A STUDY OF EPITHELIAL-MESENCHYMAL INTERACTIONS BETWEEN CELLS FROM ADJACENT RETINAL TISSUES

by

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SUMMARY.

The ability of pigmented retina epithelial cells (PRE) to spread upon a number of different cellular substrata was examined and quantitated.

PRE cells could not spread out upon sheets of cell-types which showed reduced amounts of lamellar cytoplasm, such as epithelial cells (PRE and BSC-1) and transformed fibroblasts (L-929 and CHO-K1). PRE cells could adhere to the upper surfaces of these cell sheets only in small numbers.

PRE cells did appear to be able to spread and move when seeded upon the fibroblast sheets (choroid, BHK 21/C13 and heart) although the fibroblasts themselves were unable to do so. Both classes of cells adhered equally effectively to the fibroblast sheets as measured by the monolayer collection assay.

Scanning electron microscope studies showed that the PRE cells appeared to spread upon the fibroblast cells themselves and not the culture substratum. PRE cells which attached to fibroblast sheets exhibited many filopodia whereas those on epithelium did not and possessed many blebs.

Transmission electron microscope studies confirmed that the PRE cells were using the fibroblast sheet as a substratum for spreading and not extensive extracellular matrix such as collagen or the plastic culture vessel. Examination of long-term (4 hours) experiments revealed that the PRE cells were able to 'invade' the multilayered choroid sheets they had spread upon, although no contact with the tissue culture substratum was ever noted.

Head-on collisions between choroid fibroblasts resulted in classical contact inhibition of movement by one or both of the cells. However, PRE cells were not contact inhibited on collision with the choroid fibroblasts although they induced a contact inhibitory response in the latter.

It is suggested that the ability of PRE cells to spread upon and later invade sheets of choroid fibroblasts may be related to their inability to be contact inhibited by the latter.

The results are discussed in relation to morphogenesis wound healing and carcinomatous invasion *in vivo*.

CONTENTS.

1. INTRODUCTION.

1.1 General Comments.

Interactive epithelial and mesenchymal cell movements have been shown to play an important part in developmental processes (Leplat 1912), wound healing (Lash 1955, Ordmann and Gillman 1966, Croft and Tarin 1970), and carcinomatous invasion (Wood et al 1967). Knowledge of how these movements may be controlled has been gained largely from *in vitro* studies, but perhaps it would be advisable first to describe the types of interaction which occur between epithelium and mesenchyme *in vivo*.

In the chick embryo eye (an ectodermal organ), the pigmented retinal epithelium develops from the inner wall of the optic cup (Romanoff 1960), and the choroid coat is derived from secondary mesenchyme cells which condense onto the back of this inner wall (Leplat 1912). Similar mesenchymal movements occur in the developing chick cornea, and have been observed *in situ* using Nomarski optics by Bard and Hay (1975).

During the formation of endodermal organs such as the liver (Croisille and Le Douarin 1965) and the lung (Sorokin 1965), a different type of interaction occurs whereby a mesenchymal matrix is invaded by epithelial cells derived from the endoderm.

Another example of controlled invasion of the mesenchyme by the epithelium occurs during the healing of cutaneous wounds. This has been studied by light microscopy (Ordmann & Gillman 1966), and more recently by transmission electron microscopy (Croft and Tarin 1970). After the tissue has been wounded the area around the incision dies and a ridge of epithelium accumulates at the edge of the area of necrosis. Subsequently, the epithelial cells invade the underlying mesenchyme tracing the junction between the living and dead tissue, and cutting through a considerable amount of connective tissue and extra vascular fibrin. When the two epithelial sheets meet one another the migration ceases immediately. Croft and Tarin (1970) propose that this cessation of epithelial movement may be controlled by the dermis, although they recognise that contact inhibition of cell locomotion may be relevant to the phenomenon as postulated by Abercrombie and Middleton (1968).

Invasion of the uterine mesenchyme by the giant cells of the trophoblast is believed to be curtailed by the formation of a decidual mass of mesenchymal cells which are hypertrophied (see Kirby and Cowell 1968 for a review). The central region of this decidual mass becomes necrotic and it has been postulated (Kirby & Cowell 1968) that the trophoblast can invade this dead region, but is contained by the outer layer of healthy decidual tissue.

In contrast to these controlled types of invasion carcinoma cells appear to invade their surrounding tissues in a different fashion: carcinomas are malignant tumours of epithelial cells, but their appearance and rate of invasion into the mesenchyme depends upon a number of factors (See Montgomery 1965).

A well differentiated tumour of glandular origin (e.g. adenocarcinoma) may have a tubular structure, though some cells may be replaced by mucin in colloidal tumours. Alternatively the carcinoma may be undifferentiated, the tumour consisting of solid sheets of cells which have lost their structural differentiation [anaplastic cells (See Willis 1967)].

The degree to which the invasion by the carcinoma is challenged by the host tissues is also an important consideration. Some tumours infiltrate rapidly with minimal reaction from the connective tissue (See Montgomery 1965), and these are termed *encephaloid carcinomas*. Willis (1967) would consider such a tumour to be spreading largely by infiltration. If the tumour provokes a marked response on the part of the connective tissue it becomes encapsulated (See Montgomery 1965) and is termed a *scirrhous carcinoma*. Willis (1967) would consider such a tumour to be spreading largely by expansion.

These properties of carcinomas described above are extreme examples and the properties of most carcinomas fall between these extremes.

The involvement of cell motility in invasion by carcinoma cells is open to debate but it seems probable that it plays some part. Wood et al (1967) have used time-lapse filming to investigate the motility *in vivo* of V x 2 carcinoma cells in rabbit tissues, and suggested that the locomotion of these tumour cells was non-directional. Enterline and Coman (1950) using modern tissue culture methods assisted by microcinematography found that the locomotion of a wide variety of

carcinomas was amoeboid in nature once they became detatched from the epithelial outgrowth.

Willis (1967) states that he never saw evidence of individual carcinoma cells breaking contact with their neighbours, but there is evidence that a wide variety of carcinoma cells possess weaker intercellular adhesions that their normal counterparts (Coman 1944, 1953). Coman (1953) has suggested that this reduction in intercellular adhesion might be important to invasion as it would enable the highly motile carcinoma cells to escape from the tumour cell mass and infiltrate the mesenchyme.

However the nature of invasion *in vivo* may depend on the mechanical properties of the tumour (Young 1959, Eaves 1973), increase in the levels of many enzymes (Hashimoto et al 1973, Unkeless et al 1973, Ossowski et al 1973, Sylvan 1973), and possible removal of controlling mesenchymal influences (McLaughlin 1961, Wessels 1964). Therefore the extent to which tumour cell motility contributes to the invasive behaviour of the cells is not known, but the possibility that it is an important factor makes it a worthwhile subject for study. It is well known that certain tumour cells do not display classical contact inhibition of movement (Abercrombie et al 1957, Heaysman 1970, Abercrombie and Heaysman 1976), when colliding with primary embryonic fibroblast cells in culture (See section 1.3). Little is known however, about the influence on normal epithelial movement of normal fibroblastic cells (See Abercrombie and Middleton for a review).

In order to consider the regulatory effects of cell contact on locomotion it is necessary to review current ideas on the mechanism of cell locomotion. Most of these ideas have come from *in vitro* studies using fibroblasts though other cell types which have been studied share many features with these cells.

1.2 Cell locomotion *in vitro*

The salient features of a moving fibroblast *in vitro* are as follows;

1) The cells are thinly spread at their anterior region (the leading lamella) and small projections (ruffles) arise on the dorsal surface moving backwards from the edge towards the nucleus.

2) The leading lamella moves forward by exchanging old adhesions for new ones in a more anterior position.
3) Microfilament bundles are associated with the new adhesions and are probably responsible for drawing the cell body towards these new adhesions.
4) There may be a continuous backward movement of membrane upon both cell surfaces as visualised by the backward transport of particles.

1.2.1 Spreading of the Leading Edge and Ruffling

Most of the studies relevant to this aspect of cell locomotion have been conducted on the spreading of cells from suspensions seeded onto tissue culture substrata, and this has been described by Taylor (1961). Whether this type of spreading is analogous to the forward extension of a moving fibroblast is not certain, but I propose to argue later that they are related phenomena.

Wolpert and Gingell (1968) suggested that the strength of the cell-substratum adhesions must exceed the resistance of the cell to deformation before spreading of that cell will occur. Physical properties such as the relative wettability of the culture substratum by the cell, are important in determining whether a cell may spread out upon that substratum (Carter 1967a). Relative wettability probably affects spreading by influencing the ability of cells to adhere to a given substratum (Carter 1967a). Maroudas (1973) has shown that fibroblasts can spread more easily on substrata of high wettability than low wettability, but also found that the substratum must have sufficient rigidity to resist the mechanical stresses exerted by the spreading fibroblast. Also, Harris (1973a) has shown that certain cells can spread upon silicone oil droplets of much lower viscosity than others.

Proteins deposited upon the culture substratum have been shown to influence the rate of spreading in a number of cell types. Various sera have been reported to decrease the rate of spreading on glass of rabbit conjunctiva cells (Taylor 1961), MRC-5 cells (Witowski and Brighton 1972), and mouse peritoneal macrophages (Rabinovitch and De Stefano 1973a). However, Grinnell (1976) reported that a factor purified from foetal calf serum induced spreading of several mammalian cell lines on tissue culture plastic, although its action was abolished

by prior coating of the substratum with other proteins. Taylor (1961) has shown that cell microexudates retard the rate of spreading of rabbit conjunctiva cells on glass, but in contrast to this Culp (1974) showed that exudate (or substrate-attached material) from mouse 3T3 fibroblasts, caused their transformed counterparts SVT2 cells to become more flattened upon a glass or plastic substratum. A cell surface protein isolated from cultures of chick embryo fibroblasts has been reported to increase the flattening of transformed cells in a similar fashion (Yamada et al 1976).

Medium conditioned by cultures of chick embryo neural retina, and leg muscle cells was observed to increase the spreading of chick embryo chondrocytes on serum coated plastic (Takeichi 1973). The effect was abolished by trypsin and pronase indicating that the active fraction contained protein.

It is possible that these proteins and exudates may act by increasing or decreasing the strength of adhesion between the cell and the substratum. It is doubtful whether specific exudates or substrate attached materials are required for spreading however as Rabinovitch and De Stefano (1973c) have shown that Sarcoma I cells are capable of spreading on purified gelatin in the presence of Mn^{2+} ions.

The divalent cations Mg^{2+} and Ca^{2+} have been shown to be required for cell spreading in chick scleral fibroblasts (Takeichi and Okada 1972), and nearly always for induced macrophage spreading (Rabinovitch and De Stefano 1973a,b). In all of these studies spreading was more extensive in the presence of Mg^{2+} ions than in the presence of Ca^{2+} ions. Rabinovitch and De Stefano (1973b) also reported that Mn^{2+} (an ion present in low concentrations physiologically), was an active cofactor to the induction of macrophage spreading at an external concentration of 10^{-6}M, compared with 10^{-4}M for Mg^{2+} ions, and 10^{-2}M for Ca^{2+} ions. The same workers have reported that Mn^{2+} ions can induce the spreading of macrophages (Rabinovitch De Stefano 1973b), and Sarcoma I ascites cells (Rabinovitch and De Stefano 1973c, 1975) on clean glass at concentrations of around 10^{-3}M. The authors have postulated (Rabinovitch and De Stefano 1973b), that Mn^{2+} may stimulate spreading by mimicking the action of Mg^{2+} ions to greater effect. The effects of Mg^{2+} and Ca^{2+} ions upon spreading are closely paralleled by their effects on adhesion.

Mg^{2+} has been reported to increase the adhesion of chick scleral fibroblasts to protein-coated plastic (Takeichi and Okada 1972), and rat polymorphonuclear neutrophils to serum coated glass (Garvin 1968), more efficiently than Ca^{2+} ions. It is possible that Mg^{2+} and Ca^{2+} may mediate their effect upon spreading by facilitating cell-substratum adhesion. However, Rabinovitch and De Stefano (1973b) have suggested that as the above determinations of cell substratum adhesion were done after a period of incubation at 37°C, they might not be measuring the effects on the adhesive event *per se*, but effects on spreading, as this would multiply the points of contact the cells make with the substratum, making them more difficult to detach.

Divalents may affect cell locomotion without grossly affecting the spreading of the leading lamella. The motility of mouse 3T3 fibroblasts has been shown to be dramatically inhibited by EGTA which reduces the free external Ca^{2+} ion concentration to 10^{-7}M (Gail et al 1973). There was no mention of the cells rounding up during the course of the experiment however.

Procaine (a cationic anaesthetic) has been found to inhibit mouse 3T3 fibroblast locomotion (Gail and Boone 1972b),and lower concentrations of similar anaesthetics have been found to inhibit, or reverse Mn^{2+} -induced spreading of Sarcoma-I ascites cells (Rabinovitch and De Stefano 1973c, 1975). Procaine is known to decrease K^+ and Na^+ permeability in nerves (Taylor 1959), but when Gail et al (1973) treated fibroblasts with tetrodotoxin and tetraethyl ammonium, attempting to block Na^+ and K^+ permeability no effect upon locomotion was observed. Cationic anaesthetics have been postulated to act in striated muscle by reducing Ca^{2+} permeability in the sarcoplasmic reticulum (See Feinstein and Paimre 1969 for a review), and in Sarcoma I cells by the inhibition of membrane motility (Rabinovitch and De Stefano 1975). Both Gail et al (1973) and Rabinovitch and De Stefano (1975) postulated that cationic anaesthetics might affect tissue culture cell locomotion and spreading, by inhibiting Ca^{2+} ion fluxes across the endoplasmic reticulum membranes. If the internal Ca^{2+} ion concentration fell sufficiently, this would then prevent the interaction of actin and myosin (See section 1.2.2. for evidence of these proteins in non-muscle cells) in the microfilament bundles, provided this contractile system is analogous to that of striated muscle.

It has been postulated that a freshly seeded cell requires a force to deform the cell surface in order that spreading might occur (Wolpert and Gingell 1968). Harris (1973a) has put forward a similar idea to explain the extention of the leading lamella of a moving fibroblast over the substratum, and suggests that the cells may be preferentially susceptible to deformation in this region. Harris (1973a) also suggests that the blebs and microvilli which have been seen on the surfaces of BHK 21 cells (Follett and Goldman 1970), and CHO-K1 cells (Porter et al 1973), occur at similar regions of weakness at the cell surface. Harris (1973a) thought that an excess hydrostatic pressure within cells could be a candidate for the deforming force. DiPasquale (1975b) has provided evidence in favour of this hypothesis by showing that hypertonic solutions of sorbitol could abolish blebbing and extension of the leading lamella in a number of chick embryo epithelial cells. It has been postulated that the surface protrusions noted in freshly seeded BHK 21/C13 (Follett and Goldman 1970) and dividing CHO-K1 (Porter et al 1973) cells, may be a source of membrane reserve when a cell passes from the rounded to the spread state (Wolpert and Gingell 1969, Follett and Goldman 1970). A similar idea has been put forward to explain the expansion of surface membrane which occurs during the movement of *Amoeba proteus* (Czaska and Grebecki 1966). More recently, Erickson and Trinkaus (1976) estimated the total surface area of synchronised BHK 21/C13 hamster fibroblasts at different stages of spreading, and found that nearly all the surface expansion could be accounted for by the unfolding of microvilli and blebs.

Associated with the leading margin of most moving tissue culture cells, and indeed the margins of freshly seeded cells which are spreading, are ruffles (Abercrombie and Ambrose 1958) which have been shown to be upfoldings of the leading edge (Ingram 1969, Harris 1969). These structures can be clearly seen in side views of moving cells (Ingram 1969, Harris 1969), or in fixed preparations examined by electron microscopy (Abercrombie et al 1971, Price 1972). It has been observed that ruffles form when the leading edge is changing from a withdrawal phase to a protrusion phase (Abercrombie et al 1970b). Ingram (1969) postulated that formation of ruffles and their subsequent backward movement, was due to a greater contraction on the dorsal surface as compared to the ventral surface, while Abercrombie et al (1970c) have favoured the idea that the ruffle is pushed backwards by active expansion of the cell membrane just beneath the cell margin.

It now seems likely however, that ruffling is a consequence rather than a cause of cell locomotion *in vitro* as cells will continue to move even when overlain with layers of agar or plasma clot which appear to prevent ruffling [Abercrombie cited by Harris (Harris 1974)]. This is substantiated by observations of moving corneal endothelium cells (Bard et al 1975), and moving corneal fibroblasts (Bard and Hay 1975) *in situ* which do not reveal the presence of well developed ruffles. Presumably the presence of extracellular materials might have a similar restricting effect as the layers of agar and plasma clot in Abercrombie's experiments.

1.2.2 Microfilaments and Cell Locomotion

It has been shown by particle transport studies (Harris and Dunn 1972), and side view observations (Ingram 1969), that the outermost part of the leading edge of moving fibroblasts is not in contact with the substratum. Izzard and Lochner (1976) have substantiated these findings using the interference reflection microscopy technique developed by Curtis (1964). Active protrusion and withdrawal movements have been demonstrated in this region, and subjected to careful statistical analysis (Abercrombie et al 1970a). It was discovered that the leading edge spent more time in the protrusion phase than the withdrawal phase, and Abercrombie et al postulated that this might result in a net forward displacement of the cell. The energy for the protrusion phase might come from an excess hydrostatic pressure (Harris 1973a, DiPasquale 1975b), coupled with an inward pull generated by the contractile microfilaments (Harris 1973a). This would generate pressure to force the leading lamella of moving fibroblasts over the substratum, and push out the various processes which are formed prior to the spreading of a freshly seeded cell(Rajaraman et al 1974).

Another role for the microfilaments might be to generate tension, which would be required to draw the cell body forwards towards the advancing leading edge (Abercrombie et al 1970c). Sheets of fibroblasts spreading upon silica fibres have indeed been seen to exert considerable tension (Curtis and Varde 1964), and Maroudas (1973) has shown that single fibroblasts are capable of exerting enough tension to bend a fine glass fibre they are growing on. Further evidence comes from the micromanipulation studies of Chambers and Fell (1931) using epithelia, fibroblasts and myoblasts, Vaughan and Trinkaus (1966) using epithelia, and Harris (1973c) using a variety of fibroblasts and tumour cells. It was found that when a

cell was detached from substratum at the leading edge the result was one of contraction of the detached margin into the cell body again indicating the presence of tension within the cell.

Abercrombie et al (1971) have demonstrated the presence of longitudinal bundles of microfilaments in moving fibroblasts. These bundles appear to originate from the nucleus and terminate at adhesion 'plaques' which are closely applied to the substratum (30nm. separation). Similar plaque-like areas have been seen by Izzard and Lochner (1976) using interference reflection microscopy, and these so called *focal contacts* (10-15nm. separation) were seen to be coincident with cytoplasmic fibres of the same dimensions as the contact. These *focal contacts* were distributed within a broad area of close opposition similar to that described by Curtis (1964). The presence of retraction fibres which have been demonstrated when cells detach from the substratum (Taylor and Robbins 1963, Harris 1973c), and subsequent studies using the scanning electron microscope to examine the underside of spread cells (Revel et al 1974) were suggestive of adhesions similar to those described by Abercrombie et al (1971) and Izzard and Lochner (1976).

It has been postulated that the microfilament bundles by contracting might draw the cell body forwards towards new points of adhesion (Abercrombie et al 1970c), and interference reflection studies have shown that the plaques do not move forward but are replaced by plaques in a more anterior position (Lochner and Izzard 1973). Wolpert et al (1969) and Harris (1973b) have suggested that when competition exists between different areas of the cell margin, the cell moves in the direction of the strongest cell-substratum adhesion and as a result tend to move up a gradient of adhesiveness as noted by Carter (1965, 1967a). Possibly the more recently formed cell-substratum adhesions (plaques) are strongest and persist at the expense of the weaker older adhesions.

Microfilaments then might be regarded as having two major roles in cell locomotion:

1) Generation of pressure to force the leading lamella over the substratum.

2) Contraction of microfilament bundles drawing the cell body forwards towards newly formed adhesions at the leading edge.

Two different arrangements of microfilaments have been described (Spooner et al 1971, Wessels et al 1973). The lattice or network microfilaments(5nm. diam.) which occur directly beneath the plasma membrane and the sheath microfilaments (5-7nm. diam.) which occur a little way beneath the plasma membrane. Spooner et al (1971) have reported that the drug cytochalasin B (Carter 1967b) alters the microfilament network although the arrangement of the sheath microfilaments remains unchanged.

This drug and other cytochalasins have been shown to inhibit cell movement and spreading in mouse fibroblasts (Carter 1967b), glial cells (Spooner et al 1971), epithelial cells (DiPasquale 1975a,b), chick myoblasts, chick fibroblasts, chick chondrocytes, HeLa cells (Sanger and Holtzer 1972), and Ehrlich ascites cells (Weiss 1972). Experiments using these drugs should be interpreted with caution however, as some workers have found indications that cytochalasin B affects other aspects of cell surface activity, such as cell-substratum (Weiss 1972), and cell to cell adhesiveness (Sanger and Holtzer 1972), glucose transport (Zigmond and Hirsch 1972, Estensen and Plagemann 1972) and mucopolysaccharide synthesis (Sanger and Holtzer 1972). It would therefore be dangerous to assume that the inhibition of spreading and locomotion by cytochalasins was due to the action of the drug upon the lattice microfilaments. Nevertheless there exists a body of circumstantial evidence which suggests that microfilaments *are* involved in cell movement (See earlier).

It has also been found that recovery of cell movement following treatment of cells with cytochalasin B (Spooner et al 1971, Sanger and Holtzer 1972), or trypsin (Goldman et al 1973,Goldman and Knipe 1973) could occur in the presence of cycloheximide indicating that the components for microfilament assembly were already present, and did not require resynthesis.

Huxley (1973) has advanced a theory for the contraction of microfilaments, similar to the sliding filament mechanism of striated muscle contraction. Indeed, studies using fluorescent antibodies

have demonstrated the presence of the major muscle proteins actin (Lazarides and Weber 1974), and myosin (Weber and Gröeschel-Stewart 1974), associated with the microfilament bundles. Actin has also been demonstrated in sheath microfilaments by its ability to bind heavy meromyosin (Spooner et al 1973, Wessels et al 1973). Tropomyosin which regulates the Ca^{2+} dependent interaction of actin and myosin in striated muscle has also been shown to be associated with microfilament bundles in fibroblasts by Lazarides (1975) using the fluorescent antibody technique. Similar techniques have shown the Z-line protein α-actinin to be associated with the microfilament bundles along their entire length in microvilli of epithelial brush borders (Schollmeyer et al 1974), and in rat embryo cells (Lazarides 1976). It has been postulated that the electron dense plaques seen at the attachment of microfilaments to the cell membrane contain α-actinin (Mooseker and Tilney 1975), and that they also connect the actin filaments to the membrane in the form of lateral bridges. Mooseker and Tilney (1975) state that these lateral bridges may also involve membrane bound myosin, and myosin has been demonstrated at the inner face of the plasma membrane by Painter et al (1975) using a fluorescent antibody technique.

Although the finer points of the mechanism of microfilament contraction and its involvement with cell movements remains to be elucidated it seems probable that the locomotion of tissue cells is effected by events similar to those which occur in striated muscle.

1.2.3 The involvement of microtubules and 10nm diameter filaments in cell locomotion.

Studies using colchicine and other drugs known to disrupt microtubules have shown that fibroblasts lose their triangular shape, (Vasiliev et al 1970, Gail and Boone 1971, Goldman 1971, Goldman et al 1973), and reduce their speed (Gail and Boone 1971, Goldman 1971, Goldman et al 1973) and persistence (Vasiliev 1970, Gail and Boone 1971, Goldman 1971, Goldman et al 1973) of locomotion. These cells show an epitheloid morphology where ruffling persists around the perimeter of the cells (Vasiliev et al 1970, Gail and Boone 1971, Goldman 1971, Goldman et al 1973) and some workers have noted the disappearance of microtubules in such cells (Vasiliev et al 1970, Goldman 1971). Spooner et al (1971) and Goldman (1971) have noted the appearance of 10-12nm. filaments in the region of the nucleus ('juxta nuclear cap'),

in colchizine-treated glial cells and fibroblasts respectively. Goldman and Follett (1970) also noted the appearance of a birefringent sphere shortly before spreading in BHK 21/C13 fibroblasts using polarization light microscopy. Subsequent examination under the transmission electron microscope revealed that this sphere was packed with small strands of 10-12nm filaments. The electron microscope studies revealed that as the birefringent sphere 'reeled out' its fibres (as seen by light microscopy) 10nm. filaments are simultaneously dispersed into longitudinally oriented arrays in the major cell processes during cell spreading. Goldman (1971) thought that the filaments were dependent upon the microtubules for their distribution, and that this microtubule-filament complex was involved in the determination of triangular cell shape, the formation of major cell processes and locomotion, in fibroblasts.

The spreading and locomotion of BSC-1 (Goldman et al 1973), and chick embryo (DiPasquale 1975b) epithelial cells has been shown to be unaffected by colchizine and related drugs. A juxta nuclear cap of 10-12nm. filaments does appear in colchizine-treated BSC-1 cells however (Goldman 1971), and this together with the observation that epithelial cells contain far fewer microbutubles than fibroblasts in their major cell processes (DiPasquale 1975a) has lead Goldman et al (1973) to postulate that these fibres are not involved in the spreading of fibroblasts or epithelia.

In summary it appears that microtubules and filaments are important for the maintenance of directional locomotion in fibroblasts, but their role in epithelial cells is less clear. Microtubules have been shown to be present in cytoplasm in the form of precursors (possibly as globular subunits), as their assembly does not require protein synthesis (Goldman and Knipe 1973). The 10-12nm. filaments appear to be stored in a birefringent sphere adjacent to the nucleus (Goldman and Follett 1970).

1.2.4 Retraction of the Trailing Edge.

It has been shown that when the cell moves forward it exchanges existing adhesions for those in a more anterior position (Lochner and Izzard 1973). However if the cell is to make any progress it must break posterior adhesions at the trailing edge in a similar manner, and be drawn forward actively by the contractile microfilaments or passively by elastic recoil (Harris 1974).

Some circumstantial evidence has been provided in favour of the former hypotheses by Luduena and Wessels (1973) when studying the locomotion of neurones in culture. These cells do not need the retraction phase of cell locomotion, as the nerve cell bodies do not move, and significantly it was found that these cells lacked the longitudinally oriented sheath microfilaments, which extend along the length of the fibroblast parallel to the direction of locomotion (Wessels 1971).

The observation that cells will move faster upon substrata to which their adhesion is lower, but sufficient to allow spreading (Gail and Boone 1972a), possibly reflects an increased ability to break existing adhesions and assist the detachment of the trailing edge.

1.2.5 Membrane Flow

When small particles are spread upon a culture substratum, moving fibroblasts (Ingram 1969, Abercrombie et al 1970c, Harris and Dunn 1972), epithelial cells (DiPasquale 1975a) and neurones (Bray 1970) pick these up and transport them centripetally towards the nucleus. A similar type of transport has been shown to occur on the ventral surface of cells (Harris and Dunn 1972). These experiments together with the demonstration that ruffles (Abercrombie et al 1970b), and blebs (Harris 1973a), move rearward with respect to the substratum,has lead authors to suggest that membrane flows backwards, and is disassambled near the nucleus (Abercrombie et al 1970c, Harris 1973a). This theory requires either, (1) continuous resynthesis of new membrane, or (2) Recycling of disassembled membrane from a sink near to the nucleus, and following this the membrane would have to be inserted at the leading lamella (Abercrombie et al 1970c, Harris 1973a). A similar idea has been put forward to explain particle transport in the slime mould *Polysphondylium violaceum* (Shaffer 1963).

If membrane is continuously resynthesised this would require that one tenth of the plasma membrane be resynthesised every minute (Abercrombie et al 1970c). Warren and Glick (1968) studied the overall plasma membrane turnover using ^{14}C labelled sugars and amino acids, and found the overall rate of plasma membrane turnover to be very much less than this. However, they do state that there may be a more rapid turnover between the membrane and an internal pool which would be masked by an efficient reutilisation of isotope. Also, turnover might occur between different organisational states of the membrane which would not require any macromolecular synthesis. Therefore, Warren and Glick's experiments do not rule out the possibility that the membrane may be continuously reassembled from precursors present in an internal pool.

Harris (1973a) has postulated that membrane is disassembled at the rear of the cell and transported to the leading edge in the form of vesicles or sub-units, reassembled and reinserted into the plasma membrane. However not enough vesicles have been seen to explain the rate of particle transport. Harris (1973a) also postulates that the membrane is pulled rearward by the contractile machinary of the cell and in a later paper (Harris 1976) has invoked a thermodynamic equilibrium between the membrane and its components disassembled in the cytoplasm. Harris (1976) has further proposed that local stretching, provided by the longitudinal contraction of the microfilaments at the leading edge, might shift the equilibrium in favour of membrane insertion, and disassembly might occur at regions of least stretching as proposed by Shaffer (1965) to explain the membrane flow of slime moulds. Evidence for the insertion of whole membrane at the leading lamella has been provided by marking the appearance of haemagglutinin on myxovirus-infected HeLa cells (giant cells) (Marcus 1962). The haemagglutinin was shown to enter the membrane first at the cell margin and move in to occupy the entire cell surface, and a similar appearance of newly synthesised antigens in fused hetero-karyons has been reported by H. Harris et al (1969).

Abercrombie et al (1972) also noted that peroxidase-conjugated Concanavalin A was cleared first at the leading edge in moving chick heart fibroblasts.

As it is thought that bivalent agents are capable of cross-linking the membrane components and immobilising the membrane flow in that region, the above evidence has been subject to a different interpretation. De Petris and Raff (1973) have proposed that particles, bivalent antibodies, lectins, and other entities used to mark the surface may cross-link membrane components and immobilise them allowing unlabelled molecules to move forwards, analogous to the capping of cross-linked antigens which has been observed on lymphocytes (Taylor et al 1971). The clearance of Con A observed by Abercrombie et al (1972) could then be regarded as capping of this bivalent lectin and may not be evidence that whole membrane is inserted at the leading edge. The capping phenomenon has been demonstrated in moving mouse fibroblasts using fluorescent antibody (Edidin and Weiss 1972) but this phenomenon does not occur in some other types of fibroblast (Edidin and Weiss 1973) and epithelia (Edidin and Weiss 1972).

It has been shown that capping is energy dependent in lymphocytes (Taylor et al 1971) and fibroblasts (Edidin and Weiss 1972) and is partially inhibited by cytochalasin B in lymphocytes (Taylor et al 1971). This evidence has lead to proposals that the submembranous contractile system is involved in the process of capping. A hypothesis that the insertion of microfilaments upon the inner face of the plasma membrane, might transform the membrane from a fluid state, into a more rigid structure capable of transmitting tension, was put forward by De Petris and Raff (1973).

However, addition of cytochalasin B to lymphocytes inhibits capping in half of the cells and it is only when colchicine is added simultaneously that capping is abolished (De Petris 1974). Further cytochalasin B does not have any effect on the capping of mouse fibroblast antigens (Edidin and Weiss 1972).

As a result other authors have sought an alternative explanation for membrane flow in capping and tissue cell locomotion. It is difficult to imagine how membrane can be completely reassembled in membrane flow as antigens which have been 'capped' take up to 8 hours to be replaced on the lymphocyte surface and required protein synthesis (Loor et al 1972). However the observation that blebs, (Harris 1973a) and ruffles (Abercrombie 1970b),move backwards with

reference to the substratum in moving fibroblasts is good evidence that the membrane *does* flow backwards although a counter current might exist when Concanavalin A is cleared or capped from the leading edge (Abercrombie et al 1972).

Bretscher (1976) has suggested that there might be a rapid recycling of lipid molecules in the form of vesicles as opposed to the recycling of whole membrane. Bretscher postulates that other membrane molecules such as proteins would remain randomly distributed as a result of their lateral diffusion and points out that the diffusion rates of rhodopsin in the membranes of rods (Poo and Cone 1974) support this idea. A large membrane molecule such as a cross-linked antigen or an adherent particle may not have a sufficient rate of lateral diffusion to overcome the rapid directional flow of lipid and as a result might be swept along *en masse* to be capped. Bretscher goes on to suggest that proteins might be removed at the 'sink' of the cell by a coated vesicle which would act as a molecular filter to remove membrane proteins and much of the cholesterol.

The advantage of Bretscher's hypothesis is that it requires no transduction of information(e.g. the form of a conformational change in the antigen) to the cytoplasmic face of the plasma membrane which would then be recognised by the contractile elements of the cell. Such a transduction is difficult to reconcile with the requirement for cross-linking before capping occurs. A univalent antibody would presumably cause the same conformational change whether it were cross-linked or not and should therefore be capped. Bretscher (1976) prefers to explain the role of microfilaments and microtubules as one of transporting the lipid vesicles from the 'sink' to the front of the cell where they are reinserted in the plasma membrane.

Although Bretscher's theory explains many of the observations associated with lymphocyte capping it is debatable whether the ideas can be directly applied to moving fibroblasts. The observation by Edidin and Weiss (1973) that not all moving fibroblasts form caps when moving on a tissue culture substratum is suggestive that the membrane flow may be quantitatively different at least. Further although the evidence for the involvement of microfilaments in the movement of cross-linked antigens into caps can be questioned, Abercrombie et al (1971) have clearly demonstrated the presence of bundles of microfilaments at the leading edge of chick heart fibroblasts. It is difficult to imagine how these microfilaments could move the cell body forward without inserting on the inner face of the plasma membrane, and although they may not be responsible

for membrane flow their presence cannot readily be explained by Bretscher's theory if the substratum cross-links membrane components. It would seem more likely that some sort of transduction across the plasma membrane resulting from adhesion to the culture substratum is responsible for the condensation of microfilaments. Possibly the theory might be modified to accommodate these anomalies in the future as there is a good deal of evidence in favour of a lipid flow in lymphocytes at least.

Finally, particle transport has been shown to be hindered by the presence of a large external trypsin sensitive protein (LETS protein) in mouse 3T3 fibroblasts (Albrecht-Buehler and Chen 1977). This perhaps suggests that external proteins of this type might exert some control upon the cross-linked molecules or particles being swept along in Bretscher's model.

1.2.6 The Differences Between Epithelial and Fibroblastic Locomotion *in vitro*

Epithelial cells have been observed to migrate from a cultured explant as a coherent sheet with ruffling membrane around the perimeter as distinct from fibroblasts which migrate as single cells (Vaughan and Trinkaus 1966). Subsequent micromanipulation studies by the same workers showed that these sheets are firmly attached to the substratum only at the edges. Holmes (1914) thought that epithelial sheets were pulled outwards by the cells at the perimeter but it might be that all the cells contribute to the movement of the sheet as a whole.

Epithelial cells may migrate as a sheet because of their tendency to adhere strongly by their lateral edges (Lewis 1922, Loeb 1922) and these might be stabilised by the formation of adhesive specialisations consisting of a desmosome-like component and a tight or gap junctional component. These specialisations are analogous to the terminal bar described by Farquhar and Palade (1963) in intestinal columnar epithelium, and have been described in 24 hour cultures of pigmented retina epithelial cells (Middleton 1973, Middleton and Pegrum 1976).

Isolated pigmented retina epithelial cells from the chick embryo have been reported not to exhibit any directional movement and are poorly spread upon the substratum (Middleton 1973). Similar observations have been reported for epithelioid limpet haemocytes

(Partridge and Davies 1974), and chick embryo gut and corneal epithelial cells (DiPasquale 1975a). This phenomenon has been termed contact-induced spreading (Middleton 1976).

The reason epithelial cells need to make lateral contacts with their neighbours before cell spreading and directional locomotion can proceed is obscure but the paucity of microtubules which has been reported in some cultured epithelial cells (DiPasquale 1975a) may be significant. One could argue that epithelial cells require to make intercellular contacts in order to polarise their microfilament bundles, whereas fibroblasts require intact microtubules for polarisation and directional movement.

Pigmented retina epithelial cells at the edge of cell islands have been reported to show cell-substratum adhesion plaques when examined under the electron microscope (Middleton 1973) similar to those found in fibroblasts (Abercrombie et al 1971). However, DiPasquale (1975a,b) does not report the presence of similar plaques in other chick epithelial cells.

1.2.7 The Differences Between Fibroblastic Locomotion *in vivo* and *in vitro*

Bard and Hay (1975) compared the locomotion of fibroblasts from the transparent chick cornea *in situ* with their mode of movement *in vitro*. They noticed that the flat leading lamella was not seen *in situ* but that other general features of movement was the same, namely a gross contraction or recoil of the cell body into the anterior cell process. Alternatively a more subtle flow of cytoplasm was observed without the immediate loss of the trailing cell process. Cells *in situ* were seen to lack ruffles, and blebs, indicating that these structures may be artefacts of tissue culture. Nevertheless such structures seem to enable workers to visualise a phenomenon which may well be membrane flow without the addition of any marker, so reference to them when describing cell contact phenomena is justified.

1.3 Contact Inhibition of Cell Locomotion

Contact inhibition of movement has been defined as being "the directional restriction of displacement on contact" (Abercrombie 1970) and has been demonstrated in fibroblasts (Abercrombie and Heaysman 1953, see also Abercrombie 1970 for a review), and epithelial cells (Middleton 1972). A similar phenomenon (termed 'contact inhibition of extension')

has been demonstrated for nerve fibres (Dunn 1971).

1.3.1 Classical Studies

Abercrombie and Heaysman (1953) demonstrated that the speed at which a chick heart fibroblast moved in tissue culture was inversely proportional to the number of neighbouring cells with which it made contact. More recent studies by Martz (1973) using mouse 3T3 fibroblasts yielded similar results. Abercrombie and Heaysman (1953) showed that the inverse correlation was not dependent upon the age of the culture or the local population density. In further studies on cell speed Abercrombie and Heaysman (1954a) compared the speed of the cells between two explants of chick heart, and the speed of those to the sides. They found that after the outgrowths had made contact, the speed of the cells between the explants was reduced to one quarter of the speed of the cells to the side of the explants. In the same paper Abercrombie and Heaysman (1954a) showed that cell movement between the explants changed from a predominantly outward direction to be more or less equal in all directions, and this tended to produce a uniform distribution of cells.

Working on the assumption that if contact inhibition was absent cells would tend to overlap each other, Abercrombie and Heaysman (1954a) evaluated an index for the amount of overlapping in cell cultures as a measure of the amount of contact inhibition in that culture. They chose to measure as an index the number of nuclear overlaps, as these were easier to distinguish than cytoplasmic overlaps. They expressed this number as a % of the expected number of overlaps, if the cells moved at random within the culture. The advantage of the overlap index is that the behaviour of a large number of cells can be analysed quickly as against filmed collisions which requires a much greater time to obtain a statistically significant amount of data. They found that this nuclear overlap index was about 33% between two explants of chick heart fibroblasts.

The analysis was repeated to examine the interactions between sarcoma cells (lines S37 and S180) and mouse muscle or chick heart fibroblasts (Abercrombie et al 1957). It was found that normal fibroblasts were contact inhibited on collision with normal fibroblasts *or* sarcoma cells but that sarcoma cells proceed relatively unimpeded by the fibroblasts in confronted cultures of the two cell types.

The nuclear overlap index showed that there was a significant decrease in the number of chick heart fibroblasts overlapping either their own cell type, or mouse muscle fibroblasts, from that which would be expected if the cells were randomly arranged. However, the sarcoma cells were observed to overlap both chick heart and mouse muscle fibroblasts more or less at random.

Abercrombie and his co workers have suggested that there may be a link between this "invasive" behaviour of sarcoma cells *in vitro* and the infiltrative properties of cancer cells *in vivo* (Abercrombie and Heaysman 1954b, Abercrombie 1962). Recently Abercrombie and Heaysman (1976) developed an 'invasion index' by plotting the % of cells which were not diverted upon collision with a population of chick heart fibroblasts.

It was found that there was a correlation between the degree of invasiveness *in vivo* by 3 sarcoma cell lines and their 'invasion index'. MCIM invasion index > BAS/56 > 311 > normal mouse fibroblasts.

1.3.2 Contact Paralysis

Time lapse films of fibroblasts moving in culture have shown that when two cells collide ruffling and pinocytosis cease (Abercrombie and Ambrose 1958), this phenomenon has been termed contact paralysis (Wolpert and Gingell 1968), and is similar to the paralysis observed when primary or secondary mesenchyme cells of the sea urchin *Psammechinus miliaris* contact each other (Gustafson and Wolpert 1967). It has also been reported that blebbing ceases in fibroblasts where contact is made with another cell, (Harris 1973a).

Trinkaus et al (1971) noticed that this contact paralysis was limited to the regions near where two cells were in contact and postulated that the mutual adhesion between the cells in some way inhibited the membrane activity in that area. Curtis (1960a) has suggested that when one cell moves over another, the ruffling of one cell will be moving in the opposite direction to the other and this results in an increase in shear upon the two cell membranes, leading to adhesion, and eventually contact paralysis.

When collisions between sarcoma cells and normal fibroblasts were filmed (Abercrombie and Ambrose 1958) it was noticed that neither the sarcoma cell nor the fibroblast exhibited contact paralysis.

However, subsequent studies by Heaysman (1970) have shown that in some cases contact paralysis is non-reciprocal. She noticed that when MCIM sarcoma cells collided with normal fibroblasts the latter underwent contact paralysis, withdrew and changed direction if there was a space available for them to move into. The MCIM sarcoma cells showed no contact paralysis or retraction on collision with the fibroblasts and continued their locomotion unabated.

1.3.3 Contact Withdrawal (Contact Retraction)

After cells susceptible to contact inhibition of movement contact each other and undergo contact paralysis one or both of the cells appears to undergo an abrupt contraction (Weiss, 1958) during which the attenuated leading lamella is drawn towards the nucleus leaving small punctate adhesions at the end of retraction fibrils (Taylor and Robbins 1963). Weiss (1958) called this response contact withdrawal and suggested that it was due to passive recoil of the cells when they exchange their adhesions to the substratum for adhesions with the colliding cell. Curtis and Varde (1964), Maroudas (1973) and Harris (1973c) have shown that fibroblasts can exhibit considerable tension so to this extent the Weiss hypothesis would appear to be feasible. However, subsequent studies using the interference reflection microscope by Abercrombie and Dunn (1975) showed that fibroblasts did not lose 'feet' (presumed to be adhesions with the substratum) on collision with a neighbouring fibroblast. This evidence tends to favour an active contraction rather than a passive recoil, and this view is strengthened by the work of McNutt et al (1971) who showed the presence of contractile microfilaments beneath intercellular contacts.

1.3.4 Possible Mechanisms of Contact Inhibition of Movement.

Curtis (1967) has proposed that cessation of movement in fibroblasts might be due to strong lateral adhesions which are formed between the colliding cells. Gail and Boone(1971) have suggested that the two contacting cells might pull largely in different directions and hence reduce the displacement of the pair. Gail and Boone believe this idea is supported by the finding (Gail and Boone, 1971) that the speed of mouse 3T3 fibroblasts is much less than the speed of their transformed counterparts SV 3T3, as measured by the augmented diffusion constant D* (Gail and Boone 1970), and that contacts between 3T3 cells lasted 3 times as long as contacts between SV 3T3 cells

(Gail and Boone, 1971). Harris (1974) points out that SV3T3 cells make far more contacts than 3T3 cells at high density so that Gail and Boone's model (Gail and Boone 1971) would not account for the lack of 'density dependent inhibition of locomotion' which has been observed in SV 3T3 cells (Gail and Boone 1971).

The theory of differential adhesiveness by which Steinberg (1970) has sought to explain the sorting out from one another of different cell types involves cells breaking weak adhesions in preference for stronger ones until an equilibrium is reached. Martz and Steinberg 1973) and Martz et al (1974) have tried to explain the monolayering of cells in tissue culture in terms of the differential adhesiveness hypothesis. There is indeed evidence that differential adhesion can control the degree to which cells overlap one another in tissue culture. Fibroblasts have been shown to overlap one another to greater extents when cultured on substrata of low adhesiveness (Carter 1965, 1967a, Abercrombie 1970), and Carter argued that this was because the adhesiveness of the cells to each other was now greater than that of the cells to the substratum. Carter also suggested that cells might follow gradient of adhesiveness, thereby collecting on the most adhesive surface, a phenomenon he called 'haptotaxis' (Carter 1965, 1967). Harris (1973b) has since shown however that cells will collect upon a substratum of high adhesiveness without the presence of a gradient. Harris explains his results by saying that the cell processes which adhere to the substratum of high adhesiveness will persist at the expense of those which ad here to the substratum of lower adhesiveness, and hence the cells will collect on the more adhesive substratum.

In addition to the type of contact inhibition which has been described by Abercrombie and his co-workers involving the paralysis of the locomotary machinery (Abercrombie and Ambrose 1958), there are other ways in which overlapping may be restricted. It has been shown that cells can form a monolayer without any paralysis of the ruffled membrane (Vesely and Weiss 1973) and this has been referred to as contact inhibit-:ion 'type 2'. The authors suggest that this form of contact inhibition might result from a preferential attachment of the cells to the substratum, rather than the upper surfaces of the cells. Therefore one would regard this contact inhibition 'type 2' as an example of differential adhesiveness. However differential adhesion cannot explain the "paraphenomena" of contact inhibition 'type 1', such as contact paralysis and contact retrac-:tion. This is illustrated by the observation that if cells reach an interface between a substratum of high adhesiveness and one of low adhesiveness (e.g. cellulose acetate) the cells do not stop ruffling even though they will not pass onto the cellulose acetate (Harris 1973b). The increased overlapping which is observed when cells are cultured upon substrata of low adhesiveness such as cellulose acetate (Carter 1965, 1967a), or agar (Abercrombie 1970), might be regarded as a breakdown in contact inhibition 'type 2'. However, this is not a reliable indication of a breakdown in contact inhibition 'type 1', a point which will be fully discussed in Section 1.3.5.

Nevertheless, ideas related to the differential adhesion hypothesis have been put forward to explain contact inhibition 'type 1'. Abercrombie (1970) has suggested that the cell margins might be adhesive

whereas the dorsal and ventral surfaces are non-adhesive, and certain evidence has come to light which indicates that this might indeed be the case. Dipasquale and Bell (1972, 1974) reported that sarcoma 180, Polyoma virus transformed 3T3 (fibroblast cell lines), KB cells (carcinoma cells), and chick heart fibroblasts were unable to spread or move upon epithelial sheets, or on one another's surfaces. By using time-lapse filming they were able to show that these cells would attach preferentially around the perimeter of the epithelial islands or individual fibroblast cells (Dipasquale and Bell 1974). Recently Vasiliev et al (1975) also reported that confluent sheets of epithelial cell lines were incapable of supporting the adhesion of other epithelial cells. The observation that fibroblasts are able to form monolayers when grown between two glass fibres (Curtis and Varde 1964), would appear to argue against the necessity for a substratum in some types of monolayering, but it does support the idea that cells are capable of forming strong lateral adhesions.

Low resistance junctions have been demonstrated between certain cells (Loewenstein 1968) allowing electrotonic coupling and this together with the discovery that certain cancerous cells showed reduced electrotonic coupling (Loewenstein and Kanno 1967) gave rise to the idea that a chemical message may pass from one cell to another on contact and 'switch off' the locomotary 'machinery'. Subsequent studies however, have demonstrated that other transformed cells such as Novikoff hepatoma cells (Johnson and Sheridan 1971), and virus-transformed 3T3 and BHK cell lines (Potter et al 1966), are commonly electrically coupled, so the correlation between the lack of ionic coupling and the cancerous

state does not hold in some instances. Cell metabolites have also been shown to diffuse between adhering cells *in vitro* (Subak-Sharpe et al 1969), a phenomenon known as metabolic co-operation, and both this and ionic coupling have been shown to be associated with the presence of gap junctions between the cells (Gilula et al 1972).

One candidate for the chemical messanger has been the cyclic nucleotide cyclic AMP (cAMP) which has been shown to restore the morphology of some transformed cultures to that of untransformed ones (Hsie and Puck 1971). Opposite effects have been noted by Jimenez de Asua et al (1973) using insulin which could mediate its effect by decreasing the internal cAMP levels. Johnson et al (1972) noticed that cAMP reduced the rate of cell locomotion in cultured mouse fibroblasts, but this inhibition was found to be less than that produced by contact with another cell (Ramsey 1972).

There are however, many difficulties with the idea of chemical messengers. First, it is difficult to imagine a chemical messenger which could produce an inhibition in a neighbouring cell without inhibiting the cell of its origin (Harris 1974). Second even if the cells were capable of producing a chemical to which they alone were insensitive, the observations that inhibition of ruffling is confined to the regions of contact (Trinkaus et al 1971), and that intercellular contact is necessary for contact inhibition to occur (Abercrombie and Gitlin 1965), would require any hypothetical chemical messenger to be of high molecular weight so that it could not diffuse to other regions of the contacted cell (Harris 1974).

Abercrombie (1970) suggested that contact inhibition of movement might involve some form of recognition depending upon fixed properties of the surfaces of the contacting cells.

Studies using fixed cells indicate physical impedence alone is not sufficient to account for contact inhibition 'type 1' at least in some cases. Cairns and Weiss (cited by Harris 1974) have shown that living cells can move readily onto the surfaces of cells fixed with glutaraldehyde. Similarly fixed cells have been reported to be unable to produce contact paralysis in living fibroblasts, although no increase in overlapping was noted (Vesely and Weiss 1973, Harris 1974). More recently however, Heaysman and Turin(1976) showed that chick heart fibroblasts which had been fixed with zinc chloride according to the method of Warren and Glick (1966), were capable of eliciting contact paralysis and contact retraction in living fibroblasts which collided with them. This method whereby cells are treated with .001M $ZnCl_2$, Tween 20, and .01M Zn Cl_2 in turn makes membranes 'tougher' (Warren and Glick 1966), but the result indicates that contact inhibition may depend at least partly, upon the recognition of some cell surface components which are altered by glutaraldehyde fixation but not by $ZnCl_2$ fixation.

Heaysman and Pegrum (1973) quickly fixed colliding fibroblasts, and prepared them for electron microscopy. They found that within 20 seconds of the cells contacting each other, submembranous microfilaments had accumulated beneath the junction which had a 150-200 A° separation distance between the two cell membranes. Subsequent studies revealed that sarcoma 180 cells did not possess

such filaments on collision with chick heart fibroblasts, and the latter also showed a reduced number of microfilaments Heaysman and Pegrum (1973b). Significantly, these sarcoma cells do not show a contact retraction when colliding with chick heart fibroblasts and vice versa, so there would appear to be a corelation between the lack of a contact retraction and the absence of the microfilament bundles.

This evidence together with that of Heaysman and Turin (1976), is suggestive of a mechanism of contact inhibition involving adhesion of the two leading edges, followed by recognition and transduction across the plasma membrane leading to accumulation of microfilaments at the cytoplasmic face of the plasma membrane. This possibility will be discussed at length in section 4.4.

It is possible that the large external transformation sensitive protein (LETS protein) might be involved in the adhesion and/or recognition stage of this proposed mechanism. Detection of this protein on the surface of mouse 3T3 fibroblasts showed that wherever it was present particle transport was blocked, and also that LETS accumulated at regions of intercellular contact (Albrecht-Buehler and Chen 1977). Weston and Roth (1969) demonstrated that the degree of overlapping in cultures of chick embryo fibroblasts could be increased by the addition of 0.2M urea, and have shown that this change in culture morphology is due to the removal of a cell surface protein (CSP), which is probably homologous with LETS. More recently Yamada et al (1976) showed that addition of a similar protein isolated from chick embryo fibroblasts was capable of reducing overlapping in cultures of virus-transformed

fibroblasts. This might well be effected by increasing the cell to substratum adhesion of the transformed cells as reported by Yamada et al (1976). Weston and Roth (1969) reported that cell to substratum adhesion was not affected by urea treatment although cell to cell adhesion was.

1.3.5 Increase in Overlapping May Not Necessarily Reflect Reduced Contact Inhibition.

It has been reported that fibroblasts will show reduced contact inhibition when cultured on substrata of low adhesiveness (Carter 1965, 1967a Abercrombie 1970) as measured by an increase in the degree of overlapping. Fibroblasts however, are capable of underlapping other fibroblasts under certain circumstances as demonstrated by scanning electron microscopy (Boyde et al 1969), time-lapse filming (Hlinka and Sanders 1972, Harris 1973c) and micromanipulation (Chambers and Fell 1931, Harris 1973c). Harris (1974) has suggested that the more stellate a fibroblast the greater will be the gap between cell and substratum immediately below the nucleus (Algard 1953), and the greater the chance of one fibroblast underlapping another. Increase in the nuclear overlap index may reflect an increase in the degree to which cells underlap each other, and might be influenced by phenomena other than the lack of contact paralysis etc. when two fibroblasts collide (Harris 1974).

It has been shown that cultures of mouse 3T3 fibroblasts (Bell 1972) and BHK 21 hamster fibroblasts (Erickson 1976) transformed by polyoma virus, although exhibiting increased overlapping, still display contact paralysis and contact retraction when they collide head-on. It was concluded that the criss-cross morphology in cultures of these cells was due to cells moving under one another, rather than using the upper surfaces of cells as a substratum for locomotion. Similar observations have been reported by Guelstein et al (1973), who also observed that the extent of underlapping was greater in the virus-transformed fibroblast cultures than in cultures of their untransformed counterparts.

Overlapping may increase by the phenomenon of retraction clumping (Harris 1973b) whereby sheets of fibroblasts lose contact with the substratum, and retract into a strip which may overlie other cells, and thus increase the overlap index. Retraction clumping is more likely to occur when the substratum adhesiveness is low (Harris 1974), and could be partially responsible for the observations made by Carter (1965, 1967a) and Abercrombie (1970) cited earlier.

When cultures of fibroblasts are grown past confluency more layers of fibroblasts form on top of the first layer resulting in an increase in overlapping (Hayflick and Moorhead 1961). Similar cultures have been examined under the transmission electron microscope and layers of extracellular matrix (probably collagen) were seen between the cellular layers (Yardley 1962). Elsdale and Foley (1969) found that they could prevent multi-layering in cultures of human lung fibroblasts by growing the cells in the presence of small amounts of the enzyme collagenase. They demonstrated that the enzyme removed the collagen as fast as it was secreted, and postulated that the presence of intercellular matrix 'insulates' the fibroblasts from each other, rendering them insusceptible to contact inhibition by the cells of the lower layer.

In contrast to the effects discussed above a genuine absence of contact inhibition could be defined as a situation in which:

(1) One cell actually actively moves onto the surface of another.
(2) Does not exhibit contact paralysis.
(3) Does not exhibit contact retraction.

(4) Is not using extracellular matrix (i.e. components which are not associated directly with the lipid bilayer) as a substratum for locomotion.

1.3.6. Contact Inhibition Between Epithelial Cells.

Middleton (1972) has measured the nuclear overlap index in cultures of chick embryo pigmented retina cells and has shown them to be strongly monolayered. He also found that many of the nuclei involved in overlaps were mitotic, and subsequent studies (Middleton 1973) have shown that dividing pigmented retina epithelial cells, despite the presence of retraction fibres which may anchor them to the culture substratum, are positioned on top of the monolayer.

Lash (1955) reported that when sheets of homologous epithelial cells joined movement ceased resulting in the formation of close junctions (as seen by the light microscope), and Smith (1913) made similar observations on collisions between heterologous epithelia. However, Santler (cited by Abercrombie and Middleton 1968) observed that paralysis of ruffled membranes was less marked in collisions between chick embryo crop and lung epithelia.

Middleton (1973) showed that epithelial cells exhibit contact paralysis on collision with their neighbours in culture, but whereas fibroblasts exhibit a gradual narrowing of mutual contact with their partner which results in subsequent contact withdrawal and cell separation (Weiss 1958), epithelial cells tend to broaden the area of mutual contact (Weiss 1958), resulting in the formation of stable contacts (Weiss 1958, Middleton 1973, 1976).

1.3.7. Interactions Between Epithelial Cells and Fibroblasts.

Abercrombie and Middleton (1968) reported that when chick embryo pigmented retina epithelial cells are mixed with fibroblasts (chick heart and chorid), there was some restriction in the degree of overlap between epithelial and fibroblastic cell types. Time-lapse films showed that the paralysis of both epithelium and fibroblast on contact was less marked than in a homotypic collision. When epithelial cells from the proventriculus and gizzard were confronted with chick heart fibroblasts, the mesenchymal cells were diverted by the epithelial sheet, but if they passed the epithelial 'rim' then they were capable of active locomotion.between the epithelial sheet and the tissue culture substratum (Abercrombie and Middleton 1968).

Elsdale and Bard (1975) using human kidney epithelium and lung fibroblasts stated that the fibroblasts were contact inhibited by the epithelial cells, but not vice versa. Wilbanks and Richart (1966) reported that human cervical epithelium was unaffected on collision with cervical fibroblasts and that the fibroblasts showed occasional contact withdrawal although the majority were pushed aside or detached by the advancing epithelial sheet.

Thus it appears that fibroblasts exhibit a form of contact inhibition on colliding with epithelium but the reciprocal response by the epithelium seems not to have been clearly demonstrated.

1.3.8 Cell Types Which do not Exhibit Contact Inhibition of Movement.

Chick macrophages and polymorphonuclear granulocytes (PMNs) have been reported by Oldfield (1963) to overlap chick heart fibroblasts more or less randomly, and similar observations have been reported for rabbit PMNs with chick heart fibroblasts (Armstrong and Lackie 1975).

Macrophages and polymorphs do not overlap one another in culture (Oldfield 1963), but this may be due to contact inhibition 'type 2' as human polymorphs have been reported not to paralyse one another upon contact (Ramsey and Harris 1973).

Malignant carcinoma (Santesson 1935) and sarcoma cells (Abercrombie et al 1957, Abercrombie and Heaysman 1976) have been shown to infiltrate cultures of normal mesenchymal cells. In the latter case it can be seen from time-lapse films that the sarcoma cells actually use the chick heart fibroblasts as a substratum for locomotion (Abercrombie and Ambrose 1958, Heaysman 1970).

This would appear to indicate a loss of contact inhibition in the sarcoma cells on collision with chick heart fibroblasts, but in the case of cultures which exhibit criss-cross morphology the situation is less clear (See section 1.3.5.).

Invasion of one cell population by another may take place by the invasive cell type moving between the cells of the other type (Barski and Belehradek 1965) and therefore this does not indicate a genuine breakdown in contact inhibition as defined at the end of section 1.3.5.

In one instance it has been reported (Rezinkoff et al 1970) that transformed cells exhibit contact inhibition as judged by their inability to move from an explant when confronted with a culture of normal fibroblasts. The normal fibroblasts are unaffected by the presence of the transformed cells. This situation is exactly the reverse of that demonstrated by Abercrombie et al (1957) and Abercrombie and Heaysman (1976), although Rezinkoff et al (1970) did not demonstrate that normal fibroblasts used the transformed fibroblasts as a substratum for locomotion.

1.4. Can Cells Use the Upper Surfaces of Other Cells as a Substratum for Locomotion?

Dipasquale and Bell (1972,1974) and Vasiliev et al (1975) have reported that the upper surface of epithelial cells is not capable of supporting the adhesion or locomotion of a wide variety of epithelial and fibroblastic cells. Often however, observations indicate that pigmented retina epithelial cells from the chick embryo might be able to support at least the adhesion, though not the locomotion of other pigmented retina cells (Middleton 1973, Buultjens and Edwards 1977), chick heart (Middleton 1973) and choroid (Buultjens and Edwards 1977) fibroblasts, and neural retina cells (Buultjens and Edwards 1977).

Dipasquale and Bell (1974) also reported that fibroblasts seeded upon the upper surfaces of fibroblasts sheets were never seen to spread out upon the sheet, and as fibroblasts in monolayers have many gaps between the cells, penetration to the culture substratum usually occurred. They reported that fibroblasts which appear to have adhered to the upper surface of another spread fibroblast might initially attach at the cell margins, and be transported centripetally to the upper surface. Such adhesions are the ones Dipasquale and Bell maintain are those which are measured in the cell sheet collection adhesion assays (Walther et al 1973), Buultjens and Edwards 1977). It is doubtful however if this applies to the adhesion of cells to confluent sheets of epithelium reported by Buultjens and Edwards (1977). Dipasquale and Bell's observations on the restriction of spreading upon epithelial sheets of all cells tested, and the restriction of spreading of fibroblasts on other spread fibroblasts, have been supported by other workers

using explants or single cell suspensions (Elsdale and Bard 1974) and tissue grafts (de Ridder et al 1975).

However, Bell has suggested (cited byTrinkaus see Middleton 1973 in discussion) that pigmented retina epithelial cells may be unable to spread when seeded upon sheets of chick heart fibroblasts, an observation which is not supported by other workers. Elsdale and Bard (1975) reported that explants of human kidney epithelium appeared to spread when seeded onto sheets of human lung fibroblasts, and similar observations have been made by Buultjens and Edwards (1977) using single epithelial cells, and de Ridder et al (1975) using epithelial grafts.

Dipasquale and Bell (1975) argued that it was impossible to be sure that the epithelial cells were spreading on the fibroblasts for the following reasons:

(1) It was not demonstrated that the epithelial cells were not penetrating the diffuse fibroblast sheet and using the culture surface as a substratum for spreading.

(2) Extracellular matrix may have been secreted by the cells which would insulate the two cell types from each other and provide a substratum on which the epithelial cells could spread.

(3) Epithelial cells have never been shown to translocate actively over the fibroblast sheet.

Despite these objections there are some reports which indicate that at least in some cases the upper cell surface is capable of supporting spreading. Mechanically dissociated nerve cells derived from the cerebral hemispheres of chick embryo (Sensenbenner and Mandel 1974)

have been shown to spread out upon sheets of astroblasts (glial cells). Also cells which do not display contact inhibition have been shown to translocate freely upon the upper cell surfaces of other cell types (Abercrombie and Ambrose 1958, Lackie and De Bono 1977).

1.5. Aims of Research.

The long term aim of the kind of work presented in this thesis is to examine the role of cell contact phenomena in the construction of organs, the healing of cutaneous wounds or the invasive behaviour of carcinoma cells. Cell types which are normally invasive *in vivo* usually exhibit reduced contact inhibition *in vitro*, and are capable of migrating over the surfaces of other cells (Abercrombie and Ambrose 1958, Lackie and de Bono 1977).

Dipasquale and Bell (1974) interpreted a fibroblasts failure to spread upon the upper surfaces of other cells purely in terms of their inability to adhere to these surfaces. An alternative hypothesis is that seeded cells adhere but extended processes are inhibited by the same mechanism which operates in contact inhibition 'type 1'. If it is the case that PRE spreads on choroid (Buultjens and Edwards 1977), then PRE should not be inhibited in collision with choroid fibroblasts. The immediate objectives of the study were then, to re-examine whether in fact pigmented retina cells *did* spread upon choroid fibroblasts and to examine whether pigmented retina cells are contact inhibited on collision with choroid fibroblasts.

Cell contact phenomena between epithelial and mesenchymal cells *in vitro* have been largely unexplored (see Abercrombie and Middleton 1968 for a review), and with the exception of one study (Wilbanks and Richart 1966) have employed cells derived from tissues which are widely separated *in vivo*. Therefore, to gain information in this field it would be more sensible to use cells from epithelium and mesenchyme which interact with each other

in vivo, Such a system exists in the 10 day old chick embryo retina where the pigmented retina epithelium and the choroid mesenchyme can be dissected apart (Middleton 1972, Buultjens and Edwards 1977) and cultured independently.

In vivo the retina develops from the optic vesicles which are derived from the neural tube and are therefore ectodermal in origin (See Romanoff 1960). The optic vesicle invaginates to form the double walled optic cup and secondary mesenchyme cells condense onto the outer wall of this cup giving rise to the choroid (Leplat 1912). The outer wall of the optic cup later develops into the pigmented retina epithelium and the inner wall gives rise to the neural retina. So the epithelium and fibroblasts used interacted with each other at an early stage in development. The adhesive interactions between the two cell types have been studied (Buultjens , and Edwards 1977), and a further advantage with the system is that the epithelial cells contain a natural marker in the form of melanin granules. This facilitates the detection of the pigmented retina cells when they are mixed with, or seeded upon cells devoid of this pigment, and enables the spreading of seeded PRE cells to be scored.

The question must be asked whether any results obtained with this retinal system are a general property of epithelio-mesenchymal interactions or not. In an effort to answer this question, two additional cell types from different animals were used in some experiments namely an epithelial cell line from the African green monkey kidney (BSC-1), and the hamster kidney fibroblast line BHK21/C13 (Stoker and Macpherson 1964).

2. MATERIALS AND METHODS.

2.1 Materials

Eggs.

Fertile White leghorn chicken eggs obtained from a local hatchery were maintained at 37°C in a humidified incubator for ten days, and were used on the tenth day.

Growth Media (EFT)

Growth medium was Eagle's minimal essential medium (Glasgow modification) supplemented with 10% foetal calf serum (Gibco Bio-cult Ltd.) and 10% tryptose phosphate broth (Oxoid Ltd.). For growth of cells to be labelled with ^{32}P orthophosphate, tryptose phosphate broth was omitted (EF10).

Balanced Salt Solutions.

(1) Hanks Hepes Solution (HH)

Hanks basic salt solution contained 1 gm/litre glucose and buffered to pH 7.4 with 0.01M HEPES (N-2-hydroxyethyl piper-azine-N^1-2-ethane sulphoric acid). Hanks Hepes supplemented with 10% foetal calf serum (HH10) was used as the incubation medium for experiments lasting less than six hours.

(2) Calcium and Magnesium - free Salt Solution (CMF)

This contained:-

0.12M Sodium Chloride
5mM Potassium Chloride
0.8mM Di-Sodium Hydrogen Phosphate
1.8mM Potassium Di-Hydrogen Phosphate
5.6mM Glucose
19mM Tris-Hcl.

The pH of this solution was adjusted to pH7.85 and for certain procedures was supplemented with 10% foetal calf serum (CMF10).

(3) Tris-saline (Tris).

This contained:-

25mM Tris-HCl
0.14M Sodium Chloride
5mM Potassium Chloride
0.7mH Di-Sodium Hydrogen Phosphate.

(4) Dulbecco's Phosphate Buffered Saline (PBS).

This contained:-

0.14M Sodium Chloride
2.7mM Potassium Chloride
8.1mM Di-sodium Hydrogen Phosphate
1.5mM Potassium Di-hydrogen Phosphate
0.7mM Calcium Chloride
0.5mM Magnesium Chloride

Disaggregating Agents.

(1) Versene

0.55mM solution of EDTA (Ethylene-diaminetetra-acetic acid) in phosphate buffered saline (PH7.4).

(2) Trypsin.

0.25% w/v (Difco 1:250) in Tris-saline (PH7.4).

Nitex Filters.

10,15 and 20 mesh diameter filters were placed in millipore filter assemblies and sterilised in an autoclave (150° C).

Culture Dishes.

35mm. and 60mm. diam. culture dishes were obtained from Falcon Plastics Ltd.

Linbro trays containing 24 16mm. diam. wells, arranged in a 6 by 4 pattern were obtained from Flow Laboratories, Ayrshire.

Scintillation Fluid.
Aquasol was obtained from New England Nuclear.

Silicone Fluid.
Dc 1107 was obtained from Hopkin and Williams Chemicals and made up to a 1% solution in ethyl acetate.

Fixatives.
(1) Glutaraldehyde.

TAAB glutaraldehyde was supplied as a 25% solution (MacFarlane- Robson Ltd.), and made up to 3% on the day of use by diluting in PBS.

(2) Osmium Tetroxide.

Osmium tetroxide (BDH Chemicals Ltd.) was made up to 1% w/v in PBS.

Dehydrating Agents.
Analar ethanol was obtained from William Burrough Ltd.
Amyl acetate was obtained from BDH Chemicals.

Embedding Medium.
All the components of the Spurr embedding medium were obtained from EM scope Laboratories.
The composition was as follows:-

11.5 gms. Vinyl cyclohexene dioxide (V.C.D.)
31.0 gms. nonyl succinic anhydride (N.S.A.)
7.0 gms diglycidyl ester of polypropyleneglycol (DER.736)
0.5 gms dimethyl amino ethanol (S.I.).

The Spurr was made up fresh on the day of use.

Stains For Electron Microscopy.

(1) Uranyl Acetate.

This wasobtained from BDH Chemicals and dissolved in distilled water to give a concentration of 4% w/v. Just before use this was mixed with an equal volume of absolute ethanol to give a final concentration of 2%.

(2) Lead Citrate.

Lead citrate was prepared according to the method of Reynolds (1963) and was centrifuged prior to use.

Both stains were stored in the dark at room temperature.

Radioactive Materials.

Carrier-free ^{32}P orthophosphate was obtained from the Radiochemicals Centre, Amersham, Bucks.

Histological Stains.

Ehrlich's Haemotoxylin.

This was supplied by Searle Ltd. High Wycombe, Bucks.

Eosin Yellowish

This was supplied by Searle Ltd., High Wycombe, Bucks. and made up to a 1% solution in 70% alchohol. Before use three drops of acetic acid were added to the stain.

Xylene and D.P.X.

These were obtained from B.D.H. Chemicals Ltd.

2.2 Methods.

2.2.1 Preparation of chick embryonic primary cells.

Choroid (Ch) and pigmented retina epithelial (PRE) cells were prepared using a method similar to that of Buultjens and Edwards (1977).

The eyes were removed aseptically from twenty four White Leghorn chick embryos and placed in Hanks Hepes solution (room temp.). The corneas were removed and the sclera were dissected away and discarded. The eyes were then washed once in CMF, and transferred to 0.25% trypsin for 5-7 mins. until the choroid coat was loosened. The eyes were next placed in CMF10, the choroid coats were dissected from the eyeball, and transferred to a 15 ml. centrifuge tube containing CMF10. The iris was removed and the vitreous humour and lens extracted, to leave the neural and pigmented retina layers. The PRE was peeled away from the neural retina and pooled in a similar fashion to the choroid.

Both tissues were rinsed three times in CMF (4° C) and left for 1 - 2 mins. in versene (37° C). Following this the tissues were incubated for 15 mins. in 0.25% trypsin (37° C). Both cell types were suspended in EFT and gently aspirated with a pasteur pipette until a suspension containing small clumps of cells was obtained. These were centrifuged for 5 mins. at 1000g, and the pellets resuspended in fresh medium to remove any residual trypsin activity.

The cells were then plated in 60mm. diam. plastic tissue culture dishes at the following concentrations:-

PRE 1.2×10^7 cells

Ch 1.0×10^7 cells

Cultures were maintained at 37°C in a gas phase consisting of 5% CO_2 and 95% air. The appearance of sub-confluent cultures of PRE and Ch can be seen from Plates 1 (a) and 1 (b) respectively.

2.2.2. Cell Lines.

Cell stocks were frozen in a mixture consisting of Eagles medium (13 ml), foetal calf serum (6 ml.), and glycerol (1 ml.) and stored in liquid nitrogen.

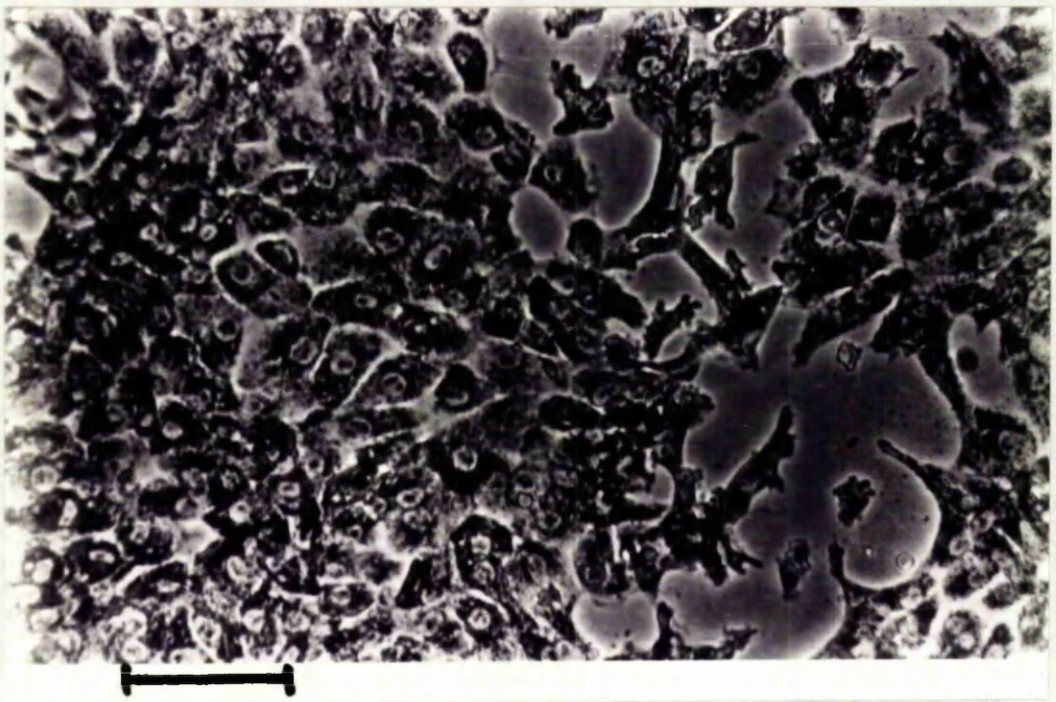
For sub-culturing, confluent monolayers were rinsed twice in Tris, once in 0.05% trypsin in versene, and left to stand until the cells detached. The cells were then shaken off the bottle surface into growth medium, aspirated gently with a pasteur pipette to give a suspension consisting largely of single cells, and replated at the required concentration. Subconfluent cultures of BSC-1 and C13 lines are shown in Plates 1 (c) and 1 (d). The culture medium was EFT, and in the case of the CHO-K1 cell line this was supplemented with 500mM proline.

The cultures were maintained at 37°C in a gas phase of 5% CO_2 and 95% air.

Cells were grown in glass bottles and passaged twice weekly. After eight passages the cells were discarded, and a new stock recovered from liquid nitrogen.

2.2.3. Preparation of Monolayers.

Monolayers were grown overnight in 16 mm. diam. Linbro wells, or on Decon-washed 13 mm. glass coverslips placed in the bottom of the wells. For confluency to be obtained in 12 hours the following concentrations were used.



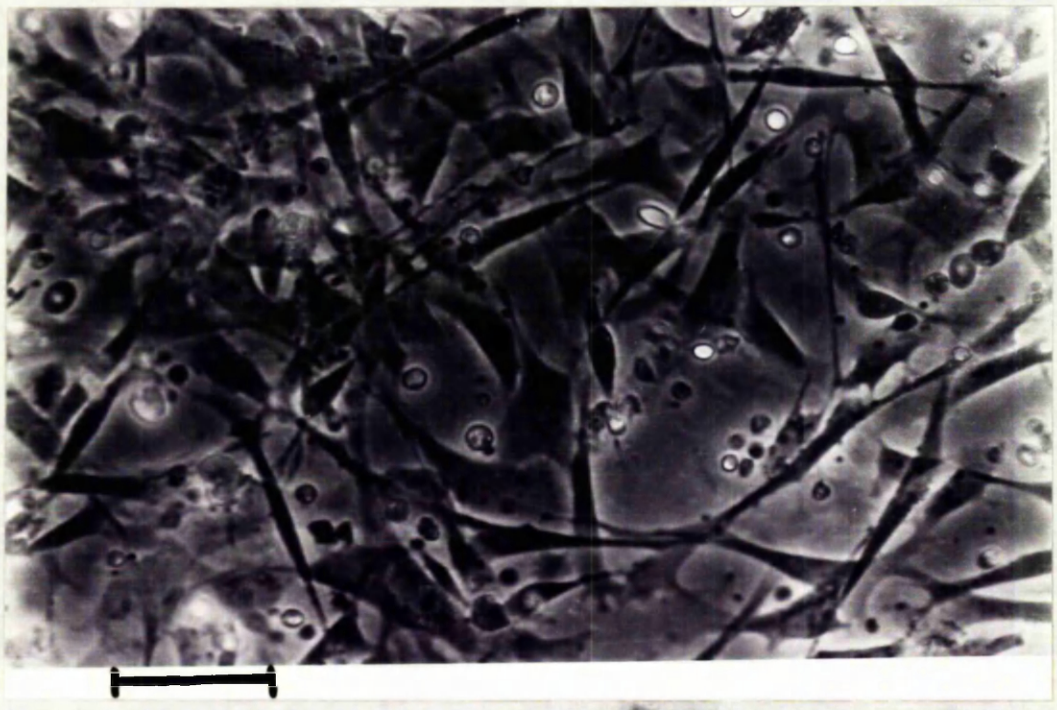


PLATE 1.

Plate 1 (a). A pigmented retina epithelial cell primary culture 16 hours after dissociation.

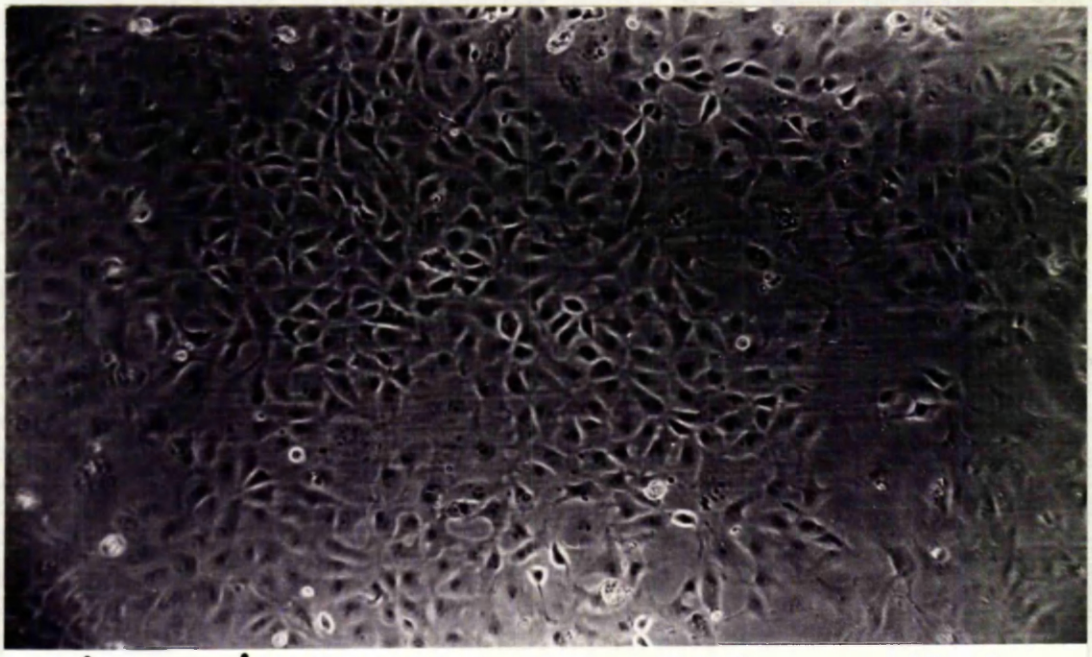
Optics Phase Contrast.

Bar = 33μ

Plate 1 (b). A choroid fibroblast primary cell culture 16 hours after dissociation.

Optics Phase Contrast.

Bar = 33μ



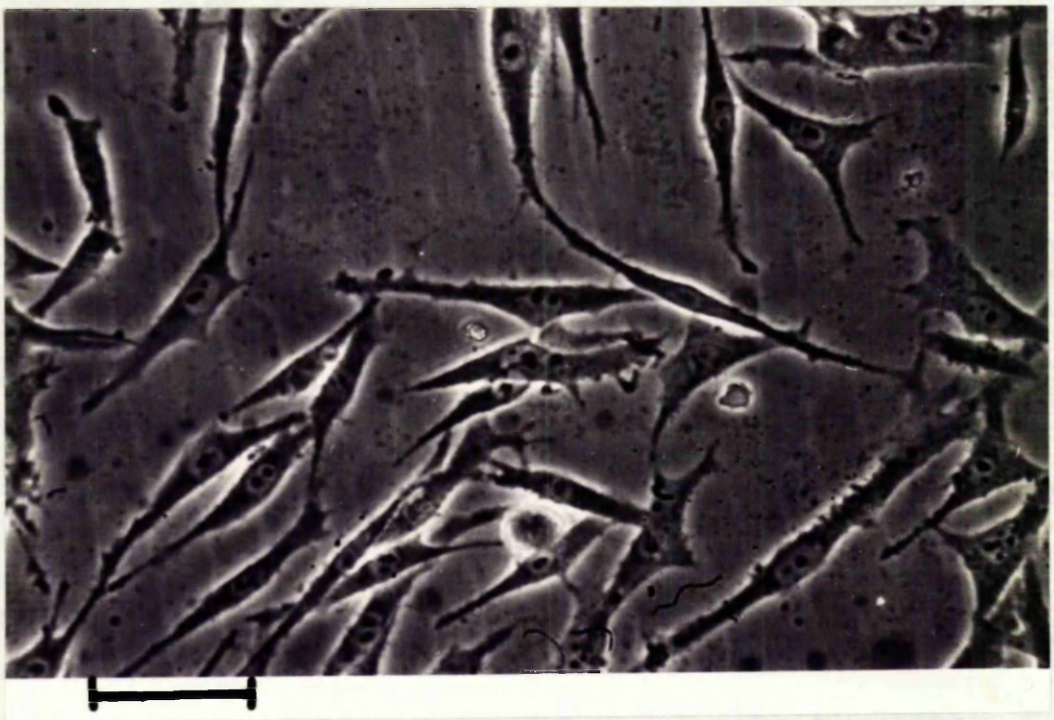


PLATE 1 (contd.)

Plate 1 (c). A BSC-1 kidney epithelial cell line culture (2nd passage) after 24 hours growth.
Optics Phase Contrast.
Bar = 67μ

Plate (d). A BHK 21/C13 hamster kidney fibroblast cell line culture (1st passage).
Optics Phase Contrast.
Bar = 33μ

PRE	1.5×10^6	cells per well
Ch	2.0×10^6	do. do.
BSC-1	0.4×10^6	do. do.
BHK21/C13	0.6×10^6	do. do.
L929	0.5×10^6	do. do.
CHO - K1	0.5×10^6	do. do.

2.2.4. Monolayer Collection Assay.

The assay was carried out in a fashion similar to that of Walther et al (1973).

Cells were labelled with ^{32}P orthophosphate 24 hours prior to the experiment and chased with 'cold' EFT 6 hours before the experiment.

The monolayers were washed three times in HH1O (37°C) and left to equilibrate with this solution for 30 mins.

The labelled cells were removed from the culture vessel in the following manner:-

Embryonic cells were rinsed for 30 seconds in versene (37°C), and then incubated for 5 mins. in 0.25% trypsin (37°C). BSC-1 cells were rinsed twice in versene (37°C), and once in 0.25% trypsin and C13 cells were rinsed twice in Tris (4°C), and once in 0.05% trypsin in versene (4°c).

Once detached, labelled cells were shaken free from the culture vessels, suspended in HH1O and centrifuged at 1000g for 5 mins. Following this the cells were re-suspended in HH1O and driven through a Nitex filter of the appropriate pore size to yield a suspension consisting largely of single cells.

The pore sizes of the filters were:-

PRE	10 µ
Ch	10 µ
C13	15 µ
BSC-1	20 µ

This cell suspension was diluted with HH10 to give 2×10^5 cells per ml., and 0.5ml. of this suspension was added to each monolayer using an automatic pipette. The monolayers and labelled cells were left at 37°C for the duration of the experiment after which the non-adherent cells were rinsed away by washing the monolayer three times in HH (37°C) dispensed in 0.5ml. aliquots from an automatic pipette.

The medium was drained from the monolayers, 1 ml. of 10% ammonium hydroxide solution was added to each monolayer and left overnight to solubilise the radioactivity. The dissolved monolayers were placed in polythylene counting vials together with 10 ml. of scintillation fluid and counted on a Beckmann scintillation counter. Triplicate samples of the cell suspension were treated in a similar fashion to give the total number of counts added to each monolayer. The results were expressed as a % of this total.

2.2.5. Quantitation of PRE Spreading.

The experiments were carried out in a similar fashion to the monolayer collection assay previously described. At the termination of the experiment 13mm. coverslips carrying the monolayers were washed three times in PBS (37°C) and then fixed in 3% glutaraldehyde for 15 mins. Following fixation the monolayers were rinsed

twice in PBS (4°C) and stored at 4°C.

The monolayers and adherent PRE cells were examined under the x 40 objective (Numerical Aperture = 0.17) of a Leitz Ortholux microscope using differential interference contrast optics (Nomarski-type) and photographed using a Canon FP camera loaded with Kodak Panotomic-X film.

The cells were selected for scoring by means of a 25-point Chalkley grid (Curtis 1960b) fitted into the eye pieces of the microscope. The cells were scored as either spread or unspread, if a cell was partially spread the portion the Chalkley grid point intersected was scored. Cells were scored as spread if the pigment granules and/or the nucleus were distinguishable. Ten fields were examined using four orientations of the grid for each field. The total of the spread cells was corrected to allow for the increase in surface area. Middleton (1976) reported that a totally spread PRE cell had approximately 1.5 x the planar surface area of a poorly spread cell. Some cells however would be totally unspread, and therefore this correction does not allow for such cells.

The results were expressed as the % of adherent PRE cells which were classed as totally spread.

2.2.6 Light Microscopy.

Cultures were routinely examined under a Leitz Diavert phase contrast inverted microscope and photographed using a Canon FP camera loaded with Kodak Panatomic-X film.

2.2.7 Scanning Electron Microscopy.

Specimens carried on 13mm diam. coverslips were fixed in 3% glutaraldehyde (PH 7.4) at 4°C for 30 mins. After fixation specimens were given two 15 min. washes in PBS (PH7.4) at 4°C, and then post-fixed in 1% osmium tetroxide (PH7.4) at 4°C for 30 mins.

Following this specimens were dehydrated in 30%, 50%. 70%, 90% and absolute ethanol (2 changes) for 30 mins. each. Absolute ethanol was substituted with amyl acetate by soaking in 50% amyl acetate in ethanol, and then 100% amyl acetate for 30 mins. each.

The specimens were then transferred to a fresh bath of amyl acetate and placed in a critical point drying apparatus. The amyl acetate was displaced from the tissue with liquid CO_2 by flushing two-three times for 5 mins. each during a 90 min. infiltration period. The specimens were then critical point dried according to the method of Anderson (1951).

Dried specimens were glued to the specimen holders using high conductivity paint, coated with a layer of gold 500 Å thick using a Polaron S.E.M. coating unit (E5000), and examined in a Phillips S.E.M.500 .

2.2.8 Transmission Electron Microscopy.

Monolayers grown on 13 mm. diam. millipore filters were washed three times in warm PBS (pH7.4) at the termination of the experiment. Specimens were then fixed in 3% glutaraldehyde (PH7.4) at 4°C for two hours, rinsed three times and soaked overnight in PBS at 4°C. After post-fixation in 1% osmium tetroxide (PH7.4) for one hour, specimens were dehydrated in 35%, 50%, 70%, 90%

ethanol, for ten mins. each followed by several changes of absolute ethanol totalling one hour.

Filters were then soaked in Spurr/ethanol mixtures of 1:1, 3:1, and finally 100% Spurr for 30 mins. each at room temperature. After changing to a fresh bath of Spurr resin the specimens were transferred to an oven at 58°C and the Spurr was polymerised for 19-24 hours.

The blocks were sectioned on a LKB Ultratome I microtome using glass knives and the sections picked up on 400 m. diam. mesh copper grids.

The sections were stained for 5 mins. each in 2% uranyl acetate, and Reynold's lead citrate followed by two 30 sec. rinses in distilled water.

Stained sections were examined in an AEl 801 transmission electron microscope.

2.2.9. Nuclear Overlap Index Measurements.

Cultures containing approximately half the number of cells required to give confluency were rinsed three times in P.B.S. (37°C), and fixed in 3% glutaraldehyde for 15 mins. Following this cells were soaked in 70%, 50% and 30% ethanol respectively for 5 mins. each and left for a further 5 mins. in distilled water. The cultures were then stained for 10 mins. in Ehrlich's Haemotoxylin and washed in tap water until they appeared blue (approximately 5 mins.). After washing in distilled water the cultures were stained for 2 mins. in Eosin, washed in distilled water, and dehydrated

in 30%, 50%, 70%, 90% and absolute ethanol (2 changes) for 5 mins. each. Following this the cultures were cleared in xylol and mounted in D.P.X.

The cultures were examined under the X40 objective of a Leitz Ortholux microscope (bright field) and the number of nuclei (n), their radius (r), the number of overlaps (o), and the total area (A) of each field was estimated. The expected number of nuclear overlaps if the cells were arranged randomly was calculated by the formula:

$$\text{expected overlaps (e)} = \frac{n\ (n-1)}{2} \cdot \frac{\pi(2r)^2}{A}$$

(Abercrombie and Heaysman 1954a).

2.2.10. Time-Lapse Filming.

Cells were grown on Decon-washed 32mm. by 32mm. square glass coverslips and were rinsed three times in HHlO prior to filming. Routinely the filming chamber was a 17mm. diam. circular aperture in a 1mm. thick steel plate with a 32 mm. coverslip sealed onto one side with silicone grease. When differential interference contrast optics were employed 0.7mm thick plates were used. This chamber was filled with HHlO, and completed by sealing the coverslip bearing the cells onto the open side of the chamber using a 50:50 mixture of paraffin wax and vaseline. The optical surfaces were rinsed with distilled water, dried and cleaned with lens tissue before being placed upon the pre-heated stage of a Leitz Ortholux microscope. The preparation was maintained at 37°C by an air curtain incubator.

The cells were filmed using a Bolex camera at an interval of one frame every 15 secs. Alternatively cells plated in 35 mm. diam. tissue culture dishes were placed on the stage of a Leitz Diavert inverted microscope and filmed with a Vinten time-lapse film unit (W. Vinten Ltd., Bury St. Edmunds, Suffolk), using Kodak Plus-X reversal film. Films were developed by Brent Laboratories Ltd. and analysed using a L and W analytical projector.

Cell speeds were estimated by measuring the displacement of the leading lamella, and time was derived from the reading of the frame counter.

2.2.11. Treatment of Glassware.

Glass coverslips 13 mm. diam. and 32 mm. by 32 mm. square were soaked in 10% Decon for 15 mins. rinsed thoroughly in distilled water followed by absolute ethanol, and stored under 70% ethanol.

Sterile universal-containers were siliconised by filling with 1% silicone fluid in ethyl acetate for 10 mins. and after drying briefly were baked in a hot oven 160°C) for 1 hour. These siliconised universals were used to contain single cell suspensions during the monolayer collection assay.

2.2.12. Statistics.

A problem in statistics is to decide whether several samples should be regarded as coming from the same population. Such samples will usually differ, and it must be decided whether these differences signify

different populations, or merely chance variations to be expected from the same population.

The Wilcoxon-Mann-Whitney test was used, and is based upon the rank sum tests of Krukal and Wallis, Wilcoxon, and Mann-Whitney (See Snedecor and Cochran 1972 for details). The only assumptions underlying the use of ranks are, that the observations are all independent, that those within a sample come from a single population, and are all of the same form, Some statistical tests assume approximate normality in addition to those mentioned above. In exploratory research when the investigator does not know much about the type of distribution being sampled, it is advisable to use non-parametric or distribution-free methods such as the test used here.

The test used here is an easy way of comparing two sets of data not necessarily containing the same number of samples, and is accurate provided the number of samples is not too small (i.e. <5 from each group). The $\hat{z}$ statistic is $\sqrt{X^2}$ and is distributed as t_{∞} The data = is arranged in ascending quantitative order, and each sample is given a rank number (1, 2 .... n etc). Following this the rank numbers for each set of data are added up and the $\hat{z}$ statistic is calculated from the formula

$$\hat{z} = \frac{R_1 - [n(n+m+1)/2]}{n.m(n+m+1)/12}$$

Where R_1 = Rank sum for n individuals

R_2 = Rank sum for m individuals

If two samples tie for example the 6th rank then each sample is assigned a rank number of 6.5. When $p \leq 0.05$ this was taken as indicating a significant difference between the two populations.

When P = 0.05 ; $\hat{z} \geq t_{\infty} = 1.96$

3. RESULTS.

3.1. Behaviour of PRE cells upon epithelial sheets.

Middleton (1973) has reported that PRE cells are unable to spread out upon sheets of their own type and similar observations have been made by Dipasquale and Bell (1972, 1974) using other chick embryonic epithelial cells. These studies have different limitations. Dipasquale and Bell used time-lapse filming, which although possessing certain advantages made it impossible to observe more than three or four cells at any one time and no quantitation was possible. Middleton on the other hand observed cultures which had been fixed, bleached and stained but there was no way of telling whether the attached cells he observed upon the upper surface of the sheet, were seeded PRE cells or mitotic cells derived from the monolayer.

Dipasquale and Bell maintain that not only do their seeded epithelial cells not spread but they are only capable of adhering at the free edges of the sheet. Middleton (1973) however, has observed PRE cells apparently firmly attached to the upper surface of a PRE sheet some distance from any free edge.

In the work reported here PRE cells were seeded onto the upper surface of a sheet of non-pigmented BSC-1 kidney epithelial cells, and can be clearly distinguished from the latter in the light microscope by virtue of the presence of melanin granules. PRE cells can be distinguished from BSC-1 cells in the scanning electron microscope by their much smaller size.

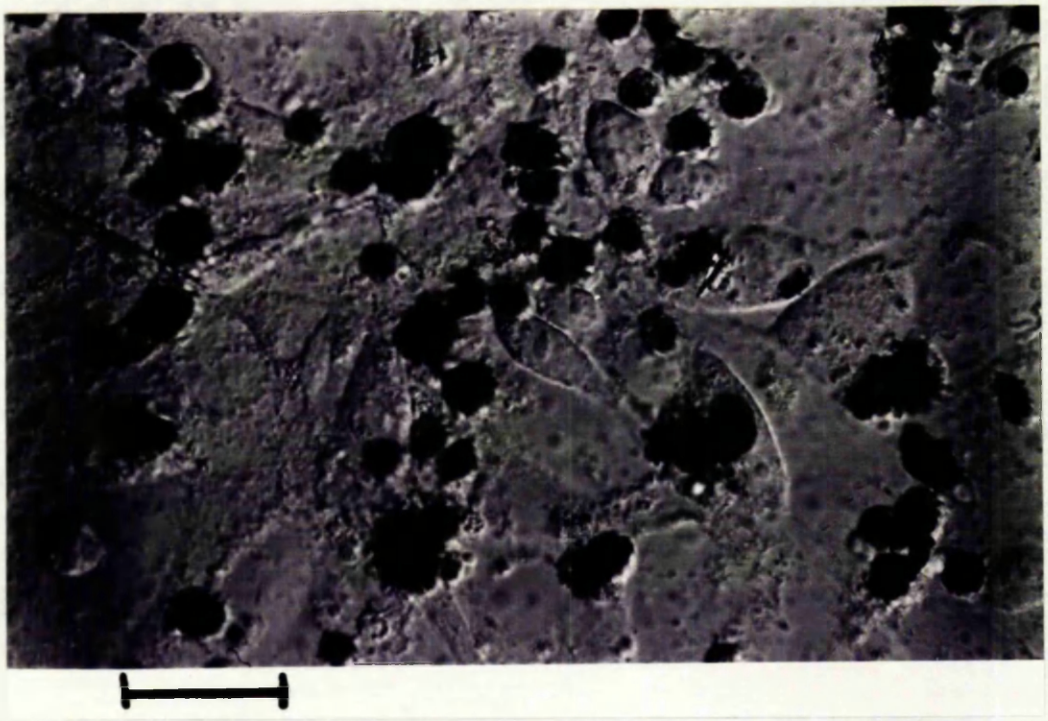
3.1.1. Light Microscope Observations.

The seeded PRE cells were observed not to attach very readily to the upper surfaces of BSC-1 cell sheets, and even after long time periods the majority of these cells were readily detached by rinsing (see section 3.5.1.). Of the cells not removed by rinsing, even the vast majority of these were unspread (see section 3.3) after four hours in culture, as can be seen from Plate 2 (a). The cells are rounded and the individual melanin granules are indistinguishable, in marked contrast to the more polygonal and flattened PRE cells seeded upon a serum coated culture substratum (Plate 2 (b)). In these cells the pigment granules can be clearly distinguished surrounding a pale central nuclear area devoid of pigment.

From time-lapse films the cells upon the BSC-1 sheet appeared to bleb vigorously even when firmly attached to the sheet.

3.1.2. Scanning electron microscopy

PRE cells when seeded onto serum-films (Plate 3(a) and 3 (b)) exhibit a flattened appearance with little evidence of blebbing except at the margins of the cells. Another characteristic of spread PRE cells in culture, is the many microvilli associated with their upper surface. In Plate 3 (a) many PRE cells in various stages of spreading may be seen and those in the early stages of the process possess many filopodia. These are similar to the ones which have been reported to grow out centrifugally at the beginning of spreading in WI38 cells and which measure 800-1600Å in diam. near their tips (Rajaraman et al 1974). For further discussion see section 3.2.2. and also Plate 8 (d).



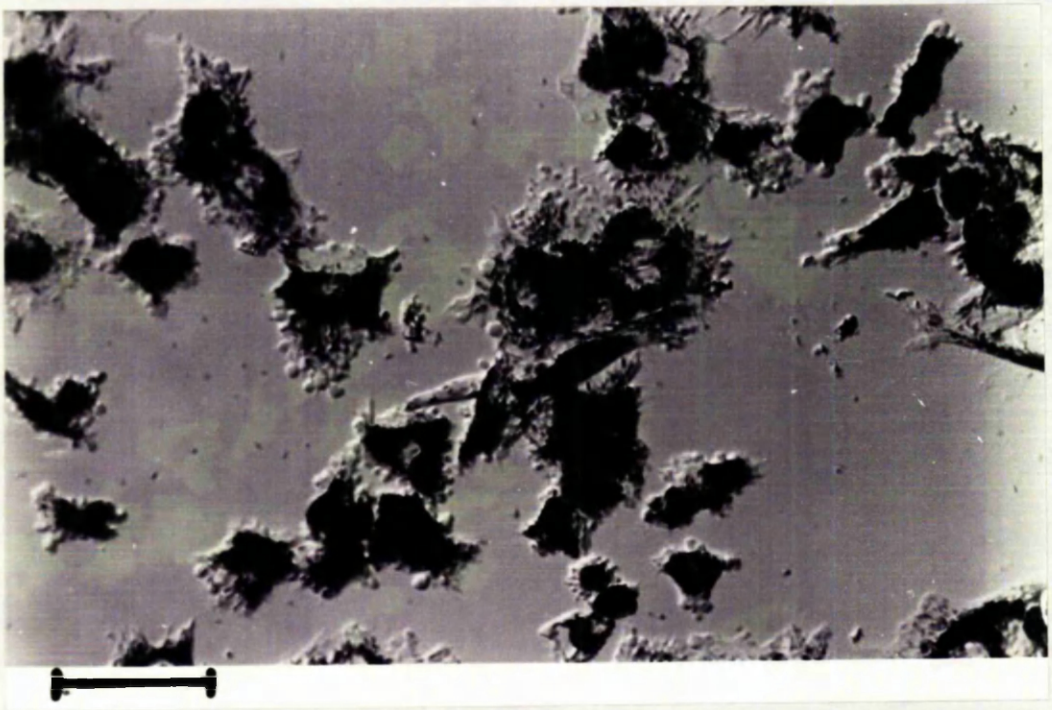
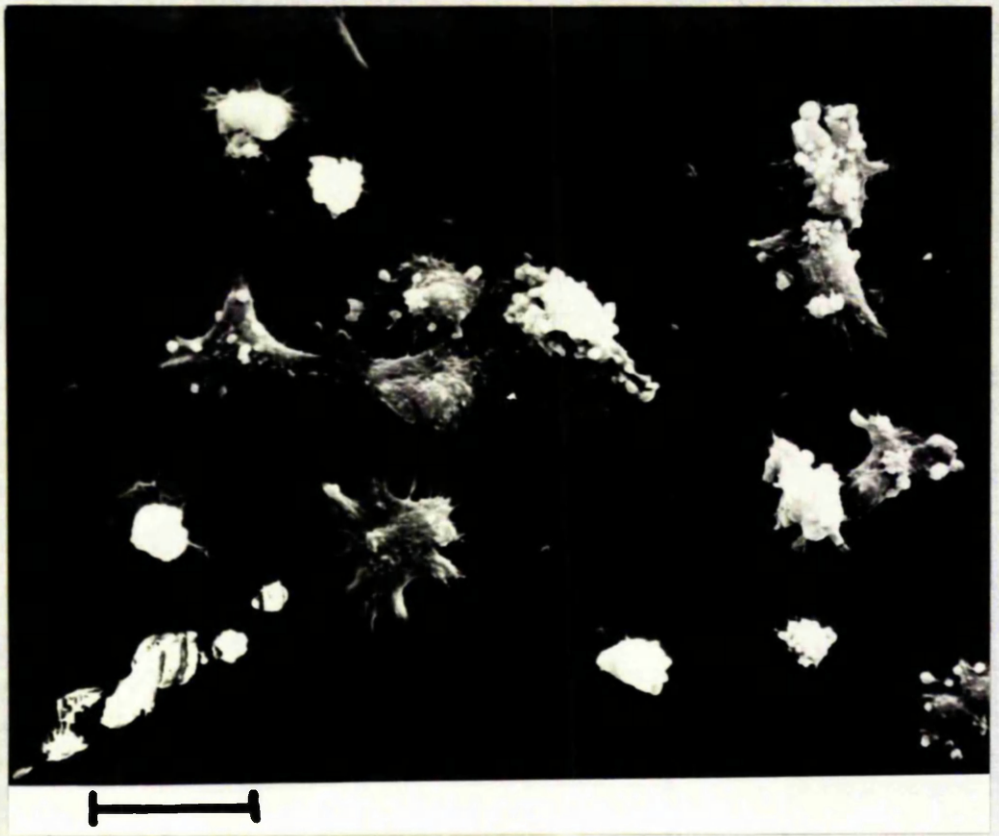


PLATE 2.

Plate 2 (a). PRE cells freshly dissociated from a confluent primary culture adhering to the surfaces of a BSC-1 epithelial cell sheet.
Culture time 4 Hours.
Optics Nomarski.
Bar = 17μ

Plate 2 (b). PRE cells freshly dissociated from the same confluent culture as 2(a) adhering and spreading out upon serum-coated glass.
Culture Time 4 Hours.
Optics Nomarski.
Bar = 17μ



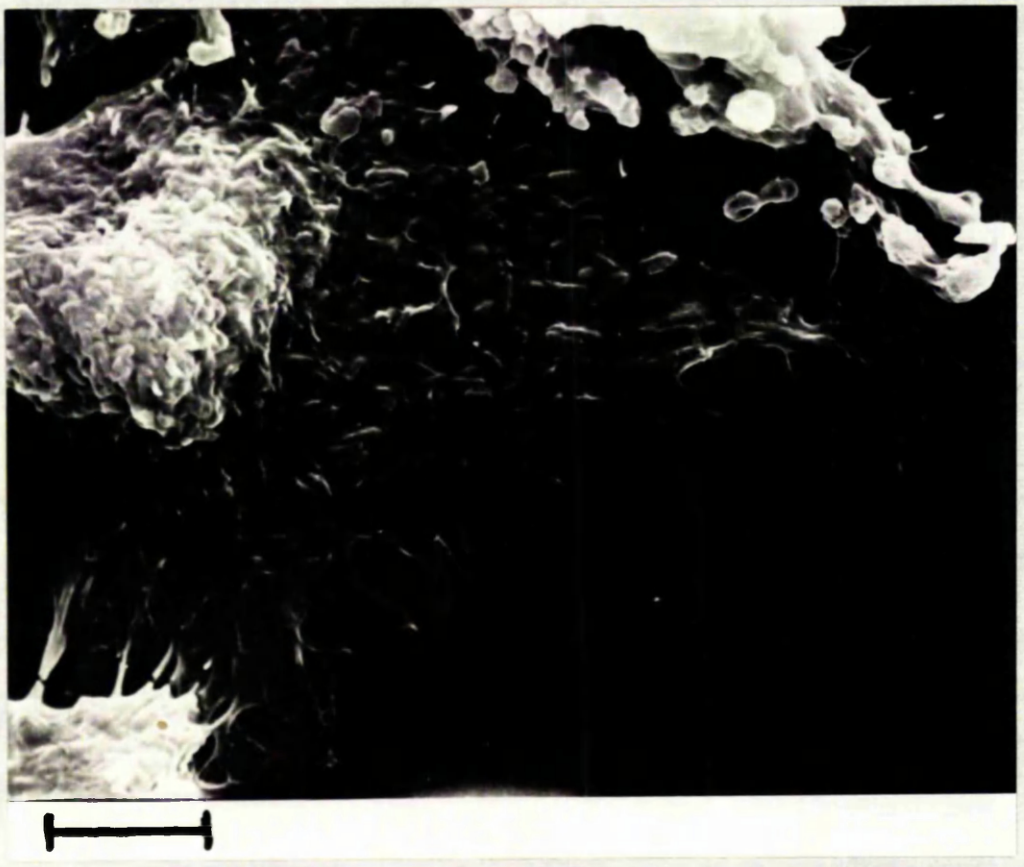


PLATE 3.

Plate 3 (a). Scanning electron micrograph of PRE cells freshly dissociated from a confluent primary culture, adhering and spreading upon serum-coated glass.
Culture Time = 1 Hour.
Bar = 15.6μ

Plate 3 (b). Scanning electron micrograph of the same culture of PRE cells as 3(a) at a slightly higher power.
Bar = 4μ

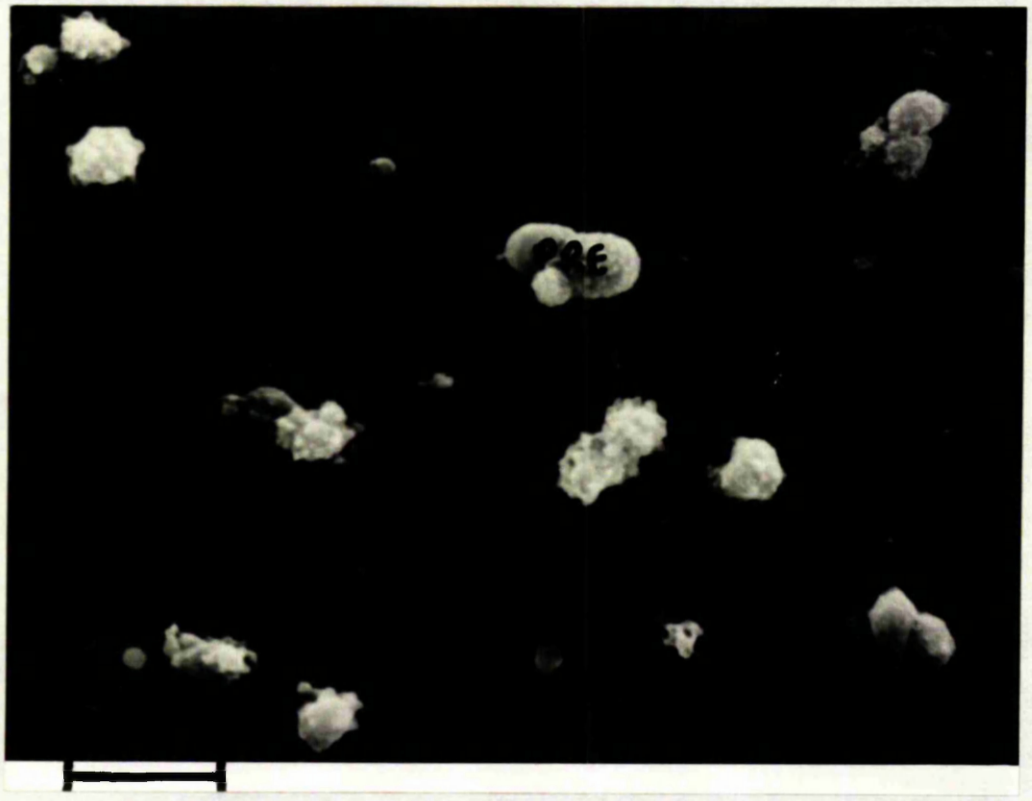
The PRE cells seeded upon sheets of BSC-1 cells exhibit a rounded appearance, their surface is covered with blebs (see Plates 4 (a) and 4 (b)), and no filopodia were ever seen except where the sheet of BSC-1 cells was damaged.

PRE cells seeded upon a sheet of their own cell type showed a similar morphology (Plates 5 (a) and 5 (b)). Although some of these cells may have been mitotic cells (reasons outlined in the introduction to this section), it is felt unlikely that such a large number of cells would be in mitosis at one time. Further the work of Buultjens and Edwards (1977) has shown that up to 30% of the cells in a ^{32}P-labelled PRE suspension are capable of adhering to a preformed PRE sheet (see also section 3.5.1.).

3.1.3. Discussion of microscopical observations.

The results presented here are in agreement with the reports by Middleton (1973) and Dipasquale and Bell (1972, 1974) that epithelial cells are unable to spread out upon preformed epithelial sheets. The evidence reported here however, indicates that PRE and BSC-1 epithelial sheets are capable of supporting the adhesion of at least a small proportion of the seeded PRE cells. These cells do not appear to originate in the epithelial sheet and are sufficiently well attached to resist rinsing, fixing and critical point drying. The results do not therefore support the claim by Dipasquale and Bell (1972, 1974) that the upper surfaces of epithelial sheets in tissue culture are totally non-adhesive.

The possibility that the seeded PRE cells may be adhering to singularities such as gaps in the sheet or


PRE

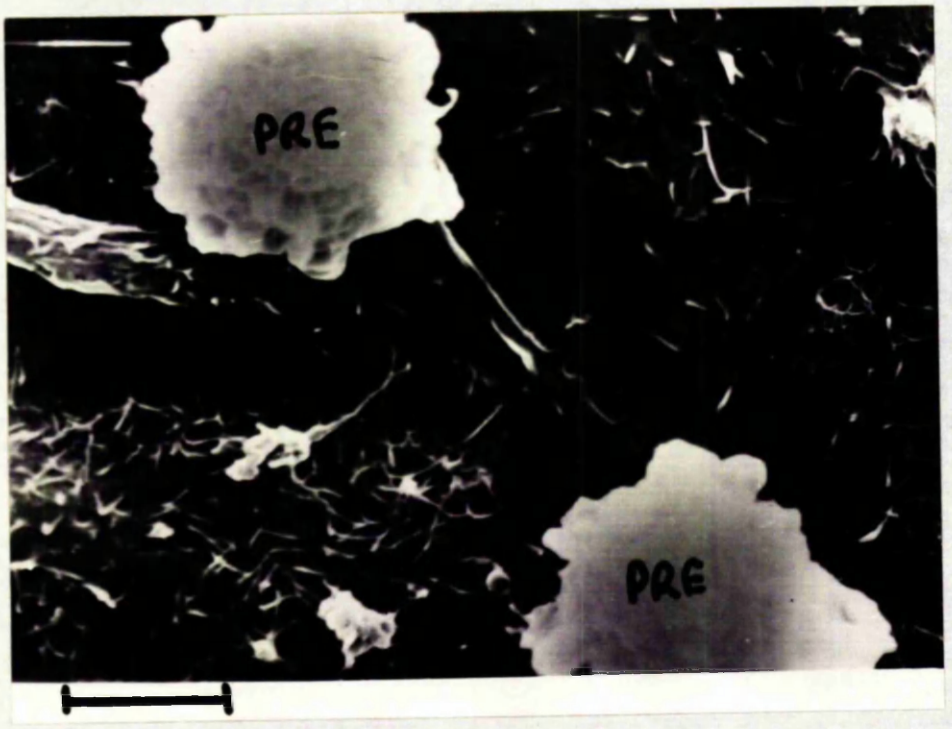
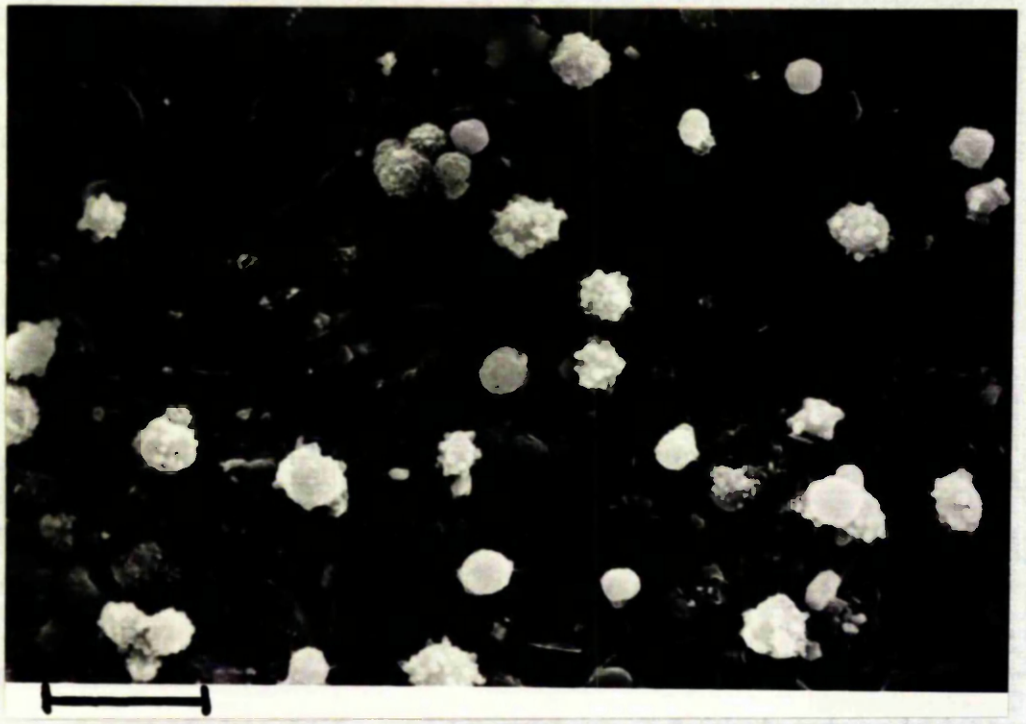

PRE
PRE

PLATE 4.

Plate 4 (a). Scanning electron micrograph of freshly dissociated PRE cells from the same primary culture as the cells in Plate 3(a) and 3(b) adhering to a sheet of BSC-1 kidney epithelial cells.
Culture Time = 1 Hour
Bar = 17.3μ

Plate 4 (b). Similar to 4(a) showing a different field at higher magnification.
Bar = 4μ
PRE = Pigmented retina cell.
BSC = BSC-1 cell sheet.



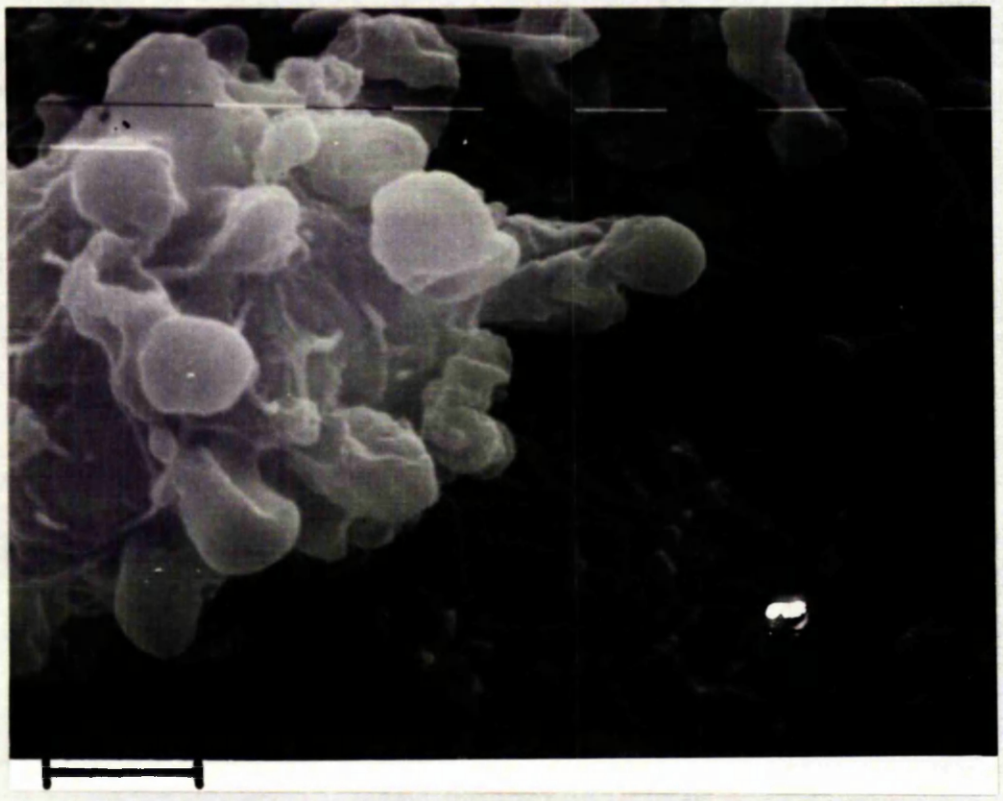


PLATE 5.

Plate 5 (a). Scanning electron micrograph of the same suspension of PRE cells as in plates 3 and 4 after adhering to a sheet of PRE cells.

Culture Time = 1 Hour

Bar = 15.6μ

Plate 5 (b). A different field of 5(a) seen at a higher magnification.

Bar = 1.6μ

the adhesive free edges of cells in damaged regions of the sheet must be considered. Buultjens and Edwards (1977) have argued against this idea however. They found that if the number of cells seeded onto each cell sheet was reduced a lower fraction of the suspension adhered to the sheet. This was the opposite result from that which would be expected if the cells were adhering to limited singularities of any kind. Vasiliev et al (1975) reported that certain epithelial cells were only able to engage in phagocytosis at their free edges in vitro. They correlated the inability of confluent sheets of epithelial cells to engage in phagocytosis, with the low adhesiveness of their upper cell surfaces. Buultjens and Edwards (1977) suggested that only epithelial cells which were phagocytic as a sheet might have adhesive upper surfaces, as PRE are known to possess this function invivo (Young and Bok 1969). The ability of BSC-1 cells to support adhesion of PRE cells would be consistent, only if these cells can also engage in phagocytosis but no information is available on this point.

The inability of PRE cells to spread out upon preformed epithelial sheets seems to becorrelated with a lack of filopodia, and the retention of a large number of blebs. The absence of filopodia in PRE cells adhering to epithelial sheets would presumably indicate that these cells do not begin to spread. The retention of blebs by the unspread PRE cells is in agreement with earlier ideas that these structures, together with microvilli, may be sources of reserve membrane which is used as the cell spreads (Follett and Goldman 1970, Erickson and Trikaus 1976). PRE cells spreading upon serum coated glass (Plates 3 (a) and 3 (b)) gradually lose their blebs

and the last blebs to disappear are those which are closest to the edges.

3.2. Behaviour of PRE cells upon fibroblast sheets.

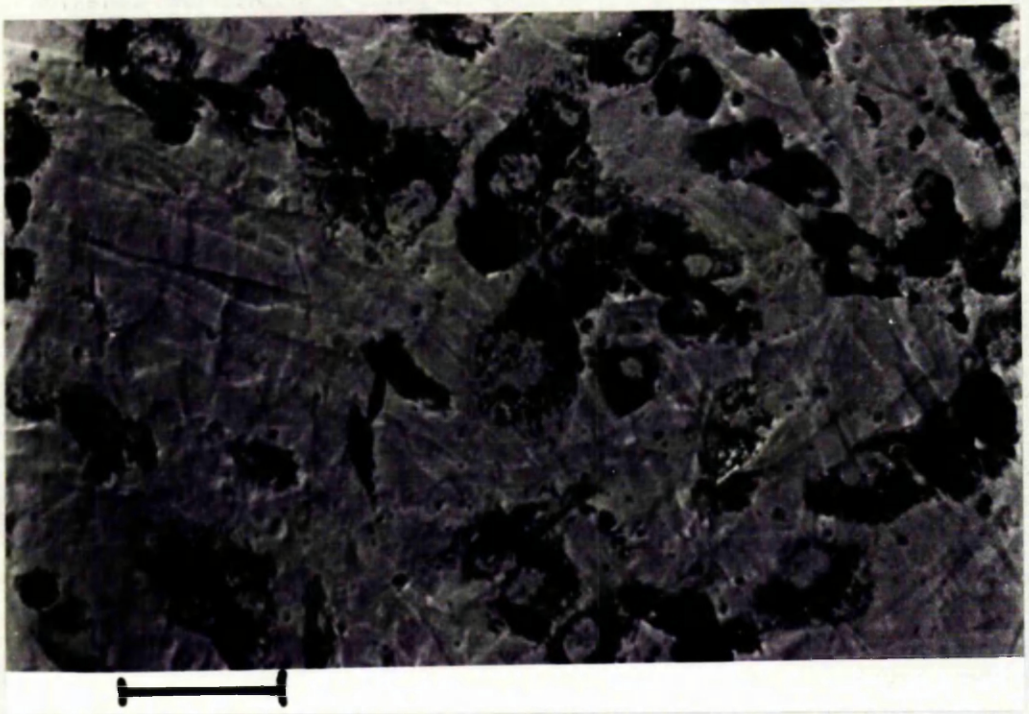
A number of epithelial cell types have been reported to be able to spread upon fibroblast sheets (see section 1.4). In apparent conflict with this Bell (cited by Trinkaus (in discussion, Middleton 1973)) reported that PRE cells do not spread when seeded upon sheets of chick heart fibroblasts.

3.2.1. Light microscope observations.

Seeded PRE cells were observed to attach to sheets of Ch and C13 fibroblasts, and to begin to spread out after about 20-30 minutes. At the end of four hours the majority of the PRE cells were fully spread out (Plates 6 (a) and 6 (b)), and were morphologically very similar to the cells seeded upon serum films (Plate 2 (b)). Large islands of up to ten cells could be seen at this stage, and these were derived from a cell suspension consisting mostly of single cells. This tendency of PRE cells to congregate into islands seemed to be a result of the phenomenon of contact induced spreading, which has been described by Middleton (1976) for PRE cells on a tissue culture substratum. The PRE cells did not appear to spread upon the fibroblasts until they made lateral contact with a neighbouring PRE cell.

3.2.2. Scanning electron microscope observations.

The results confirm that the PRE cells do spread out upon choroid fibroblast sheets (Plates 7 (a) and 7 (c)), and that the edges of these cells were clearly



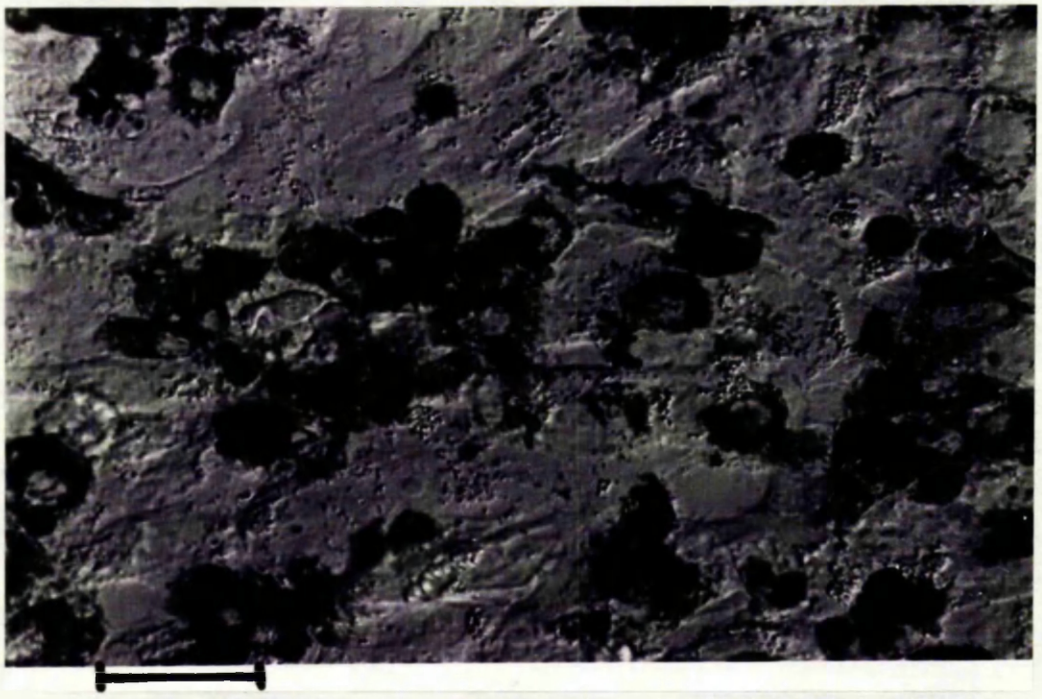
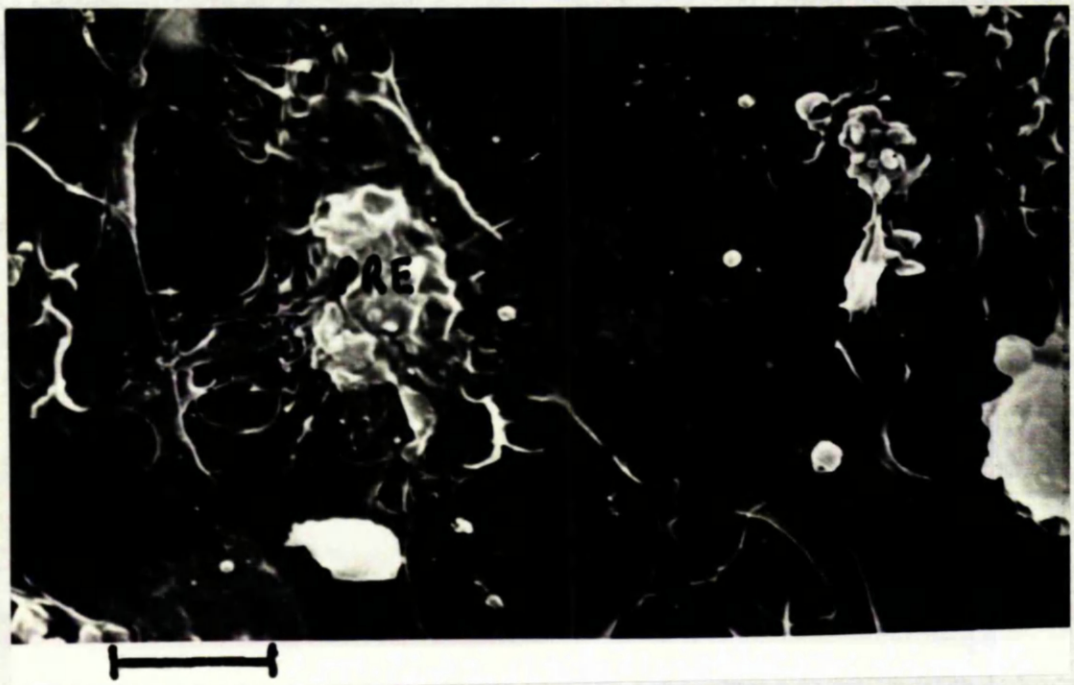


PLATE 6.

Plate 6 (a). A light micrograph of the same PRE cell suspension as in Plate 2 after adhering and spreading upon a sheet of choroid fibroblasts.
Culture Time = 4 Hours.
Optics Nomarski.
Bar = 17μ

Plate 6 (b). The PRE cell suspension after adhering and spreading upon a sheet of BHK 21/C13 fibroblasts.
Culture Time = 4 Hours.
Optics Nomarski.
Bar = 17μ



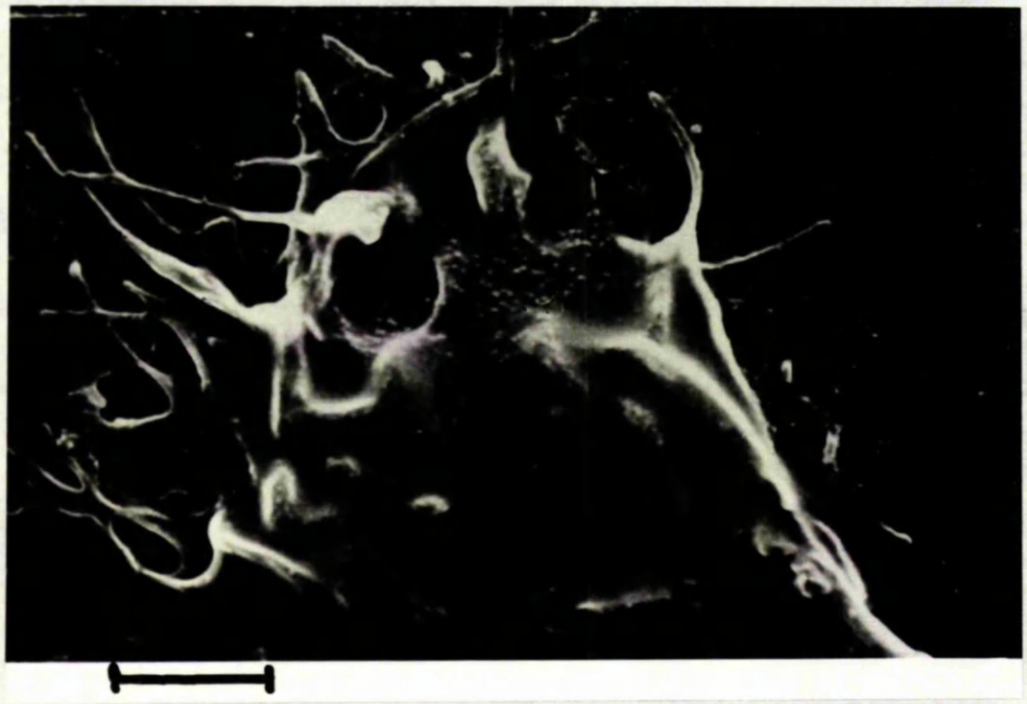


PLATE 7.

Plate 7 (a). Scanning electron micrograph of a PRE cell suspension after adhering to and spreading upon a sheet of choroid fibroblasts.
Culture Time 40 mins.
Bar = 7μ
PRE = Pigmented Retina Cell.
Ch = Choroid Sheet

Plate 7 (b). Scanning electron micrograph of the same field as 7(a) at a higher power.
Bar = 1.7μ
PRE = Pigmented Retina Cell.
Ch = Choroid Sheet.

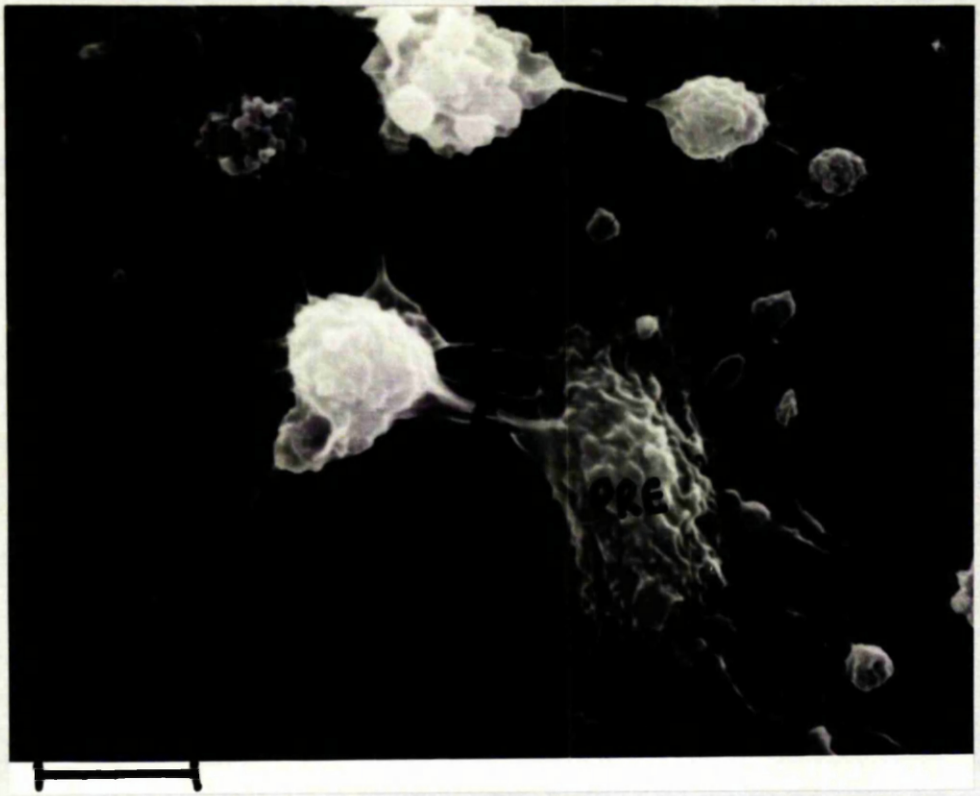

PRE

PLATE 7 (contd.)

Plate 7 (c). Scanning electron micrograph of the same preparation as 7(a): A different field.

Culture Time = 1 Hour.

Bar = 7μ

PRE = Pigmented Retina Cell.

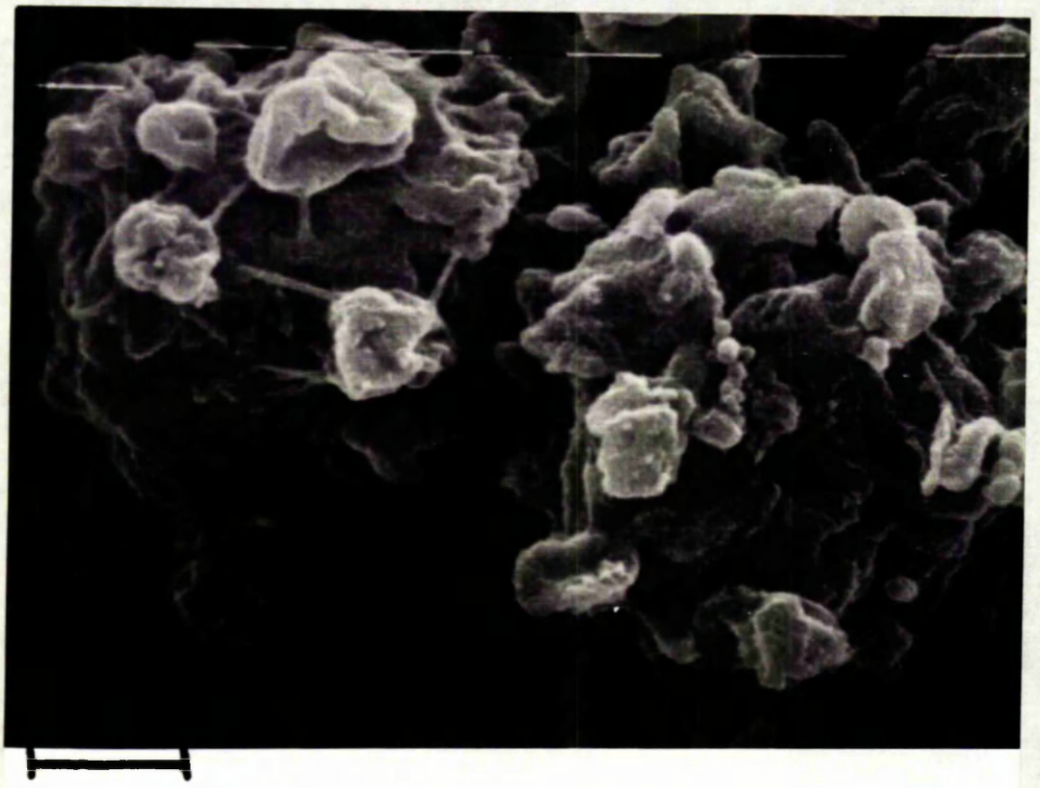
Ch = Choroid Sheet.

adhering to the backs of the choroid cells (Plate 7 (b)). The fully spread PRE cells on the fibroblast sheet were similar in dimensions and morphology to those spreading upon the serum-films (Plates 3 (a) and 3 (b)). The PRE cells could be clearly distinguished from the smooth choroid cells, by the presence of microvilli on the upper surface giving the former cell type a more roughened appearance.

PRE cells fixed at earlier times on sheets of choroid (Plate 8 (a)) were found to be rounded, and to be covered with blebs similar to the cells seeded upon epithelial sheets (Plates 4 and 5). However it can be seen that cells adhering to the fibroblast sheets begin to produce filopodia as early as ten minutes after attachment (Plate 8 (b)), and these increase in number as spreading proceeds (Plate 8 (c)). The filopodia when seen under high power (Plate 8 (d)) measured 1000A° in diam. near their tips, and in this respect were similar to those described for WI 38 cells by Rajaraman et al (1974).

3.2.3. Comparison with the behaviour of choroid cells upon choroid sheets.

When the behaviour of PRE cells upon a choroid sheet, was compared with the behaviour of a choroid cell on the same or a similar sheet a number of differences were noticed. A choroid fibroblast seeded upon a sheet of similar fibroblast cells adheres rapidly but does not spread out. Processes of the seeded cells appear to penetrate the spaces between the constituent cells of the sheet as observed by high power phase contrast microscopy. The seeded cells were then seen to squeeze



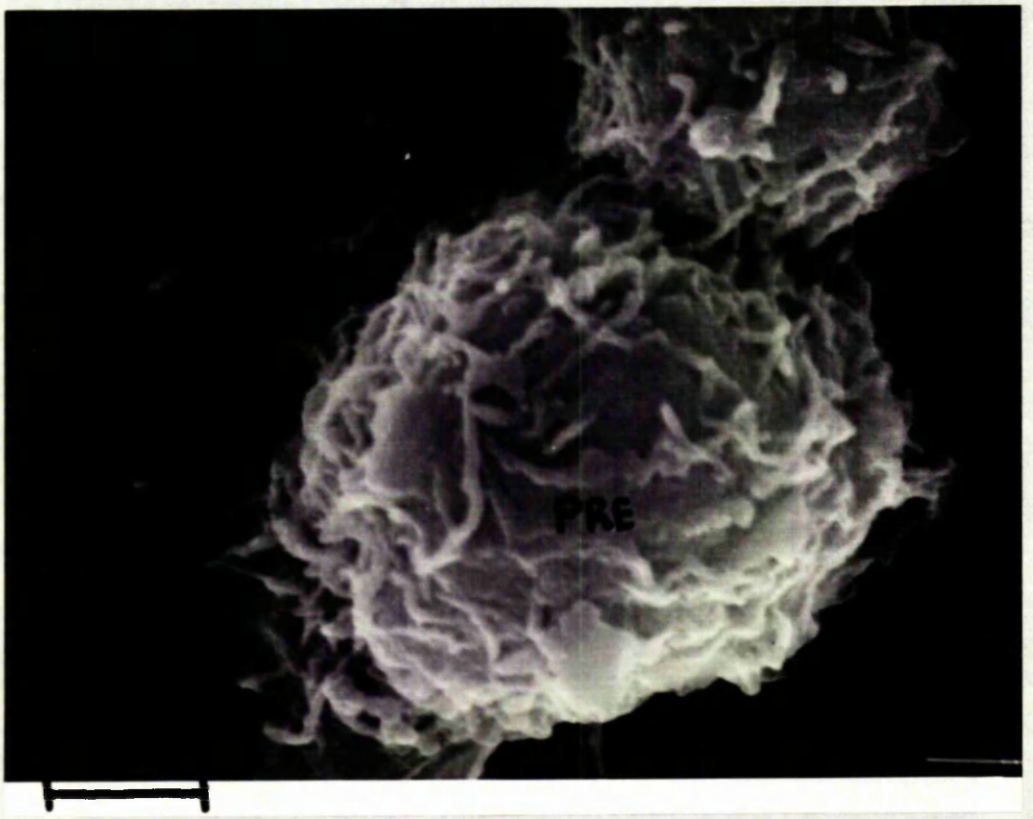
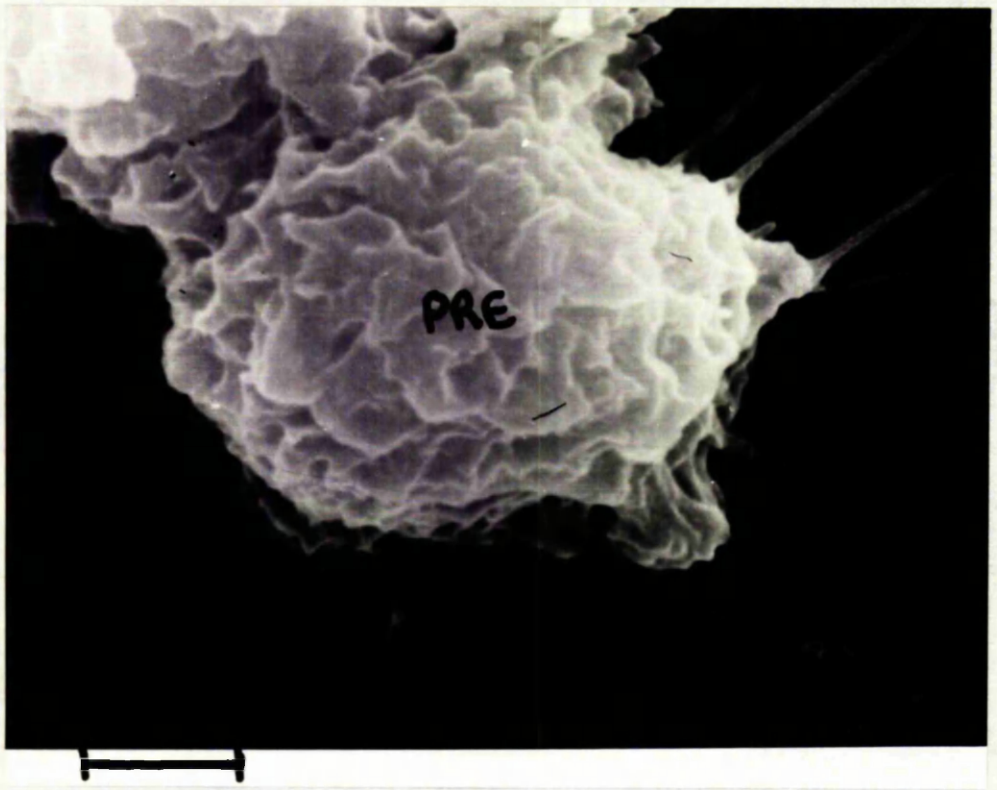

PRE

PLATE 8.

Plate 8 (a). Scanning electron micrograph of the same PRE cell suspension as Plate 7 after adhering to a sheet of choroid fibroblasts.
Culture Time = 10 mins.
Bar = 1.5μ
PRE = Pigmented Retina Cell.
Ch = Choroid Sheet.

Plate 8 (b). As Plate 8(a).
Bar = 1.5μ


PRE

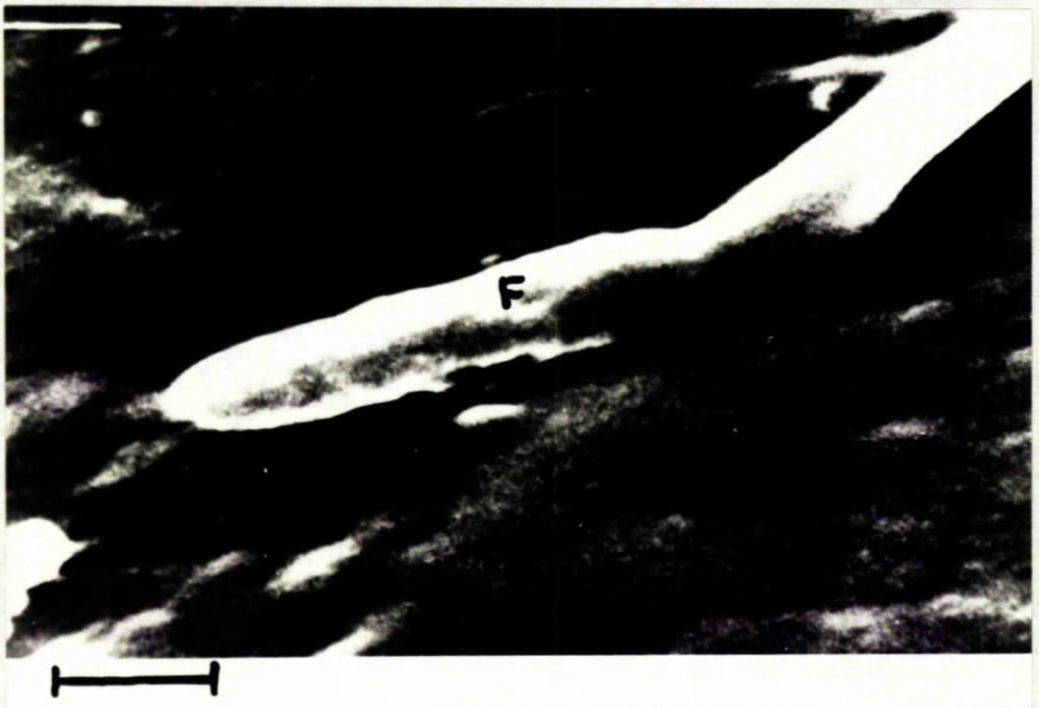

F

PLATE 8 (contd.)

Plate 8 (c). As Plate 8(a).

Culture Time = 30 mins.

Bar = 1.5μ

Plate 8 (d). Scanning electron micrograph of a filopodium of a PRE cell after adhering to a sheet of choroid fibroblasts.

Culture Time = 30 mins.

Bar = 0.4μ

F = Filopodium of a PRE cell.

Ch = Choroid Sheet.

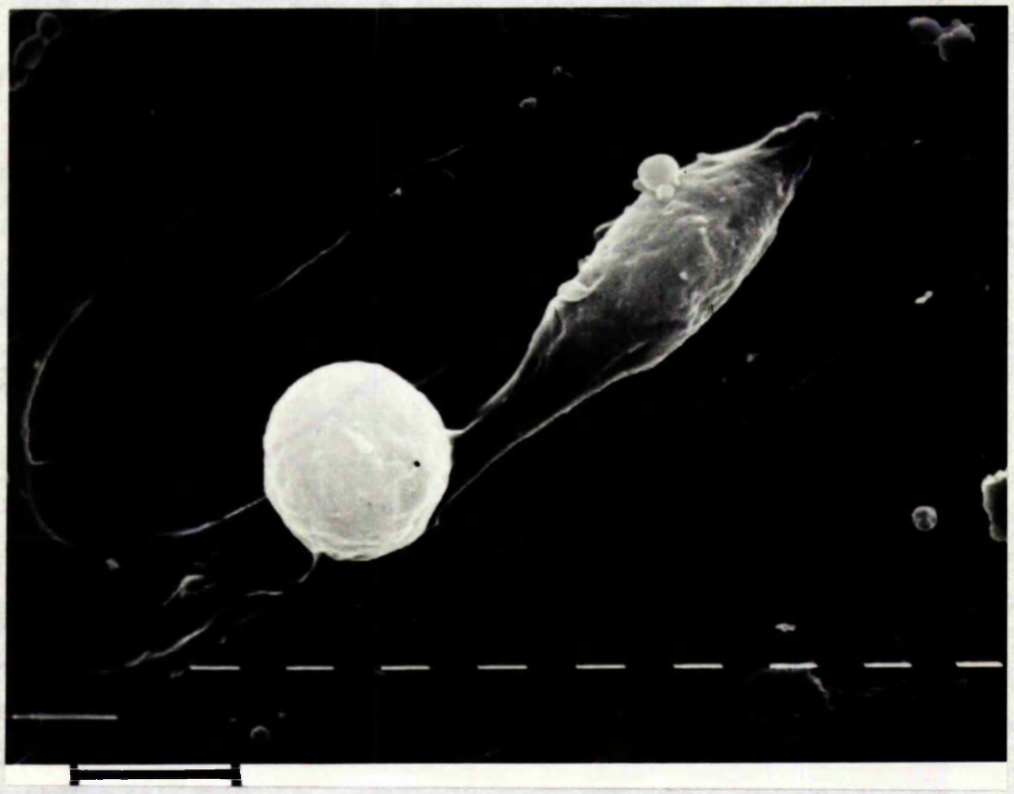
between the cells of the sheet, and to become a part of that sheet. Seeded cells began to sink into the sheets after forty five minutes of incubation, and incorporation into the sheet was usually complete after ninety minutes.

Examination of similar cultures under the scanning electron microscope revealed that at early times seeded choroid cells on choroid sheets exhibited few filopodia, and when these were present they appeared to be probing between two of the constituent cells of the sheet. These results confirmed the observations gained from high power light microscopy. After one hour's incubation few seeded cells could be seen on top of the sheet, and those that remained exhibited a rounded or a spindle-shaped morphology (Plate 9 (a)). The spindle-shaped cells appeared to be adhering at either end to singularities; either to the culture substratum, or the edges of the fibroblasts within the sheet. In such cells no lateral spreading onto the sheet or laterally oriented filopodia were ever seen. These cells were morphologically distinct from choroid cells spreading upon serum-films (See Plate 9 (b)).

3.2.4. Discussion.

The behaviour of PRE cells and choroid fibroblasts when seeded upon serum-films and choroid sheets is summarised in Figs. 1 and 2 respectively. The two cell types exhibit a marked difference in behaviour in the latter situation (see section 3.2.1 to 3.2.3. for details).

Dipasquale and Bell (1975) have suggested that such apparent behavioural differences might be due to



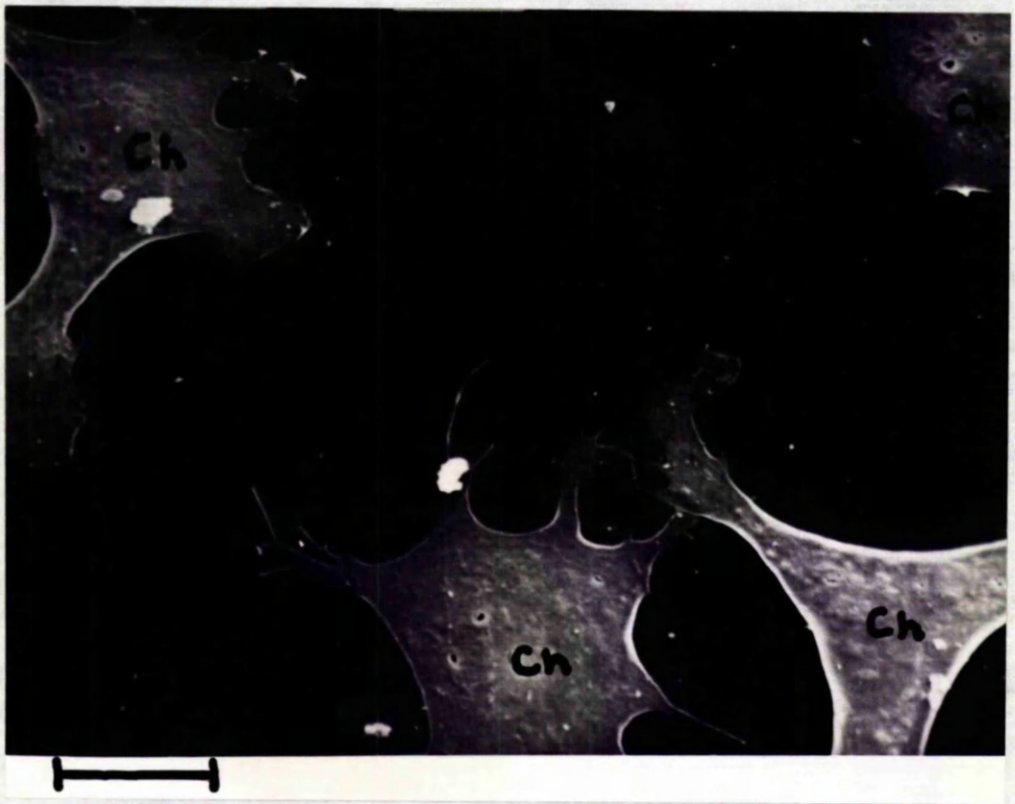

Ch
Ch
Ch

PLATE 9.

Plate 9 (a). Scanning electron micrograph of freshly dissociated choroid fibroblasts derived from a confluent primary culture after adhering to a sheet of their own cell type.

Culture Time = 1 Hour.

Bar = 3.3μ

Plate 9 (b). The same choroid fibroblast suspension as in 9(a) after adhering and spreading upon serum-coated glass.

Culture Time = 1 Hour

Bar = 15.6μ

Ch = Choroid Cell.

Fig. 1

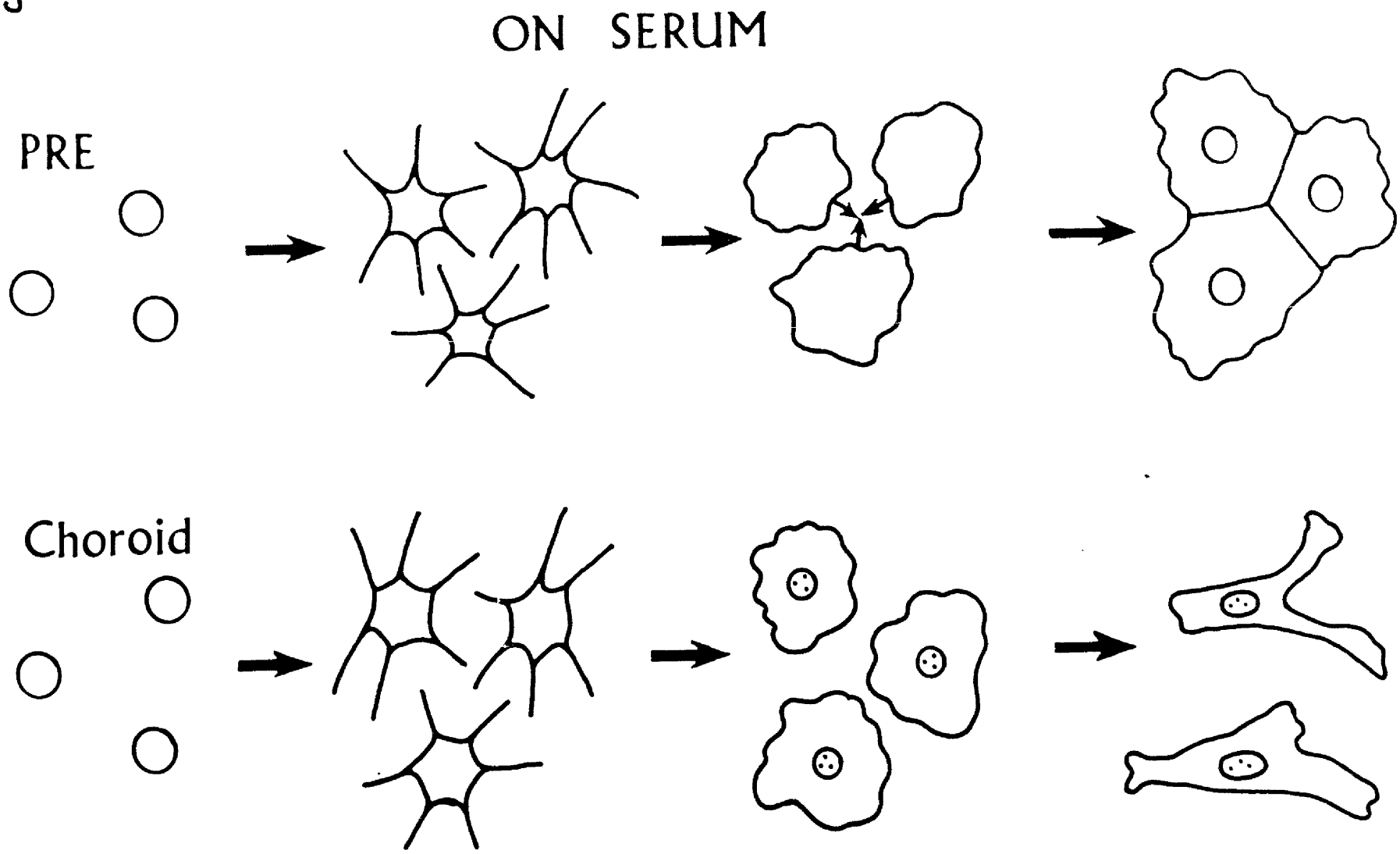
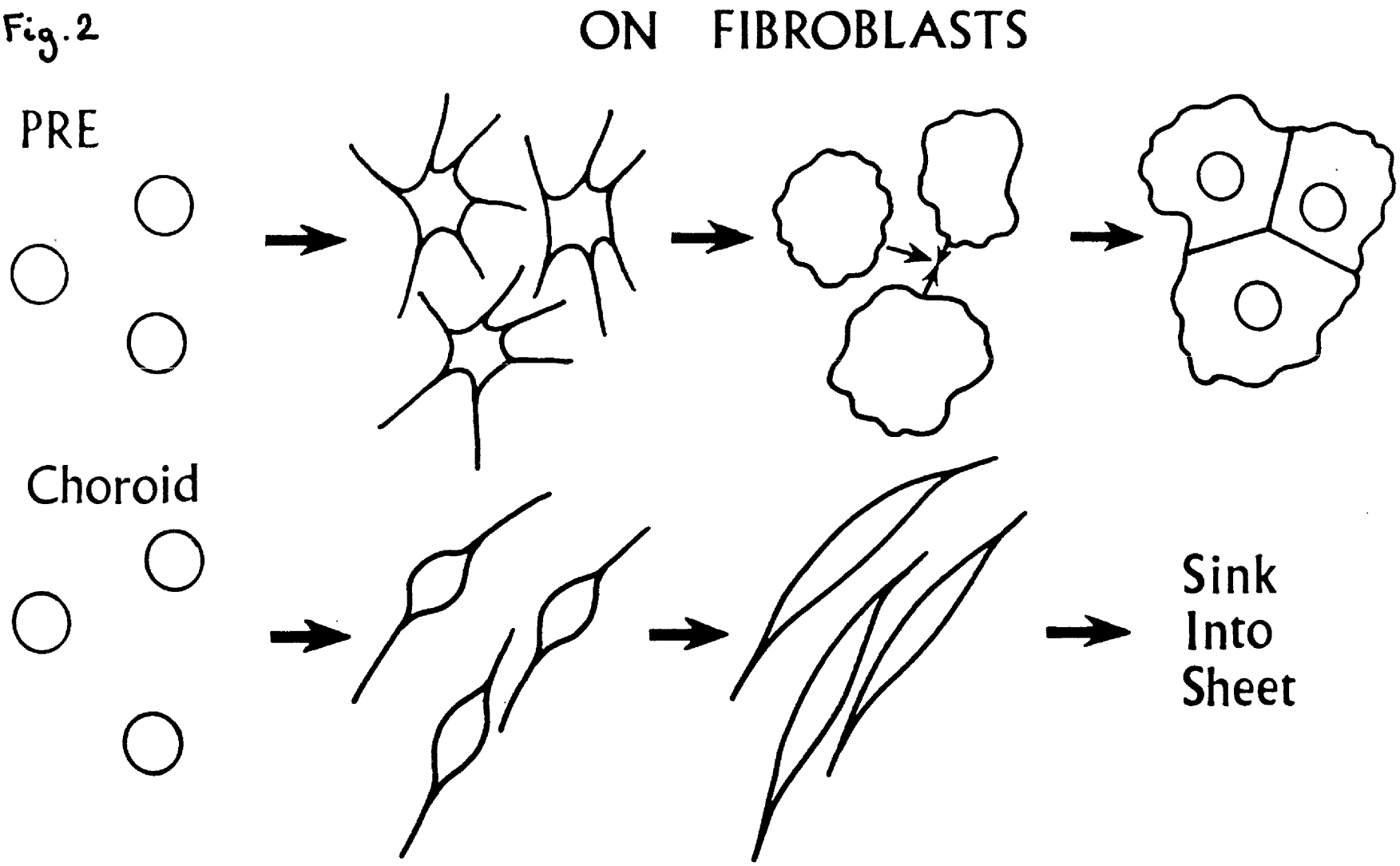


Fig. 2



epithelial cells gaining access to the culture substratum, or spreading upon basement membrane and other extracellular material secreted by the cells.

The scanning electron micrographs reveal that the PRE cells appear to spread initially upon the upper surface of the spread choroid cells themselves rather than the culture substratum (Plate 7). The presence of extracellular matrix cannot be investigated by this method but the effect any such matrix may have on cell behaviour is open to question. When PRE cells and Ch cells were seeded together onto the same choroid sheet, they behaved exactly as when they were seeded upon separate choroid sheets. It would seem therefore, that if extracellular matrix were present it would have to allow the spreading of PRE cells but not that of choroid cells. Such an effect would be unprecedented, and indeed likely candidates for this matrix (i.e. collagen) have been used as substrata for the culture of PRE cells (Middleton 1972), and corneal fibroblasts (Bard and Hay 1975) so this would appear to be an unlikely explanation for the observations.

3.3. Quantitation of PRE cell spreading.

3.3.1. Ability of PRE cells to spread upon Ch, BSC-1 and C13 cell sheets.

The appearance of PRE cells upon these three cell types has already been described (sections 3.1 and 3.2), and the quantitative estimates of the degree of spreading in nine experiments are listed in Table 1. As the rate of spreading probably depends on the rate of recovery from dissociation, the extent of spreading of the PRE cells on the cell sheet was divided by the

extent of spreading on the serum-film control to give a ratio. The extent of spreading on the serum-film was defined as being 1.0 and each experiment was thus standardised against the control in an effort to reduce variability due to differential dissociation. The results so expressed are given in Table 2, and the statistical analysis of the data at the one hour time point is given in Tables 2 (a), 2 (b) and 2 (c) (see also section 2.2.12.). Significance was taken as being the 5 % confidence level where $\hat{z} = 1.96$. The high $\hat{z}$ value for each combination indicates that the PRE cells are behaving as three separate populations upon the three different cell sheets, and therefore the results are significantly different from each other. The results are expressed in histogram form in figure 3 and it can be seen that the spreading of PRE cells is greater upon the choroid fibroblast sheet than the C13 fibroblast sheet although the difference becomes less significant after two and four hours incubation. The spreading upon both fibroblast sheets is significantly higher than on the BSC-1 sheet at all time points.

3.3.2. Comparison of PRE spreading upon choroid fibroblasts with spreading upon chick heart fibroblasts.

It was decided to reinvestigate the claim made by Bell (cited by Trikaus (see Middleton 1973 in discussion)) that PRE cells were incapable of spreading upon chick heart fibroblasts.

Spreading upon choroid sheets and serum-film controls was compared with the spreading upon chick heart and the results are given in Table 3 and Fig. 4.

TABLE 1

SPREADING OF PRE CELLS ON CELL SHEETS. I

CELL SHEET	TIME/EXPT.NO.	1	2	3	4	5	6	7	8	9	Mean	± STD.DEV.
Ch	60	57.2	57.0	67.6	34.2	35.4	42.6	51.7	62.0	23.9	48.6	±13.5
	120	65.4	61.1	76.2	47.7	50.9	53.8	67.0	62.9	43.5	58.7	±10.5
	240	62.1	74.4	74.4	50.4	52.4	59.9	65.7	73.0	56.9	63.2	± 9.3
BSC 1	60	2.6	6.4	1.4	-	0.0	-	-	2.7	0.0	2.2	±2.4
	120	9.0	4.3	8.3	-	1.6	-	-	11.3	1.0	5.9	±4.2
	240	17.5	6.0	13.1	-	1.0	-	-	12.6	9.0	9.9	±5.8
C13	60	22.2	25.8	-	18.5	18.3	26.7	33.1	28.7	-	24.8	±5.4
	120	56.5	47.1	-	36.7	28.3	38.4	44.6	51.1	-	43.2	±9.5
	240	56.5	58.0	-	44.8	48.3	41.2	53.3	72.3	-	53.5	±10.3
Serum	60	35.4	52.8	52.1	27.2	20.3	34.8	52.2	58.1	33.9	40.8	±13.3
	120	51.1	42.5	50.8	35.2	37.5	31.4	50.2	62.1	40.5	44.6	±9.7
	240	56.0	65.7	43.4	48.7	45.5	54.4	49.3	71.4	54.5	54.3	±9.2

Fig. 3

SPREADING OF PIGMENTED RETINA CELLS ON CELL SHEETS

(SERUM = 1.0)

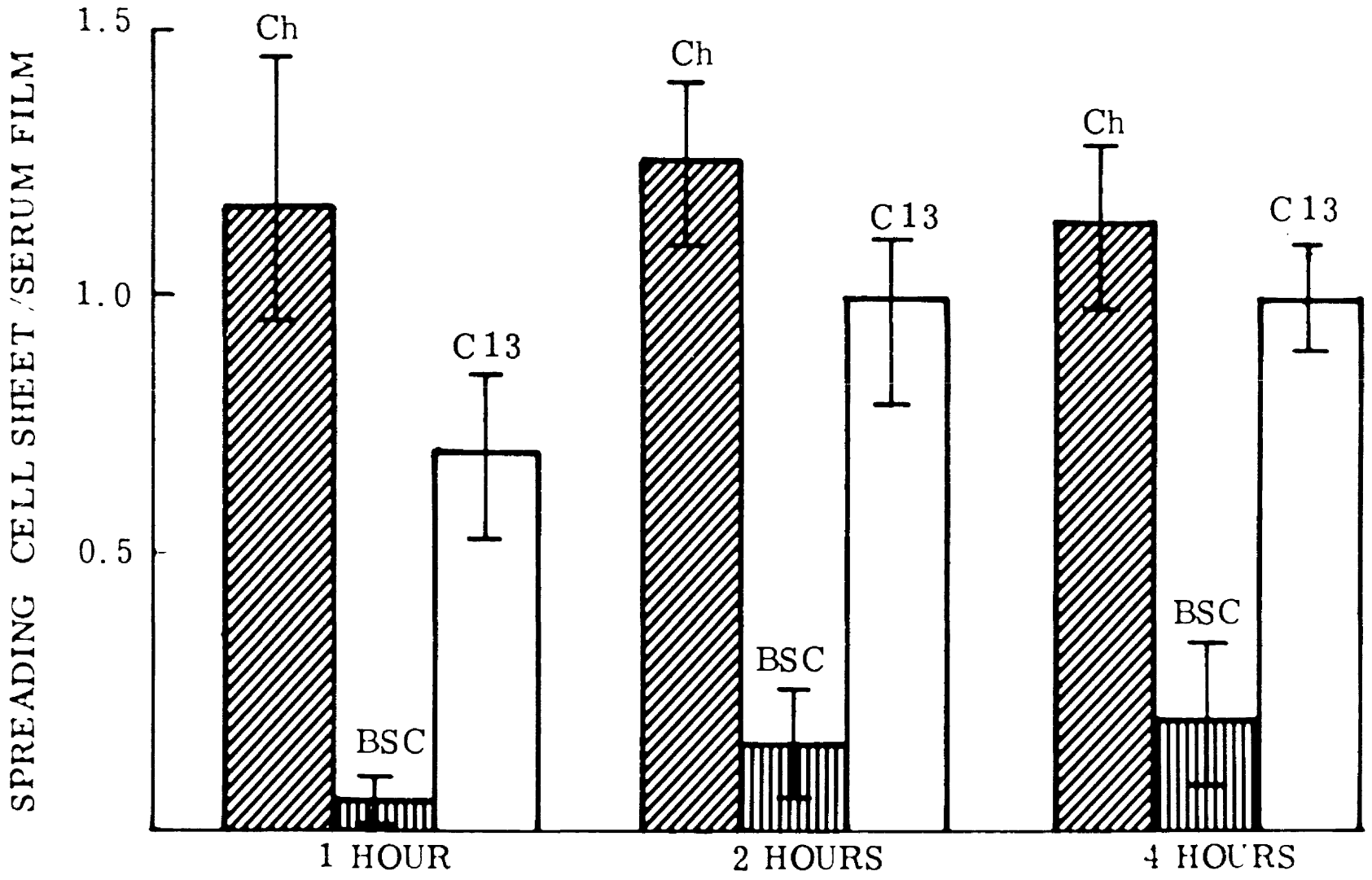


TABLE 2

SPREADING OF PRE CELLS ON CELL SHEETS / SPREADING ON SERUM FILM

CELL SHEET	TIME/EXPT.NO.	1	2	3	4	5	6	7	8	9	Mean	±STD DEV
Ch	60	0.88	1.08	1.62	1.30	1.26	1.74	1.22	0.99	1.07	1.24	±0.28
	120	1.07	1.44	1.28	1.52	1.36	1.36	1.71	1.33	1.01	1.34	±0.21
	240	1.04	1.13	1.11	1.71	1.03	1.15	1.10	1.33	1.02	1.18	±0.22
BSC 1	60	0.00	0.12	0.07	0.03	-	0.00	-	-	0.05	0.05	±0.05
	120	0.02	0.10	0.18	0.16	-	0.04	-	-	0.18	0.11	±0.07
	240	0.17	0.10	0.31	0.30	-	0.02	-	-	0.18	0.18	±0.11
C13	60	-	0.49	0.63	-	0.68	0.90	0.77	0.63	0.49	0.66	±0.15
	120	-	1.11	1.11	-	1.04	0.75	1.22	0.89	0.82	0.99	±0.17
	240	-	0.88	1.01	-	0.92	1.06	0.76	1.08	1.01	0.96	±0.11

TABLE 2 (a)

Wilcoxon-Mann-Whitney Tests on Spreading Data.

(1) Comparison of Spreading on Choroid with Spreading on BSC-1 Combined order statistic on cell sheet/serum film ratios.

Time = 60 MINS.

Choroid						0.88	0.99	1.07	1.08	1.22	1.26	1.30
10.00	0.00	0.03	0.05	0.07	0.12							
1	2	3	4	5	6	7	8	9	10	11	12	13

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-

1.62 1.74

l

14 15

Choroid	n	= 9		R_{ch}	= 99
BSC-1	m	= 6		R_{BSC}	= 21
Totals	T	= 15			

$$\hat{z} = \frac{99 - (9(9 + 6 + 1)/2)}{\sqrt{6 \times 9(9 + 6 + 1)/12}} = \frac{99 - 72}{\sqrt{72}} = \frac{27}{8.49}$$

$\hat{z} = 3.182$ ∴ Signif.

TABLE 2 (b)

(2) Comparison of Spreading on Choroid with Spreading on BHK21C13

Choroid							0.88		0.99	1.07
C13	0.49	0.49	0.63	0.63	0.68	0.77		0.90		
Rank	1.5	1.5	3.5	3.5	5	6	7	8	9	10
Choroid	1.08	1.22	1.26	1.30	1.62	1.74				
C13										
Rank	11	12	13	14	15	16				

Choroid n = 9 $R_{cn} = 107$

BHK21C13 m = 7 $R_{c13} = 29$

Totals T = 16

$$\hat{z} = \frac{107 - [9(9 + 7 + 1)/2]}{\sqrt{7 \times 9(9 + 7 + 1)/12}} = \frac{107 - 76.5}{\sqrt{89.25}} = \frac{30.5}{9.45}$$

$\hat{z} = 3.228$ $\therefore$ Signif.

Table 2 (c)

(3) Comparison of Spreading on BHK21C13 with Spreading on BSC - 1

HK 21 C13							0.49	0.49	0.63	0.63
SC-1	0.00	0.00	0.03	0.05	0.07	0.12				
ank	1.5	1.5	3	4	5	6	7.5	7.5	9.5	9.5

HK21 C13	0.68	0.77	0.90
SC-1			
ank	11	12	13

BHK21C13 n = 7 R_{c13} = 70

BSC-1 m = 6 R_{BSC} = 21

Totals T = 13

$$\hat{z} = \frac{70 - [7(7 + 6 + 1)/2]}{\sqrt{6 \times 7(7 + 6 + 1)/12}} = \frac{70 - 49}{\sqrt{49}} = \frac{21}{7}$$

$\hat{z} = 3.000$ $\therefore$ Signif.

Fig. 4

SPREADING OF PIGMENTED RETINA CELLS ON CELL SHEETS (SERUM =1.0)

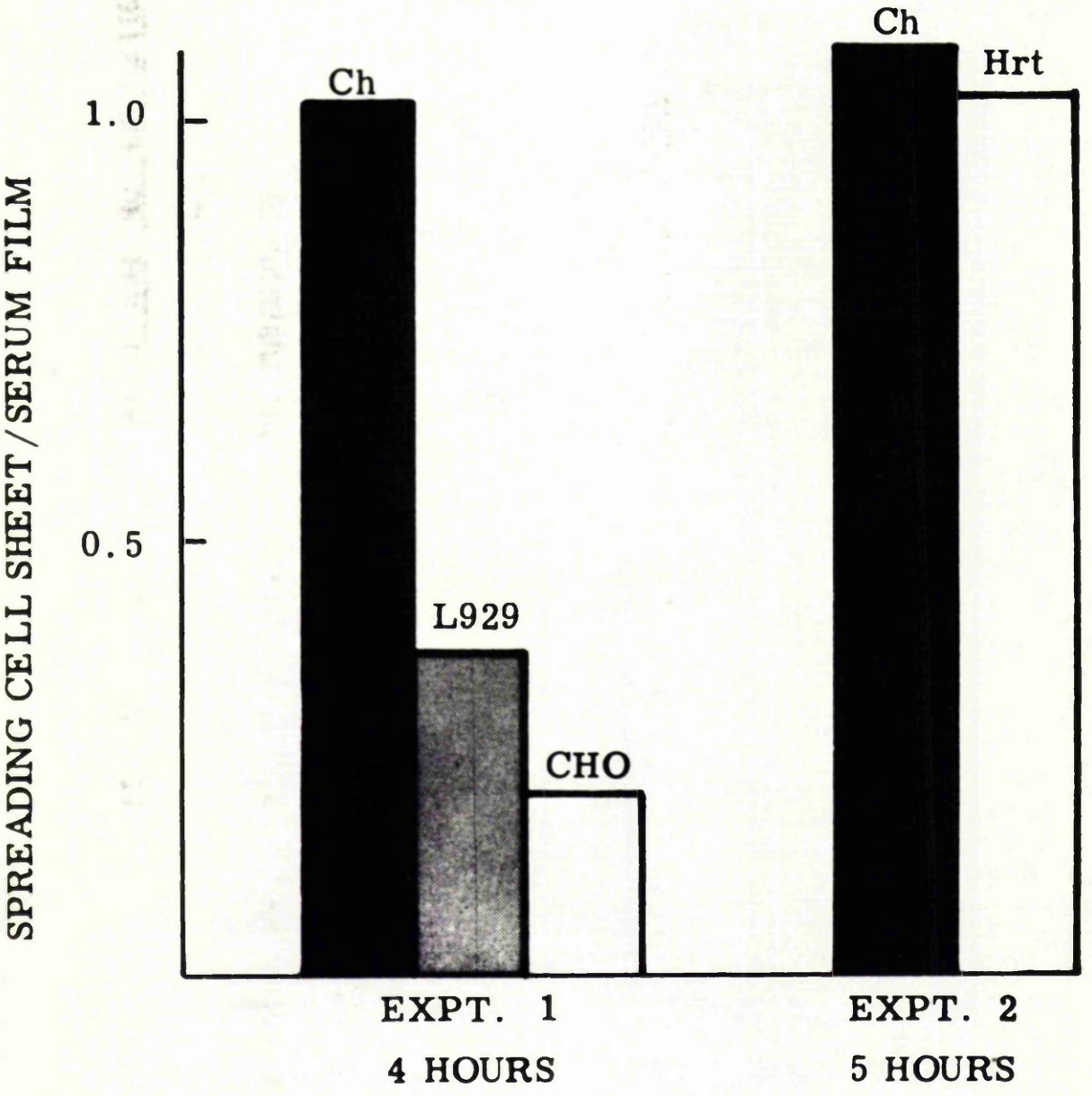


TABLE 3

SPREADING OF PRE CELLS ON CELL SHEETS II

T = 240 mins n = 1

CELL SHEET	% ADHERENT CELLS SPREAD	CELL SHEET/SERUM FILM RATIO
CHO K1	15.9	0.21
L929	28.6	0.38
Ch	76.6	1.02
Serum Film	75.4	-

SPREADING OF PRE CELLS ON CELL SHEETS III

T = 300 mins n = 1

CELL SHEET	% ADHERENT CELLS SPREAD	CELL SHEET/SERUM FILM RATIO
Ch	78.6	1.08
Heart	74.1	1.02
Serum Film	73.0	-

There appeared to be no difference after long periods of incubation (five hours) in the extent of PRE spreading upon any of the substrata.

3.3.3. Comparison of PRE spreading upon choroid Fibroblast sheets with spreading upon transformed fibroblast sheets.

It has been suggested (Vasiliev et al 1976) that cell types which possess a reduced area of lamellar cytoplasm do not support the adhesion of many cells when growing as sheets. To investigate this problem two cell lines CHO-K1 and L-929 (transformed fibroblasts) were used which although they possess a reduced area of lamellar cytoplasm, do not form tightly coherent sheets in vitro as epithelial cells do, and gaps still exist between the cells.

The results of the experiment (Table 3 and Fig. 4) show that even after four hours, spreading upon the transformed fibroblast sheet is very much less (25 - 50%) than that upon the choroid sheet or serum-film control.

3.3.4. Discussion.

PRE cells appear to spread out efficiently when seeded upon fibroblast cell sheets (Ch, C13, Hrt), but a statistically significant difference was noted between the rate of spreading of PRE cells upon Ch and C13; being faster upon the former cell type. PRE cells show little ability to spread out upon sheets of transformed fibroblast cell lines deficient in lamellar cytoplasm (L-929,CHO-K1).

Broadly, PRE cells spread on sheets of cells which possess lamellar cytoplasm (Ch, C13 and Hrt), but poorly or not at all upon sheets of cells which do not possess lamellar cytoplasm (L-929, CHO-K1 and BSC-1). These results are difficult to explain, but it is possible that a number of factors are involved in the restriction of spreading. Perhaps the cells lacking lamellar cytoplasm may be less adhesive than those which possess it, and as spreading possibly depends upon intercellular adhesion overcoming the PRE cell's resistance to deformation (see Wolpert and Gingell 1968), this could be an important factor. Qualitatively L-929 and CHO-K1 cells appear to collect fewer cells than the fibroblast sheets, and a quantitative study was conducted though not with these cell types (see section 3.5 for further discussion). Alternatively, the cells which do not possess lamellar cytoplasm may possess a different distribution of microtubules, and this may render these cells insufficiently rigid to support the spreading of the PRE cells. Maroudas (1973) has suggested that cells may not spread upon substrata of low rigidity. Certain epithelial cells which do not possess lamellar cytoplasm have been shown to possess very few microtubules (Dipasquale 1975a). Finally, BSC-1 epithelial cells and the transformed fibroblast lines (CHO-K1 and L-929) may restrict PRE cell spreading by a mechanism similar to contact inhibition 'type 1' (see section 3.6 for further details).

3.4. Transmission electron microscopy.

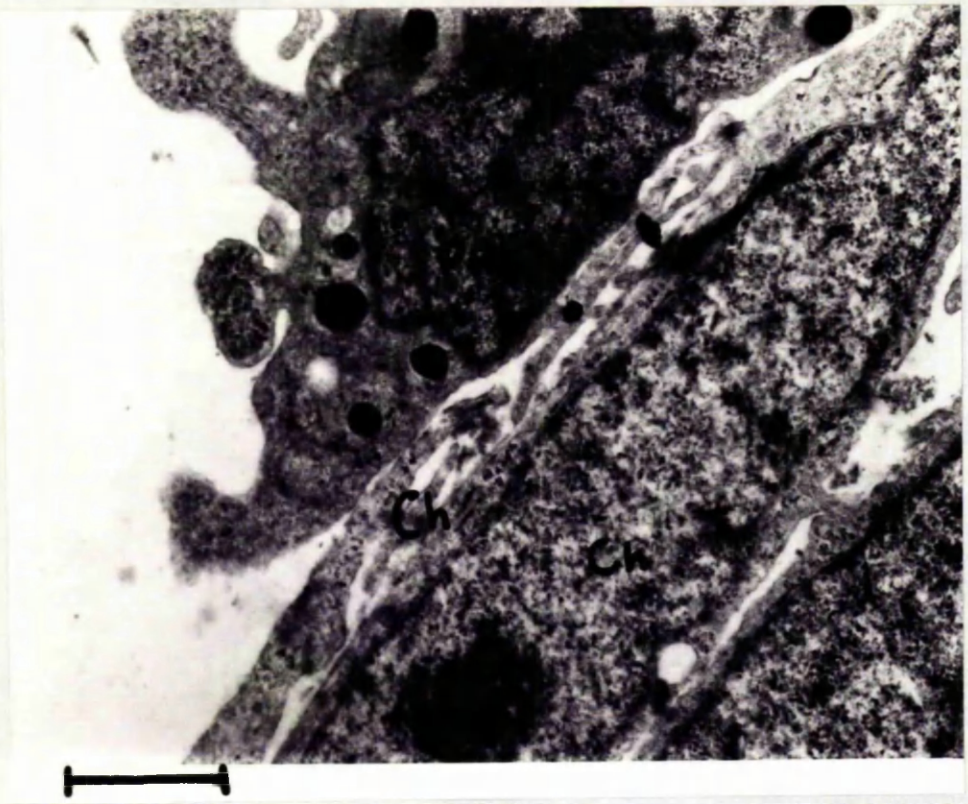
In view of the possibility that extracellular matrix might be present in cultures of cells on which PRE cells were observed to spread (see section 3.2.4), it was decided to examine thin sections of such cultures

under the transmission electron microscope. In these experiments only the PRE cells spreading upon the choroid fibroblasts were examined.

3.4.1. Short-term experiments (1 hour).

Most of the PRE cells appeared to be flattening on top of the choroid fibroblasts (Plates 10 and 11) and resembled PRE cells seeded upon serum-films (Plate 12) as indicated by the earlier experiments (see section 3.2). High power micrographs (Plates 10b, 10c, 11a, 11b) reveal the presence of many close contacts between the under-surface of the PRE cells, and the upper surfaces of choroid fibroblasts the separation distance being between 150A° and 200A° so these regions may be sites of intercellular adhesion between the two cell types. In certain areas microfilaments (Plate 11(a)) and microtubules (Plate 11 (b)) were seen just beneath the plasma membrane in the choroid cells, but not in the PRE cells. The microfilaments did appear to be associated with the membrane in the region of the intercellular contact but their exact point of insertion was difficult to detect. No evidence of extracellular matrix was seen anywhere in the culture even though the fibroblast sheet was up to four cells thick in some places, neither was there any evidence of a basement lamina associated with the PRE cells. In certain areas of the fibroblast sheet close contacts were present between the choroid cells (Plates 10 (b) and 10 (c)), and these were accompanied by microfilaments from both cells.

PRE cells can be readily identified in the sections not only by the presence of pigment granules but also by virtue of their darkly staining cytoplasm. Based on this criterion it is possible to identify PRE


Ch
Ch

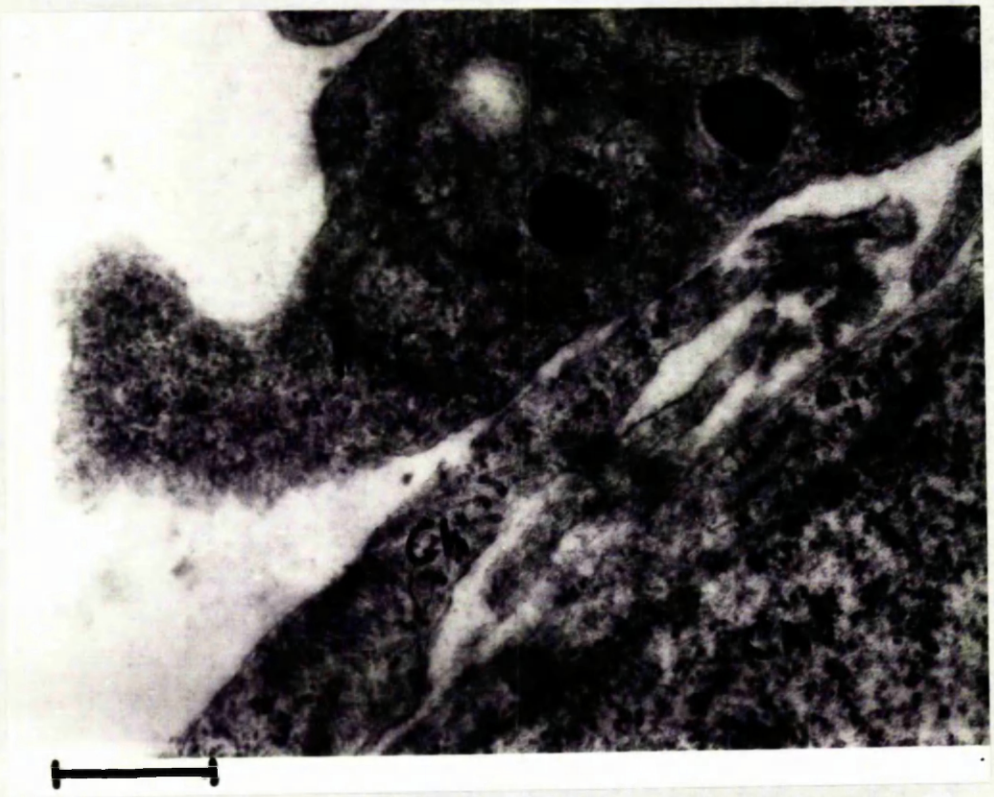

Ch

PLATE 10.

Plate 10 (a). Transmission electron micrograph of PRE cells freshly dissociated from a confluent primary culture after adhering and spreading upon a sheet of choroid fibroblasts.
Culture Time = 1 Hour.
Bar = 1.1μ
PRE = Pigmented Retina Cell.
Ch = Choroid Cell.

Plate 10 (b). As for 10(a).
Bar = 0.53μ

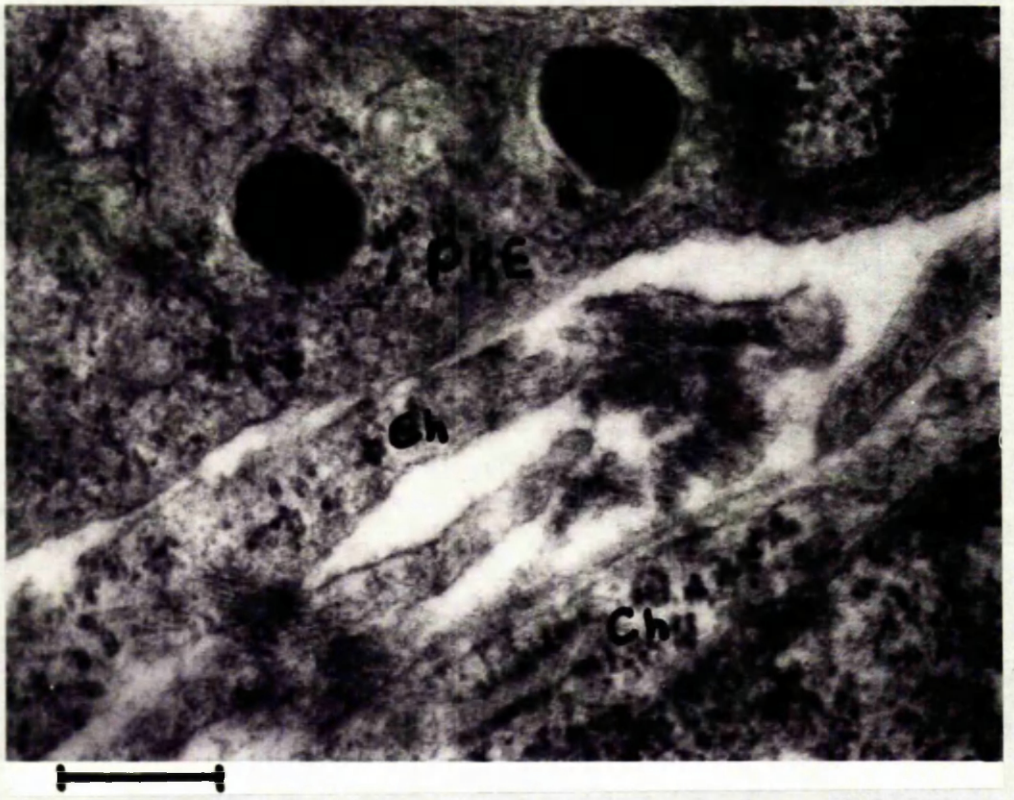
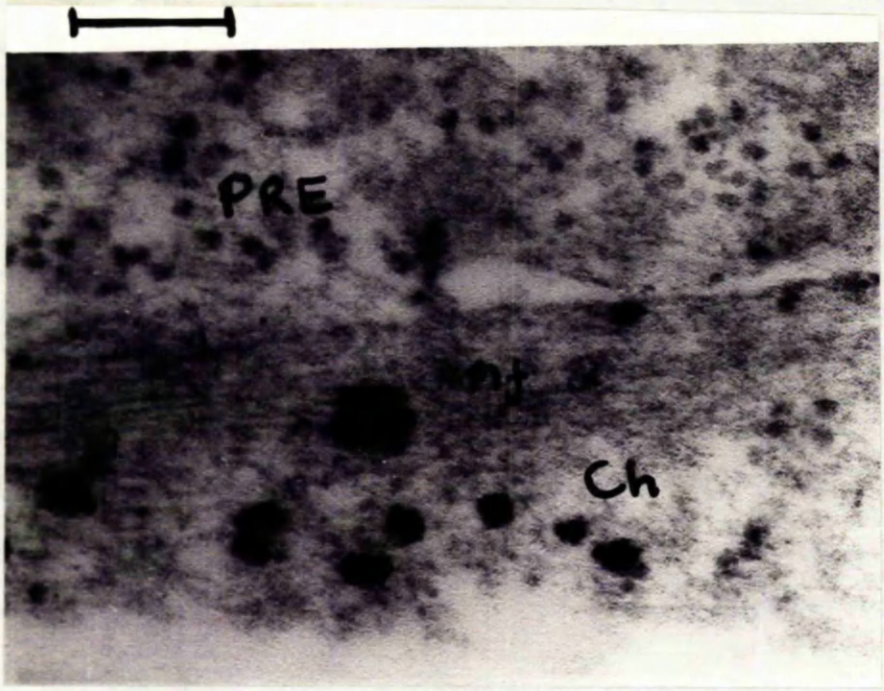

PRE
Ch
Ch

PLATE 10 (contd.)

Plate 10 (c). As for 10(a).

Bar = 0.27μ


PRE
Mt
Ch

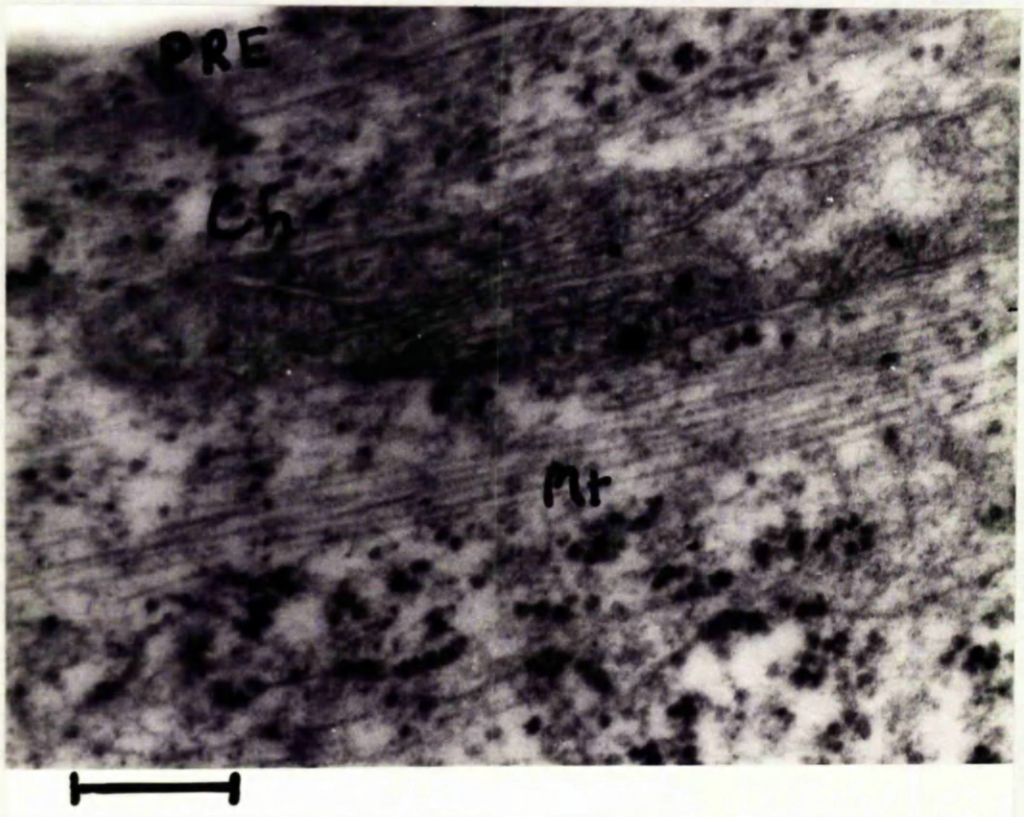

PRE
Ch
Mt

PLATE 11.

Plate 11 (a). Transmission electron micrograph of contact between a seeded PRE cell and the choroid cell sheet.

Culutre Time = 1 Hour.

Bar = 0.12μ

PRE = Pigmented Retina Cell.

Ch = Choroid Cell.

Mf = Microfilaments.

Plate 11 (b). As 11 (a) : A different section

Bar = 0.17μ

PRE = Pigmented Retina Cell.

Ch = Choroid Cell.

Mt. = Microtubules.

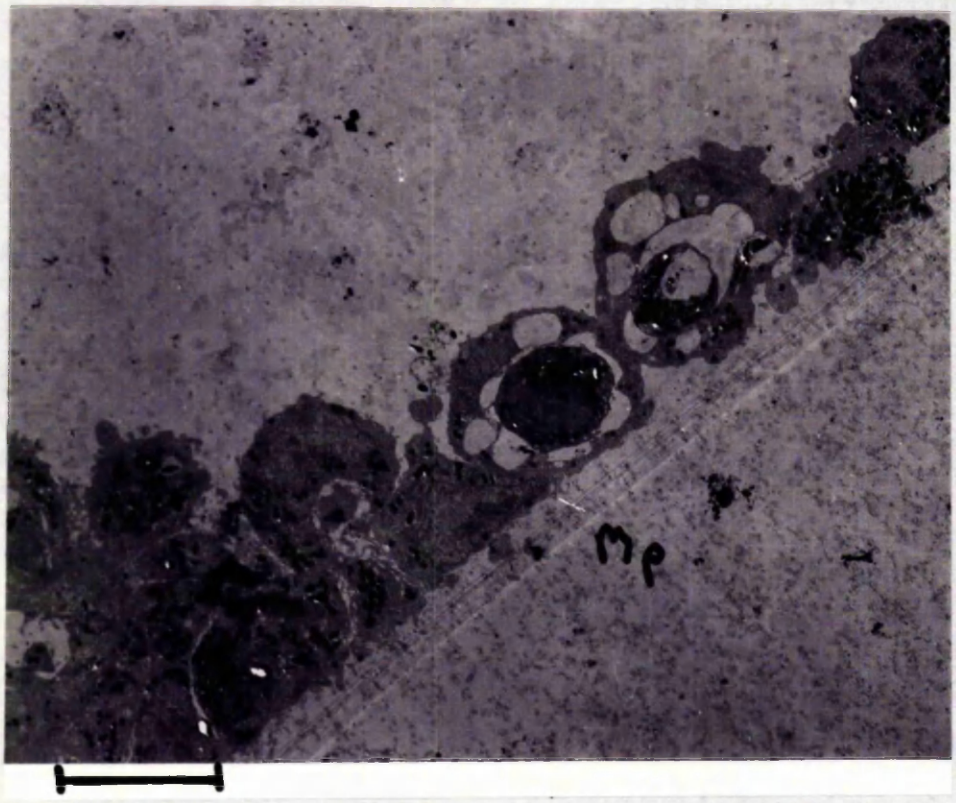
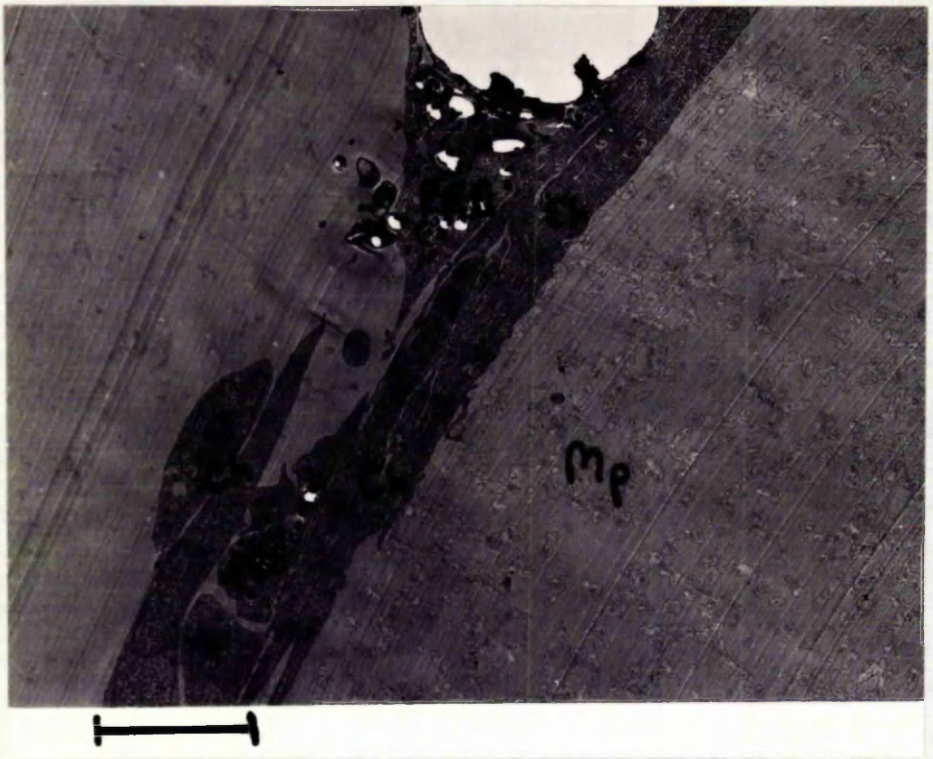

Mp

PLATE 12.

Plate 12. Transmission electron micrograph of a PRE cell suspension freshly dissociated from a confluent primary culture after adhering and spreading upon a serum-coated millipore filter.
Culture Time = 1 Hour.
Bar = 10μ
PRE = Pigmented retina Cell.
Mp. = Millipore Filter.


Mp


ER
Mp

PLATE 13.

Plate 13 (a). Transmission electron micrograph of a PRE cell suspension (prepared as in plates 10 and 11) after spreading on a sheet of choroid fibroblasts.

Culture Time = 4 Hours.

Bar = 2.7μ

PRE = Pigmented Retina Cell.

Ch = Choroid Cell.

Plate 13 (b). As for Plate 13(a).

Bar = 1.1μ


Ch
Cl
Mp

PLATE 13 (contd.)

Plate 13 (c). As for Plate 13(a).
Bar = 0.5μ

cell processes as distinct from the choroid, and no such processes were ever seen to be contacting the tissue culture substratum (millipore filter).

3.4.2. Long-term experiments (4 hours).

The PRE cells now appear to occupy a position between two layers of fibroblasts (Plate 13) and appear to be invading the sheet. The PRE cells do not appear to be making contact with the culture substratum, and many areas of close contact between the PRE cells and the choroid cells can be seen.

3.4.3. Discussion of transmission electron microscope observations.

The results show that any observed spreading by the PRE cells upon sheets of choroid fibroblasts cannot be attributed to the presence of any gross extracellular matrix or basement lamina and no evidence was provided to indicate that the PRE cells were finding gaps in the fibroblast sheet and using the culture substratum for spreading. This supports the view that the differential spreading exhibited by the PRE and Ch cells upon fibroblast sheets is a genuine difference in cell behaviour and requires explanation.

Possible explanations might be:

(1) Differential adhesiveness to the choroid sheet.
(2) Differential deformability.
(3) Differences in the ability of PRE and Ch to spread upon cell sheets of low rigidity.
(4) Lack of contact inhibition of the PRE cells on contact with the choroid fibroblasts.

Wolpert and Gingell (1968) have suggested that spreading might result when the strength of adhesion between a seeded cell and its substratum overcame the cell's resistance to deformation. The strength of adhesion between the PRE cells and the fibroblast sheet might be stronger than that for choroid cells to a similar sheet, and this possibility is discussed in section 3.5.

Equally the PRE cells may have less resistance to deformation than Ch cells but although no quantitative data is available on this subject some evidence would suggest that epithelial cells are less rigid than fibroblasts. Epithelial cells have been shown to possess far fewer microtubules than fibroblasts (Dipasquale 1975a), and as these structures are responsible for the maintenance of cell shape (Goldman et al 1973) it may be that PRE cells are more extensible than choroid fibroblasts.

Alternatively PRE cells may be able to spread out upon substrata of lower rigidity than choroid cells, as fibroblast sheets are not very rigid compared to a tissue culture substratum or a collagen gel. No quantitative information exists on this aspect, but the possibility should not be ignored.

The long term experiments indicate that PRE cells are very capable of invading a sheet of choroid fibroblasts, and this perhaps suggests that the former are not being contact inhibited by the latter. If this were shown to be the case, then possibly PRE cells might be able to spread upon fibroblast sheets because they are not paralysed by the latter after attachment, and this idea is discussed in detail in section 3.6.

Any cell which is spreading upon another would be expected to adhere to the latter, and evidence has been presented in this section that PRE cells do form close contacts with the choroid fibroblasts upon which they are spreading. Microfilaments were seen to be associated with these contacts on the choroid side. This together with the separation distance (≏20nm) would indicate that these regions of close contact are zonula-adhaerens-type junctions, and could be points of intercellular adhesion between the spreading PRE cells and the choroid fibroblasts. Similar junctions were seen between the constituent cells of the choroid sheet, but these were confined to the edges of the cells and the main body of the fibroblasts were separated from each other by a wide gap of >100 nm.

3.5. Cell sheet collection assays.

The cell sheet collection assay was used to measure the adhesion of the cells PRE, BSC-1, Ch and C13 to sheets of each of these types. The assay was modified from that of Walther et al (1973) and was carried out in a similar fashion to that of Buultjens and Edwards (1977) as described in Section 2.

3.5.1. Adhesion of PRE cells to cell sheets.

The results of the experiments are given in Table 4 (a) and each experiment was standardised as far as possible by expressing the cell sheet collection as a ratio of the serum-film control (see Table 4 (b)). The data from Table 4 (b) is expressed in histogram form in Fig. 5 (a), and the statistical analysis of the 60 min. data is given in Table 4 (c).

TABLE 4

ADHESION OF PRE CELLS TO CELL SHEETS

(a) % Collection by Cell Sheets.

CELL SHEET	Time(Mins)/ Expt. No.	1	2	3	4	5	6	7	8	Mean ± Std.Dev.
PRE	30	39.5	25.9		17.4	32.3	37.8	26.4	43.5	31.8 ± 9.2
	60	25.8	38.7		32.6	35.5	37.8	30.0	51.3	36.0 ± 8.1
Ch	30	59.4	49.9	39.0	46.8	54.1	46.9	50.4	61.0	50.9 ± 7.2
	60	60.6	69.9	65.1	74.4	69.8	62.0	64.2	74.6	66.3 ± 7.0
BSC-1	30		43.3	15.4	27.0	33.6	36.9	54.4	16.2	32.4 ± 14.2
	60		29.2	15.4	43.4	56.4	44.5		20.2	34.9 ± 15.8
C13	30	72.6	72.7	53.6	43.1	63.8		70.3	39.1	59.3 ± 14.1
	60	77.3	81.9	66.1	69.9	70.1		80.0	50.0	70.8 ± 10.8
Serum Film	30	46.7	42.9	62.1	31.0	49.2	40.2	52.1	35.2	44.9 ± 9.9
	60	48.4	67.2	55.2	58.5	58.2	52.1	59.1	50.8	56.2 ± 5.9

Fig. 5(a)

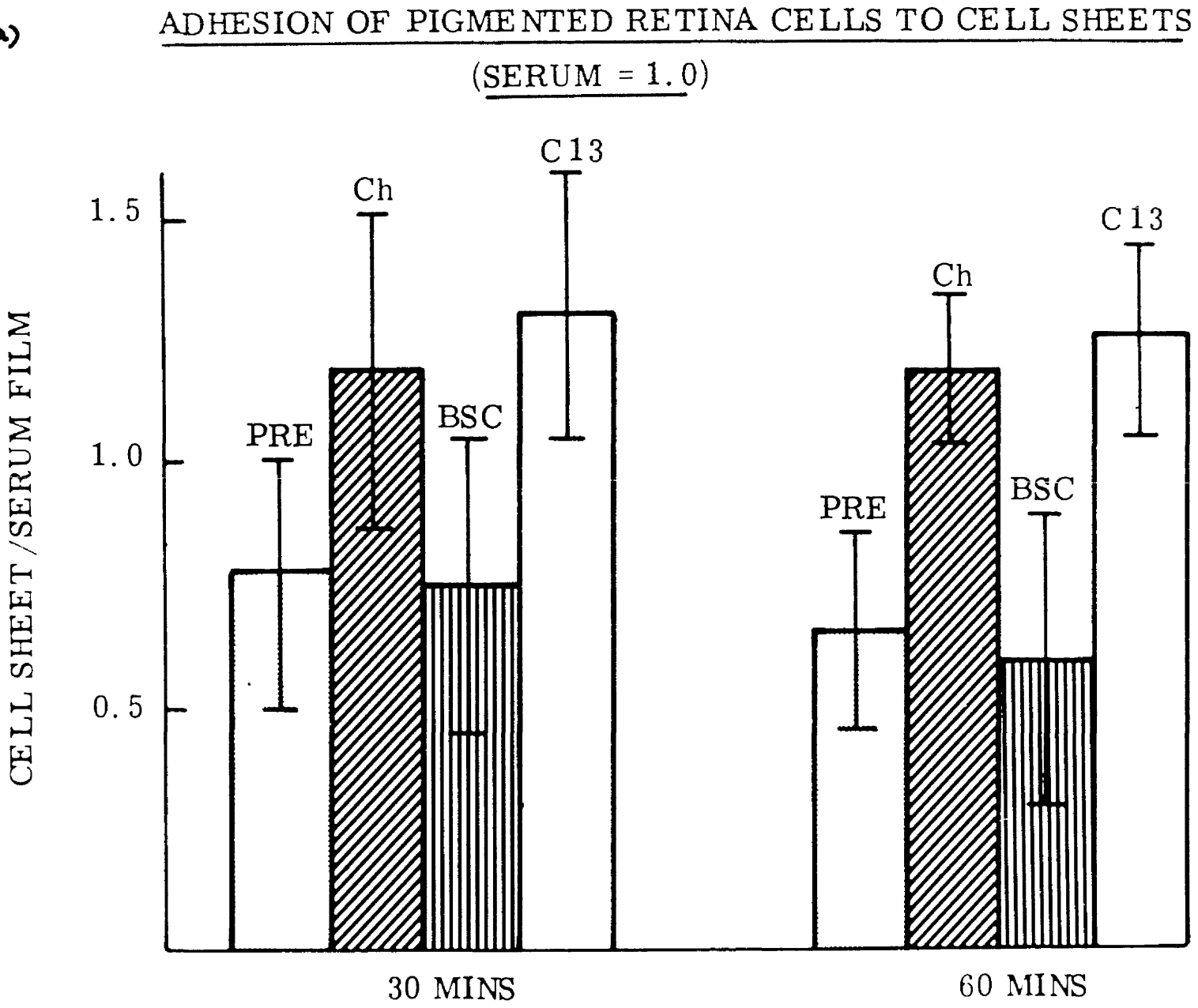

ADHESION OF PIGMENTED RETINA CELLS TO CELL SHEETS
(SERUM = 1.0)
CELL SHEET /SERUM FILM
1.5
1.0
0.5
PRE
Ch
BSC
C13
PRE
Ch
BSC
C13
30 MINS
60 MINS

TABLE 4 (continued)

(b) Cell Sheet/Serum Film Ratio

CELL SHEET	Time / Expt No.	1	2	3	4	5	6	7	8	Mean ± Std.Dev.
PRA	30	0.85	0.60		0.56	0.66	0.94	0.51	1.24	0.77 ±0.26
	60	0.53	0.58		0.56	0.61	0.73	0.51	1.01	0.65 ±0.18
Ch	30	1.27	1.16	0.63	1.51	1.10	1.17	0.97	1.73	1.19 ±.33
	60	1.25	1.04	1.00	1.27	1.20	1.19	1.09	1.47	1.19 ±0.15
BSC-1	30		1.01	0.25	0.87	0.68	0.92	1.04	0.46	0.75 ±0.30
	60		0.43	0.28	0.74	0.97	0.85		0.40	0.61 ±0.28
C13	30	1.55	1.69	0.86	1.39	1.30		1.35	1.11	1.32 ±0.27
	60	1.60	1.22	1.20	1.19	1.20		1.35	0.98	1.25 ±0.19

TABLE 4 (c)

ADHESION OF PRE CELLS TO CELL SHEETS

Wilcoxon-Mann-Whitney Test Applied to Data at T = 60 Mins for Cell Sheet/Serum Film Ratios

(a) Comparison of Adhesion to PRE sheets and Ch sheets.

PRE	0.51	0.53	0.56	0.58	0.61	0.73		1.01							
Ch							1.00		1.04	1.09	1.19	1.20	1.25	1.27	1.47
Rank	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15

PRE	n = 7	R_1 = 29	
Ch	m = 8	R_2 = 91	
Totals	T = 15	120	

$$\hat{z} = \frac{29 - [7(7+8+1)/2]}{\sqrt{8 \times 7(7+8+1)/12}}$$

$$= \frac{29 - 56}{\sqrt{74.66}} = \frac{-27}{8.64}$$

$\hat{z} = -3.125$ $\therefore$ signif.

TABLE 4 (c) continued

(b) Comparison of Adhesion to PRE sheets and BSC-1 sheets.

PRE				0.51	0.53	0;56	0.58	0.61	0.73				1.01
BSC-1	0.28	0.40	0.43							0.74	0.85	0.97	
Rank	1	2	3	4	5	6	7	8	9	10	11	12	13

PRE n = 7 R_1 = 52
BSC-1 m = 6 R_2 = 39
Totals T = 13 91

$$\hat{z} = \frac{52 - [7(7+6+1)/2]}{\sqrt{6x7(7+6+1)/12}}$$

$$= \frac{52 - 49}{\sqrt{49}} = \frac{3}{7}$$

$\hat{z} = 0.4285$ $\therefore$ not signif.

TABLE 4 (c) continued

(c) Comparison of Adhesion to PRE sheets and C13 Sheets.

PRE	0.51	0.53	0.56	0.58	0.61	0.73		1.01					
C13							0.98		1.19	1.20	1.20	1.22	1.35	1.60
Rank	1	2	3	4	5	6	7	8	9	10	11	12	13	14

PRE	= n = 7	R_1 = 29
C13	m = 7	R_2 = 76
Totals	T = 14	105

$$\hat{z} = \frac{29 - [7(7+7+1)/2]}{\sqrt{7x7(7+7+1)/12}}$$

$$= \frac{29 - 52.5}{\sqrt{61.25}} = \frac{23.5}{7.83}$$

$\hat{z} = 3.001$ $\therefore$ Signif.

TABLE 4 (c) continued

(d) Comparison of Adhesion to Choroid Sheets with BSC-1 Sheets.

Ch							1.00	1.04	1.09	1.19	1.20	1.25	1.27	1.47
BSC-1	0.28	0.40	0.43	0.74	0.85	0.97								
Rank	1	2	3	4	5	6	7	8	9	10	11	12	13	14

Ch n = 8 R_1 = 84
BSC-1 m = 6 R_2 = 21
Totals T = 14 105

$$\hat{z} = \frac{84 - [8(8+6+1)/2]}{\sqrt{6 \times 8(8+6+1)/12}}$$

$$= \frac{84 - 60}{\sqrt{60}} \qquad \frac{24}{7.75}$$

$\hat{z} = 3.097$ $\therefore$ Signif.

TABLE 4 (c) (continued)

(e) Comparison of Adhesion to Choroid Sheets with that to C13 Sheets.

Ch		1.00	1.04	1.09	1.19	1.20			1.25	1.27		1.47	
C13	0.98				1.19	1.20	1.20	1.22			1.35		1.60
Rank	1	2	3	4	5.5	7.7	7.7	10	11	12	13	14	15

Ch $n = 8$ $R_1 = 59.2$

C13 $m = 7$ $R_2 = 59.9$

Totals $T = 15$ 119.1

$$\hat{z} = \frac{59.2 - [8(8+7+1)/2]}{\sqrt{7 \times 8(8+7+1)/12}}$$

$$= \frac{59.2 - 64}{\sqrt{74.66}} \qquad \frac{-4.8}{8.64}$$

$\hat{z} = -0.5555$ $\therefore$ not signif.

TABLE 4 (c) continued

(f) Comparison of Adhesion to BSC-1 sheets with that to C13 sheets.

BSC-1	0.28	0.40	0.43	0.74	0.85	0.97							
C13							0.98	1.19	1.20	1.20	1.22	1.35	1.60
Rank	1	2	3	4	5	6	7	8	9.5	9.5	11	12	13

BSC-1	n = 6	R_1 = 21
C13	m = 7	R_2 = 70
Totals	T = 13	91

$$\hat{z} = \frac{21 - \lceil 6(6+7+1)/2 \rceil}{\sqrt{7 \times 6(6+7+1)/12}}$$

$$= \frac{21 - 42}{\sqrt{49}} = \frac{21}{7}$$

$\hat{z} = 3.000$ $\therefore$ signif.

The data at 30 mins and 60 mins show a similar pattern of collection although the former data are more variable. The PRE cells stick rapidly and firmly to the fibroblast sheets (Ch and C13), but weakly to the epithelial sheets (PRE and BSC-1). Attachment to the fibroblast sheets was more efficient than to the serum-film controls, whereas attachment to the epithelial sheets was less efficient.

When the data was tested statistically it was found that the attachment of PRE cells to Ch and C13 sheets was significantly greater than to both epithelial sheets. The collection by the Ch sheets was not significantly different from that by the C13 sheets, and similarly there was no significant difference between the collections by the BSC-1 sheets and the PRE sheets.

3.5.2. Adhesion of Choroid cells to cell sheets.

The results are given in Tables 5 (a) and 5 (b), and the data from Table 5(b) are shown in histogram form in Fig. 5 (b).

The pattern of collection by the Ch, BSC-1 and C13 sheets is the same as for the PRE cells but the collection of the Ch cells by the PRE sheet is somewhat higher than the collection of PRE cells. Statistical testing of the 60 min. data (Table 5 (c)) shows that the collecting sheets Ch and C13 behave as one population, and the PRE and BSC-1 sheets behave as a separate population. The result is therefore similar to that for the PRE cell suspension.

3.5.3. Adhesion of C13 cells to cell sheets.

The results are given in Tables 6 (a) and 6 (b),

TABLE 5

ADHESION OF CHOROID CELLS TO CELL SHEETS

(a) %COLLECTION BY CELL SHEETS

CELL SHEET	TIME/ EXPT NO.	1	2	3	4	5	6	7	Mean ± Std.Dev.
PRE	30	48.9	40.8		39.4	28.1	26.2	37.1	36.8 ± 8.5
	60	58.4	43.9		46.0	59.2	40.6	55.6	50.6 ± 8.1
Ch	30	54.9	53.6	51.6	57.4	56.5	59.0	39.6	53.2 ± 6.5
	60	63.2	67.5	71.2	75.0	84.6	79.1	75.0	73.7 ± 7.1
BSC-1	30	45.0		22.0	19.8	42.7	28.5	17.0	29.2 ± 12.0
	60	39.2		22.8	25.8		61.3	24.7	34.8 ± 16.2
C13	30	60.0	72.0	58.1	76.1	71.0	54.6	40.9	61.8 ± 12.2
	60	67.3	82.2	73.5	87.9	86.1	81.0	68.7	78.1 ± 8.3
Serum Films	30	51.6	44.6	57.0	39.7	46.6	52.4	22,8	45.0 ± 11.3
	60	67.6	63.5	60.2	68.2	56.2	80.6	47.1	63.3 ± 10.5

Fig. 5(b)

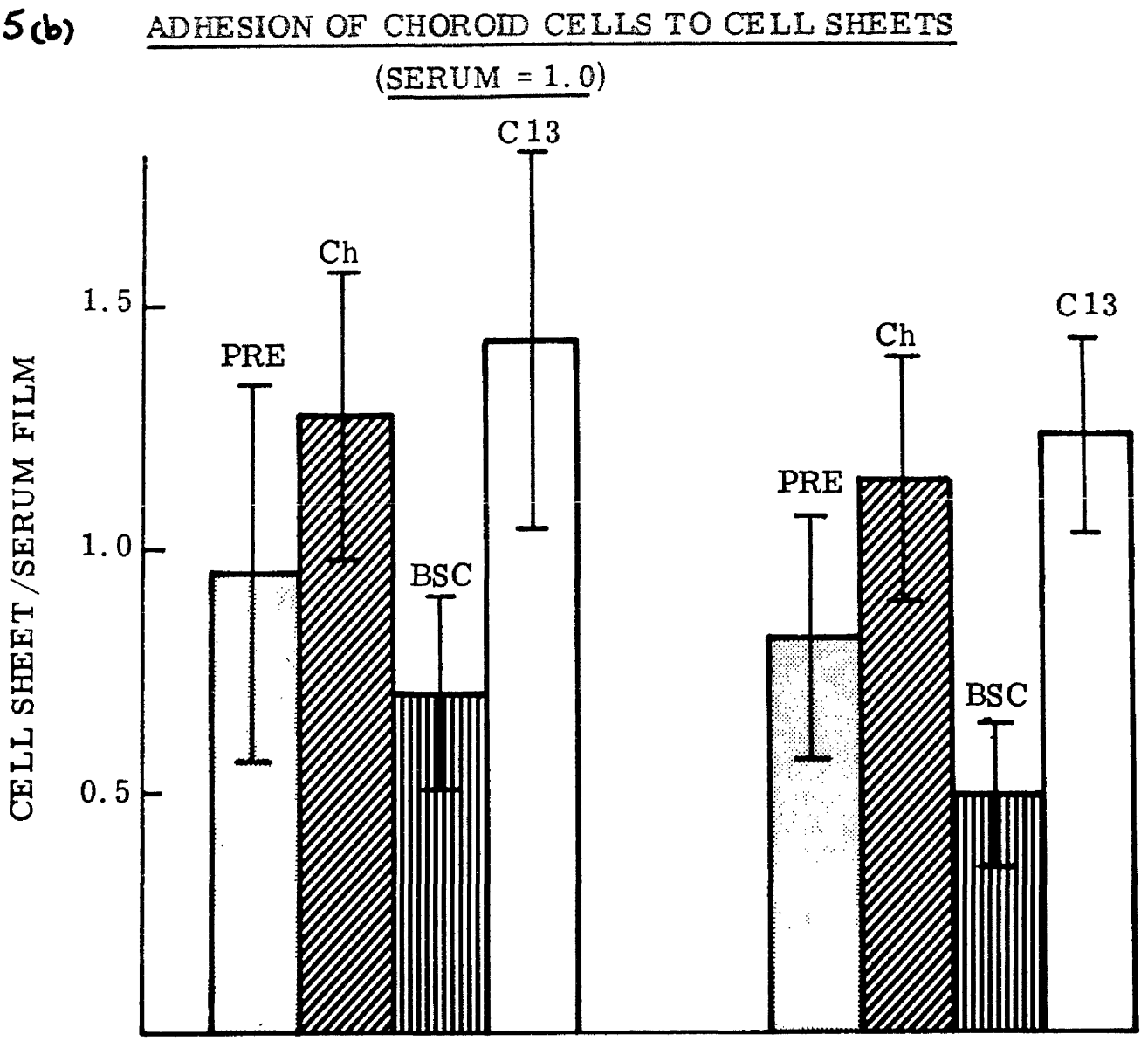

ADHESION OF CHOROID CELLS TO CELL SHEETS
(SERUM = 1.0)
CELL SHEET /SERUM FILM
1.5
1.0
0.5
PRE
Ch
BSC
C13
PRE
Ch
BSC
C13

TABLE 5 continued

(b) CELL SHEET/SERUM FILM RATIO

CELL SHEET	TIME/ EXPT NO.	1	2	3	4	5	6	7	Mean	± Stnd. Dev.
PRc	30	0.95	0.91		0.99	0.60	0.50	1.63	0.93	± 0.40
	60	0.86	0.69		0.67	1.05	0.50	1.18	0.83	± 0.26
Ch	30	1.06	1.20	0.91	1.45	1.21	1.13	1.74	1.24	± 0.27
	60	0.93	1.06	1.18	1.10	1.51	0.98	1.59	1.19	± 0.26
BSC-1	30	0.87		0.39	0.50	0.92	0.54	0.75	0.66	± 0.22
	60	0.58		0.38	0.38		0.76	0.52	0.52	± 0.16
C13	30	1.16	1.61	1.02	1.92	1.52	1.04	1.79	1.44	± 0.37
	60	1.00	1.29	1.22	1.29	1.53	1.00	1.46	1.26	± 0.20

TABLE 5 (c)

ADHESION OF CHOROID CELLS TO CELL SHEETS

(a) Comparison of Adhesion to PRE sheets with that to Ch sheets.

PRE	0.50	0.67	0.69	0.86			1.05			1.18		
Ch					0.93	0.98		1.06	1.10	1.18	1.51	1.59
Rank	1	2	3	4	5	6	7	8	9	10.5	12	13

PRE	n = 6	R_1 =	27.5
Ch	m = 7	R_2 =	63.5
Totals	13		91

$$\hat{z} = \frac{27.5 - [6(6+7+1)/2]}{\sqrt{7 \times 6(6+7+1)/12}}$$

$$= \frac{27.5 - 42}{\sqrt{49}} = \frac{-14.5}{7}$$

$\hat{z} = 2.071$ ∴ Signif.

TABLE 5 (c) continued

(b) Comparison of Adhesion to PRE sheets with that to BSC-1 sheets.

PRE			0.50			0.67	0.69		0.86	1.05	1.18
BSC-1	0.38	0.38		0.52	0.58			0.76			
Rank	1.5	1.5	3	4	5	6	7	8	9	10	11

PRE	n = 6	R_1 = 46
BSC-1	m = 5	R_2 = 20
Totals	T = 11	66

$$\hat{z} = \frac{46 - [6(6+5+1)/2]}{\sqrt{5x6\ (6+5+1)/12}}$$

$$= \frac{46 - 36}{\sqrt{30}} = \frac{10}{5.47}$$

$\hat{z}$ = 1.828 $\therefore$ not signif.

TABLE 5(c)continued

(c) Comparison of Adhesion to PRE sheets with that to C13 sheets.

PRE	0.50	0.67	0.69	0.86			1.05	1.18					
C13					1.00	1.00			1.22	1.29	1.29	1.46	1.53
Rank	1	2	3	4	5.5	5.5	7	8	9	10.5	10.5	12	13

PRE	n = 6	R_1 = 25
C13	m = 7	R_2 = 66
Totals	T = 13	91

$$\hat{z} = \frac{25 - [6(6+7+1)/2]}{\sqrt{7 \times 6(6+7+1)/12}}$$

$$= \frac{25 - 42}{\sqrt{49}} = \frac{-17}{7}$$

$\hat{z} = 2.429$ $\therefore$ signif.

TABLE 5 (c) continued

(d) Comparison of Adhesion to Choroid sheets with that to BSC-1 sheets

Ch						0.93	0.98	1.06	1.10	1.18	1.51	1.59
BSC-1	0.38	0.38	0.52	0.58	0.76							
Rank	1	2	3	4	5	6	7	8	9	10	11	12

Ch n = 7 R_1 = 63

BSC-1 m = 5 R_2 = 15

Totals T = 12 78

$$\hat{z} = \frac{63 - [7(7+5+1)/2]}{\sqrt{5x7(7+5+1)/12}}$$

$$= \frac{63 - 45.5}{\sqrt{13}} = \frac{17.5}{3.6}$$

$\hat{z}$ = 4.891 ∴ signif.

TABLE 5 (c) continued

(e) Comparison of adhesion to choroid sheets with that to C13 sheets.

Ch	0.93	0.98			1.06	1.10	1.18					1.51		1.59
C13			1.00	1.00				1.22	1.29	1.29	1.46		1.53	
Rank	1	2	3.5	3.5	5	6	7	8	9.5	9.5	11	12	13	14

Ch n = 7 R_1 = 47

C13 m = 7 R_2 = 58

Totals T = 14 105

$$\hat{z} = \frac{47 - \left[7(7+7+1)/2\right]}{\sqrt{7 \times 7(7+7+1)/12}}$$

$$= \frac{47 - 52.5}{\sqrt{61.25}} = \frac{-5.25}{7.826}$$

$\hat{z} = 0.6708$ ∴ not signif.

TABLE 5 (c) Continued

(f) Comparison of adhesion to BSC-1 sheets with that to C13 sheets.

BSC-1	0.38	0.38	0.52	0.58	0.76							
						1.00	1.00	1.22	1.29	1.29	1.46	1.53
Rank	1	2	3	4	5	6	7	8	9	10	11	12

BSC-1 =	n = 5	R_1 =	15
C13	m = 7	R_2 =	63
Totals	T = 12		78

$$\hat{z} = \frac{\left[15 - 5(5+7+1)/2\right]}{\sqrt{7 \times 5(5+7+1)/12}}$$

$$= \frac{15 - 32.5}{\sqrt{13}} = \frac{17.5}{3.6}$$

$\hat{z} = 4.891$ ∴ signif.

and expressed in histogram form in Fig. 5 (c). The pattern of collection was very similar to that of the PRE cells.

3.5.4. Adhesion of BSC-1 cells to cell sheets.

The results are given in Tables 7 (a) and 7 (b) and in histogram form in Fig. 5 (d). Although the collection pattern of the BSC-1 cells was essentially similar to that of the previous cell types the data was extremely variable making the detection of statistically significant differences difficult. Further, the ability of the BSC-1 cells to adhere to any cell sheet was very much less than the ability to adhere to a serum-film control. The latter gave normal collection values which would indicate that the cells were not merely dead or damaged.

3.5.5. Discussion.

The epithelial cell sheets (PRE and BSC-1) do not collect any of the cell types tested very well with one exception. This lower collection value might possibly explain why no cell has ever been observed to spread upon the upper surface of an epithelial sheet (see section 3.1, Middleton (1973), Dipasquale and Bell (1972, 1974)) because the strength of adhesion to the sheet is too small to allow spreading. However it was shown in section 3.1 that cells which do adhere well to the upper surfaces of epithelial sheets are still incapable of spreading. A possible reason may be that the epithelial sheets may not possess enough adhesive sites to allow spreading, and that these are quickly saturated by the cell sheet collection assay. Buultjens and Edwards (1977) tested this possibility for PRE

TABLE 6

ADHESION OF C13 CELLS TO MONOLAYERS

(a) % COLLECTION BY CELL SHEETS

CELL SHEET	TIME/ EXPT NO.	1	2	3	4	5	6	7	Mean	± Std.Dev
PRS	30	25.2	30.9	23.9	51.9	49.8	30.6	32.0	34.9	± 11.3
	60.	22.2	49.3	44.9	55.7	42.3	43.6	49.4	43.9	± 10.6
Ch	30	70.5	62.7	57.2	83.5	66.2	67.2	56.2	66.2	± 9.2
	60	88.8	84.6	86.5	86.2	81.0	80.6	88.2	85.1	± 3.3
BSC-1	30	27.7	27.2	38.0	39.7	22.4	14.3	29.9	28.5	± 8.7
	60	51.7	29.6	48.9	51.2	36.4	23.9	43.7	40.8	± 11.0
C13	30	63.1	62.5	70,5	80.2	42.3	60.8	48.2	61.0	± 12.8
	60	82.7	80.8	84.9	85.9	69.5	78.9	76.5	79.9	± 5.6
Serum Film	30	74.2	51.8	79.4	72.1	70.1	72.9	46.0	66.6	± 12.6
	60	76.4	62.9	81.6	78.5	76.4	79.5	69.5	75.0	± 6.5

Fig. 5 (c)

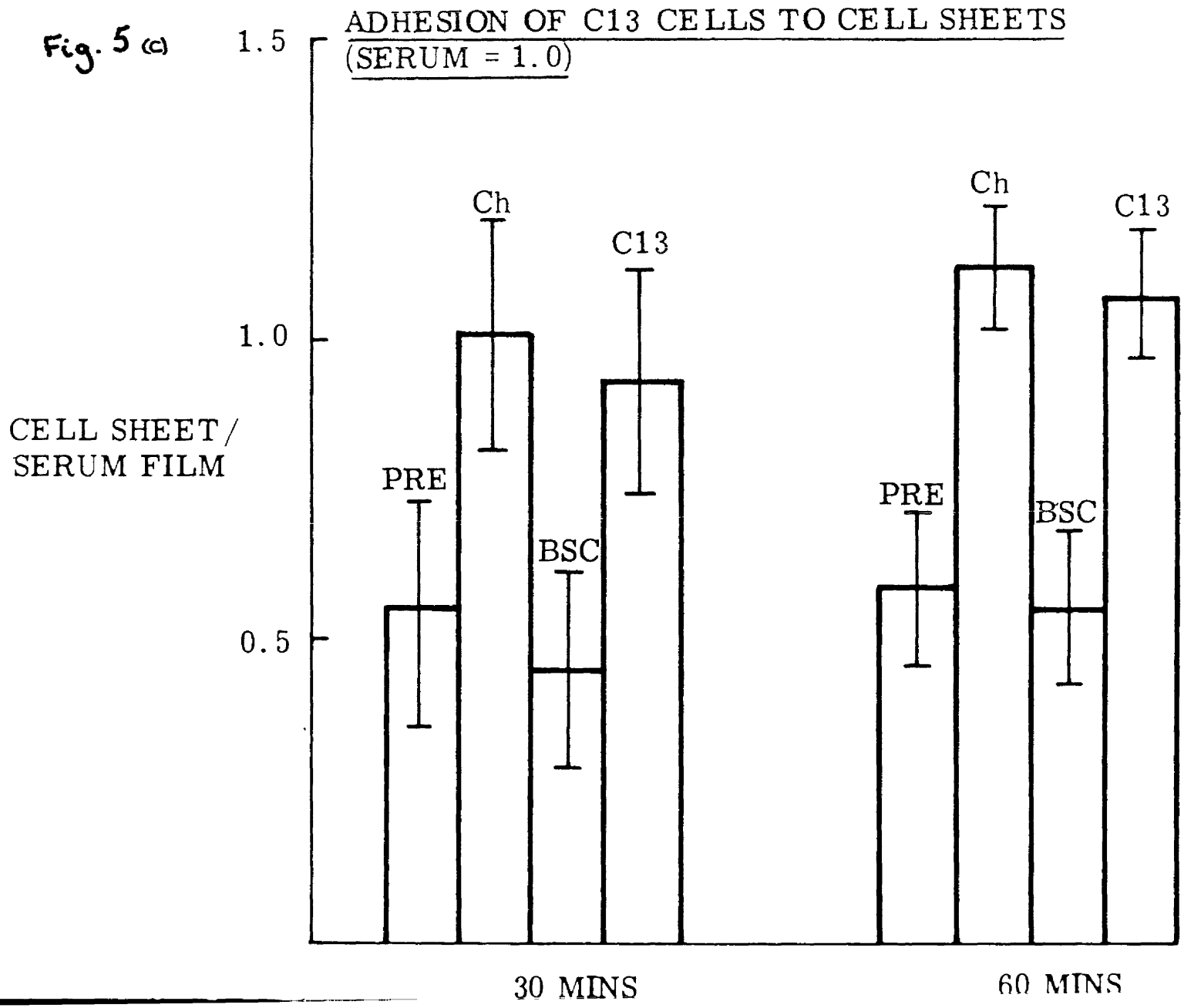

ADHESION OF C13 CELLS TO CELL SHEETS
(SERUM = 1.0)
1.5
1.0
0.5
CELL SHEET/
SERUM FILM
PRE
Ch
BSC
C13
PRE
Ch
BSC
C13
30 MINS
60 MINS

TABLE 6 continued

(b) CELL SHEET / SERUM FILM RATIO

CELL SHEET	TIME/ EXPT. NO.	1	2	3	4	5	6	7	Mean ± Std.Dev
PRE	30	0.34	0.60	0.30	0.72	0.71	0.42	0.70	0.54 ± 0.18
	60	0.29	0.78	0.55	0.71	0.55	0.55	0.71	0.59 ± 0.16
Ch	30	0.95	1.21	0.72	1.16	0.94	0.92	1.22	1.02 ± 0.19
	60	1.16	1.34	1.06	1.10	1.06	1.01	1.27	1.14 ± 0.12
BSC-1	30	0.37	0.53	0.48	0.55	0.32	0.20	0.65	0.44 ± 0.15
	60	0.70	0.57	0.62	0.65	0.48	0.30	0.63	0.56 ± 0.14
C13	30	0.85	1.21	0.89	1.11	0.60	0.83	1.05	0.93 ± 0.20
	60	1.08	1.29	1.04	1.09	0.91	0.99	1.10	1.07 ± 0.12

TABLE 7

ADHESION OF BSC-1 CELLS TO CELL SHEETS

(a) % COLLECTION BY CELL SHEETS

CELL SHEET	TIME EXPT. NO.	1	2	3	4	5	6	Mean ± Std. Dev
PRS	30	9.0	4.3	6.2	3.0	2.7	11.7	6.2 ± 3.6
	60	25.6	18.1	19.2	10.8	15.9	26.7	19.4 ± 6.0
Ch	30	22.5	9.5	3.9	7.1	15.4	33.9	15.4 ± 11.2
	60	47.7	34.2	33.9	21.6	28.6	55.3	36.9 ± 12.4
BSC-1	30		4.9	6.0	1.5	3.1	5.7	4.2 ± 1.9
	60		27.6	26.7	5.4	9.5	17.1	17.3 ± 10.0
C13	30	37.9	6.0	7.2	6.7	14.2	32.5	17.4 ± 14.2
	60	59.1	18.4	26.0	15.5	22.8	42.6	30.7 ± 16.8
Serum Film	30	71.4	57.4	71.9	67.0	86.9	83.4	73.0 ± 10.8
	60	81.6	71.2	68.1	69.3	83.2	83.2	76.1 ± 7.3

Fig. 5(d)

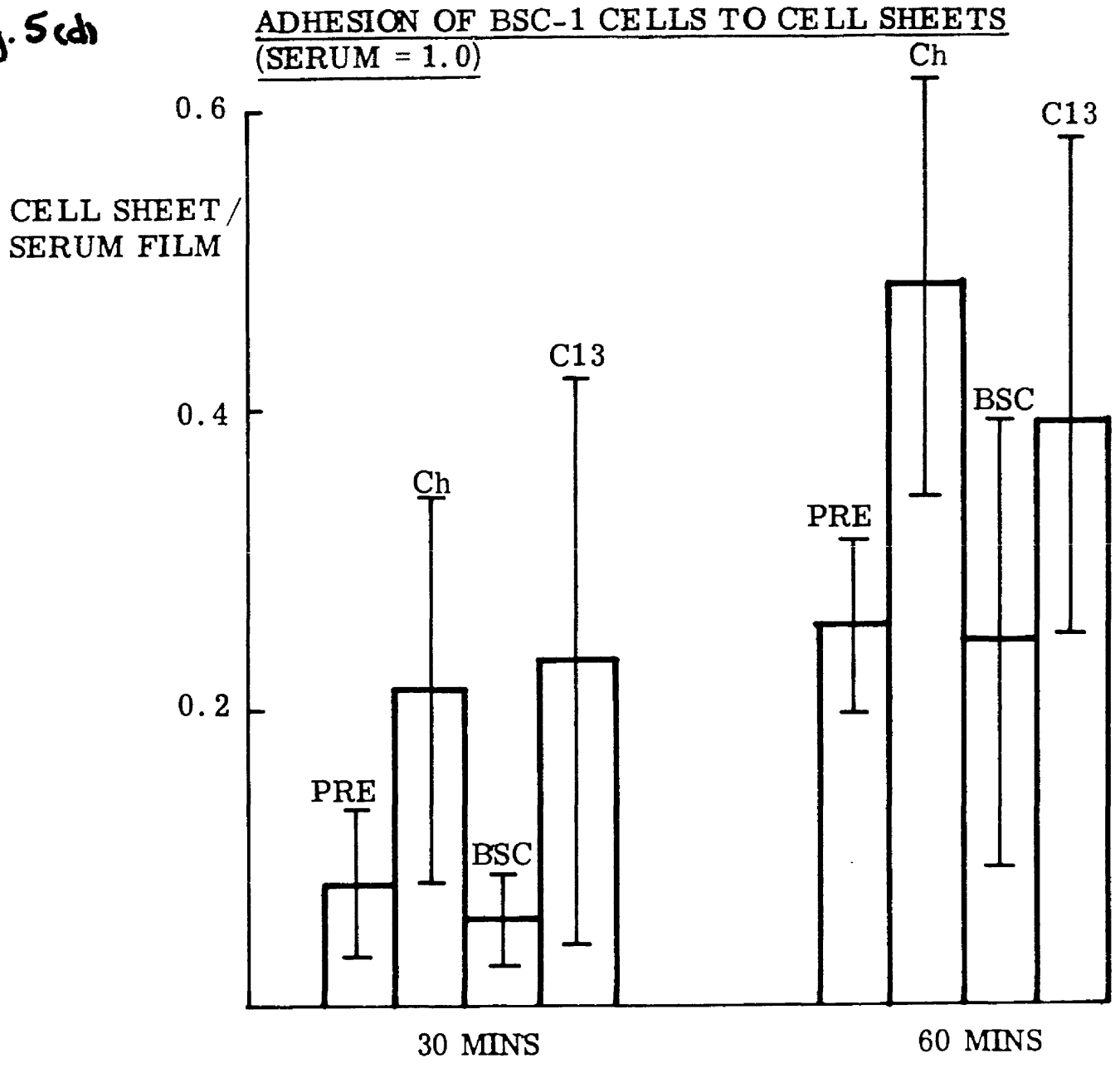

ADHESION OF BSC-1 CELLS TO CELL SHEETS
(SERUM = 1.0)
0.6
0.4
0.2
CELL SHEET / SERUM FILM
PRE
Ch
BSC
C13
PRE
Ch
BSC
C13
30 MINS
60 MINS

TABLE 7 continued

(b) CELL SHEET/SERUM FILM RATIO

CELL SHEET	TIME EXPT. NO.	1	2	3	4	5	6	Mean ± Std.Dev.
PRE	30	0.13	0.07	0.09	0.04	0.03	0.14	0.08 ± 0.05
	60	0.31	0.25	0.28	0.16	0.19	0.32	0.25 ± 0.06
Ch	30	0.32	0.17	0.05	0.11	0.18	0.41	0.21 ± 0.13
	60	0.58	0.48	0.50	0.31	0.34	0.66	0.48 ± 0.14
BSC-1	30		0.09	0.08	0.02	0.04	0.07	0.06 ± 0.03
	60		0.39	0.39	0.08	0.11	0.21	0.24 ± 0.15
C13	30	0.53	0.10	0.10	0.10	0.16	0.39	0.23 ± 0.19
	60	0.72	0.26	0.38	0.22	0.27	0.51	0.39 ± 0.19

sheets (see section 3.1) and found that there was no evidence for the above hypothesis.

The exceptionally high collection of Ch cells by PRE sheets as compared to the collection of other fibroblasts is interesting. Bueltjens and Edwards (1977) thought that the result was unlikely to be explained by quick penetration of the PRE sheet by the choroid cells as the latter were never observed to be spread during the cell sheet collection assay. This feeling is supported by Middleton (1973 in discussion) who reported that chick heart fibroblasts took up to twenty four hours to penetrate a confluent PRE sheet. The increased affinity of choroid fibroblasts for PRE cell sheets is interesting because these two cell types are juxtapposed *in vivo*. However, in development the choroid fibroblasts condense onto the *basal* surface of the presumptive PRE layer. The results described in this section are probably testing adhesion to the apical surface, as PRE monolayers show apical microilli upon their upper surfaces similar to the cells in Plate 3. Bearing this in mind, it may still be important that a "foreign" fibroblast such as a Cl3 cell cannot attach so efficiently, and that neither fibroblast cell type attaches well to the unrelated BSC-1 kidney epithelium. It is possible that there is some retinal-specific contribution to adhesion which increases the adhesiveness of chick embryo fibroblasts, or even choroid fibroblasts alone, but further work is needed to examine this possibility.

No differences were detected in the collection values to suggest that the rate or strength of adhesion of the PRE cells to the fibroblast sheets (Ch and Cl3) was any greater than that of Ch cells to similar sheets.

Moreover there was no correlation between the rate of PRE spreading upon Ch and Cl3 sheets (see section 3.3), and the rate of adhesion to those sheets. It must be remembered that the cell sheet collection assay may not be sufficiently sensitive to detect the minor differences in adhesive strength, which might be sufficient to explain differential spreading upon cell sheets. A further problem might be, that as PRE cells spread out on fibroblast sheets, their resistance to detachment might be due in part to increasing their area of contact with the fibroblast sheets as suggested by Jones et al (1976) to account for the stabilisation of contacts between aggregating limpet haemocytes. However this might indicate the PRE cells are actually less firmly attached in the absence of spreading, than the Ch cells. It is not possible to dismiss the involvement of differential adhesion in the processes described in sections 3.1 - 3.4 but no evidence was provided to explain the observed differential spreading behaviour in these terms.

It seems likely that spreading in these cases may depend upon a number of parameters, and another possible controlling influence; that of contact inhibition of cell spreading is discussed in the next section.

3.6. Time-lapse filming of collisions between retinal cells.

Abercrombie (see Middleton 1973 in discussion) has suggested the contact inhibition might be responsible for cells failing to spread upon the surfaces of other cells. If this is indeed the case then it follows that cells which <u>are</u> able to spread out upon other cells

would be expected not to be contact inhibited by those cells. Epithelial cells seem unable to spread out upon cell sheets of their own cell type and in accord with this Middleton (1973) has shown that PRE cells are contact inhibited on collision with each other. Abercrombie and Middleton (1968) have described experiments which suggest that heterologous epithelia may be subject to contact inhibition when colliding with each other, and PRE cells have been shown to be unable to spread when seeded onto sheets of kidney epithelium (BSC-1). (see section 3.1).

Fibroblast cells appear unable to use their own cell type or epithelial sheets as a substratum for spreading (Dipasquale and Bell 1974, Elsdale and Bard 1975). In agreement with this homologous and heterologous contact inhibition has been demonstrated for a wide range of fibroblast cell types (see Abercrombie 1970 for a review). Also, chick heart fibroblasts have been shown to be contact inhibited when they collide with a PRE sheet (Abercrombie and Middleton 1968). If Abercrombie's explanation is correct, then PRE cells which do spread upon choroid sheets should not be contact inhibited by the latter, and choroid cells which cannot spread upon PRE cells should be contact inhibited. Choroid cells cannot spread upon a cell sheet of their own cell type so they would be expected to contact inhibit one another.

Collision between epithelial cells and fibroblast cells have not been examined in much detail (see section 1.3). None of the authors present quantitative data from their time-lapse filming, or select head-on collisions for interpretation. The latter point is especially pertinent to collissions between choroid fibroblasts.

3.6.1 Overlap index analyses of cultures of Ch and BHK21/C13 fibroblasts.

The culture morphology of Ch fibroblasts (see Plate 1 (b)) is of a criss-cross pattern similar to that of SV-3T3 mouse fibroblasts. The C13 fibroblasts exhibit a more ordered appearance in culture (see Plate 1 (d)), and at confluency are arranged in parallel assays. The overlap index analyses for the two cell types are listed in Tables 8 (a) and 8 (b). It can be seen that the Ch fibroblasts have an overlap index of around two and a half times that of the C13, as suspected from the culture morphology.

3.6.2 Collisions between Ch fibroblasts.

The results of eleven collisions are presented in Table 9 (a) and are summarised in Table 9 (b). It was noticed that in the majority of these collisions only one of the colliding pair of cells exhibited contact withdrawal, so the speed estimation values were split into two groups;

(1) cells exhibiting contact withdrawal
(2) cells which do not.

From the results it appears that the cells exhibiting contact withdrawal show a greater reduction in speed (30% of initial speed) than the cells which did not (50% of initial speed). This impression is probably given by an unusually high initial speed for one of these cells (262.9μ/hr.). In spite of the great variability in cell speed when the data is subject to statistical analysis (see Table 9 (c)) $\hat{z}$ is high for both groups of cells, indicating that there is a significant drop in speed on contact whether contact

TABLE 8

NUCLEAR OVERLAP INDEX OF FIBROBLAST CULTURES

(a) Choroid Cells

Sample (5 fields)	o	n	r(mm.)	2r(mm.)	A(mm^2)	e	o/e
1	19	172	0.0023	0.0046	0.1418	34.8	0.547
2	20	173	0.0026	0.0052	do.	46.0	0.434
3	23	174	0.0021	0.0042	do.	31.3	0.734
4	15	160	0.0025	0.0050	do.	35.5	0.422
5	12	142	0.0024	0.0048	do.	26.2	0.459

Mean Overlap Index 0.519

Std.Dev ±0.130

TABLE 8 (continued)

(b) BHK21/C13 Cells

Sample (5 fields)	o	n	r(mm)	2r(mm)	A(mm^2)	e	o/e
1	5	68	0.0073	0.0146	0.1571	9.6	0.521
2	2	85	0.0072	0.0144	do.	15.2	0.132
3	4	58	0.0091	0.0182	do.	10.9	0.367
4	0	56	0.0046	0.0092	do.	2.6	0.000
5	2	57	0.0079	0.0158	do.	8.1	0.247
6	1	79	0.0064	0.0128	do	10.0	0.100

Mean Overlap Index = 0.228

Std.Dev = ±0.191

TABLE 9 (a)

COLLISIONS BETWEEN TWO CHOROID CELLS

Collision Number.		Speed M./hr Cell 1	Speed M./hr Cell 2	Contact Time mins.	Result of Collision
1	Before contact	41.8	87.9		No reversal of cells or contact with-
	During contact	32.9	24.9	84.25	drawal contact paralysis.
2	Before contact	10.4	29.4		Contact paralysis
	During contact	2.2	13.4	64.00	Cell 2 changed direction.
3	Before contact	43.1	18.0		Contact paralysis No change in direction
	During contact	22.7	20.6	53.50	Cell 1 contact retracts
4	Before contact	45.1	18.8		Contact rectraction Cell 2 Contact paralysis
	During contact	31.9	15.1	37.00	do. do. do
5	Before contact	51.7	37.9		do. do. do.
	During contact	17.8	22.7	12.75	do. do. do.
6	Before contact	24.7	11.1		do. do. do.
	During contact	9.3	5.0	33.25	do. do. do.
7	Before contact	5.9	44.3		do. do. do.
	During contact	25.5	49.7	16.25	do. do. do.
8	Before contact	25.9	57.9		do. Cell 1 do.
	during contact	24.3	17.0	42.50	do. do. do.
9	Before contact	37.6	67.7		Both Cells retract. do.
	During contact	7.6	9.5	21.75	do. do.

10/

Table 9 (a) (continued)

10.	Before Contact	34.5	40.5		Contact Retraction Cell 2 Contact Paralysis
	During contact	17.2	25.9	12.00	do. do. do.
11.	Before contact	34.3	262.89		Cell 2 retracts and reverses Cell 1 changes direction
	During contact	13.8	12.8	14.50	

Table 9 (b)

COLLISIONS BETWEEN TWO CHOROID CELLS

1. Cell Speeds.

	Speed μ/hr ± Stad.Dev.		%Collisions resulting in speed reduction.	
(a) Cells showing contact withdrawal.				
Before contact	60.0	± 72.9	90	10
During contact	19.5	± 13.0		
(b) Cells not showing contact withdrawal.				
Before contact	36.8	± 22.5	83.3	12
During contact	18.9	± 9.0		

2. Contact Time.

Mean contact time (mins)	± Std.Dev.
35.6	± 23.7

3. % Collisions Showing Contact Withdrawal.

45.5

TABLE 9 (c)

COLLISIONS BETWEEN TWO CHOROID FIBROBLASTS

(a) Cells showing contact withdrawal.

Before contact				11.1			18.8					35.9	37.6	37.9	40.5	43.1	44.3
During contact	5.0	7.6	9.5		12.8	15.1		22.7	22.7	24.3	25.9						
Rank	1	2	3	4	5	6	7	8.5	8.5	10	11	12	13	14	15	16	17

Before contact	49.7	67.7	262.9			
During contact	49.7					
Rank	18	19	20			

Before contact $n = 10$ $R_1 = 137$

During contact $m = 10$ $R_2 = 73$

Totals $T = 20$ 210

$$\hat{z} = \frac{137 - \left[10(10+10+1)/2\right]}{\sqrt{10 \times 10(10+10+1)/12}} = \frac{137 - 105}{\sqrt{175}} = \frac{32}{175}$$

$\therefore \hat{z} = \underline{2.418}$ $\therefore$ Signif.

TABLE 9 (c) continued

(b) Cells not showing contact withdrawal

Before contact		5.9		10.4						18.0		24.7			29.4		
During contact	2.2		9.3		13.4	13.8	17.0	17.2	17.8		20.6		24.9	25.5		31.9	32.9
Rank	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17

Before contact	34.3	34.5	41.8	45.1	51.7	57.9	87.9
During contact							
Rank	18	19	20	21	22	23	24

Before contact $n = 12$ $R_1 = 190$

During contact $m = 12$ $R_2 = 110$

Totals $T = 24$ 300

$$\hat{z} = \frac{190 - \left[12(12+12+1)/2\right]}{\sqrt{12 \times 12(12+12+1)}} = \frac{190 - 150}{\sqrt{300}} = \frac{40}{17.32}$$

$$\underline{\hat{z} = 2.309} \quad \therefore \text{ Signif.}$$

withdrawal occurs or not. Contact paralysis occurred in all collisions analysed, and contact withdrawal occurred in at least one of the colliding cells in all but two of the collisions.

3.6.3 Collisions between PRE cells and Ch fibroblasts.

The results of eleven collisions are given in Table 10 (a), and summarised in Table 10 (b). A tracing of one of these collisions is shown in Fig. 6 and as the cells were filmed from beneath the culture it was easy to detect that the choroid fibroblast was underlapping the PRE sheet as in Fig. 6 (b). After twenty minutes the choroid cell leading lamella narrowed (Fig. 6 (c)) and retracted in less than forty five seconds to leave retraction fibrils attached to the lower surface of the PRE sheet (Fig. 6 (d)). The PRE sheet in the region of contact within the Ch fibroblast was carefully examined in each collision and no contact paralysis or contact withdrawal was ever noted.

This impression that the fibroblast is contact inhibited by the PRE cells, but that the latter are insensitive to the former is supported by the quantitative data. The data (Table 10 (c)) show that the choroid fibroblasts slow to 30% of their initial speed ($\hat{2}$ = 2.003) on collision with the PRE sheet. The PRE slow to 75% of their initial speed, and this drop in speed was not significant ($\hat{2}$ = 0.7550). A lower fraction of the PRE cells than the Ch cells experienced a speed reduction of any kind.

Collisions between PRE cells and BHK21/C13 hamster fibroblasts are qualitatively similar to the combination described, although the C13 cells move much more slowly than the choroid cells.

TABLE 10 (a)

COLLISIONS BETWEEN CHOROID FIBROBLASTS AND PRE SHEET

Collision No.		Cell Speeds µ/hr.		Contact Time mins.	Result of Collision
		Choroid	PRE		
1	Before contact	16.3	73.5	30.25	Contact Paralysis Contact Retraction and reversal of Ch
	During contact	1.4	1.4	30.25	No effect on PRE
2	Before contact	82.0	47.2		As collision 1
	During contact	0.75	17.4	47.50	
3	Before contact	22.9	83.8		
	During contact	3.0	30.4	23.50	As collision 1
4	Before contact	64.3	7.1		No paralysis reversal or retraction of Ch.
	During contact	-	15.6	64.0	PRE appeared to move underneath choroid cell
5	Before contact	23.3	29.2		
	During contact	27.5	82.4	13.00	As collision 1
6	Before contact	11.4	5.7		Tenuous contact made between 2 cells no retraction or reversal of choroid cell
	During contact	7.6	12.2	30.00	
7	Before contact	7.6	45.7		
	During contact	0.0	27.4	7.5	As collision 1
8	Before contact	23.7	11.5		
	During contact	13.9	10.8	14.75	As collision 1
9	Before contact	9.6	14.4		

TABLE 10 (a) (continued)

10	Before contact	12.1	27.3		
	After contact	6.6	23.4	30.50	As Collision 1
11	Before contact	99.8	22.3		
	After contact	14.1	9.4	13.25	As collision 1

Fig 6

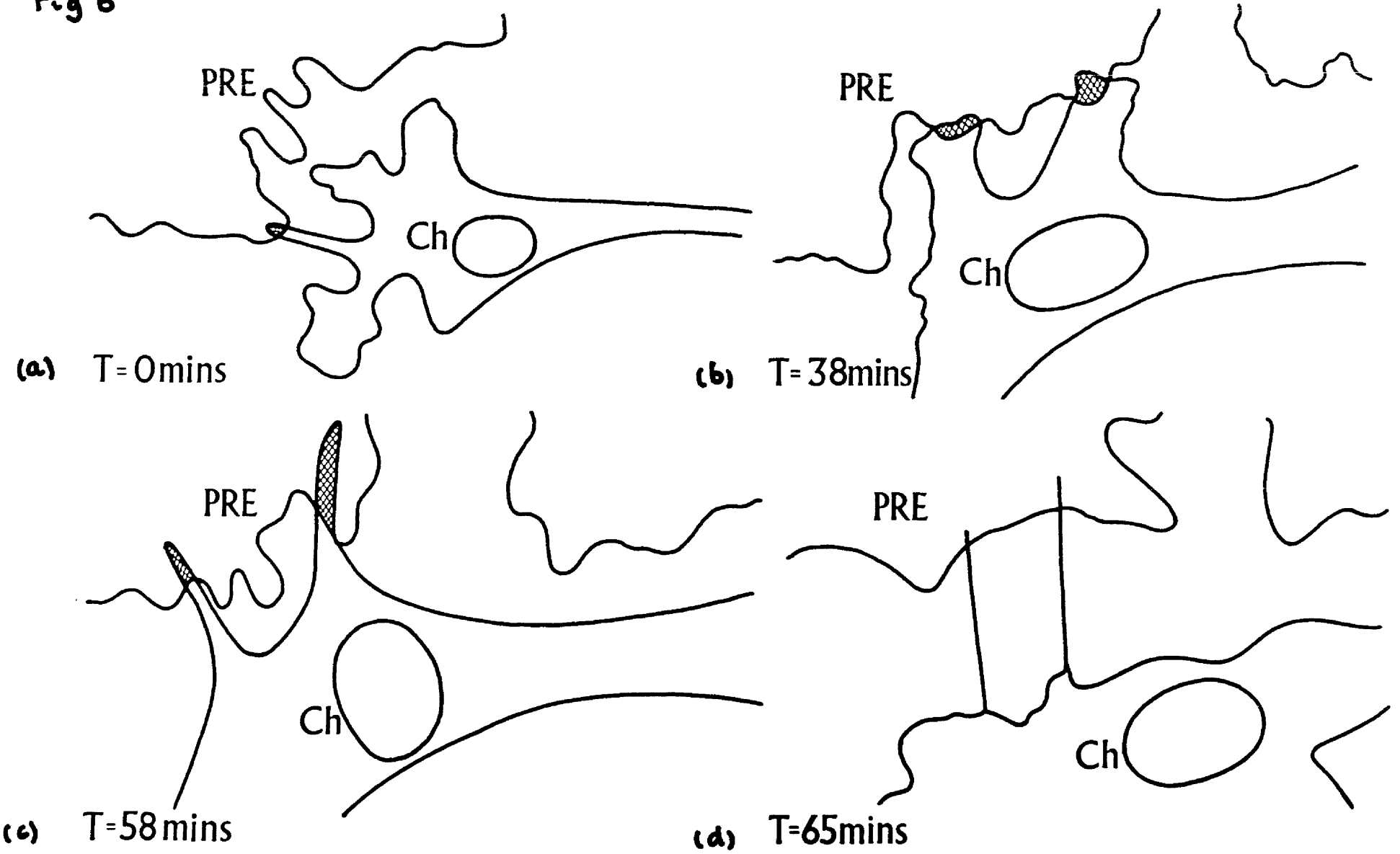

PRE
Ch
(a) T = 0mins
PRE
Ch
(b) T = 38mins
PRE
Ch
(c) T = 58 mins
PRE
Ch
(d) T = 65mins

TABLE 10 (b)

COLLISIONS BETWEEN CHOROID FIBROBLASTS AND PRE SHEET

1. Cell Speeds

	Speed μ/hr	± Stnd.Dev.	% of collisions resulting in (a) speed reduction	(b) contact withdrawal
(a) Choroid cells				
Before contact	30.9	±32.4	80	83.3
During contact	9.8	± 9.7		
(b) PRE cells				
Before contact	33.4	±26.4	63.6	0
During contact	24.1	±21.8		

2. Contact time mins

Mean Contact Time between PRE and Ch Cells	± Std.Dev.
24.1	±17.7

TABLE 10 (c)

COLLISIONS BETWEEN CHOROID FIBROBLASTS AND PRE SHEETS

(a) Choroid Cells.

Cell speeds	Before Contact						7.6	9.6	11.4	12.1				
	During Contact	0.0	0.75	1.4	3.0	6.6	7.6					13.9	14.1	
	Rank	1	2	3	4	5	6.5	8	9	10		11	12	
	Before contact	16.3	22.9	23.3		23.7		82.0	99.8					
	During contact				23.4		27.5							
	Rank	13	14	15	16	17	18	19	20					

Before contact n = 10 R_1 = 131.5

During contact m = 10 R_2 = 78.5

Totals $\underline{T}$ = 20 210

$$\hat{z} = \frac{131.5 - \left[10(10+10+1)/2\right]}{\sqrt{10 \times 10(10+10+1)/12}} = \frac{131.5-105}{\sqrt{175}} = \frac{26.5}{13.23}$$

$\underline{\hat{z} = 2.003}$ $\therefore$ Signif.

TABLE 10 (c) (continued)

(b) PRE CELLS.

Cell speeds													
Before contact			5.7	7.1			11.5		14.4			22.3	
During contact	1.4				9.4	10.8		12.2		15.6	17.4		23.4
Rank	1	2	3	4	5	6	7	8	9	10	11	12	
Before contact	27.3		29.2			45.7	47.2	73.5		83.8			
During contact		27.4		30.4	35.2				82.4				
Rank	13	14	15	16	17	18	19	20	21	22			

Before contact	n = 11	R_1 =	137
During contact	m = 11	R_2 =	116
Totals	T = 22		248

$$\hat{z} = \frac{137 - [11(11+11+1)/2]}{\sqrt{11 \times 11(11+11+1)/12}} = \frac{137 - 126.5}{\sqrt{231.9}} = \frac{11.5}{15.23}$$

$\hat{z}$ = 0.7550 $\therefore$ Not Signif.

TABLE 10 (d)

DIFFERENCES IN CONTACT TIME BETWEEN HOMOTYPIC (CH-CH) and HETEROTYPIC (PRE-CH) COLLISIONS

PRE-CH	7.5	9.75			13.0	13.25		14.75			23.5	30	30.25	30.5
Ch-Ch			12.0	12.75			14.5		16.25	21.75				
Rank	1	2	3	4	5	6	7	8	9	10	11	12	13	14

PRE-Ch				47.5		64.0	
Ch-Ch	33.25	37.0	42.5		53.5	64.0	84.25
Rank	15	16	17	18	19	20.5	22

PRE-Ch	n = 11	R_1 =	110.5
Ch-Ch	m = 11	R_2 =	142.5
Totals	T = 22		253

$$\hat{z} = \frac{110.5-\left[11(11+11+1)/2\right]}{\sqrt{11\times11(11+11+1)/12}} = \frac{110.5-126.5}{\sqrt{231.9}} = \frac{16}{15.23}$$

$\hat{z} = 1.051$ $\therefore$ Not Signif

3.6.4 Contact time in homotypic and heterotypic collisions

The duration of contact in Ch-Ch collisions (Table 9(b)) is somewhat greater than the duration of contact in PRE-Ch collisions (Table 10 (b)). The $\hat{z}$ statistic was found to be low (1.051) and therefore the difference in contact time must be regarded as not significant (see Table 10 (d)).

3.6.5. Discussion.

The results support the hypothesis that cells which can spread upon sheets of other cells are not contact inhibited by the latter. PRE cells can be adjudged not to be contact inhibited by Ch fibroblasts on three counts.

(1) PRE cells do not reduce their speed significantly on contact with Ch cells, and what reduction there is could be explained by physical hinderance due to the fibroblast.

(2) PRE cells exhibit no contact paralysis on collision with choroid fibroblasts.

(3) PRE cells invade confluent sheets of Ch fibroblasts onto which they have been seeded (see section 3.4).

The absence of contact withdrawal is less important as PRE cells do not exhibit contact withdrawal when they collide with other PRE cells, by which they <u>are</u> contact inhibited.

Ch fibroblasts on the other hand <u>are</u> contact inhibited by the PRE sheet, and according to Abercrombie's hypothesis (see Middleton 1973 in discussion), they should not be able to spread upon sheets of PRE, as was found by Buultjens and Edwards (1977). Contact inhibition of chick fibroblasts (choroid and heart) by

PRE sheets has been previously reported by Abercrombie and Middleton (1968). Also, human embryo lung Fibroblasts appear to be contact inhibited on collision with human kidney epithelium (Elsdale and Bard 1975). These observations suggest that contact inhibition of fibroblasts by epithelial sheets may be a general phenomenon, although Wilbanks and Richart (1966) failed to observe a consistent contact withdrawal of cervical fibroblasts on collision with human cervical epithelium. Perhaps in the latter instance the workers did not take steps to analyse head-on collisions, and also the fibroblasts in many regions were very dense making interpretation difficult.

Even if the behaviour exhibited by the PRE and Ch proves to be a general one, there are still some objections which can be raised against the role of contact inhibition of movement in suppressing the spreading of cells upon the upper surfaces of other cells.

(1) Contact inhibition has never been shown to be induced in other cells by the upper surfaces (i.e. not lamellar cytoplasm) of spread cells.

(2) Epithelial cells do not migrate over the upper surfaces of fibroblasts in confronted cultures (Elsdale and Bard 1975).

It has never been shown that the cell surface above non-lamellar cytoplasm can induce a contact inhibitory response in another cell. However there is some evidence to suggest that cells falling upon a cell sheet in fact adhere to the ruffling margins (Dipasquale and Bell 1974), which <u>can</u> induce a contact inhibitory response. Possibly filopodia which have been associated with the early stages of spreading (Rajaraman et al 1974) may originate at the point of contact with the

cell sheet, and are prevented from forming owing to contact paralysis by the latter. PRE cells, which do spread on choroid sheets and are not contact inhibited by them are seen to form filopodia as early as ten minutes after attachment to the sheet (see Plate 8 (b)).

If epithelial cells can generally spread upon fibroblast sheets, and preliminary observations suggest that PRE can move on choroid at 70% of their speed on serum-films why then do they not crawl over such a sheet after contact is made instead of pushing back the fibroblasts as observed by Elsdale and Bard (1975) using human cells?

There are two possible answers: First as the fibroblasts usually retract on collision with epithelial sheets, then possibly the epithelia may not have a chance to crawl on top of the faster moving fibroblast. When the culture is dense the epithelium may pass between the fibroblasts and the substratum causing the latter to be detached. Secondly, Dunn and Heath (1976) have shown that chick heart fibroblasts are subject to mechanical restraints which prevent them from crawling over apices of more than 4^{o} to the horizontal. The reason for these restraints appears to involve the microfilament bundles which insert at the leading lamella, and are thought to be responsible for pulling the cell body forwards towards new points of adhesion. (Abercrombie et al 1970c, 1971). When the angle of the prism used in Dunn and Heath's experiments exceded 4^{o}, the microfilaments could apparently no longer insert on the membrane at new points of adhesion across the ridge. Transmission electron microscopy revealed that the microfilament bundles terminated at the ridge. Dunn and Heath stress that for the inverse situation (i.e.

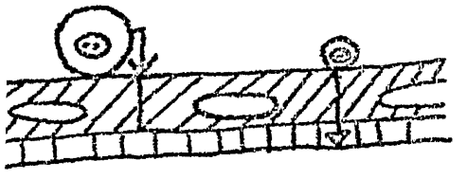
negotiating slopes or climbing on top of other cells), and for shorter cells (i.e. PRE cells), the restraints may be quantitatively different. However, it is possible that similar restraints may act upon epithelial sheets preventing them from overlapping the fibroblast sheets, as observed in human epithelia by Elsdale and Bard (1975).

Even if these objections are satisfied it must be recognised that factors other than contact inhibition may well have strong influences in determining whether one cell type can spread and move upon another cell type. Other possible influences have been discussed earlier. Namely differences between PRE and Ch in:

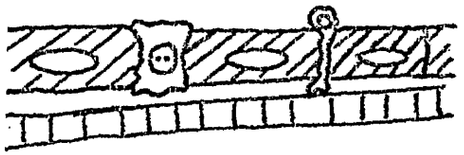
(1) Their adhesiveness to fibroblast sheets.
(2) Their resistance to deformation.
(3) Their ability to spread upon substrata of low rigidity.

BEHAVIOUR OF EPITHELIAL AND MESENCHYMAL CELLS ON CELL SHEETS.

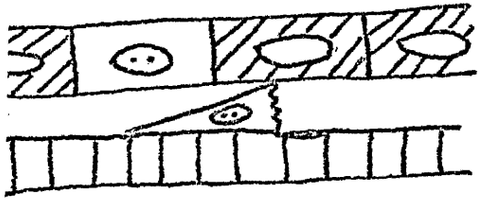
a) ON EPITHELIAL SHEETS.



(1) ZERO TIME



(2) INTERMEDIATE STAGE



(3) 24 HOURS

KEY

CULTURE SUBSTRATUM

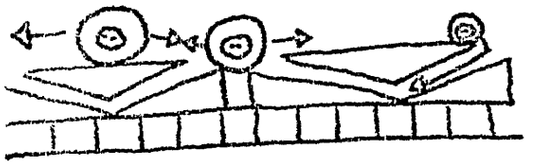
EPITHELIAL SHEET

FIBROBLAST SHEET

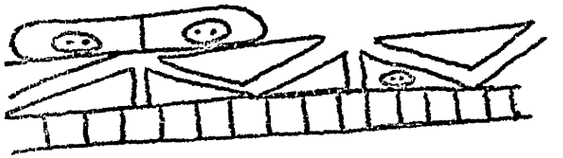
SEEDED EPITHELIAL CELL

SEEDED FIBROBLASTIC CELL

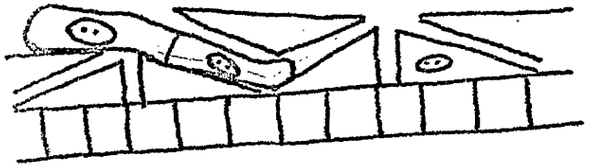
(b) ON MESENCHYMAL SHEETS.



(1) ZERO TIME



(2) 1 HOUR



(3) 4 HOURS

4. GENERAL DISCUSSION

4.1. Differential spreading and organ construction.

The spreading behaviour of epithelial and fibroblastic cell types on different cellular substrata is summarised in Fig. 7. In the present study epithelial cells were found to be unable to spread upon their own cell type and other epithelia, although some cells were eventually seen to become incorporated into the preformed sheets. Middleton (1973 in discussion) has reported that fibroblasts are capable of penetrating an epithelial sheet and spreading out upon the culture substratum beneath. Epithelial cells (PRE) were able to spread out upon sheets of fibroblasts but fibroblasts themselves were unable to do so. The ability of epithelial cells to spread upon fibroblasts correlates with the inability of the former to be contact inhibited by the latter (see section 3.6.5. for details). This differential ability to spread upon cellular substrata of different origin may be important in developing organs.

In the eye, presumptive choroid fibroblasts condense onto the basal surface of the developing PRE (Leplat 1912). If the choroid cells were to accidently arrive at the apical surface of the PRE their inability to spread, coupled with their possible ability to penetrate the PRE, would lead to a restoration of order within the developing organ. The inability of PRE cells to spread and move upon sheets of their own cell type may similarly prevent multilayering of the PRE. If this behaviour is general for epithelial cells it is curious how a multilayered epithelium can arise, as for example in skin.

It is recognised that the presence of intercellular matrix, basement lamina etc. may well insulate the cells from each other's effects, as both epithelial and fibroblastic cells are known to be able to spread upon such substances as collagen. Overton (1977) has shown that epithelial cells were capable of spreading upon the isolated basement membrane of Xenopus tadpole skin, but that mesenchymal cells (chick fibroblasts and myoblasts) were not. The mesenchymal cells were seen to penetrate the lamina and occupy a similar position to that which they would occupy *in vivo*. Therefore, this differential ability of epithelial and fibroblastic cells to spread upon basement lamina may serve to control cell movements *in vivo*. However, the events described earlier during the development of the eye occur at day five (see Romanoff 1960), and at this stage of development the presence of much intercellular matrix or a basement lamina is unlikely. So, it appears that the differential ability of epithelial and mesenchymal cells to spread upon other cells, in addition to their differential ability to spread upon basement lamina, could be important during the construction of organs such as the eye.

4.2. Invasion of choroid by the PRE: its possible relevance to morphogenesis, wound healing and carcinomatous invasion.

The apparent invasion of choroid fibroblast sheets by PRE cells (see section 3.4) is possibly an important observation and is presumably a consequence of the failure of PRE cells to be contact inhibited by choroid fibroblasts (see section 3.6). However, PRE cells do not invade choroid fibroblast sheets in the developing chick eye. The PRE develops from a coherent sheet of cells derived from the ectoderm (neural tube), and this

sheet is contiguous with the pigmented iris epithelium so that there are no free edges. As the PRE differentiates the cells presumably acquire epithelial junctional complexes ((terminal bars) Farquhar and Palade 1963), consisting of a gap or tight junction and a zonula adhaerens. These junctional complexes have been demonstrated in cultures of PRE cells (Middleton and Pegum 1976), and presumably the zonula adhaerens serve to 'lock' the cells together, since the function of the gap junctional component is to allow passage of molecules between the cells of the sheet (Gilula et al 1972), and the function of the tight junctional component is to form a seal preventing passage of most molecules via spaces between the epithelial cells (Cohen 1965). The cells of a PRE sheet in vivo would not be able to present a free edge to the choroid, as it has been demonstrated that PRE cells contact inhibit one another (Middleton 1972), and that collisions between these cells result in the formation of stable contacts (Middleton 1973) so there is no ruffling membrane in the sheet. Possibly it is only when PRE cells possess a free edge that they are'potentially invasive' to the choroid sheet. If this behaviour is common to all epithelial-mesenchymal systems then it might explain other features of epithelial behaviour towards fibroblasts.

Free edges in an epithelial monolayer can be produced by:

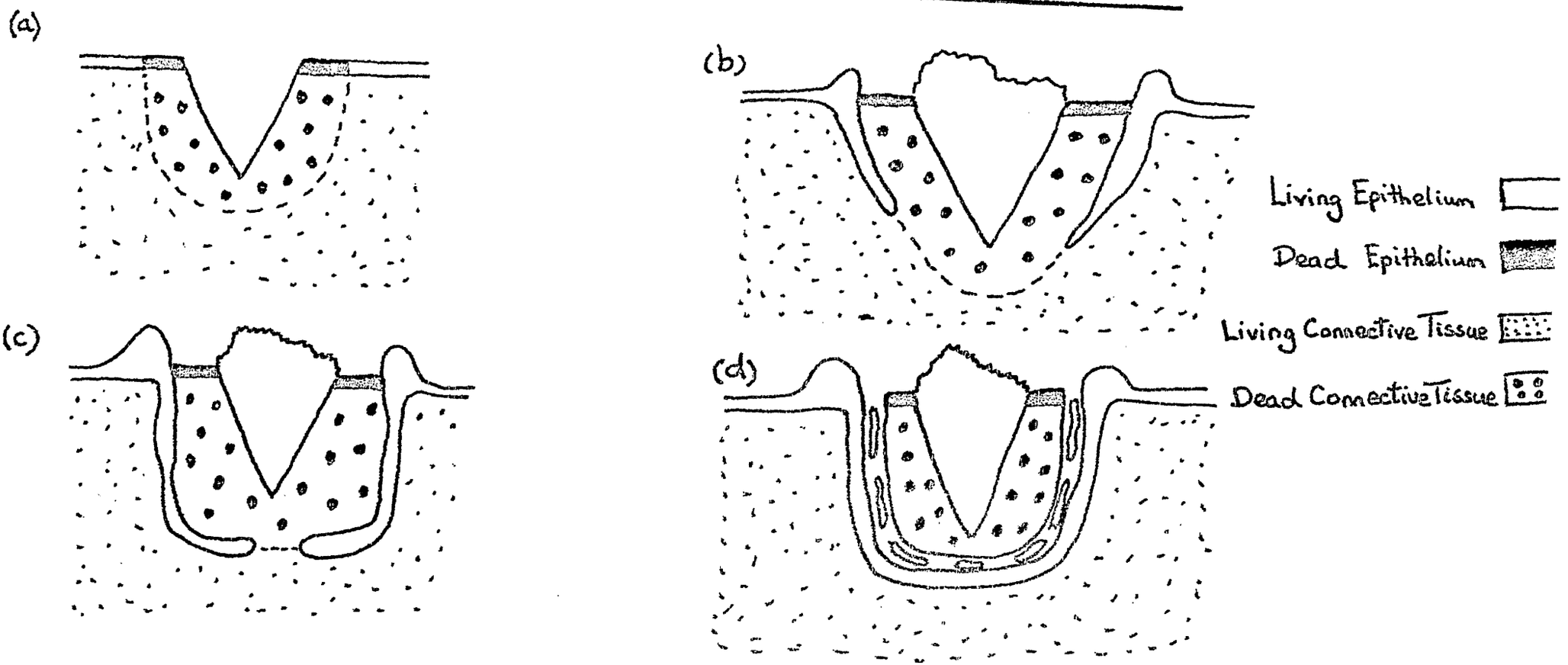
(1) Dissociating the cells as in the experiments described in section 3.4.

(2) Wounding the epithelium.

(3) Carcinogenesis; if this indeed results in reduction of intercellular adhesions between epithelial cells and interference with the contact stability (Coman 1944, 1953).

Fig. 8 [After Croft and Tarin (1970)]

EPITHELIAL MIGRATION DURING WOUND HEALING.



The events following the wounding of mouse skin have been described by Croft and Tarin (1970) see Fig.8. Croft and Tarin (1970) have shown that the migrating epithelium follows the boundary between living and dead tissue in the wound, and that deposits of extra vascular fibrin are present beneath the epithelium which does not penetrate living mesenchyme . These results suggest that the migrating epithelial sheets may be guided towards one another by the living tissue/dead tissue interface or by the deposits of extra vascular fibrin. The epithelial movement stops when the two sheets meet presumably due to the influence of homotypic contact inhibition of movement which has been demonstrated for epithelial cells (Vaughan and Trinkaus 1966, Middleton 1972,1973). The results described in section 3 suggest that epithelial cells can spread upon mesenchyme but this does not explain why there is no invasion of the connective tissue by wounded skin epithelium. Similar types of 'controlled invasion' of mesenchyme by epithelium occurs in the development of certain endodemal organs such as the liver (Croisille and Le Douarin 1965), and the lungs (Sorokin 1965) in the form of epithelial cords. Controlled invasion of the uterus by the trophoblast (see Kirby and Cowell 1968 for a review) is possibly effected by a thin layer of decidualised mesenchyme which remains healthy. In this case and in that of wound healing, the invasive epithelium can apparently only invade dead or degenerating tissue. The production of a free epithelial edge by carcinogenesis results in a completely uncontrolled type of invasion, and some of the reasons for this will be discussed in the next section.

4.3. Invasion of mesenchyme by carcinoma cells: the possible importance of loss of intercellular adhesion.

4.3.1. General

As human kidney (Elsdale and Bard 1975), human cervix (Wilbanks and Richart 1966), and chick embryo PRE (see section 3.6) seem not to be contact inhibited by fibroblasts *in vitro*, any reduction in the strong lateral adhesions which exist between the epithelial cells (Farquhar and Palade 1963, Middleton and Pegrum 1976) allowing individual cells to break free would be instrumental towards the infiltration of these cells into the mesenchyme. The junctional complexes (terminal bars) have been shown to be attenuated or absent in some malignancies (see Weinstein et al for a review).

A reduction in intercellular adhesion between squamous carcinoma cells as compared to their normal counterparts, was demonstrated by Coman (1944), and many similar examples have been reported (see Coman 1953 for a review). The movement of isolated PRE cells has been said to be amoeboid random and slow (Middleton 1973, 1976) and could be regarded as being similar to the locomotion of carcinoma cells *in vitro* (Enterline and Coman 1950), and *in vivo* (Wood et al 1967).

To what extent then are carcinoma cells 'normal' epithelial cells with reduced intercellular adhesion (or homotypic contact inhibition)? The controlled invasion which occurs during the healing of wounds (Croft and Tarin 1970), and the fact that benign tumors which display reduced intercellular adhesion cannot infiltrate cultures of fibroblasts *in vitro* (Santesson 1935), would tend to argue against this point of view. Clearly other parameters must be present in normal tissue to restrain these 'potentially invasive' epithelial cells, and

prevent them from producing adherent structures during development and following wounding. The PRE cells used in the present study were embryonic and it should be borne in mind that adult epithelia may not behave in the same manner. However proteolysis may also play a part in the invasive behaviour of many tumors and recent evidence is discussed in the next section.

4.3.2. Proteases and the reduction in intercellular adhesion.

Fibrinlysins have been shown to be present in cultures of chick fibroblasts transformed by RNA viruses, in much higher levels than their normal counterparts (Unkeless et al 1973), and similar reports have been made for virus-transformed mammalian cells (Ossowski et al 1973). Subsequent studies have shown that the fibrinlysin exists in an inactive form (Plasminogen), in the sera of a number of animals including humans (Quigley et al 1974). This proenzyme (plasminogen) was then shown to be activated by a factor (the plasminogen activator) produced by virus-transformed fibroblasts (Unkeless et al 1974 , Christman and Acs 1974).

The plasminogen-activator has been shown to be released by virus-transformed fibroblasts in much greater amounts than their normal counterparts as tested by the caesinolysis assay of Goldberg (1974). Preliminary observations suggest that similar high levels of plasminogen-activator are produced by several types of carcinoma cells (Unkeless et al 1974). Also, Strickland et al (1976) have reported that the invading trophoblast is capable of producing high levels of plasminogen-activator.

In addition to plasmin a trypsin-like protease (Cathepsin B) has been demonstrated at the surface of

invading tumor cells but not their normal counterparts (Sylvan 1973), and the potential significance of the presence of these enzymes is clear.

Not only would these enzymes assist in the loosening of intercellular contacts between epithelial cells but would also assist in the removal of extracellular matrix, and weaken contacts between the cells of the tissue being invaded. Ossowski et al (1974) have demonstrated that when normal and transformed fibroblasts are co-cultured, the high plasmin levels of the transformed cells bestow a transformed cell morphology upon the normal fibroblasts. This morphology could well be associated with reduced adhesion of these cells to the culture substratum.

4.3.3. Collagenases and invasion by carcinoma cells.

Extracellular matrix (e.g. collagen fibres) may provide a natural barrier, so that when normal epithelium is wounded, or should individual epithelial cells break free, invasion of the connective tissue will not occur and the epithelial monolayer will be stabilised. There have been reports of collagenase activity in migrating wound epithelium (Grillo and Gross 1967), and also in skin carcinoma (Hashimoto et al 1973). The latter enzyme has been shown to be tumor-specific, and to be capable of breaking down the basement lamina of the epithelium. This evidence is in agreement with the observations of Franks (1973), who reported that breakdown of the basement lamina always preceded invasion. Transmission electron microscope studies by Tarin (1967) suggested that the first step in skin carcinogenesis was a gradual thickening of the basement membrane with eventual

separation from the epithelial cells, followed by a breakdown of the basement membrane. Subsequent studies (Tarin 1968) have shown that non-carcinogenic irritant chemicals do not produce these changes. The difference between wounded skin and carcinomatous skin may lie in the relative levels of the collagenases described by Grillo and Gross (1967) and Hashimoto et al (1973). Alternatively, the tumor-specific collagenase (Hashimoto et al 1973) may be a different, more effective enzyme, or it may be the same enzyme working more efficiently due to the high levels of other proteases which are associated with tumor cells.

4.3.4. Influence of the mesenchyme upon the epithelium.

The influence of the mesenchymal cells of the connective tissue may play an important part in the maintenance of epithelial integrity. Connective tissue has been shown to affect the rate of epithelial cell division in chick embryo epidermis (McLaughlin 1961, Wessels 1964), and also to affect the differentiation of the epithelium in mouse salivary gland (Grobstein 1953), mouse thymus gland (Auerbach 1960), chick embryo epidermis (McLaughlin 1961), and chick embryo pituitary (Sobel 1958).

Carcinoma cells usually exhibit an increased rate of cell division and decreased morphological differentiation (anaplasia) to varying degrees (see Willis 1967). This might well be due in part to the removal of the influence of the mesenchymal cells beneath the tumor. Indeed the studies of Tarin (1967) on experimentally-induced skin cancer revealed that there was a marked destruction of the connective tissue and the basement lamina in the region of carcinogenesis. This

destruction might also reduce the degree to which the tumor is encapsulated by the connective tissue, and hence will promote its expansion and infiltration. The destruction of the connective tissue does not occur when the skin is treated with non-carcinogenic irritants (Tarin 1968).

4.3.5. How do these results relate to the mechanism of invasion in vivo ?

The evidence presented in section 3 suggests that there may be a correlation between the ability of PRE cells to spread upon and later 'invade' sheets of choroid fibroblasts, and the lack of contact inhibition of cell locomotion exhibited by PRE cells in collisions between the two cell types. 'Invasion' in this sense is defined as being the active movement of one cell type so that it occupies space formerly occupied by another cell type.

In the system described in this thesis the absence of connective tissue allows the two cell types (PRE and Ch) to contact one another freely, and in this sense it differs from the in vivo situation. However the influence of the mesenchyme cells was still present and there was no evidence of the fibroblasts being lysed or destroyed by the invading PRE cells (see section 3.4). The fibrinolysins and collagenases described in sections 4.3.2. and 4.3.3. would remove the connective tissue during carcinogenesis perhaps permitting free contact between the free epithelial edges and the mesenchyme cells. It seems possible that the cell contact phenomena described in section 3 if general to all epithelio-mesenchymal systems could play a part in the direct spread of carcinoma cells. If normal epithelial cells when possessing a free edge, are potentially invasive to mesenchymal cells in the sense that they are not contact inhibited by

the latter, then the main parameters affecting carcinomatous invasion of the mesenchyme might be summarised as follows:

(1) Appearance of high levels of proteases and fibrinolysins at the tumor cell surface resulting in diminished intercellular adhesion and assisting the breakdown of intercellular matrix.
(2) Removal of natural barriers to invasion such as collagen and the basement lamina by tumor-specific collagenases.
(3) Loss of intercellular contact between epithelial cells allowing individual cells and/or small groups of cells to infiltrate the mesenchyme by migrating over and between the fibroblasts, unimpeded by contact inhibition. The latter statement clearly assumes that the results described in section 3 are general phenomena and the PRE and Ch cells are 'normal' cells.

4.4. Contact Inhibition: its relevance to in vivo events.

To what extent do the processes of contact paralysis and contact retraction relate to the in vivo events outlined in section 1.1? Only Bard and Hay (1975) have observed contacts between fibroblasts in situ, and found that no recognisable paralysis or withdrawal occurred after the cells made contact. This would tend to argue that contact inhibition is unimportant in vivo. However, Bard and Hay (1975) did demonstrate that corneal fibroblasts in situ do reverse their locomotion, so that a contact inhibition of sorts does occur in vivo. It would be impossible to see contact paralysis in Bard and Hay's system, as ruffling and blebbing were not apparent, but the absence of contact withdrawal is harder to explain. Perhaps the sudden contraction (which occurs in less than forty five seconds in vitro) is much

less abrupt *in vivo* owing to the surrounding extracellular matrix. Bard and Hay would not have been aware of such slow changes as they were unable to employ time-lapse filming in their system.

Another major objection in applying information gained from studies *in vitro* is that the presence of large amounts of extracellular matrix would render direct contact of cells *in vivo* unlikely. However, some evidence has been provided by Bluemink et al (1976) that epithelial cells *do* make contact with mesenchymal cells, even when a basement membrane is fully developed in the epithelium, and large amounts of collagen fibres have been secreted by the fibroblasts. Close contacts between the epithelial cells and the mesenchymal cells of 10nm. separation distance were observed in regions of cellular proliferation. In these regions, the basement lamina and connective tissue appeared to be absent, and as the two cell types were seen to make contact with each other this would allow contact inhibition and other phenomena to affect subsequent events.

4.5. Contact inhibition of movement: possible mechanisms.

In this thesis it was suggested that the ability of normal epithelial cells to invade fibroblast sheets *in vitro* may be seen as a natural consequence of the lack of contact inhibition of the former by the latter (see section 3.6). The mechanism of contact inhibition of movement has not been fully resolved although a number of theories have been proposed (see section 1.3). Some previous models have been advanced with the notion that it is the avoidance of overlapping which needs

explanation, and this may be the result of phenomena other than contact inhibition of movement 'type 1' (see section 1.3.5.). In addition the results outlined in section 3 would tend to argue against hypotheses based on the inability of the upper cell surface to support spreading and locomotion (Dipasquale and Bell 1974). Also, this and theories based upon the differential adhesiveness hypothesis (Martz and Steinberg 1973, Martz et al 1974) suffer from another objection. In cases of non-reciprocal contact inhibition (see section 3.6) and in some cases of homotypic contact inhibition (Abercrombie 1970), cells which are underlapping are the ones which are inhibited, and no change in substratum is ever noted. It is recognised that cells in confluent monolayers do change position even when surrounded on all sides by other cells (Garrod and Steinberg 1975, Steinberg and Garrod 1975, Martz 1973). These cells may change position by exchanging weak intercellular adhesions for strong ones, and if this is the case then this type of movement cannot be regarded as being the same as the directional movement exhibited by fibroblasts and epithelial sheets outwith a confluent monolayer.

Any future model must:

(1) Account for non-reciprocal contact inhibition between epithelial cells (or MCIM sarcoma cells), and normal fibroblasts.

(2) Allow contact paralysis but not contact withdrawal when two epithelial cells collide.

(3) Allow contact paralysis and contact withdrawal when two fibroblasts collide.

(4) Explain contact paralysis and contact withdrawal.

(5) Accomodate the following recent evidence and ideas:

Evidence has recently been provided by Heaysman and Turin (1976) which might indicate that there is a cell surface component on one cell which is recognised by another cell leading to contact inhibition in the latter. If one cell was fixed using glutaraldehyde a living cell colliding with it showed no contact paralysis or withdrawal. However there was no such alteration if the first cell was fixed by the zinc chloride method of Warren ahd Glick (1966), and the colliding cell still exhibited contact paralysis and retraction. If contact inhibition 'type 1' is dependent upon the recognition of a glutaraldehyde sensitive molecule (GSM) such recognition might result in transduction of information across the plasma membrane, triggering the assembly of adhesion plaques and microfilament bundles. These plaques and bundles have been shown to appear within twenty seconds of contact between two chick heart fibroblasts (Heaysman and Pegrum 1973a). The finding that such 'adhaerens-type' junctions do not form in sarcoma 180 cells on collision with normal chick heart fibroblasts (Heaysman and Pegrum 1973b), strengthens the view that formation of these junctions might account for some of the features of contact inhibition 'type 1' as sarcoma 180 cells show no contact paralysis or contact withdrawal when colliding with chick heart fibroblasts. Contraction of the microfilament bundles associated with these junctions might well be responsible for the contact withdrawal which occurs in contact inhibited fibroblasts. So why then does a similar event not occur in collisions between epithelial cells? Early contacts between colliding epithelial cells have not yet been examined with the electron microscope, but Middleton and Pegrum (1976) have reported 'adhaerens-type' junctions between pigmented retina epithelial cells which have been in

culture for twenty four hours. It seems likely that 'adhaerens type' junctions do form between colliding epithelial cells, but possibly the strength of adhesion might be greater between the lateral edges of epithelial cells than between the lateral edges of fibroblasts. Thus the strength of adhesion between the epithelial cells might be able to resist the contraction of the microfilament bundles, and pull the two cells together broadening the area of mutual contact between the cells. This extension of the area of mutual contact was actually seen to occur in cultures of epithelial cells by Weiss (1958).

It has recently been postulated that cell to substratum adhesion and assembly of microfilament 'plaques' requires a membrane-located molecule linked to the microfilaments which is clustered by a second trysin-sensitive molecule which can be replaced by certain lectins (Rees et al 1977). A possible candidate for the latter component might be the large external transformation-sensitive protein (LETS) (Hynes 1973) which is highly trypsin-sensitive in a number of systems (see Hynes 1976 for a review). Albrecht-Buehler and Chen (1977) have shown that LETS protein appeared to predominate at regions of contact between balb/3T3 mouse fibroblasts, indicating LETS protein may be associated with inter-cellular contact. Recent studies have shown that whilst some epithelial cells possess LETS protein others do not (Chen et al 1977), so the involvement of this protein in the assembly of 'adaerens-type' junctions may not be general. However, junction assembly is likely to form the basis of the contact retraction which is observed to occur between colliding fibroblasts, and should LETS be shown to play a part in this assembly there may also be a link with contact paralysis. Albrecht-Buehler and

Chen (1977) have shown a correlation between the presence of LETS protein, and the inhibition of particle transport in mouse fibroblasts, so it is conceivable that LETS may block membrane flow at regions of intercellular contact. However, the lack of LETS protein in some epithelial cell types (Chen et al 1977), and its absence in certain virus-transformed cell lines which have since proved to contact inhibit one another when colliding head-on (Bell 1972, Erickson 1976), would seem to argue against the involvement of LETS protein in contact inhibition 'type 1'. Nevertheless, the association of this protein within intercellular contacts is of great interest.

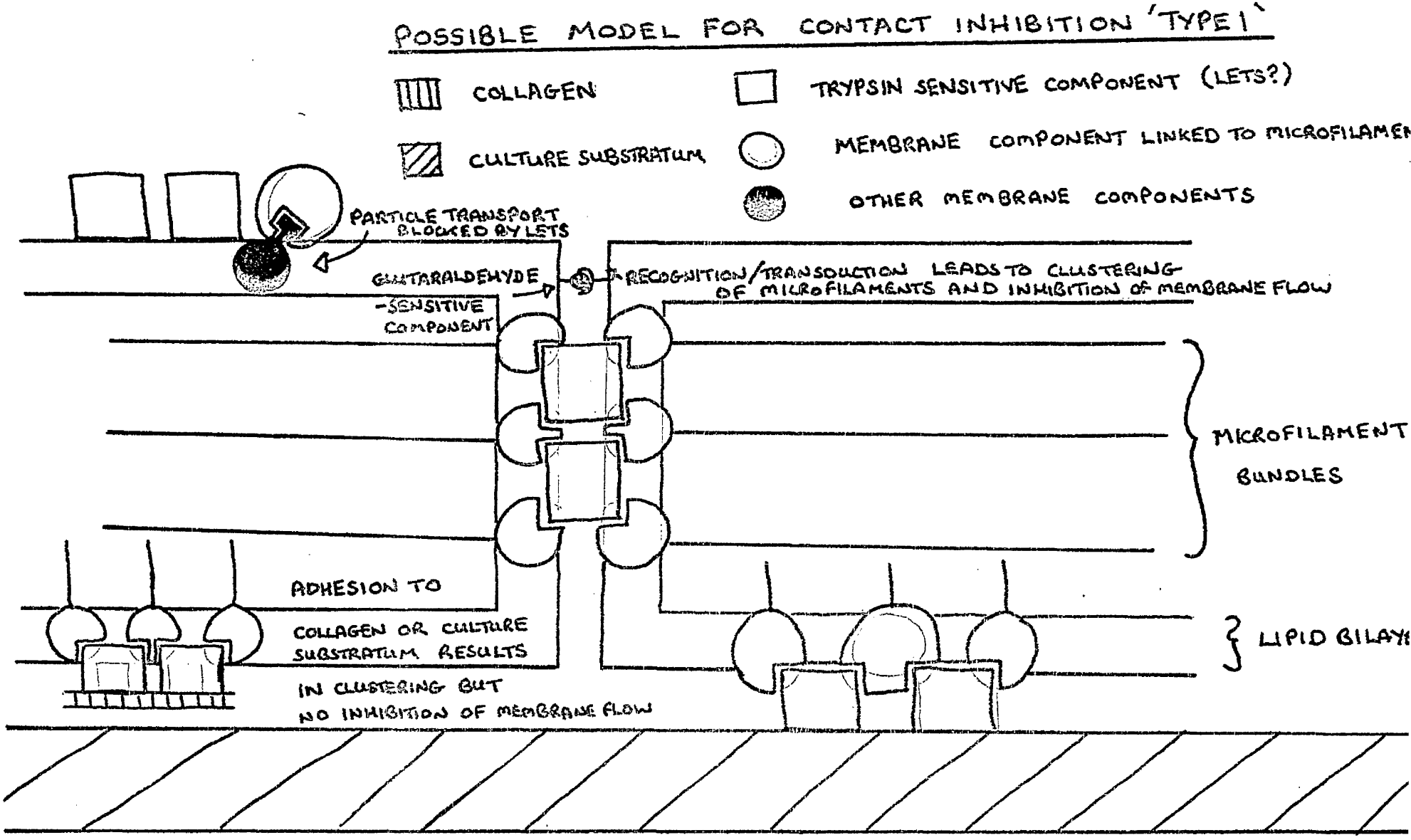
If contact inhibition 'type 1' functions in the same way as intercellular adhesion then it is difficult to explain contact paralysis, as adhesion plaques to the culture substratum are morphologically similar (Abercrombie et al 1971) to those seen between colliding fibroblasts by Heaysman and Pegrum (1973a). Yet assembly of such junctions does not seem to prevent extension of the leading edge ahead of the plaque. One must postulate an extra recognition mechanism activated only by intercellular contact which inhibits the assembly of membrane at the point of contact. Adhesion of the leading lamella to the culture substratum must be strong enough to resist the contraction of the microfilament bundles as the cell body moves forward towards new points of adhesion and does not undergo a contact retraction.

To explain non-reciprocal contact inhibition 'type 1' one could postulate that a fibroblastic cell is capable of recognising another fibroblastic cell (or epithelial cell), leading to assembly of an adhesion

plaque and associated microfilament bundles by a mechanism similar to that suggested by Rees et al (1977) for cell to substratum adhesion. The same recognition mechanism may lead to inhibition of membrane flow. The epithelial cell must then only be able to recognise another epithelial cell, so one would not expect to see microfilament bundle assembly in an epithelial cell after contact with a fibroblast, as the former do not exhibit contact withdrawal on collision with the latter (see section 3.6).

Tumor cells are subject to great variation as regards the way they behave in culture. MCIM sarcoma cells elicit a contact inhibition 'type 1' response in normal fibroblasts but are not contact inhibited by the fibroblasts (Heaysman 1970), but in collisions between sarcoma 180 cells and normal fibroblasts neither cell undergoes contact inhibition (Abercrombie and Ambrose 1958). Many changes in cell surface glycoproteins, proteins (See Nicholson 1976 for a review), and glycolipids (see Hakomori 1975 for a review) occur after malignant transformation. Also many of the more externally situated proteins are altered or deleted following such a change (see Hynes 1976 for a review). How a tumor cell behaves towards a normal fibroblast, would depend upon which of the molecules shown in Fig. 9 are deleted, altered or masked. The MCIM cell might be unable to recognise a normal fibroblast but can still elicit a response in the latter, so the glutaraldehyde-sensitive molecule (GSM) should be unaltered in these cells. However, in sarcoma 180 cells both GSM and the recognition site should be lacking. Within the confines of the scheme outlined in Fig. 9, it would still be possible for a transformed cell to exhibit contact inhibition 'type 1' (see Bell (1972), Erickson (1976)), provided the molecules shown in Fig. 9

Fig. 9


POSSIBLE MODEL FOR CONTACT INHIBITION 'TYPE I'
COLLAGEN
CULTURE SUBSTRATUM
TRYPSIN SENSITIVE COMPONENT (LETS?)
MEMBRANE COMPONENT LINKED TO MICROFILAMEN
OTHER MEMBRANE COMPONENTS
PARTICLE TRANSPORT BLOCKED BY LETS
GLUTARALDEHYDE
-SENSITIVE COMPONENT
RECOGNITION/TRANSDUCTION LEADS TO CLUSTERING OF MICROFILAMENTS AND INHIBITION OF MEMBRANE FLOW
MICROFILAMENT BUNDLES
ADHESION TO
COLLAGEN OR CULTURE SUBSTRATUM RESULTS
IN CLUSTERING BUT
NO INHIBITION OF MEMBRANE FLOW
LIPID BILAY

were not altered sufficiently to impair their function.

In summary, contact inhibition 'type 1' may involve an interaction which is similar to the mechanism of cell to substratum adhesion proposed by Rees et al (1977). However contact inhibition 'type 1' must also possess an additional cellular recognition mechanism, which may or may not be responsible for inhibition of membrane flow on collision with another contact inhibiting cell. The model is summarised in Fig. 9.

The substantiation of this model (or the lack of it) depends upon advances in the understanding of molecular species such as LETS, and the generality of its application depends on the assumption that the results described in section 3.6 are common to all collisions between epithelial and fibroblastic cells. Previous work by Wilbanks and Richart (1966), Abercrombie and Middleton (1968), Elsdale and Bard (1975) is suggestive that the behaviour of PRE cells described in section 3.6 may be a general property of epithelial cells. These authors did not present quantitative data however, and more of this will be required before any generalisations can be made about interactions between epithelium and fibroblasts.

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