CELL BEHAVIOUR DURING EPITHELIAL WOUND CLOSURE IN THE CHICK EXTRA-EMBRYONIC EPIBLAST

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ABSTRACT

Cell Behaviour during Epithelial Wound Closure in the Chick Extra-Embryonic Epiblast.

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Light and electron microsope techniques have been used to investigate the normal stage 4-5 epiblast periphery and wound closure at the epithelial margin.

The stage 4-5 epiblast is attached to the vitelline membrane by an association of flattened 'edge cells'. Basal 'edge cells' possess lamellipodia oriented in the direction of epiblast expansion. Adjacent cells are connected by junctions and appear to retain their respective positions during the 'gliding' movement of the attached periphery. Fixation at low temperatures produces an alteration in 'edge cell' morphology consistent with a retraction of the epiblast margin.

After the excision of approximately 200μm of epiblast periphery, the wound gapes due to tensions within the proximal epithelium. The epiblast cells respond to the trauma of wounding by rounding. Wound closure commences within 1 hour of 'edge cell' excision as epiblast cells at the margins attach to the membrane in distal-proximal sequence. The attached wound margins migrate toward each other across the membrane and close the wound at around 10 hours reincubation. Junctions are not observed at the wound margins and the cells appear to employ a 'rolling and sliding' form of locomotion. These results suggest that 'edge cells' are intrinsically different from those of the proximal epiblast.

The normal stage 4-5 epiblast is overlain by a basement membrane. This structure is not observed at the early wound margin. The migrating wound margins deposit extracellular materials on the vitelline membrane and these resemble substances associated with the normal stage 4-5 epiblast basement membrane. Similar materials are also produced by explants and isolated cells of the epiblast periphery when cultured on the vitelline membrane. It is suggested that these materials may represent an attempt to reconstitute a basement membrane during wound closure.

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STATEMENT.

The accompanying thesis submitted for the degree of Ph.D. entitled

Cell Behaviour during Epithelial Wound Closure in the Chick Extra-Embryonic Epiblast

is based on work conducted by the author in the Department of Anatomy of the University of Leicester mainly during the period between October, 1982 and October, 1986.

All the work recorded in this thesis is original unless otherwise acknowledged in the text or by references.

None of the work has been submitted for a degree in this or any other University.

Signed: ...Mark Goodwin..... Date: 25th November, 1986.

This thesis is dedicated to Sister Cecelia Mary and to the memory of Mr Maurice Wiggins

CONTENTS.

I. INTRODUCTION.

A. The Extra-Embryonic Epiblast.

1. Epiblast Expansion.

The early avian embryo develops as a disc of cells between the central yolk mass and surrounding vitelline membrane. A central portion of the blastoderm, classically termed the area pellucida, gives rise to the embryonic axis and structures of the chick (Hamburger and Hamilton, 1951; Eyal-Giladi and Kochav, 1976; Azar and Eyal-Giladi, 1981; Bellairs, 1982). Surrounding the area pellucida is the annular area opaca, the source of the extra-embryonic membranes which maintain the embryo throughout pre-natal life. During the first 3-4 days of incubation, the area opaca of the chick embryo (Gallus domesticus) expands across the inner surface of the vitelline membrane to encompass the entire yolk mass (Downie, 1976).

The vitelline membrane consists of three distinct layers (Bellairs et al, 1963; Bellairs et al, 1969; Jensen, 1969; Rhea and Rosenberg, 1971; Chernoff and Overton, 1977). The inner layer, formed around the yolk before ovulation (Bellairs, 1965), is a three-dimensional fibrous meshwork containing a substructure of fine fibrils embedded within a granular matrix material (Bellairs et al, 1963; Bellairs et al, 1969; Jensen, 1969; Chernoff and Overton, 1977). Proteins found within this inner membrane layer are similar to those associated with collagen in the connective tissues of the adult hen (Bellairs et al, 1963). The outer layer of the vitelline membrane comprises a varying number of sublayers, each of which consists of a meshwork of fine fibrils (Bellairs et al, 1963; Bellairs et al, 1969; Chernoff and Overton, 1977). In this case, the proteins present resemble materials occurring

within the surrounding egg albumen (Bellairs et al, 1963). Between the outer and inner layers of the vitelline membrane is a thin granular structure labelled the 'continuous membrane' by Bellairs et al (1963).

Early investigations led some authors to believe that the periphery of the area opaca was a syncytium (Duval, 1884; Harper, 1904; Blount, 1909; Patterson, 1907; Olsen, 1942), with cell proliferation the driving force behind its epibolic expansion across the vitelline membrane (Schlesinger, 1958; Haas and Spratt, 1968). New (1959), without ignoring the importance of cell proliferation, was the first to suggest that the activities of a peripheral band of migratory 'edge cells' were responsible for the expansion of the area opaca. The morphology of the peripheral area opaca has since been investigated using a variety of techniques including transmission electron microscopy (T.E.M.) (Bellairs, 1963; Bellairs et al, 1969; Downie and Pegrum, 1971; Chernoff and Overton, 1979; Andries and Vakaet, 1985a; Andries et al, 1985a) and scanning electron microscopy (S.E.M.) (Bellairs et al, 1969; Chernoff and Overton, 1977, 1979; Andries and Vakaet, 1985b). Several studies have also been performed to elucidate the mechanisms of area opaca expansion (Bellairs et al, 1969; Downie, 1974, 1975, 1976; Mareel et al, 1984).

The unincubated blastoderm is a loose assemblage of rounded cells fringed by filopodia - long thread-like cellular processes (Downie, 1974; Chernoff and Overton, 1979). After 5-6 hours incubation, the peripheral cells become attached to the inner surface of the vitelline membrane (Vakaet, 1962; Downie, 1974) and begin to migrate outwards, pulling the remaining blastoderm behind them as a cohesive epithelial sheet (Downie, 1976). At stages 4-5 (Hamburger and Hamilton, 1951), following 20-24 hours incubation, the outer regions of the blastoderm consist of two distinct cell layers (Bellairs, 1963). The dorsal layer, or epiblast, is a squamous epithelium continuous both with the

ectoderm of the central area pellucida and with the migratory 'edge cells' at the periphery of the area opaca. The ventral hypoblast layer, a loose association of rounded cells, lies between the epiblast and the yolk mass. During the expansion of the area opaca, the epiblast precedes the hypoblast creating a peripheral ring of free epiblast bordered by the attached 'edge cells'. This peripheral region was labelled the 'margin of overgrowth' by Bellairs and New (1962).

The migration of the epiblast 'edge cells' begins slowly, reaches a maximum of 500 um/hour after 3 days incubation and then slows as the coverage of the yolk mass nears completion (Downie, 1976). At stages 4-5, the attached 'edge cells' form a multilayered association at the epiblast periphery (Downie and Pegrum, 1971; Andries and Vakaet, 1985b; Andries *et al*, 1985a). Adjacent 'edge cells'are connected by tight junctions, gap junctions and desmosomes (Andries and Vakaet, 1985a; Andries *et al*, 1985a). 'Edge cells' in contact with the membrane surface are extensively flattened and possess lamellipodia oriented in the direction of epiblast expansion (Bellairs, 1963; Bellairs *et al*, 1969; Downie and Pegrum, 1971; Andries and Vakaet, 1985b). Small processes of the dorsal lamellipodial surface extend between the fibres comprising the inner layer of the vitelline membrane (Bellairs, 1963; Andries and Vakaet, 1985ab) and attachment plaques are observed toward the lamellipodial margins (Downie and Pegrum, 1971).

The exact nature of 'edge cell' movement is still the subject of debate. Bellairs *et al* (1969), using both S.E.M. and time-lapse cinematography, observed a regular series of protusions and withdrawals in the lamellipodia of actively migrating 'edge cells'. A similar 'lamellipodial' locomotion has been described for some fibroblastic (Abercrombie *et al*, 1977; Abercrombie, 1982) and epithelial (DiPasquale, 1975ab; Brown and Middleton, 1985) cell types moving across *in vitro* substrata. In a later S.E.M. study of the epiblast edge,

Chernoff and Overton (1977) observed long filopodial processes at the margins of 'edge cell' lamellipodia at stages 7-9. Similar processes were also found on 'edge cells' isolated *in vitro*. These observations led the authors to propose that 'edge cell' locomotion was 'filopodial/lamellipodial', a process resembling the spreading of fibroblasts in culture (Rajaraman *et al*, 1974; Heaysman *et al*, 1982). The lamellipodial cytoplasm contains well-defined bundles of cortical microfilaments and occasional arrays of unoriented microtubules (Downie and Pegrum, 1971), but the role of these cytoskeletal elements in 'edge cell' locomotion remains unresolved (Downie, 1975; Chernoff and Overton, 1979; Mareel *et al*, 1984).

Izzard and Lochner (1980) referred to the entire flattened protusion of actively migrating fibroblasts as the 'leading lamella', and resticted the term 'lamellipodium' to the active marginal regions of such a process. In the following study, lamellipodium will be used in the sense that Izzard and Lochner defined leading lamella, a practice widely shared by others discussing epithelial cell movement (DiPasquale, 1975ab; Radice, 1980ab; Donaldson and Dunlap, 1981).

For the first 20-24 hours of incubation (Downie, 1976), the migration of the attached 'edge cells' is accompanied by an increase in the tension across the simple squamous epithelium of the proximal epiblast (Bellairs, 1963; Chernoff and Overton, 1977). This tension rises to a peak at stages 4-5 and is subsequently reduced through cell proliferation and changes in cell shape (Downie, 1976). There is some evidence that the tension is not merely a passive result of 'edge cell' movement, but is generated by a band of cells toward the middle of the proximal epiblast (Kucera and Burnand, 1984).

Cell division was originally thought to be confined to a region just behind the advancing 'edge cells' (New, 1959), but current theory holds that mitoses are scattered evenly throughout the epiblast until

shortly before the closure of the yolk sack at stages 17-18 (Downie, 1976). Mitotic figures are not observed in 'edge cells' (Downie and Pegrum, 1971) and the epiblast continues to expand after treatment with aminopterin, a drug which prevents cell division without affecting microtubules (Bellairs, 1954; Downie, 1975). Downie (1976) has postulated that recruitment from the proximal epiblast provides the additional 'edge cells' required for continued epibolic expansion.

The unincubated epiblast is a monolayer of tall columnar cells (Downie, 1974). As the attached 'edge cells' move across the vitelline membrane, the cells of the proximal epiblast flatten with the result that each contributes a larger surface area to the expanding epithelium (Downie, 1976). This shape change may be produced by the direct action of intracellular microtubules (Downie, 1975), or may be a passive reaction to the tensions resulting from 'edge cell' migration (Downie, 1976; Mareel et al, 1984).

During the normal expansion of the area opaca, only the peripheral 'edge cells' of the epiblast attach to, and migrate across, the inner surface of the vitelline membrane (Downie and Pegrum, 1971; Downie, 1976). New (1959) put forward two hypotheses to account for the differential behaviour of epiblast 'edge cells': firstly, that "the cells of the blastoderm edge are intrinsically different as regards their surface structure"; and secondly, that 'edge cells' are intrinsically the same as those of the proximal epiblast "but behave differently as a result of their position". The attached epiblast periphery forms a distinct morphological unit (Andries and Vakaet, 1985ab; Andries et al, 1985a), and several authors have proposed that that the cells involved are inherently different from those of the unattached proximal epiblast (New, 1959; Bellairs and New, 1962; Bellairs, 1963; Bellairs et al, 1969; Chernoff and Overton, 1977, 1979). These differences may have been overstated, despite New's

(1959) observation that cells of the proximal epiblast would not attach to the vitelline membrane when given a free edge. Under certain conditions, explants of area opaca epiblast will attach to the inner surface of the vitelline membrane although, in contrast to the expansion of the intact epiblast, the entire underside of the explant adheres to the membrane substratum (Downie, 1971; Downie and Pegrum, 1971). Similar results have been obtained *in vitro* using explants of epiblast epithelium from the central area pellucida (Voon, 1980; Bellairs *et al*, 1981; Al-Nassar and Bellairs, 1982; Ireland and Stern, 1982).

The epiblast periphery exhibits a certain degree of specificity for its natural substratum, the inner surface of the vitelline membrane. Blastoderms explanted onto the inner face of the vitelline membrane *in vitro*, reattach and expand at rates similar to those observed *in ovo* (New, 1959; Chernoff and Overton, 1977; Downie, 1979). In contrast, blastoderms cultured on the outer surface of the vitelline membrane (New, 1959; Chernoff and Overton, 1977) or on plasma clots (Waddington, 1932) attach to the underlying substratum but do not expand.

2. Extracellular Materials.

Extracellular materials are thought to regulate many cellular interactions (Toole, 1981; Solursh *et al*, 1984) and the basement membranes (Hay, 1981) that underlie epithelial cell sheets contain a number of extracellular substances including glycosaminoglycans (GAGs), various collagens, especially type IV, and the glycoproteins fibronectin, laminin and entactin (Fisher and Solursh, 1977; Kleinman *et al*, 1982; Laurie *et al*, 1982; Laurie and Leblond, 1983; Tuckett and Morris-Kay, 1986).

In structural terms, basement membranes consist of an amorphous

sheet, the basal lamina, overlain by a layer of collagen fibrils (Hay and Dodson, 1973). A clear space, the lamina lucida, separates the basal lamina from the underlying epithelial cell surfaces (Low, 1961).

Small globular deposits of extracellular material are present on the ventral surface of the unincubated epiblast (Low, 1967; Sanders, 1979). During the first 20-24 hours of incubation, the epiblast produces large amounts of GAGs, especially hyaluronic acid (Solursh, 1976; Fisher and Solursh, 1977; Sanders, 1979; Vanroelen et al, 1980), and some striated fibrils, presumably collagen precursors (Frederickson and Low, 1971; Sanders, 1979). By stages 4-5, the ventral surface of the epiblast is covered by an amorphous basal lamina and an associated fibrous meshwork (Ebendal, 1976; England and Wakely, 1977; Wakely and England, 1979). Treating this basement membrane with hyaluronidase removes the hyaluronic acid, leaving only the fibrillar component in situ (Sanders, 1979).

No basement membrane is associated with the 'edge cells' at the epiblast periphery (Bellairs, 1963). A dark line is present on the ventral surface of some attached 'edge cells' by T.E.M., but is discontinuous and was thought to represent hydrated yolk material by Bellairs (1963).

All materials found within basement membranes were originally thought to be produced by the underlying epithelium (review: Kefalides et al, 1979), but several studies have suggested that some of these materials may come from adjacent tissues (Brownell and Slavkin, 1980; Brownell et al, 1981; Sariola et al, 1984). Harrisson et al (1985a) demonstrated that extracellular materials are transferred from the stage 3 hypoblast to the developing epiblast and Vanroelen et al (1982), at the same stages, found that removal of the hypoblast resulted in the absence of a normal basal lamina. Tufts of extracellular material have been observed on the dorsal surface of the area pellucida endoderm at

stage 5 (England, 1981).

Recent reports have suggested that, in the absence of glycoproteins, GAGs and collagens are unable to promote cell adhesion or migration (Fisher and Solursh, 1979; Rich et al, 1981; Newgreen et al, 1982; Erickson and Turley, 1983; Tucker and Erickson, 1984), although certain GAGs may act to open up tissue spaces prior to cell movement (Solursh, 1976; Toole, 1981; Anderson and Meier, 1982).

Fibronectin, first identified as a cell-surface glycoprotein by Hynes (1973), is believed to influence the behaviour of many cell types both in vivo and in vitro (reviews: Hynes, 1981; Hynes and Yamada, 1982; Hynes, 1986). Through interactions at the cell surface (Yamada, 1983), the glycoprotein is thought to regulate certain cell-substrate interactions (Duband et al, 1986), including attachment (Couchman et al, 1982) and migration (Yamada, 1983).

Fibronectin fibrils form parallel to the long axis of spreading fibroblasts (Hynes and Destree, 1978) and, on the basis that these fibrils affect the organisation of the cytoskeleton (Pearlstein et al, 1980), Yamada (1983) has proposed that they might determine the direction of future cell migration. Oriented fibrillar deposits of fibronectin can direct cell migration in vitro (Chiquet et al, 1981; Turner et al, 1983) and extracellular collagen matrices containing fibronectin are known to guide the in vivo movements of mesoderm (Wakely and England, 1979), neural crest cells (Newgreen and Thiery, 1980; Greenberg et al, 1981; Newgreen et al, 1982; Rosavio et al, 1983; Tucker and Erickson, 1984), primordial germ cells (Wakely and England, 1979; England, 1980a; Bronner-Fraser, 1981; England, 1982) and developing corneal epithelium (Kurkinen et al, 1979; Fujikawa et al, 1981).

Laminin (Timpl et al, 1979), another glycoprotein commonly associated with epithelial basement membranes (Foidart et al, 1980;

Laurie et al, 1983; Meier and Drake, 1984), is thought to mediate the attachment of epithelial cells to the collagen fibrils contained within their basal laminae (Terranova et al, 1980).

From immunocytochemical studies, it appears that fibronectin is present on the stage 4-5 epiblast basal lamina (Wakely and England, 1979; Duband and Thiery, 1982; Mitrani, 1982; Mitrani and Faberov, 1982; Sanders, 1982; Harrisson et al, 1984ab; Harrisson et al, 1985b). Laminin has also been detected toward the periphery of the basal lamina of the extra-embryonic epiblast (England and Wakely, personal communication). As yet, no investigations have reported the presence of entactin within the epiblast basal lamina.

Extracellular materials form a fibrillar network on the basal lamina of the stage 4-5 extra-embryonic epiblast, the pattern of which changes from the centre to the periphery of the area opaca (Wakely and England, 1979; Monnet-Tschudi et al, 1985). These fibres contain fibronectin (Mayer et al, 1981; Monnet-Tschudi et al, 1985) and, at later stages, are thought to provide some form of contact guidance (Weiss, 1945; Dunn, 1982) for the outward expansion of the area opaca mesoblast (Mayer and Packard, 1978; Mayer et al, 1981; Monnet-Tschudi et al, 1985).

Small spherical aggregations of extracellular materials are also associated with the ventral surface of the epiblast basal lamina (Mayer and Packard, 1978; Mayer et al, 1981). These 'interstitial bodies' (Low, 1962, 1968, 1970) contain GAGs and fibronectin (Cohen and Hay, 1971; Mayer and Packard, 1978; Mayer et al, 1981). Similar structures have also been observed with the S.E.M. during the movement of neural crest cells through extracellular matrices (Tosney, 1978; Mayer et al, 1981).

Fibronectin is associated with the migratory 'edge cells' at the periphery of the extra-embryonic epiblast and has been detected both on

the outer plasma membrane and within the 'edge cell' cytoplasm (Critchley et al, 1979; Monnet-Tschudi et al, 1985). Several authors have proposed that active cells may internalise fibronectin in order to deactivate an excess (Hatten et al, 1982; Chen et al, 1984).

As in the epiblast of the area opaca, extracellular materials are arranged in a series of distinct fibrillar patterns on the contiguous basal lamina of the adjacent stage 4-5 area pellucida epiblast (Critchley et al, 1979; Wakely and England, 1979; England, 1980b, Kordylewski and England, 1980; England, 1981; Harrisson et al, 1984b; Andries et al, 1985b; Harrisson et al, 1985b). It has been proposed (Critchley et al, 1979; Wakely and England, 1979; Andries et al, 1985b) that a 'fibrous band' at the anterior boundary of the area pellucida may be responsible for directing both the outward expansion of the newly formed embryonic mesoblast and the movement of primordial germ cells (Wakely and England, 1979; England, 1980a; Bellairs, 1982; England, 1982). This structure has been shown to contain type I collagen, GAGs and fibronectin (Critchley et al, 1979; Wakely and England, 1979; England et al, 1982).

Posterior to the 'fibrous band', at early stage 5, an additional fan-shaped collection of fibres is thought to perform a similar function in guiding the anterior movement of the pre-notochordal mesoderm (England, 1981; Harrisson et al, 1985b). These processes also contain fibronectin and, like the fibres in the area opaca, are associated with 'interstitial bodies' (Sanders, 1982, 1984; Harrisson et al, 1985b). There is evidence that the deep layer (Sanders et al, 1978) covering the fibres may play some part in their maintenance (Kordylewski and England, 1980; Harrisson et al, 1985a) and Sanders (1984) has suggested that the 'interstitial bodies' represent certain turnover products of the basement membrane.

Fibrillar systems also appear to guide the presumptive mesoderm

following gastrulation in some urodele amphibians (Nakatsuji et al, 1982; Nakatsuji and Johnson, 1984; Darribere et al, 1985). The fibres involved contain fibronectin (Boucat and Darribere, 1983ab) and antibodies to this glycoprotein can reversibly prevent normal gastrulation (Boucat et al, 1984).

B. The Closure of Epithelial Wounds.

Wound closure, as distinct from wound healing, is a process comprising the spreading of epithelia and other phenomena immediately associated with a discontinuity in the cell sheet (Trinkaus, 1976). Wound healing is a more complex process and implies an almost complete restoration of the original epithelial morphology (McMinn, 1976). The closure of epithelial wounds, through the lateral movement of cells at the newly formed wound edge, appears to be a general characteristic of metazoan epithelia, leading Rand (1915) to hypothesise that 'an epithelium will not tolerate a free edge'.

Epithelial wound closure has been extensively studied over the years (review: Wright and Alison, 1984) due, no doubt in part, to it's direct and obvious clinical relevance (McMinn, 1976). However, the observation that such epithelial spreading restores the continuity of the cell sheet both in adult epithelia (Donaldson and Dunlap, 1981), larval epithelia (Radice, 1980a), embryonic epithelia (Stannisstreet *et al*, 1980) and epithelia isolated *in vitro* (Voon, 1980) suggests that wound closure may also represent an expression of underlying morphogenetic principles (Stannisstreet and Jumah, 1983).

As a restorative morphogenetic movement, wound closure offers several distinct advantages to the investigator; it can be induced at will through direct manipulation (Trinkaus, 1976), can be studied in a simple epithelial system supplied with a suitable natural (Gipson *et al*, 1984) or artificial (Takeuchi, 1976) substratum, and is amenable to observation both *in vivo* (Radice, 1980ab) and *in vitro* (Gibbins, 1978).

Following Peters' (1885) original observation of the rapid movement of epithelial cells over a fresh wound surface, Barfurth (1891) established that the cells effecting wound closure originate

from areas bordering the wound. Despite this early start, and the intensive investigations that have been performed since, many of the basic mechanisms underlying wound closure, and epithelial expansion in general, are still open to debate.

Wound closure, as a process in time, may be divided into three distinct, though contiguous, stages:

(i) The reactions of cells at, or near, the wound edge to the epithelial discontinuity;

(ii) The lateral movements of these marginal cells;

(iii) The fusion of apposing wound margins to effect wound closure.

Initial reactions to the trauma of wounding are similar in most of the epithelia studied. The wound gapes and the cells at the newly formed wound margin become rounded (England and Cowper, 1977; Radice, 1980a, Stanisstreet et al, 1980), possibly due to a local decrease in epithelial tensions (England and Cowper, 1977; Stanisstreet et al, 1980; Smedley and Stanisstreet, 1984). Marginal cells may increase in size (McMinn, 1976; England and Cowper, 1977) and possess blebs, microvilli and filopodia on their exposed surfaces (Van Horn et al, 1976; England and Cowper, 1977; Pang et al, 1978; Stanisstreet et al, 1980).

In contrast, the lateral movement of the epithelium at and around the wound margin is both one of the most variable and least understood aspects of wound closure. Epithelial wounds may close through changes in cell shape (Stanisstreet et al, 1980; Smedley and Stannisstreet, 1984), epithelial proliferation and minor alterations in cell position (Rafferty, 1976; Rafferty and Smith, 1976) or the migration of

marginal cells across an underlying substratum (Pang et al, 1978; Radice, 1980a).

There is some evidence that the cell behaviours employed during wound closure depend largely on the nature of the wound inflicted (Wright and Alison, 1984) and the organisation of the epithelium involved (Stannisstreet et al, 1980). In the embryonic ectoderm of Xenopus and the chick, wound closure is effected by changes in cell shape - with limited mitoses and cell rearrangement (Stanisstreet and Panayi, 1980; Stanisstreet et al, 1980; Stanisstreet, 1982; Stanisstreet et al, 1986). There is considerable evidence that, in Xenopus at least, these shape changes may be linked to the actions of calcium-activated microfilaments (Nakatsuji, 1979; Stanisstreet and Panayi, 1980; Stanisstreet, 1982; Stanisstreet and Jumah, 1983; Smedley and Stanisstreet, 1985b). In the endoderm of the chick embryo, however, wound closure is bought about by active cell migration across the ectodermal basement membrane (England and Cowper, 1977; Mareel and Vakaet, 1977). Stanisstreet et al (1980) proposed that differences in the cell behaviours observed could arise from differences in the structures of the epithelia involved. At the stages used, the chick endoderm, unlike the ectoderm, has no coherent basement membrane to act as a substratum for wound margin migration (Low, 1967). In addition, the ectodermal cells are linked by highly developed junctional complexes (Trelstad et al, 1967) which might preclude the cellular independance required for migration.

In most adult stratified epithelia, the lateral migration of epithelial cells at the wound margin is the process predominantly responsible for wound closure (Croft and Tarin, 1970; Pfister, 1975; Gabbiani et al, 1978; Gibbins, 1978; Radice, 1980ab; Gipson and Kiorpes, 1982). Although mitoses in the surrounding epithelium may accompany cell locomotion (Takeuchi, 1976; Selden and Schwartz, 1979), and

result in a certain amount of passive cell translocation (Matoltsky and Viziam, 1970), migration and proliferation appear to act independantly during the closure of epithelial wounds by cell movement (Dunlap and Donaldson, 1978; Yamanaka and Eguchi, 1981; Wright and Alison, 1984).

The evidence suggests that the inward movement of the newly formed epithelial edge is an active process (Donaldson and Mahan, 1984) mediated by marginal cells attached to the underlying substratum, be it basement membrane (Radice, 1980a), connective tissue (Tarin and Croft, 1970) or fibrin clot (Repesh and Oberpriller, 1978). The migrating epithelial cells possess filopodia, lamellipodia and ruffled membranes (Pfister, 1975; Gibbins, 1978; Pang *et al*, 1978; Repesh and Oberpriller, 1978), which are processes characteristic of cell movement both *in vivo* and *in vitro* (Abercrombie *et al*, 1971; DiPasquale, 1975ab; England, 1983). Microfilaments arise *de novo* in epithelial cells at the wound margin (Krawczyk, 1971; Gabbiani and Ryan, 1974; Gabbiani *et al*, 1978; Repesh and Oberpriller, 1980) and Cytochalasin B, a drug which disrupts microfilaments (Wessels *et al*, 1971) and inhibits *in vitro* cell movement (Miranda *et al*, 1974; Atlas and Lin, 1978), can prevent the closure of epithelial wounds by cell migration (Donaldson and Dunlap, 1981; Pascolini *et al*, 1984).

The exact mechanisms by which epithelial wound margins migrate during wound closure remain open to debate (Trinkaus, 1976; Pang *et al*, 1978; Repesh and Oberpriller, 1980; Wright and Alison, 1984). Epithelia expanding *in vitro* are attached to the underlying substratum through a single row of migratory cells at the epithelial periphery (Vaughan and Trinkaus, 1966; DiPasquale, 1975ab; Vasiliev and Gelfand, 1977). Proximal cells retain their ranks during the spreading of the cell sheet, and there is little alteration in the relative positions of adjacent submarginal cells (Vaughan and Trinkaus, 1966). The chick extra-embryonic epiblast appears to expand in a similar fashion

(Downie, 1976), although the attached periphery is a multilayer of flattened 'edge cells' (Downie and Pegrum, 1971; Andries and Vakaet, 1985ab; Andries et al, 1985a). Several authors have proposed that this 'gliding' form of epithelial locomotion effects wound closure in a range of amphibian epithelia (Lash, 1955; Radice, 1980ab) and mammalian epithelia (Odland and Ross, 1968; Croft and Tarin, 1970; Martinez, 1972).

In contrast, most mammalian epidermal wounds (Winter, 1972; Krawczyk, 1971, 1972; Beerens et al, 1975; Winstanley, 1975) and mucosal wounds (Sciubba, 1977; Gibbins, 1978) appear to close through the submarginal cells 'rolling or sliding' over their neighbours at the epithelial edge. A similar form of epithelial expansion has also been described during wound closure in some amphibian epithelia (Derby, 1978; Repesh and Oberpriller, 1978, 1980).

Although these models of epithelial advancement are based largely on the static cell morphologies observed in wound margins fixed for light or electron microscopy, both patterns have also been observed during time-lapse cinematography of wound closure in living tissue (Gibbins, 1978; Radice, 1980a). In addition, wound closure in the cornea of some amphibians appears to involve elements of both patterns of epithelial expansion (Yamanaka and Eguchi, 1981)

The cells at an epithelial wound margin generally cease migration when they contact their counterparts from the apposing wound margin (Radice, 1980a), although a slight delay in the quiescence of the migrating submarginal cells often results in some thickening of the cell sheet surrounding the closed wound (Derby, 1978; Repesh and Oberpriller, 1978). One of the initial stimuli for epithelial expansion at a wound margin could be a localised release of 'contact inhibition' (Abercrombie and Heaysman, 1954; Middleton, 1977; Heaysman, 1978) at the newly formed edge and its cessation, although probably influenced by

many factors (Croft and Tarin, 1970), may result from cell-cell contacts at the closed wound restoring 'contact inhibition' of movement throughout the cell sheet (Martinez, 1972; Winter, 1972).

The expansion of epithelial sheets forms an integral part of morphogenesis (England and Wakely, 1977; Mayer and Packard, 1978) and the recapitulative process of wound closure (Repesh and Oberpriller, 1978; Radice, 1980a). As a result, the expansion of the chick extra-embryonic epiblast is of interest, not only for it's direct relevance to avian developmental biology, but also as a readily accessible model system with which to investigate epithelial migration over a natural substratum (New, 1959; Chernoff and Overton, 1979). In addition, the limited area of epiblast attachment (Downie and Pegrum, 1971) and the identification of the specialised function of the 'edge cells' (Downie, 1976; Andries and Vakaet, 1985ab) make the system especially suitable for the study of cell-substratum relationships at the periphery of an actively expanding epithelium (Downie and Pegrum, 1971; Chernoff and Overton, 1977, 1979).

This study has been undertaken with several aims:

(i) To re-examine the morphology of the extra-embryonic epiblast edge, with special reference to the influence of fixation temperature;

(ii) To investigate the effects of removing a small portion of the attached epiblast periphery and, during the subsequent wound closure, to compare the organisation of the wound margin with that of the normal epiblast edge;

(iii) To examine the morphology of the extracellular materials produced by the epiblast cells at the wound margin and the 'edge cells' of the normal epiblast edge.

To further investigate the cell behaviours involved, explants and isolated cells from the epiblast periphery were reincubated on the inner surface of the vegetal pole vitelline membrane. Explants were prepared with and without 'edge cells', and the single cell suspensions used contained both 'edge cells' and epiblast cells of the adjacent proximal epithelium.

II. MATERIALS AND METHODS.

A. Specimen Preparation.

Fertilized hens' eggs of the White Leghorn sub-species of Gallus domesticus were used for this study. The eggs were incubated in a Trimline incubator at a temperature of 37.5°C (+/- 1°C) and a humidity of 60-65%. The embryos were dissected out as for New culture (New, 1955) and assigned stages according to Hamburger and Hamiltons' normal series of embryonic development (Hamburger and Hamilton, 1951).

1. New Culture.

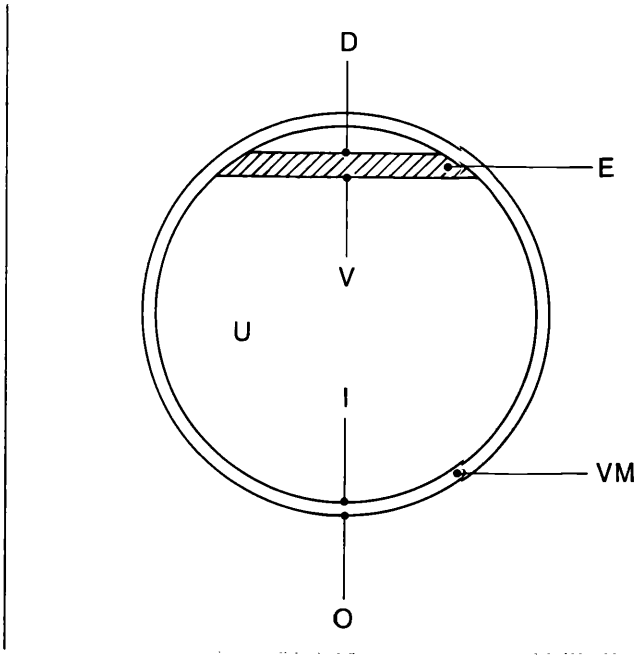
All the embryos studied were isolated in vitro using a slightly modified version of the New technique (New, 1955). The eggs were incubated for a period designed to produce the developmental stage required and dissected out using sterile instruments.

Eggs were opened by gently tapping the shell at the narrow end and removing the top-piece with attendant shell membranes. The gelatinous albumen and chalazae were discarded and the fluid albumen retained for future use as a nutrient medium. The yolk was poured into a glass crystallising bowl containing enough normal saline (Pannet and Compton, 1924) to cover it completely and any gelatinous albumen remaining on the outer surface of the vitelline membrane removed by pipette. A shallow watch-glass was placed in the glass bowl and scissors used to cut the vitelline membrane around the equator of the yolk. Using forceps, the vitelline membrane hemisphere containing the embryo was gently peeled from the yolk and transferred to the watch-glass with the embryo uppermost. A glass ring was placed over the vitelline membrane, with the embryo positioned centrally, and the watch-glass and contents

lifted into a Petri-dish lined with a moist tissue-paper ring. Saline was removed from the preparation until the liquid level fell below the top of the glass ring. The free edge of the vitelline membrane was trimmed with scissors and, using forceps, folded over the glass ring so as to produce an even tension across the membrane. Excess yolk was removed from the preparation by gentle pipetting with saline under a Kyowa dissecting microscope.

Care was taken not to dislodge the embryo from the vitelline membrane or disrupt the epithelial integrity of the epiblast. Only those specimens which appeared intact were used. For reincubation excess saline was removed from above the preparation and fluid albumen placed beneath the vitelline membrane. Specimens were reincubated in closed petri-dishes at a temperature of 37.5^{o}C and a humidity of 60-65 % using a Trimline incubator.

In the present study the terms 'dorsal', 'ventral', 'inner' and 'outer' refer to <u>in ovo</u> relationships (diagram 1).



(Diagram 1). The <u>in ovo</u> arrangement of the inner (I) and outer (O) layers of the vitelline membrane (VM), and the dorsal (D) and ventral (V) surfaces of the embryo (E).
Y - Yolk.

2. The Normal Epiblast Periphery.

Chick embryos of stages 4-5 were prepared as for New culture. The ventral surface of the epiblast periphery and adjacent vitelline membrane were cleared of loosely adherent yolk by gentle pipetting with saline. The embryos were then examined under a Kyowa dissecting microscope, to ensure that the epiblast epithelium remained intact, and fixed in Karnovsky's fluid at a temperature of 20°C (+/- 3°C) for a period of 6-12 hours.

As a control, some stage 4-5 embryos were reincubated at a temperature of 37.5°C for 30 minutes to 1 hour before fixation in Karnovsky's. This reincubation period allowed the epiblast to recover from any previously undetected disturbances resulting from the New culture procedure, and produced active epiblast expansion accessible to immediate fixation. Such embryos also act as controls for any changes in cell morphology resulting from reincubation.

3. The Epiblast Periphery after Cold Fixation.

Initial preparation was as described for the study of normal epiblast morphology (Section A. 2), but the embryos were fixed using Karnovsky's fluid at a temperature of 5°C (+/- 1°C) for 6-12 hours.

4.Wounding and Wound Closure in the Epiblast Periphery.

Stage 4-5 chick embryos were isolated as for New culture and cleared of excess yolk by gentle pipetting with saline. Only those specimens which appeared undamaged were used to investigate wounding and subsequent wound closure.

Embryos were wounded under saline to avoid the effects of surface

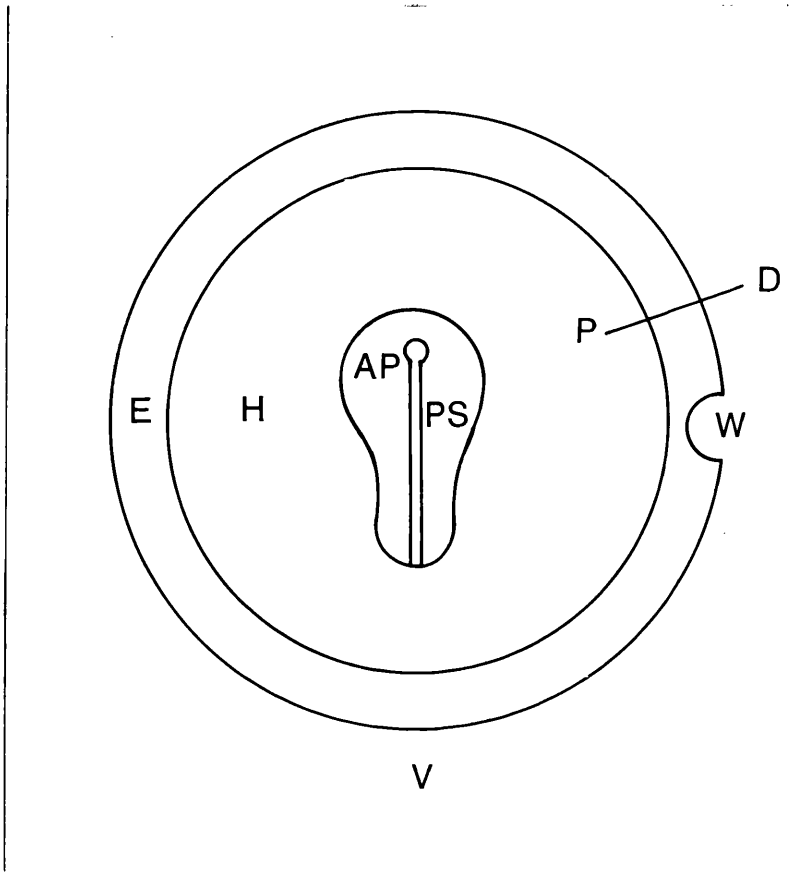
tension. The wounding was performed using a cactus needle (Rebutia hybrid) mounted on a cocktail stick with the colloidal silver paint Electrodag 915 (England, 1981). A 200μm portion of the attached epiblast periphery was lifted from the surface of the vitelline membrane, excised and removed from the preparation by pipette. The resultant wound appeared as a 200μm long indentation of the epiblast periphery (diagram 2). Wounds were confined to the epiblast periphery lateral to the midpoint of the area pellucida, and were made at right-angles to the line of the primitive streak. Measurements of initial wound size were taken in vitro using a calibrated 1 cm eyepiece graticule at a fixed magnification of approximately 50X.

In the present study, those structures lying toward the epiblast periphery are defined as distal, while those lying toward the embryonic axis are defined as proximal. All measurements of wound size were made parallel to the epiblast periphery, at right-angles to the proximal-distal axis.

At stages 4-5 the chick embryo is bilaterally symmetrical about the primitive streak. In some specimens wounds were made in the epiblast periphery lateral to both sides of the streak. In other specimens, one side of the epiblast was left undisturbed as a control.

To examine epiblast movements during wound closure, carbon particles were placed on the ventral surface of the epithelium surrounding 0-hour epiblast wounds. Individual wounds were photographed before reincubation and again after reincubation for 1, 5 and 10 hours at a temperature of 37.5 ^{o}C.

Of the remaining wounded embryos, some were immediately fixed by immersion in Karnovsky's fluid at 20 ^{o}C for 6-12 hours (0-hour wounds). Others were reincubated at 37.5 ^{o}C for periods of 1, 5 and 10 hours before fixation in Karnovsky's as described above.



(Diagram 2). Ventral view of a wounded stage 4 chick embryo. The proximal-distal axis (P-D) lies at right-angles to the epiblast periphery.
AP - Area pellucida, E - Epiblast, H - Hypoblast, PS - Primitive streak, V - Vitelline membrane, W - Wound.

5. Explants of the Epiblast Periphery ('Edge Cells' and Proximal Epiblast).

Stage 4-5 chick embryos were isolated as for New culture. The remaining vegetal pole vitelline membranes were also lifted from the yolks and mounted according to the New technique. All *in vitro* preparations were cleared of excess yolk by gentle pipetting with fresh saline.

Explants were obtained by the excision of small pieces of the peripheral extra-embryonic epiblast. The operation was performed under saline with a mounted cactus needle and resembled the wounding procedure detailed in Section A. 4. For each cultured embryo, short distances of the attached epiblast periphery, lateral to the midpoint of the area pellucida, were lifted from the surface of the vitelline membrane and excised. Similar excisions were made from both sides of the epiblast and up to 8 explants were removed from any one embryo. Each explant was approximately 200μm square and contained a length of previously attached 'edge cells' and a small portion of the adjacent proximal epiblast (diagram 3.I). Measurements of explant size were taken *in vitro*, both during and after excision, using a calibrated 1cm eyepiece graticule at fixed magnification.

Excised explants were removed from the preparation by pipette, washed in fresh saline to remove loosely adherent yolk and transferred, in a small volume of saline, to the corresponding vegetal pole vitelline membrane. The explants were positioned equidistant and manoeuvered so that they were ventral surface uppermost (the ventral surface may be identified by the presence of yolk globules).

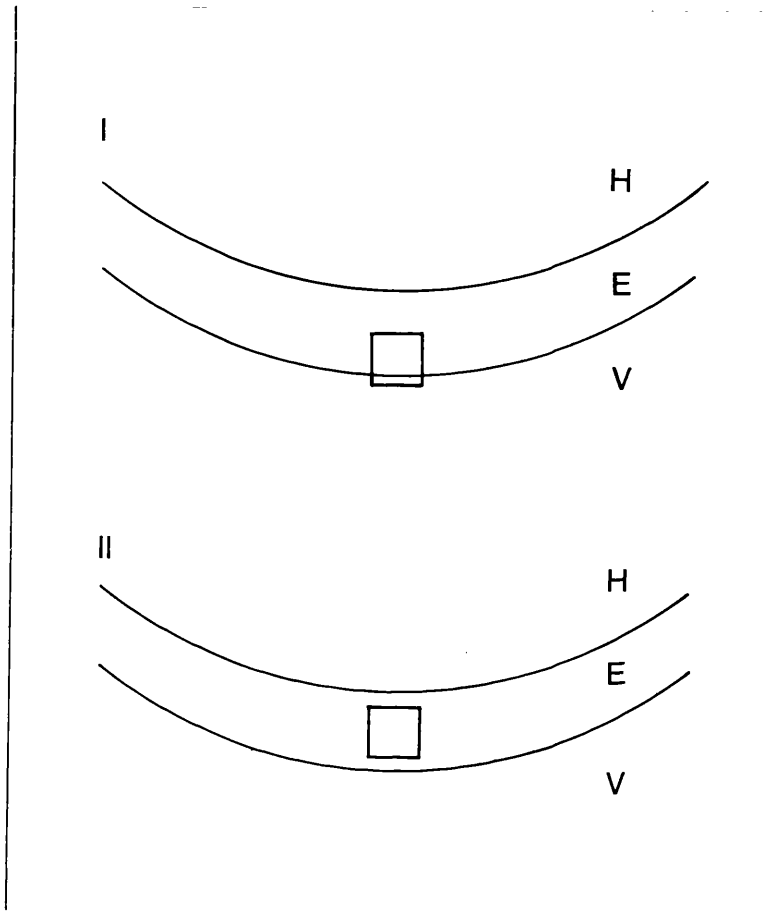
To follow explant movements relative to the vitelline membrane, a number of carbon particles were placed on the membrane surface adjacent to the explants. Excess saline was then slowly removed from the New culture ring, and the explant-membrane preparations reincubated at 37.5

^{o}C for 1, 2 and 3 hours before fixation in Karnovsky's fluid for 6-12 hours at 20 ^{o}C. Individual explants were photographed under a Kyowa dissecting microscope after 1, 2 and 3 hours of reincubation and, using the carbon particles as reference points, tracings were made of each explant after successive reincubation periods. These tracings were taken from the negatives using a Durst Bimaneg enlarger.

6. Explants of the Epiblast Periphery (Proximal Epiblast Only).

Stage 4-5 embryos were isolated as for New culture and the vegetal pole vitelline membranes mounted as described in Section A. 5. Explants were also prepared from individual embryos, but consisted of proximal epiblast from the 'margin of overgrowth' adjacent to the attached 'edge cells' (diagram 3.II). These 200μm square explants were mounted, ventral surface uppermost, on the inner surface of the corresponding vegetal pole vitelline membrane and reincubated for 2 hours at 37.5 ^{o}C before fixation in Karnovsky's fluid for 6-12 hours at 20 ^{o}C.

Tracings were made of explant outline and position after reincubation for 1 and 2 hours as previously described (Section A. 5).



(Diagram 3). Diagram of the stage 4-5 extra-embryonic epiblast periphery showing the areas excised to prepare explants containing 'edge cells' and proximal epiblast (I) and proximal epiblast only (II).
E - Epiblast, H - Hypoblast, V - Vitelline membrane.

7. Isolated Cells of the Epiblast Periphery ('Edge Cells' and Proximal Epiblast).

Stage 4-5 chick embryos were removed from their yolks and mounted as for New culture. The vegetal pole vitelline membranes were also mounted according to the New technique.

Under saline, the lateral extra-embryonic epiblast periphery - or 'margin of overgrowth' - was dissected from individual embryos using a mounted cactus needle. The tissue removed contained 'edge cells' and adjacent proximal epiblast. Excised portions were removed from the embryo preparation by pipette and transferred to a petri-dish containing fresh saline. The tissue was then washed in saline to remove excess yolk globules and transferred to a second petri-dish containing a freshly made solution of 1% trypsin in saline (pH adjusted to 7.4 with 0.1N sodium hydroxide). After immersion in the trypsin solution for 15-30 minutes at room temperature (approximately 20 ^{o}C), the tissue was transferred to a small volume of fresh saline and dissociated by gentle pipetting. The cell suspension was then spun down for 5 minutes at 1200 r.p.m. in a MSE Centaur 1 centrifuge, re-suspended in 2ml of saline and placed on the inner surface of the corresponding vitelline membrane. The prepared cell-membrane preparations were reincubated for 1 and 3 hours at 37.5 ^{o}C before fixation in Karnovsky's for 6-12 hours at 20 ^{o}C.

B. Specimen Examination.

All specimens were initially fixed in Karnovsky's fluid, as previously detailed in Section A, Specimen Preparation. The specimens were then washed in several changes of 0.2M sodium cacodylate buffer, pH 7.4 (Plumel, 1948) for 6 to 12 hours and post-fixed in cacodylate-buffered 1-2% osmium tetroxide for 30-40 minutes. After fixation the specimens were dehydrated, while still stretched over the New culture ring, in the following ascending series of graded ethanol-distilled water concentrations.

(i)	30% ethanol-distilled water.	15-20 minutes.
(ii)	50% ethanol-distilled water.	15-20 minutes.
(iii)	70% ethanol-distilled water.	12-24 hours.
(iv)	90% ethanol-distilled water.	15-20 minutes.
(v)	100% ethanol.	30 minutes.

1. Scanning Electron Microscopy (S.E.M.).

After dehydration the specimens were transferred from 100% ethanol to 100% acetone, previously dried using a molecular sieve. The areas of the specimen to be examined by S.E.M. were dissected from the New culture ring, under a Kyowa dissecting microscope, using a Borradaile knife and watch-makers forceps. The dissected specimens were placed in gelatine capsules with holes bored at each end (a modification of Carri and Suburu, 1979, by England, 1981) and stored in dried 100% acetone, with several changes, for 12-24 hours.

(a). Critical Point Drying of Specimens.

The gelatine capsules containing the specimens were transferred, in 100% dried acetone, to the chamber of a Polaron critical point drying apparatus. The acetone was replaced by liquid carbon dioxide, at a temperature of 20°C and a pressure of 800 PSI, by a flushing procedure repeated 3 times over a period of 10 minutes. The specimens were left for a period of 20 minutes, to allow the carbon dioxide to completely infiltrate the tissues, and any residual acetone removed by repeating the flushing procedure a further 3 times over a period of 10 minutes. The whole preparation was then slowly heated until, at a temperature of 31.5°C and a pressure of 1200 PSI, the carbon dioxide reached its critical point and moved from the liquid to the gaseous phase. The gaseous carbon dioxide was then slowly leaked from the preparation and the dried specimens, still in their gelatine capsules, were removed from the apparatus.

(b). Mounting of Specimens.

After critical point drying, the specimens were removed from the gelatine capsules and mounted on aluminium Cambridge stubs using double-sided sticky tape or the colloidal silver paint Electrodag 915.

To study the ventral surface of the intact peripheral extra-embryonic epiblast, embryos and attached vitelline membrane were mounted on stubs with the embryo lying uppermost. A similar specimen orientation was also used for the ventral surface of the peripheral epiblast after cold fixation, the ventral surface of the intact epiblast wound and the ventral surface of the epiblast explants and single cells on the vegetal pole vitelline membrane.

To expose the proximal margin of the attached epiblast 'edge cells'

and adjacent membrane surface, the proximal epiblast was excised from specimens mounted - ventral surface uppermost - as described above. The unattached epithelium was dissected away using a mounted cactus needle (Section A. 4). A similar procedure was used to expose the membrane surface surrounding epiblast wound margins after reincubation for 5 and 10 hours.

To examine the dorsal surface of the extra-embryonic epiblast periphery, the vitelline membrane was removed from the embryo in one of two ways. Some embryos were initially mounted ventral surface upwards. The dried epiblast periphery was then gently teased from the surface of the underlying vitelline membrane, using a mounted cactus needle. The isolated epiblast was then remounted, using colloidal silver paint, with its dorsal surface uppermost. Alternatively, the specimens were mounted dorsal surface upwards, and the overlying vitelline membrane carefully dissected away. Both techniques were successfully used to observe the proximal dorsal surface of the epiblast, but resulted in some damage to those peripheral regions of the epiblast originally attached to the surface of the vitelline membrane. This limitation was exaggerated during the study of 5 and 10 hour epiblast wound reincubations as the wound margins, and some of the proximal epiblast, were also attached to the surface of the vitelline membrane.

(c). Gold-Coating of Specimens.

Mounted specimens were stored overnight in a dessicator, to allow the colloidal silver paint to fully evaporate. The specimens were then coated with 20-25 nm of gold in a Polaron sputter coater.

Coated specimens were examined under an International Scientific Instruments (I.S.I.) 60 scanning electron microscope with an accelerating voltage of 30 kV, and an I.S.I. DS-130 S.E.M. with varying

accelerating voltages.

2. Thick Sections.

The initial specimen preparation, to 100% ethanol, is as previously described for scanning electron microscopy in Section B. 1. The specimens were left in 100% ethanol for a period of 2-6 hours, with several changes, to allow complete infiltration of the tissues and the removal of any residual water. The areas of the specimen to be examined were then dissected from the New culture ring, under a Kyowa dissecting microscope, using a Borradaile knife and watch-makers forceps.

(a) Embedding of Specimens.

The dissected specimens were removed from the 100% ethanol, and embedded in araldite (Glauert and Glauert, 1958). The araldite was taken from a stock solution, and thoroughly mixed with a polymerisation accelerator (Benzyldimethylamine, BDMA). The specimens were then placed in foil dishes, containing the araldite-accelerator mixture which was allowed to polymerise over a period of 48 hours, at a temperature of 60^{o}C, in a B & T (Searle) oven.

(b). Thick Sectioning of Specimens.

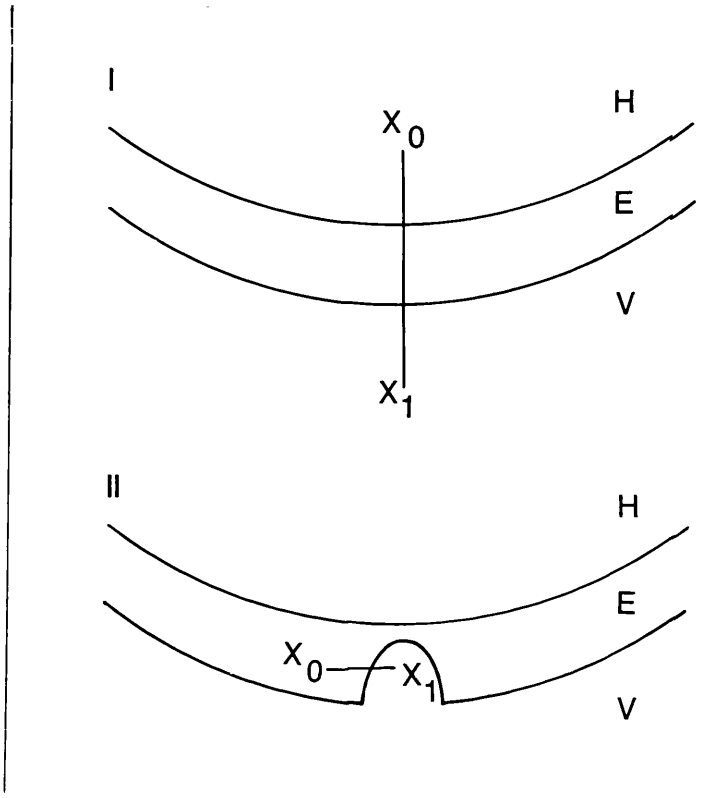
After polymerisation the araldite was removed from the foil dishes and cut into rectangular pieces, each containing a single specimen. The smaller blocks were trimmed with a razor blade, under a binocular dissecting microscope, to produce a rectangular block-face with the specimen positioned centrally. The block was then placed in the chuck of a Reichert-Jung OMU-4 or Huxley (Cambridge) Ultramicrotome and 1-2 um

thick sections cut from the long axis of the block-face, using glass knives previously prepared on a LKB 7800 Knifemaker. The sections were transferred to drops of 10% acetone-distilled water on glass slides, and placed on a B & T (Searle) hotplate, at a temperature of 60^{o}C, until the 10% acetone evaporated and the sections flattened. The staining procedure involved covering the sections with 1% toluidine blue in 1% borax and heating the slides on the hotplate for 15-30 seconds, at which point the stain began to evaporate. Excess toluidine blue was then removed with distilled water, and the slides returned to the hotplate until dry.

(c). Mounting of Sections.

Stained sections were mounted in araldite-accelerator, under glass coverslips. The slides were then left for 14-21 days, in the dark and at room temperature, to allow the araldite to polymerise slowly. Finally excess araldite was removed from the slides using 100% dried acetone and the mounted specimens viewed, in transmitted light, under a Zeiss Ultraphot.

In the normal and cold-fixed extra-embryonic epiblast, sections were cut longitudinally, at right-angles to the epiblast periphery, to demonstrate the morphology of the epiblast periphery and adjacent proximal epiblast. To demonstrate the morphology of the wound margin, sections were cut transversely, parallel to the epiblast periphery (diagram. 4).



(Diagram 4). Diagrams of the ventral surface of the epiblast periphery.
I. Normal epiblast periphery showing the orientation (X_o-----X_1) of longitudinal sections through the attached edge.
II. Wounded epiblast periphery showing the orientation (X_o-----X_1) of transverse sections through the wound margin.
E - Epiblast, H - Hypoblast, V - Vitelline membrane.

3. Transmission Electron Microscopy (T.E.M.).

Initial specimen preparation was as previously described for thick sections in Section B. 2.

(a). Thin Sections (Ultramicrotomy) of Specimens.

After polymerisation the araldite was removed from the foil dishes and cut into rectangular pieces containing individual specimens. These smaller blocks were then trimmed with a razor blade, under a binocular dissecting microscope, to give a four-sided truncated pyramid with a

0.5mm square block-face. After examining a preliminary series of thick sections (Section B. 2), additional cuts were made to produce a trapezoidal block-face with the specimen positioned centrally or toward the wider portion. Sections were cut, using a Reichert-Jung OMU-4 ultramicrotome and glass knives produced as previously described (Section B. 2), with the wider edge of the block-face meeting the knife first. The sections were collected in troughs filled with distilled water and their thickness determined by the observation of interference colours. Ribbons of silver (60-90 nm) or gold (90-150 nm) sections were flattened onto the surface of the distilled water, by exposure to 100% chloroform vapour, and picked up on uncoated 3mm HF34 200 or 300 mesh copper grids. Excess water was removed using velin tissue paper.

(b). Staining of Sections.

The sections were stained with uranyl acetate and lead citrate (Reynolds, 1963).

The grids were initially immersed, sections uppermost, in drops of 10% uranyl acetate-100% methanol for a period of 30 seconds to one minute. The stain was placed on dental wax, within a petri-dish, and a fresh drop used for each grid. The grids were then washed for 30 seconds in each of 4 wash-pots containing 100% methanol, rinsed briefly with distilled water and dried using velin tissue paper.

The second stage of the staining procedure involved floating the grids, sections lowermost, on individual drops of lead citrate solution for a period of 4-6 minutes. The stain was placed on fresh dental wax,within a covered petri-dish, and sodium hydroxide pellets added to absorb atmospheric carbon dioxide. The grids were subsequently washed for 30 seconds in each of 4 wash-pots containing distilled water, the

first wash-pot also containing a few drops of ammonia solution. Finally, the washed grids were dried using velin tissue paper and placed in a dessicator overnight.

Stained grids were examined under a Jeol 100-S or Jeol 100-CX Transmission Electron Microscope with an accelerating voltage of 80 kV.

The normal and cold-fixed extra-embryonic epiblasts were sectioned longitudinally, at right-angles to the epiblast periphery, to demonstrate the morphology of the epiblast periphery and adjacent proximal epiblast. In the wounded extra-embryonic epiblast, sections were cut transversly, parallel to the epiblast periphery, to demonstrate the morphology of the epiblast cells comprising the wound margin (diagram. 4).

III. RESULTS.

A. The Normal Epiblast Periphery.

1. Scanning Electron Microscopy.

(a). The Ventral Surface of the Attached 'Edge Cells' and Proximal Epiblast.

At stages 4-5 the inner surface of the vitelline membrane surrounding the yolk is a meshwork of randomly orientated fibres (fig. 1). These fibres are flattened in the membrane plane, and appear coated with an amorphous matrix material. Occasionally, smaller fibrils may be seen extending across the numerous apertures in the membrane surface.

The periphery of the stage 4-5 epiblast is bordered by a continuous band of flattened 'edge cells' (fig. 2). These cells lie on the inner surface of the vitelline membrane, and form the distal margin of the expanding epiblast. The remaining epiblast lies above the surface of the membrane and is generally obscured by yolk globules, some of which may be intracellular. Individual cells of the hypoblast are commonly observed on the ventral epiblast surface proximal to the attached periphery.

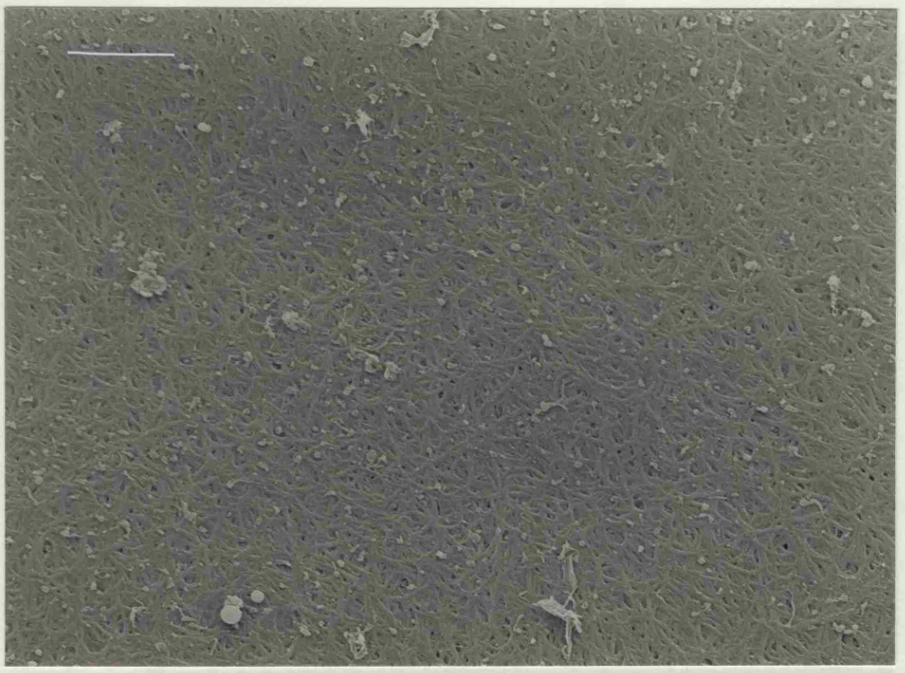
The attached 'edge cells' possess lamellipodia, which thicken proximally to the main body of the cell (fig. 3). These lamellipodia are long, broad, and extend at right-angles to the epiblast periphery. They are extensively flattened in the plane of the vitelline membrane, to which they are closely apposed, and have a free distal margin. There are small bleb-like processes on the ventral lamellipodial surface, with occasional microvilli and filopodia. Larger hemispherical processes are also commonly observed toward the cell body. The distal lamellipodial

margin may have a scalloped appearance, with the concave regions lifted above the surface of the vitelline membrane. In general, however, the lamellipodial margins appear closely apposed to the surface of the membrane, and occasionally extend into short filopodial processes which lie at right-angles to the epiblast edge. Ruffle-like processes are occasionally observed on the ventral surfaces of those 'edge cell' lamellipodia attached to the vitelline membrane.

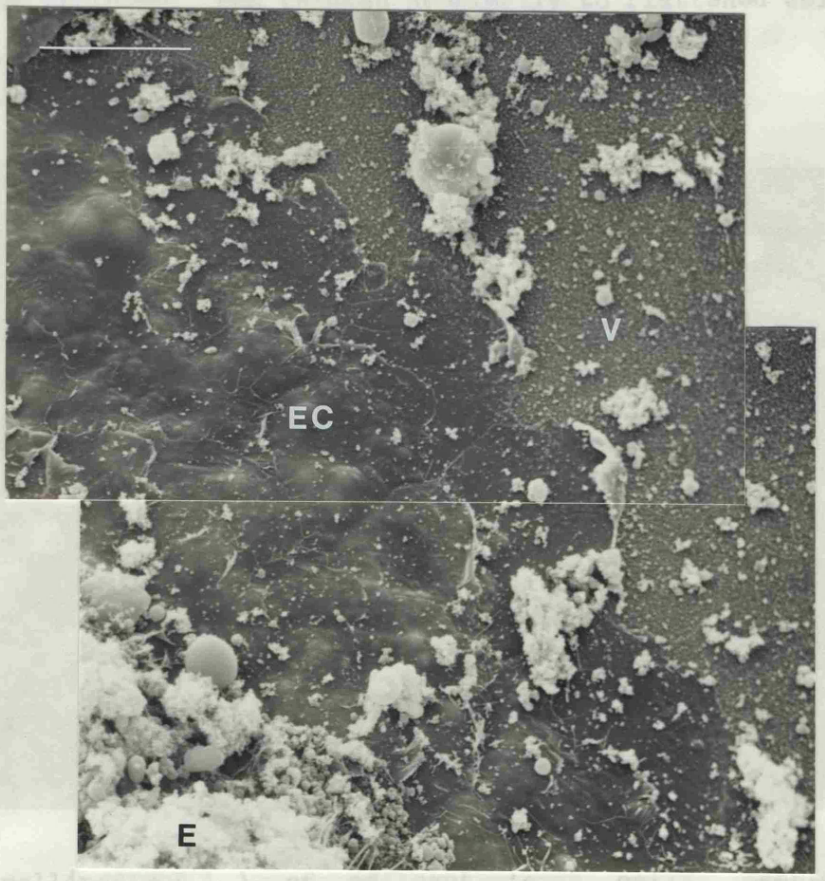
The main 'edge cell' body, situated proximal to the lamellipodium, is also flattened in the plane of the vitelline membrane. Hemispherical processes, and smaller bleb-like processes, occur on the cell surfaces, as do occasional microvilli and filopodia. Adjacent 'edge cells' are closely apposed, and their cell bodies and lamellipodia overlap to form a complete border of attached cells at the epiblast periphery (fig. 4).

The cell body of each attached 'edge cell' is also wholly, or partly, overlain by the lamellipodia of proximally situated cells. These lamellipodia extend radially from central cell bodies, and may have ruffle-like processes at their margins. The majority of the lamellipodial margin is closely apposed to the surface of the underlying cells, and occasionally extends into short filopodial processes.

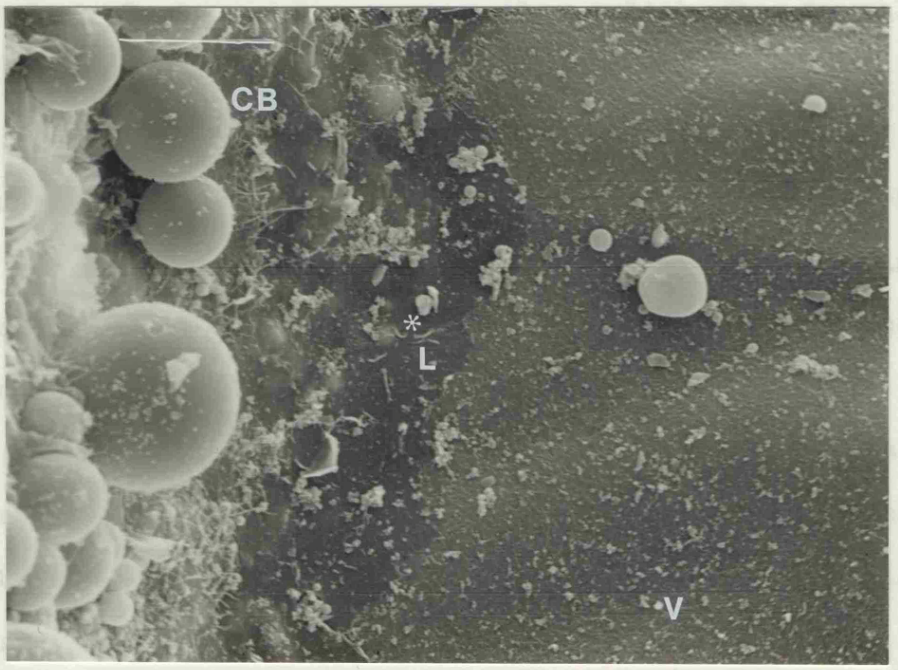
Specimens fixed immediately after New culture are similar in morphology to those reincubated for 30 minutes to 1 hour before fixation and preparation for the S.E.M.



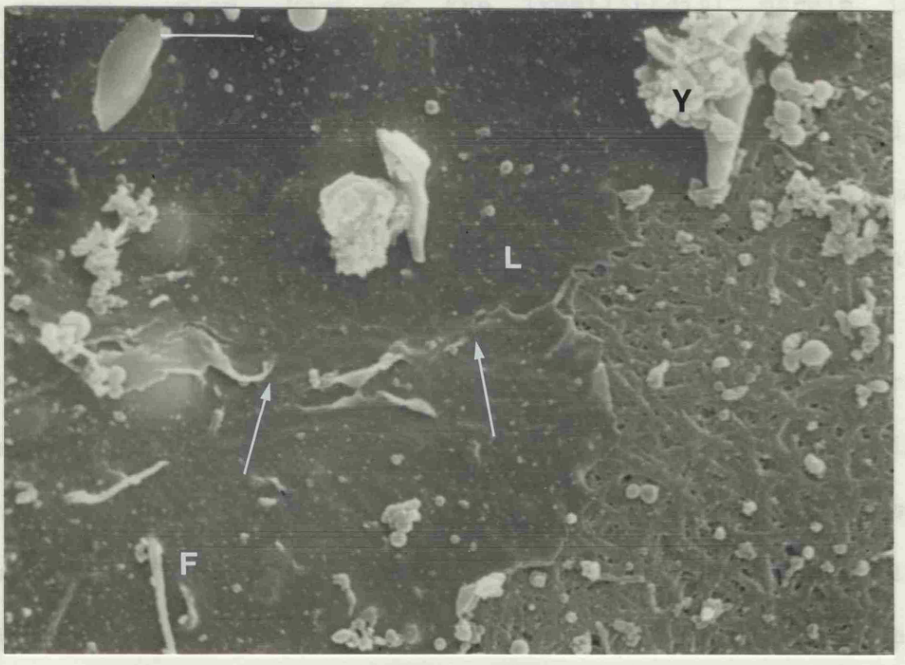
(Fig. 1). The inner surface of the stage 4-5 vitelline membrane. The membrane appears as a meshwork of randomly orientated fibres.
Bar = 10µm.



(Fig. 2). Ventral view of the stage 4-5 epiblast periphery. The epiblast (E) is bordered by a band of flattened 'edge cells' (EC) attached to the vitelline membrane (V).
Bar = 20µm.



(Fig. 3). Ventral view of a group of stage 4-5 epiblast 'edge cells' The lamellipodia (L) extend at right-angles to the epiblast periphery, and thicken proximally to flattened cell bodies (CB).
V - Vitelline membrane.
Bar = 20µm.



(Fig. 4). The lamellipodia (L) of adjacent 'edge cells' overlap (arrows) - * in fig. 3.
F - Filopodium, Y - Yolk.
Bar = 2µm.

(b). The Ventral Surface of the Proximal 'Edge Cells' after Removal of the Overlying Epiblast.

A mounted cactus needle can be used to remove the unattached stage 4-5 epiblast from specimens dried by the critical point method. This operation exposes the 'edge cells' at the proximal margin of the attached epiblast periphery.

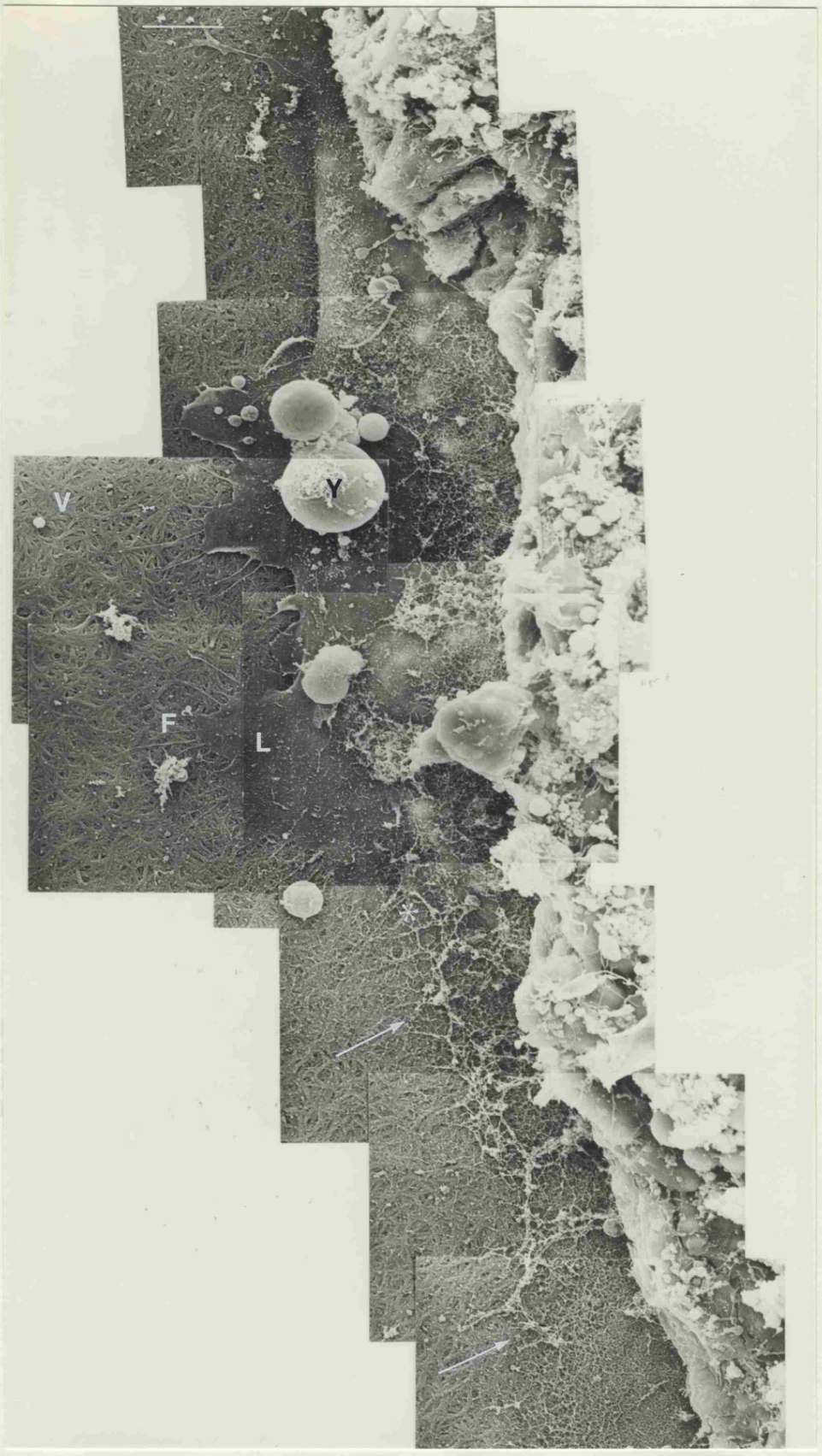
Most of the cells at the proximal rear of the attached epiblast periphery are rounded, and some appear to lie above the surface of the vitelline membrane. Small bleb-like processes and occasional microvilli are observed on the exposed cell surfaces.

'Edge cells' in contact with the membrane surface may possess one or more short lamellipodia. These processes are oriented proximally, away from the epiblast periphery, and lie in the opposite direction to the movement of the attached cells (fig. 5). The lamellipodia of adjacent cells rarely overlap. Most of the lamellipodial margin is closely apposed to the surface of the vitelline membrane although some regions, especially those adjacent to the cell body, appear unattached. Filopodial processes extend from both the attached and unattached lamellipodial margins and lie on the membrane surface, predominantly at right-angles to the epiblast periphery.

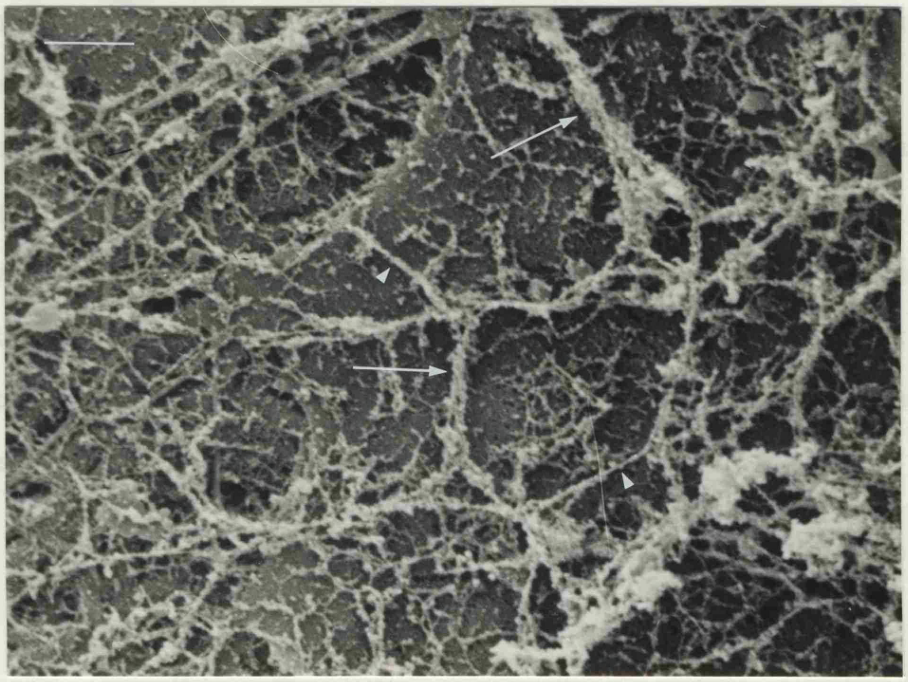
The proximal 'edge cells', and adjacent vitelline membrane, are frequently overlain by an irregular meshwork of fine fibrils. This fibrillar material may occur as a thin layer or in occasional dense patches, and similar aggregations are commonly observed on nearby yolk globules. Adjacent fibrils are often aligned to form fibre-like bundles (fig. 6). These bundles vary in thickness and length depending on the number and length of the fibrils involved. Fibres appear to lie above the cell surface or vitelline membrane for much of their length and tend to run parallel, or at right-angles, to the epiblast periphery. Both

fibrils and fibres are commonly associated with a granular material which may occur in localised aggregations along a given process. A similar particulate material is also present in spherical aggregations on the exposed cell surfaces and adjacent vitelline membrane.

The vitelline membrane exposed by the excision of the proximal epiblast appears relatively clear. Small amounts of granular material, some cell debris and the occasional yolk globule are observed on the membrane surface.



(Fig. 5). Ventral view of the proximal margin of the attached epiblast periphery at stages 4-5. The 'edge cells' possess short lamellipodia (L) with filopodial processes (F) at their margins. A meshwork of fibrillar material (arrows) is associated with the attached cells and the adjacent vitelline membrane (V).
Y - Yolk.
Bar = 10µm.

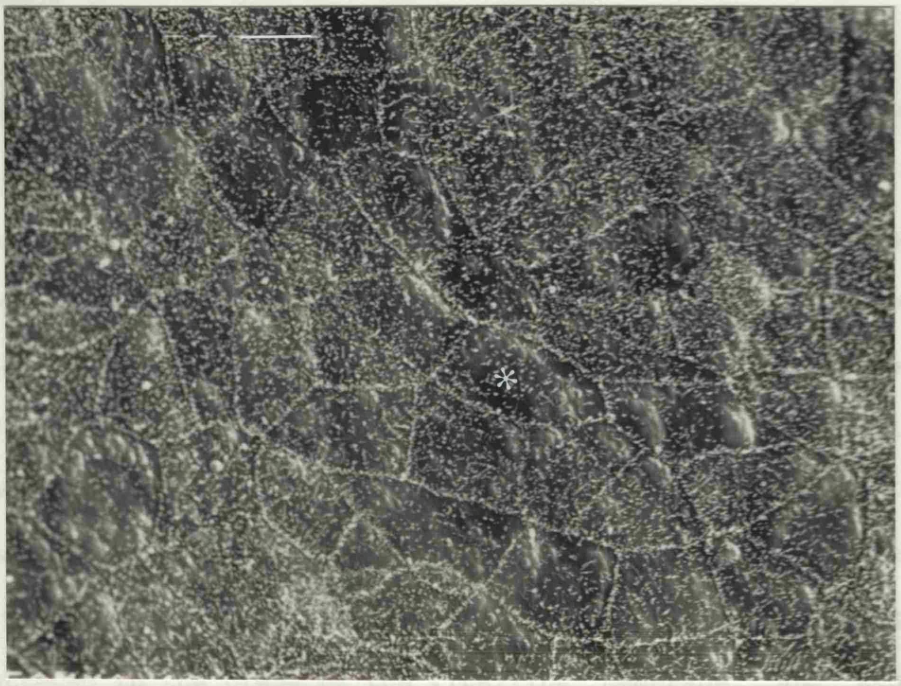


(Fig. 6). Detail of fibrillar meshwork (* in fig. 5). The fine fibrils (arrowheads) of the meshwork often lie parallel, forming fibre-like bundles (arrows).
Bar = 1µm.

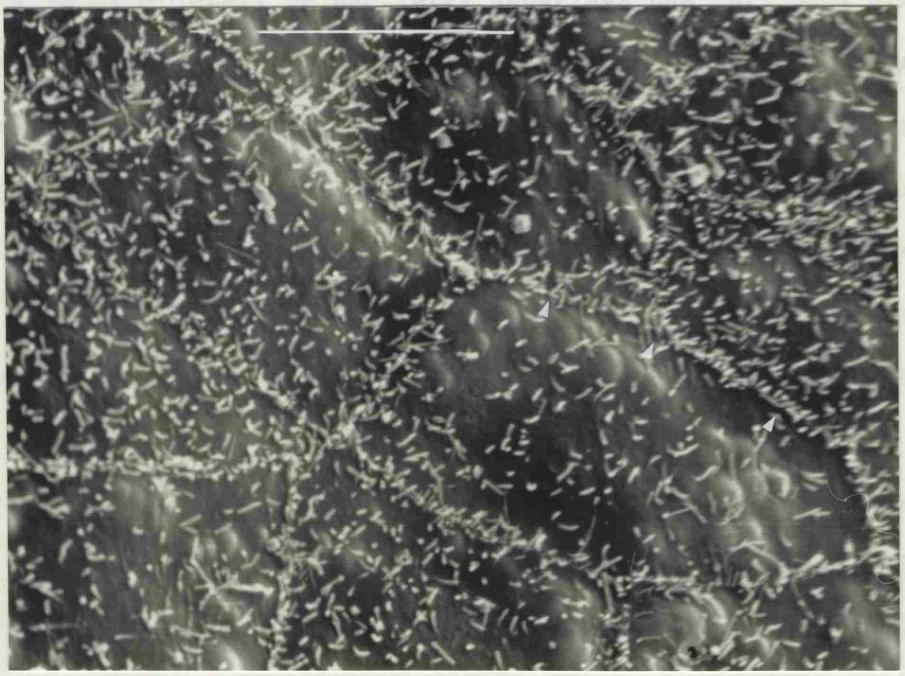
(c). The Dorsal Surface of the Proximal Epiblast.

The dorsal surface of the attached epiblast periphery is intimately asssociated with the inner surface of the vitelline membrane, and cannot be removed without considerable distortion of the normal 'edge cell' morphology. In contrast, the unattached proximal epiblast may be excised using a mounted cactus needle and inverted to allow observation of the dorsal surface.

At stages 4-5 the dorsal epiblast surface lying proximal to the attached 'edge cells' is composed of densely packed polygonal cells (fig. 7). Many short microvilli occur on the dorsal cell surfaces, and rows of similar processes delineate the cell boundaries. The cell surfaces also possess large hemi-spherical processes and smaller bleb-like processes. Filopodia are infrequently observed.



(Fig. 7a). Dorsal view of the stage 4-5 epiblast lying proximal to the attached periphery. The cells are densely packed and polygonal in shape.
Bar = 10μm.



(Fig. 7b). Detail of an individual cell (* in fig. 7a). Microvilli are present on the cell surface and delineate the cell boundary (arrowheads).
Bar = 10μm.

2. Thick Sections.

Longitudinal sections, cut as a radius of the embryo, were taken through the attached periphery of the stage 4-5 epiblast periphery.

In section, the vitelline membrane consists of a thick inner layer which is underlain dorsally by a variable number of thinner outer layers (fig. 8). The epiblast is attached to the surface of the inner layer by a group of cells at its distal extremity. The cells proximal to this region lie above the membrane surface.

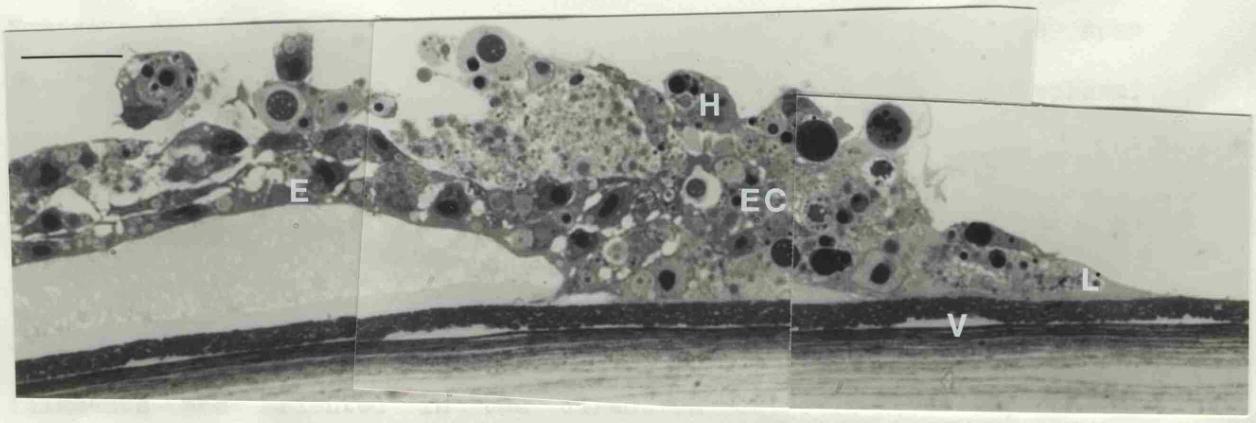
The attached epiblast periphery continues proximally, at right-angles to the epiblast edge, for some 3-5 cells. These 'edge cells' are flattened in the plane of the vitelline membrane, and each has a lamellipodium which extends towards the epiblast edge. The lamellipodia taper distally, and appear closely apposed to the membrane. Each lamellipodium completely or partially underlays the cell body and lamellipodium of its neighbour. An exception is the cell at the extreme epiblast edge, which has a long thick lamellipodium not fully overlain or underlain by that of any other cell. The morphology of this cell has been previously described under the S.E.M. The most proximal 'edge cell', at the junction between attached and unattached epiblast, has an additional short lamellipodium which extends proximally, away from the epiblast edge.

The inner vitelline membrane layer that lies beneath the attached 'edge cells' is frequently elevated above the outer layers. The small spaces created by this separation are commonest under the lamellipodia of the cells at the distal and proximal edges of the attached group.

The 'edge cells' are themselves overlain by additional flattened cells of the epiblast periphery, forming an association 2-3 cells deep. These cells do not make contact with the vitelline membrane, and their orientation could not be determined under the light microscope. The

upper cells of the peripheral group are overlain by yolk, and branching cellular processes often extend from the ventral cell surfaces to lie between the yolk globules.

The unattached proximal epiblast appears as a simple epithelium of flattened cells. The majority of the cell-cell contacts appear to be restricted to the lower, dorsal, surface of the epithelium. At high magnifications, structures resembling a terminal bar are occasionally resolved. Small gaps between adjacent cells are frequently observed toward the ventral surface, which is overlain by a loose association of hypoblast cells, yolk globules and fine membraneous material.



(Fig. 8). Longitudinal thick section through the stage 4-5 epiblast periphery. 'Edge cells' (EC) attach the distal extremity of the epiblast (E) to the vitelline membrane (V).
H - Hypoblast cell, L - Lamellipodium.
Bar = 10µm.

3. Transmission Electron Microscopy.

Longitudinal thin sections, cut at right-angles to the epiblast edge, were taken through the stage 4-5 epiblast periphery in order to examine the fine structure of the inner vitelline membrane layer, the attached 'edge cells' and the adjacent proximal epiblast.

The inner layer of the vitelline membrane is a meshwork of solid fibres. In section, most of these fibres appear to lie parallel to the membrane surface. Individual fibres may extend into thin fibrils, and are often associated with a granular matrix material. A similar material is also observed in the spaces separating adjacent fibres. The inner membrane layer is separated from the dorsal outer layers by a thin membrane, a structure rarely resolved under the light microscope.

The 'edge cells' at the distal margin of the attached epiblast periphery are flattened in the plane of the vitelline membrane and possess lamellipodia which extend in the direction of cell movement (fig. 9). The 'edge cell' nuclei are oval in shape and are usually situated towards the proximal rear of the cell. The nuclear material is largely euchromatic with a single dense, heterochromatic, nucleolus. Numerous rounded vesicles, a number of elongate mitochondria and some smooth endoplasmic reticulum are observed within the cell cytoplasm. Most of the cytoplasmic vesicles appear to contain either yolk material or cell debris.

Microfilaments, either singly or in small bundles, are also present in the 'edge cell' cytoplasm adjacent to the cell surface. Most of these filaments are oriented in the direction of cell movement, i.e. at right-angles to the epiblast edge. Isolated microtubules are observed throughout the cytoplasm, but appear to lack a preferred orientation.

Finger-like processes from the dorsal surface of the 'edge cell' lamellipodia extend into the spaces separating the fibres of the

vitelline membrane (fig. 10). Areas of apparent contact between process and membrane fibre are associated with an electron-dense thickening of the cell surface. Similar plaque-like structures are also observed toward the lamellipodial margins.

The 'edge cells' attached to the surface of the vitelline membrane are overlain by flattened epiblast cells. Apposed cell membranes usually lie slightly apart, although larger gaps are occasionally observed. In some areas, the adjacent cell membranes appear intimately apposed, and are associated with aggregations of a densely stained material. These junctional structures are prevalent between neighbouring cells throughout the multilayered epiblast periphery. The exposed cell surfaces of the overlying cells often possess processes which extend into the surrounding yolk, and yolk material is also associated with semicircular indentations of the free cell surface. In some sections, a fine layer of lightly stained material is observed on the ventral surfaces of the overlying cells.

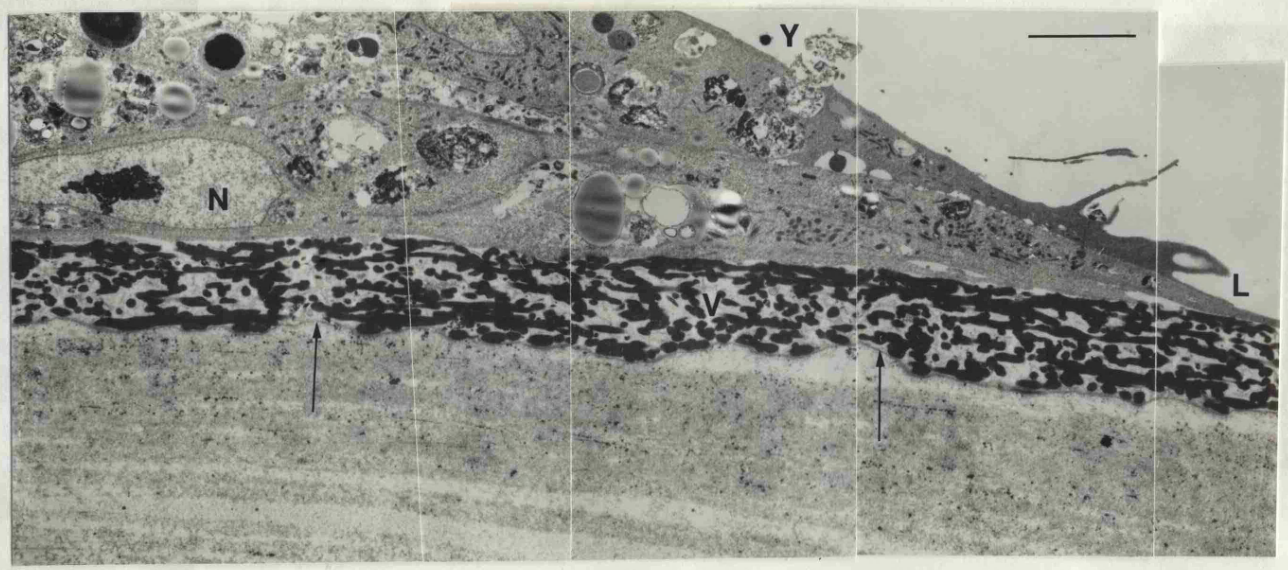
The 'edge cells' at the proximal margin of the attached epiblast periphery often appear rounded in comparison with their distal counterparts (fig. 11). Most attached cells possess a lamellipodium oriented in the direction of cell movement, and a few possess an additional short process oriented proximally - away from the epiblast edge. The main cell body often lies above the surface of the vitelline membrane. Junctional structures resembling those described for the distal 'edge cells' are observed between adjacent cells, and desmosomes, both large and small, are also present. In a few specimens, a lightly stained material is associated with the exposed cells surfaces and adjacent vitelline membrane.

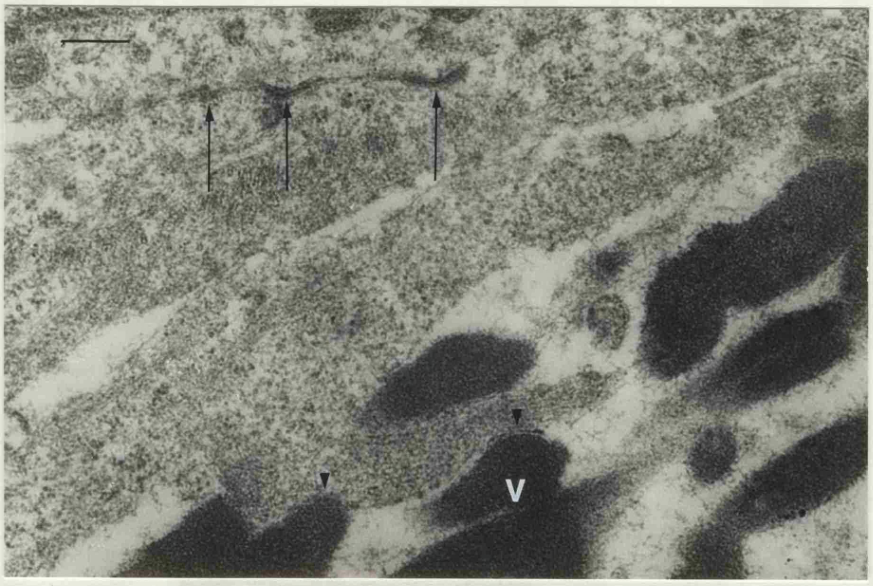
The epiblast immediately adjacent to the attached 'edge cells' consists of 1-2 layers of rounded cells and lies above the membrane surface. Further proximally, distant to the attached periphery, the

epiblast is a single layer of flattened cells.

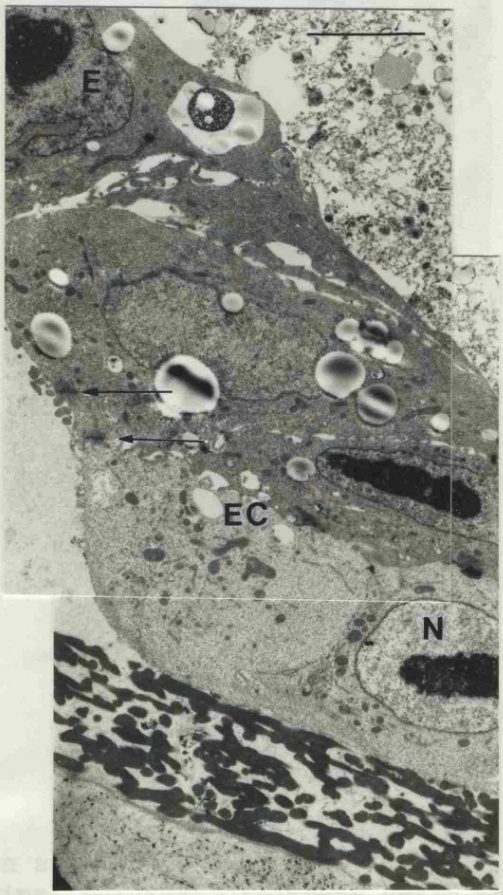
The nuclei of the attached cells are oval in shape and possess a single dense nucleolus. The cell cytoplasm contains mitochondria and a number of small rounded inclusions (fig. 12). A thin basement membrane is associated with the exposed cell membranes at the ventral surface of the epithelium some distance from the attached 'edge cells'. Neighbouring cells are connected by a number of discrete junctional structures situated toward the dorsal cell surface. At the dorsal cell extremity, the adjacent cell membranes are closely apposed and are associated with an electron-dense material. The intercellular space is absent and close contacts of this sort were present in all the sections examined. Ventral to the apical junction, one or more desmosomes are observed.

(Fig. 9). Longitudinal thin section through the 'edge cells' bordering the stage 4-5 epiblast. The attached cells possess lamellipodia (L) which extend across the vitelline membrane (V) in the direction of epiblast expansion.
Arrows - Continuous membrane, N - Nucleus, Y - Yolk.
Bar = 5µm.

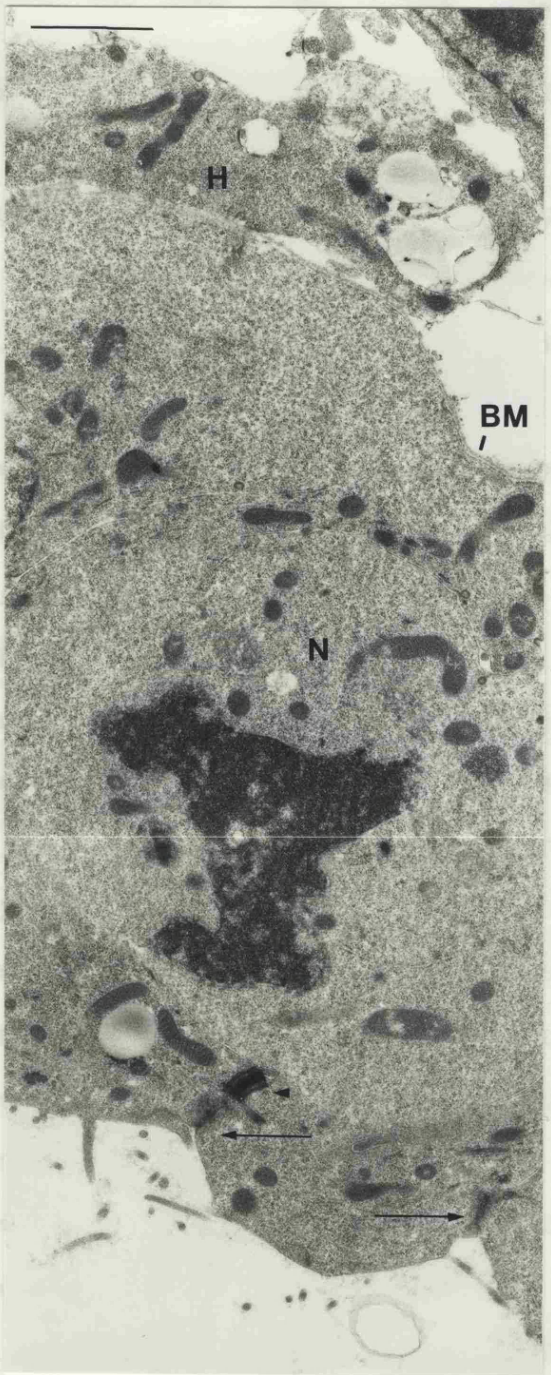




(Fig. 10). Longitudinal section through 'edge cell' and adjacent vitelline membrane (V). A short lamellipodial process lies between the membrane fibres.
Arrows - Cell-cell junctions, Arrowheads - Attachment plaques.
Bar = 1µm.



(Fig. 11). Longitudinal section through the 'edge cells' (EC) at the proximal rear of the attached epiblast periphery. Desmosomes (arrows) connect 'edge cells' to other attached cells and unattached cells of the proximal epiblast (E).
N - Nucleus.
Bar = 5µm.



(Fig. 12). Longitudinal thin section through a cell of the unattached stage 4-5 proximal epiblast. Adjacent cells are connected by a dorsal junctional structure (arrows) and desmosomes (arrowheads).
BM - Basement membrane, H - Hypoblast, N - Nucleus.
Bar = 2μm.

B. The Epiblast Periphery after Cold Fixation.

1. Scanning Electron Microscopy.

(a). The Ventral Surface of the Cold-Fixed Epiblast Periphery.

The morphology of the stage 4-5 epiblast is altered from that previously described as normal by the use of Karnovsky's fluid at a temperature of 5 ^{o}C. The morphology of the vitelline membrane is similar to that previously described.

After treatment with cold fixative, the epiblast periphery has a crenated appearance with short distances of the epithelial margin attached to the vitelline membrane by flattened 'edge cells' (fig. 13). Some of these attached cells possess a single broad lamellipodium and appear similar to the 'edge cells' of the normal epiblast periphery (Section A). In others, the lamellipodia are shorter and narrower than those previously described. The majority of the distal lamellipodial margin lies above the surface of the vitelline membrane and often extends into long, radially orientated, thread-like processes. Individual thread-like processes may be straight or branched, lie above the membrane for most of their length and end in one or more points of contact with the membrane surface. The ventral lamellipodial surface is uneven with short folds, small bleb-like processes, microvilli and filopodia. These short lamellipodia often widen proximally, and thicken abruptly to rounded cell bodies. The overlapping of adjacent lamellipodia is reduced from that previously described for the normal epiblast periphery.

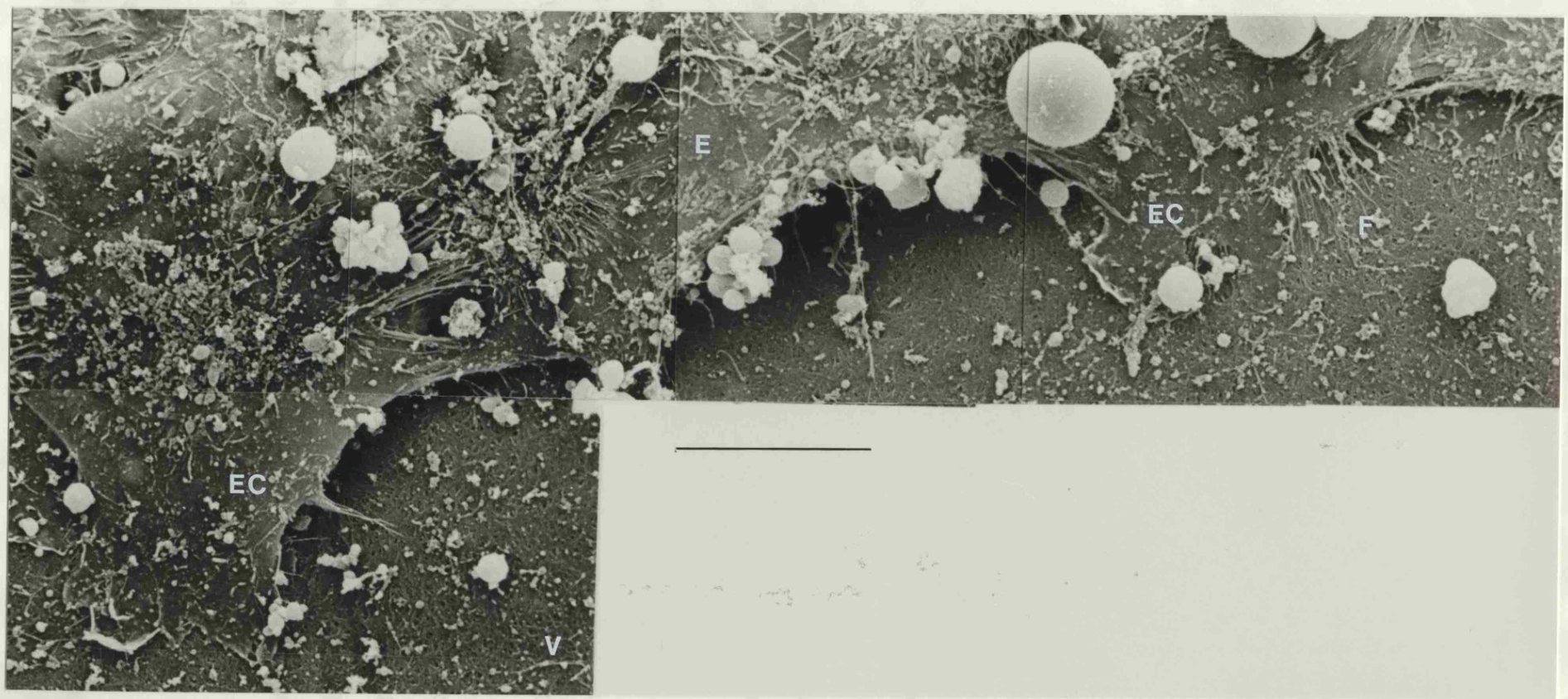
The peripheral epiblast lying between the groups of attached 'edge cells' is recessed proximally as concave portions which lie above the surface of the vitelline membrane. These unattached 'edge cells' are

often rounded and appear elongated parallel to the epiblast periphery. Numerous bleb-like processes, of varying size, are observed on the ventral cell surfaces, as are microvilli and occasional filopodia. Adjacent cells overlap and the junctions are marked by short lamellipodial and filopodial processes. These filopodial processes extend radially from the lamellipodial margin and make contact with the surface of the neighbouring cell. Both the unattached cells at the epiblast periphery and the filopodial processes of the attached cells may be underlain by lamellipodia closely apposed to the surface of the vitelline membrane. Filopodial processes, often branched, are also observed at the junctions between attached and unattached 'edge cells'. These processes occur in rows which extend, at right-angles to the epiblast periphery, from the cell body to the underlying vitelline membrane.

Most cells of the proximal epiblast are rounded, with many appearing almost spherical. There are numerous bleb-like processes, of varying size, on the ventral cell surfaces with occasional microvilli and filopodia. Adjacent cells are connected by short filopodial and lamellipodial processes.

Cell debris and yolk globules are present on both the epiblast and vitelline membrane. 'Thread-like' processes are also frequently observed on that surface of the vitelline membrane lying adjacent to the epiblast periphery.

(Fig. 13). The ventral surface of the stage 4-5 epiblast periphery, after treatment with cold fixative. The epiblast (E) is bordered by small groups of attached 'edge cells' (EC).
F - Filopodial process, V - Vitelline membrane.
Bar = 20µm.

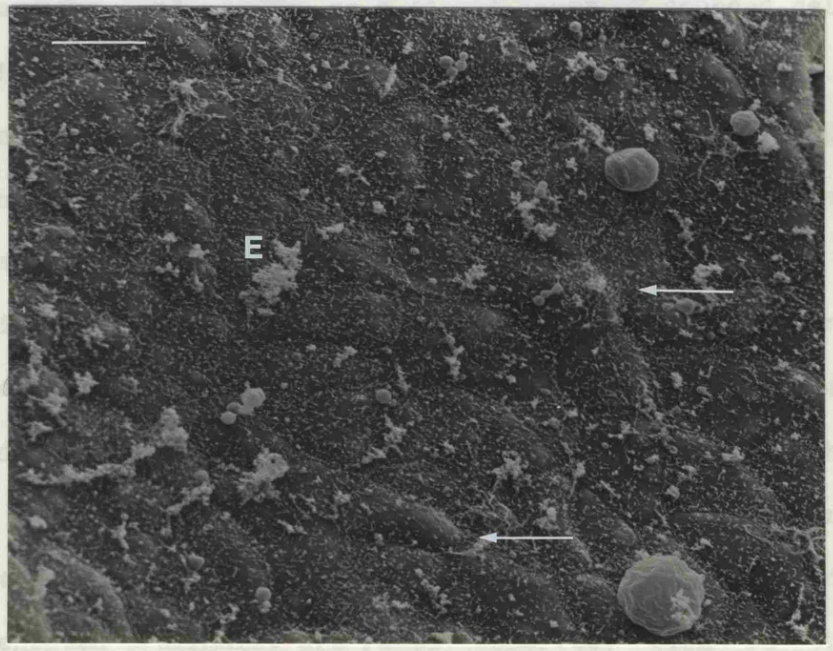


(b). The Dorsal Surface of the Proximal Cold-Fixed Epiblast.

After treatment with cold fixative, the dorsal surface of the stage 4-5 proximal epiblast appears as a sheet of densely packed cells (fig. 14).

Some of these cells are flattened in the plane of the epithelium, and resemble the polygonal cells previously described for the normal dorsal epiblast (Section A). However, the microvilli on the exposed cell surfaces appear slightly longer than those previously described, and may occur in larger numbers. Short filopodia are also occasionally observed.

In contrast, many other cells, or small groups of cells, appear rounded in the plane of the epiblast. These cells are no longer polygonal, and the epithelium has an uneven appearance. Adjacent cells generally remain closely apposed but their boundaries may, or may not, be delineated by microvilli. Numerous such processes, of varying length, are observed on the dorsal cell surfaces. These microvilli often occur in localised concentrations on the cell surface, and occur in larger numbers than described for the normal dorsal epiblast. Small bleb-like processes, and occasional short filopodia, are also observed.



(Fig. 14). The dorsal surface of the stage 4-5 proximal epiblast (E) after exposure to cold fixative. Some of the epiblast cells (arrows) appear slightly rounded in the plane of the epithelium.
Bar = 10μm.

2. Thick Sections.

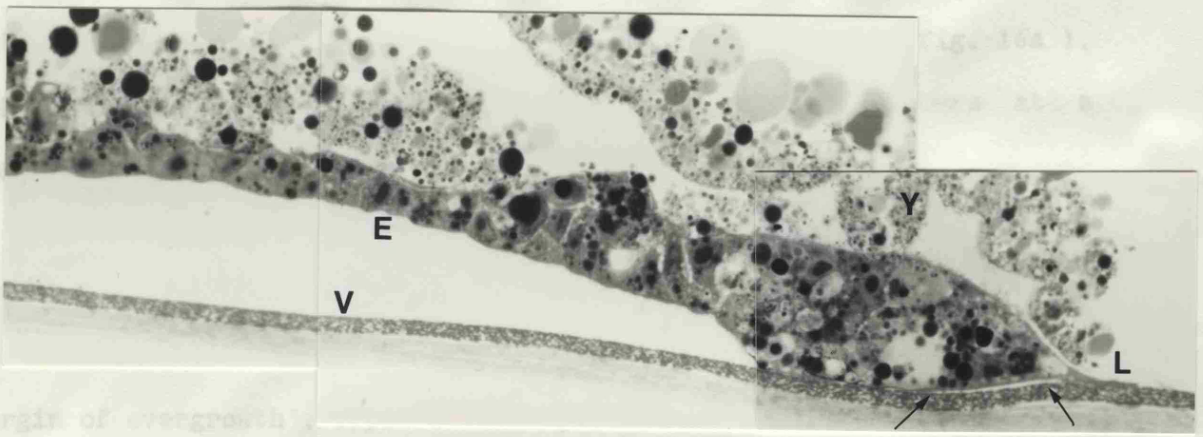
Longitudinal thick sections were taken, at right-angles through the stage 4-5 epiblast periphery, after treatment with cold fixative. The morphology of the vitelline membrane resembles that previously described after fixation at room temperature (Section A).

In some specimens, the epiblast periphery lies above the surface of the vitelline membrane and appears as a single layer of rounded cells. Neighbouring cells appear to remain closely apposed.

In other specimens, the epiblast is attached to the inner surface of the vitelline membrane by a small group of peripheral cells (fig. 15). This attached group is often narrower, at right-angles to the epiblast edge, than that previously described for the normal epiblast and some peripheral cells may lie above the membrane surface. These unattached cells lack lamellipodia and are usually rounded. Those peripheral cells that remain attached to the membrane are also rounded and often possess lamellipodia which extend toward the epiblast edge. Lamellipodia orientated proximally are rarely observed, even on those cells toward the rear of the attached group.

In some cases, the attached periphery is reduced to a single rounded cell at the extreme epiblast edge. Contact between this cell and the membrane may be further restricted to the tip of a long, distally orientated, lamellipodium. The inner layer of the vitelline membrane situated beneath this cell often lies some distance above the adjacent outer layers. Both attached and unattached cells of the peripheral group are usually overlain by rounded epiblast cells to form an association 2-4 cells deep. These overlying cells rarely possess lamellipodia.

The unattached proximal epiblast is a single layer of rounded cells. Adjacent cells appear to remain closely apposed, and the epithelium is overlain by yolk material and rounded hypoblast cells.



(Fig. 15). Longitudinal thick section through the stage 4-5 epiblast periphery after treatment with cold fixative. The epiblast cells (E) are rounded, and some cells (arrows) of the peripheral group lie above the vitelline membrane (V).
L - Lamellipodium, Y - Yolk.
Bar = 10µm.

C. Light Microscopy of Epiblast Wound Closure.

Stage 4-5 chick embryos, mounted as for New culture (New, 1955), were wounded through the removal of approximately 200 µm of the attached epiblast periphery. Immediately after 'edge cell' excision, seven carbon particles were positioned around the newly formed wound (fig. 16a). Prepared specimens were then reincubated for a total of 10 hours at a temperature of 37.5 ^{o}C (+/- 1 ^{o}C).

The newly formed wound margin is irregular in outline and short distances of epiblast periphery, on both sides of the wound, lie above the surface of the vitelline membrane. The remaining epiblast, or 'margin of overgrowth', appears undamaged and may be distinguished from the opaque proximal hypoblast as a transparent ring of tissue at the area opaca perimeter.

After reincubation for 1 hour, the epiblast wound is smooth in outline and appears slightly larger than the newly formed wound (fig. 16b). Both the epiblast periphery adjacent to the wound and the distal wound margins, i.e. those portions of the wound margin next to the epiblast periphery, are attached to the inner surface of the vitelline membrane. Using the carbon particle at the proximal wound extremity (1) as a point of reference, the attached epiblast periphery adjacent to the wound appears to have moved - at right-angles to the epiblast edge - across the vitelline membrane.

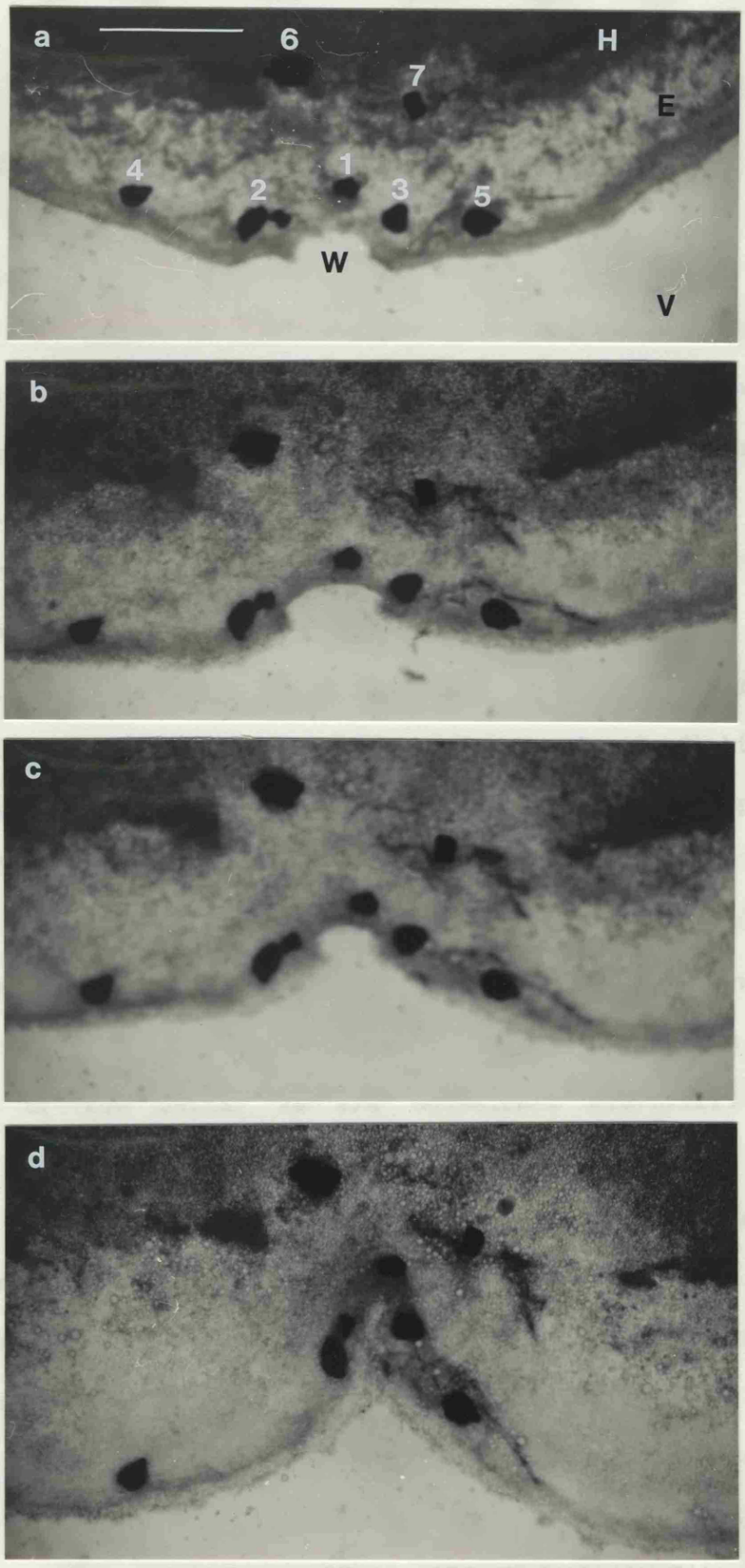
After reincubation for 5 hours, wound margin attachment appears to have continued in a proximal direction (fig. 16c). The distal portion of both wound margins is attached to the vitelline membrane and only a short distance of the proximal margin appears to lie above the membrane surface.

Finally, after 10 hours reincubation, both wound margins are attached to the vitelline membrane for their entire length (fig. 16d).

The proximal wound margins are closely apposed and the distance between the carbon particles at the lateral wound margins (2-3) is reduced from that observed after reincubation for 5 hours. In contrast, the distance between the carbon particle at the proximal wound extremity (1) and those originally placed on the epiblast periphery adjacent to the wound appears to have increased. It is now more difficult to distinguish the epiblast periphery, i.e. the 'margin of overgrowth', from the hypoblast.

With continued reincubation, the closed wound increases in length - at right-angles to the epiblast periphery - until the edge of the New culture ring prevents further expansion.

The morphology of the epiblast wound after 0, 1, 5 and 10 hours reincubation is described in more detail in Sections D-G.



(Fig. 16). Light micrographs of an epiblast wound after reincubation for 0 (a), 1 (b), 5 (c) and 10 (d) hours. Carbon particles (1-7) have been placed on the ventral surface of the epiblast (E) and hypoblast (H) adjacent to the wound (W).
V - Vitelline membrane.
Bar = 200µm.

D. The 0-Hour Epiblast Wound.

1. Scanning Electron Microscopy.

(a). The Ventral Surface of the Epiblast Wound.

The epiblast wound, fixed immediately after the removal of the attached 'edge cells', appears as a rectangular indentation of the epiblast periphery (fig. 17). The wound margin is irregular in outline, and lies above the surface of the vitelline membrane. Small numbers of 'edge cells', situated on either side of the wound, may also lie above the membrane surface. Distant to the wound, the epiblast periphery remains attached to the vitelline membrane by a continuous band of flattened cells. The vitelline membrane exposed by the 'edge cell' removal appears relatively free of yolk.

The epiblast cells bordering the wound generally appear rounded and, toward the newly torn edge, may be elongated parallel to the wound margin. Bleb-like processes, of varying size, are observed on the ventral surfaces of the cells, as are occasional microvilli and filopodia.

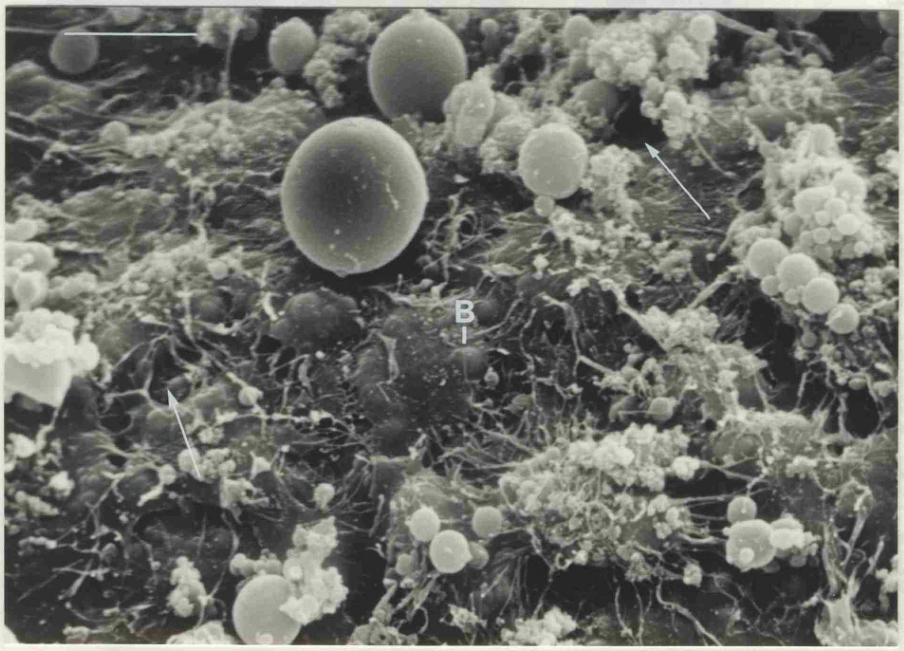
The epiblast cells lying adjacent to the wound are closely apposed, although occasional gaps occur at the cell boundaries (fig. 18). The cells appear coated with a smooth layer of amorphous material. Filopodia-like processes often connect neighbouring cells, and originate directly from the cell surface or are carried on short lamellipodia. Small bleb-like processes, and larger hemispherical processes, are observed on the ventral cell surfaces. Microvilli, and short filopodia, are also occasionally present.

The attached 'edge cells' directly adjacent to the wound differ from those previously described for the normal epiblast periphery

(fig. 19). The lamellipodia are shorter, and portions of the distal margin often lie above the surface of the vitelline membrane as flap-like processes. The lamellipodial margin is uneven, and may extend into filopodial processes which lie across the membrane. The ventral lamellipodial surfaces are frequently folded into short ridges which lie predominantly at right-angles to the epiblast periphery. Small bleb-like processes and microvilli are also present. The overlapping of adjacent lamellipodia is less evident than was previously described for the normal epiblast periphery, and contact is frequently reduced to discrete points of overlap and connecting filopodia-like processes. The main 'edge cell' body is rounded, with small bleb-like processes, occasional microvilli and filopodia. Larger hemispherical processes are also commonly present toward the perimeter of the cell body. Adjacent 'edge cells' usually remain closely apposed, their junctions marked by short lamellipodia with crenelated margins. Filopodial processes often extend radially from these lamellipodial margins, to form single or multiple points of contact with the surface of the underlying cell.

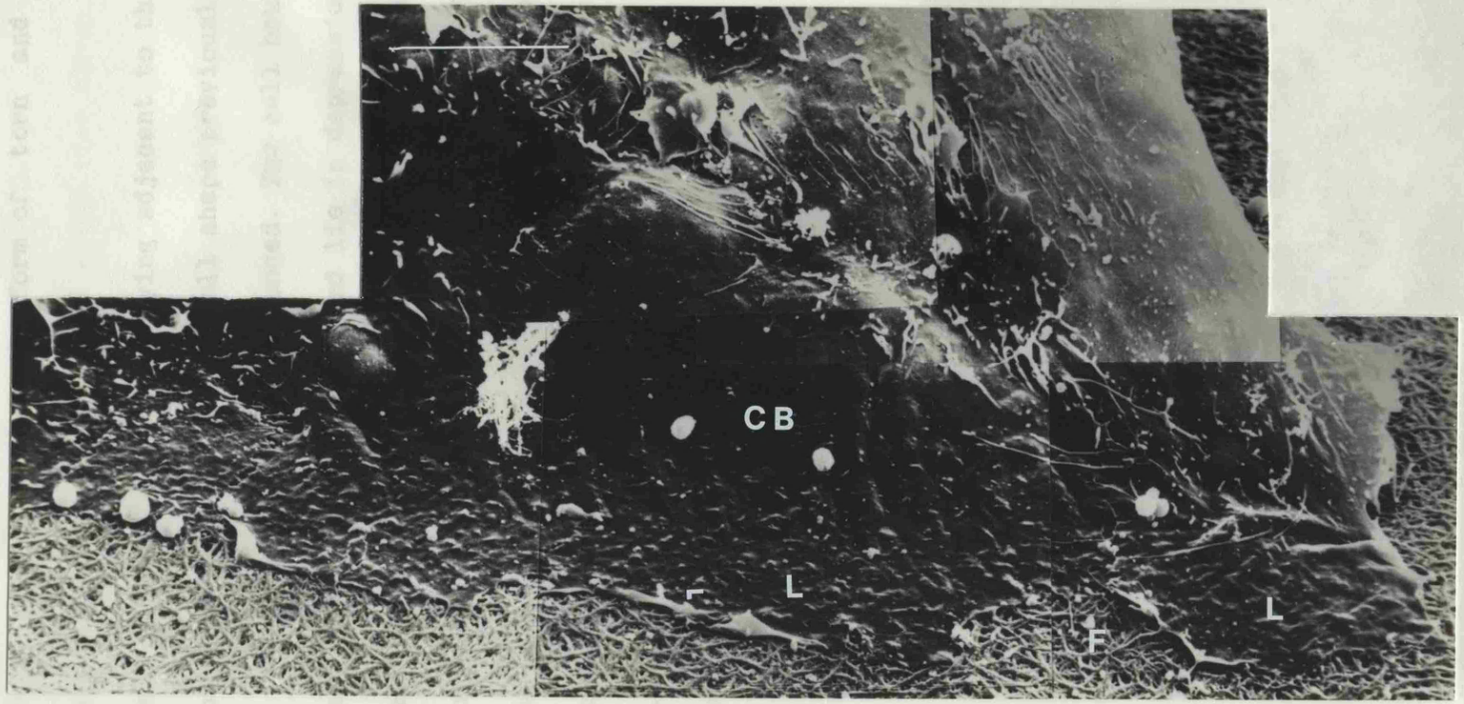
Yolk globules, other yolk material, and cell debris may be observed on both the epiblast periphery and adjacent vitelline membrane.

(Fig. 17). The ventral surface of the 0-hour wound (W). The wound margin (arrows) is uneven, and lies above the surface of the vitelline membrane (V).
E - Epiblast.
Bar = 100μm.



(Fig. 18). View of the ventral epiblast surface, adjacent to the proximal margin of the 0-hour wound. The epiblast cells possess bleb-like processes (B). Small gaps (arrows) are observed between adjacent cells.
Bar = 10μm.

(Fig. 19). View of the attached epiblast 'edge cells' adjacent to the 0-hour wound. The cells have a short lamellipodium (L), and a rounded cell body (CB).
F - Filopodial process.
Bar = 10μm.



(b). The Dorsal Surface of the Epiblast Wound.

The epiblast cells at the dorsal margin of the 0-hour wound are rounded. Cell damage is frequently observed in the form of torn and incomplete cells (fig. 20).

Those cells of the dorsal epiblast lying adjacent to the wound are also slightly rounded, and the polygonal cell shape previously described for the normal epiblast is less pronounced. The cell boundaries are generally delineated by fewer microvilli and lie in depressions of the epithelial surface. Numerous small bleb-like processes, often occurring in localised groupings, are present on the exposed cell surfaces. Many short microvilli are also observed on the cell surfaces, as are occasional filopodia.

Cell debris, and small numbers of yolk globules, lie on the dorsal epiblast in the vicinity of the wound.

(Fig. 20). The dorsal surface of the epiblast (E) bordering the proximal margin of the 0-hour wound (W). The cells are slightly rounded and the epithelium has an uneven appearance.
Bar = 10µm.

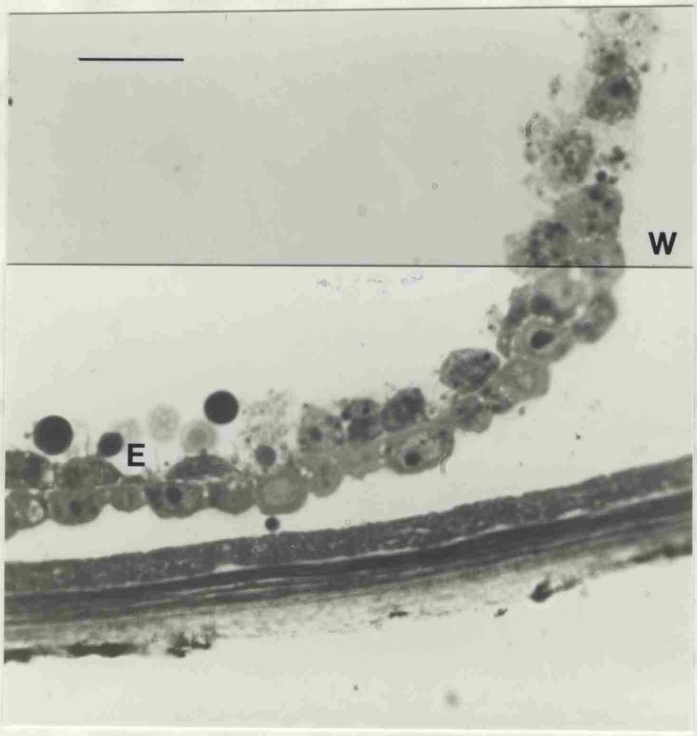
2. Thick Sections.

Transverse sections, cut parallel to the epiblast periphery, were taken through the cells bordering the newly formed wound.

The epiblast surrounding the proximal wound lies above the surface of the vitelline membrane, with the separation usually increasing toward the wound margin (fig. 21). The cells adjacent to the wound are rounded and appear loosely connected to each other. These cells are often larger, and stain less densely, than those of the proximal epiblast distant to the wound. The proximal epiblast cells are also rounded and the epithelium has an uneven appearance. Neighbouring cells remain closely apposed although small gaps are occasionally observed. Some portions of the epithelium appear to consist of dead or dying cells.

The proximal epiblast may be 2-3 cells thick directly adjacent to the wound margin. The remaining unattached epiblast resembles that previously described adjacent to the wound.

The ventral surface of the epiblast epithelium is overlain by rounded hypoblast cells, yolk globules and membraneous material.



(Fig. 21). Transverse thick section through the epiblast (E) bordering the 0-hour wound (W). The cells at the wound margin are rounded and appear larger than those distant to the wound.
Bar = 10µm.

3. Transmission Electron Microscopy.

Transverse thin sections, cut parallel to the epiblast periphery, were also taken through the cells bordering the 0-hour wound margin.

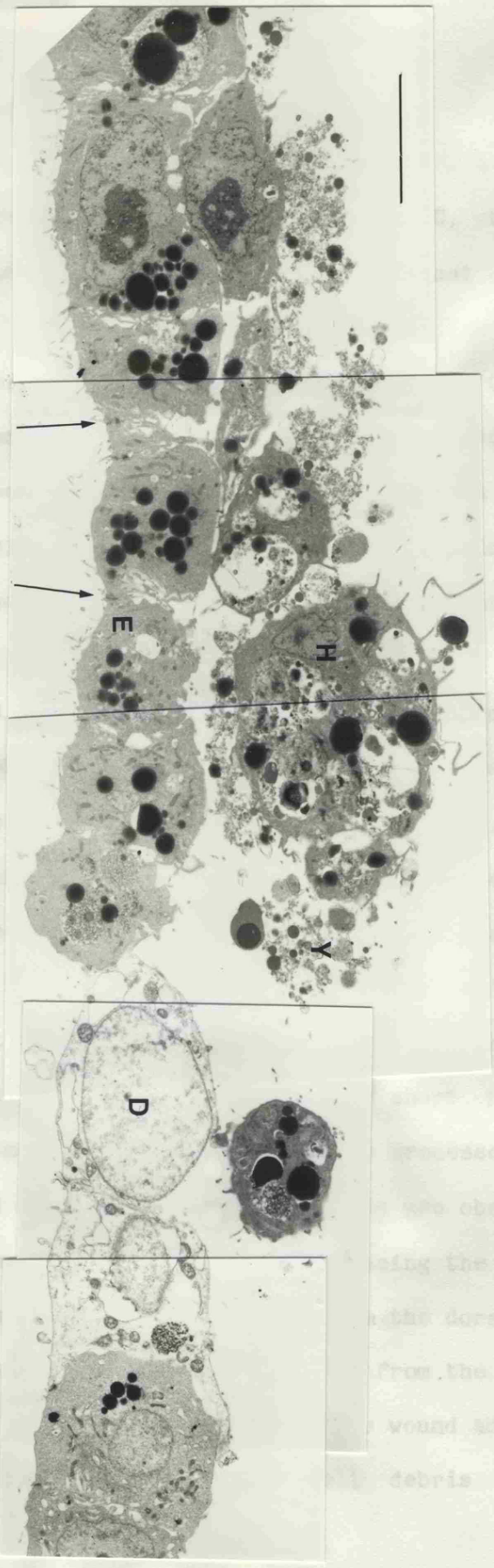
The epiblast cells bordering the wound are rounded, and have no discerible basement membrane (fig. 22). Numerous microvilli extend from the dorsal cell surfaces, and lie toward the vitelline membrane. Similar microvilli are also occasionally observed on the ventral cell surfaces.

Adjacent cells are connected by a junctional complex at the dorsal cell surface. Ventral to this region, one or more desmosomes are also observed. These desmosomes may directly connect apposing cell sufaces, or be present at the tip of a short cellular process extending between the surfaces of neighbouring cells. The majority of the remaining cell surfaces lie apart, although several interdigitating microvilli-like processes commonly extend ventrally from the apposed surfaces of neighbouring cells.

The nuclei of the epiblast cells are relatively large and appear irregular in shape. A single large nucleolus, and some small patches of dense heterochromatin, are commonly present within an otherwise largely euchromatic nucleus. The cell cytoplasm contains several darkly staining vesicles of varying size, elongated mitochondria, and a small amount of endoplasmic reticulum.

Dead and dying cells are frequently observed toward the wound margin. The cell cytoplasm is often loosened or displaced from the nucleus, and may be present in smaller amounts than for normal epiblast cells. There is a general lack of structural detail within the cytoplasm and nucleus, and both the cell membrane and nuclear membrane may be torn or incomplete. The dying cells usually contact their neighbours through the junctional complex at the dorsal cell surface.

Hypoblast cells, and patches of yolk material, overlay the epiblast cells adjacent to the wound.



(Fig. 22). Transverse section through the 0-hour wound margin. Adjacent epiblast cells (E) are connected at the dorsal surface of the epithelium (arrows). Dying cells (D) are observed toward the wound margin. H - Hypoblast cell, Y - Yolk material. Bar = 10µm.

E. The Epiblast Wound after Reincubation for 1 Hour.

1. Scanning Electron Microscopy.

(a). The Ventral Surface of the Epiblast Wound.

After reincubation for 1 hour, at a temperature of 37.5 ^{o}C, the wound appears as a semi-circular indentation of the epiblast periphery (fig. 23).

The distal extremity of the wound margin is attached to the inner surface of the vitelline membrane by a small group of flattened epiblast cells. The epiblast cells bordering the remaining wound margin lie above the membrane surface. This wound margin is smooth in outline, and is continuous with the attached epiblast periphery lying adjacent to the wound.

The unattached wound margin is composed of 2-4 layers of rounded cells (fig. 24). The epiblast cells exposed at the wound edge may also be elongated at right-angles to the wound margin. Thin fibrils, commonly associated to form fibre-like processes, are occasionally observed on the exposed cell surfaces. In some specimens, these processes form a fibrillar meshwork similar to that observed on the 'edge cells' at the proximal region of the attached epiblast periphery. Neighbouring cells are often separated by small spaces, and are linked by short filopodial processes and flap-like lamellipodia. Small bleb-like processes, larger hemispherical processes and occasional membranous folds are observed on the exposed cell surfaces. In contrast, the cells facing the vitelline membrane possess numerous microvilli which extend from the dorsal wound margin. The unattached wound margin often curls away from the vitelline membrane, exposing cells of the dorsal epiblast at the wound edge.

At the extreme wound edge dying cells, cell debris and yolk

globules are commonly observed. Dying cells are usually rounded and may be covered with large bleb-like processes.

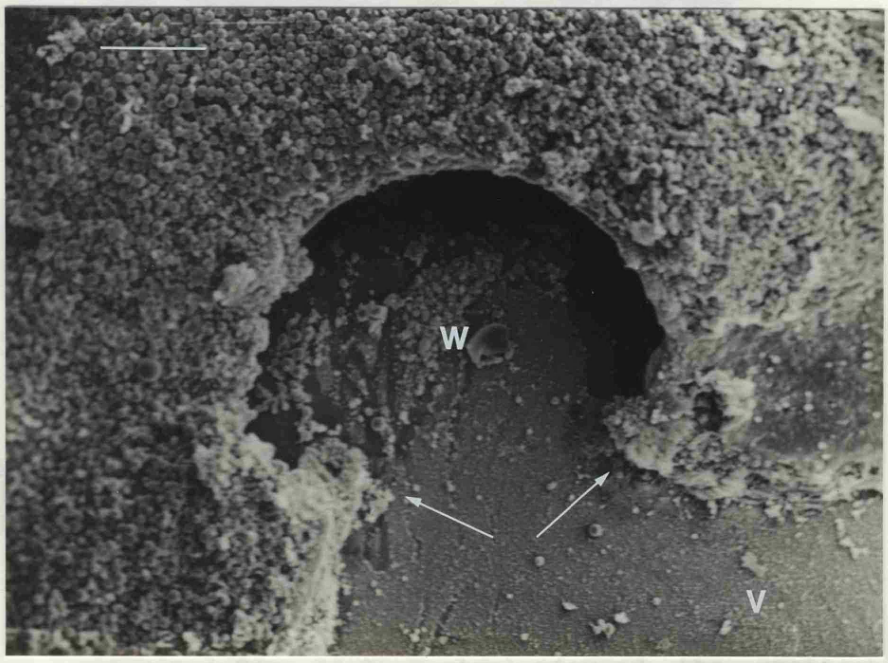
The epiblast cells adjacent to the wound appear flattened in comparison with those at the wound margin and are covered with a thin layer of membranous material. Filopodial processes often extend from the cell surface, or from short flap-like lamellipodia, to the surfaces of neighbouring cells. The ventral cell surfaces are uneven with small bleb-like processes, large hemispherical processes, and occasional membranous folds. Microvilli and filopodial processes are also commonly observed.

The attached 'edge cells' of the epiblast periphery, directly adjacent to the wound, possess lamellipodia which extend at right-angles to the epiblast periphery. The lamellipodia appear closely apposed to the surface of the vitelline membrane, and are generally shorter than those previously described for the normal epiblast periphery. There are numerous small bleb-like processes, and some microvilli, on the ventral lamellipodial surfaces. Short ridges, lying predominantly along the length of the lamellipodia, are also observed toward the lamellipodial margin.The majority of the distal lamellipodial margin is usually closely apposed to the surface of the vitelline membrane, and the lamellipodia thicken abruptly to rounded cell bodies. The cell body has an uneven surface and often displays large hemi-spherical processes at its junction with the lamellipodium. The ventral cell surface displays small bleb-like processes and microvilli. Filopodia are infrequently observed.

The attached distal wound margin is continuous with the attached epiblast periphery on either side of the wound, and the attached epiblast cells bordering the distal wound are similar in morphology to those of the attached epiblast periphery adjacent to the wound (fig. 25). The cell lamellipodia, however, are shorter than those of

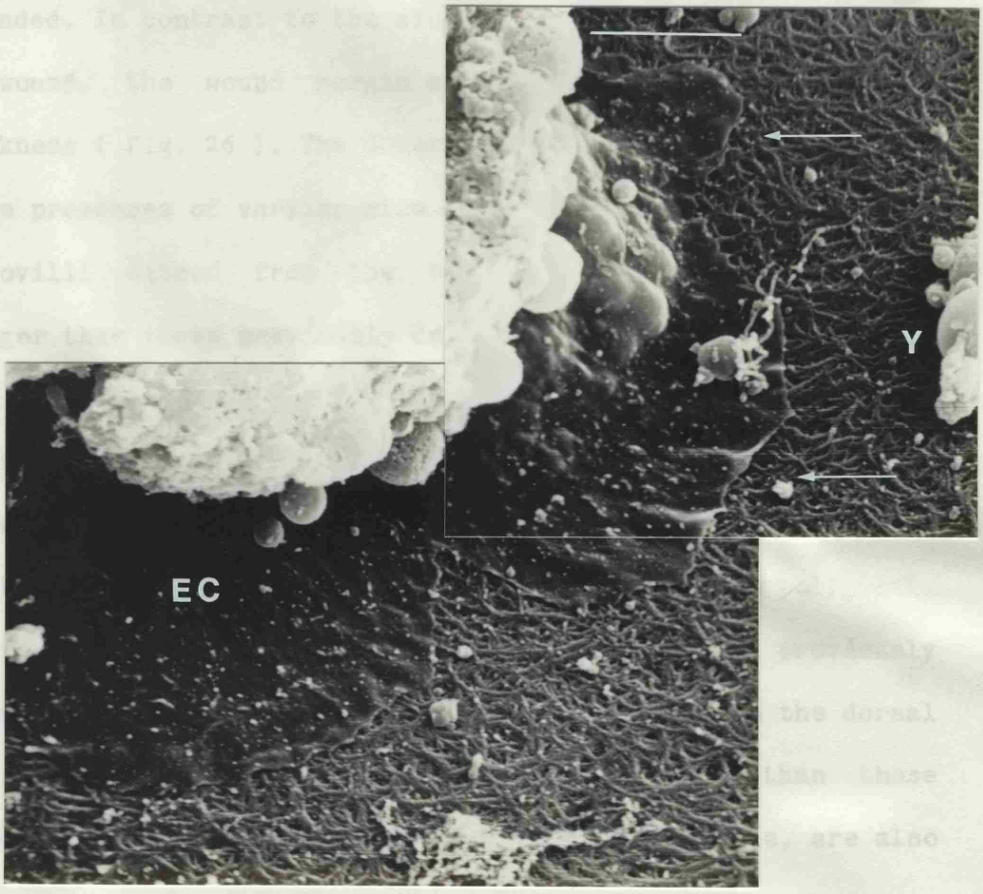
the attached epiblast periphery and extend across the vitelline membrane at right-angles to the wound edge. The lamellipodial margins also often extend into filopodial processes which run centrifugally across the surface of the vitelline membrane. Individual cells, at varying points along the wound edge, may also appear attached to the surface of the vitelline membrane and have a similar morphology to those at the distal wound extremity.

Yolk globules, and occasional cell debris, may be observed on both the epiblast and vitelline membrane.



(Fig. 23). The ventral surface of the 1-hour reincubated wound (W). The distal extremity of the wound margin (arrows) is attached to the vitelline membrane (V) by a small group of flattened cells.
Bar = 100µm.

(Fig. 24). Ventral view of the unattached wound margin after reincubation for 1 hour. The wound is bordered by several layers of rounded cells. Dead and dying cells (D) are commonly observed toward the wound edge.
Y - Yolk.
Bar = 5µm.



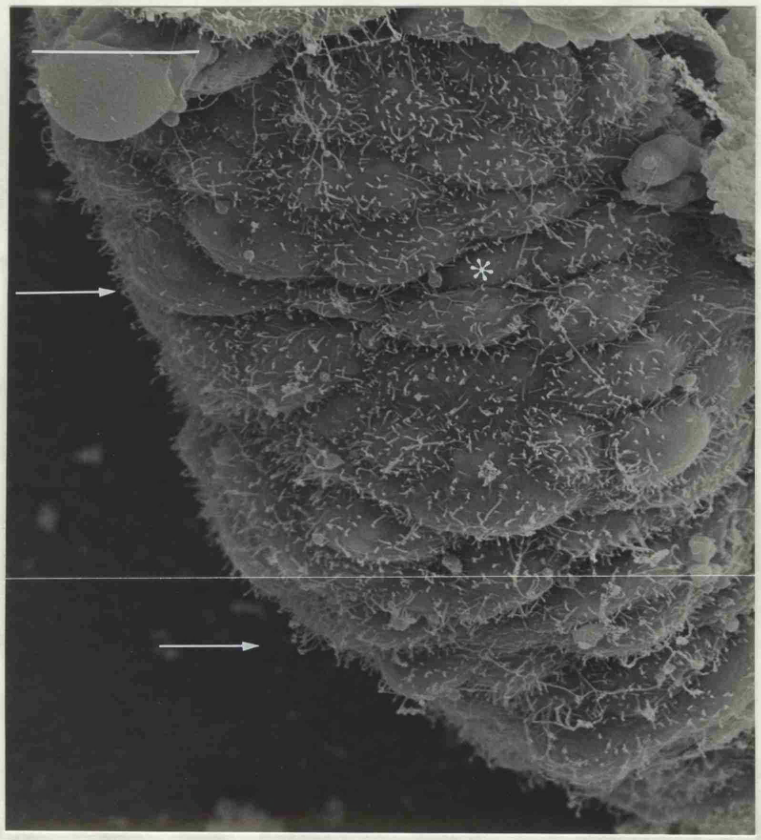
(Fig. 25). View of the distal attached wound margin extremity after 1 hour reincubation. The epiblast cell at the wound margin (arrows), and an adjacent 'edge cell' (EC), are attached to the surface of the vitelline membrane.
Y - Yolk.
Bar = 5μm.

(b). The Dorsal Surface of the Epiblast Wound.

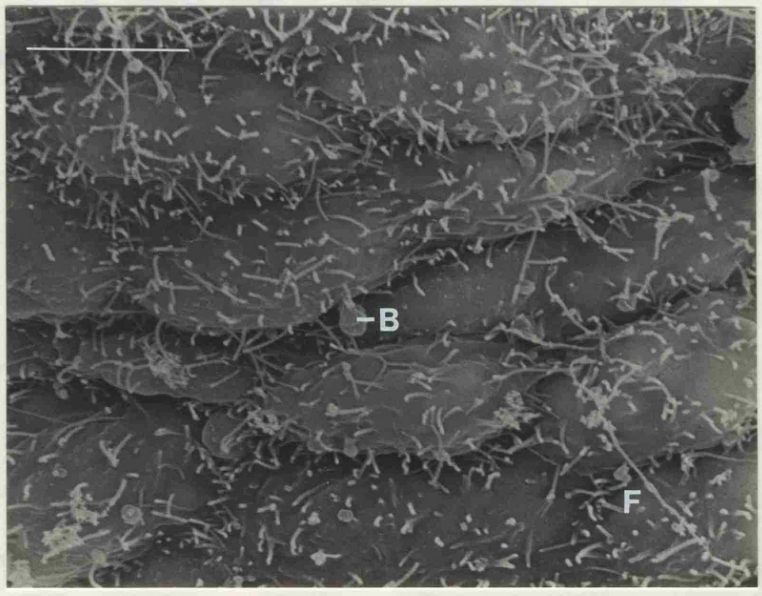
The epiblast cells bordering the dorsal margin of the 1 hour reincubated wound are rounded. In contrast to the single layer of cells bordering the 0-hour wound, the wound margin after 1 hour reincubation is 3-5 cells in thickness (fig. 26). The dorsal cell surfaces are uneven, with bleb-like processes of varying size and occasional short filopodia. Numerous microvilli extend from the cell surfaces, and these are generally longer than those previously described for the dorsal epiblast bordering the 0-hour wound (Section D). These processes are often distributed unevenly, with localized concentrations on the cell surface. Dead cells, and cell debris, are frequently observed at the wound edges.

The epiblast cells lying adjacent to the wound are polygonal, flattened in the plane of the epithelium, and resemble those previously described for the normal dorsal epiblast. The microvilli on the dorsal cell surfaces, however, are longer and more numerous than those previously described. Bleb-like processes, and short filopodia, are also frequently present.

Cell debris, and yolk globules, commonly lie on the dorsal epiblast adjacent to the wound.



(Fig. 26a). The dorsal surface of the proximal epiblast bordering the 1 hour reincubated wound. The cells bordering the wound are elongated toward the free edge (arrows).
Bar = 10µm.



(Fig. 26b). Detail of the cells bordering the dorsal wound margin (* in fig. 26a). The cells are rounded, with bleb-like processes (B), numerous long microvilli, and occasional filopodia (F).
Bar = 5µm

2. Thick Sections.

Transverse sections, cut parallel to the epiblast periphery, were taken through the unattached proximal wound margin, and the attached group of epiblast cells bordering the distal wound after reincubation for 1 hour at 37.5 ^{o}C.

The unattached wound margin consists of 3-5 layers of rounded cells (fig. 27). Towards the wound edge, the epithelium may curl away from the vitelline membrane. The epiblast cells bordering the wound margin generally appear larger than those of the epiblast distant to the wound, and are often elongated toward the wound edge. Adjacent cells may contact each other at any point on the cell surface.

The epithelium remains several cells thick for a short distance back from the wound edge, and numerous gaps are observed between neighbouring cells. The epiblast cells adjacent to the margin may be elongated at right-angles to the vitelline membrane. Distant to the wound edge, the epiblast is a single layer of cells. These cells are also rounded, but to a lesser degree than those at the wound margin. Neighbouring cells appear to be connected at, or near, the dorsal cell surface.

The attached distal wound margin is 1-7 cells wide, at right-angles to the wound edge (fig. 28). The epiblast cells in contact with the vitelline membrane are less flattened than the 'edge cells' of the normal epiblast periphery. Many of the attached cells have short lamellipodia which extend toward the wound and underlap adjacent cells. The cell at the extreme wound edge has a short lamellipodium which thickens abruptly to a rounded cell body.

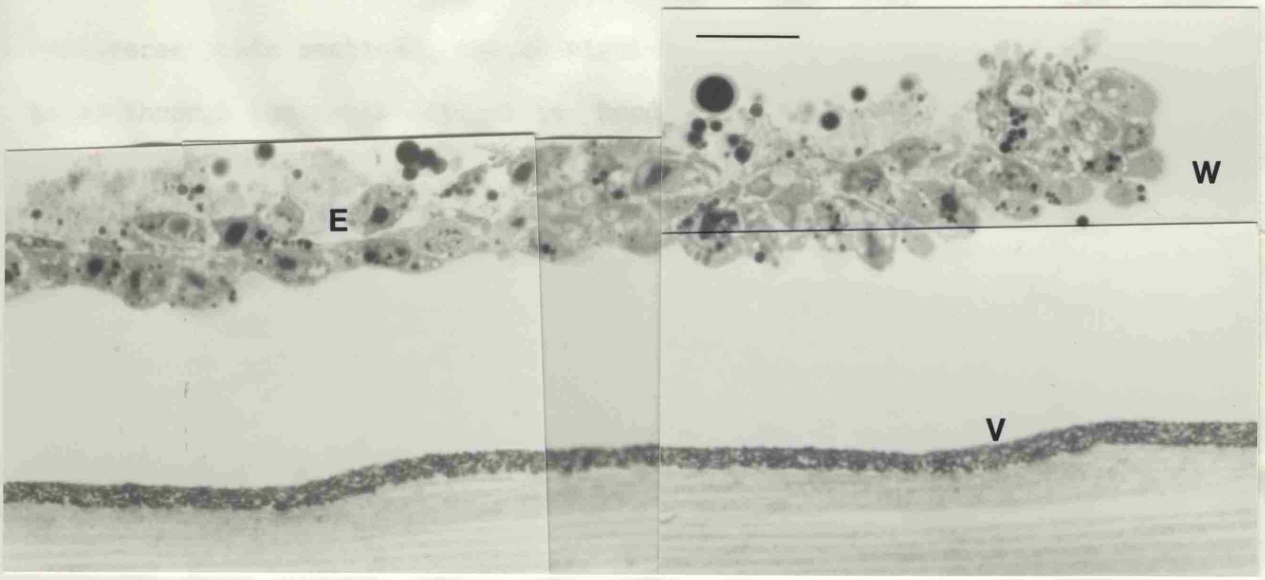
The attached cell furthest from the wound usually has an additional short lamellipodium directed away from the wound. The inner layer of the vitelline membrane lying beneath the attached epiblast cells is often

separated from the outer layers by a number of small gaps.

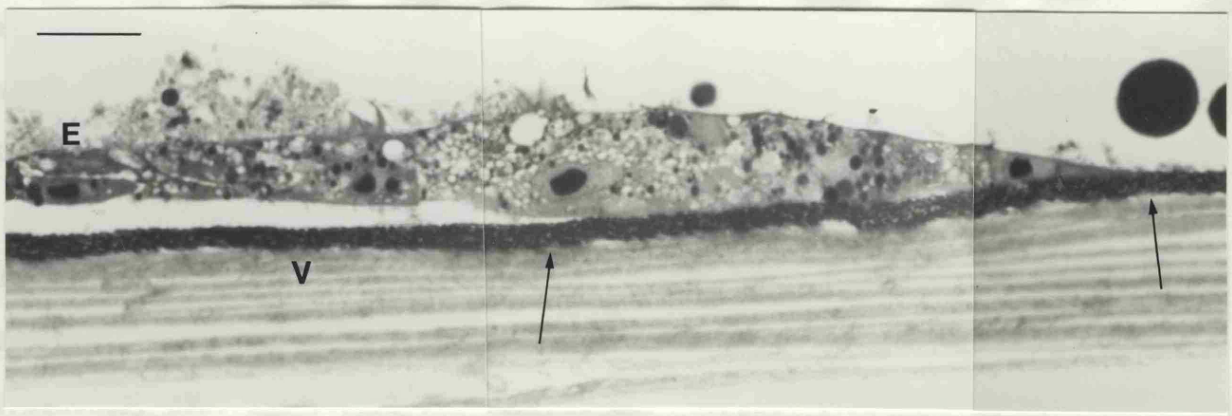
The attached cells are overlain by rounded epiblast cells, forming an association 2-4 cells deep above the vitelline membrane. The orientation of these cells could not be determined under the light microscope. The cells of the attached group appear closely apposed, and gaps are seldom observed between neighbouring cells. The adjacent unattached epiblast is composed of 2-3 layers of loosely connected cells. These cells are rounded and, toward the attached wound margin, may be elongated parallel to the vitelline membrane.

Individual cells, or small groups of cells, are occasionally independently attached to the vitelline membrane underlying the wound. These cells are flattened, to varying degrees, on the membrane surface.

Hypoblast cells, and yolk material, are observed on the upper, ventral, surface of the epiblast adjacent to the wound.



(Fig. 27). Transverse thick section through the unattached proximal wound margin after reincubation for 1 hour. The epiblast cells (E) adjacent to the wound (W) are rounded, and appear elongated toward the wound edge .
V - Vitelline membrane.
Bar = 10µm.



(Fig. 28). Transverse thick section through the distal wound margin after reincubation for 1 hour. A group of rounded cells (arrows), wider than that previously described for the normal epiblast periphery, attach the epiblast (E) at the wound edge to the vitelline membrane (V).
Bar = 10µm.

3. Transmission Electron Microscopy.

Transverse thin sections, cut at right-angles to the wound margin, were taken through the epiblast cells bordering the distal wound after reincubation at 37.5 ^{o}C for 1 hour.

The attached cells are elongated at right-angles to the wound edge, and appear rounded in comparison with the 'edge cells' previously described for the normal epiblast periphery. The cell at the wound edge has a short, thick lamellipodium which extends toward the wound. The submarginal cells possess thinner lamellipodia which also lie toward the wound and underlap adjacent cells (fig. 29).

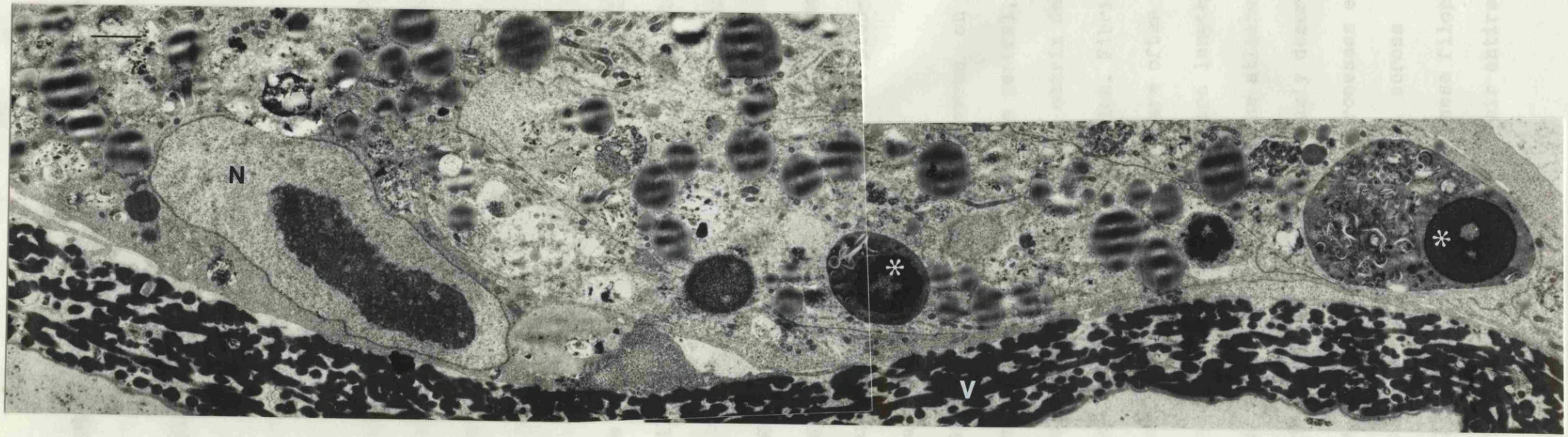
The cells are usually closely apposed to the vitelline membrane, although small gaps are occasionally observed. Small rounded processes often extend from the lower surfaces of the lamellipodia and lie between the fibres comprising the vitelline membrane. Neighbouring cells, and their underlying lamellipodia, are closely apposed. Discrete junctional structures are rarely observed, but the cell membranes are frequently separated by a continuous thin layer of densely stained material.

The cell nuclei are relatively large and, in section, appear elongated with the cell at right-angles to the wound margin. The nuclear material is mainly euchromatic, although most cells possess a single large nucleolus.

The cell cytoplasm contains many elongated microvilli and numerous darkly stained vesicles. Endoplasmic reticulum, both rough and smooth, is also present. Structures resembling microfilament bundles are frequently observed toward the ventral and dorsal cell surfaces. The fibres are usually aligned parallel to the long axis of the cell, and are especially common within the lamellipodial cytoplasm directly adjacent to the vitelline membrane. Single microtubules are also observed scattered throughout the cytoplasm.

Small rounded cellular structures are frequently observed within the cytoplasm of the perinuclear region and lamellipodial base. The nuclei of these engulfed cells are circular in outline. The nuclear material is almost totally heterochromatic, although small patches of euchromatin are occasionally observed toward the centre of the nucleus. The cell cytoplasm is dense and often fibrous in appearance.

(Fig. 29). Transverse thin section through the attached epiblast cells bordering the distal wound after reincubation for 1 hour. The attached cells possess lamellipodia which lie toward the wound and underlap adjacent cells. Dead cells (*) are observed within the cytoplasm of the epiblast cells at the wound margin.
N - Nucleus, V - Vitelline membrane.
Bar = 2µm.



F. The Epiblast Wound after Reincubation for 5 Hours.

1. Scanning Electron Microscopy.

(a). The Ventral Surface of the Epiblast Wound.

After 5 hours reincubation, at a temperature of 37.5 ^{o}C, the wound appears as a 'key-hole' shaped indentation of the epiblast periphery (fig. 30). The epiblast periphery adjacent to the wound, and epiblast cells bordering the distal wound, are attached to the surface of the vitelline membrane.

The epiblast cells bordering the proximal wound lie above the surface of the vitelline membrane, although the separation is smaller than that previously described after 1 hour reincubation. This unattached wound margin is semi-circular in outline, with the cells rounded and often elongated parallel to the wound edge. Microvilli are present on the cell surfaces, with occasional small bleb-like processes and larger hemispherical processes.

A tenuous meshwork of fine fibrils is observed on many of the unattached cells at the proximal wound margin. In general, this material is present in larger quantities than the similar matrix described on the unattached wound edge after 1 hours reincubation. Fibrils contact the underlying cell surface at one or both ends and are often associated to form fibre-like processes of variable thickness and length.

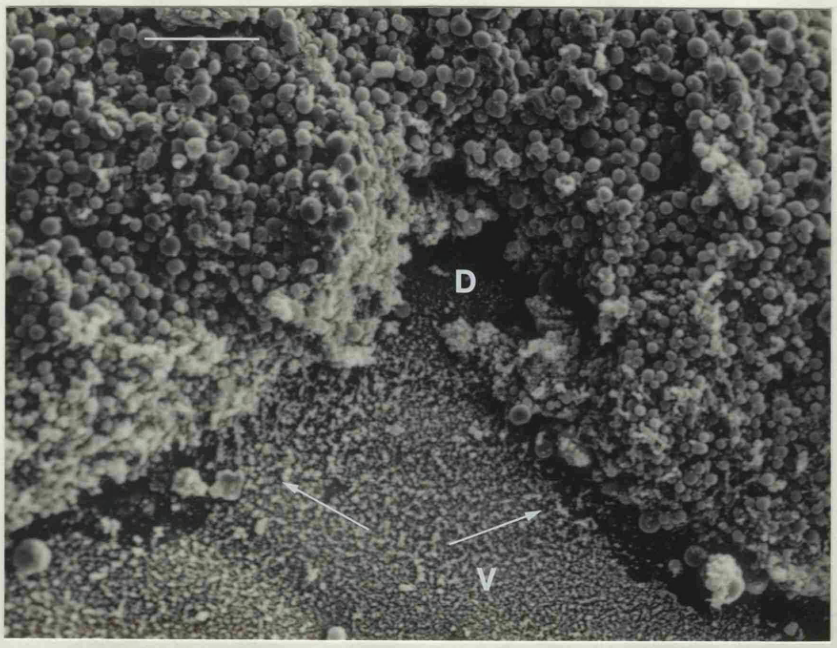
The cells at the proximal extremity of the attached wound margin are less flattened (fig. 31) than those previously described for the normal epiblast periphery. Numerous filopodial processes extend from the cell body, or from short lamellipodia, and lie across the vitelline membrane at right-angles to the wound margin. These filopodia appear to be in contact with the vitelline membrane for their entire length, and

are usually unbranched. The cell body is rounded and has an uneven ventral surface with large hemispherical processes, small bleb-like processes and occasional filopodia. Microvilli are less prevalent than was previously described for the rounded cells of the unattached wound margin after 1 hour reincubation.

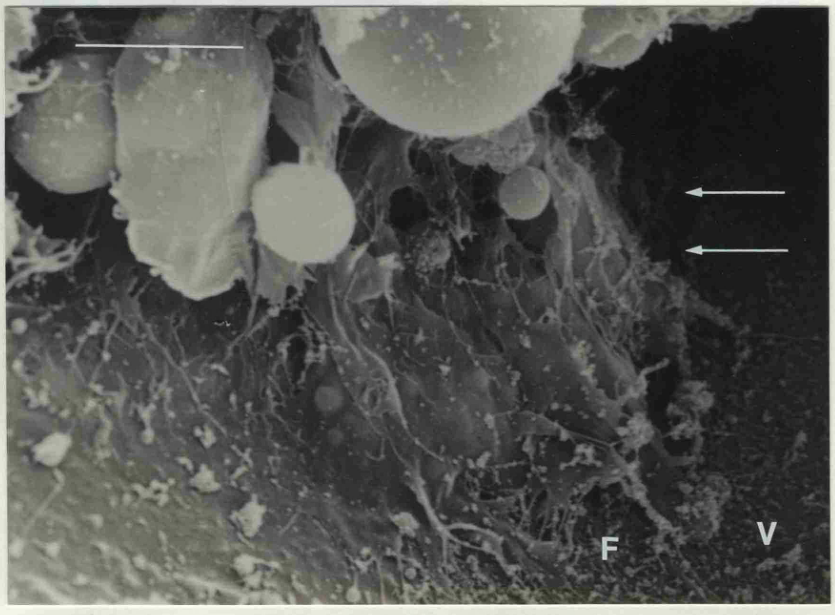
Moving distally along the attached wound margin, the cells are extended into short flap-like processes, and lamellipodial processes with short filopodia at their margins (fig. 32). These processes also lie at right-angles to the wound margin, and appear closely apposed to the vitelline membrane. The cell bodies resemble those previously described for the cells at the proximal extremity of the attached wound margin.

The cells at the distal wound margin are flattened and resemble the 'edge cells' of the attached epiblast periphery with which they are continuous. The cells have a single broad lamellipodium, extended across the vitelline membrane at right-angles to the wound margin. Adjacent cells, and their lamellipodia, overlap to form a complete border of attached cells at the distal wound margin.

Yolk globules, and occasional cell debris, may be observed on both the epiblast and vitelline membrane.



(Fig. 30). Ventral view of the epiblast wound after 5 hours reincubation. The distal wound (D) is attached to the vitelline membrane (V) by a row of flattened cells at the wound margin (arrows).
Bar = 50µm.



(Fig. 31). The ventral surface of the attached wound margin after 5 hours reincubation. The most proximal cell (arrows) extends into several filopodial processes (F). Cell debris is observed on the attached cells and adjacent vitelline membrane (V).
Bar = 10µm.

(Fig. 32). The attached cells adjacent to the proximal wound extremity (PW) are flattened and possess broad, flat, lamellipodia (L).
C - Cell body, F - Filopodia, V - Vitelline membrane,
Y - Yolk.
Bar = 4µm.

(b). The Vitelline Membrane Surrounding the Wound Margins after Removal of the Overlying Epiblast

A mounted cactus needle can be used to remove the unattached epiblast from wounds reincubated for 5 hours. This operation exposes the inner surface of the vitelline membrane surrounding the closed wound. Cells at the distal wound margins, and adjacent epiblast periphery, remain attached to the surface of the vitelline membrane. The unattached proximal wound margin is removed along with the surrounding epiblast epithelium.

After 5 hours reincubation, the vitelline membrane bordered by the wound margin may be covered with a thin layer of yolk material. In contrast, the adjacent membrane surface, previously overlain by epiblast cells, appears relatively free of yolk. As a result, the original wound outline is often apparent even after removal of the unattached wound margin (fig. 33).

In some of the specimens examined, a number of thread-like fibres were observed at the proximal extremity of the wound. These fibres extend radially from the wound margin and most lie at right-angles to the epiblast periphery. Adjacent fibres often run parallel and are regularly spaced. Similar processes are occasionally present at the lateral wound margins, but fibres of this sort are not observed in the vicinity of the attached epiblast periphery.

Additional short fibres may occur some distance from the wound margin. These processes often lie next to longer fibres and, like them, are oriented toward the wound margin. In some specimens, individual fibres are distally continuous with the lamellipodia of flattened cells at, or near, the wound margin. In all other cases, both ends of the fibre lie on, or slightly above, the surface of the vitelline membrane.

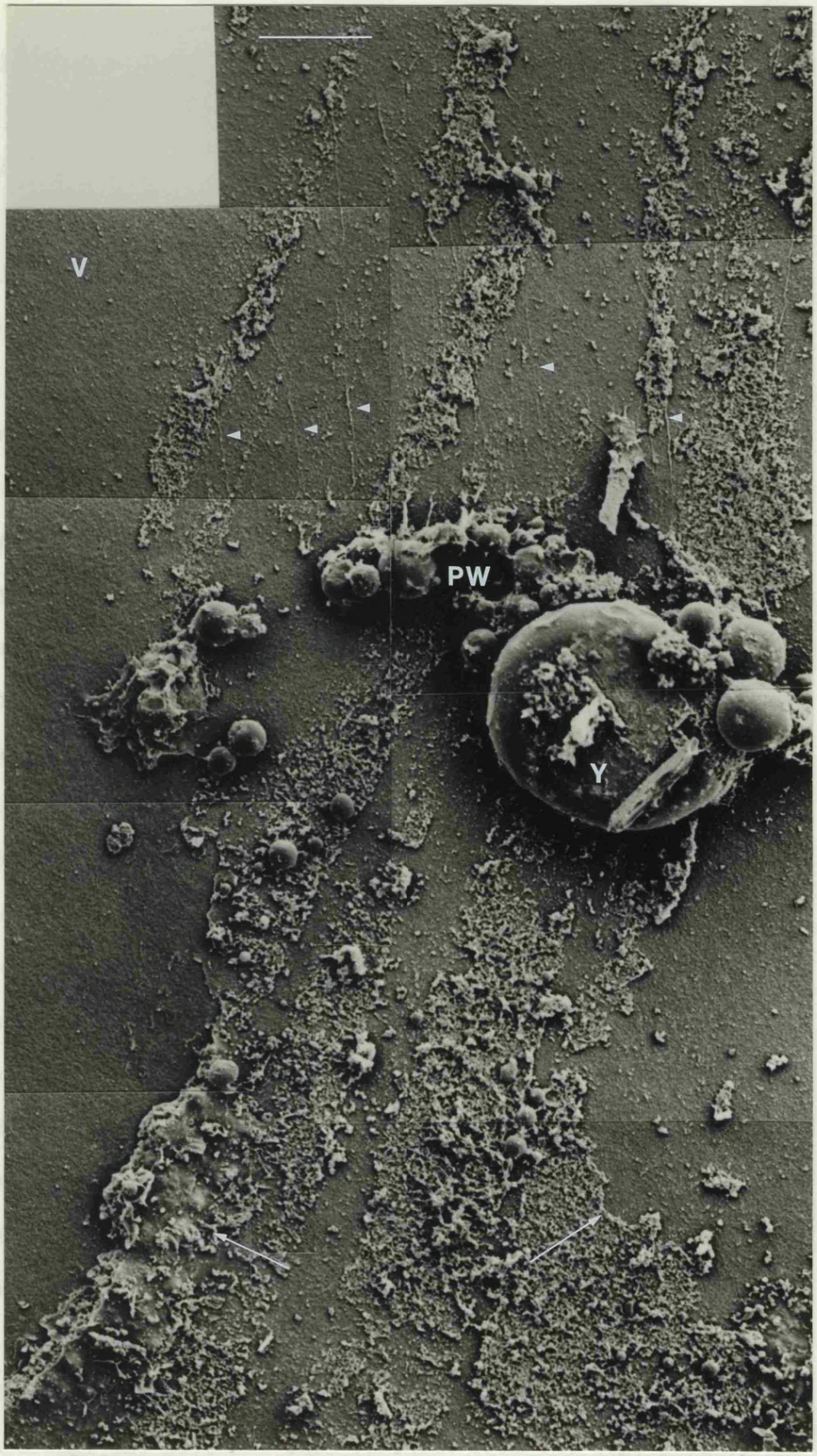
Individual fibres are relatively straight, and most appear closely

apposed to the surface of the vitelline membrane. In some instances, a fibre may be seen to consist of several fine fibrils associated with an amorphous or particulate material. The thickness of a fibre tends to vary with length and, occasionally, a single fibre divides into two or more processes.

Small spherical aggregations of an amorphous material are commonly observed along a given process and a particulate matter is associated with the adjacent vitelline membrane.

Yolk material and small amounts of cellular debris are associated with the vitelline membrane adjacent to the fibres. Flattened cells are also occasionally observed on the vitelline membrane proximal to the wound margin.

A more detailed account of the fibres associated with the wound margins is given in Section G.



(Fig. 33). The 5-hour epiblast wound after removal of the unattached proximal wound margin and surrounding epiblast. Thin fibres (arrowheads) lie on the vitelline membrane (V) adjacent to the proximal wound extremity (PW).
Arrows - Distal wound margins, Y - Yolk.
Bar = 50µm.

2. Thick Sections.

After 5 hours reincubation at 37.5 °C transverse thick sections, cut at right-angles to the wound margin, were taken through the unattached epiblast cells bordering the proximal wound, the attached cells adjacent to the proximal wound margin, and the attached cells of the distal wound margin.

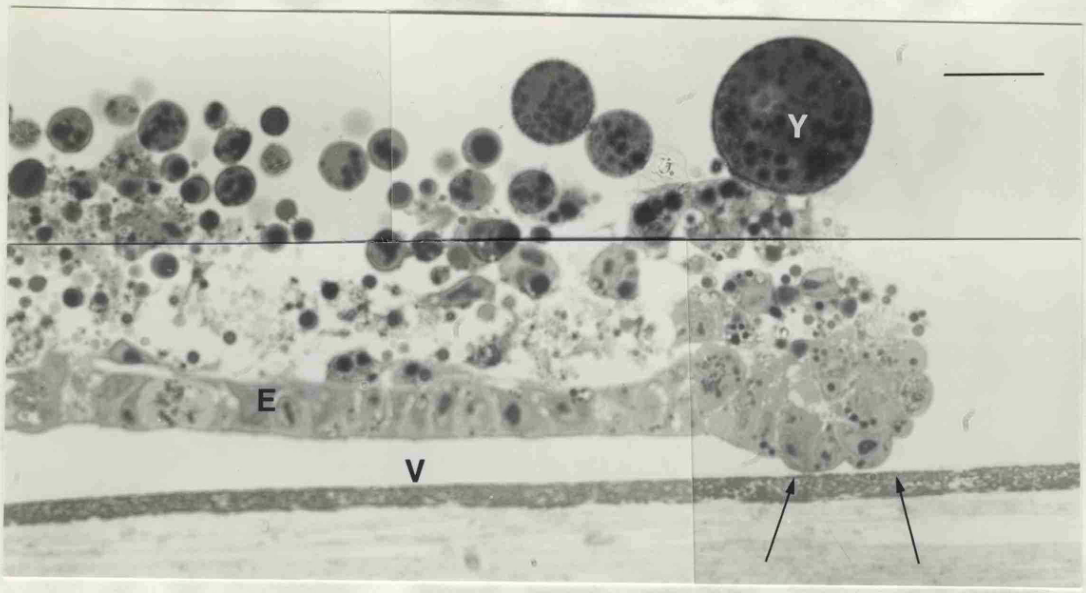
The unattached epiblast of the proximal wound margin is generally 1-3 cells thick. The cells bordering the wound lie above the surface of the vitelline membrane and are rounded. In section, the cells at the wound edge appear slightly larger than those distant to the wound, and may be elongated toward the vitelline membrane. The wound margin often curls away from the membrane surface, and the epiblast cell closest to the membrane may be several back from the edge. Toward the attached wound margin the cells may lie very close to the surface of the vitelline membrane, and their dorsal surfaces facing the membrane often appear flattened parallel to its surface (fig. 34). Small gaps are frequently observed between neighbouring cells. The epiblast distant to the wound margin is a single layer of cells. These cells also lie above the surface of the vitelline membrane and are rounded or cuboidal in shape. Neighbouring cells appear to be connected at their dorsal surfaces, and small gaps are observed between the adjacent cells.

The proximal extremity of the attached wound margin is connected to the vitelline membrane by a small group of 1-3 cells at the wound edge (fig. 35). The cell at the extreme wound edge has a lamellipodium which extends toward the wound. This process may be short and blunt, or long and tapering. The attached cell furthest from the edge usually has a similar lamellipodium directed away from the wound. The attached cells are rounded and often appear elongated at right-angles to the wound margin. Adjacent cells are closely apposed, although small gaps are

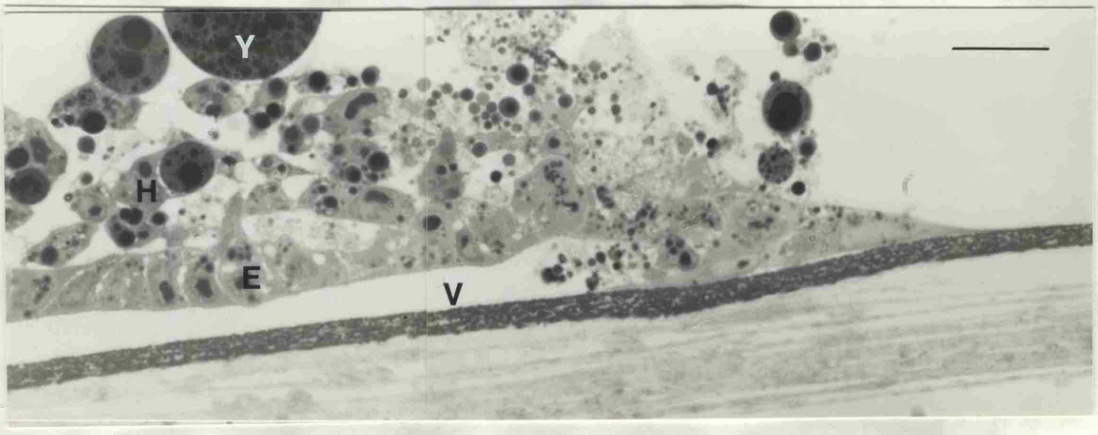
occasionally observed between the cells and their underlying vitelline membrane. The cells on the membrane surface are overlain by additional rounded cells, forming an association 2-3 cells deep. The epiblast distant to the wound margin resembles that previously described for the unattached proximal wound margin.

The distal wound margin is also attached to the vitelline membrane by a group of cells at the wound edge (fig. 36). However, this attached region is usually 4-5 cells long in the plane of the section. The cells in contact with the membrane are extensively flattened, and appear elongated at right-angles to the wound margin. The epiblast cell at the extreme wound margin has a long, thin, lamellipodium which tapers gradually to its tip. This process is closely apposed to the surface of the vitelline membrane and lies toward the wound. The submarginal cells possess similar lamellipodia which also extend toward the wound and underlap neighbouring cells. The attached cell furthest from the wound has an additional short lamellipodium directed away from the margin. The attached cells of the wound edge appear loosely connected, and gaps are observed between adjacent individuals. The inner vitelline membrane layer beneath the attached wound margin often lies a short distance above the outer membrane layers. The attached wound margin is overlain by 2-3 layers of flattened cells. The precise orientation of these overlying cells could not be determined under the light microscope. The unattached epiblast distant to the wound is often 2-3 cells thick. These cells are less rounded than those previously described for the proximal wound and, adjacent to the attached margin, may be elongated at right-angles to the wound edge.

Yolk material, and numerous hypoblast cells, are present on the upper, ventral surface of the epiblast surrounding the wound.

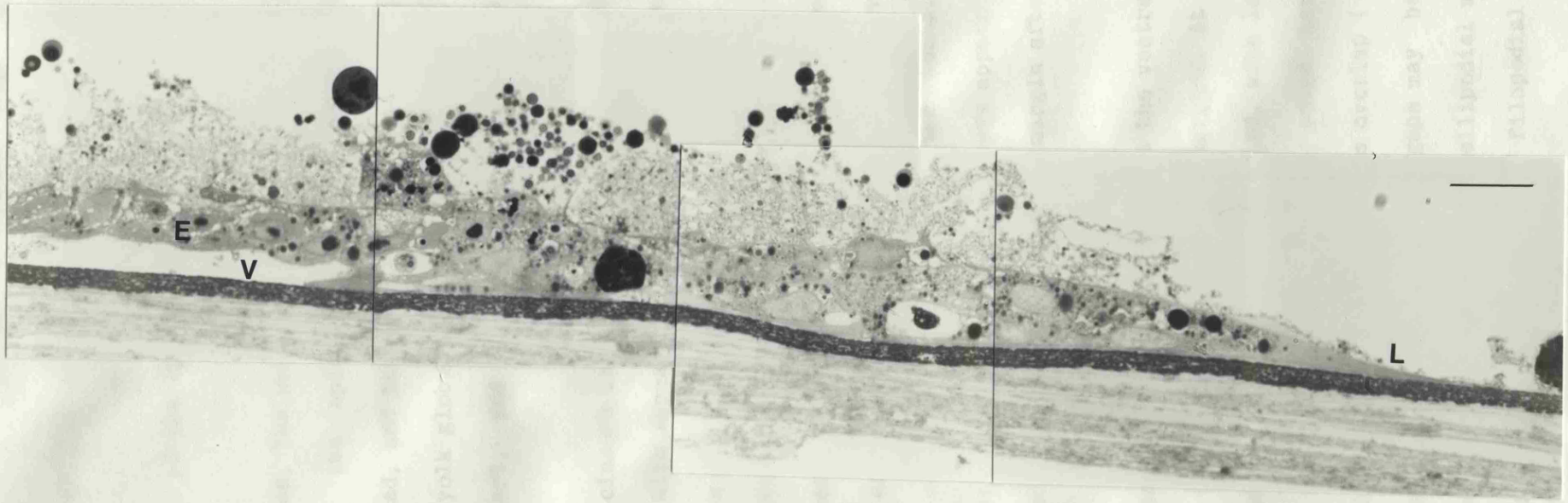


(Fig. 34). Transverse thick section through the unattached proximal wound margin after 5 hours reincubation. Epiblast cells (arrows) adjacent to the wound edge lie close to the surface of the vitelline membrane (V).
E - Epiblast, Y - Yolk.
Bar = 10µm.



(Fig. 35). Transverse thick section through the attached proximal wound margin after 5 hours reincubation. The epiblast (E) is attached to the surface of the vitelline membrane (V) by a small group of cells at the wound edge.
H - Hypoblast cell, Y - Yolk.
Bar = 10µm.

(Fig. 36). Transverse thick section through the attached distal wound margin after 5 hours reincubation. A group of flattened cells attach the epiblast (E) at the wound edge to the surface of the vitelline membrane (V). The cell at the extreme wound edge has a long, thin, lamellipodium (L) which lies toward the wound.
Bar = 10µm.



G. The Epiblast Wound after Reincubation for 10 Hours.

1. Scanning Electron Microscopy.

(a). The Ventral Surface of the Epiblast Wound.

After 10 hours reincubation, the wound appears as a shallow groove in the ventral surface of the epiblast (fig. 37). The proximal wound margins are closely apposed, and the groove marking the closed wound contains cell debris and yolk globules. Distal to this region of closure the wound margins lie apart, and are continuous with the attached epiblast periphery.

The initial wound closure usually occurs at an intermediate point along the wound margins, with apposition then proceeding sequentially away from the site in both directions.

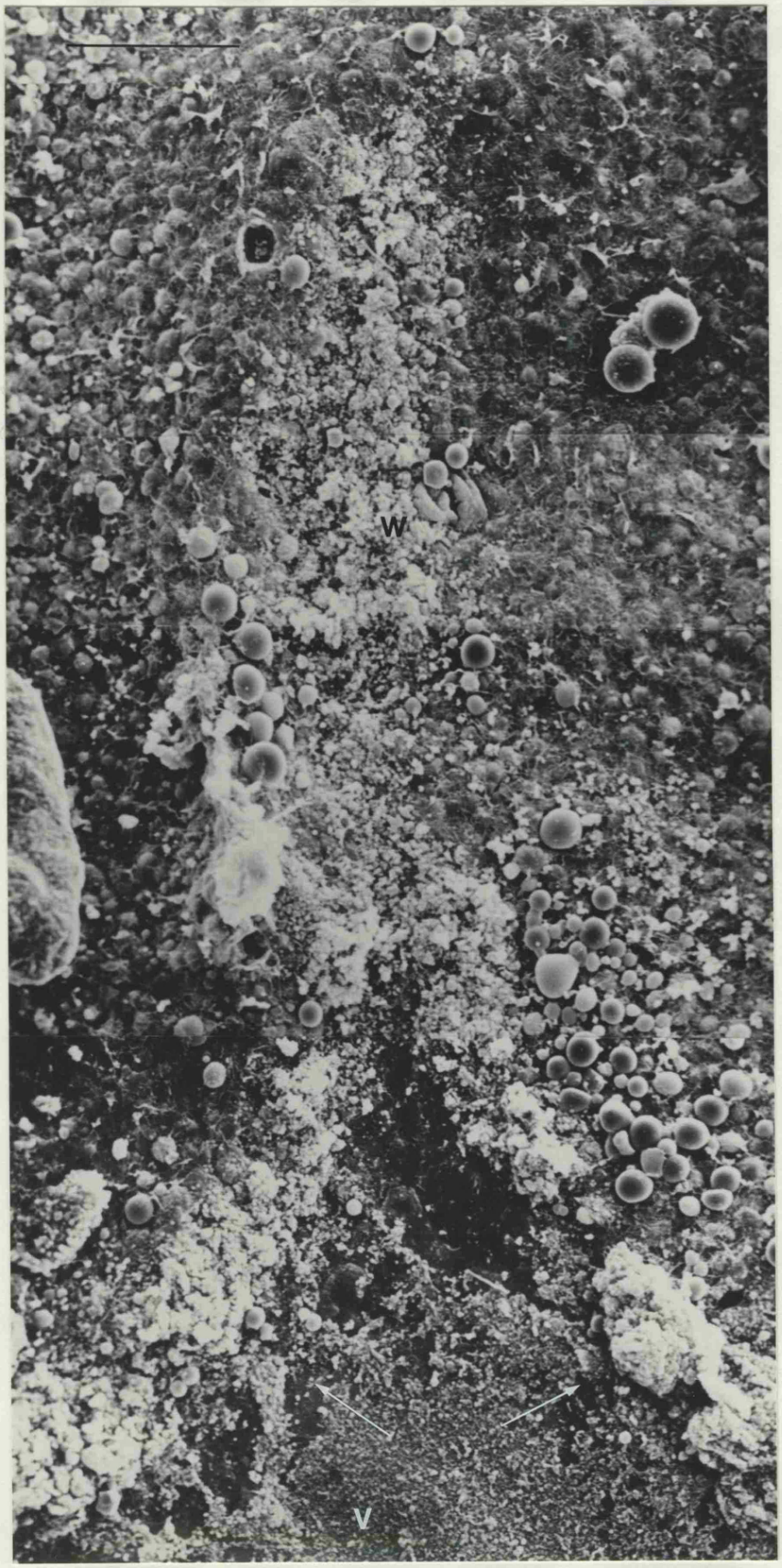
The epiblast lying directly adjacent to the closed wound is raised, and the cells often appear slightly rounded. In many specimens, a meshwork of thin fibrils and composite fibre-like processes overlies the ventral surface of the epithelium surrounding the wound. At high magnifications, the individual fibrillar processes appear similar to those previously described at the unattached wound margin after 1 and 5 hours reincubation.

Numerous filopodia and beaded threads occur on the ventral surfaces of the epiblast cells bordering the apposed wound margins. At the distal extremity of the closed wound the cells are flattened, with lamellipodia extended predominantly at right-angles to the wound margin. The lamellipodia of cells from apposing wound margins overlap (fig. 38). This overlapping is often only partial, and small gaps may be observed between the apposed lamellipodia. Both the lamellipodial margin, and ventral lamellipodial surface, possess numerous filopodial processes

which contact the lamellipodia of the cells from the adjacent wound margin, and often terminate in rounded processes. There are also small bleb-like processes on the ventral lamellipodial surface, with occasional microvilli toward the lamellipodial margin.

Distal to this region of closure the wound margins lie apart. The cells of the distal wound margin are attached to the surface of the vitelline membrane, and resemble those previously described for the normal epiblast periphery.

Cell debris may be observed on both the epiblast and vitelline membrane. Yolk globules are present in large numbers on the vitelline membrane, but appear less evident on the ventral epiblast surface than was previously described for reincubation periods of 1 and 5 hours.



(Fig. 37). Ventral view of the epiblast wound after 10 hours reincubation. The proximal wound (W) is closed, but the distal wound margins lie apart (arrows).
V - Vitelline membrane.
Bar = 100µm.

(Fig. 38). The distal extremity of the closed epiblast wound. The lamellipodia of cells from apposing wound margins overlap at their margins. Numerous filopodial processes (F) extend from the lamellipodial margins.
Y - Yolk.
Bar = 10µm.

(b). The Ventral Surface of the Vitelline Membrane Surrounding the Closed Wound after Removal of the Overlying Epiblast.

After 10 hours reincubation, the wound margins appear closely apposed and are attached to the inner surface of the vitelline membrane for their entire length. In some specimens, the tissue adjacent to the closed wound is seen to consist of two distinct cell layers - a dorsal eptihelium of flattened cells and an overlying association of yolk globules and rounded hypoblast cells.

The newly exposed vitelline membrane is relatively free of yolk, although occasional patches of cell debris are observed in the vicinity of the attached wound margins.

The proximal extremity of the closed wound is usually slightly wider, at right-angles to the line of wound closure, than is the rest of the attached margin. Flattened cells border this attached proximal margin and possess lamellipodial processes oriented away from the closed wound (fig. 39). These lamellipodia are closely apposed to the vitelline membrane and often extend into short filopodial processes which also lie across the membrane surface. Bleb-like processes, and occasional microvilli, are observed on the lamellipodia and adjacent cell bodies.

The attached cells at the proximal wound extremity are overlain by similarly flattened cells, hypoblast cells and yolk material. Isolated flattened cells, and cell debris, are observed on the vitelline membrane adjacent to the proximal wound margins. Yolk globules are rarely observed.

A number of thin fibre-like processes lie on the vitelline membrane proximal to the closed wound. These processes resemble those previously described on the vitelline membrane surrounding the epiblast wound after 5 hours reincubation.

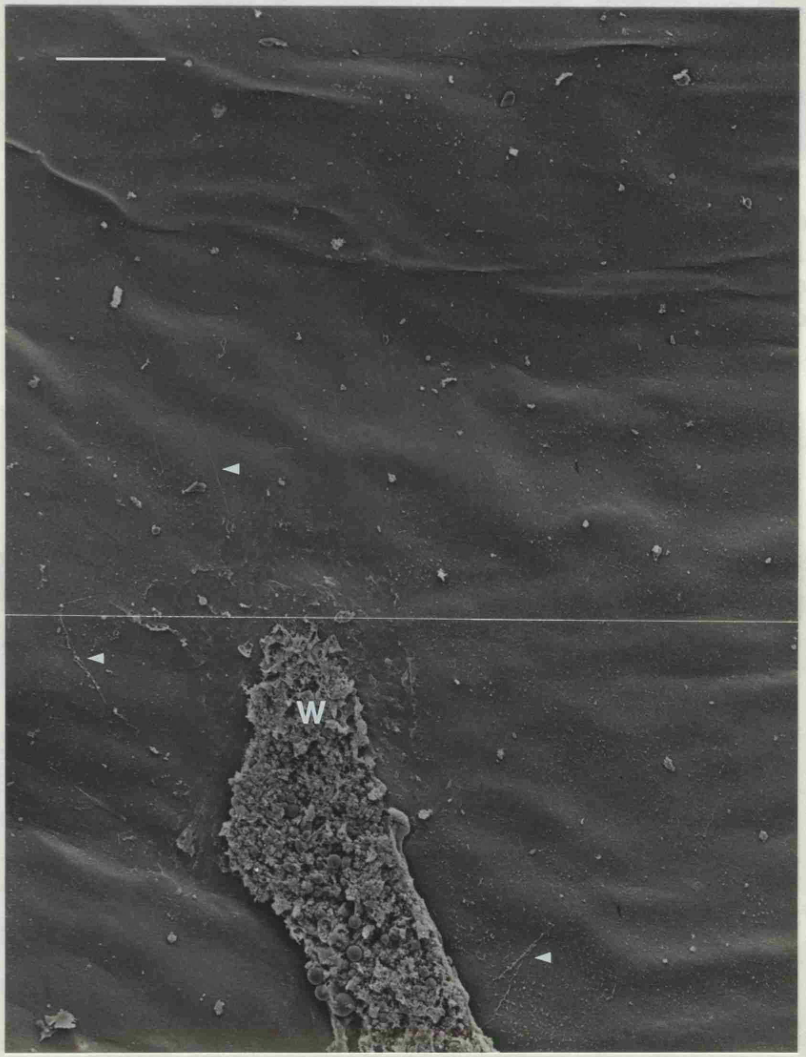
Most of the fibres observed are situated directly adjacent to the proximal extremity of the closed wound. The fibres extend radially from the attached wound and the majority lie at right-angles to the epiblast periphery. In general, the fibres after 10 hours reincubation are longer than those described for the epiblast wound after 5 hours reincubation. Fibres similar to those at the proximal wound are also observed at the lateral wound margins and additional short processes are observed some distance from the wound. Most of these fibres are oriented in the direction of the attached wound margins. Fibres are not associated with the attached 'edge cells' at the epiblast periphery.

One end of a fibre-like process often lies close to the attached wound margin and many such processes are attached to the lamellipodia or cell bodies of flattened cells at the proximal extremity of the closed wound (fig. 40). The other end, distant to the wound, may consist of two or more finer processes and often curls back upon itself. Short fibres are observed on the exposed ventral surfaces of the attached cells, and these processes are occasionally continuous with the longer fibres on the adjacent vitelline membrane.

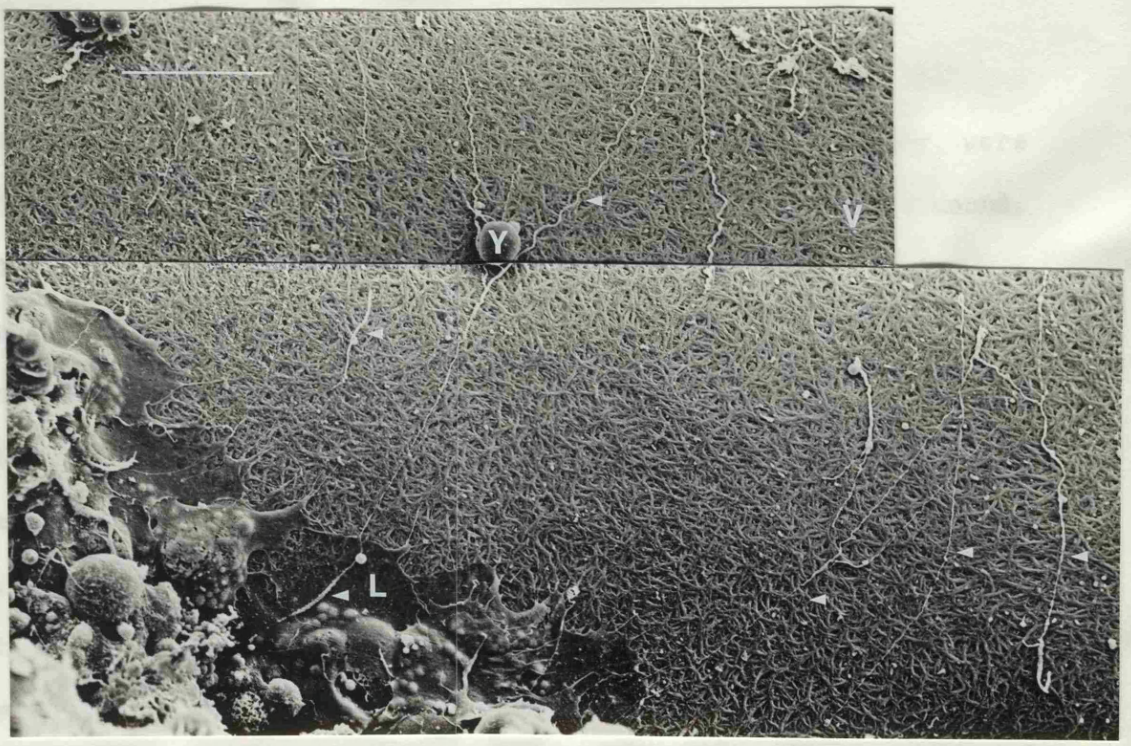
As in the wound after 5 hours reincubation, adjacent fibres usually lie approximately parallel and are regularly spaced. The fibres adjacent to the proximal wound are closely apposed to the surface of the vitelline membrane, although a given fibre may overlie a number of similar processes. Each fibre appears to consist of one or more fine fibrils coated with an amorphous matrix material. Rounded aggregations of this material often lie at irregular intervals along a given process and similar spherical bodies are often observed on the surrounding vitelline membrane (fig. 41).

A fine particulate matter is also associated with the fibres and adjacent membrane surface. In some cases, the fibrils comprising a fibre lie approximately parallel for their entire length. In others, the

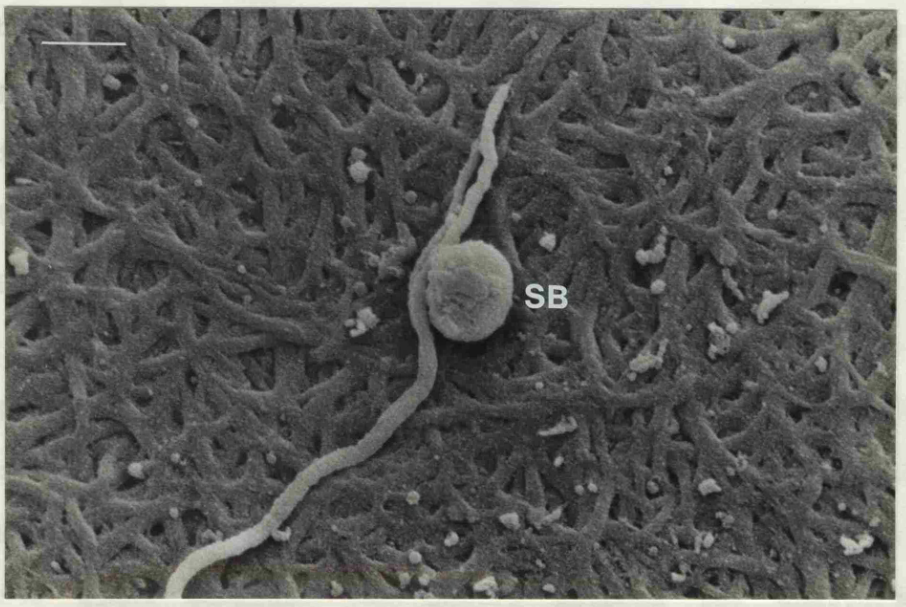
fibrils are wrapped around each other in a helical arrangement. At certain points along some fibres, the amorphous matrix material appears to be absent or reduced and the adjacent fibrils lie slightly apart. Fibres may also split into two or more finer processes which lie, usually at a slight angle to the original fibre, across the surface of the vitelline membrane.



(Fig. 39). The proximal extremity of the closed epiblast wound (W) after reincubation for 10 hours. Fibres (arrowheads) are present on the inner surface of the vitelline membrane (V) surrounding the wound.
Bar = 100µm.



(Fig. 40). Flattened cells at the proximal wound extremity possess short lamellipodia (L) oriented away from the closed wound. Fibres (arrowheads) are observed on the exposed lamellipodial surfaces and adjacent vitelline membrane (V).
Y - Yolk.
Bar = 20µm.



(Fig. 41). Detail of a fibre end and adjacent spherical body (SB).
Bar = 2µm.

2. Thick Sections.

Transverse thick sections, cut parallel to the epiblast periphery, were taken through the closed proximal wound, and the attached distal wound, after 10 hours reincubation at 37.5 ^{o}C.

The epiblast of the closed wound is attached to the surface of the vitelline membrane by 2-4 layers of flattened cells, while the epiblast adjacent to the closed wound lies above the surface of the vitelline membrane (fig. 42). The attached cells are tightly packed and, in section, appear elongated at right-angles to the wound. There is no apparent discontinuity between the attached epiblast cells from apposing wound margins. At the junction between the attached and unattached epiblast, additional cells extend across the vitelline membrane from both sides of the wound. These cells are elongated away from the wound, and possess lamellipodia which lie at right-angles to the apposed wound margins. Cells adjacent to the wound are overlain by others further from the region of closure, forming an association 3-4 cells thick. These cells are closely apposed, although small gaps are occasionally observed between neighbouring cells in the vicinity of the closed wound. These lateral groups of cells are observed by S.E.M. after the removal of the unattached epiblast surrounding the proximal wound.

The unattached epiblast directly adjacent to the closed wound is usually a single layer of cuboidal or columnar cells. Neighbouring cells are joined at their dorsal surfaces. Adjacent ventral cell surfaces often lie apart, or are attached at a number of discrete points. The epithelium bordering the closed wound lies some distance above the vitelline membrane, although it remains connected to the epiblast cells of the apposed wound margins. The groove between the unattached epiblasts of the adjacent wound margins is filled with a loose association of rounded hypoblast cells and yolk globules. This material

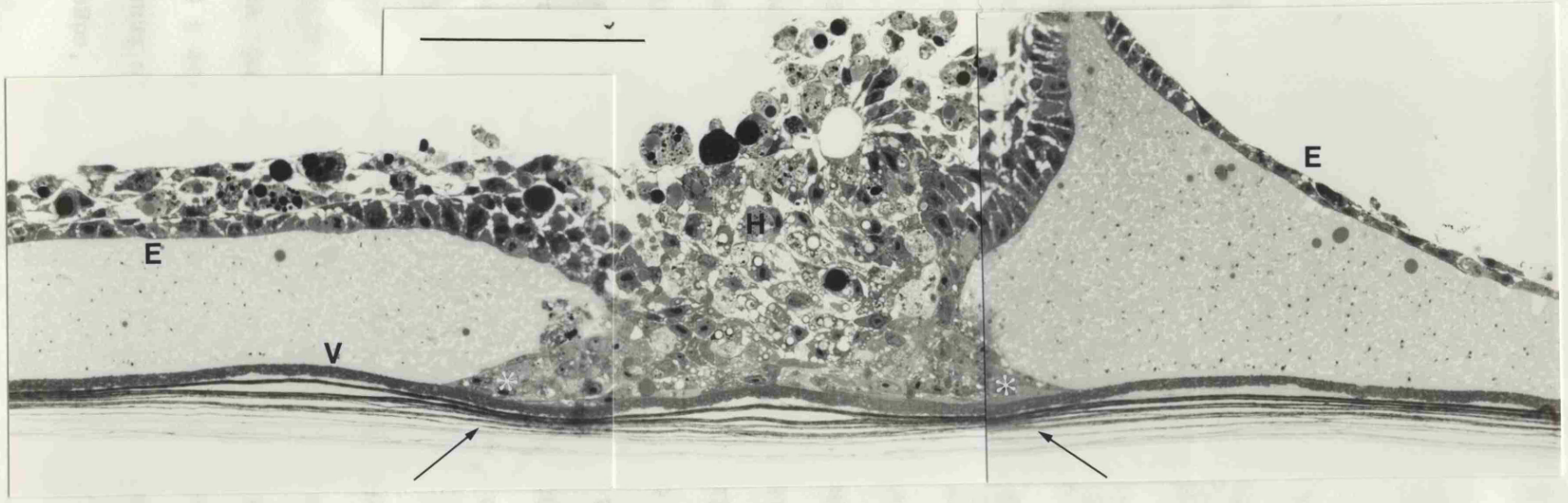
overlies the attached epiblast cells of the closed wound.

The epiblast distant to the wound lies closer to the vitelline membrane than that adjacent to the wound, and is composed of a single layer of flattened cells. Hypoblast cells and yolk globules are observed on the upper, ventral surface of the epithelium.

The distal wound margins lie apart, and each is attached to the surface of the vitelline membrane by a group of extensively flattened cells. These attached cells appear to be elongated at right-angles to the wound, and continue for 5-9 cells back from the wound edge. The cells are closely apposed to the vitelline membrane and possess long, thin lamellipodia which extend toward the wound and underlap neighbouring cells. The attached cell furthest from the edge has an additional lamellipodium directed away from the wound. Adjacent cells appear closely apposed, and are overlain by 1-3 layers of similarly flattened cells.

Yolk material is observed on the upper surfaces of the attached epiblast cells, and the vitelline membrane between the wound margins.

(Fig. 42). Transverse thick section through the closed proximal wound after 10 hours reincubation. The region of wound closure (arrows) is marked by a group of flattened epiblast cells attached to the surface of the vitelline membrane (V). At the edge of this group, elongated cells (*) extend away from the wound.
E - Epiblast, H - Hypoblast cells.
Bar = 50µm.



H. Light Microscopy of Explant Movement ('Edge Cells' and Proximal Epiblast.

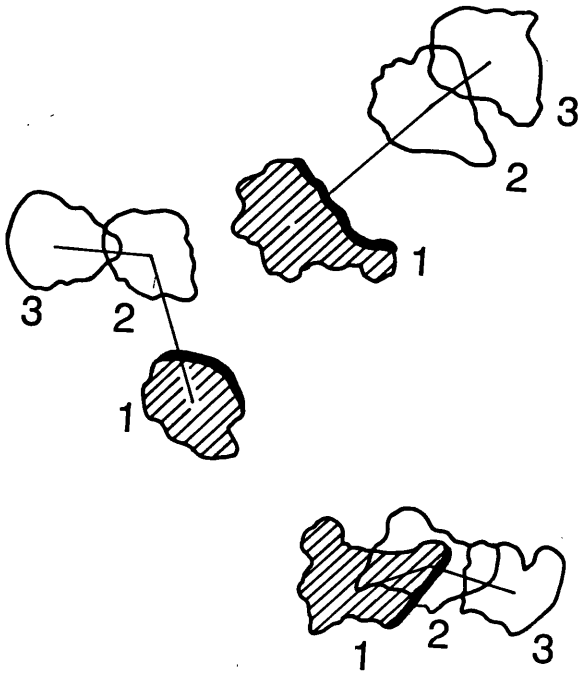
Explants of the epiblast periphery containing a row of 'edge cells' and a portion of the proximal epiblast were placed on the inner surface of the vegetal pole vitelline membrane, and reincubated for 3 hours at 37.5 ^{o}C. Carbon particles were placed around the explants and, using these as points of reference, tracings were made of each explant after 1, 2 and 3 hours reincubation (diagram 5).

After reincubation for 1 hour, all the epiblast explants are attached to the vitelline membrane by the explant margin containing the original 'edge cells'. This explant margin appears relatively smooth, in contrast to the newly cut explant edges which are irregular in outline and appear to lie either on or just above the membrane surface.

After 2 hours reincubation, most explants have moved relative to the carbon particles on the surface of the vitelline membrane. This movement is in the direction of the original attached epiblast margin, i.e. that region containing the epiblast 'edge cells'.

A similar explant movement is detected after reincubation for 3 hours. This movement is also in the direction of the attached epiblast margin and may be parallel, or at an angle to, the previous translocation. Over the full three hour reincubation period, some explants move a distance several times their own length.

The morphology of the epiblast explants after 1, 2 and 3 hours reincubation is described in more detail in Sections I-K.



(Diagram 5). Tracings of explants of the epiblast periphery ('edge cells' and proximal epiblast) after reincubation for 1, 2 and 3 hours. The position of explants after 1 hour is illustrated by the hatched outlines. The heavy line denotes the position of the original 'edge cells'.

I. Explants of the Epiblast Periphery ('Edge Cells' and Proximal Epiblast) after Reincubation for 1 Hour.

1. Scanning Electron Microscopy.

After incubation for 1 hour at 37.5 °C, the explants of epiblast periphery appear as rectangular pieces of tissue on the inner surface of the vegetal pole vitelline membrane. Most explants are attached to the vitelline membrane by a row of cells at the distal edge, i.e. that margin containing the original epiblast 'edge cells' (fig. 43). The explant proximal to this attached region usually lies above the membrane surface.

The flattened cells at the attached edge possess lamellipodia which lie at right-angles to the explant margin. These processes are generally smaller than those previously described for the 'edge cells' of the normal epiblast periphery. The lamellipodia of adjacent cells overlap toward the proximal cell body and the attached edge is irregular in appearance. Most of the lamellipodial margin appears closely apposed to the vitelline membrane and extends into short filopodia which lie across the membrane surface. Those portions of the lamellipodial margin which lie above the membrane are often reflexed toward the proximal explant. The main cell bodies appear less flattened than those of the normal epiblast periphery and are overlain by the lamellipodia of adjacent marginal cells and processes from cells of the proximal explant. Bleb-like processes, microvilli and occasional filopodia are observed on the lamellipodial surfaces and rounded cell bodies.

At either side of the distal explant edge, i.e. at the junction between attached and unattached explant margin, the cells on the surface of the membrane appear less flattened than those described above. These cells are surrounded by a short lamellipodial border of flattened

cytoplasm which extends from the region of cell-membrane contact. Excepting areas of cell-cell overlap, this lamellipodial margin appears closely apposed to the surface of the vitelline membrane and extends into long filopodial processes which lie across the membrane surface. Bleb-like processes and occasional microvilli are observed on the rounded cell bodies.

If explants are viewed from the lateral, unattached, margin, a number of thin thread-like processes are observed on the vitelline membrane underlying the proximal explant (fig. 44). At higher magnifications, these fibres are seen to be similar to those previously described on the vitelline membrane surrounding the epiblast wound after 5 (Section E) and 10 (Section F) hours reincubation. Some fibres extend from short lamellipodial processes of attached cells at the lateral explant margins. Others fibres begin and end on the surface of the vitelline membrane. Fibres are not attached to the flattened cells at the distal explant edge, i.e. the original epiblast 'edge cells'.

Individual fibres may vary in thickness and, at high magnifications, some processes are seen to consist of several fine fibrils. These fibrils are approximately the same diameter as a filopodial process, but extend for considerably longer distances across the membrane surface. Fibrils usually become associated to form fibres on the vitelline membrane adjacent to the lamellipodial margin. The fibrils comprising a given fibre appear to lie parallel or are wrapped around each other in a helical arrangement.

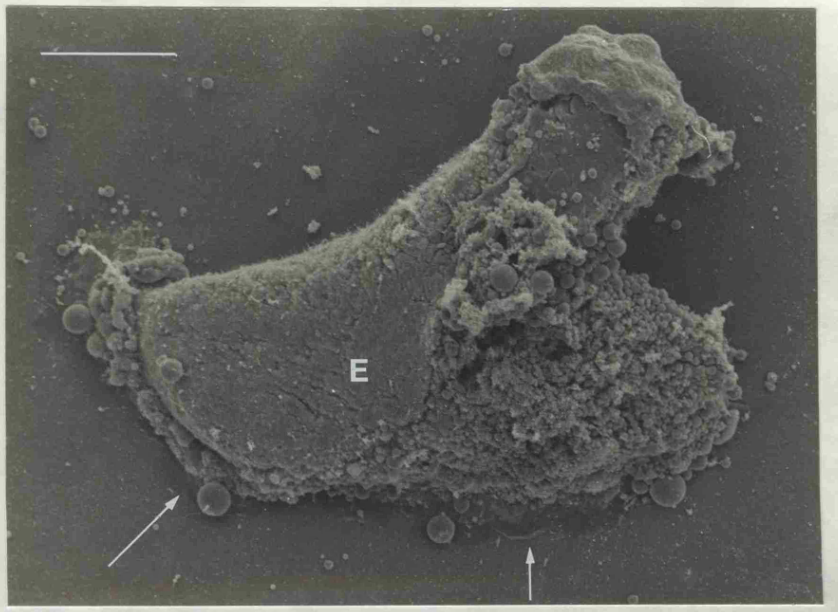
In general, fibres are oriented at right-angles to the attached explant margin. Fibres vary in length and the longest may extend almost half the length of the explant. Over short distances, the fibres appear irregular and often broken. Over longer distances, and at low magnifications, they appear relatively straight. Adjacent fibres lie parallel, or may overlap each other at one or more points of contact.

Small spherical bodies, yolk globules and patches of cell debris are associated with the fibres and adjacent vitelline membrane (fig. 45).

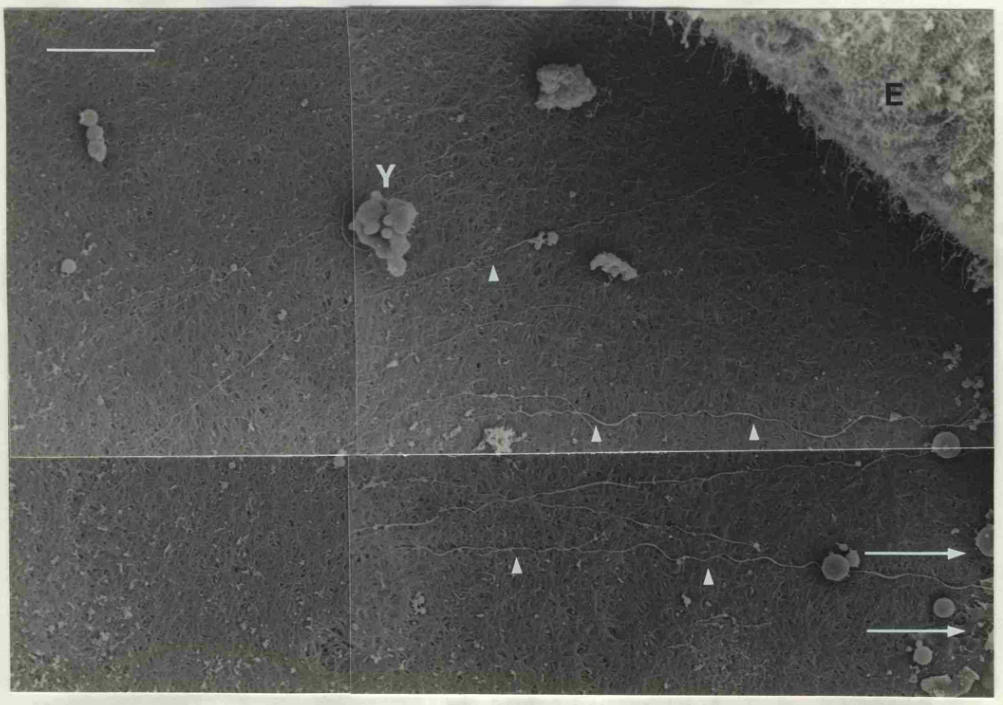
The proximal explant lies above the surface of the vitelline membrane and appears several cells thick. Those cells exposed at the ventral explant surface are rounded with bleb-like processes, microvilli and large numbers of filopodia (fig. 46). Adjacent cells are closely apposed and, at low magnifications, the explant surface has a 'cobble-stoned' appearance.

Cells at the unattached explant margins are overlain by a loose meshwork of fine fibrils, resembling that described on the cells at the proximal margin of the attached epiblast periphery and the cells at the epiblast wound margin. A similar, though attenuated, meshwork is also observed on the cells at the ventral explant surface. As previously detailed, adjacent fibrils appear to lie parallel, or are wrapped around each other, to form fibres of variable thickness and length. Fibres, and constituent fibrils, are usually attached to the cell surface at one or both ends. The remaining process lies above the cell and may extend over the surfaces of neighbouring cells. Small spherical bodies are observed on the fibrils, fibres and underlying cell surface.

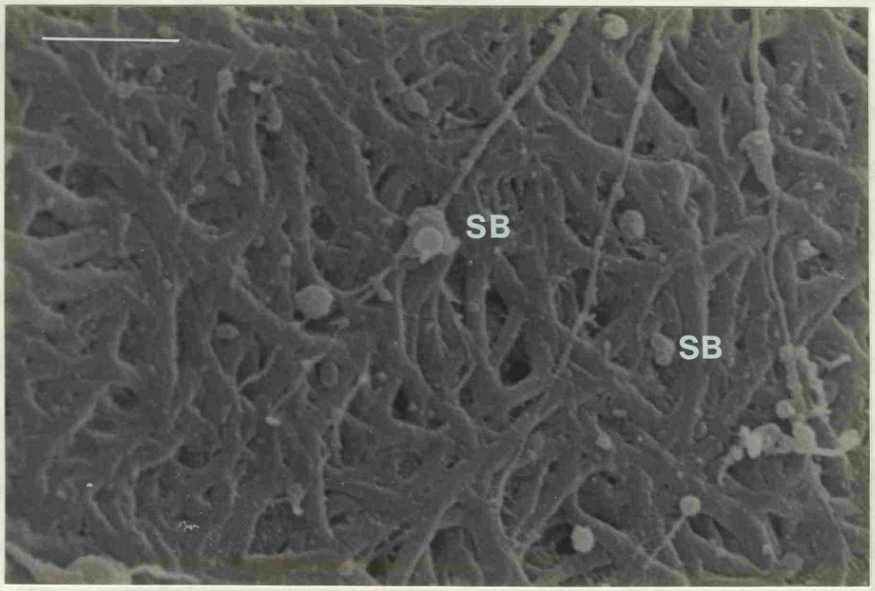
In many explants, the unattached margins curl toward the surface of the vitelline membrane. The fibrils, fibres and filopodia on the unattached explant edge contact the membrane surface next to the attached cells at the distal margin.



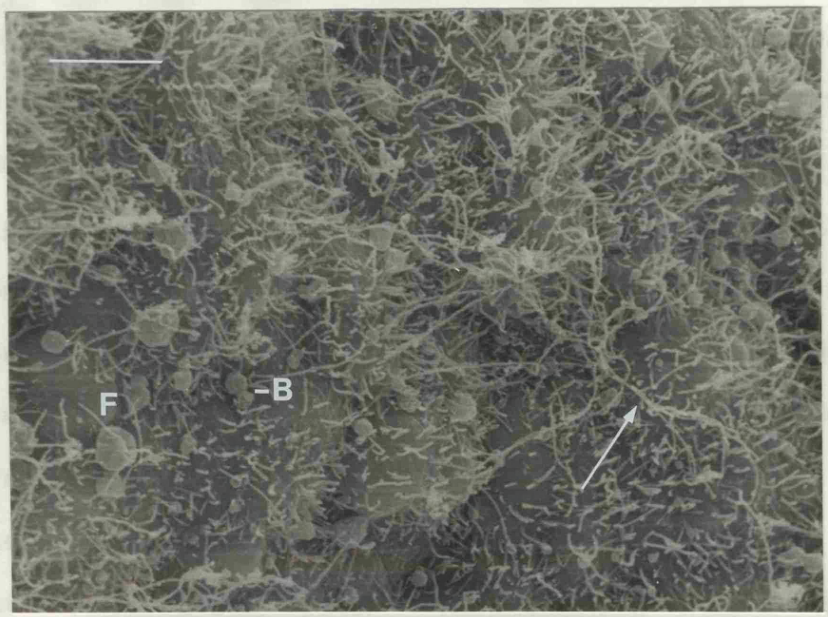
(Fig. 43). Stage 4-5 epiblast periphery explant after reincubation for 1 hour. Flattened cells (arrows) attach the distal explant edge to the inner surface of the vitelline membrane.
E - Explant.
Bar = 100µm.



(Fig. 44). Fibres (arrowheads) are closely apposed to the inner surface of the vitelline membrane (V) beneath the explant (E) and lie at right-angles to the attached explant margin (arrows).
Y - Yolk.
Bar = 20µm.



(Fig. 45). Small spherical bodies (SB) are associated with the fibres and vitelline membrane.
Bar = 2µm.



(Fig. 46). The cells at the unattached explant margins are rounded with blebs (B), microvilli and filopodia (F). Fibrils - often associated to form fibres (arrows) - are observed on the exposed cell surfaces.
Bar = 5µm.

J. Explants ('Edge Cells' and Proximal Epiblast) after Reincubation for 2 Hours.

1. Scanning Electron Microscopy.

After reincubation for 2 hours at 37.5 °C, the attached explants generally appear less rectangular than those described after reincubation for 1 hour. In many, the attachment of the lateral margins appears to have continued in a proximal direction and the explant periphery is bordered by a semi-circular row of flattened cells (fig. 47). The cells of the remaining explant edge are rounded and lie on, or slightly above, the inner surface of the vegetal pole vitelline membrane.

The cells at the centre of the attached explant periphery, i.e. the original distal margin, appear flattened in comparison with those described after reincubation for 1 hour. Each of the attached cells has a single broad lamellipodium which lies at right-angles to the explant edge and extends into one or more short filopodia. Much of the remaining lamellipodial margin is situated above the membrane surface as 'ruffle-like' processes. Small blebs, microvilli and occasional filopodia are observed on the lamellipodial surfaces and flattened cell bodies. Neighbouring cells are closely apposed and adjacent lamellipodia and cell bodies overlap to form a continuous row of attached cells resembling the 'edge cells' of the normal epiblast periphery.

The attached epiblast cells at the lateral explant edges, i.e. the previously unattached margins, appear less flattened than those described above. Those at the proximal extremity of the attached explant periphery possess a number of long filopodia which extend from the cell body or from several short lamellipodia. These processes are oriented radial to the cell body and appear closely apposed to the membrane

surface.

Cells situated further toward the distal explant margin have one or two large lamellipodial processes fringed by short filopodia. The adjacent cell bodies appear rounded and are partially overlain by the flattened cells of the remaining explant. Blebs of varying size, microvilli and occasional filopodia are observed on the exposed cell surfaces.

In some of the larger explants, the rounded cells of the remaining explant periphery also lie on the surface of the vitelline membrane. Unlike those described above at the distal and lateral margins, these cells possess few processes at the region of cell-membrane contact. Small flap-like lamellipodia are occasionally observed at the cell perimeter but, excepting blebs and microvilli, many of the cells appear almost spherical. The unattached cells of the explant edge generally resemble those described after reincubation for 1 hour.

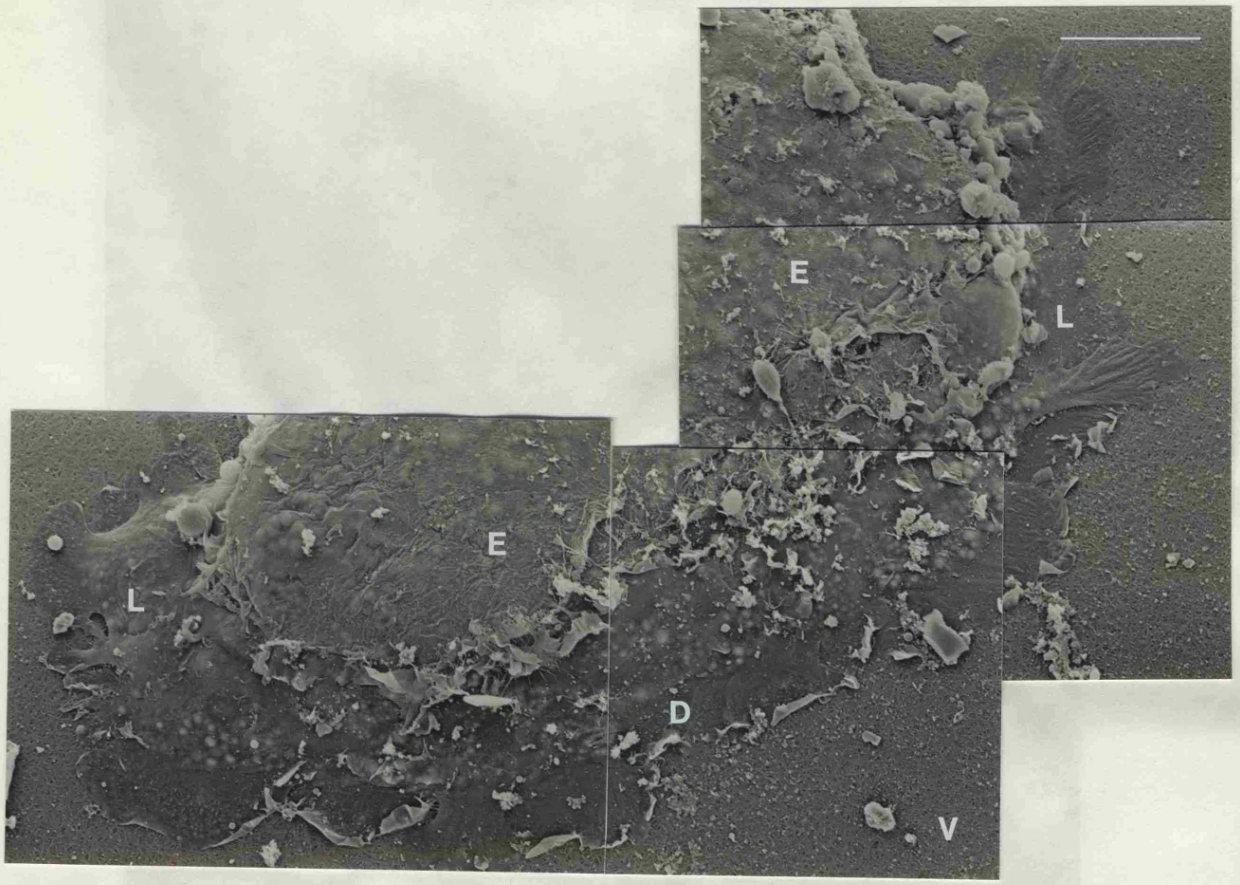
Long thread-like fibrils and fibre-like processes extend from attached epiblast cells at the lateral and proximal explant margins (fig. 48). These processes resemble those observed after reincubation for 1 hour, but are longer and more numerous than those previously described. Individual fibrils are often attached to the rounded cell bodies and flap-like lamellipodia of epiblast cells and become associated to form fibre-like processes on the adjacent membrane surface. Many fibrils may extend from one attached cell and some are longer than the main body of the explant. The fibres lie predominantly parallel to the lateral explant margin, i.e. at right-angles to the flattened cells of the distal edge, and appear closely apposed to the surface of the vitelline membrane (fig. 49). Adjacent fibres lie approximately parallel, although neighbouring processes occasionally overlap.

Individual fibres consist of several thin fibrils covered in an

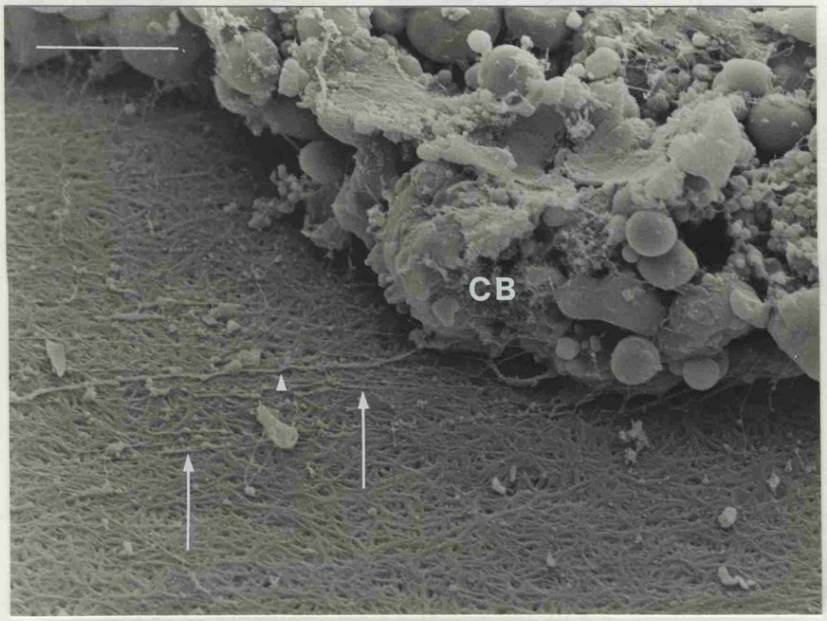
amorphous matrix material. Small spherical bodies and isolated fine fibrils are associated with the fibres and underlying vitelline membrane (fig. 50).

A meshwork of fine fibrils, often associated to form fibre-like processes, is also present on both the attached and unattached epiblast cells at the lateral and proximal explant edge. Small spherical bodies are observed on the fibrillar processes and exposed cell surfaces. A similar material has been described on the unattached explant edge after reincubation for 1 hour. After 2 hours reincubation, the material appears to be present in larger amounts, especially toward the proximal explant margin.

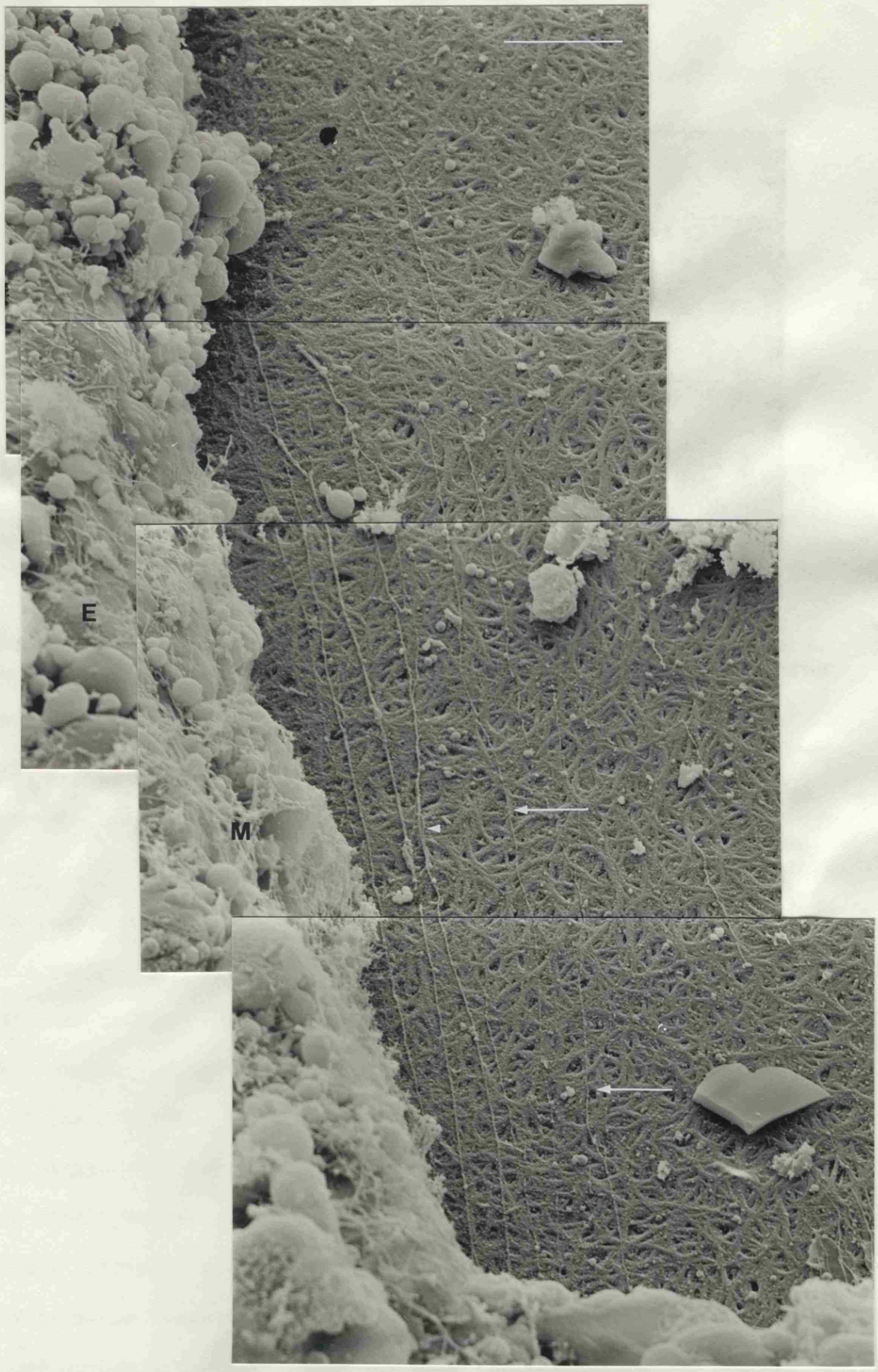
The cells at the ventral explant surface are overlain by a thin layer of amorphous matrix material. The underlying cell surfaces and cell boundaries are indistinct, although bleb-like processes are occasionally observed through the membranous structure. This smooth material often covers the entire explant, with the exception of the attached and unattached cells at the explant edge. Yolk globules and patches of cell debris occur on the exposed explant surface.



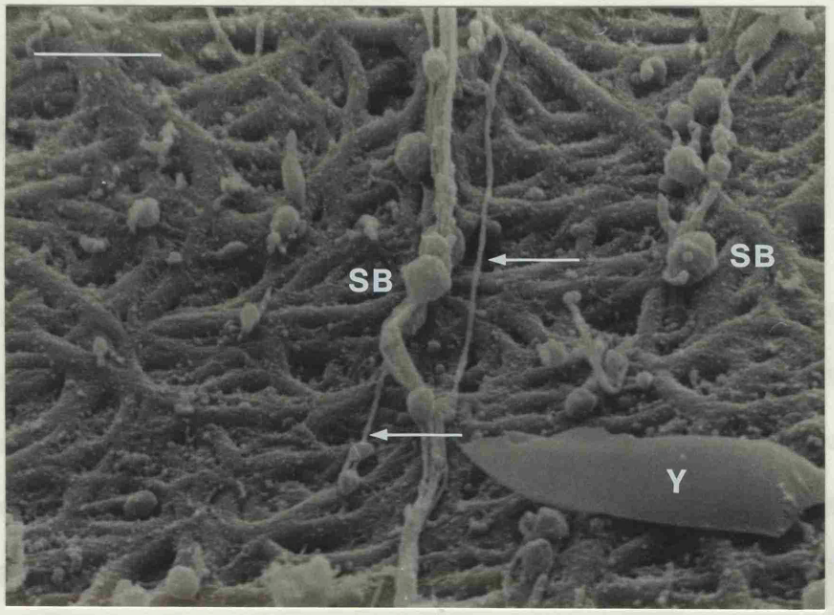
(Fig. 47). The stage 4-5 explant (E) after reincubation for 2 hours. Flattened cells at the distal (D) and lateral (L) explant margins are attached to the inner surface of the vitelline membrane (V).
Bar = 50µm.



(Fig. 48). Fibrils (arrows) extend from the cell bodies (CB) of attached epiblast cells at the lateral explant margins. These fibrils often become associated to form fibre-like processes (arrowheads) on the adjacent vitelline membrane.
Bar = 10µm.



(Fig. 49). Fibrils (arrows) and fibres (arrowheads) lie on the vitelline membrane parallel to the lateral explant margin. A fibrillar meshwork (M) is observed on the rounded epiblast cells at the explant edge.
E - Explant.
Bar = 10µm.



(Fig. 50). Each fibre consists of several fine fibrils (arrows). Small spherical bodies (SB) are observed on the fibres and adjacent vitelline membrane.
Y - Yolk.
Bar = 2µm.

K. Explants ('Edge Cells' and Proximal Epiblast) after Reincubation for 3 Hours.

1. Scanning Electron Microscopy.

After 3 hours at 37.5 ^{o}C, many of the explants on the inner surface of the vegetal pole vitelline membrane appear similar to those described after reincubation for 2 hours. The attached explants are approximately rectangular in shape and possess a continuous row of flattened cells at the distal explant edge and adjacent lateral margins. The rounded cells of the remaining explant periphery lie on or slightly above the surface of the vitelline membrane.

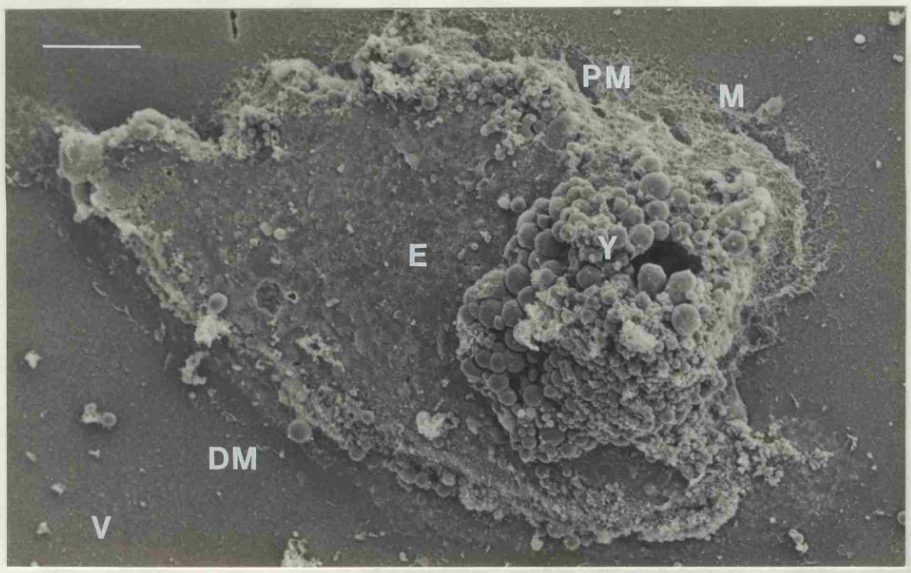
A number of long thread-like processes are attached to many of the epiblast cells in contact with the membrane surface. These processes lie at right-angles to the flattened cells of the distal explant margin and are often longer than the fibrils and fibres described after reincubation for 2 hours.

In some explants, especially larger pieces of tissue, the entire explant periphery appears closely apposed to the inner surface of the vitelline membrane (fig. 51). The flattened cells at the distal and lateral explant margins are as previously described, but the rounded cells of the remaining periphery are coated with a dense fibrillar matrix. This material consists of numerous fine fibrils, often aligned to form fibre-like processes, and a number of small spherical bodies. Individual processes appear similar to those previously described at the rear of attached 'edge cells' and on the surface of epiblast cells at the wound margin and explant edge. In many explants, this fibrous mat is sufficiently dense to completely obscure the underlying marginal cells and may extend across the surrounding membrane surface. The adjacent

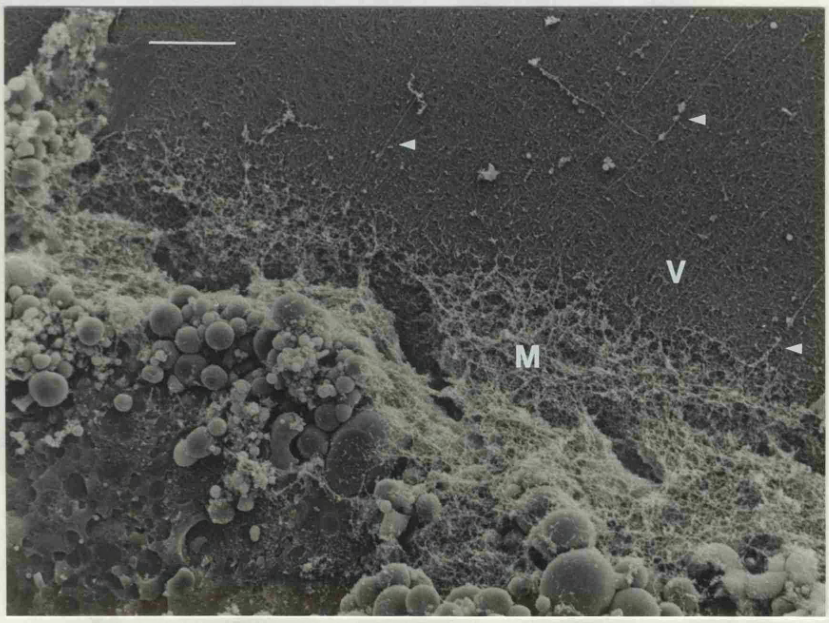
explant surface is covered with a thin sheet of smooth membranous material and resembles that described after reincubation for 2 hours.

Individual fibrils and fibre-like processes also extend from the meshwork at the explant edge and lie for considerable distances across the membrane surface (fig. 52). These processes are oriented at right-angles to the flattened cells at the distal margin and are commonly 3-5 times the length of the main explant body. Similar, though in most cases shorter, processes have previously been described on the vitelline membrane surrounding the closing epiblast wound, and the epiblast explant after reincubation for 1 and 2 hours. The longitudinal processes, fibrils and fibres, are often continuous with the fibrillar meshwork overlying the attached marginal cells. The fibrils comprising a given fibre usually separate and are intermingled with the fibrils of the matrix. The processes on the membrane surface are relatively straight and often lie in parallel rows (fig. 53).

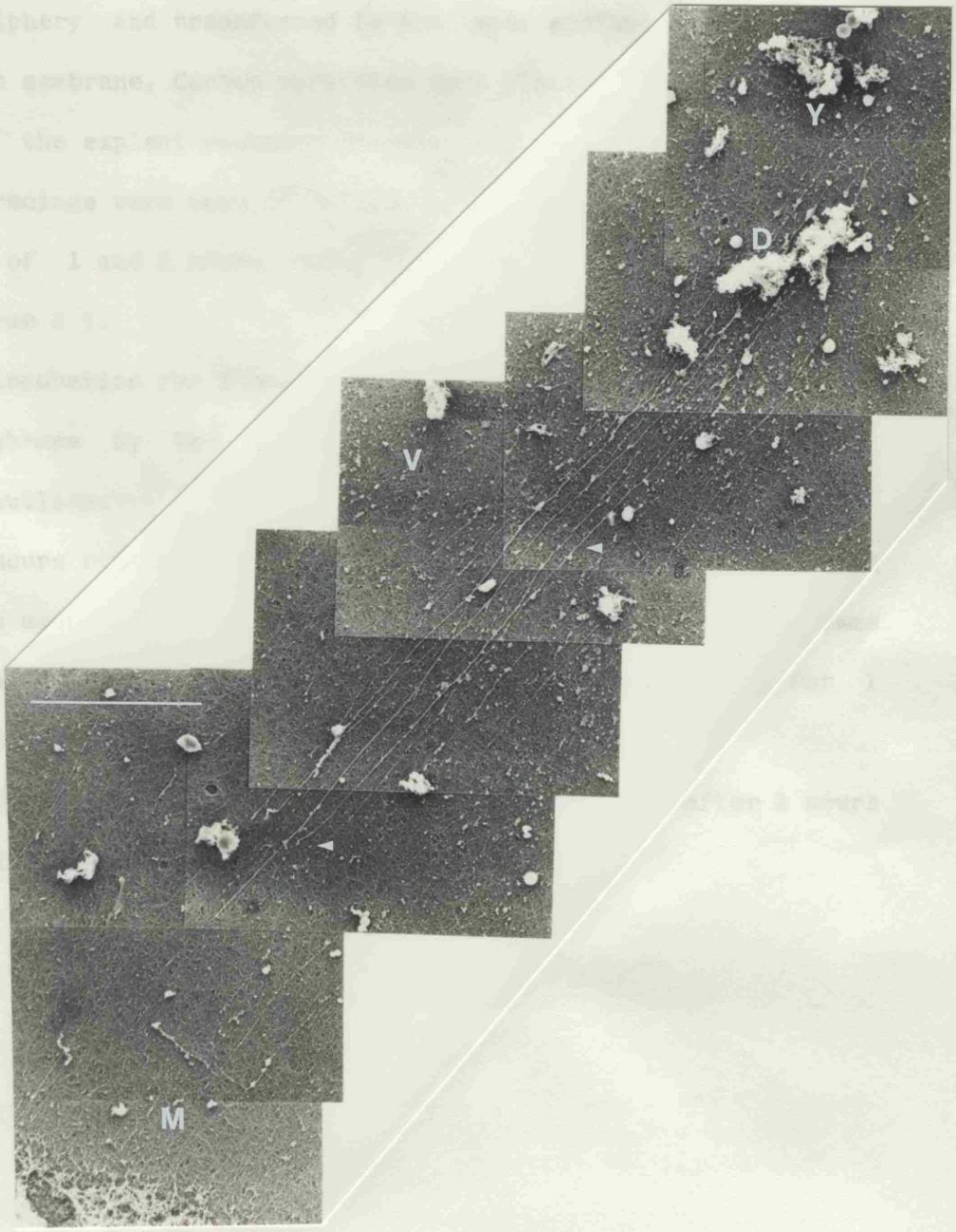
Individual processes may vary in thickness and appear closely apposed to the underlying vitelline membrane. Small spherical bodies are observed along the length of a given process and on the surrounding membrane surface. The other ends of the fibrils and fibres, i.e. those distant to the explant edge, are commonly associated with a region of cellular debris and yolk globules. Cell debris, yolk globules and patches of fibrillar material are also occur on and around the fibres themselves. Isolated flattened cells are occasionally observed under, or next to, the fibrils and fibres on the membrane surface.



(Fig. 51). The stage 4-5 explant (E) after reincubation for 3 hours. The distal margin (DM) is attached to the vitelline membrane (V) by flattened cells. The proximal margin (PM) is coated with a fibrillar meshwork (M).
Y - Yolk.
Bar = 50µm.



(Fig. 52). Long fibrils and fibre-like processes (arrowheads) extend from the fibrillar meshwork (M) at the proximal explant periphery.
V - Vitelline membrane.
Bar = 20µm.



(Fig. 53). The fibrillar processes (arrowheads) on the vitelline membrane (V) are relatively straight and lie in parallel rows. Cell debris (D) and yolk globules (Y) are associated with the fibre ends distant to the meshwork (M) at the explant margin.
Bar = 50µm.

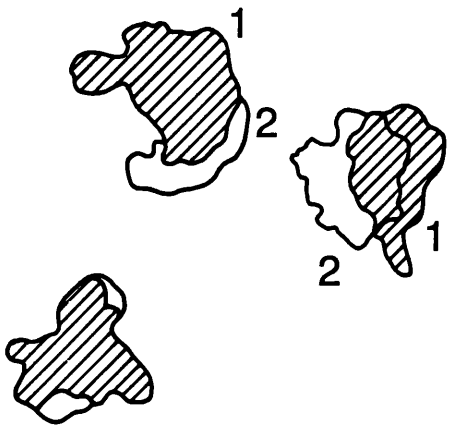
L. Light Microscopy of Explant Movement (Proximal Epiblast Only).

Explants containing proximal epiblast only were excised from the epiblast periphery and transferred to the inner surface of the vegetal pole vitelline membrane. Carbon particles were placed adjacent to the explants and the explant-membrane preparations reincubated for 2 hours at 37.5 ^{o}C. Tracings were made of individual explants after reincubation for periods of 1 and 2 hours, using the carbon particles as reference points (diagram 6).

After reincubation for 1 hour, the explants appear attached to the vitelline membrane by the entire explant margin. The explants are irregular in outline.

After 2 hours reincubation, a few explants appear to have moved a short distance across the membrane surface. The majority are in the same position, relative to the carbon particles, as after reincubation for 1 hour. Large-scale explant translocations are not observed.

The morphology of the proximal epiblast explants after 2 hours reincubation is described in more detail in Section M.



(Diagram 6). Tracings of explants of the epiblast periphery (proximal epiblast only) after reincubation for 1 and 2 hours. The position of the explants after 1 hour is illustrated by the hatched outlines.

M. Explants of the Epiblast Periphery (Proximal Epiblast Only) after Reincubation for 2 Hours.

1. Scanning Electron Microscopy.

After reincubation for 2 hours at 37.5 ^{o}C, most explants containing only proximal epiblast are attached to the inner surface of the vegetal pole vitelline membrane. The explants are irregular in shape and the entire explant margin appears closely apposed to the membrane surface. The main body of the explant is surrounded by single cells and small groups of cells which are also attached to the membrane surface (fig. 54). The numbers of these isolated cells decreases with distance from the explant periphery.

Some of the isolated cells are rounded and a few appear almost spherical. Long filopodial processes extend from the region of cell-membrane contact and lie radial to the cell perimeter. These processes may be attached to the cell body or to one or more short lamellipodia at the base of the cell. Individual processes are relatively straight and appear closely apposed to the membrane surface. Blebs of varying size, microvilli and occasional short filopodia are observed on the rounded cell bodies.

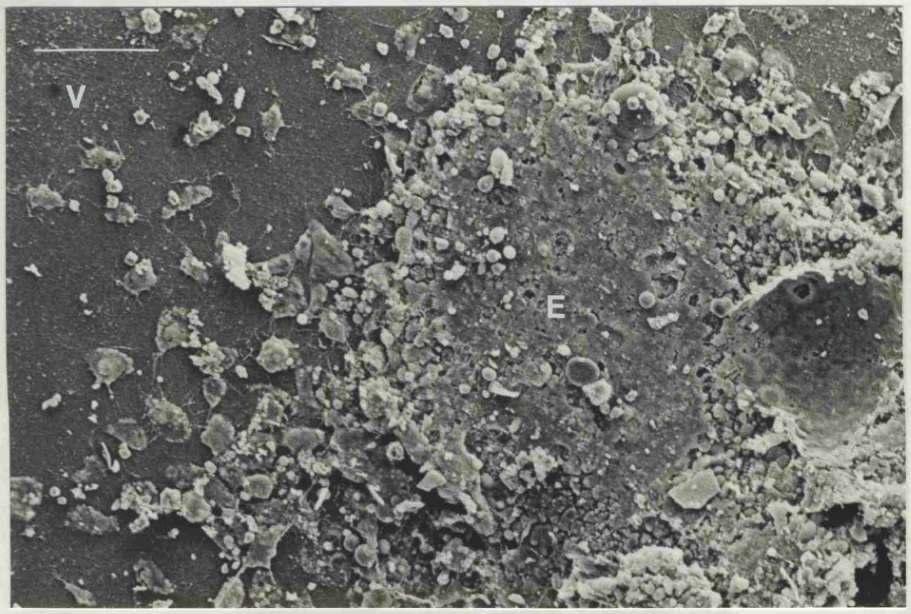
Other single cells in the vicinity of the explant margin are less rounded than those described above (fig. 55). In general, the further from the explant edge, the flatter the isolated cells. Each flattened cell has a single large lamellipodium which extends into several short filopodia. The majority of the remaining lamellipodial margin lies above the surface of the vitelline membrane as 'ruffle-like' processes. Some cells possess a smaller flap-like lamellipodium at the opposite side of the cell to the main cytoplasmic extension. This shorter process is

usually attached to a number of long and branched filopodia which lie above the membrane surface for much of their length. The cell body is flattened with large hemispherical processes at the exposed ventral surface. Small blebs, microvilli and occasional short filopodia are also observed on the lamellipodial surfaces and cell bodies.

Adjacent epiblast cells often overlap, forming small groups of connected cells on the vitelline membrane next to the main body of the explant. The region of cell-cell contact is generally small and, in most cases, restricted to the lamellipodial margins. Connected cells, and those situated a short distance apart, are commonly linked by long filopodial processes attached to the cell bodies or lamellipodial margins. These processes may be straight or branched and are also observed on the surrounding vitelline membrane. Some of the thinner filopodia resemble the fibrils previously described at the epiblast wound margin and epiblast explant periphery.

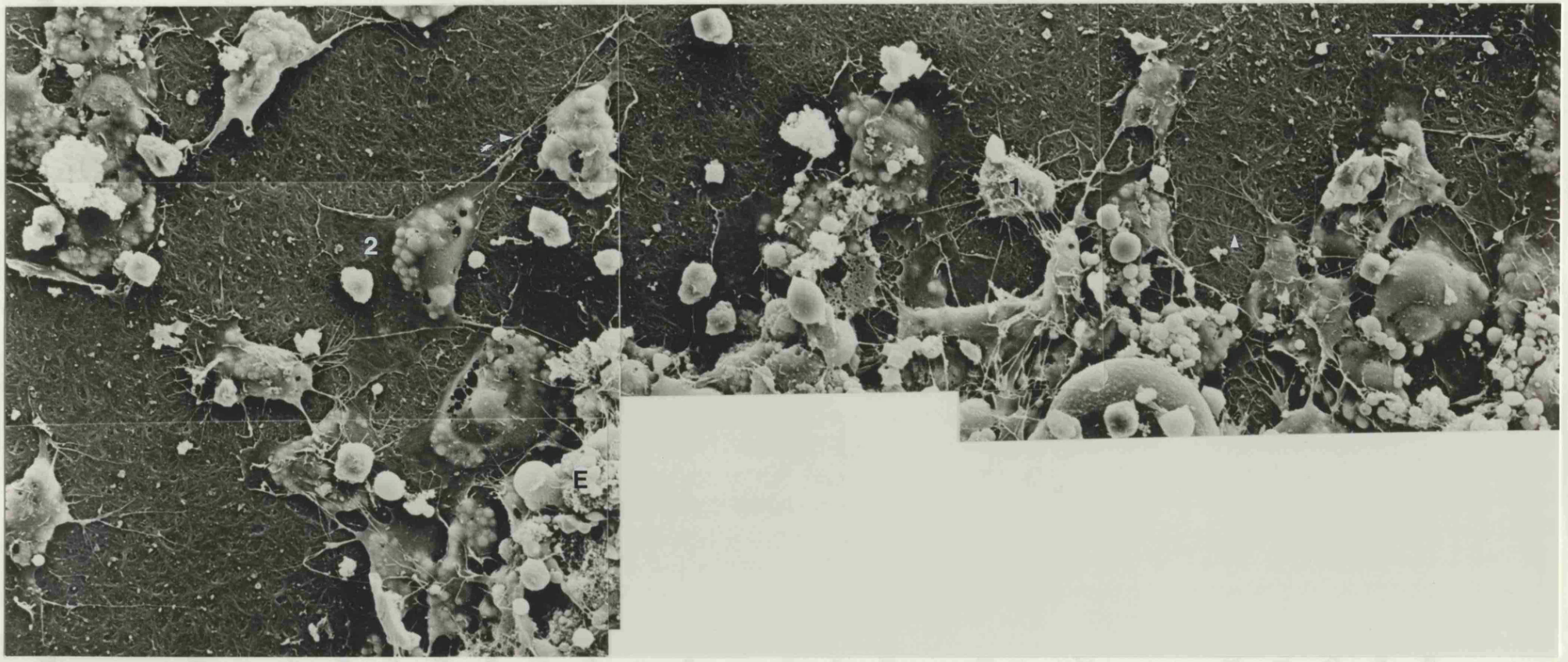
The centre of the explant appears several cells thick. Those at the ventral surface are often coated with a thin sheet of membranous material similar to that described on epiblast periphery explants. The explant margin generally consists of a single layer of rounded and flattened cells. Adjacent cells are loosely apposed and large hemispherical processes, blebs and microvilli are observed on the exposed cell surfaces. Small patches of a fibrillar meshwork and thin filopodial processes may connect neighbouring cells. At high magnifications, this fibrillar material resembles that previously observed, often in larger quantities, on the cells at epiblast wound margin and epiblast periphery explant.

Cell debris, damaged cells and yolk globules are common in the vicinity of the attached explant.



(Fig. 54). Stage 4-5 epiblast explant (no 'edge cells') after reincubation for 2 hours. The attached explant (E) is surrounded by single cells and isolated groups of cells.
V - Vitelline membrane.
Bar = 10μm.

(Fig. 55). Some cells adjacent to the explant edge (E) are rounded (1). Others appear extensively flattened in the plane of the vitelline membrane (2). Neighbouring cells are often connected by thin thread-like processes (arrowheads).
V - Vitelline membrane.
Bar = 20µm.



N. Isolated Cells of the Epiblast Periphery ('Edge Cells and Proximal Epiblast) after Reincubation for 1 Hour.

1. Scanning Electron Microscopy.

After reincubation for 1 hour at 37.5 °C, many of the cells from the original suspension are attached to the inner surface of the vegetal pole vitelline membrane.

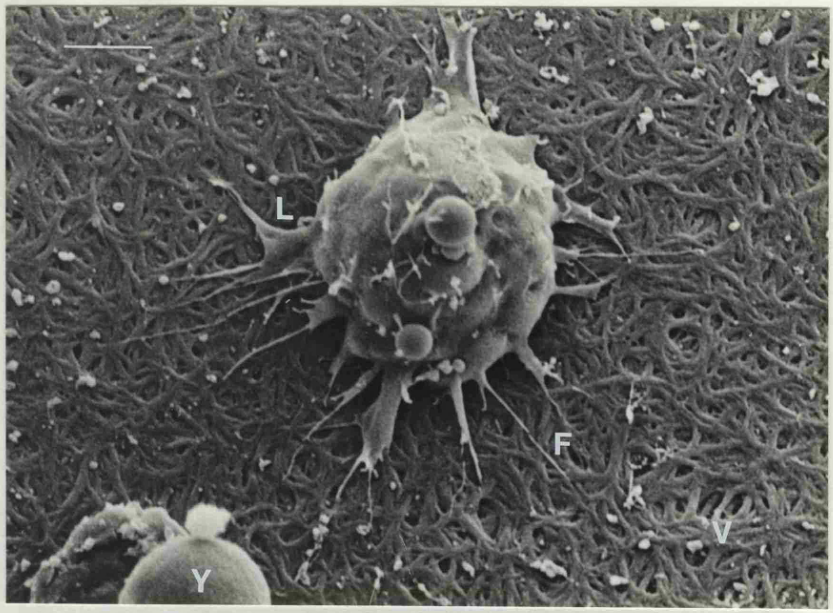
Some of the attached cells are rounded and a few appear almost spherical (fig. 56). The exposed cell surfaces are uneven with bleb-like processes of varying size, microvilli and short filopodial processes. Longer filopodia extend from the region of cell-membrane contact or from short lamellipodia at the cell periphery. These processes appear closely apposed to the membrane surface and lie radial to the main cell body. Small spherical swellings are frequently observed at the tips of the filopodia. Individual processes are often branched and may vary in thickness.

Other cells appear less rounded than those described above (fig. 57). Small bleb-like processes, microvilli and occasional filopodia are observed on the cell bodies. Lamellipodial processes border the attached cell periphery and often extend into one or more short filopodia. In general, both lamellipodial and filopodial processes appear closely apposed to the inner surface of the vitelline membrane.

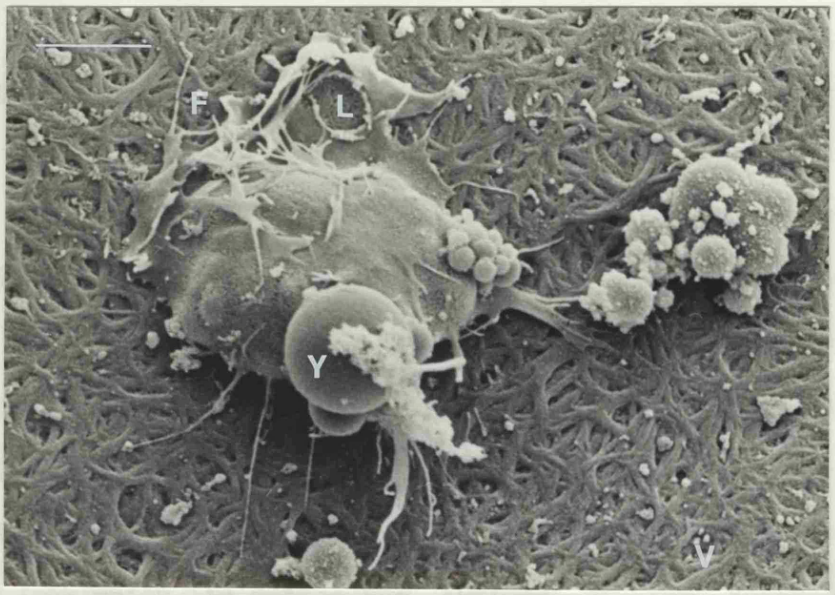
A third group of cells is attached to the inner surface of the vitelline membrane by a single large lamellipodium (fig. 58). The cell body is flattened in the plane of the vitelline membrane. Occasional small bleb-like processes, microvilli and filopodia are observed on an otherwise smooth cell surface. The lamellipodium is broad, flat and extends from one side of the cell. The majority of the lamellipodial margin lies above the surface of the vitelline membrane as 'ruffle-like'

processes. Individual filopodia are occasionally observed at the lamellipodial margin. Opposite to the attached lamellipodium, the cell periphery extends into a number of branched filopodial processes, of varying thichness, which make contact with the vitelline membrane some distance from the cell body. These processes may be attached to the cell body or to short flap-like lamellipodia. Unlike the filopodia of the rounded cells, these largely unattached processes do not possess terminal swellings.

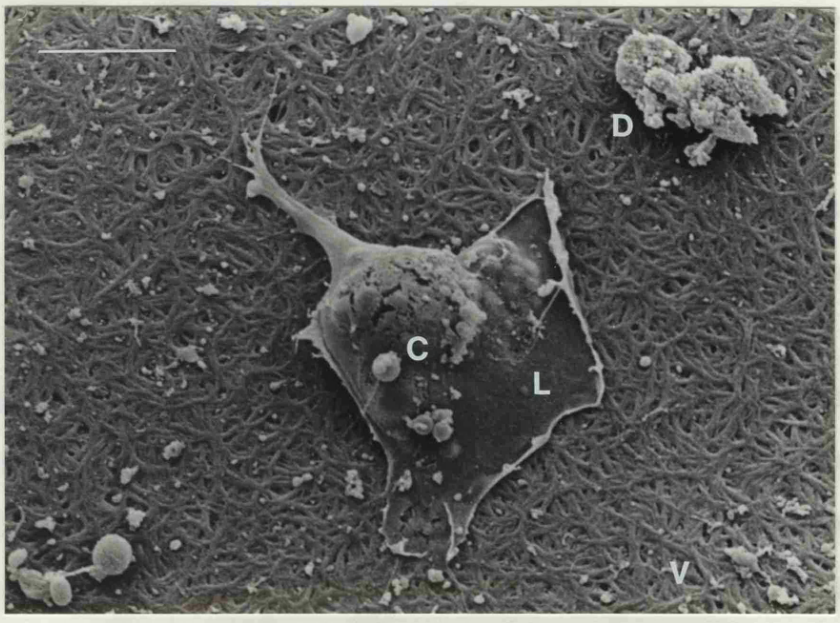
Small yolk globules are often associated with the exposed cell surfaces and adjacent vitelline membrane. Patches of cell debris are also observed on the membrane adjacent to the attached cells.



(Fig. 56). Stage 4-5 epiblast cell after reincubation for 1 hour on the inner surface of the vitelline membrane (V). The rounded cell is fringed by filopodia processes (F) and short lamellipodia (L).
Y - Yolk.
Bar = 5µm.



(Fig. 57). Partially flattened cell with filopodial processes (F) extending from short lamellipodia (L).
V - Vitelline membrane, Y - Yolk.
Bar = 5µm.



(Fig. 58). Flattened cell with a single large lamellipodium (L).
C - Cell body, D - Debris, V - Vitelline membrane,
Bar = 10µm.

0. Isolated Cells ('Edge Cells' and Proximal Epiblast) after Reincubation for 3 Hours.

1. Scanning Electron Microscopy.

After 3 hours reincubation at a temperature of 37.5 ^{o}C, the number of epiblast cells attached to the inner surface of the vitelline membrane is larger than after reincubation for 1 hour. The attached cells appear similar in morphology to those previously described, although a larger proportion are flattened in the plane of the membrane surface.

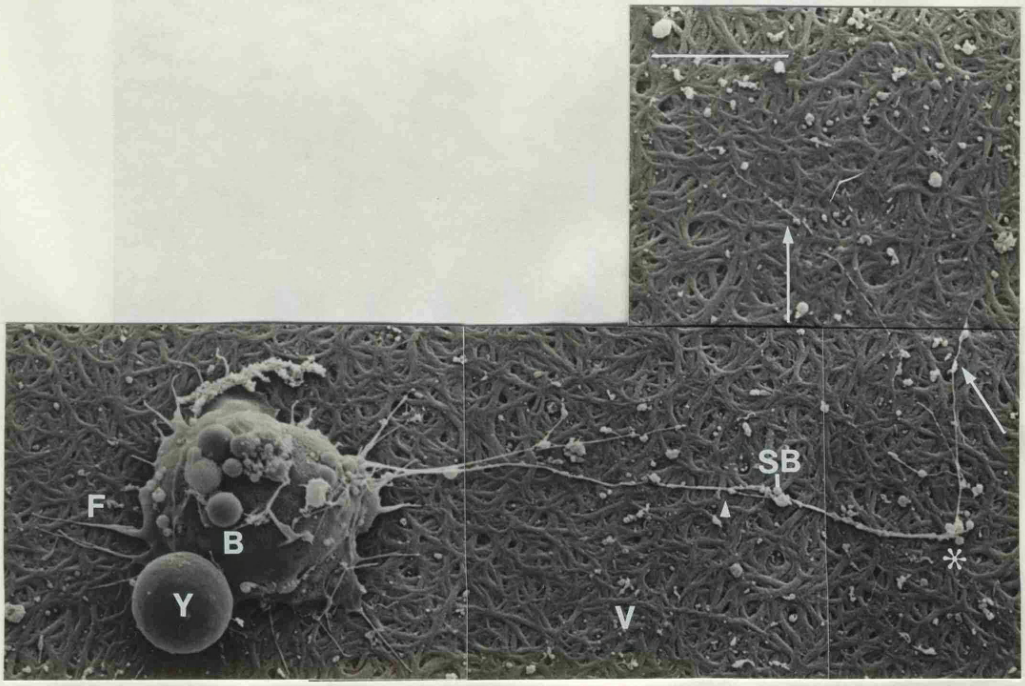
Many of the attached epiblast cells possess long fibrils and fibre-like processes which lie across the surface of the surrounding vitelline membrane (fig. 59a). Individual cells may have one or more of the processes attached to the cell body or to short flap-like lamellipodia at the cell perimeter. Unlike the fibrils and fibres observed at the closing epiblast wound and attached epiblast explant, these processes are rarely straight and appear to possess no preferred orientation. Many are bent or curled through angles of 90^{o} or more and gaps are common along the length of a given process. Blebs of varying size, microvilli and occasional short filopodia are observed on the exposed lamellipodial surfaces and cell bodies.

At high magnifications, individual fibre-like processes are seen to consist of several fine fibrils associated with an amorphous matrix material. Small spheres of a similar material are also observed on the fibrils (fig. 59b) and on the surface of the cell bodies and surrounding vitelline membrane. Fibrils and fibres vary both in thickness and in length, although the diameter of any given fibre is determined to a large extent by the number of fibrils involved. In some cases, the fibrils separate a short distance from the cell and lie at various angles to the original fibre-like process. Most fibrils and

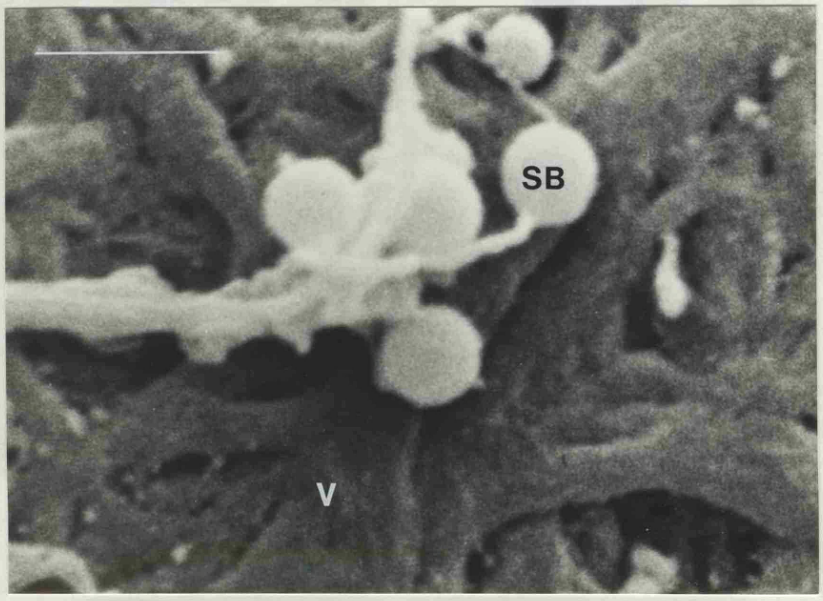
fibres appear closely apposed to the surface of the vitelline membrane and may thicken slightly toward their point of contact with the cell surface.

A number of flattened epiblast cells lie in the vicinity of the fibrils and fibres on the inner surface of the vitelline membrane (fig. 60). Those close to a fibril are usually oriented toward the process, i.e. the long axis of the cell lies at right-angles to the line of the fibril. Some cells possess a large lamellipodium which is oriented toward an adjacent fibril and appears closely apposed to the membrane surface. The lamellipodial margins are fringed by filopodia, many of which have small spherical swellings at their distal tips. Some of these filopodia may contact the fibril (fig. 61), and those that do lie parallel to the thread-like process.

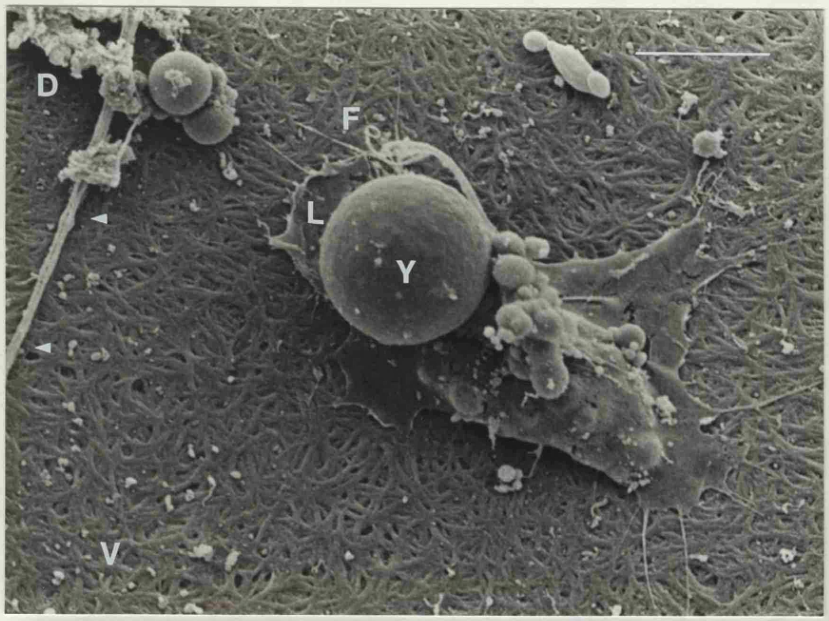
Other epiblast cells lie between the fibrillar processes and the surface of the vitelline membrane (fig. 62). Several such cells may underlie some of the longer processes on the membrane surface. The fibrils or fibres appear closely apposed to the underlying cell body or lamellipodium, and the cell itself is extensively flattened in the membrane plane. Some of these flattened cells are elongated parallel to the overlying process and resemble those observed next to the fibrils (fig. 63). Others cells are approximately circular and appear to lack to a preferred orientation. No epiblast cells overlie any of the fibrils or fibre-like processes attached to the inner surface of the vitelline membrane.



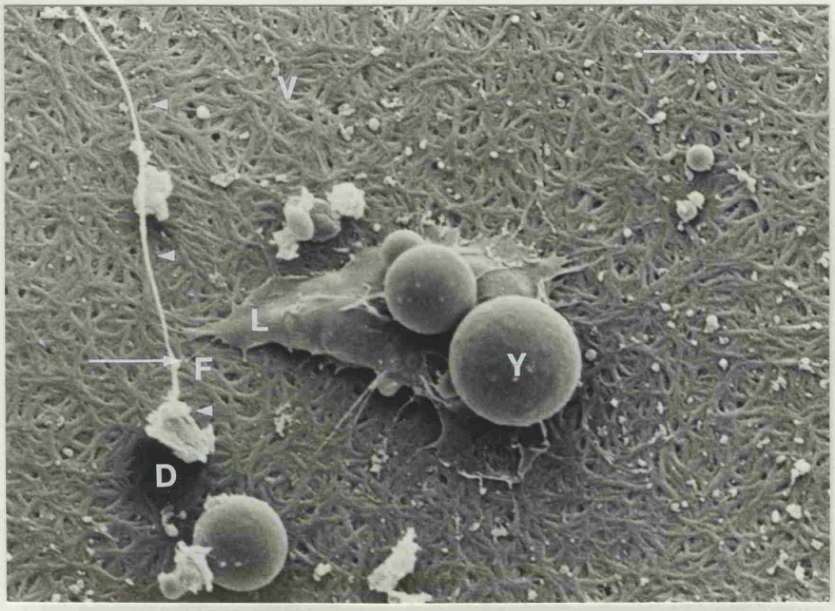
(Fig. 59a). Stage 4-5 epiblast cell after reincubation for 1 hour on the inner surface of the vitelline membrane (V). The cell is attached to a long fibre-like process (arrowheads) which extends into several thin fibrils (arrows).
B - Bleb, F - Filopodia, SB - Spherical Body, Y - Yolk.
Bar = 10µm.



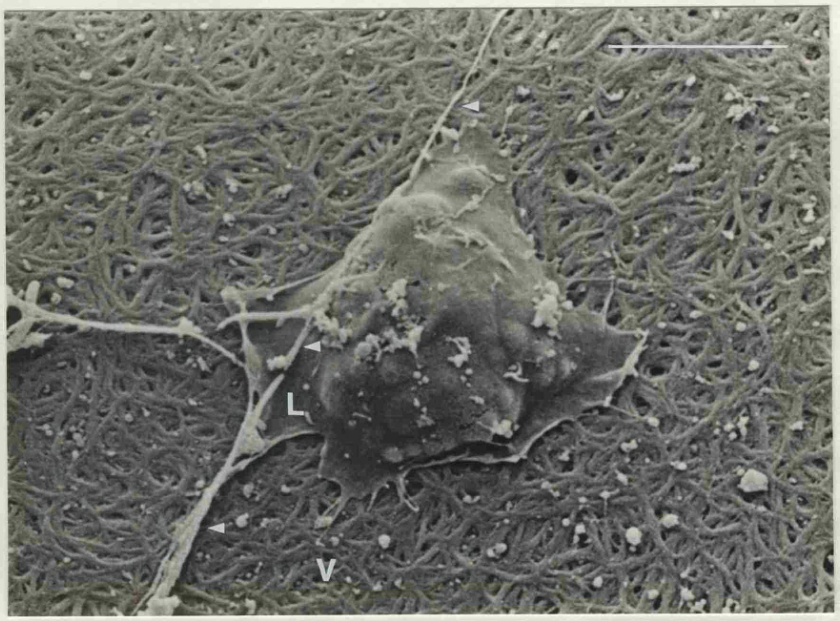
(Fig. 59b). Detail of a fibre and attached spherical bodies (SB) - * in fig. 59a.
V - Vitelline membrane.
Bar = 1µm.



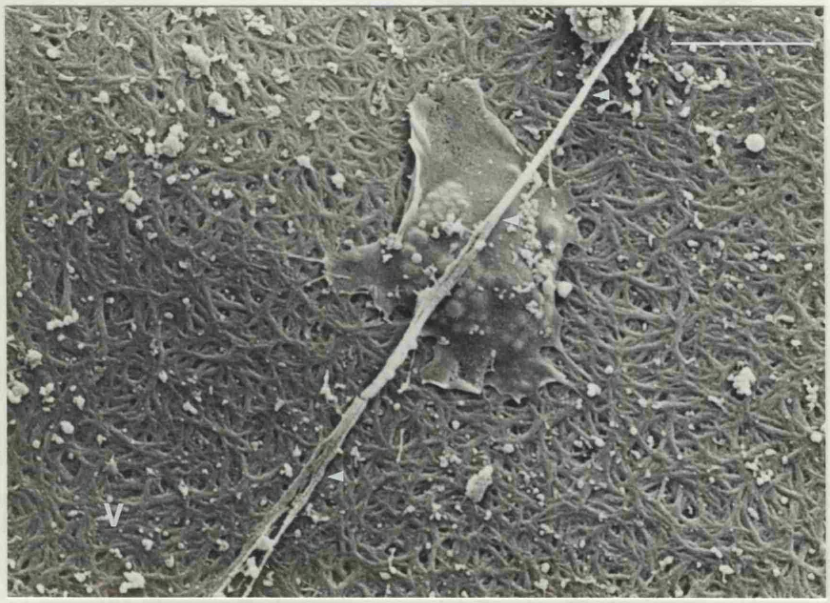
(Fig. 60). An epiblast cell on the vitelline membrane (V) close to a fibre-like process (arrowheads). A lamellipodium (L) with filopodia (F) is oriented toward the fibre.
D - Debris, Y - Yolk.
Bar = 10µm.



(Fig. 61). Epiblast cells may contact nearby fibres (arrowheads) through filopodial processes (F) attached to short lamellipodia (L) at the cell perimeter. Many such filopodia have small spherical swellings at their tips (arrows).
D - Debris, V - Vitelline membrane, Y - Yolk.
Bar = 10µm.



(Fig. 62). An epiblast cell aligned with a fibre-like process (arrowheads). The fibre lies parallel to the long axis of the cell and overlies a lamellipodium (L) attached to the inner surface of the vitelline membrane (V).
Bar = 10µm.



(Fig. 63). A flattened epiblast cell positioned between a fibre (arrowheads) and the vitelline membrane (V). The fibre appears closely apposed to the surface of the underlying cell.
Bar = 10µm.

IV. DISCUSSION.

A. The Normal Epiblast Periphery.

The stage 4-5 extra-embryonic epiblast periphery has been examined by S.E.M., the light microscopy of thick sections and T.E.M.

The vitelline membrane observed in the present study appears similar to that described by previous authors (Bellairs *et al*, 1963; Bellairs *et al*, 1969; Jensen, 1969; Chernoff and Overton, 1977). The inner membrane surface, the substratum for 'edge cell' migration, consists of a three-dimensional meshwork of fibres flattened in the membrane plane. By S.E.M. and T.E.M, a substructure of fine fibrils was observed both between and within the larger fibres. Jensen (1969) has proposed that the presence of these fibrils may represent a progressive weakening of the membrane structure as epiblast expansion continues, prior to rupture after coverage of the yolk mass.

The stage 4-5 extra-embryonic epiblast consists of an epithelial sheet one cell thick attached to the inner surface of the vitelline membrane by a peripheral association of 'edge cells'.

Since New (1959) first proposed that these 'edge cells' were responsible for epiblast expansion, the morphology of this peripheral region has been the subject of a number of investigations (Bellairs, 1963; Bellairs *et al*, 1969; Downie and Pegrum, 1971; Chernoff and Overton, 1977, 1979; Andries and Vakaet, 1985ab; Andries *et al*, 1985a). At least three different models of 'edge cell' structure and organisation have resulted from these studies.

In a T.E.M. examination of epiblast 'edge cells' through stages 3-12, Bellairs (1963) reported that the 'edge cell' bodies were indistinguishable from those of the adjacent proximal epiblast. The special feature of attached 'edge cells' was a long distal process (or

processes) extending for up to 500um across the vitelline membrane. By S.E.M. (Bellairs et al, 1969), these thin processes were observed as a flattened cytoplasmic sheet at the epiblast periphery.

Downie and Pegrum (1971), in a later T.E.M. study, found no trace of the long processes described by Bellairs (1963). These authors reported a narrow multilayered association of flattened 'edge cells' at the stage 4-5 epiblast periphery. Basal 'edge cells' were attached to the underlying vitelline membrane through distally oriented lamellipodia 40-60um long.

A third model was proposed by Chernoff and Overton (1977, 1979). In S.E.M. investigations of the stage 7-9 epiblast periphery, these authors observed rows of radially oriented filopodial processes on attached 'edge cells'. The filopodia averaged 20um in length and were connected to the 'edge cell' body or to short lamellipodial processes at the cell perimeter. Similar filopodia were also present at the perimeter of isolated 'edge cells' cultured on the vitelline membrane or in vitro (Chernoff and Overton, 1979).

In general, the results obtained in this study support the observations of Downie and Pegrum (1971). The stage 4-5 peripheral epiblast appears as a single layer of flattened cells, and this epithelial sheet is attached to the vitelline membrane by a peripheral association of multilayered 'edge cells'. Basal 'edge cells' possess broad lamellipodia oriented in the direction of epiblast expansion. These cells are overlain by flattened suprabasal 'edge cells' which appear to play no direct role in the migration of the attached edge.

The 500um processes reported by Bellairs (1963) were not observed in this study, either by S.E.M., T.E.M. or in thick sections of the stage 4-5 epiblast periphery. However, it was noted that the distally oriented 'edge cell' lamellipodia form a continuous border at the epithelial edge. It is possible that the elongated structures described

by Bellairs may represent tangential sections through a number of adjacent lamellipodia.

The long filopodia recorded by Chernoff and Overton (1977, 1979) were absent from stage 4-5 'edge cells' examined after fixation at 20 °C. However, 'edge cell' morphologies resembling those described by these authors were obtained following the use of Karnovsky's at 5 °C. The influence of fixation temperature on the morphology of the stage 4-5 epiblast periphery is discussed at greater length in Section B.

The basal 'edge cells' observed in this study possess many structures characteristic of actively migrating epithelial cells. The broad distal lamellipodia, oriented in the direction of cell movement, are processes characteristic of both fibroblasts (Abercrombie et al, 1977; Abercrombie, 1982) and epithelial cells (Dispasquale, 1975ab; Garrod and Steinberg, 1975; Middleton,1977, 1982; Brown and Middleton, 1985) migrating across planar substrata in vitro.

Isolated epithelial cells often adopt an unpolarized morphology in culture, with lamellipodial cytoplasm encircling the entire cell perimeter (Brown and Middleton, 1985). In basal 'edge cells', the distal orientation of the lamellipodia may be due to the microtubules observed within the lamellipodial cytoplasm both in this study and previously by Downie and Pegrum (1971). These cytoskeletal elements restrict the formation of lamellipodia to limited portions of the fibroblast cell margin (Vasiliev and Gelfand, 1977), and are thought to play a similar role in some epithelial cells (DiPasquale, 1975b; Middleton, 1982). The importance of microtubules to 'edge cell' morphology is discussed in Section B.

The extensive lateral contacts made by basal 'edge cells' may also act to restrict lamellipodial formation to the distal cell margin (DiPasquale, 1975b; Radice, 1980a). Downie and Pegrum (1971) have reported that distal 'edge cells' lack specialized junctions, although

desmosomes are common between proximal 'edge cells'. Other authors have detected tight junctions, gap junctions and desmosomes throughout the attached epiblast edge (Andries and Vakaet, 1985a; Andries et al, 1985a). In the present investigation, discrete junctional structures have been observed in T.E.M. sections of basal 'edge cells' just behind the advancing distal margin. These local accumulations of densely stained material at the apposed plasma membranes probably represent incipient cell-cell junctions (Dembitzer et al, 1980; Andries et al, 1985a). Tight junctions and desmosomes are known to play a role in intercellular cohesion (Staehelin, 1974; Stolinski et al, 1981). These structures may coordinate the movements of basal 'edge cells' and assist in distributing the tensions produced by epiblast expansion.

In T.E.M. sections of some basal 'edge cells', short finger-like processes were observed on the underside of the distally oriented lamellipodia. These protusions extend for short distances between the fibres of the membrane surface and are often associated with fibrous attachment plaques similar to those found at the margins of migrating fibroblasts and epithelial cells (Abercrombie et al, 1971; Heath and Dunn, 1978; Heath, 1982). Other authors have described similar structures beneath 'edge cell' lamellipodia and have suggested that these cell-substratum adhesions play an important role in enabling the migrating cells to grip the underlying membrane (Bellairs, 1963; Downie and Pegrum, 1971; Andries et al, 1985a)

The most proximal basal 'edge cells', i.e. those next to the unattached epiblast, often possess additional short lamellipodia oriented away from the advancing epithelial edge. Other authors have reported similar processes in T.E.M. sections of the epiblast margin (Downie and Pegrum, 1971) and S.E.M. micrographs of 'edge cells' detached from the vitelline membrane (Andries and Vakaet, 1985b). Downie and Pegrum (1971) have suggested that these extra cell

processes result from the presence of available substratum on both sides of the cell body. In contrast, Andries and Vakaet (1985b) have proposed that the proximal edge is a trailing edge, and that the processes observed correspond to those which occur at the rear of epithelial cells migrating in vitro (Albrecht-Buehler, 1979).

In this investigation, the proximal 'edge cells' have been studied in situ after removal of the overlying epiblast. The short lamellipodia observed in section were not observed on all proximal 'edge cells' and, when present, were often associated with thread-like 'retraction processes' (Chen, 1981; Trinkaus and Erickson, 1983). Those 'edge cells' without lamellipodia or retraction processes were rounded and, in some cases, appeared to lie slightly above the vitelline membrane. These observations support the hypothesis of Andries and Vakaet (1985b), that the proximal margin of the attached epiblast edge represents a region of 'edge cell' detachment from the membrane surface.

B. The Epiblast Periphery after Cold Fixation.

In this study, the stage 4-5 epiblast periphery after cold fixation differs from that observed after fixation at room temperature. The cold-fixed 'edge cells' are rounded to varying degrees and the broad lamellipodia observed after fixation at 20 ^{o}C are largely absent. Some portions of the epiblast periphery lie above the membrane surface and are consistent with a partial retraction of the cell sheet. The cells of the unattached proximal epiblast are also rounded, although neighbouring cells remain closely apposed.

Some of the rounded 'edge cells' remain attached to the vitelline membrane through thread-like retraction fibres (Chen, 1981; Trinkaus and Erickson, 1983). These processes may connect the cell body to the lamellipodial attachment plaques (Abercrombie et al, 1971; Revel and Wolken, 1973; Heath, 1982) observed both in this study and in an earlier T.E.M. investigation by Downie and Pegrum (1971). Similar retraction fibres have been described after epiblast withdrawals due to dehydration (Bellairs et al, 1969) and cytochalasin B, a drug which affects cytoplasmic microfilaments (Chernoff and Overton, 1979).

Isolated fibroblasts and epithelial cells are known to round up when incubated at low temperatures on in vitro substrata (Revel et al, 1974; Lonchampt et al, 1976). Revel et al (1974) have proposed that this cell rounding could result from the disruption of cytoplasmic microtubules. Several authors have reported that low temperatures can cause the partial disaggregation of microtubules (Tilney and Porter, 1967; Weber et al, 1975; Brinkley et al, 1975) and these cytoskeletal elements are thought to influence cell shape and locomotion in a variety of cell types (Wells and Mallucci, 1978; Solomon, 1980; Middleton, 1982; Maro and Pickering, 1984). It is possible that in this study, cold fixation causes the 'edge cells' to round up by disrupting

cytoskeletal microtubules.

Microtubules have been observed within 'edge cells' in this and other studies (Downie and Pegrum, 1971) and microtubule inhibitors (MTIs) are known to prevent epiblast expansion and produce a retraction of the cell sheet similar to that observed after cold fixation (Downie, 1975; Mareel et al, 1984).

Two conflicting hypotheses have been put forward to account for the role of microtubules in epiblast expansion. Downie (1975) reported that 'edge cells' exposed to the MTI colchicine could continue to migrate after excision of the remaining epiblast, and proposed that epiblast retraction was due to a loss of microtubules from the cells of the proximal epithelium. These cells flatten as the epiblast expands (Downie, 1976) and, if this flattening is even partially dependant on microtubules, their loss through cold or MTIs would result in a retraction of the cell sheet.

In contrast, Mareel et al (1984) found that the MTIs nocodazole and taxol could prevent 'edge cell' migration even after removal of the proximal epithelium. On the basis of this observation, Mareel et al (1984) proposed that intact cytoplasmic microtubules are necessary for 'edge cell' locomotion per se.

The results obtained in this study support the view of Mareel et al (1984) that microtubules are necessary for normal 'edge cell' form and function. The rounding of proximal epiblast cells after cold fixation is more likely to be a result, rather than the cause, of 'edge cells' detaching from the surface of the vitelline membrane. Several authors have reported that the migration of the attached 'edge cells' is responsible for epiblast tension, and that lifting the attached edge from the vitelline membrane causes a retraction of the epithelial margin and cell rounding (New, 1959; Bellairs et al, 1967; Downie, 1976; Andries and Vakaet, 1985b). A similar cell rounding has been observed

in the present investigation at the margin of the newly formed epiblast wound.

The stage 4-5 epiblast periphery after cold fixation in this study resembles that described as normal at stages 7-9 by Chernoff and Overton (1977). These same authors also reported that incubation at low temperatures produced no alteration in the morphology of the attached epiblast periphery, although dissociated 'edge cells' showed some filopodial branching and an increase in the number of processes on exposed surfaces (Chernoff and Overton, 1979).

In the present study, epiblasts have been incubated in New culture (New, 1955) to stages 7-9 and, although ruffles become more prominent at the lamellipodial margin, the epiblast periphery resembles that described at stages 4-5. The similarity between the morphology observed as normal by Chernoff and Overton (1977, 1979), and that described in this study after cold fixation, suggests that the epiblast margin they studied may have already undergone some form of epiblast retraction.

C. The Closure of Wounds at the Epiblast Periphery.

1. Cell Reactions to the Trauma of Wounding.

The reactions of cells in the epiblast epithelium to a discontinuity in the cell sheet are similar to those observed in a number of embryonic and adult epithelia.

In the present study, the excision of a portion of attached 'edge cells' and adjacent proximal epiblast produces a rectangular wound at the stage 4-5 epiblast periphery. With reincubation, the wound becomes semi-circular and gapes as the outward migration of the remaining 'edge cells' increases the tension within the epithelial sheet (New, 1959; Bellairs et al, 1967; Downie, 1976). This delay between wounding and the widening of the wound margins suggests that epiblast tension, which rises to a peak at stages 4-5 (Downie, 1976), is reduced during New culture and only restored after reincubation and continued 'edge cell' movement.

The initial gaping of the wound, prior to the inward movement of the wound margins, is a response characteristic of epithelia under tension. Many embryonic epithelia are under tensile stress during early morphogenesis and Stanisstreet et al (1980) have reported the gaping of freshly made ectodermal wounds in stage 3-5 chick embryos and Xenopus neurulae. A similar response has also been observed in the stage 3-5 chick endoderm (England and Cowper, 1977) and lateral mesencephalon of the rat embryo (Smedley and Stanisstreet, 1984).

Wound enlargement is rarely observed in flaccid epithelia, such as the extra-embryonic membranes of the rat embryo (Smedley and Stanisstreet, 1984). Radice (1980a) also observed little gaping of epidermal wounds in the tailfin of Xenopus larvae, and attributed this to a lack of tension caused by the strong hemidesmosomal contacts

between the epidermal cells and their underlying basement membrane.

The epiblast cells at the newly formed wound margin are rounded and may curl out of the plane of the epithelial sheet. In the normal stage 4-5 epiblast, these cells are extensively flattened in the plane of the epithelium (Bellair, 1963; Downie, 1976) and their rounding may be attributed to a localized release of tension at the wound edge. Bellairs et al (1967) have described a similar cell rounding by light microscopy after the release of normal epiblast tension and, in the present study, 'edge cell' detachment due to cold fixation has been seen to cause cell rounding throughout the proximal epiblast. The speed with which the epiblast cells round up, most 0-hour wounds were fixed within 30 seconds of 'edge cell' excision, is further evidence to support the view that this change in cell shape represents a largely passive reaction to a rapid local reduction in epithelial tensions.

McMinn (1976) has described the cells at an epidermal wound margin as 'hypertrophied' and the rounded epiblast cells at the wound edge often appear slightly larger than those of the remaining epithelium. This swelling may be related to cell rounding, or may represent a general cellular reaction to stressful conditions. Several authors have noted a certain degree of cell enlargement under conditions of osmotic or toxic strain (Carter et al, 1976; Harding and Morris, 1976; Litke and Low, 1977).

The thin basement membrane which normally overlies the stage 4-5 proximal epiblast (Bellairs, 1963) appears to be absent at the wound margin. Blebs occur on the newly exposed ventral cell surfaces and microvilli, which are prevalent on the dorsal epithelium (Chernoff and Overton, 1977), are also frequently observed. Several authors have reported that epithelial cells produce protusions, including blebs, after treatment with enzymes known to digest basement membranes (Silver and Wakely, 1974; Wakely, 1977). At the epiblast wound margin, both

these processes may represent a reaction to the loss of overlying extracellular materials, or may act as reserves of excess membrane after cell rounding (Trinkaus and Erickson, 1974; Knutton et al, 1976). Brown and Middleton (1985) have noted that poorly spread epithelial cells bleb vigorously on in vitro substrata, and it may be that this represents a similar reaction to the a loss of a flattened cell shape.

Rounded and enlarged cells appear to be a general characteristic of newly formed wound margins in a range of epithelia (Croft and Tarin, 1970; England and Cowper, 1977; Repesh and Oberpriller, 1980; Stanisstreet et al, 1980). Radice (1980a) has suggested that, for some marginal cells, these changes may represent a prelude to cell death and eventual extrusion from the cell sheet. Some epiblast cells are extensively damaged during wound formation and, in the present study, dead cells and cell debris are frequently observed at the newly formed wound margin. These remnants remain connected to the epithelium by a dorsal tight junction and residual desmosomes.

Other epiblast cells are traumatized to a lesser extent by the wounding procedure and die after a period of reincubation. As in other epithelial wounds, these dying cells are irregular in shape and possess dark pycnotic nuclei, a decreased or loosened cytoplasmic mass and show a general reduction in internal structural detail (Croft and Tarin, 1970; Repesh and Oberpriller, 1978, 1980; Radice, 1980a).

2. The Attachment of the Epiblast Wound Margins.

The closure of wounds at the epiblast periphery begins as early as one hour after 'edge cell' excision, as the epiblast cells at the distal wound margins and the displaced 'edge cells' either side of the wound become attached to the surface of the vitelline membrane. The attached epiblast cells then migrate in a lateral direction, at right-angles to

the normal movement of the epiblast periphery, until the wound is closed at around 10 hours reincubation.

The epiblast wound margin becomes attached to the inner surface of the vitelline membrane in a distal-proximal progression. As a result, cells at all stages of the attachment process may be observed in linear sequence along a single fixed wound margin. This allows the observer to determine the order of the events involved in cell attachment but not their timing, as there in no evidence to suggest attachment proceeds at the same rate along the entire wound edge.

The first epiblast cells to become attached to the vitelline membrane are those at the distal wound extremity. These cells come close to the membrane surface as the adjacent 'edge cells' continue their outward migration. Thereafter, attachment proceeds proximally as successive epiblast cells are brought into contact with the membrane through the spreading of their distal neighbours.

Initial cell contact with the vitelline membrane probably occurs through one or more of the microvilli on the dorsal surface of the epiblast. Microvilli have been described on the normal dorsal surface of the epiblast in this study and by previous authors (Bellairs, 1963; Chernoff and Overton, 1977). Those on the rounded epiblast cells of the wound margin are more prevalent and appear slightly longer. Newly attached epiblast cells possess long filopodia which extend radially from the region of cell-membrane contact. Similar processes have also been observed at the perimeter of fibroblasts attaching to planar substrata *in vitro* (Witkowski and Brighton, 1971, 1972; Rajaraman *et al*, 1974; Heaysman *et al*, 1982). On the epiblast cells, these processes may represent elongated microvilli (Witkowski and Brighton, 1971), or be processes created *de novo* by the assembly of new membrane material and/or the withdrawal of cell surface microvilli (Trinkaus and Erickson, 1974).

In the early stages, cell attachment is brought about by physical forces acting on a largely passive cell body. In vitro, the fibroblast cell surface and glass substratum have been shown to have net negative charges and hence repulse each other (King et al, 1979). Pethica (1961), on physiochemical bases, predicted that this repulsion could be overcome by cellular probes with dimensions similar to those of microvilli and filopodia. At the epiblast wound margin, these processes may play a similar role in establishing the first focal contacts (Heaysman et al, 1982) and may also have an exploratory function in cell-membrane interactions (Albrecht-Buehler, 1976). Similar filopodia have been observed on endodermal cells attaching to the ectodermal basement membrane during wound closure in the stage 3-5 chick area pellucida (England and Cowper, 1977).

Epiblast cells at the wound margin which have been in contact with the membrane surface for some time possess lamellipodia oriented at right-angles to the wound margin. From observations of cells at different regions along an attaching wound margin, it appears that these lamellipodia are formed by cytoplasmic 'webbing' between the peripheral filopodia. A similar cell flattening has been described for fibroblasts attaching in vitro (Witkowski and Brighton, 1971, 1972; Rajaraman et al, 1974).

Epithelial cells undergoing contact-induced spreading in culture develop lamellipodia from that part of the cell not contacted by other cells (Middleton, 1977, 1982). A similar form of contact inhibition (Heaysman, 1978) could be responsible for orienting cell movement at the epiblast wound margins. The restriction of lamellipodia to the edges of migrating epithelia has been recorded in a wide variety of in vitro cultures (Vaughan and Trinkaus, 1966; DiPasquale, 1975a; Abro and Lingaas, 1977; Miettinen et al, 1978). Similarly, the appearance of lamellipodia from newly exposed cell margins at spontaneously or

artificially created gaps in the cell sheet has been well documented (Vaughan and Trinkaus, 1966; Voon, 1980).

The morphologies of the attaching cells at the wound edge are similar to those observed in this investigation at the lateral explant margins and in isolated cells of the epiblast periphery. Downie and Pegrum (1971) reported that fragments of the proximal epiblast would attach to the vitelline membrane but that, unlike the normal epiblast periphery, all the cells of the explant were in contact with the membrane surface. A similar result has been obtained with explants of the stage 4-5 proximal epiblast in this study.

In the present investigation, the flattened cells of the normal stage 4-5 proximal epiblast are seen to be connected by a dorsal tight junction and several lateral desmosomes. These junctions have been observed by other authors (Bellairs, 1963; Andries et al, 1985a) and such structures are thought to play an important role in the maintenance of intercellular cohesion (Staehelin, 1974; Stolinski et al, 1981). Both tight junctions and desmosomes are retained at the newly formed wound edge but, during the attachment of the epiblast margin, these structures are disassembled. Under the T.E.M., no definite junctional structures were detected between the attached epiblast cells at the wound margin.

Desmosomes are often internalised by rounding cells, preparatory to cell attachment and locomotion (Overton, 1968; Kartenbeck, 1982). At the epiblast wound margin, the loss of cell-cell junctions may enable the epiblast cells to undergo the extensive alterations in cell shape required during attachment and the onset of cell locomotion. In addition, as these structures play a role in maintaining cell position within the cell sheet, the absence of tight junctions and desmosomes may permit the multilayering observed at the attaching wound margin.

The fate of desmosomes during wound closure in other epithelial

systems is far from clear. Desmosomes have been detected between migratory cells at wound margins (Krawczyk, 1971; Pang et al, 1978), but are generally present in smaller numbers than in non-migratory epithelia (Gibbins, 1968; Croft and Tarin, 1970; McMinn, 1976; Gabbiani et al, 1978; Repesh and Oberpriller, 1980; Ortonne et al, 1981). Beerens et al (1975) have observed the phagocytosis of hemidesmosomes during wound closure in the adult epidermis.

Desmosomes and tight junctions are present between the 'edge cells' at the normal stage 4-5 epiblast periphery (Andries and Vakaet, 1985a; Andries et al, 1985a). This suggests that either the wound margin migrates differently to the epiblast periphery, or that the wound margin aquires these junctional structures some time after cell attachment. Tight junctions require little protein synthesis and can be assembled within 1-15 minutes (Meldolesi et al, 1978; Cohen et al, 1985). Desmosome formation takes 1-2 hours (Dembitzer et al, 1980; Overton and DeSalle, 1980; Hennings and Holbrook, 1983).

For many tissue cells, polarised spreading constitutes the first stage of locomotion (Vasiliev and Gelfand, 1977). The unincubated blastoderm of the chick embryo is not attached to the vitelline membrane and exists as a loose assemblage of rounded cells between the yolk mass and the inner membrane surface (Vakaet, 1962; Downie, 1974). It is interesting to note that the attachment of the presumptive 'edge cells' (Downie, 1974, Chernoff and Overton, 1979), at around 6-10 hours reincubation, is similar in morphology to the spreading of epiblast cells at the wound margin, and that both these events are immediately followed by cell locomotion.

In the next section, the lateral migration of the attached wound margins is discussed in greater detail, and compared with the epibolic expansion of the normal stage 4-5 epiblast periphery.

3. The Migration of the Epiblast Wound Margins.

The lateral movement of the wound margins during wound closure has been described in a variety of embryonic and adult epithelia (Pfister, 1975; Rafferty, 1976; Rafferty and Smith, 1976; Takeuchi, 1976; England and Cowper, 1977; Repesh and Oberpriller, 1978, 1980; Radice, 1980a; Stanisstreet et al, 1980; Smedley and Stanisstreet, 1984; Matsuda et al, 1985).

In general, epithelial wounds close through one or a combination of three basic cell behaviours; alterations in cell shape or position, localized or general cell proliferation within the cell sheet and cell migration across a suitable substratum.

Recent research has suggested that the mechanisms used to close wounds in an epithelium are largely dependant on the organisation and structure of the cell sheet. For example, in the embryonic ectoderm of the chick area pellucida, the Xenopus neurulae and the lateral mesencephalon of the rat, coordinated changes in cell shape are important in effecting wound closure (Stanisstreet et al, 1980; Smedley and Stanisstreet, 1984). Limited mitoses and some minor alterations in cell position are observed in the chick ectoderm, but there is no evidence for active cell migration at the epithelial wound margin. Stanisstreet et al (1980) have proposed that this dependance on cell elongation and proliferation reflects the organisation of the early chick ectoderm. At the stages used (3-5), the chick ectoderm is not underlain by a coherent endodermal basement membrane, or any other substratum suitable for cell locomotion (Sanders, 1979). The ectodermal cells are connected by highly developed junctional complexes (Trelstad et al, 1967) and, in the short-term, these could preclude the cellular independance required for active cell movement. In contrast, endodermal wounds in the stage 3-5 chick area pellucida close

through rapid cell migration across the intact ectodermal basement membrane (England and Cowper, 1977; Mareel and Vakaet, 1977).

The epithelial conditions which determine the mechanism of wound closure are not always as obvious as in the above example. Some apparently similar epithelia may react differently during wound closure in different species. Wounds in the corneal endothelium of the adult rabbit are closed through a combination of proliferation and cell flattening, with limited migration at the wound margin (Van Horn et al, 1976; Matsuda et al, 1985). Wounds in the corneal endothelium of the cat are closed primarily through cell migration (Van Horn et al, 1976). Similarly, an injury to the central portion of the mouse lens evokes a single burst of proliferative activity in the cells immediately surrounding the newly formed wound (Rafferty, 1976; Rafferty and Smith, 1976). In the lens epithelium of the amphibian Rana pipiens, a similar proliferative response follows an initial period of cell migration (Rafferty, 1971).

These results imply that factors other than simple epithelial structure, possibly chemical differences, may influence the mechanisms employed during wound closure. Calcium has been implicated in the promotion of wound closure in amphibian embryos (Stanisstreet, 1982; Stanisstreet and Jumah, 1983; Stanisstreet et al, 1986), but calcium chelators do not inhibit closure in mammalian embryos unless used at concentrations capable of dissociating the embryo (Smedley and Stanisstreet, 1984).

Cell proliferation and cell migration are the processes predominantly responsible for wound closure in the stratified epithelia of the adult organism (Lash, 1955; Croft and Tarin, 1970; Krawczyk, 1971; Beerens et al, 1975; Pang et al, 1978; Dunlap and Donaldson, 1981; review: Wright and Alison, 1984). As previously discussed, a suitable substratum is a prerequisite for cell locomotion and epithelial wound

margins migrate across basement membranes (Krawczyk, 1971; Beerens et al, 1975; Derby, 1978; Pang et al, 1978; Radice, 1980a), including the corneal Descemet's membrane (Van Horn et al, 1976), and fibrin meshworks (Repesh and Oberpriller, 1978, 1980; Yamanaka and Eguchi, 1981).

In this study, the migration of the epiblast wound margin across the vitelline membrane is the process predominantly responsible for wound closure. This membrane is the natural substratum for the movement of epiblast 'edge cells' (New, 1959; Bellairs, 1963; Bellairs et al, 1969; Downie and Pegrum, 1971; Downie, 1976) and the present investigation has shown that cells of the proximal epiblast will attach to the membrane surface.

A diversity of opinion exists regarding the mode of epithelial migration during wound closure (Trinkaus, 1976; Pang et al, 1978; Repesh and Oberpriller, 1980; Wright and Alison, 1984).

Several authors have suggested that the cells in an epithelial sheet, either in vivo or in vitro, maintain their relative positions as the sheet migrates (Vaughan and Trinkaus, 1966; DiPasquale, 1975a; Trinkaus, 1976; Vasiliev and Gelfand, 1977; Stoker et al, 1978). Proximal cells retain their ranks during the movement of the cell sheet and there is little alteration in the arrangement of submarginal cells. It has generally been assumed that this 'gliding' form of locomotion results from the presence of contact specialisations holding the cells together (Trinkaus, 1976; Middleton, 1982). Ultrastructural studies appear to confirm this suggestion since lateral adhesions, including tight junctions and desmosomes, have been described in a wide variety of epithelia (DiPasquale, 1975a; Lonchampt et al, 1976; Stoker et al, 1978; Middleton, 1982).

Lash (1955) first described 'gliding' epithelial wound closure in Urodele epidermis. Similar observations have since been made in other

amphibian (Radice, 1980a; Donaldson and Dunlap, 1981) and mammalian epithelia (Weiss, 1961; Odland and Ross, 1968; Croft and Tarin, 1970; Martinez, 1972).

In contrast, most mammalian epidermal wounds (Winter, 1972; Krawczyk, 1971, 1972; Rovee et al, 1972; Beerens et al, 1975; Winstanley, 1975) and mucosal wounds (Sciubba, 1977; Gibbins, 1978) appear to close through the submarginal cells 'rolling and sliding' over attached marginal cells. Once in contact with the substratum, the cells at the wound margin cease locomotion and form desmosomes and hemidesmosomes (Krawczyk, 1971; Pang et al, 1978). Additional evidence for this 'leap-frog' hypothesis was obtained by Ortonne et al (1981) using immunocytochemical methods. These authors found that suprabasal epidermal cells, which stain for keratin, migrated laterally, while the non-stained basal cells did not. Epithelial morphologies reminiscent of 'rolling and sliding' have also been observed in some amphibiam wound closure (Derby, 1978; Repesh and Oberpriller, 1978, 1980).

A third model of epithelia wound closure, the sliding and intermingling of suprabasal and basal cells in the cornea of Triturus (Yamanaka and Eguchi, 1981), can be seen to contain elements of both the above models.

The evidence presented in this study suggests that the normal stage 4-5 epiblast periphery moves across the inner surface of the vitelline membrane using a 'gliding' form of locomotion, a view shared by Andries et al (1985a). The epiblast is attached to the membrane by a narrow band of peripheral 'edge cells', and this region appeared approximately the same length in all the radial sections studied. The proximal epiblast lies above the membrane surface as a single layer of flattened cells.

If the epiblast periphery employed a 'rolling and sliding' style of locomotion, as described above, the membrane surface overlain by the

normal stage 4-5 proximal epiblast should be covered in attached 'edge cells' left behind by the advancing epithelial front. This was not observed to be the case in the present study, although some extracellular materials were produced by migrating explants of the epiblast periphery (see Extracellular Materials). In addition, tight junctions and desmosomes were observed between the proximal 'edge cells', and between these cells and the adjacent unattached epithelium. Similar structures have been described by Andries and his co-workers (Andries and Vakaet, 1985a; Andries et al, 1985a). Given the presence of these junctions, it is difficult to see how these peripheral cells could move relative to one another, as would be required by the 'rolling and sliding' model.

Andries et al (1985a) have also reported the presence of gap junctions between migrating 'edge cells'. These structures have been implicated in intercellular communication (Gilula et al, 1972) and may play an important role in coordinating 'edge cell' activity.

Although superficially similar to the normal stage 4-5 epiblast periphery by S.E.M., the migrating epiblast wound margin described in this study appears to utilise a different mode of epithelial locomotion.

As previously discussed, the attaching epiblast wound margin is 2-3 cells deep and, in static T.E.M. images of the advancing wound margin, the suprabasal cells appear to be moving over those attached to the vitelline membrane. Unlike ventral 'edge cells', which have no preferred orientation and are only attached to other cells, these suprabasal epiblast cells are elongated toward the wound and may contact the membrane surface beyond the basal cells through lamellipodial processes. Lamellipodia oriented in the direction of cell movement are characteristic of cell locomotion (Abercrombie, 1982; Brown and Middleton, 1985).

The attached wound margin also appears to increase in length, at

right-angles to the normal epiblast edge, as wound closure proceeds. This increase in length has been observed both between reincubation periods and in proximal-distal serial sections along a given wound margin. In general, the length of the attached wound margin is equal to the approximate distance covered by the wound edge during closure. This observation, in conjunction with the morphology of the attached epiblast cells, suggests that the epiblast wound margin moves according to the 'rolling and sliding' model of epithelial expansion.

Other evidence obtained during the present investigation supports the above conclusion. Unlike 'edge cells', the epiblast cells at the attached wound margin are not connected by discrete cell-cell junctions. The lack of tight junctions and desmosomes may allow the epiblast cells to move over and around each other at the advancing wound edge.

By T.E.M., a thin layer of densely stained material separates the migratory epiblast cells at the wound margin. Similar cell coats have been observed around 'rolling and sliding' cells during wound closure in amphibian and mammalian epithelia (Krawczyk, 1976; Repesh and Oberpriller, 1980). Most epithelial cells are unable to move over the exposed surfaces of similar cells on *in vitro* substrata (Middleton, 1982), and form monolayers held together by strong lateral adhesions (Middleton and Pegrum, 1976; Parkinson and Edwards, 1978; Miettinen *et al*, 1978). At the epiblast wound margin, the material observed around the attached cells may make their surfaces a suitable substratum for the movement of other epiblast cells, and prevent the formation of cell-cell adhesions.

The observations made at the wound margin in the present investigation were supported by the behaviour of explants from the stage 4-5 epiblast periphery cultured on the inner surface of the vitelline membrane. Explants containing 'edge cells' attached to, and migrated across, the membrane surface. The explants were in contact with the

membrane at that edge containing the original 'edge cells' and appeared to move through an uniform row of flattened cells. Adjacent migratory cells appeared closely apposed. In contrast, explants containing only the proximal epiblast became attached to the membrane surface at the entire explant periphery. Isolated epiblast cells, and small groups of cells, were observed on the vitelline membrane some distance from the main explant mass. This lends credence to the idea that the cells of the newly formed epiblast edge adhere to each other less, or to the membrane more, than do the 'edge cells' of the epiblast periphery.

Dead and dying cells were observed at the migrating epiblast wound margin in the present investigation. Some of the cell debris may result from damage inflicted during the wounding procedure, but the persistence of this material suggests a functional role in the migration of the wound margin (McMinn, 1976). Glucksmann (1951) has proposed that cell elimination is necessary during morphogenesis to allow changes in cell shape and position which would otherwise be prevented by close cell-cell apposition within epithelial sheets. A similar freedom may be necessary for alterations in cell position at the epiblast wound margin.

Rounded inclusions containing material remininscent of cell debris were observed inside the migrating epiblast cells at the wound margin. Several authors have described phagocytic behaviour at wound edges in other epithelia (Gibbins, 1968; Odland and Ross, 1968; Croft and Tarin, 1970; Tarin and Croft, 1970, Krawczyk, 1971). The 'edge cells' of the normal epiblast periphery are also phagocytic (Bellairs and New, 1962) and it is possible that such behaviour is common to spreading epithelia.

Alterations in cell shape, other than those required for cell attachment and migration, do not appear to be important in the lateral movement of the epiblast wound margins. As described in this study and by previous authors (Bellairs, 1963; Downie, 1976), the unattached cells of the normal stage 4-5 proximal epiblast are already extensively

flattened in the plane of the epithelium.

Similarly, cell proliferation appears to be of secondary importance to cell migration during the closure of wounds in the epiblast periphery. Beaded threads, which represent residual mitotic mid-bodies (Bellairs and Bancroft, 1975), were observed on the ventral surface of the epithelium surrounding the closed epiblast wound. However, it was difficult to determine whether these processes arose from the epiblast itself, or from the closely packed cells of the overlying hypoblast.

In many small wounds, there is a lag period between the onset of cell migration at the newly formed epithelial margins and the compensatory mitotic activity in the surrounding epithelium (Yamanaka and Eguchi, 1981). It is also possible that the stimulus for cell proliferation within the epithelial sheet is a result of tensions set up by the migration of the wound margins.

4. Wound Closure.

The inhibition of lamellipodial formation and cessation of cell movement between epithelia colliding *in vitro* has been well documented (Vaughan and Trinkaus, 1966; Abercrombie and Middleton, 1968; Middleton, 1972; DiPasquale, 1975a; Stoker *et al*, 1978). The 'gliding' movement of the cells at the epithelial periphery is substantially inhibited and there is little superimposition of the apposing epithelia. In most cases, apposition results in a seamless junction between the two cell sheets and, although there may be some oscillation of cells, there is no evidence for any cell intermingling (Stoker *et al*, 1978). Initial epithelial cell-cell contacts lead to the formation of strong and long-lasting lateral adhesions which bind the cell sheets together (DiPasquale, 1975a; Lonchampt *et al*, 1976; Middleton and Pegrum, 1976; Miettinen *et al*, 1978; Stoker *et al*, 1978).

Evidence from studies of wound closure suggests that those wound margins which move in a 'gliding' fashion are similarly 'contact inhibited' (Lash, 1955; Radice, 1980a). The apposing wound margins stop moving once contact is established and the attached cells remain monolayered at the centre of the wound. In these cases, as originally suggested by Abercrombie (1964), the phenomenon of 'contact inhibition' (Abercrombie and Heaysman, 1954; Heaysman, 1978) can account for both the initiation and cessation of cellular migration during wound closure.

However, as discussed in the previous section, the attached epiblast wound margins in the present investigation appear to migrate across the vitelline membrane using a 'rolling and sliding' form of locomotion. In other epithelia, the apposition of attached wound margins that move in this manner is often associated with an accumulation of cells at the closed wound (Derby, 1978; Repesh and Oberpriller, 1980). This apparent lack of contact inhibition, and the resultant piling up of cells, is to be expected between such wound margins, given the hypothesis that the cells involved habitually move over each other during the lateral expansion of the wound edge.

The closed epiblast wound, after 10 hours reincubation, consists of 2-4 layers of flattened cells on the surface of the vitelline membrane. There is no apparent discontinuity between the cells from apposing wound margins.

Static images, as are obtained by S.E.M. or from thick sections, make it difficult to determine to what extent the cells at the epiblast wound margins become superimposed or intermingled. The migrating wound margins are several cells thick, and the morphologies observed at the closed wound may result from their simple apposition followed by contact inhibition and some minor alterations in cell position. Bellairs et al (1981) reported that explants of area pellucida epiblast confronted

in vitro failed to interpenetrate a formed a barrier-like region. Although no such barrier was observed at the closed epiblast wound, the apposed wound margins could still remain essentially separate. The additional thickening of the cell sheet surrounding the closed wound could be attributed to a slight delay in the quiescence of the submarginal epiblast cells (Derby, 1978; Repesh and Oberpriller, 1978).

Alternatively, the epiblast wound margins could meet and the cells involved move over and around each other at the site of apposition. This behaviour would be similar to that thought to occur at the expanding wound edge (see previous section). As already noted, the cell coat on the migrating epiblast cells may allow these cells to move over other such cells, and prevent the formation of the strong lateral adhesions that would otherwise develop.

Numerous long filopodia are observed on the attached epiblast cells at the closed wound. These processes often connect cells from apposing wound margins, and several authors have suggested that filopodia may be responsible for establishing initial cell-cell contacts in a variety of situations (Gouda, 1974; Bancroft and Bellairs, 1975; Waterman, 1976).

Dead cells and cell debris are extruded from the closing epiblast wound. Similar behaviour has been reported during wound closure in other epithelial systems (England and Cowper, 1977; Stanisstreet et al, 1980; Smedley and Stanisstreet, 1984). As previously discussed in terms of the advancing wound margin, this cell death may play an important role in allowing alterations in cell shape and position (Glucksmann, 1951).

Wounds at the epiblast periphery were never observed to regenerate the normal stage 4-5 proximal epithelium. Both wound margins remained attached to the membrane surface for their entire length and, with continued reincubation, the closed wound merely became longer at right-angles to the epiblast periphery.

D. Extracellular Materials.

Basement membranes are layers of extracellular materials situated at the basal surfaces of almost all coherent epithelia (Hay, 1981). At the ultrastructural level, these membranous structures are composed of an amorphous sheet, the basal lamina, overlain by collagen fibrils and associated extracellular materials (Hay and Dodson, 1973). By T.E.M., the basal lamina appears to consist of an electron dense layer, the lamina densa, situated between two less dense layers, the laminae rarae externa (or lucida) and interna (Low, 1961; Hay, 1981; Harrisson et al, 1985b). In the following discussion, the term basal lamina will be used to denote basement membrane constituents other than collagen.

Under the S.E.M., the basement membrane of the stage 4-5 chick epiblast is also seen to consist of a textureless sheet and overlying fibrillar meshwork (Bancroft and Bellairs, 1974; Ebendal, 1976; England and Wakely, 1977; Wakely and England, 1979). Some longer fibres appear as continuous strands which form a series of stage-dependant patterns on the basal laminae of both the area pellucida and area opaca (Critchley et al, 1979; Wakely and England, 1979; England, 1980b; Kordylewski and England, 1980; England, 1981; Harrisson et al, 1984b; Andries et al, 1985b; Monnet-Tschudi et al, 1985). These thread-like processes are associated with glycosaminoglycans (GAGs) and fibronectin, and are thought to guide the movements of mesoderm cells and primordial germ cells over the epiblast basement membrane (Mayer and Packard, 1978; Critchley et al, 1979; Wakely and England, 1979; England, 1981; Mayer et al, 1981; England, 1982; Harrisson et al, 1984ab; Andries et al, 1985b; Harrisson et al, 1985b; Monnet-Tschudi et al, 1985).

GAGs, especially hyaluronic acid, appear to be a major constituent of the epiblast basal lamina (Solursh, 1976; Fisher and Solursh, 1977; Sanders, 1979; Vanroelen et al, 1980). Treating the epiblast basement

membrane with hyaluronidase removes hyaluronic acid, leaving only the fibrillar component intact (Sanders, 1979)

In the present investigation, no basement membrane was detected on the 'edge cells' at the normal stage 4-5 epiblast periphery. A discontinuous layer of amorphous material was observed on the exposed ventral cell surfaces in some T.E.M. sections, but did not appear to constitute an coherent basal lamina. Bellairs (1963) described a similar material in an earlier T.E.M. study of the 'edge cells', and suggested that this substance might represent hydrated yolk lipid. After removal of the overlying unattached proximal epiblast, a fibrillar meshwork was often seen to be associated with the 'edge cells' at the proximal margin of the attached edge and adjacent vitelline membrane. The function and origin of this material is not known.

The 'edge cells' form a distinct morphological unit at the epiblast periphery (Andries and Vakaet, 1985ab; Andries et al, 1985a) and the absence of a basement membrane may represent an adaptation to active cell movement (Sanders, 1984). Monnet-Tschudi et al (1985) have identified internalized fibronectin within stage 4-6 'edge cells' and have proposed that these cells may play some role in the regulation of extracellular materials at the expanding epithelial margin.

In contrast to the 'edge cells', an intact basement membrane was associated with the normal stage 4-5 epiblast just proximal to the 'edge cells'. This membranous structure was only observed by T.E.M., as a dense layer of overlying yolk globules prevented S.E.M. examination. A similar structure has previously been described in this region by Bellairs (1963).

No coherent basement membrane was observed by S.E.M. or T.E.M. on the rounded cells at the attaching epiblast wound margin. This apparent dissolution of the epiblast basement membrane may represent a reaction to the loss of normal epithelial organisation and/or an early adaptation

to the onset of cell movement during wound closure.

The loss of basement membrane components, especially basal lamina GAGs, may be a general mechanism for regulating epithelial cell populations during morphogenesis (Bernfield and Banerjee, 1982). Disruption of the basal lamina precedes the invagination of epiblast cells during gastrulation in the stage 4-5 chick embryo (Sanders, 1984) and is associated with morphogenesis in the mammary epithelium of the mouse embryo (Kratochwil, 1977; Durnberger and Kratochwil, 1980). A reduced or interrupted basal lamina may also accompany the invasion of certain metastatic tumours (Liotta et al, 1980).

Basal lamina GAGs show a rapid turnover (Sanders, 1984) and their removal from the basement membrane, with the resulting disruption of the basal lamina (Sanders, 1979), might result from alterations in the rate of synthesis and/or degradation (Bernfield and Banerjee, 1982). Basement membrane Type IV collagen has a much slower rate of turnover (Laurie and Leblond, 1983) and the dissolution of this fibrillar membrane component may require enzymatic action by the underlying epithelial cells.

Several authors have reported that the presence of an intact basement membrane is essential for the maintenance of tissue integrity (Banerjee et al, 1977; Cohn et al, 1977). The evidence presented in this investigation suggests that the reverse may also be valid, i.e. that epithelial continuity is important in the maintenance of normal basement membrane morphology.

As previously discussed (Section C.3), continued reincubation leads to wound closure through the lateral migration of the epithelial margins. When, in the present study, the unattached proximal epiblast surrounding closed or partially closed wounds was removed, long thin fibres and small spherical bodies were observed on the vitelline membrane proximal to the wound. The position and orientation of these

fibres suggests that they are formed by the movement of epiblast cells at the closing wound margins.

By S.E.M., the processes on the vitelline membrane resemble the thread-like structures and 'interstitial bodies' described by other authors on the basal lamina of the stage 4-5 area pellucida (England and Wakely, 1977; Wakely and England, 1979; England, 1981). Analogous objects have also been observed in T.E.M. and immunocytochemical studies of the stage 12 area opaca basal lamina (Mayer and Packard, 1978; Mayer et al, 1981; Monnet-Tschudi et al, 1985). On the basis of this resemblance, it is possible that the fibres and spherical bodies adjacent to the wound represent basement membrane constituents produced by epiblast cells at the wound margins.

During the normal development of the epiblast basement membrane, the fibrillar membrane component is produced as a meshwork on the exposed ventral cell surfaces (Sanders, 1979). A similar cell surface material was observed at the reincubated epiblast wound margins in the present investigation, and this material could be responsible for the formation of the elongated fibres observed adjacent to the closing epiblast wound. Although individual fibres were occasionally connected to attached epiblast cells at partially closed wounds, the majority were situated radial to the unattached proximal wound extremity. The wound margin attached in a distal-proximal progression (see Section C.2) and before flattening, the cells at the wound margin may be dragged some distance across the membrane surface by attached distal neighbours and continued epiblast expansion. Any fibrillar material on these marginal cells could adhere to the membrane and be pulled into elongated fibres behind the moving cells.

At high magnifications, many fibres were seen to contain a number of fine fibrils. These composite fibrous bundles are probably the result of fibrils within the meshwork being aligned by the tensions involved in

fibre formation. Embryonic mouse lung epithelial cells produce a meshwork of Type IV collagen fibrils when cultured on rat tail collagen substrata (Chen and Little, 1985). In vitro, the same cells produce bundles of Type IV collagen fibrils aligned parallel to the long axes of the cells. Although, in this instance, the bundles are probably produced by tensions reulting from 'tractional structuring' beneath the attached cells (Harris et al, 1981; Stopack and Harris, 1982), these results indicate the tendency of collagen fibrils to become aligned under conditions of tensile stress.

A similar process may be responsible for producing the fibrillar patterns observed on the basal laminae of the chick area pellucida and area opaca. Monnet-Tschudi et al (1985) have already suggested that the radial fibronectin-containing fibres within the area opaca may be oriented by the tensions created within the epithelium by 'edge cell' migration (New, 1959; Bellairs et al, 1967; Downie, 1976).

Basal lamina GAGs are secreted in globular form during the formation of the epiblast basement membrane (Sanders, 1979). The 'interstitial bodies of the normal stage 4-5 basement membrane have similarly been shown to contain GAGs and are thought to represent turnover products of the basal lamina (Wakely and England, 1979; Mayer et al, 1981; Sanders, 1984; Harrisson et al, 1985b). The spherical bodies associated with the fibres adjacent to the epiblast wound probably represent similar aggregations of basal lamina materials, presumably GAGs, produced by epiblast cells in an attempt to reconstitute a basement membrane. Several investigations have demonstrated the close association of GAGs and collagen within epithelial basement membranes (Banerjee et al, 1977; Cohn et al, 1977; Sanders, 1979).

To further investigate the production of basement membrane materials by epiblast cells , explants containing a row of 'edge cells'

and some proximal epiblast were cultured on the inner surface of the vitelline membrane. Individual explants became attached to the membrane surface by that margin containing the original 'edge cells' and moved a distance several times their own length over a 3 hour reincubation period.

After reincubation for 1 hour, no coherent basement membrane could be detected on the ventral explant surface. With continued reincubation, a dense fibrillar meshwork was formed on the exposed surfaces of the rounded epiblast cells. Increasing numbers of spherical bodies were also associated with the fibrillar meshwork and underlying cells surfaces until, at 3 hours reincubation, the ventral explant was overlain by a smooth basement membrane. These results suggest that the fibrillar membrane component is covered by an amorphous layer of coalesced spherical bodies during the reconstitution of the epiblast basement membrane. Sanders (1979) has reported a similar sequence of events during the development of the basement membrane overlying the chick area pellucida, and Harrisson et al (1985b) have described fibrillar processes compressed between the epithelial cell surface and basal lamina.

Some epiblast cells immediately adjacent to the explant 'edge cells' become attached to the vitelline membrane during explant reincubation. This widening of the attached explant edge, and the action of gravity on the proximal explant, often result in the lateral and proximal explant margins coming to lie on the membrane surface. Numerous fibres and spherical bodies are observed on the vitelline membrane behind these explants, i.e. at the opposite side to the attached explant edge. The fibres are oriented away from the direction of explant movement and are connected to the cell surface or, in older explants, extend from the fibrillar meshwork attached to the cells. These processes are presumably formed from the fibrillar meshwork during

explant movement through a process similar to that thought to occur at the wound margins.

Isolated cells of the epiblast periphery also produced fibres and spherical bodies when incubated on the inner surface of the vitelline membrane. As individual 'edge cells' and cells of the proximal epiblast were indistinguishable in the present study, it was not possible to determine whether these processes were associated with one or both cell types. Not all the cells attached to the membrane were connected to fibres or spherical bodies and these processes, though present after 3 hours reincubation, were largely absent from cells reincubated for only 1 hour. The latter observation suggests that fibres and spherical bodies were produced by the cells concerned after dissociation of the epithelial tissue and cell attachment to the membrane surface.

Isolated cells situated adjacent to fibrils and fibres were often elongated at right-angles to the long axis of the thread-like structure. Other single cells contacted these fibrillar processes through short filopodial or lamellipodial protusions. Similar contacts have previously been observed between migrating mesodermal cells and the fibres of the stage 4 area pellucida basal lamina (England and Wakely, 1977). That isolated cells some distance from a fibre were oriented toward the process suggests the presence of a chemical signal, possibly in the form of a diffusion gradient.

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