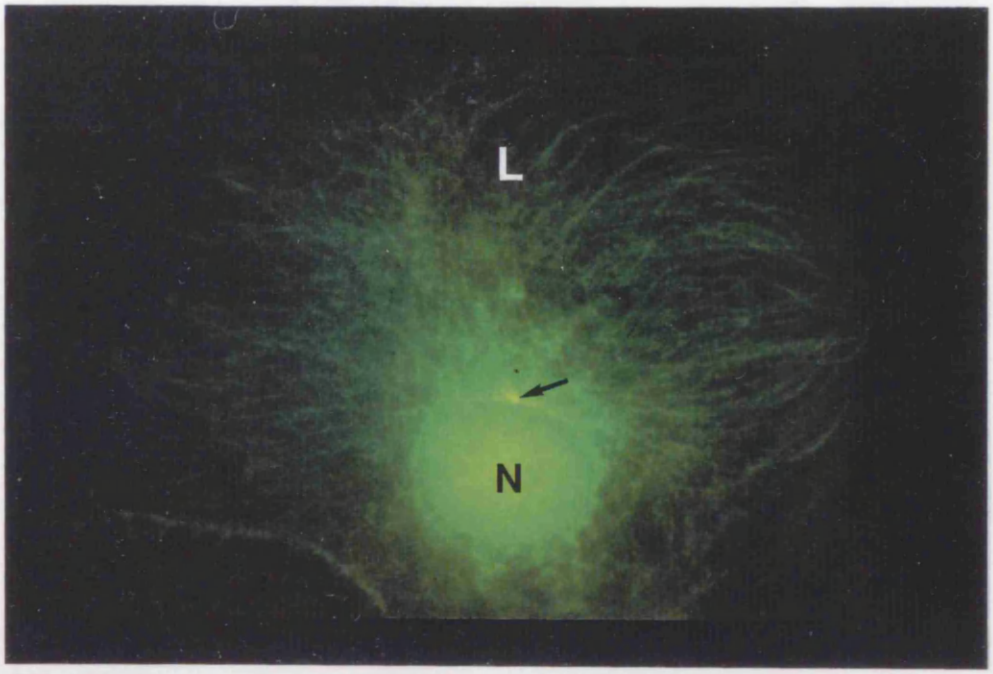

Fig.7.3a

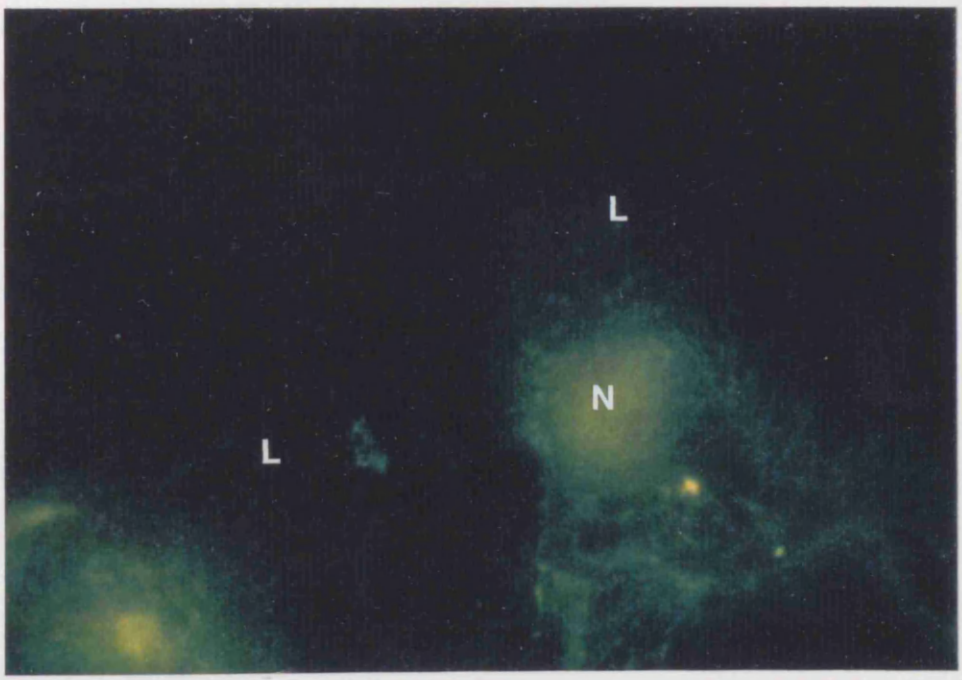

Fig.7.3b

Fig.7.4 a- Immunofluorescent micrograph showing the distribution of microfilaments in cultured leading edge cells. Notice the presence of strong immunofluorescent staining at some points of the leading lamellae (arrows) which may be points of attachment of the lamellae to the substratum. L= leading lamellae. Bar= 50 μm.

Fig.7.4 b- Immunofluorescent micrograph similar to (a) but at the deep layer cell side. L= leading lamellae. Bar= 50 μm.

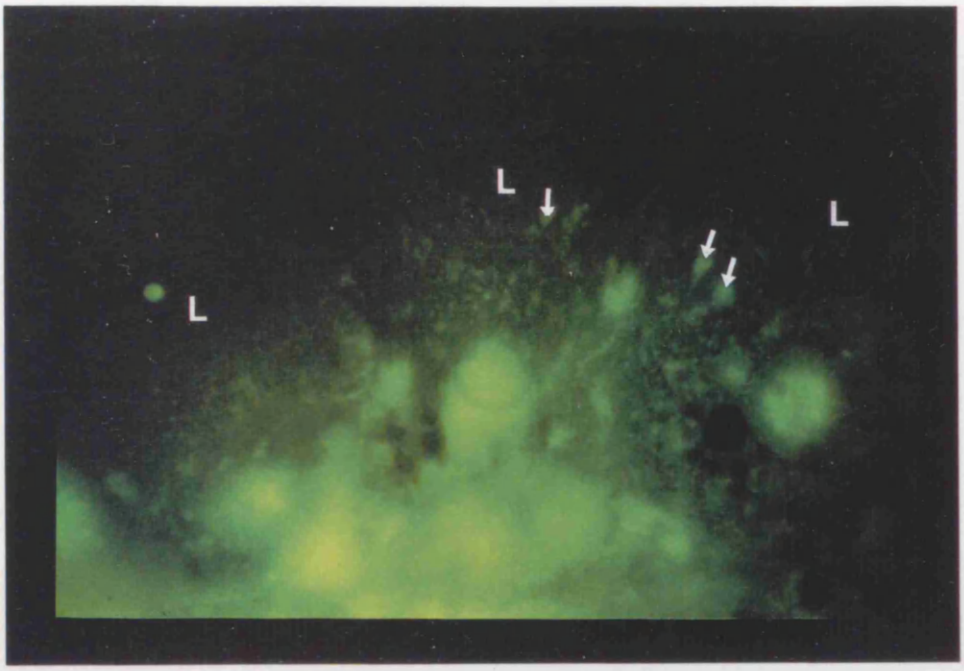

Fig.7.4a

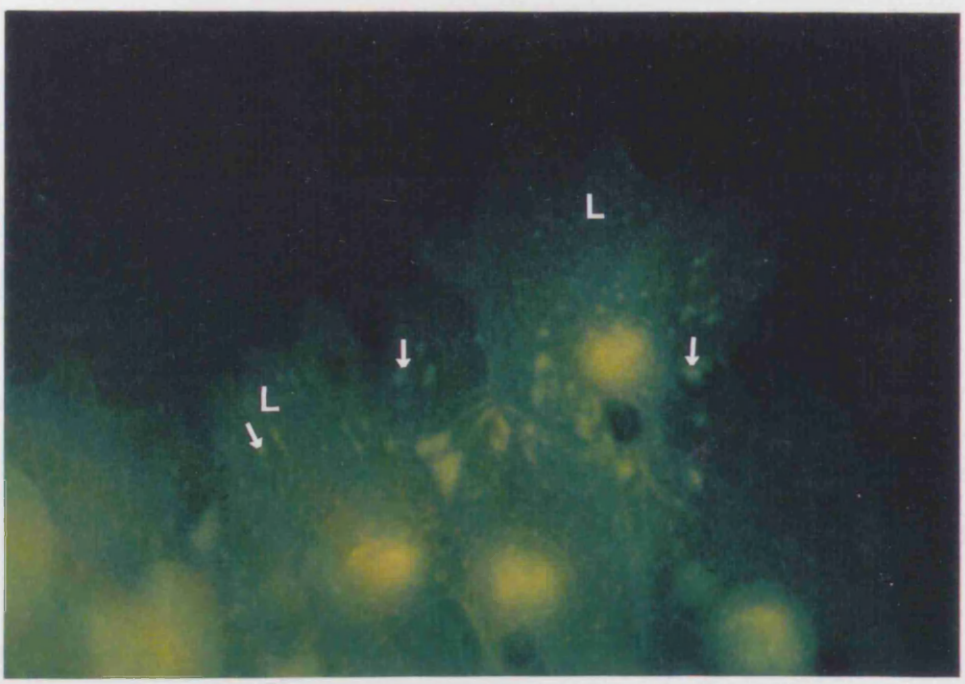

Fig.7.4b

Fig.7.5 (a&b)- Immunofluorescent micrograph showing the effect of cytochalasin D on the shape of edge cells (a) and deep layer cells (b). Notice the collapse of the usual cell shape with the withdrawal of the leading lamellae except for a few processes extending on the substratum. Arrows show points of attachment. Bar= 50 μm.

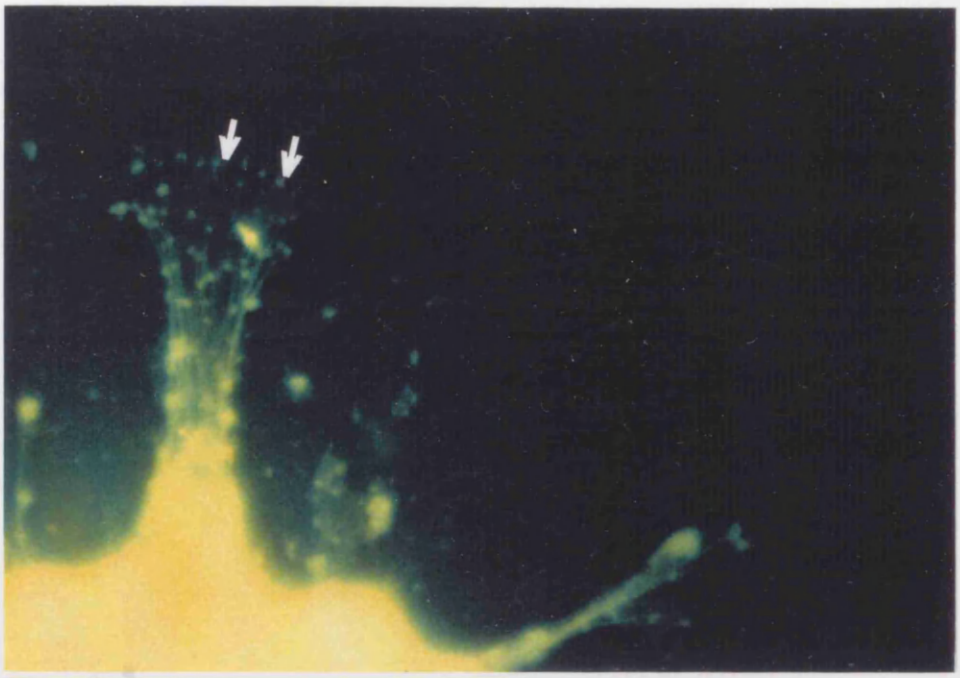

Fig.7.5a

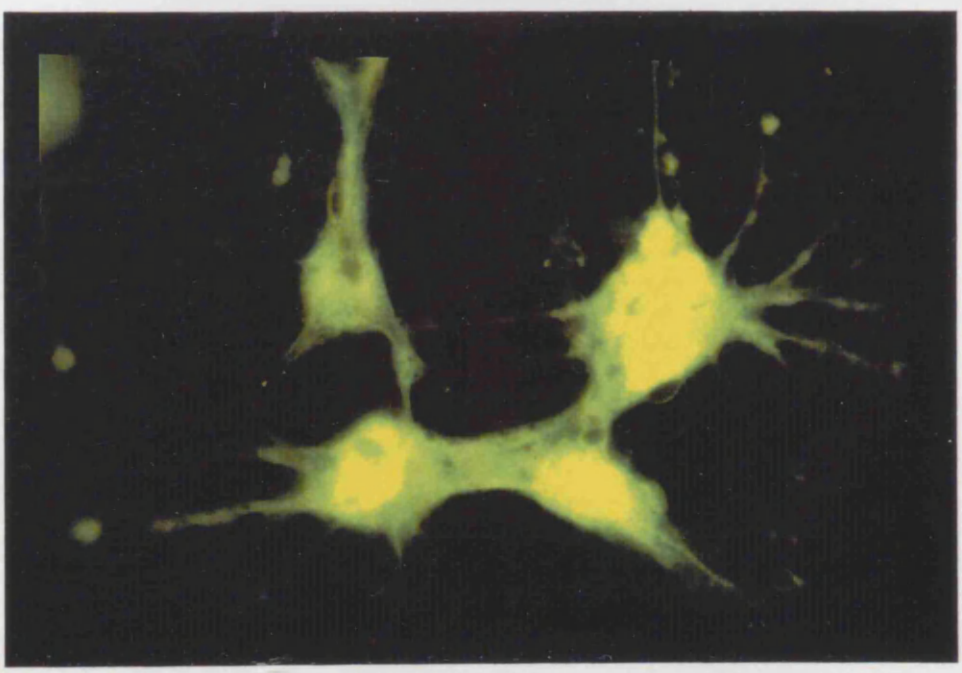

Fig.7.5b

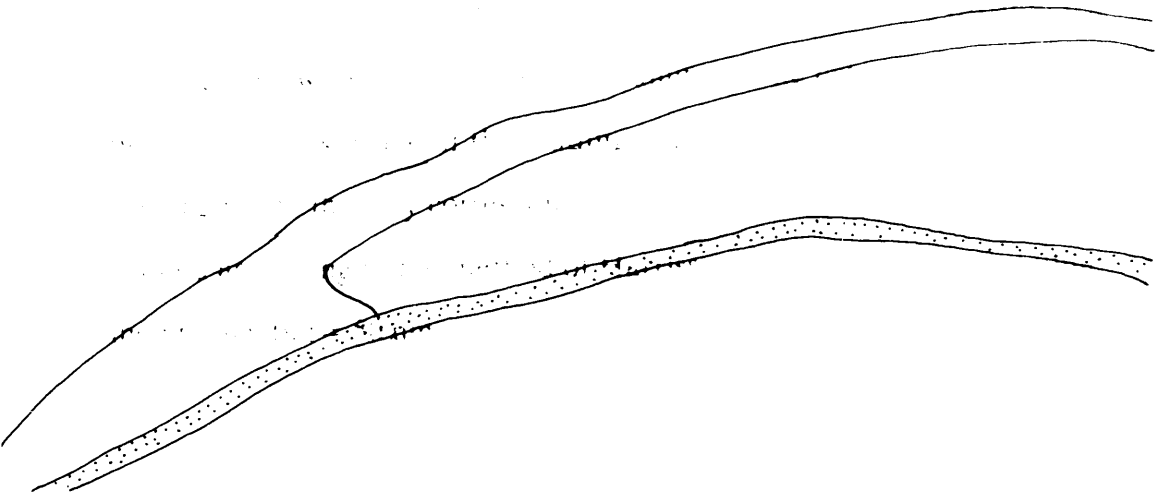

Fig.7.6 b- Another immunofluorescent micrograph showing similar distribution of fibronectin (arrow) on the basal side of the ectodermal cells (E) and inside some edge cells (Le) of a stage 6 blastoderm, using the same method of staining as above. Arrowhead= fibronectin staining inside the leading edge. V= vitelline membrane. Bar= 50 µm.

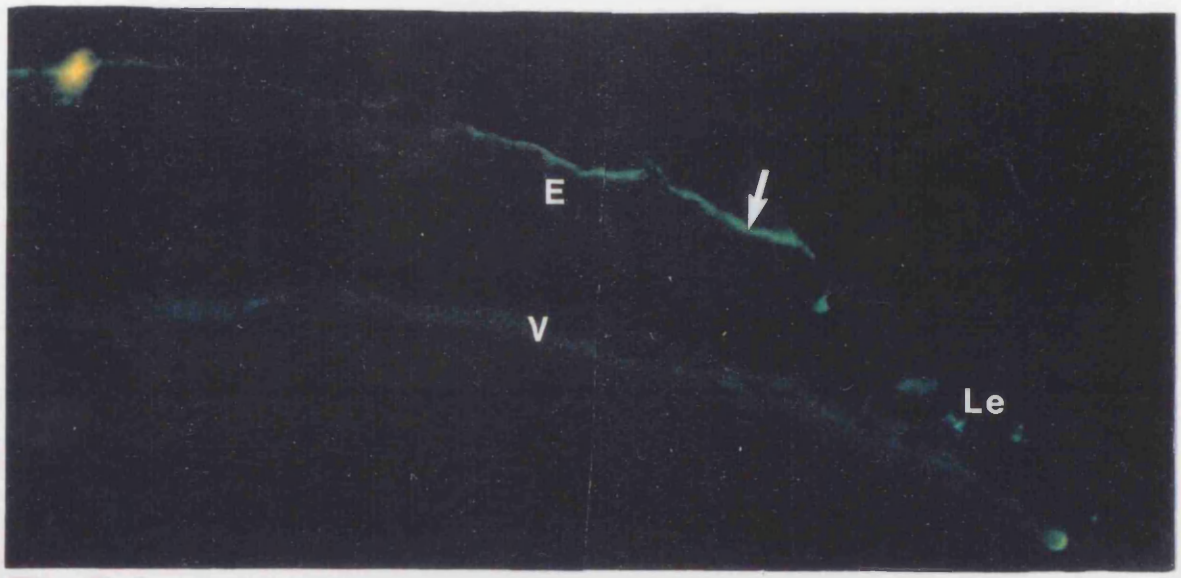

Fig.7.6a

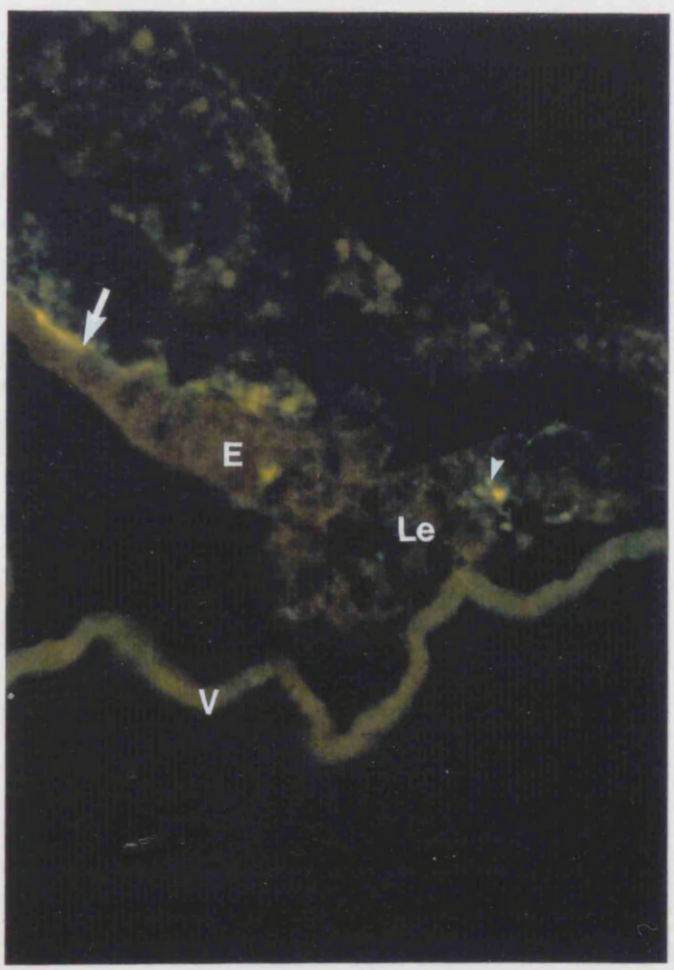

Fig.7.6b

Fig.7.7 (a&b)- Immunofluorescent micrographs showing the distribution of fibronectin in a stage 8 the chick blastoderm edge using the method of Ostrovsky (1983). Here again, staining for fibronectin is present on the basal lamina (arrow) of the ectodermal cells (E) and in some points inside some leading edge cells (Le) (arrowheads). V= vitelline membrane. Bar= 50 μm.

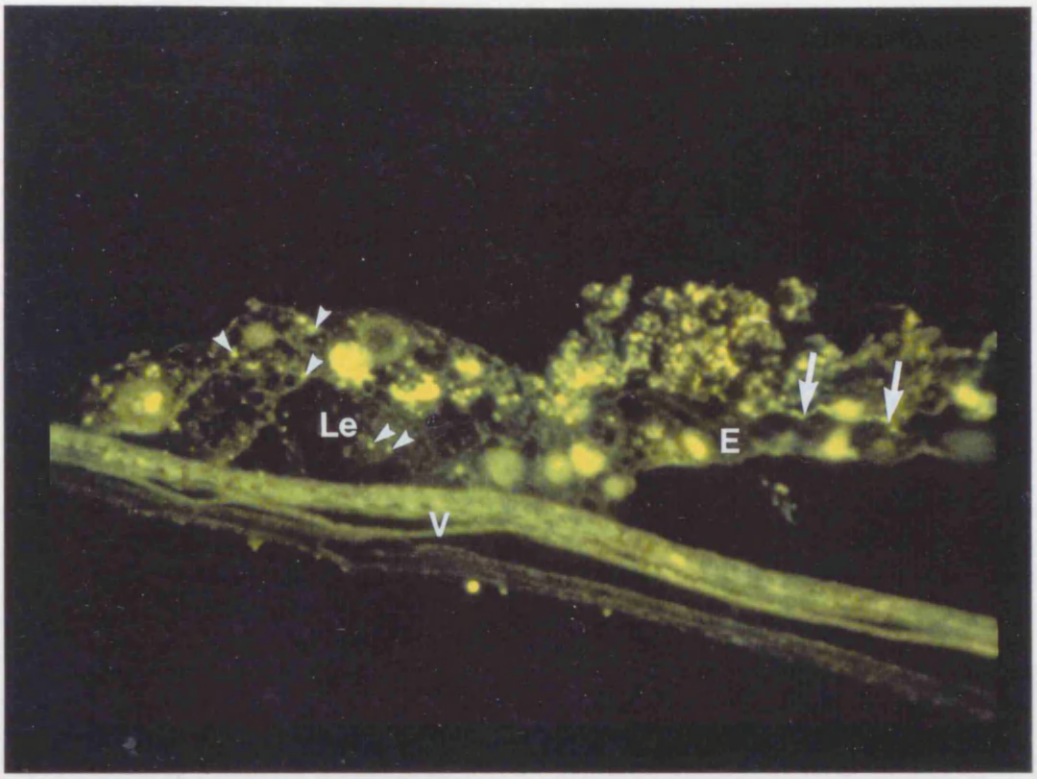

Fig.7.7a

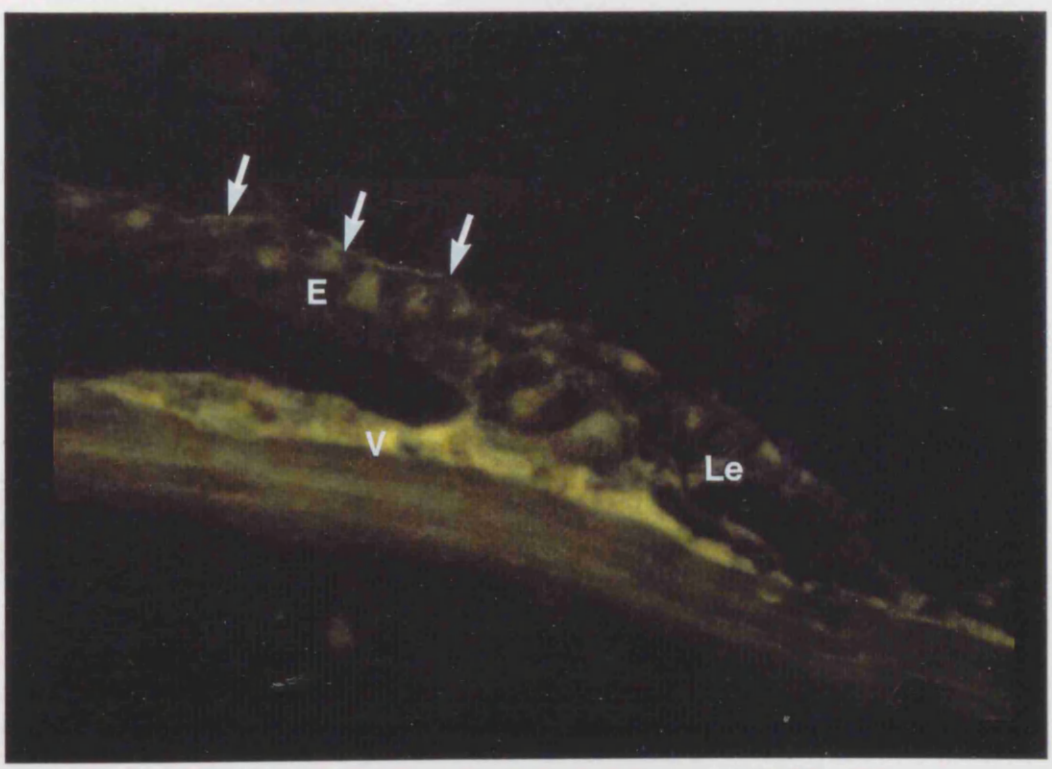

Fig.7.7b

Fig.7.8 (a&b)- Immunofluorescent micrograph showing the presence of fibronectin staining (arrows) on the inner surface of the vitelline membrane (V) to which stationary edge cells (S) of a 3-day old blastoderm are attached. No similar staining was detected between the edge cells and the vitelline membrane during the earlier stages. (E)= ectoderm. Bar= 50 μm.

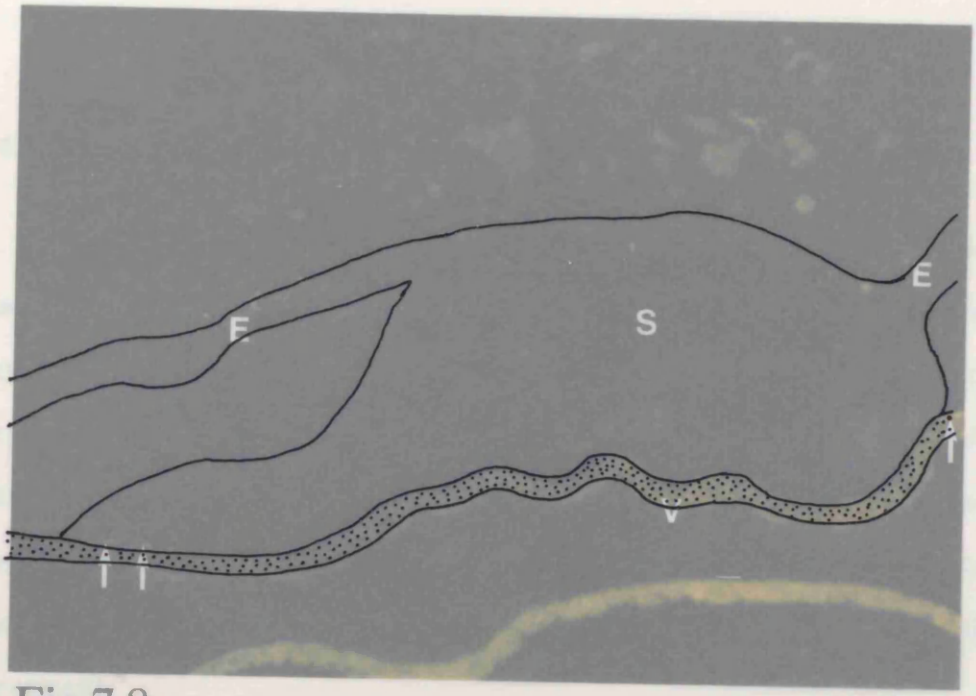

Fig.7.8a

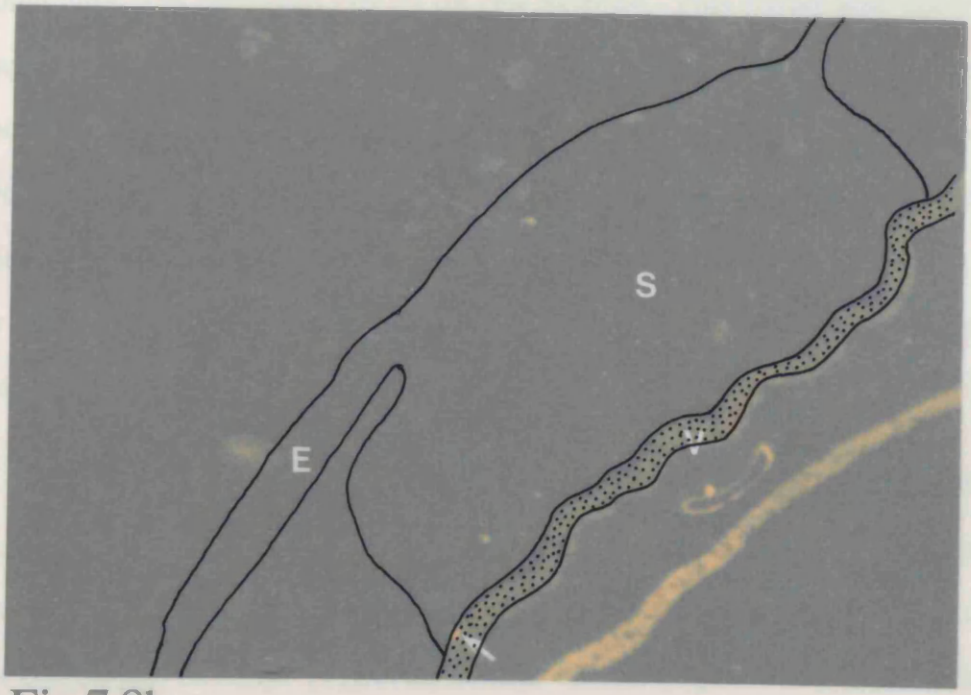

Fig.7.8b

7.4. DISCUSSION

7.4.1 Microtubules:

The results reported here concern cell shape in explants of intact blastoderm edges cultured on glass in the presence or absence of colchicine. Colchicine appears to have no effect on cell shape, or on the ability of the explants to adhere to and spread on the glass substratum. My indirect immunofluorescence observations confirm that the distal edge cells normally contain a distally-radiating array of microtubules, with their organising centre facing the direction of migration, and that this array is eliminated by colchicine treatment.

Previous studies have reported similar results, in somewhat different circumstances, and therefore these results are largely confirmatory. Downie (1975) cultured whole blastoderms and blastoderm edges in the presence of colchicine, and observed effects on cell motility. This paper also referred to unpublished preliminary observations (Downie, 1971- thesis) on cell shape and motility in colchicine treated cultures of extraembryonic ectoderm on glass. Downie (1975) was not able to give a detailed description of microtubule distribution in intact cells. Chernoff and Overton (1979) found that the presence of colcemid (a relation of colchicine) did not prevent the normal flattening of the initially rounded cells taken from unincubated blastoderms.

My study has not dealt with the controversy on whether microtubules are necessary for epithelial cell orientated movement. Vasiliev (1970) showed that intact microtubules are not essential for fibroblast motility, but are necessary for directional motility. However, various studies (e.g. Euteneuer and Schliwa, 1984) have suggested that the same is not true of epithelial cells. Interesting work by Gottlieb (most recently Wong and Gottlieb, 1988) using "wound-

healing" in confluent endothelial cell cultures, suggest that microtubules are not necessary for directional migration, but are necessary when cells have to change direction.

In the case of blastoderm edges on the vitelline membrane, Downie's work (Downie, 1975) suggested that disruption of microtubules in edge cells did not stop edge cell migration. More recently, however, Mareel *et. al.* (1984) using both taxol and Nocodazole got the opposite results. The reason for the discrepancy in these results is not clear.

7.4.2 Microfilaments:

Similarly, my results on the importance of intact microfilaments are essentially confirmatory. The only previous study using cytochalasin (B, not D as in my work) to disrupt blastoderm microfilaments is by Chernoff and Overton (1979). They either incubated intact blastoderms in cytochalasin for up to 20 minutes, or incubated dissociated cells from the edge region in cytochalasin for 30 minutes (both using stage 7-9 blastoderms), or dissociated cells from unincubated blastoderms in cytochalasin for varying periods.

My experiments used explants of intact edges from 1-2 day embryos on glass. Results were very similar to Chernoff and Overton's. If cytochalasin D was present from the start, cells failed to adhere to the substratum. If added later, cells retracted, leaving filopodia attached to the substratum at their distal ends.

7.4.2 Fibronectin:

In this case, my results appear to be at variance with previous investigations. The reason for carrying out this work was to check whether the fibronectin distribution found by other workers (Monnet-Tschudi *et. al.* 1985; Lash *et. al.*, 1990) in the blastoderm edge during the early stages of expansion continued into the later stages, given that my work (Chapter 5) showed

considerable changes in edge cell organisation during the later stages.

However, my results even for early blastoderm edges seem to differ from those of earlier authors. Monnet-Tschudi *et. al.* (1985) used two methods to discover fibronectin distribution in the blastoderm (including the edge). First, they treated paraformaldehyde-fixed complete 1 day blastoderms on their vitelline membranes with a chick monoclonal fibronectin antibody, or a human polyclonal. After staining, the vitelline membrane was removed and blastoderms were spread on a coverslip, covered in mounting medium and observed. Edge zones were said to contain a coarse network of fibronectin, mostly orientated parallel to the periphery. Unfortunately, from the technique used, it is impossible to be sure where this fibronectin actually is. Edge cells tend to curl up when removed from the vitelline membrane, and it is entirely unclear whether the fibronectin stained in the relevant figure (6) is actually at the blastoderm edge, or in the distalmost basal lamina area. Indeed, the text suggests that the authors believe they are looking at basal lamina fibronectin whereas there is no basal lamina in the attached edge (See this thesis, chapter 3).

The second method used colloidal gold techniques to localise fibronectin at electron microscope level. The morphology of the edge cells presented in their illustration (Fig. 8) looks abnormal, but they do find a small amount of fibronectin in the edge. A very little appears at the cell surface (unfortunately, the authors muddle their terminology between dorsal-ventral and basal-apical, so that their description is difficult to follow) and rather more internally.

Although the summary of Monnet-Tschudi *et. al*'s paper claims to have demonstrated that fibronectin is all round the actively moving cells of the edge, the evidence for this is very poor.

The later paper to report fibronectin in the blastoderm edge is Lash *et. al.* (1990). A curiosity of this paper is a completely misleading cartoon drawing of

the edge (Fig. 1B). For indirect immunofluorescent localisation of fibronectin in sections of the edge, Lash *et. al.* used the method of Ostrovsky *et. al.* (1983), with anti-fibronectin of un-named specificity. They present one figure (7) demonstrating fibronectin in the edge with a description stating that fibronectin is all round the cells of the edge, with apparent lamellar arrangement. The discussion, however, states that fibronectin was not found at the extreme tips of the edge cells. In my view, the verbal description presented by Lash *et. al.* does not fully accord with their illustration. There is no sign of a lack of fibronectin at the distal tip, nor does the fibronectin appear to be distributed all round all the edge cells.

Monnet-Tschudi *et. al.* (1985) and Lash *et. al.* (1990) both suggest that edge cells use fibronectin as a means of attaching to the vitelline membrane, and therefore as part of their locomotory mechanism. As I have indicated above, the evidence for this is not compelling: an additional problem is that neither paper shows any fibronectin staining at the vitelline membrane surface after the edge cells have moved over it. If fibronectin is indeed used as an adhesive intermediate here, some would be expected to be left at the matrix (vitelline membrane) surface.

The differences between my results and the others may be due to differences in antibody specificity : the fibronectins are molecules with multiple forms (Kornblihtt and Gutman, 1988). However, it is clear that my experiments, using two different preoperative methods, give repeatable positive results, showing fibronectin in the basal lamina proximal to the attached edge but not around edge cells, only occasionally within them at early rapidly expanding stages. As in other studies, I found no fibronectin in the vitelline membrane at the early stages. Extracellular edge fibronectin is only found in the stationary streaks.

These findings accord well with results from experiments on fibroblast motility which show that blocking of fibronectin-receptor interaction has little

effect on cell motility, but that stationary cells accumulate fibrillar fibronectin at their adhesion sites (Duband *et. al.*, 1988 ; Akiyama *et. .al.*, 1989).

CHAPTER EIGHT

GENERAL DISCUSSION AND CONCLUSIONS

The expansion of the chick blastoderm provides the best example of a co-ordinated epithelial movement which covers a considerable area over a short of time.

The present work has been designed to further our knowledge of changes in the organisation and behaviour of the leading edge cells of the blastoderm during early and late stages of development and to relate these changes to the ability of these cells to migrate at different speeds on the vitelline membrane, leading to the expansion of the whole blastoderm.

Since each individual chapter has its own discussion section, this chapter will deal with the main findings of the present work on the blastoderm edge and then try to put these findings in the context of what we know about other examples of epithelial movement.

8.1 Major findings:

Chapter 3:

Results presented in this chapter provide a clearer picture as to the behaviour of the cells of the leading edge from as early as possible after their attachment to the vitelline membrane to the time they reach the equator of the egg. They retain a multilayer organisation throughout and show a progressive increase in their width, especially around the second day of development. Their number was also found to increase around the same time. However, there was a drop in the height of the edge around that time. Cell size was found not to follow a particular pattern.

This chapter also reveals that throughout these stages of development the proximal cells of the edge are connected together by well developed desmosomes as well as points of close membrane apposition. Desmosomes become scarce between the midpart cells and are totally absent between the distal cells.

Chapter 4:

This chapter provides some evidence that the "deep layer" cells in the vicinity of the leading edge are the cells responsible for the increase in edge cell number reported in chapter 3. Light and trans-mission electron microscopy revealed that these cells incorporate themselves into the edge after extending their leading lamellae into the edge area. Some evidence for the unlikeliness of ectodermal cells being added to the leading edge is discussed.

Chapter 5:

This chapter describes for the first time the events that take place in the chick blastoderm in general and the leading edge in particular as the edge passes the equator of the egg. These events include: a) the transformation of edge organisation from being multilayer to monolayer. b) Some of the leading edge cells stop their migration leading to the appearance of stationary points distributed around the circumference of the edge. c) More cells are added to the stationary cells resulting in the formation of stationary streaks. These streaks differ from each other in time of appearance and therefore in their length. d) Cell death appears in the old stationary cells which are then eliminated and replaced by the nearby ectodermal cells. e) Blastoderm expansion at these later stages of epiboly is a very slow process as can be deduced from the fact that it takes more than 6 days for the blastoderm edge to migrate from the equator of the egg to cover the rest of the egg. The extent of blastoderm expansion also differs from one blastoderm to another even at the same stage of development. f) Deep layer cells persist even at the very late stages of blastoderm expansion when they form what seems like another layer

of cells on the monolayer of edge cells which seals the blastoderm.

Chapter 6:

This chapter provides a detailed study for the behaviour of pieces of the blastoderm lacking the edge cells cultured on the inner surface of the vitelline membrane. Results showed that deep layer cells attach to the vitelline membrane soon after culturing and start centrifugal migration. Ectodermal cells of the cultured pieces do not attach to or move on the vitelline membrane unless they lose their apical-basal polarity. When they do so, together with the deep layer cells, they form a rapidly moving mass of cells called the "regenerated edge" which possesses several of the features of the normal blastoderm edge.

The chapter also gives light and transmission electron microscopical observations on the behaviour of pieces of blastoderm (including an edge or lacking it) on the outer surface of the vitelline membrane. It reports that such pieces lose their ability to expand on such a substratum due to the increased adhesion of the cells to the fluid fibrous material of the substratum. Bundles of microfilaments, or stress fibres, which end at points of contact between the cells and the substratum are a common feature of the cultured cells.

Chapter 7:

This chapter reports on immunofluorescent staining of chick edge cells cultured on glass, to study the effect of microtubule and microfilament inhibitors on the shape of the edge cells. It supports previous reports (Dipasquale, 1975; Chernoff and Overton, 1979; Euteneuer and Schliwa, 1984 and Middleton *et. al.*, 1988) on other epithelia, that maintenance of the morphology of cultured epithelial cells depends on an intact system of microfilaments rather than microtubules. The chapter also reports that fibronectin near the blastoderm edge is found mainly in the basal lamina of the ectodermal cells. Edge cells do not show fibronectin staining except for some

points inside their cytoplasm. During the late stages of epiboly, localised fibronectin staining was present on some areas of the vitelline membrane to which stationary cells attached. No such fibronectin was ever observed in such areas during the earlier stages of epiboly i.e before the formation of the stationary streaks.

8.2 How do epithelia move?

Much of our knowledge about epithelial cell movement *in vivo* comes from studies on the movement of epidermal cells during wound healing. Based on ultrastructural observations of wound healing in mammalian epidermis a pattern of "rolling and sliding" movement of these cells has been proposed (Krawczyk, 1971; Gibbins, 1978; Repesh and Oberpriller, 1980). It was suggested that the marginal cells i.e. those at the leading edge, adhere to the substratum and the submarginal cells move over them and in turn attach to the substratum via hemidesmosomes. The pattern is repeated till the closure of the wound. This pattern concerns stratified epithelia where the basal layer cells are regarded as stationary, and only the cells of the upper layers move.

Radice (1980 a&b) investigated cell movement during wound healing in *Xenopus* tadpoles with time-lapse cinematography and TEM and suggested another pattern for epithelial movement. He suggested that the cells do not roll or slide but retain their position relative to each other so that as the marginal cells move forward, the submarginal cells immediately occupy their position, without intermingling between cells of marginal and submarginal areas. In Radice's view all cells of the moving epithelium contribute actively to epithelial spreading.

The epidermis of *Xenopus*, as used by Radice, is essentially a monolayer with an additional surface periderm only, and so it is likely in any case to differ in behaviour from the multilayered mouse epidermis studied by Krawczyk.

Both of these patterns of epithelial cell movement differ from

predominant ideas put forward for epithelia in tissue culture, where Vaughan and Trinkaus (1966) and later DiPasquale (1975) suggested that epithelia are actively attached to the substratum only at the margin and that detachment of marginal cells from the substratum at one area of the epithelial sheet leads to retraction of the sheet at that area.

The organisation of the leading edge of the blastoderm reported in this thesis does not support the suggestion of Vaughan and Trinkaus, since the cells of this edge are not a single peripheral band but an increasing number of cells attaching to a wide area of the substratum. In fact, the organisation of the edge cells of the chick blastoderm reported here reveals that it contains elements of both Radice's and Krawczyk's patterns of epithelial cell movement.

Supporting Radice's model of epithelial edge organisation, there is both indirect and direct evidence that edge cells mainly retain their position relative to each other. The indirect evidence comes from the finding that, on one hand, well developed desmosomes and areas of tight junctions are present between the proximal edge cells, suggesting that these cells retain their organisation and position relative to cells of the other parts of the edge. Indeed, support for this comes from the behaviour of these cells during the late stages of blastoderm expansion. While cells of the rest of the edge change their organisation into a monolayer, the proximal cells retain their original multilayered organisation. On the other hand, the infrequent presence of desmosomes between the mid-part cells of the edge and their total absence between the distal cells makes it likely that the cells of these two areas may be able to change their position relative to one another. However, direct evidence exists to suggest otherwise i.e. distal cells seem to retain their position relative to mid-part cells. This direct evidence comes the from time-lapse photography which I did on blastoderms cultured on the vitelline membrane (Chapter 4). Because of the difficulty of filming the whole edge of the blastoderm, only the distal cells were filmed during their migration. Analysis of the films revealed that these cells always

remained distal and did not retreat to a more proximal position and that there was no sign that cells behind them moved over them and in turn became distal. Whether mid-part cells are able to insert themselves between the distal cannot be excluded, since visualisation of these was not good enough.

Although the above findings make it unlikely that the mid-part cells overtake the distal cells, as proposed in Krawczyk's model, it may be possible for these cells to shift their positions relative to each other since, as mentioned above, desmosomes which may greatly impede such shifting are scarce between these cells. Also, the tight junctions present between these cells have been shown to be of the discontinuous type which allows shifting of position to take place (Andries *et.al.*, 1985). In fact, support for the idea that these cells are able to shift position relative to each other comes again from the organisation of edge cells during the late stages of epiboly, e.g. during the third day of development. While the proximal part of the edge at these stages retains its multilayered organisation, the cells of the mid-part become organised into a monolayer, indicative of the ability of the cells to shift positions relative to each other.

Another feature of Radice's model, distinguishing it from Vaughan and Trinkaus, is that many cells attach to the substratum and contribute actively to expansion, not merely a simple peripheral band. The blastoderm edge starts with a rather narrow band of attached cells, but as expansion continues, more and more cells attach to the vitelline membrane. Their morphology strongly suggests that these attached cells all contribute to active epithelial spreading.

Krawczyk's model suggests that basal marginal epidermal cell are stationary and that epidermal spreading involves the movement of upper layer more proximal cells which move more distally over the stationary cells and make contact with the basal lamina. The process is repeated with the recruitment of new cells into the margin from submarginal positions. It is in the recruitment of new cells into the edge that blastoderm expansion resembles

this model.

Results presented in chapter 4 showed that deep layer cells are recruited into the edge after leaving the basal lamina of the ectodermal part of the blastoderm. Many of these cells extend leading lamellae which make contact with the ventral surface of the proximal and the mid-part cells of the leading edge. I suggest that the deep layer cells extend these lamellae to overtake the proximal area of the edge, where cells are very stable, and attach to mid-part cells. Since cells of this area are capable of shifting position, it may be possible for the newly recruited cells to be incorporated easily into this part of the edge. Whether or not the deep layer cells can overtake the mid-part area of the edge and become distal cells is not clear but filming of the edge never showed deep layer cells or the original mid-part cells overtaking the distal cells and becoming marginal themselves. It is possible, however, that recruited cells as well as mid-part ones are able to become distal by inserting themselves between the original distal cells rather than by overtaking them.

8.3 The rate of epithelial movement:

Although no changes have been detected in junction distribution between edge cells, other changes do occur as the edge moves progressively towards the equator. These changes include a steady increase in the width of the edge and the number of its cells as expansion progresses. This increase in these two parameters was found to be statistically significant around 2-2.5 days of incubation. This coincides with the reported increase in the rate of expansion of the blastoderm both *in vitro* and *in vivo* (Downie, 1976).

Another finding of this thesis is the existence of a consistent pattern concerning the ability of edge, or non-edge, cells to migrate at one rate or another on the inner surface of the vitelline membrane. Basically, for cells to migrate rapidly on the vitelline membrane they must form a multilayered edge. Normal edge cells of up to 2.5 day blastoderms migrate at their fastest speed as long as they maintain their multilayered organisation. However, as these same

cells reorganise themselves into a monolayer after the third day of incubation their rate of migration is greatly reduced, as can be judged from the time they take to cover the other half of the egg - a further 6 days at least. Similarly, when pieces of the blastoderm lacking an edge were cultured on the inner surface of the vitelline membrane, the outgrowing sheet of deep layer cells initially organised into a monolayer, migrates at the rate of 71 um/hr., but after the formation of the "regenerated edge" which has a multilayered organisation, the rate of migration increases to 217 um/hr.

I suggest that the explanation for the relationship between the rate of movement of an edge with changes in cell organisation is the degree to which the different cells of the edge or sheet can co-ordinate their movement. It may be that co-ordination of movement over an extensive monolayer of cells is more difficult to achieve than in cells organised as a multilayer and covering a relatively smaller area.

Another obvious suggestion to make is that the greater the number of well-co-ordinated actively moving cells, the faster the rate of expansion. Therefore, at the earliest stages of blastoderm expansion, there is a multilayered edge, but rather few cells are attached. Expansion rate increases as the edge increases in width and cell number.

8.4 Reduction in edge cell numbers:

As the edge passes the equator of the egg it starts to reduce its circumference by the development of stationary points in which some of the edge cells stop moving forward. These stationary points are distributed within the edge without any fixed pattern. As the edge between the stationary points moves forward, more of the edge cells neighbouring these points are added to original stationary cells and become stationary themselves. This leads to the formation of long stationary streaks and to the transformation of the smooth edge into crescent-like areas.

The question arises as to what makes the cells of the stationary streaks stop movement. One answer to this question could be related to contact inhibition of movement similar to that noticed in cultured fibroblasts (See Heaysman, 1978, for a general review of contact inhibition, and Middleton,1982 for a discussion of contact inhibition in epithelia). Collision experiments reported in chapter 4 showed that once two blastoderm edges collide they immediately cease any movement and it is very likely that the same thing happens during the late stages of epiboly, after the formation of the first stationary points. As stationary points appear, cells of the edge located on both sides of these points can be considered as "two" edges moving towards each other to cover the V-shaped area of the vitelline membrane. The cells of these "two" edges extend their lamellae towards each other and, on making contact, become stationary. More cells are added to the stationary streaks in the same way.

The set of events suggested above may explain the addition of stationary cells to the streaks but does not show what makes the cells which initiate the stationary points stop movement in the first place. One possibility for this is that once the edge passes the equator, crowding within the edge will start to occur, and this may cause the lamellae of adjacent cells to inhibit one another and to initiate stationary points within the edge. The formation of these points will ease crowding within the edge for a while, but as the moving parts of the edge extend to further narrow spaces on the vitelline membrane, crowding occurs again and new stationary points are formed, and so on. In fact, it was found that in all blastoderms studied, stationary streaks of different lengths exist in every blastoderm, indicative of their different initiation times.

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