INVESTIGATIONS ON THE CONTROL OF CELL BEHAVIOUR AND THE CELL CYCLE

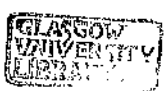
by

SATISH CHANDRA TRIPATHI

M.Sc., M.Phil.

A THESIS SUBMITTED TO THE UNIVERSITY OF GLASGOW FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

August, 1984



ACKNOWLEDGEMENTS

I Would like to thank :

Professor A.S.G. Curtis, my supervisor, for helpful discussion, encouragement and valuable criticism of the work, and the following organisations for financial assistance :

(i) R. D. Birla Smarak Kosh, Bombay Hospital Trust, Bombay, India for awarding me a fellowship.

(ii) Education Division of Police Department, Ministry of Home Affairs, State Government of Madhya Pradesh, Government of India.

(iii) Committee of Vice-Chancellors and Principals of the Universities of the United Kingdom.

(iv) University of Glasgow for a Bursary.

(v) Dorab Tata Trust, Bombay, India.

I would also like to thank :

Dr. G. R. Moores for valuable discussion.

Dr. A. J. Lawrence for introducing me to enzyme assay.

Dr. J. G. Edwards for introducing me to autoradiography.

Dr. J. Kerr and Mr. C. Mucci for introducing me to Scanning and Transmission Electron Microscopy and carrying out these procedures for me.

Mr. R.B. Gallagher and Dr. J.M. Lackie for reading the manuscript.

Mr. M. F. McGrath invented the means of culturing cell suspension on grids.

Finally, I would like to thank J.N.K. University, Jabalpur, India for granting me a study leave during the period of this work.

ABBREVIATIONS.

Å	Angstrom.
AD	Anchorage dependence.
AM	Actomyosin.
AR	Araldite Resin.
ATP	Adenosine triphosphate.
BAEE	Nα-Benzoyl-L-Arginine Ethyl Ester.
°C	Centigrade.
CHFs	Chick heart fibroblasts.
Cm.	Centimetres.
CMF	Calcium and magnesium free salt solution.
CMF 10	CMF supplemented with 10 % foetal calf serum.
DDI	Density dependent inhibition.
DNA	Deoxyribonucleic Acid.
DNase-I	Deoxyribonuclease-I.
EDTA	Disodium-ethylene-diamino-tetra-acetate.
EM	Electron Microscopy.
F-Actin	Filamentous (Polymerised) Actin.
FCS	Foetal Calf Serum.
G_1	Period (Gap) between mitotic (M) and start of DNA synthetic (S) phases.
G_2	Period (Gap) between the completion of DNA synthetic (S) phase and next mitotic (M) phase.
G-Actin	Nonfilamentous (Unpolymerised) Actin.
ger	Granular endoplasmic reticulum.
HCl	Hydrochloric Acid.

HEFT	Eagle's minimal essential medium in Hepes buffer supplemented with 10% foetal calf serum and 10% tryptose phosphate broth.
HH	Hanks Hepes.
Ig	Immunoglobulin.
MFs	Microfilaments.
$MgSO_4$	Magnesium sulphate.
µgm.	Micrograms.
m	Microprocess-like structures.
µm.	Micrometres.
mm.	Millimetres.
M	Molar (Solutions).
M-phase	Mitotic phase of the cell cycle.
ng.	Nanograms.
NaH_2PO_4	Sodium dihydrogen phosphate.
NBD-Ph.	Nitrobenzooxadiazole-Phallacidin.
nm.	Nanometres.
PBS	Dulbecco's Phosphate Buffered Saline.
PCA	Perchloric Acid.
PO	Propylene Oxide.
PRE	Pigmented Retinal Epithelia.
S-phase	Synthetic (DNA) phase of the cell cycle.
SEM	Scanning Electron Microscopy.
T	Total cell cycle time.
TEM	Transmission Electron Microscopy.
TCA	Trichloroacetic Acid.

SUMMARY

Little work has been done previously on cells grown as sail-sheets. This thesis describes the morphology, behaviour and movement of chick heart fibroblasts (CHFs) in sail-sheets and the effects of mechanical tension on actin content and the cell cycle of these cells.

Abercrombie *et al.* (1970 a) quantified the measurements on the features of movement of CHFs on glass coverslips (or, conventional cultures). Since the sail-sheet cultures appeared to resemble more closely the *in vivo* situation than the conventional cultures, it seemed appropriate that the features of cell movement in sail-sheets be studied and compared with those in conventional cultures. The work presented in this thesis suggests that CHFs in sail-sheets do exhibit such features as described for conventional cultures (Abercrombie *et al.*, 1970 a) but at a relatively lower speed.

Mechanical tension occurs within and between cells during embryogenesis, wound healing and in the repetitive contractile processes performed by various muscles of the body. Curtis and Seehar (1978) found that short-term tensing of sail-sheets with a low frequency oscillator shortened the duration of the cell cycle in CHFs. This thesis investigates whether tensing of sail-sheets for longer durations and at much lower frequencies produces any different effects than those found by Curtis and Seehar (1978). The work from these experiments suggested that on the whole, tension causes a reduction in the duration of the cell cycle. The effects of tension in a rectangular

cell sheet differ from corners, edges and centres perhaps because of local stress concentration.

The hypothesis that the effect of tension on the cell cycle may be due to its effect on the microfilaments was investigated. Results were inconclusive.

CONTENTS

I N T R O D U C T I O N

Since the beginning of this century, many investigators have attempted to culture cells *in vitro* and developed techniques to the extent that cell culture has become an indispensible tool for biological and medical research. The methods routinely employed involve growing cells either as single cell suspensions or as monolayers attached to substrates. Both these systems are highly artificial because *in vivo* the cells grow in intimate contact with other cells and often in the absence of any continuous non-living substratum (collagen, bone etc.), for example, in embryogenesis. Various attempts to grow cells in three-dimensional systems have been made to obviate these problems, e.g. on commercial cellulose sponges (Leighton, 1951), on collagen (Ehrman & Gey, 1956) etc.

In one system, cells can be grown in culture in the form of "Sail-Sheets" within the holes of various inert meshes, a form which probably more closely approximates to the *in vivo* organisation of cells. In these cultures, the cells grow as sheets supported only at the edges. This can be likened to the 'sail' of a yacht, hence the name "Sail-Sheet". This method of culture was used as long ago as 1914 by Harrison. He cultured a wide variety of cell types from chicken and frog embryos on spider webs and studied their locomotory behaviour. Harrison's type of culture system was apparently unused until Weiss (1945) cultured Schwann cells and nerve fibres in plasma clots with or without the inclusion of fibres of various kinds (glass, textiles, synthetic resins) and dimensions (8-500 µms.). Curtis and Varde (1964) grew chicken heart explants on silica fibres as sail-sheets. The term cell "sail-sheets" was introduced by Curtis and Seehar (1978) to

describe similar cultures grown within holes of nylon meshes.

Mechanical tension may occur in embryogenesis (Bellairs et al., 1967) and wound healing (Abercrombie et al,1954) and, therefore, an experimental system to study tension effects is needed. In sail-sheet cultures, the mesh may resemble the situation of intercellular material and tension may be applied naturally by one group of cells through the intercellular material to a second group. Curtis and Seehar's (1978) method of growing cells as sail-sheets also affords artificial means of applying mechanical tension.

This thesis aims to describe some features of the sail-sheet culture system and addresses a comparision of the locomotory behaviour of chick heart fibroblasts (CHFs) in sail-sheets and conventional cultures. The work presented in this thesis attempts to investigate the effects of mechanical tension (stretching of sail-sheets) on cellular morphology and the duration of the cell cycle in the sail-sheet cultures of CHFs and to see whether the effects of mechanical tension are localised at any particular part in these cultures. The distribution of actin (G and F) within the cells was studied in conventional and sail-sheet cultures and the effect of mechanical tension on the actin (G and F) content within the cells in sail-sheets was also studied. The following major questions were addressed : (i) does tension affect cellular morphology (which might affect the behaviour and movement of cells) ? (ii) does tension affect the duration of the cell cycle and if so then which phase is altered ? (iii) do various frequencies of mechanical tension (stretching) applied for different periods have different effects on the cell cycle ? (iv) Are the effects of tension on the cell

cycle related to the distribution of actin (G and F) in these cultures?

This Introduction section is divided in to three sub-sections :

[A] Cell Behaviour & Movement.

[B] The Cell Cycle.

[C] The Microfilament System.

[A] CELL BEHAVIOUR & MOVEMENT.

This sub-section will be considered in to the following subheadings :

(a) Locomotory Behaviour of Fibroblasts in Conventional Cultures.

(b) Locomotory Behaviour of Fibroblasts in Sail-Sheet Cultures.

(c) Difference in Fibroblast Locomotion *in vivo* and *in vitro*.

(d) Contact Inhibition of Locomotion.

(e) Contact Guidance of Locomotion.

Before describing fibroblast behaviour and movement in sail-sheet cultures, it may be appropriate to discuss it in conventional cultures:

(a) Locomotory Behaviour of Fibroblasts in Conventional Cultures.

The standard way of culturing cells in two-dimensional form on solid substrata such as glass and polystyrene may be termed conventional cultures.

The account of the locomotion of fibroblasts in culture can be started with the seeding of a cell suspension over a non-living substratum. The cells in suspension are rounded and, in order to show net displacement, they are required to spread and develop adhesive contacts with the substratum. Locomotion of these fibroblasts consists

of production of ruffles (Abercrombie et al., 1970 b) or, occasionally, blebs or microspikes (Harris, 1973 a), usually at the leading edge of the locomotory fibroblasts. If these protrusions persist, they are usually passed back towards the nucleus by contraction (Ingram, 1969) leaving retraction fibres behind, followed by formation of distal (new) and decay of old attachments, resulting in forward movement. Actomyosin (Isenberg et al., 1976 ; Badley et al.,1978 ; Rees et al.,1980) or the microfilament system (Heath & Dunn,1978) has been reported to be involved in fibroblast movement. Guidotti (1972) reported actomyosin-like material from the membranes of red blood cells, Komnick et al. (1973) reviewed the literature on the occurrence of actomyosin in amoebae while, Poglazov reported actomyosin from muscles earlier in 1966.

Cells, after being plated out on the substratum, might make their initial contacts through their microvillar extensions and then flatten and spread. The cell perimeter may then protrude in two forms: cylindrical filopodia or microspikes (tail) and broader flattened lamellipodia or, the leading lamella (Albrecht-Buehler,1976 ; Abercrombie et al.,1977). Activity at the margins of these cells causes filopodia and lamellipodia to bend, wave, undulate and contact the substratum and often to be restored or retracted in to the cell margin (Abercrombie et al.,1977). The protrusion may be accompanied by contraction toward the centre of the cell (Harris,1973 a). Ingram (1969) suggested that these activities were due to cytoplasmic contraction.

Side view observations (Ingram, 1969) showed that the outermost part of the leading edge of moving fibroblasts may not be in contact with

the substratum. These findings have been confirmed by Izzard and Lochner (1976) using the interference reflection microscopy technique developed by Curtis (1964). Abercrombie et al. (1970 a) demonstrated active protrusion and withdrawal movements in the region of leading lamella, and subjected their study to careful statistical analysis. They discovered that the leading edge spends more time in the protrusion phase than the withdrawal phase. Abercrombie et al. (1970 a) postulated that this might result in a net forward displacement of the cell.

The microfilaments might generate tension which would be required to draw the cell body forwards towards the advancing leading edge. Single fibroblasts have been shown to be capable of exerting enough tension to bend a fine glass fibre (Maroudas, 1973 b) and micromanipulation studies performed by Chambers and Fell (1931) using epithelia, fibroblasts and myoblasts and, Harris (1973 b) using a variety of fibroblasts and tumour cells, have shown that when a cell is detached from the substratum at the leading edge, the detached margin of the cell is contracted into the cell body indicating that the tension existed within the cell.

Abercrombie et al. (1971) have shown the presence of longitudinal bundles of microfilaments in moving fibroblasts. These bundles appear to originate from the region of the nucleus and terminate at adhesion 'plaques' which are closely applied to the substratum and separated from the latter by 30 nm. Izzard and Lochner (1976) using interference reflection microscopy, demonstrated similar plaque like areas, also known as focal contacts (10-15 nm. separation). These focal contacts are distributed within a broad area of close apposition similar to the one described by

Curtis (1964). In addition to focal contacts, Izzard and Lochner (1976), also reported the occurrence of two more types of cell-substratum contacts in fibroblasts viz. close contacts (30 nm. separation) and the regions of greater cell-to-substratum separation (100-140 nm.). The focal (Heath & Dunn, 1978) and close contacts (Heath & Dunn, 1978 ; Izzard & Lochner, 1980) have been reported to be associated with locomotory adhesion in fibroblasts. This was based upon the fact that close contacts have been found to be often associated with a concentration of loosely organised microfilaments in the cytoplasm (Heath & Dunn, 1978). Izzard and Lochner (1980) reported close contact as playing a primary role in marginal spreading and movement of fibroblasts. This finding is based upon the following evidence : close contact is lost when spreading ceases and is reformed when spreading resumes ; the advance of the margin of the leading lamella follows that of the close contact ; the advance of both the leading lamella as well as the close contact is independent of existing focal contacts and associated stress fibres. The studies conducted by Taylor and Robbins (1963) and Harris (1973 b) have demonstrated the presence of retraction fibres when the cells are detached from the substratum. Revel et al. (1974) who used the scanning electron microscope to examine the underside of spread cells, suggested the occurrence of adhesions similar to those described by Abercrombie et al. (1971) and Izzard and Lochner (1976).

Abercrombie et al. (1971) postulated that the microfilament bundles by contracting might draw the cell body in a forward direction towards new points of adhesion. Interference reflection studies (Lochner & Izzard, 1973) have shown that the plaques themselves do not move

forward but are replaced by plaques in a more anterior position (newer plaques). Harris (1973 c) has suggested that when competition exists between different areas of the cell margin, the cell moves in the direction of the strongest cell-substratum adhesion. Possibly the more recently formed adhesions persist at the expense of the weaker (older) adhesions. High Voltage Electron Microscopic (HVEM) studies performed by Heath and Dunn (1978) reveal that in fibroblasts, each focal contact corresponds with a bundle of microfilaments and that these microfilament bundles are formed synchronously with their associated focal contacts. They speculated that the formation of a focal contact provides a fixed point with reference to the substratum which immediately enables the development of contraction in the cytoplasm between this adhesion and a relatively stable fibrillar system surrounding the nucleus. They suggested that such a contraction may cause a rapid organisation of a loose meshwork of cytoplasmic contractile elements into a straight bundle extending from the focal contact to the perinuclear area (Abercrombie et al., 1976). The occurrence of microfilaments (or, filamentous F-actin) in two distinguishable structural organisations such as the linear fibrillar bundles (stress fibres) and the meshworks or networks (confined to the motile lamella zones and ruffling membranes and considered to be generally present beneath the plasmalemma in a subcortical layer), has been demonstrated (Goldman et al.,1976 ; Small et al., 1982). There is evidence that isolated stress fibres (Isenberg et al.,1976) and the stress fibres of permeable cell models (Kreis & Birchmeier, 1980) can contract in Mg^{++}-ATP, though Herman et al. (1981) working on living HeLa cells demonstrated that stress fibres may not be essential for motility.

Heath and Dunn (1978) showed that some microfilament bundles are straight and seem to be under tension between the two points (focal contacts) and the spreading of the bundles into smaller elements, seen at the focal contacts, is also seen at the perinuclear termination. Weber (1976) has reported that the nucleus of a cell remains together with the microfilament system after treating with non-ionic detergents and inferred the existence of a cytoskeleton of which the nucleus is an integral part. The nucleus is often distorted into a shape which would be expected if it were embedded in an elastic gel pulled on by the oblique microfilament bundle. Thus, the microfilament bundles become organised in a manner which enable them, by contracting, to move the main cell mass forwards towards the anterior focal contacts.

Other hypotheses have also been put forward to explain locomotion in fibroblasts : (i) Lipid Flow Hypothesis. (ii) Surface Flow Hypothesis.

(i) Lipid Flow Hypothesis.

Bretscher (1976) proposed that the polar property shown by many eukaryotic cells in the movement of particles adsorbed on their surfaces can be explained at the molecular level by an oriented and continuous flow of lipid molecules in the plasma membranes of the cells. For example, in lymphocytes (and possibly in fibroblasts also), there seems to be a lipid flow from one end of the cell to the other. This idea of membrane flow is based upon the work of Shaffer (1963, 1965), Harris (1973 a), and Bray (1973). Bretscher (1976) explained that capping is caused due to the cross linking of membrane proteins by attached antibodies and lectins causing them to be swept along by the lipid current and collected at the "uroid" portion of the cell surface, where the membrane lipid is taken into the cytoplasm (by means of a

molecular filter) for return to the opposite side of the cell as cytoplasmic vesicles. Harris (1976) proposed that capping results from a directional flow of the entire plasmalemma, including not only its lipid but also its integral protein and carbohydrate components.

(ii) Surface Flow Hypothesis.

Abercrombie et al. (1970 c, 1972) suggested that the surface of the lamella of a locomotory fibroblast continuously moves towards the nuclear region, where it is disassembled and recirculated to the periphery via the cytoplasm. They proposed that a reassembly of the surface components (which are close to the cell margin) accounts for the formation of ruffles and the closely related process of forward spreading of the cell margin. Harris (1973 a, 1976) proposed that the cortical arrays of microfilaments pull the whole lamellar surface (plasma membrane) towards the centre (nucleus) thus exerting a force on the substratum. He attributed protrusions to the flow of membranes. Dunn (1980) explained forward protrusion of the leading lamella and the exertion of the force on substratum by a single mechanism, the circulation of lamellar surface. Dunn (1980) also suggested a change in the emphasis in this 'surface-flow hypothesis' from the lamellar surface to a cytoplasmic contractile meshwork.

(b) Locomotory Behaviour in Sail-Sheet Cultures.

Harrison's (1914) spider web cultures showed firm attachment of cells on to the substratum, active cell movement and accumulation of cells at the places where web fibres cross each other. Curtis and Varde (1964) used time-lapse cinematography to demonstrate how development of sheets occurred after the silica fibres in contact with substratum had been

coated with a single layer of cells. In Curtis and Varde's (1964) cultures, single cells spanned the gap at the intersection of two fibres. Cells arriving along a fibre adhered to that fibre and one of the spanning cells. By this means, bridges of cells between the two fibres were built up which were gradually enlarged by addition of cells either by movement along the fibre in to the sheet or mitosis, so forming the sheet. At the border of a fibre with an explant and at the places where the fibres were bent, sheets formed in the same way. When a gap, between fibres arranged as a quadrilateral, was filled, it was begun at the four corners, these individual sheets gradually joining up leaving a circular hole which was also filled by gradually decreasing in diameter. They described closure of this empty 'gap' as closely resembling certain types of wound healing, e.g., in chick blastoderm.

(c) Difference in Fibroblast Locomotion in vivo and in vitro.

Using Nomarski light microscopy, Bard and Hay (1975) compared the locomotion of fibroblasts from developing chick cornea *in situ* and *in vitro*. *In situ*, fibroblasts have one or two long pseudo-podia which end in multiple fine processes or filopodia, do not show ruffles and lack the flat leading lamella. *In vitro*, they take up a flattened morphology and have broad, flat leading lamella. Bard and Hay (1975) attributed this difference to the cell's adaptation to different environments. However, the general mechanism of cell movement is the same *in situ* as that observed *in vitro* i.e. either gross contraction or recoil of the cell body in to the forward cell process, or more subtle flowing of cytoplasm into the forward cell process without immediate loss of the trailing cell process. Bard and Hay (1975) pointed out that dividing cells *in situ* do not show blebbing although they do extend

filopodia at telophase while, in vitro, blebbing of the cell surface is an important component of the end stage of mitosis.

(d) Contact Inhibition of Locomotion.

Abercrombie and Heaysman (1954) introduced the term 'contact inhibition of movement' for 'the directional restriction of displacement on contact.' This has been demonstrated in fibroblasts (Abercrombie & Heaysman, 1953,1954) and epithelial cells (Middleton, 1972). Dunn (1971) demonstrated a similar phenomenon while working on nerve fibres and termed it 'contact inhibition of extension'.

Two types of contact inhibition have been identified (Abercrombie, 1970 a,b) and have been termed Type 1 and Type 2 (Vesely & Weiss, 1973) : (i) Type-1 is the one in which a complex contractile response occurs following a collision between the leading edges of two cells (Abercrombie & Ambrose, 1958 ; Abercrombie, 1970 a). Inhibition of locomotion in the original direction is accompanied by a rapid, localised cell-cell adhesion (Abercrombie & Ambrose, 1958 ; Heaysman & Pegrum, 1973) and by suppressed localised ruffling, pinocytosis and the fluctuations of extensions of the leading edge (Abercrombie , 1970 a). Weiss (1958) and Gustafson and Wolpert (1967) discovered a general inhibition of observable membrane activities on contact and described it as 'contact paralysis'. (ii) Type-2 involves directional inhibition of cell locomotion after cell-cell collision and no concurrent exhibition of contraction. To explain this, Abercrombie (1970 b), Harris (1974) and Heaysman (1978) suggested that directional inhibition of locomotion occurs because of inability of a contacting cell to move from a tissue culture substratum to a less adhesive exposed dorsal surface of another cell. Heaysman and Pegrum (1973) suggested that

microfilaments may play a role in type-1 contact inhibition of movement by contracting the cytoplasm after collision of one cell with another. Dunn and Heath (1978) suggested that the oblique bundles of microfilaments might cause contraction.

(e) Contact Guidance of Locomotion.

That certain physical properties of the substratum can influence the direction of locomotion of cells *in vitro* has been known since the beginning of this century (Harrison, 1912 ; Loeb & Fleisher, 1917 ; Weiss, 1929). Weiss (1941) used the term 'contact guidance' for the first time to describe the orientation of cell locomotion as a response to the shape of the substratum.

Dunn and Heath (1976) developed the hypothesis that contact guidance is due to the shape of the substratum imposing mechanical restrictions on the formation of certain linear bundles of microfilaments which are involved in cell locomotion. Dunn and Heath (1976) used this hypothesis to predict fibroblast behaviour on prism-shaped substrata and the prediction was confirmed observationally by examining whole spread chick heart fibroblasts using transmission electron microscopy. These studies revealed discontinuities in the microfilament bundle system which coincided with a discontinuity in the shape of the substratum. Dunn and Ebendal (1978) demonstrated the phenomenon of contact guidance of locomotion by growing fibroblasts from heart and nerve axons from spinal ganglia of chicken embryo on collagen gels. Dunn and Ebendal (1978) took two types of collagen substrata : the original wet gel and the one whose shape was changed, without substantially altering their orientation, by air drying them on coverslips. The original wet gels

had a three-dimensional shape and exhibited strong contact guidance response when used as a substratum for both heart fibroblasts as well as nerve axons. The air-dried gels, however, due to being flattened on to the plane support, were much less effective in guiding the cells. Treatment of the dried gels with dilute acetic acid restored their three-dimensional shape and hence, contact guiding property, to a certain extent. Dunn and Ebendal (1978) interpreted their findings as indicating that contact guidance on oriented fibrillar matrices is a direct cellular response to the shape of the substratum.

The next sub-section briefly describes the ways by which the duration of the cell cycle can be determined and discusses the various theories which have been put forward to explain control of the cell cycle *in vivo* as well as *in vitro*.

[B] THE CELL CYCLE.

The life of a cell begins with its formation by division of a parent cell and ends either with the formation of daughter cells or with its death. This life time is called the cell cycle. Howard and Pelc (1953), working on ^{32}phosphate labelling of bean roots with NaH_2PO_4, were the first to divide the cell cycle in to the four phases G_1, S, G_2 and M. G_1 is the post mitotic phase, characterised by the synthesis of RNA and proteins ; the S phase is that period during which the duplication of DNA occurs while protein and RNA synthesis continues ; G_2 is the post synthetic phase and is characterised by no synthesis of DNA but continuation of RNA and protein synthesis as in G_1, and M is the mitotic phase during which no DNA synthesis and reduced protein synthesis occurs. This phase begins from prophase and continues up to

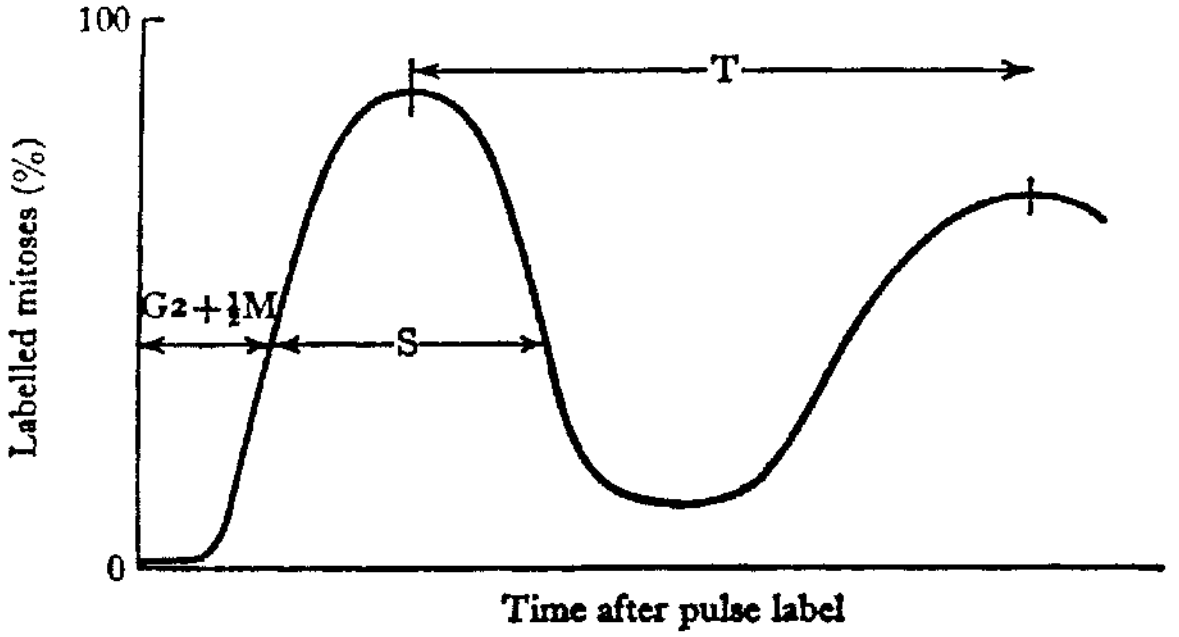


Fig. 1 Diagram of labelled mitoses (metaphases) in successive cell samples after a pulse of tritiated thymidine.

telophase of mitotic division.

Howard and Pelc (1951a,b, 1953) treated the actively growing tisue of the root meristem of *Vicia faba* with ^{32} phosphate. The ^{32} phosphate became incorporated into newly synthesised DNA which was detected by its presence in autoradiographs as developed photographic grains over the cell nucleus. This technique was described fully by Quastler and Sherman (1959) and was named the 'Percentage Labelled Mitosis' (PLM) by Baserga (1965), and by Cleaver (1967) and Mitchison (1971) who substituted tritiated thymidine [^{3}H] in place of ^{32} phosphate. This method consisted of pulse labelling with tritiated thymidine either *in vivo* or *in vitro*, sampling the cells at various time intervals and then counting the proportion of labelled mitoses. The proportion of labelled mitoses increased to a peak (figure 1), as the cells which were in S phase at the time of pulse labelling began to divide. This peak was followed by trough as the cells originally in G_1 came to the end of their cycle. The second cycle showed a similar wave, but it was lower due to the spreading cycle times. The average S period was considered to be the time between two points in the first wave where 50% of the mitoses were labelled. The time between the beginning of the experiment and first of these points was regarded as G_1 +1/2 M. Half the mitotic time was added to G_2 since the mitoses were scored in metaphase, meaning that the cells had to go through not only G_2 but also prophase before they could be scored. Prophase lasted rougly half the time of mitosis. The total cell cycle time (T) was the time between two peaks in the first and second cycle. The G_1 +1/2 M was obtained by the formula [G_1 +1/2 M ≈ T-S-G_2 -1/2 M].

The duration of the cell cycle can also be determined using other

methods (Hilscher & Maurer, 1962 ; Pilgrim & Maurer, 1962 ; Baserga & Nemeroff, 1962 ; Wimber & Quastler, 1963 ; Puck & Steffen, 1963 ; Barranco et al., 1977 ; Gray et al., 1977 ; Meselson & Stahl, 1955 ; Heck et al., 1977 ; Van Dilla et al., 1969) but, Howard and Pelc's (1951 a,b) method, was chosen for present study as being more convenient especially for sail-sheet cultures where the size of the sample is relatively small.

Control of the Cell Cycle

It is necessary to explain the changes in the cell cycle as maturity is approached or why it speeds up around wounded areas. Cell growth may be affected by factors such as temperature, pH of the growth medium, osmolarity, free ions, serum, growth factors, mitogens, lectins, proteases, hormones and chalones (see review articles by Berridge, 1976 ; Bhargava, 1977 ; Whitfield et al, 1979 ; Hochhauser et al., 1981 ; Rozengurt, 1983).

Control of the cell cycle can be studied in two systems i.e. in vivo and in vitro. Both these systems alongwith various theories proposed to describe the control of the cell cycle in them will be discussed.

(a) Control of the Cell Cycle in vivo :

The cell cycle in vivo is a controlled process, but how it is controlled is not fully understood. For example, mammalian liver shows a low rate of mitosis and its cells are characterised by a relatively long life. However, when a large piece of liver is surgically removed (partial hepatectomy), a sudden burst of mitotic activity gets started. Consequently, cells will grow and divide rapidly and the original mass

of the liver will be regained. Another example can be cited from the cells of the epidermis of the body. These cells grow, divide and differentiate at a rate to ensure a continued supply of cells to replace those that normally die. When the epidermis is wounded, the tissue begins the process of wound healing with an increased mitotic rate. Contrary to these examples, nerve cells in the body do not divide once the animal becomes adult.

The only theory that has been established so far to explain the control of the cell cycle in vivo is "chalone theory" which is based upon the assumption that the cell cycle in vivo may be controlled by growth inhibitory substances (chalones).

(b) Control of the cell cycle in vitro :

The in vitro study of the control of the cell cycle has an advantage over the in vivo due to the fact that in vitro techniques are easier (than in vivo), yield a homogeneous population of cells and that the cultured cells are more suitable for carrying out tension experiments.

In a culture of normal cells in stationary phase, the cells form a monolayer in which each cell is completely surrounded by its neighbours. The cessation of growth of each cell due to being surrounded on all sides by other cells may be due to either contact inhibition of growth (also known as density dependent inhibition = DDI) or depletion of growth factors or both. The theories which can be grouped under this type of controlling mechanism are : "diffusion boundary" and "topoinhibition". Alternatively, The cell

cycle may be anchorage dependent (AD) i.e. controlled by the way cells actually anchor over the substratum. Theories such as "cell shape", "availability of substratum" and "tension" may be grouped under this category. "Lack of space for growth" theory may be placed in between the DDI and AD since it includes both diffusion (e.g. in fibroblasts) as well as anchorage (e.g. in epithelia) dependence.

Theories explaining control of the cell cycle *in vivo* and *in vitro* will be described in the following order :

1. the Chalone theory.
2. the Diffusion Boundary Theory.
3. the Topoinhibition Theory.
4. Lack of Space for Growth Theory.
5. the Cell Shape Theory.
6. the Availability of Substratum Theory.
7. the Tension Theory.

1. The Chalone Theory.

The term 'chalone' was probably first used by Schaffer in 1913 (Iversen, 1976) and was derived from 'chalao' meaning to loose or to lower. It was then adopted by Bullough (1962) for tissue-specific anti-mitotic substances. The concept was used to explain the decline and cessation of growth in adult tissue. These are the substances whose existence is disputed. For example, epidermis is claimed to contain substances (Iversen, 1981) which have a specific growth inhibitory effect on keratinocytes. They are effective on both normal tissue and squamous carcinomas. They are known as epidermal G_2 and G_1 (Elgjo *et al.*, 1971, 1972) chalones since they act on these phases in the cellular cycle.

Lymphocytes contain factor(s) with chalone properties which inhibit lymphoblastic transformation and proliferation in a cell-line-specific manner. However, these chalones have yet to be purified and their exact role in growth regulation has to be established.

Criticism of Chalone Theory : The chalone theory has been criticised due to the fact that no chalone has yet been purified. Allen and Smith (1979) criticised it on the grounds that there had been no firm ruling in the chalone concept on what exactly constituted a 'tissue' for a particular chalone to act upon. They pointed out that an epidermal chalone could act on epithelial cells, keratinocytes, Langerhans cells etc., all of which are present in epidermis. Another criticism which has ~~from~~ come from the work of Allen and Smith (1979) questions how the wound healing mechanism postulated for epidermal operates in other tissues ; if homeostasis is maintained by a given chalone concentration, how is this concentration altered when the tissue is partially excised ? Fitchen and Cline (1981) re-examined the subject of granulocyte chalones using purified populations of human neutrophils as starting material and a variety of target tisues and assays of cell proliferation (Herman et al., 1978) and failed to find neutrophil-derived inhibitor substances with the cellular specificities required of granulocyte chalone.

2. The Diffusion Boundary Theory.

This theory (Stoker, 1973) is based upon the principle that the convective movement of most fluids approaches zero near solid surface and the transfer of molecules to and from the surface becomes largely limited to the relatively slow process of diffusion. This would result

in a diffusion boundary layer which can act as a barrier close to the reactive surface. Stoker (1973) visualized that when a cell takes up a molecule essential for growth from medium, the concentration near the cell surface would fall to a level determined by the uptake rate and the rate of replenishment by diffusion from the bulk of the medium. If the supply of such a molecule becomes limiting then the growth process becomes diffusion controlled. A similar phenomenon may arise if a cell is unable to secrete, a waste product which must be absent if division is to occur or, alternatively, if the inhibitory molecules can not diffuse away sufficiently rapidly from the vicinity of the cell, a similar problem may arise. In the microenvironment, a stagnant layer (the diffusion boundary layer) could act as a barrier close to the reactive surface. Stoker (1973) used a 'Riddle Pump' to recirculate the culture medium over the cells (and also to reduce the diffusion boundary layer) and found that the growth in confluent cultures was stimulated in the region where the pump agitated the medium. This experiment suggested that cell division is limited by diffusion.

Froehlich and Anastassides (1975) showed that if the cultures of human skin fibroblasts were put on an oscillatory platform then a greater number of cells in density inhibited cultures could resume growth in fresh medium. However, in spite of greater stimulation after a single treatment with fresh medium, refeeding the cells on the platform every other day over a 12-day period did not affect the final saturation density achieved in these cultures. They suggested that while diffusion may act as a limiting factor under certain circumstances, it is not entirely responsible for density dependent inhibition.

Evidence against the diffusion boundary theory comes from the work of Whittenberger and Glaser (1978) who postulated that if growth is a diffusion limited process, then increasing viscosity of the cell culture medium should decrease the saturation density. In a series of experiments, they showed that increasing viscosity had no effect on either the saturation density or on the rate of cell growth. Maroudas (1978) suggested that increase in the viscosity of culture medium does increase the molecular diffusivity although the effect is not as great as claimed by Whittenberger and Glaser (1978).

3. The Topoinhibition Theory.

Todaro et al. (1965), Dulbecco (1970) and Dulbecco and Stoker (1970) discovered that when a monolayer culture of cells, is grown on a solid substratum at cell densities where most cells do not synthesize DNA, wounding allowed DNA synthesis at the edges of the denuded area. The initiation of DNA synthesis seemed to depend in part on the release of cells from extensive contacts with other cells. Dulbecco (1970) termed this contact inhibition of division as 'topoinhibition'. It has been suggested (Dulbecco & Stoker 1970) that topographical relationships between cells play an important part in controlling initiation of DNA synthesis. Studies on the effect of serum on DNA synthesis in wound healing and in other cellular phenomena showed (Dulbecco, 1970) that the serum concentration markedly affected S-phase of the cell cycle in various cell types.

4. Lack of Space for Growth Theory.

Dulbecco and Elkington (1973) challenged the theory of contact

inhibition of growth. They suggested that cessation of growth occurs simply because of a lack of space. They demonstrated that the multiplication of epithelial cells (under usual culture conditions) and fibroblastic cells (at extraordinary high densities) was limited by the amount of plastic surface available per cell. They, however, found that multiplication of fibroblasts was limited by the availability of medium factors under usual conditions. They suggested that the stimulation of division in wounded areas in fibroblastic and epithelial cells is due to two different reasons: in fibroblasts due to their ability to utilise medium more efficiently while in epithelia due to availability of more space per cell.

5. The Cell Shape Theory.

Folkman and Moscona (1978) put forward the idea that an appropriate cell shape is critical for DNA synthesis in normal cells. This theory was proposed to explain 'anchorage dependence', the inability of normal cells to grow unless attached to a substratum (Maroudas, 1973 a, 1974 ; Stoker et al., 1968).They varied the adhesiveness of tissue culture plastic by applying different concentrations of poly (2-hydroxyl-ethyl methacrylate) to form a substrate and consequently, they could accurately control the extent of cell spreading. Cells cultured on these substrata could be held at any one of a graded series of quantifiable cell shapes. Cell shape was found to be tightly correlated to DNA synthesis and growth in non transformed cells. As the cells of various lines were brought from the extremely flat shape toward a spheroidal conformation, fewer cells incorporated [^{3}H]-thymidine. When the cells were almost completely spherical, but still barely attached

to the substratum, they failed to enter the S phase.

Folkman and Moscona (1978) suggested that growth control could be associated with cell shape and that the sensitivity of cells to growth factors might be linked to this. Thus, higher concentration of growth factors would be required for DNA synthesis as cell conformation was converted from flat towards hexagonal, and finally to spheroidal shapes, where no concentration of serum would be sufficient for growth. Such a relationship between cell shape and its role in DNA synthesis would explain why cells at the edge of a wound require less serum for DNA synthesis than cells a few rows behind them (Dulbecco, 1970). Also, this concept would explain why BHK21 cells grown on plastic were sixty times more sensitive to serum than the same cells grown in suspension culture (Clarke et al., 1970) and why the serum requirement is reduced in anchored cells (Paul et al., 1974).

6. The Availability of Substratum Theory.

This is basically a topoinhibition theory put forward by Todaro et al. (1965), Dulbecco (1970) and Dulbecco and Stoker (1970) described earlier (page 20) except that the experimental approach is different.

Campisi and Medrano (1983), working with mouse, hamster or human fibroblasts, discovered that a short period of detachment of these cells from the substratum inhibited them from entering the DNA synthetic (S) phase of the cell cycle for 8-12 hrs. This was despite the fact that the cells were reattached within an hour of replating and attained a spread morphology 5 to 8 hrs. later. They suggested that the delay in the cell cycle was mainly because of detachment of cells from the substratum, although the rounded morphology of the cells after

trypsinisation might have played some role. They found that the decline in the proportion of cells in S phase was accompanied by an accumulation of cells in G_1, as observed by autoradiography and flow microfluorometry. They suggested that the attachment of cells to a substratum could be attributed to the presence of some unidentified protein(s) within the cells which is necessary for the normal transit through G_1. This protein may be quite sensitive to the presence of an anchoraging substratum and alterations in cell shape and attachment could have rapid metabolic consequences.

7. The Tension Theory.

Studies on effects of tension on cells began as early as 1932 when Cameron and Oakley found that the bile duct shows increased division when stretched. Reynold (1949) observed that mitotic activity was induced in both the endometrium and myometrium of the uterine wall by distention of the uterine lumen, an effect not due to hormonal stimulus. Billingham and Medawar (1955) pointed out that the expansion of skin is possible because of the readjustment and growth of the fibrous endoskelton of the corium (a layer underneath epidermis) in response to chronic tension. It is well known that fibroblast cells adopt an orientation that conforms to the lines of superimposed tensions (Weiss, 1929,) and that the growth of many kinds of mesenchymal matrix is enhanced by mechanical stimulation (Levy, 1904 ; Glucksmann, 1942). Mechanical stimulation of dorsal mouse skin by skin massage or removal of the horny layer resulted in an increase (in comparision to the non-stimulated control) in DNA-labelling and mitotic activity (Bertsch et al., 1976). Reichhart and Thiebold (1977) reported

a possible role of mechanical factors in controlling proliferation of mesothelial cells in the mullerian ducts of chick embryos. Curtis and Seehar (1978) showed that low frequency mechanical stretching of sail-sheets shorten the duration of the cell cycle in chick heart fibroblasts (CHFs).

The last sub-section will be devoted to a brief description of the microfilament system and its role in controlling cell behaviour and the cell cycle.

[C] THE MICROFILAMENT SYSTEM .

The previous sub-sections indicated that tension may exist in cells at the time of movement and also that artificial stretching (tensing) of cells may affect the cell cycle. Since tensions may be developed by microfilaments (MFs), it seems likely that they in turn may respond to the tension applied through some artificial source. Thus, the reason for describing microfilament reactions is twofold: (i) to elucidate microfilament relations to cell behaviour and, (ii) to determine their part (if any) in controlling the nuclear activity of cells.

This sub-section can be divided in to following parts :

(a) Role of MFs in Cellular Architecture & Behaviour.

(b) Organisation of MFs in Cultured Cells.

(c) Role of MFs in Controlling the Cell Cycle.

(d) Relationship between the MFs and mechanical tension.

(a) Role of MFS in Cellular Architecture & Behaviour.

Muscle-like proteins in fibroblasts are localised in the form of

bundles or 'cables' of microfilaments lying approximately in line with the direction of the cell movement. These 'cables' include actin (Goldman et al.,1975) as the main constituent, myosin (Weber & Groeschel-Stewart, 1974), tropomyosin (Lazarides, 1975), α-actinin (Lazarides & Burridge, 1975) and M-protein (Schollmeyer et al., 1976), which are constituents of the myofibril of striated muscle, and atleast one fibrillar protein, filamin, which is found in smooth but not in striated muscle (Wang et al., 1975). These complex cables appear to be contractile but their precise organisation is unknown.

Wessels et al. (1971) showed that microfilaments are involved in controlling cellular and developmental processes and that the drug cytochalasin B can be used as a tool to study these filaments. This drug causes the disappearance of the "contractile ring", a band of microfilaments located just beneath the cleavage furrow which is thought to be the contractile agent responsible for pinching the cell in two (Schroeder, 1968,). When the drug is removed, filaments reappear and the previously inhibited activity is resumed. Cloney (1966,1969) showed that the remarkably rapid (about 6 min. in some species) shortening of ascidian tadpole tails during metamorphosis is apparently due to the involvement of the microfilament system in caudal epidermal (Cloney, 1966) or notochordal (Cloney, 1969) cells. Isenberg et al. (1976) cut out individual cables (Actomyosin or AM fibrils) from glycerinated cells using laser beam microdissection and showed that they contracted when ATP was applied. Heath and Dunn (1978) using interference-reflection microscopy and high-voltage electron microscopy, confirmed the joining of front ends of these cables with focal contacts, thus, showing the alignment of the microfilaments in

the cells. Using simple geometry, Dunn and Heath (1976) demonstrated that the microfilament system can directly detect a curvature on the substratum. They took substrata, having a sharp convex change in inclination in the range 1 to 32°, and grew fibroblasts on them. They discovered that the longest oblique bundles in a typical fibroblast can not bend more than 4°. Thus, if a cell needed to bend its microfilaments through a greater angle in order to move on an irregular surface, it is faced with an impossible task. They believed that the fibroblast partly accomodates to convex substratum curvature by reducing the maximum length of newly formed bundles in the direction of greatest curvature. Results from the work of Rieber and Rieber (1982) suggest an important role for actin-associated proteins that participate in interactions with cell surface proteins, in events that influence cell adhesion to substratum.

It has been suggested that microfilament system plays an important role by providing structural and mechanical support to the plasma membrane (PM). The PM being a weak and deformable structure may need a MFS as a stabilising structure (Singer, 1971 Szabo, 1972). The evidence for the involvement of microfilaments with the PM have been reviewed recently (Weatherbee, 1981). The first line of evidence comes from the studies carried out at the cellular level, for example, concentration of microfilaments in areas of active phagocytosis (Stossel & Hartwig, 1976 ; Berlin et al.,1979) or endocytosis (Albertini & Anderson,1977), the insertion of microfilament bundles in to the membrane-associated adhesion plaques of cultured cells (Abercrombie et al.,1971), more frequently involving intermediary elements such as the electron dense material in to which microfilament

bundles sometimes insert end-on (Abercrombie et al.,1971 ; Mooseker & Tilney,1975) or the cross bridges that sometimes form connections between membranes and microfilaments running parallel to them (Mooseker & Tilney,1975) in intestinal epithelial cells, alignment of membrane components being capped has been seen to coincide quite strikingly with the actin-containing stress fibres of the cell (Ash & Singer,1976 ; Ash et al.,1977). A second line of evidence is derived from the study of isolated membrane preparations which always contain actin as a component, e.g., in chinese hamster ovary (CHO) fibroblasts (Kochhar,1979). The presence of actin has also been demonstrated by biochemical characterisation of filaments such as preparations from Hela and 3T3 cells (Gruenstein et al.,1975) and PM of chick fibroblasts (Tannenbaum & Rich, 1979). A third line of evidence has been demonstrated from the ability of purified actin to form complexes with membrane preparations in vitro. For example, binding of actin to secretory granules (Burridge & Phillips,1975 ; Ostlund et al.,1977), plasma membrane (Taylor et al.,1976), and synaptosomes (Puszkin et al.,1976). A fourth line of evidence can be visualised from the studies on the effects of cytochalasin B on microfilaments (Wessels et al., 1971 ; Badley et al.,1980) suggesting a transmembrane relationship between the carbohydrate bearing molecules on the cell exterior and actin bundles on the inside. A fifth line of evidence may be cited from the association between the major histocompatibility antigen H-2 and actin (Koch & Smith, 1978) in P815 cells. Also, Flanagan and Koch (1978), working on lymphocytes and P3 myeloma cells, demonstrated a specific association between surface Ig and cellular actin, induced as a result of cross-linking of surface Ig by the capping and patching phenomenon.

(b) Organisation of MFS in Cultured Cells.

This subject has been extensively reviewed (Buckley & Porter, 1967 ; Spooner et al., 1971 ; Goldman et al., 1976 ; Small et al., 1982). The picture that emerges is that F-actin occurs in cells in vitro in two structures : (i) in linear fibrillar bundles known as "stress fibres" and (ii) in meshworks or networks in the ruffling membranes. In further studies, the elucidation of the general organisation of stress-fibre bundles, and the identification of F-actin as the microfilament type within them has been achieved using the electron (Goldman et al., 1976) and immunofluorescence microscopy (Lazarides & Weber, 1974 ; Lazarides, 1976 a,b). Studies using Triton-100 extraction, glutaraldehyde fixation, and negative staining have made it possible to directly visualise such meshworks (Brown et al., 1976 ; Small et al., 1978 ; Osborn et al., 1978). These techniques allowed the preservation of a broad and relatively well-ordered meshwork of actin filaments and filament bundles from the peripheral, normally convex region of cells, which correspond to the "leading edge" (Abercrombie et al., 1970 a). DeRosier and Tilney (1982) demonstrated how the actin filaments are packed into bundles. They suggested that the attachment of macromolecular bridges on actin filaments should take place at specified positions on the surface of the filaments and that there must be some degree of bending of the bridges or of the sub units that make up the actin filament. Small et al. (1978) suggested that the controlled polymerisation of actin forms the basis for the forward extension of the leading membrane of cultured cells. They proposed that the growth resulting from inward polymerisation of actin filaments

initiates at the membrane sites or foci. Small et al. (1982) proposed that this filament growth is accompanied by a cross-linking of actin filaments by proteins such as filamin and α- actinin to form a continuous, temporarily stable network. This growing network (Small et al., 1982) could exert traction against the inner cytoskeleton in order to effect forward movement.

(c) Role of MFS in Controlling the Cell Cycle.

It has been established that cellular metabolic factors influence cell division by affecting the S-phase. But, it is not known whether or not the microfilament system plays a role in the nuclear activities of cells. In fact, this subject has not been deeply investigated, whereas the involvement of microtubules with cell division has been well established. Pickett-Heaps et al. (1982) suggested that filamentous actin was not involved in mitosis.

Sanger (1975) demonstrated that heavy meromyosin can be coupled with fluorescein isothiocyanate in a manner which protects the actin-binding site and leaves unaltered the binding specificity of the heavy meromyosin. He used fluorescently labelled heavy meromyosin for light microscopic identification of actin in glycerinated chick fibroblasts. He demonstrated a change in cellular distribution of actin during the course of division. He recognised four different patterns of actin distribution during cell division viz. interphase, rounding up, furrow and cell separation. Interphase is characterised by the localisation of cellular actin in discrete bundles. The long bundles of actin fibres disappear as the cell progresses from interphase to division (i.e. rounds up). The long bundles of actin fibres disappear and a diffuse

staining pattern is formed. When the cleavage furrow forms, the cellular actin becomes concentrated mainly in the cleavage furrow (though some diffused staining over the two halves of the cell can also be seen). The presence of actin filaments in the cleavage furrow is also demonstrated by early workers (Perry et al., 1971 ; Schroeder, 1973). Actin filaments have also been observed in non furrow regions in dividing cells (Forer & Behnke, 1972) though their quantitative relationship to the furrow elements has remained uncertain. After the completion of cleavage, the actin becomes concentrated in the distal poles, where pseudopods form to pull the daughter cells apart. After separation, the daughter cells flatten and actin is again found in bundles. These different actin patterns are not likely to be due to localised synthesis and breakdown because the synthetic activity of dividing cells is very low (Mazia, 1961). However, it may be due to recycling of actin within the cell, as described above (Sanger, 1975).

(d) Relationship Between the MFS and Mechanical Tension.

Curtis and Seehar (1978) showed that tension shortened the cell cycle in chick heart fibroblasts. One possible explanation of this is that the tension orients microfilaments or changes their number and/or content, these alterations affecting nuclear activity. Work presented here will test this hypothesis which could have considerable implications in the study of wound repairs and in oncology.

Involvement of mechanical tension in the activities of the microfilament system has gained a considerable amount of attention recently. For example, the contractile structures formed during lamellisation (formation of leading lamella) probably include both

meshwork filaments and microfilament bundles (Vasiliev, 1982). Possibly, the first stage in the formation of these bundles is a parallel alignment caused by mechanical stress in the microfilament meshwork between the focal contact and the nuclear area (Fleischer & Wohlfarth-Botterman, 1975 ; Abercrombie et al., 1977). DeRosier and Tilney (1982) pointed out that the clusters or bundles of actin filaments are prominent in those parts of the cells that detect mechanical stimulation and transduce to an electrical impulse. Abercrombie (1980) suggested that the contractile proteins of non-muscle cells can apply tension to cell's attachment and cause moulding of tissues during morphogenesis in the embryo.

M A T E R I A L S & M E T H O D S.

This section can be divided in to three sub-sections :

[A] Materials.

[B] Methods.

[C] Quantitative Microscopy.

[A] M A T E R I A L S.

(a) SOURCES OF CELLS.

Fertile White Leghorn Chicken eggs were obtained from Ross Poultry Great Britain Ltd.(Aberdeenshire). They were incubated for 9 days for material for Heart Fibroblasts cultures and for 10 days for Pigmented Retinal Epithelia (PRE). Mice (BIO.BR) were obtained from the departmental stock kept at the animal house of the Southern General Hospital, Glasgow.

(b) SOLUTIONS FOR CELL CULTURE : All the solutions described below were sterilised by either autoclaving or filtration.

(i) Balanced Salt Solutions :

1. Hanks Hepes Solution (HH).

Hanks basic salt solution (Hanks & Wallace, 1949) contained 1 gm/litre glucose and was buffered to pH 7.4 with 0.01 M Hepes (N-2-hydroxyethyl piperazine-N'-2-ethane sulphonic acid).

2. Calcium and Magnesium Free Salt Solution (CMF).

Composition

0.12 M Sodium Chloride
5 mM Potassium Chloride
0.8 mM Di-sodium Hydrogen Phosphate
1.8 mM Potassium Di-hydrogen Phosphate 5.6 mM Glucose
19 mM Tris-HCl

This solution was adjusted to pH 7.8 with 5N NaOH and for certain procedures (PRE culturing from chick embryo) was supplemented with 10%

foetal calf serum (CMF 10).

3. Tris saline (Tris).

Composition

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

pH = 7.5

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

Composition

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

(ii) Growth Media :

1. HEFT.

The growth medium for Chick Heart Fibroblasts was Eagle's minimal essential medium (E) in 20 mM Hepes Buffer (H) supplemented with 10% Foetal calf serum (F) (-Gibco-Bio-Cult-Ltd.) and 10% Tryptose phosphate broth (T) (-Oxoid Ltd.). 2.5% GPS (Glutamine 1:4 Penicillin/ Streptomycin) was added to it.

2. Hams Medium.

Hams F10 medium in 20 mM Hepes buffer supplemented with 10% foetal calf serum (FCS) and 10% tryptose phosphate broth. For succeeding media changes 3% FCS and Insulin Transferrin Selenite (Insulin - 5 µgm./ml., Transferrin - 5 µgm./ml., Selenite 5 ngm./ml.) were supplements.

This growth medium was used for culturing glial cells from embryonal chick and endothelia and fibroblasts from mouse.

(iii) Disaggregating Agents :

1. Trypsin.

0.25 % W/V (Difco 1:250) in Tris-saline (pH 7.4) ; Activity 1000 BAEE units/ml.

2. Versene.

0.55 mM solution of Disodium-ethylene-diamino-tetra-acetate (EDTA) in phosphate buffered saline (pH 7.6).

(c) MATERIALS FOR CONVENTIONAL CULTURES :

(i) Coverslips.

Chance No.1 (20X20 mm.) and No.1 1/2 (16 mm.diameter) glass cover slips were used for culturing.

(ii) Culture Dishes.

Sterilised polystyrene tissue culture dishes (55 mm. diameter) were obtained from Sterilin Ltd.

(iii) Nitex Filters.

Nylon fabric (or mono filament nylon mesh) of 10 µm mesh diameter was purchased from Plastok Ltd., Oxton, Birkenhead, England and cut in to 25 mm circles to fit Millipore filter assemblies. These filter assemblies were sterilised in an autoclave (160° C.) and used for filtering the cell suspension to remove aggregates.

(d) MATERIALS FOR SAIL-SHEET CULTURES :

As described in the Introduction, the Sail-Sheet Cultures were those in which only cells at the edge were in contact with a substrate i.e. the mesh. The great majority of cells had contact solely with other cells. These cultures resemble the *in vivo* situation more than those grown on plastic or glass substrate. Various types of fabric and metal meshes were used to give a wide variety of materials for cell attachment.

(i) Nitex Mesh.

Nitex (a trade name for a mono filament nylon mesh) with 200 and 400 µm. mesh size obtained from Plastok Associates Ltd.,Oxton, Birkenhead, England.

(ii) Chiffon.

A nylon mesh (200 µm. size) obtained from a local fabric shop.

(iii) Gold Grids.

Square (200 and 400 µm. size), Hexagonal (150 and 200 µm. size) and slotted (R 200 size) gold grids were purchased from Veco Electroforming Photoetching Ltd., Caterham, Surrey.

(iv) Carbon Fibres.

Carbon Fibre Implants (Commercial name: Jenkins ligaments) were obtained as a gift from Johnson & Johnson Ltd., Slough, England.

(v) Polystyrene Coverslips.

Polystyrene plastic tissue culture coverslips (20 mm. Round No.1 1/2) purchased from Lux Scientific Corporation.

(vi) Quick-Set Epoxy Resin.

This adhesive was purchased from RS Components Ltd., London, England.

(vii) Collodion.

Necol Collodion solution was purchased from BDH Chemicals Ltd., Poole.

(e) CHEMICALS AND STAINS FOR HISTOLOGY :

(i) Light Microscopy :

1. Fixative.

Formol Saline containing 4% formaldehyde and 0.15 M sodium chloride in phosphate buffer (0.05 M) at pH 7.6.

2. Embedding Material.

Araldite CY212 Resin Kit purchased from EMscope Laboratories

Ltd., London had following composition:

Araldite Resin CY 212	10.00 ml.
Dodecenyl Succinic Anhydride (D.D.S.A.)	10.00 ml.
Dibutyl Phthalate (D.B.Pth.)	0.25 ml.
Benzyl Dimethyl Amine (B.D.M.A.)	0.15 ml.

3. Dehydrating Agent.

Analor ethanol obtainded from BDH Chemicals Ltd., Poole, England.

4. Staining, Clearing and Mounting Agents.

Haematoxylin, Ponceau fuchsin and Light Green were purchased from Gurr-Searle, High Wycombe, Bucks., England ; Phospho-molybdic acid from Koch-Light Laboratories Ltd., Colnbrook, Bucks., England ; Azure II from BDH Chemicals Ltd., Poole, England ; Cedarwood oil from Gurr-Searle and Xylene Puriss.A.R. from Koch-Light. Clearmount was obtained from Gurr-Searle. They were made up according to Silverton and Anderson (1961).

(ii) Electron Microscopy :

1. Fixatives :

Glutaraldehyde.

TAAB glutaraldehyde was supplied as a 25% solution (Macfarlane-Robson Ltd.), and made up to 2.5% on the day of use by diluting in PBS.

Osmium tetroxide.

Osmium tetroxide was obtained from BDH Chemi cals Ltd. and was made up to 1% W/V in PBS.

2. Embedding Media :

Araldite Resin.

The components of this resin were the same as described in light microscopy section.

Spurr Resin.

The components of this resin were obtained from EMscope laboratories Ltd. The composition was as follows:

11.5 gms. Vinyl cyclohexane 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 resin was made up fresh each time on the day of use.

3. Stains :

Uranyl acetate.

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

Lead citrate.

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

These stains were stored in the dark at room temperature.

(f) CHEMICALS FOR AUTORADIOGRAPHY :

(i) Radioactive material.

[6- ^{3}H] Thymidine (TRA61) ; specific activity : 20.7 mCi/mg. (766 MBq/mmol) ; Radioactive Concentration : 1.0 mCi/ml., was purchased from The Radiochemical Centre Ltd., Amersham (later Amersham International PLC), Buckinghamshire, England.

(ii) Emulsion.

Nuclear research emulsion K2 (grain diam. 0.20 μ m) was obtained from Ilford Ltd., Essex, England.

(iii) Non-radioactive thymidine.

Thymidine (non-radioactive) was purchased from Sigma Ltd.

(iv) Processing agents :

1. Developer.

Phenisol was obtained ready made from Ilford Ltd. and diluted 1:4 with distilled water before use.

2. Fixer.

Amfix was obtained from May and Baker Ltd., Dagenham, England and diluted 1:3 with distilled water before use.

(g) CHEMICALS FOR DNA MEASUREMENTS.

DNA (Calf thymus type 1) was purchased from Sigma, London ; Acetaldehyde from Hopkins & Williams, Chadwell Heath, Essex ; Trichloroacetic acid from May & Baker Ltd., Dagenham, England. Perchloric acid and Diphenylamine were purchased from Riedel-Dehaenag Seelze-Hannover, West Germany. All these chemicals were of analytical grade.

(g) CHEMICALS FOR MICROFILAMENT MEASUREMENTS :

(i) Fluorescence.

Nitrobenzooxadiazole-Phallacidin (NBD-Ph.) was purchased from Molecular Probes,Inc.,Plano,Texas, U.S.A.

(ii) Immunofluorescence.

Rabbit anti-actin antibody was given to me by Dr. P. Sheterline of Cell Biology Department of University of Liverpool, Liverpool, England. FITC-labelled goat anti rabbit Fab was purchased from Nordic Immunological Laboratories Ltd., Maidenhead, Berks, England.

(iii) DNase-I inhibition.

Highly polymerised calf thymus (type-I) DNA, non crystalline DNase-I from beef pancreas (DN 100), Actin from chicken muscle (lyophilized powder), Phenyl methyl sulfonyl fluoride PMSF (P-7626), Guanidine hydrochloride grade I (G-4505), and Adenosine triphosphate (ATP) were purchased from Sigma Ltd.

[B] M E T H O D S.

(a) CULTURING FROM CHICK EMBRYO :

Embryos were removed aseptically from the incubated eggs and placed in ice cold Hanks Hepes (HH).

(i) Heart Fibroblasts.

Hearts were dissected from embryos and transferred in to petri dish containing HH. Using a Swann-Morton's surgical knife and oven sterilized fine forceps, the arteries and pericardia were removed and the remaining ventricles chopped in to small pieces (0.5 - 1.0 mm.). These explants were washed throughly in ice cold HH to remove adherent blood.

(ii) Pigmented Retinal Epithelia.

The Pigmented retinal epithelial (PRE) cells were cultured using a method similar to that of Buultjens and Edwards (1977). The corneas were removed and the sclera dissected away. The eyes were then washed in CMF and transferred to 0.25% trypsin for 5-8 min. until the choroid coat was loosened. The eyes were then placed in CMF10 (Calcium and Magnesium-free salt solution supplemented with 10% foetal calf serum) and the choroid coats removed. The iris was removed and the vitreous humour and lens extracted. This procedure left only the neural and pigmented retina layers. The PRE was peeled away from neural and pigmented retina layers and pooled. Some of the pooled cells were used directly as explants for various sail-sheet cultures while the remaining tissue was rinsed three times in cold CMF and left for two min. in EDTA. It was then incubated for 15 min. in 0.25% trypsin (37°C.).The cells were re-suspended in HEFT and aspirated gently with a pasteur pipette to obtain small clumps of cells. These cells were

centrifuged for 5 min. at 1000g. and the pellet re-suspended in HCFT to stop any residual trypsin activity. The cells were then plated in 55 mm. diam. plastic tissue culture dishes at $8.5X10^{6}$ cells/ml. concentration.

(iii) Glial Cells.

Glial cells were obtained from embryonic brain by gently aspirating the dissected tissue with a pasteur pipette and plating it in plastic culture dishes containing Hams medium. After 3-4 days of incubation, a mixed population of glial and nerve cells was attained from which the glial cells were purified by passaging the cultures 2-3 times.

(b) CULTLURING FROM MOUSE LUNG :

The mice were killed by cervical dislocation and the thoracic cavity was opened along its midline. Both lungs were dissected out and immediately placed in ice-cold HH which was changed 2-3 times to wash away as much blood as possible. The tissue was then chopped down to the appropriate size for explants, and repeatedly washed with HH before transfer to Hams medium containing 10% tryptose phosphate broth and 10% foetal calf serum (FCS). For succeeding media changes, Insulin-transferrin-selenite and 3% FCS were supplements.

After about a week, the cultures had developed outgrowing sheets of cells of either fibroblastic or endothelial type.

(i) Endothelia.

Endothelial cells were purified by treating cultures with trypsin plus EDTA for 3-5 min., thus removing fibroblasts. This process was repeated once or twice everyday for a couple of days.

(ii) Fibroblasts.

Fibroblasts were purified by plating cells released by the short

trypsinisation in to fresh culture vessels. This process was repeated 3-5 times at intervals of several hours.

(c) CULTURING FROM PIG AORTA.

Endothelial cells were cultured from pig aortas. Aortas from freshly slaughtered pigs were obtained from the local abattoir.The aortas were tied off at one end and cannulated at the other and then, filled with BSS using a 10 ml. syringe. They were then stripped of any excess tissue and the intercostal arteries clipped off to prevent leakage ; rinsed twice with BSS, filled with collagenase (0.5 mg/ml in BSS) and left for 15-20 min. at 37°C. The collagenase was then removed and aortas were rinsed again with BSS, rubbed gently to loosen sheets of endothelium, filled with growth medium, poured out in to 25 cm. tissue culture flasks and topped up with medium. Medium was first changed after 24 hrs. and later on every alternate day. The cell sheets were grown to confluence in about a week's time.

(d) CONVENTIONAL CULTURES :

Explants from the various tissues mentioned above were used to set up conventional cultures on solid substrata such as glass coverslips and plastic culture dishes. However, some cultures (Chick heart fibroblasts) were set up by dissociation of the tissue in trypsin and DNase for 12-15 min. at 37°C. These tissue were then aspirated with a pasteur pipette to break large clumps of cells. This suspension was filtered through Nitex mesh (10 µm.) using a millipore filter assembly, and the single cells suspended in HEFT and plated on glass coverslips or plastic culture dishes at 10^6 cells/ml.

(i) Coverslips.

Cells from explants were plated on to both circular (16 mm. diam.) and square (22 X 22 mm.) glass coverslips by the suspension method

described above and by the hanging drop method. In the later method, the explants themselves were placed on coverslips in a drop of growth medium. The coverslips were then sealed to a cavity slide with paraffin wax-vaseline mixture.

(ii) Culture dishes.

Explants were cultured in plastic tissue culture dishes of 55 mm. diam. in order to obtain higher yields. These higher yields were necessary if separation of the two or more cell types, usually found in explants, was to be achieved rapidly, and were also needed for the culture technique described on page 43.

(e) SAIL-SHEET CULTURES

Sail-Sheet cultures were those in which cells were grown as sheets suspended on mesh in the absence of any continuous non living substrate.

(i) Nitex and Chiffon Meshes.

These meshes were cut in to 2X2 cm. squares which were sterilised by autoclaving. Explants of the various tisues were put on to these meshes which had been kept in sterile plastic petri dishes (35 and 55 mm. diam.) containing HEFT. Glass rings (20 mm. diam.) were placed over the mesh squares to prevent explants from falling in to the petri dish.

(ii) Carbon Fibres.

Holes (1.0 cm. diam.) were drilled in the polystyrene coverslips (2.5 cm. diam.) and carbon fibres (2 cm. long, 15 µm. diameter) were attached across the holes by gluing the fibres to the edges with cellulose acetate cement. The coverslips were then sterilized using 70% ethanol and soaked in HEFT for 2-3 min. before use. The explants from the chick heart were cultured on these carbon grids. After 3-4 days, sail-sheets were observed.

Despite the irregular array of the fibres, the cells behaved in a similar way to those on Nitex and chiffon. That is they gradually covered the space by growing inwardly from the fibre, completely filling the holes within a day or two.

(iii) Metallic Meshes.

Metallic meshes such as gold grids (3 mm. diam.), with holes of various shapes and sizes, were attached to the edge of holes drilled in polystyrene coverslips by using collodion cement. The explants were cultured on these grids as described in above paragraph.

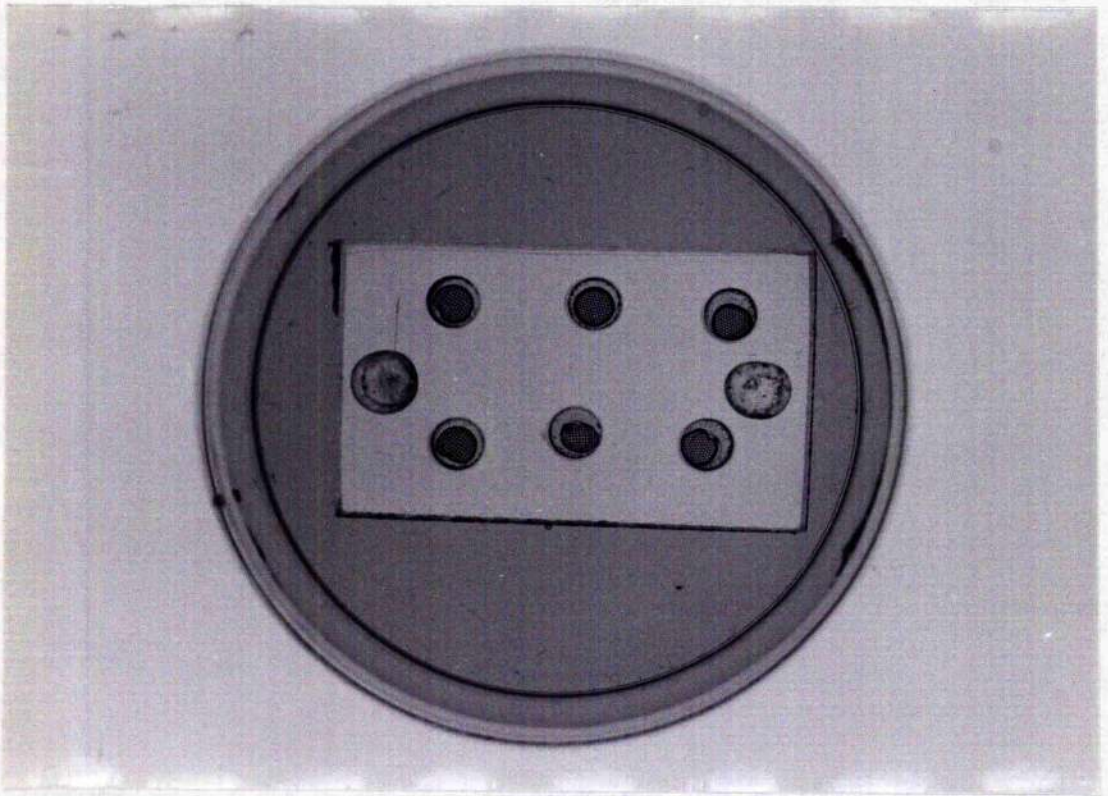
The glass rings (2 cm. diam.) having crosses of glass threads on their bottom fixed with 'quick set' epoxy resin were placed on the top of the grid above the explant to ensure that the explant did not fall off the grids.

(f) CELL SHEETS FROM DISPERSED CELLS.

In this type of culturing, the use of explants was replaced by single cell suspensions.

Six grids were held in the middle of specially prepared grid holders (plate 1) which were perspex blocks through which six holes had been drilled. Each hole had a larger diameter than the grids at the top but half way through the block this hole was narrowed so that the grid was held in place. This ensured that the cells grew as a sail-sheet.

Drops of the cell suspension ($1X10^{6}$ cells/ml) were placed on top of these grids using a thin needle (size 26 G 3/8.10-45) on a 1 ml syringe. Several drops had to be added at intervals of a few min. for a sufficient number of cells to be attached to the grid. Within 12 to 48 hours, all the gaps in the grid had been completely filled with cells.



10mm

PLATE 1.

A Perspex block with gold grids sitting in six wells. These blocks were used in culturing cell sheets from dispersed cells (see page 43).

(g) HISTOCHEMISTRY :

(i) Preparation of Epoxy Resin Blocks.

Araldite (Epoxy Resin) blocks were prepared in exactly same way as described for transmission electron microscopy.

(ii) Sectioning and Staining.

Semi-thin sections (1.2 - 3.0 µm.) were cut in a Reichert Jung Autocut Microtome, deresinised with saturated sodium ethoxide, stained in Azur-II, dehydrated in ethanol, cleared in xylene and mounted in DPX (McGadey & Reed, 1981).

(iii) Scanning Electron Microscopy.

The sail-sheet cultures on Nitex mesh and gold grid (hole diam. 200 µm.) were fixed for two hours at 4°C. in 2.5% glutaraldehyde in PBS. They were then washed in phosphate buffer (pH 7.4) for two hours at 4°C., several times. The cultures were then dehydrated through a graded acetone series to absolute acetone. They were dried using a Polaron E 3000 Critical Point Drier, mounted on 15 mm. diam. aluminium stubs and coated with gold at a thickness of approximately 200 A using a Polaron E 5000 Diode Sputter Coater. The coated cultures were then viewed using a Phillips Scanning Electron Microscope 500 with a manual stage goniometer operating at 25 or 12 Kv. Ilford FP4 120 Roll film was used for the photomicrography.

(iv) Transmission Electron Microscopy.

The sail-sheet cultures on Nitex mesh (Hole size 200 X 200 µm.) were fixed in 2.5% glutaraldehyde at 4°C. and washed in phosphate buffer (pH 7.4) at 4°C. for an hour and then overnight after buffer change. The cultures were post-fixed in 1% osmium tetroxide (phosphate buffered) for an hour at 4°C., dehydrated in absolute ethanol (5-6 changes over 2 hrs.) and immersed in Propylene Oxide (PO) twice for 20

min. then passed through a graded Araldite Resin (AR) series, 6 to 8 hrs. in 1:1 AR/PO, 8 hrs. in 3:1 AR/PO and overnight (min. 8 hrs.) in AR and were finally embedded in AR (also see page 36-37).

The conventional cultures, grown in plastic culture dishes, were embedded in Spurr resin following the above method except that instead of using PO as a solvent for the resin, absolute ethanol was used.

Sections of 300 A thickness were cut using an LKB Ultratome III. The sections were stained for 5-10 min. each in uranyl acetate and Reynold's lead citrate followed by two 30 sec. rinses in distilled water. The stained sections were examined in a Siemens Elmiskop IA transmission electron microscope. This was done with help of Mr. C. Mucci.

(h) AUTORADIOGRAPHY :

(i) Subbing of Slides.

Slides were cleaned in a mixture of ethanol and conc. nitric acid (70% ethanol was added drop by drop in the conc. nitric acid until the acid becomes brownish in colour), throughly washed in distilled water and then subbed by dipping in a solution containing 2.5 gm. of gelatin and 0.25 gm. chrom alum in 500 ml. of distilled water. The slides were dried at room temperature in slide carriers wrapped in tin foil (to keep them dust-free) and stored in refrigerator. The subbing of slides makes them more adhesive not only for nylon mesh but for emulsion also (Rogers 1969).

(ii) Radio-active Labelling.

The sail-sheet cultures were pulse-labelled with tritiated thymidine (4 µCi/ml HEFT) for an hour followed by chasing with non radioactive thymidine (2 mg./ml HEFT) for another hour to ensure that unincorporated radio-active thymidine is removed. The cultures were

subsequently grown in HEFT and fixed in formol saline (pH 7.6) for 20 min. after 3-12 hours of labelling.

After fixation, cultures were transferred to 70% ethanol and then dehydrated with absolute ethanol.

Conventional cultures were treated in the same way up to the stage of fixation and then incubated in 10% trichoroacetic acid (TCA) for 10 min. to remove unincorporated tritiated thymidine. These cultures were then washed with absolute ethanol, air dried, stuck face upwards using Clearmount and stored for autoradiography.

(iii) Preparation of Emulsion.

The emulsion was melted at 50°C. in a small glass beaker and then diluted 1:2 with pre-warmed distilled water. The melted emulsion was mixed gently to avoid formation of any air bubbles. This procedure was carried out in a dark room with a safe light on.

(iv) Sail-Sheet Cultures (Whole Mounts).

These cultures were dipped in to the melted emulsion and put on to the subbed slides. The slides were kept on an ice tray for 15-20 min. and then dried at room temperature for one hour. Throughout the process, the slides were kept in plastic boxes which were placed in light-proof tin boxes containing a small amount of dry silica gel. The tin boxes were sealed with tape, wrapped twice with tin foil and kept in cold room at 4°C. The slides were exposed for three weeks.

(v) Conventional Cultures.

The conventional cultures were dipped into the emulsion and excess emulsion was drained off immediately. The slides were placed (nearly vertical) over the foil until the emulsion had set. The slides were then treated in the same way as described for sail-sheet cultures.

(vi) Development of Autoradiograpphs.

The tin containing emulsion-coated slides which had been exposed at 4°C. was allowed to warm slowly to room temperature so that the emulsion did not crack.

The slides with conventional cultures were developed in Phenisol developer for 4 min. at 20°C. followed by a 30 sec. wash in 2% acetic acid in distilled water and fixed in Amfix for 4 min. The developing and fixing time was increased to 5 min. for sail-sheet cultures because a thicker layer of emulsion had been used to increase the number of silver grains per labelled nucleus. The slides were then gently washed several times with distilled water.

(vii) Staining the Autoradiographs.

The slides were stained by Masson's trichome procedure i.e. stained in Weigert's Iron Haemotoxylin (30-40 min. for conventional culture and 0.5-1.0 min. for sail-sheet cultures), differentiated with a quick wash in acid alcohol and then stained in Ponceau Acid Fuchsin C.I. 42685 (5min. for conventional and 15 sec. for sail-sheet cultures). This was followed by further differentiation in 1% phosphomolybdic acid, dehydration in ethanol, clearing in xylene or cedar wood oil and mounting with no.1 coverslips in Clearmount.

Staining through the emulsion with haemotoxylin is known not to affect the silver grains (Le Blond et al., 1963). Sometimes, slides were counter stained with Light Green C.I. 42095 (1-2 min.) followed by quick wash in distilled water.

(viii) Counting Autoradiographs :

1. Counting Labelled Cells.

The cells were considered to be labelled if they had more than twenty silver grains over the nuclei. The use of Phenisol as a

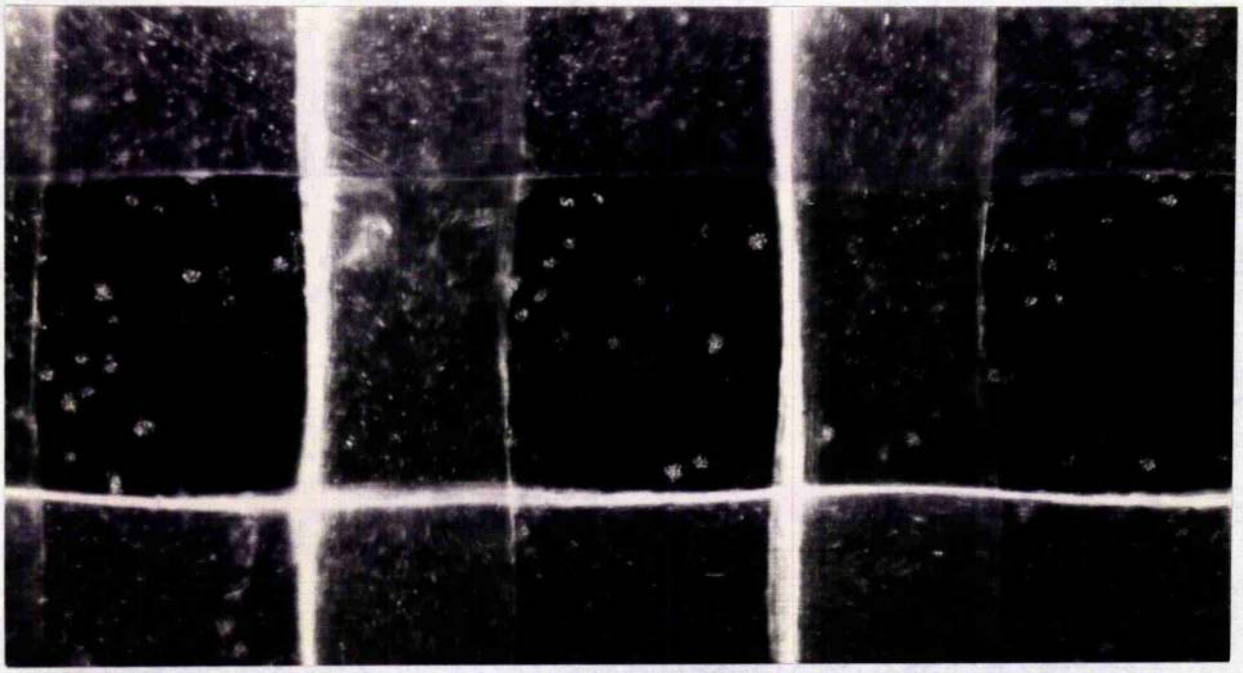
developer made the silver grains large which was useful for counting. However, because tritium is a beta emitter, the thickness of the material can affect the efficiency of silver grain formation (Rogers, 1969). In fact, if the thickness of the material is greater than emitter's path length, self absorption may even occur. Thus, in sail-sheet cultures, the self absorption of particles could be higher than in conventional cultures.

Miller (1980) stated that the background count (due to extra cellular labelling) need not be made if the cellular labelling is heavy and, therefore, background was not counted.

Plate 2 shows some of the autoradiographs which were used for counting number of labelled nuclei in the cultures.

2. Counting Total Cell Number using a Nuclear Stain.

Because conventional histological stains do not stain all nuclei in these cultures, a special method was devised on the suggestion of Professor Curtis. In this method, the autoradiographs, after counting the number of labelled cells in individual mesh holes, were transferred to xylene for 24 hours in order to free samples from the slides. The samples were then placed on a filter paper to remove xylene and then, transferred to absolute ethanol (6-8 changes, 30 minutes each change). They were then passed through a descending ethanol series to distilled water, stained with bisbenzimid fluorochrome (a DNA staining fluorescent stain) for 60-90 minutes, washed rapidly three times in PBS and mounted on the slides in a 25% solution of glycerol in PBS. The edge of the coverslips were sealed with nail polish to prevent evaporation. The specimens were then examined under a fluorescence microscope and total number of nuclei for individual mesh holes was counted.



50 μm.

PLATE 2.

Chick heart fibroblasts, autoradiographs, viewed by dark ground. [^{3}H] thymidine labelling 1 hour, showing distribution of labelled nuclei in sheets between mesh.

(i) DETERMINATION OF CELL CYCLE DURATION IN SAIL-SHEET CULTURES OF CHICK HEART FIBROBLASTS BY TOTAL DNA/ [^{3}H] -THYMIDINE INCORPORATION COUNTS.

Because of special problems in measuring the cell cycle duration in these cultures the following method was devised following a suggestion from Professor Curtis. Basically the method measures the rate of DNA doubling in the cultures but because different cultures contain different numbers of nuclei at the start of the experiment a base line was established by [^{3}H]-thymidine labelling.

The sail-sheet cultures of Chick heart fibroblasts grown on nitex mesh were pulse labelled with [^{3}H]-thymidine (4 µCi/ml. of growth medium) for 3 hours and then chased for an hour and half with cold thymidine (2 mg./ml.). The cultures were incubated for zero to 54 hours in growth medium and the DNA concentration of these cultures was determined at six hour intervals from 18 to 54 hours as well as 0 hour (4 1/2 hours from the time of labelling) using following method:

(i) Extraction of Acid Soluble Compounds.

The cultures were washed with chilled Hepes saline and homogenised in cold 6 % perchloric acid (PCA) in an ice-jacketed homogeniser (Schneider et al., 1950). These were then washed in 6 % cold PCA to remove inorganic phosphate and organic phosphorous compounds of low molecular weight, to remove sugars and polysaccharides that are likely to affect sugar reaction for nucleic acids and, to remove nucleotide co-enzymes whose purine and pyrimidine components might increase the recovery of these bases in the nucleic acid fractions.

(ii) Extraction of phospholipids.

After acid extraction, the samples were centrifuged for 5 minutes

at 2000 rpm. The pellet suspended in absolute ethanol, 2X ethanol-chloroform (3:1), ethanol ether (3:1) and, finally in ether and centrifuged every time for 2 minutes and at 2000 rpm.

(iii) Extraction of RNA.

The tissue residue obtained after extraction of phospholipids was digested in 1 N potassium hydroxide at 37°C. for 19 - 24 hours. The digest was neutralised with HCl (Tyner et al., 1953) and was acidified with 10 % tri-chloroacetic acid (TCA) in order to bring the pH down to 1. The samples were then kept in ice for 30 minutes to settle the precipitate which would have DNA and protein, centrifuged at 5000 rpm for 5 minutes. The supernatant fluid (containing ribo-nucleotides or RNA fractions) was discarded. The pellet was suspended in 5 % TCA twice and the supernatant fluid was, again, discarded.

(iv) Extraction of Proteins.

Proteins were extracted using Schneider's (1946) modification of the Schmidt-Thannhauser (1945) procedure. In this procedure, the fraction is extracted with hot PCA to hydrolyse the DNA. The pellets were suspended in 5 % PCA and were heated at 90°C. for 15 minutes (Volkin & Cohn, 1954).

(v) Determination of DNA Concentration.

The amount of DNA present in the extract was determined by measuring the deoxypentose levels with the Disch reagent. Burton's (1968) modification to Disches' method (1930) increases its sensitivity three and a half times more and was used.

Acetaldehyde, diphenylamine and standard DNA solutions were prepared using Burton's (1968) method. Suitable portions of the extract were mixed with 2 volumes of diphenylamine reagent in test tubes. Standards (having known concentrations of DNA) and blank (having no DNA) were

prepared in the same way bearing in mind that all tubes (the experimental as well as controls) should contain same amount of PCA. These tubes were incubated for 24-30 hours at 31°C. and the absorbance measured at 600 nm. DNA contents of cell extracts for various duration of cultures were determined by comparing their absorbance with that of standard tube having known concentration of DNA.

(vi) Determination of [^{3}H]- Thymidine Incorporation Counts.

After measurement of absorbance, the samples were neutralised with saturated solution of sodium hydroxide and then, transferred to scientillation vials (3.5 ml. in each vial) . 10 mls. of scintillation fluid (PICO - FLUOR^{TM} 30). were added to each vial. Beta emission was measured in a Packard Tri-Carb 300 scintillation counter with a tritium efficiency of around 75 % as determined from the SCR. Counting was done for 5 minutes for each sample and background levels were automatically removed from the final counts received.

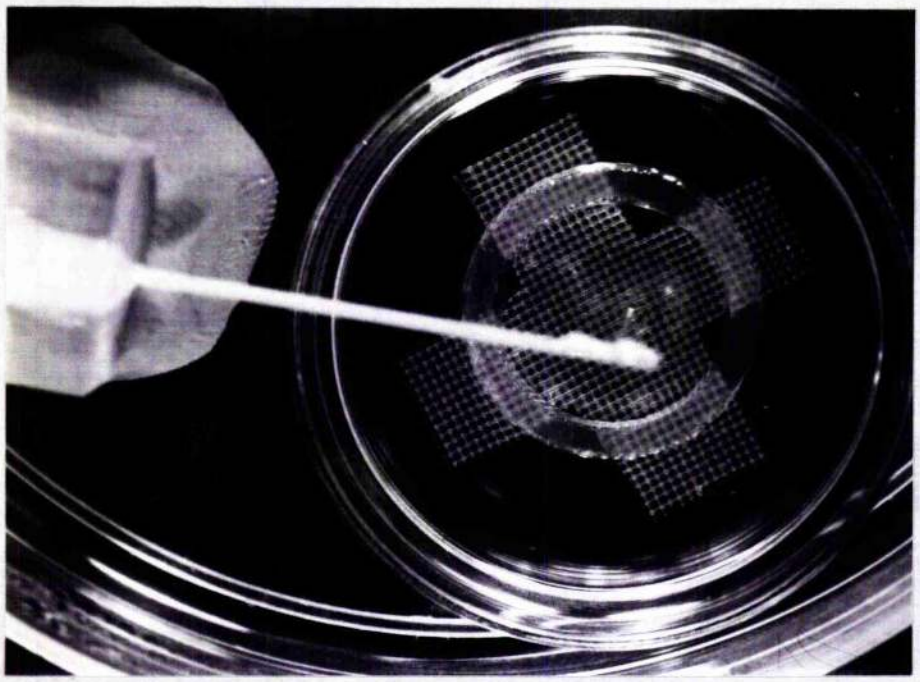
(j) TENSION EXPERIMENTS :

(i) Preparation of Culture System for Tension Experiments.

Only the sail-sheet cultures on Nitex mesh were found to be appropriate for tension experiments. Four small squares of 1.5 cm. length were cut from the corners of larger nitex mesh squares (size of holes : 200 X 200 µm.) leaving a cross shape. These crosses of nitex were fixed betwen two glass rings of the same size (as shown in plate 3) using cellulose acetate cement.

Explants from ventricles of chick hearts from 9 days incubated embryos were cultured on the grids described above.

The medium was changed the day after culturing and subsequently every third day until a final change 12 hrs. before starting the tension



5µm.

PLATE 3.

A piezo electric ceramic probe inserted into a sail-sheet culture for tension experiments. Mesh size 400 X 400 μm^2.

experiments.

(ii) Preparation of Low Frequency Oscillator and Piezo Ceramic Probe.

1. The Oscillator.

A very low frequency sine wave oscillator with a built in amplifier was designed by Dr. A. J. Lawrence and constructed by Mr. W. MacLeod of Cell Biology Department of the University of Glasgow. This equipment is based on that used by Seehar (1978). The maximum output of the amplifier was 60 V.

Three frequencies were used for the tension experiments viz. 0.01, 0.1 and 1.0 Hz.

2. The Probe.

Two wires were soldered to opposite sides of one end of a piezoelectric ceramic probe (Mullard, type MB 8004) and a fine glass rod (solid) was attached to the other end using quick-set epoxy resin. The soldered end was attached to a piece of perspex which was supported in plastic box with plasticine.

The tip of the probe moved about 60 μm when a current of 60 V.from the oscillator is passed through the poles of the element but stretched nylon mesh sheets to a lesser extent.

(iii) Application of Tension and Design of Experiments.

When the thin glass rod was inserted in one of the nitex mesh squares and the sine-wave oscillator was switched on, a potential difference of 40 V. was applied across the poles of the ceramic probe. The oscillations of the probe caused stress in the sail-sheets resulting in to the intermittent distortion of the mesh.

Three types of controls were carried out :

(a) cultures without a probe.

(b) cultures with a probe stirring the medium but not touching the

cells.

(c) cultures with a probe neither stirring the medium nor stressing the cells.

The first series of experiments was designed to determine the effect of three frequencies on the following :

1. the duration of the cell cycle,

2. the distribution of cells in S-phase within the mesh holes, and

3. the positional localisation of the proportion of labelled cells.

These experiments were carried out by applying tension for various durations such as 3, 6 and 12 hrs. and pulse labelling the cultures with triliated thymidine (4 µCi/ml. in HEFT) during the first and the final hour of deformation. The cultures were then fixed for 20 min. in formol saline (pH 7.6) and stored in 70% ethanol awaiting further preparation for whole mount autoradiography.

Curtis and Seehar (1978), showed that the small mechanical tensions applied to cells shortened the cell cycle duration. One possible explanation for this is that tension might orient microfilaments or increase their number in such a way that the nuclear activity is affected. The second experiment was designed to test this hypothesis which may be of considerable importance in the control of division in, for example, wound repair, malignancy and atherosclerosis. This experiment was performed by applying mechanical tension and carefully looking its effects in a number of ways such as :

(a) detection of actin with labelled phallacidin.

(b) by determining the amount of actin by DNase-I inhibition method,

(c) by anti-actin antibody staining, and

(d) by transmission electron microscopy.

A third class of experiments, to examine the effect of mechanical tension on cell orientation and surface protrusion in sail-sheet cultures. To do this, unstained cultures and controls were examined by scanning electron microscopy.

(k) MICROFILAMENT SYSTEM :

(i) Fluorescence Assay.

Nitro-benzooxadiazole-Phallacidin (NBD-Ph) was used for fluorescence assay of actin microfilament system. It is a rapid *in vitro* stain for the detection of different cell types. This stain may be used for *in vivo* studies to investigate the role of f-actin in cellular processes.

Cells from both the sail-sheet and the conventional cultures were washed twice in phosphate buffered saline (PBS) and were fixed in a 3.7% formaldehyde solution in PBS for 10 min. at room temperature. They were then washed twice in PBS, placed in a glass petri dish and extracted with acetone at -20°C. for 3-5 min. The samples were air dried.

10 microlitres (33 ng.) of NBD-Ph was dried in a small test tube under a nitrogen gas stream and redissolved in 200 microlitres of PBS. This solution was placed on the samples of cells for 20 min. at room temperature. Any temperature over the range 4 to 37°C. was found to be suitable. The samples were then washed rapidly twice in PBS and mounted on the slides in a 10% solution of glycerol in PBS. The edge of the coverslips were sealed with nail polish to prevent evaporation. Specimens prepared in the above manner retain actin staining for periods of at least 5-6 months when stored in the dark at 4°C.

(ii) Determination of Actin by DNase I Inhibition Method.

Principle:This method has been extensively described by Blikstad et

al. (1978) and is based upon the fact that unpolymerised actin (G-actin) inhibits the depolymerisation of DNA by DNAase and thus, this enzyme assay may be used as the basis of a method of determining G-actin. DNAase-I may be used to depolymerise DNA and the rate of this reaction can be measured by measuring the difference in absorbance at an optical density (O.D.) of 260 nm between unpolymerised and polymerised DNA.

The total filamentous (F-actin) and G-actin may be determined by determining the extent of inhibition of DNase-1 before and after treating the sample with 1.5 M guanidine hydrochloride, which depolymerises actin filaments. The amount of actin measured before treatment with guanidine hydrochloride is G-actin and the total amount of actin found after depolymerisation subtracting the G-actin gives a measurement of the total F-actin present.

Method: 1 ml. of 40µg/ml. solution of highly polymerised DNA was suspended in 0.1 M tris/HCl (pH 7.5) buffer containing 4 mM $MgSO_4$ and 1.8 mM $CaCl_2$ and was mixed with 3.35 µl. of 0.1 mg./ml. DNase-I dissolved in 50 mM tris/HCl (pH 7.5) containing 0.1-0.5 mM $CaCl_2$ and incubated in a Varian Techtron 635 UV-VIS Spectrophotometer. The O.D. of DNA solution and DNA plus DNase were measured separately for 3-5 min. each at a wavelength of 260 nm. and in a 1 cm. light path.

The rate of rise of O.D. (after mixing of DNase-I in to the DNA solution) was measured to give a blank value for zero inhibitor. Samples of pure actin were added at dilutions which gave 35-65% inhibition of DNase-I activity. A standard Actin inhibition curve was obtained. Samples of control (untensed) and experimental (tensed) cells were treated in the same way and the amount of G-actin present in both of these samples was determined by comparing them with the standard

actin inhibition curve as described above.

Total actin content in these samples was determined after depolymerising the F-actin by incubating the samples with equal volumes of 1.5 M guanidine hydrochloride, 1 M Sodium acetate, 1 mM Calcium chloride and 1 mM ATP in a 20 mM tris/HCl (pH 7.5) buffer for greater than 5 minutes each at 4°C. One activity of actin inhibition is defined as that amount which gives 1% inhibition of a standard amount of DNAase.

Total protein content in these samples was determined using Bradford's (1976) method.

(iii) Transmission Electron Microscopy (TEM).

TEM studies were carried out in a similar way as described on page 44 for the control (untensed) and the experimental (tensed) sail-sheet cultures with the help of Mr. C. Mucci.

(iv) Anti-actin Antibody Staining.

Conventional and sail-sheet cultures were washed in Hepes saline (pH 7.5), fixed in cold methanol at -60°C. for one min. and then washed thoroughly in Hepes saline. They were stored until use in Hepes saline with a few crystals of sodium azide to avoid contamination with micro-organisms.

These cultures were stained with Rabbit-anti-actin antibody for 2-4 hrs., then washed 5 times in Hepes saline and stained with FITC labelled goat anti-rabbit FAB for an hour. They were then washed in Hepes saline 5 times, mounted in Hepes saline and observed under UV light in Vickers M 17 Fluorescence Microscope.

(1) TIME-LAPSE MICROSCOPIC STUDIES :

Behavioural studies were carried out for both the conventional and

the sail-sheet cultures using a National Panasonic time-lapse VTR (NV-8030) Video recorder which was connected to a Leitz Diavert Inverted Microscope through a National Panasonic camera with a Chalnicon Camera tube. Scotch Video-tape was used for filming.

Filming.

Culture dishes containing cultured tissue (conventional or sail-sheet) were sealed with tape, placed on the stage of the inverted microscope and focussed. The camera was switched on and the timer adjusted to a speed of shot/10 seconds. The culture was maintained at 37°C. with a heater placed at an appropriate distance from the stage of the microscope which was fitted with a thermometer. The heater was switched on 30-40 min. prior to filming. Filming was done for periods of 12 hrs. up to several days.

Tapes were replayed 560 times faster than the filming.

Quantitative Interpretation of Data.

The tape was played very slowly and stopped at an interval of 15 seconds to 2 min. depending upon the type of movement being examined ; an individual cell or whole sheet. The television screen was calibrated using a stage micrometer and corresponding lines were drawn on the screen. These were then transferred on to the transparent sheets. Distances travelled by individual cells and whole sheets were measured using a Map Measure and a Ruler. Time taken was also noted down from the timer display on the screen. From these measurements, the distance travelled in unit time gave the speed of movement of individual cells and whole sheets.

The percentage frequency of presence of protrusions in the conventional and sail-sheet cultures was obtained by directly observing tape carefully.

[C] Q U A N T I T A T I V E M I C R O S C O P Y.

Autoradiographs from sail-sheet cultures alone were used for statistical analyses ; since the conventional culture controls are not strictly comparable.

Analysis of Localisation of Tension Effects.

Data from tables 5-10 showed an increase in the proportion of labelled cells in the tensed (in comparision with their respective controls) sail-sheet cultures of chick heart fibroblasts (CHFs). The question is whether this increment in the proportion of labelled cells in tensed cultures is localised at a particular part in the culture. This question was addressed by testing data on the proportion of labelled cells at various parts in the cultures by analysis of variance using the randomised complete-block design and 3^2 X 2 factorial experiment (Steel & Torrie,1960).

Procedure.

Mesh holes from both experimental (tensed) as well as control (untensed) sail-sheet cultures which were filled with cells were considered for this study. Each such mesh hole was considered as an individual field and all these fields were divided in to 9 equal compartments using a graticule. The number of labelled cells in each of these compartments was counted. These compartments were arbitrarily (on the basis of their positions within the mesh hole) grouped in to three regions: Corners (consisting of 4 compartments occupying the corners), Edges (consisting of 4 compartments located at the edge) and Centre (consisting of just one compartment lying at the centre). Counts obtained from the individual compartments at the corners and at the edges were added separately and averaged. The centre had only one compartment and hence, the number of labelled nuclei falling in this

position was itself considered as its average. The percentage frequency of labelled nuclei at these positions (corner,edge and centre) was determined by dividing their average number of labelled nuclei with total number (labelled as well as unlabelled) of nuclei multiplied by 100. The total number of nuclei was obtained by staining these samples for their DNA using the bisbenzimid fluorochrome method (see page 48). In order to analyse the effects of various frequencies of tension, data on the positional distribution of the proportion of labelled cells from the experimental (tensed for 3-12 hours) and control (untensed) sail-sheet cultures was tested by analysis of variance using a 95% confidence interval for analysing the significance of position effects of tension (Steel & Torrie,1960).

R E S U L T S

This section can be divided in to the following sub-sections :

[A] The Culture System.

[B) Cell Behaviour and Movement.

[C] The Cell Cycle.

[D] The Microfilament System.

[A] THE CULTURE SYSTEM

This sub-section can be divided in to following parts :

(a) General Features of Sail-Sheet Cultures.

(b) Histological Studies :

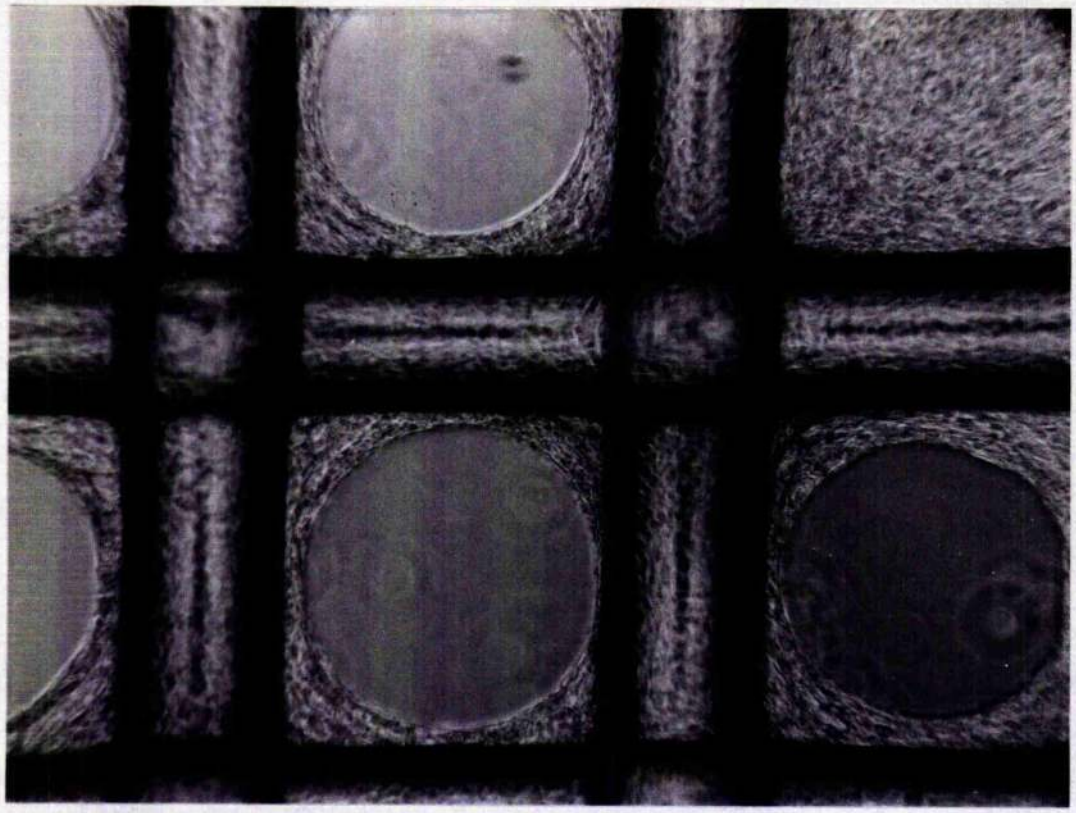
(i) Optical Microscopy.

(ii) Electron Microscopy.

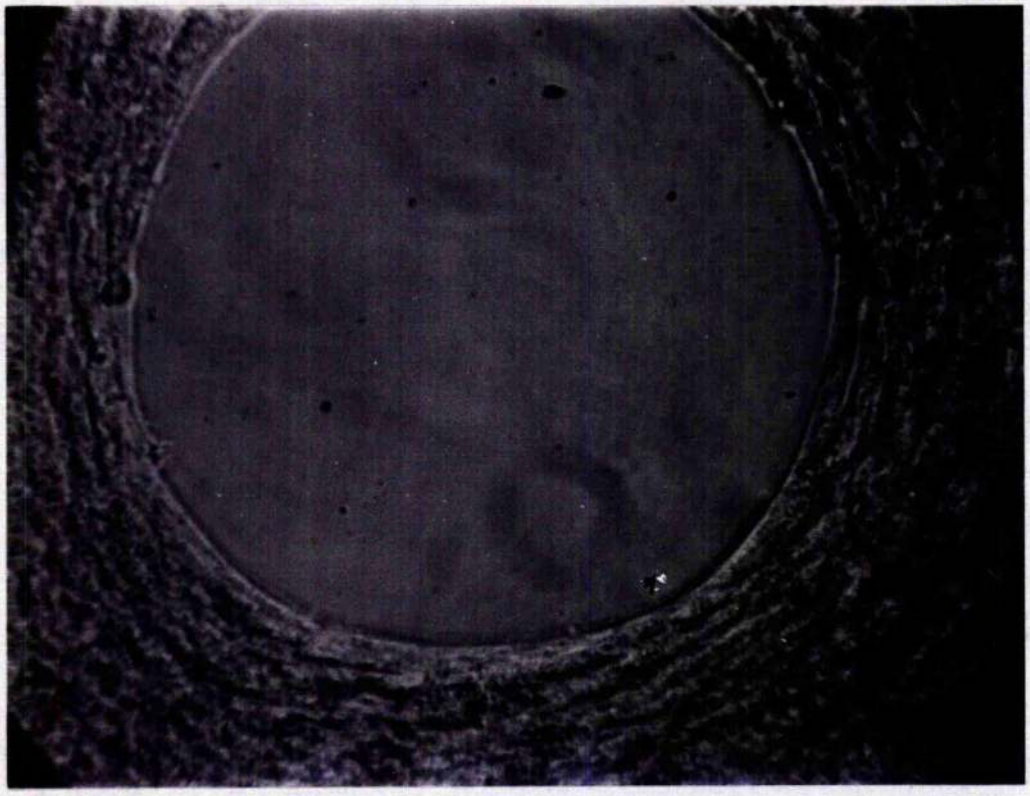
(c) Effects of Mechanical Tension on Cellular Morphology.

(a) General Features of Sail-Sheet Cultures.

The Sail-Sheet Cultures described in this thesis are cultures in which cells are grown within mesh holes without any solid support except for attachment at their sides to other cells and to the mesh threads (plates 4-7, 8 c, 9). The majority of cells, in these cultures, are supported only at the edge by other cells forming a 'sail' like that of a yacht. In this culture system, these 'sails' grow as sheets, hence the name 'sail-sheet' (Curtis & Seehar, 1978). The cells remain suspended in growth medium and initially are in contact with growth medium on both upper and lower sides, unlike traditional cultures on solid substrata (plate 8 a, 8 b). Subsequently, because of multilayering of cells in the sail-sheet cultures, only the upper side



100 μm

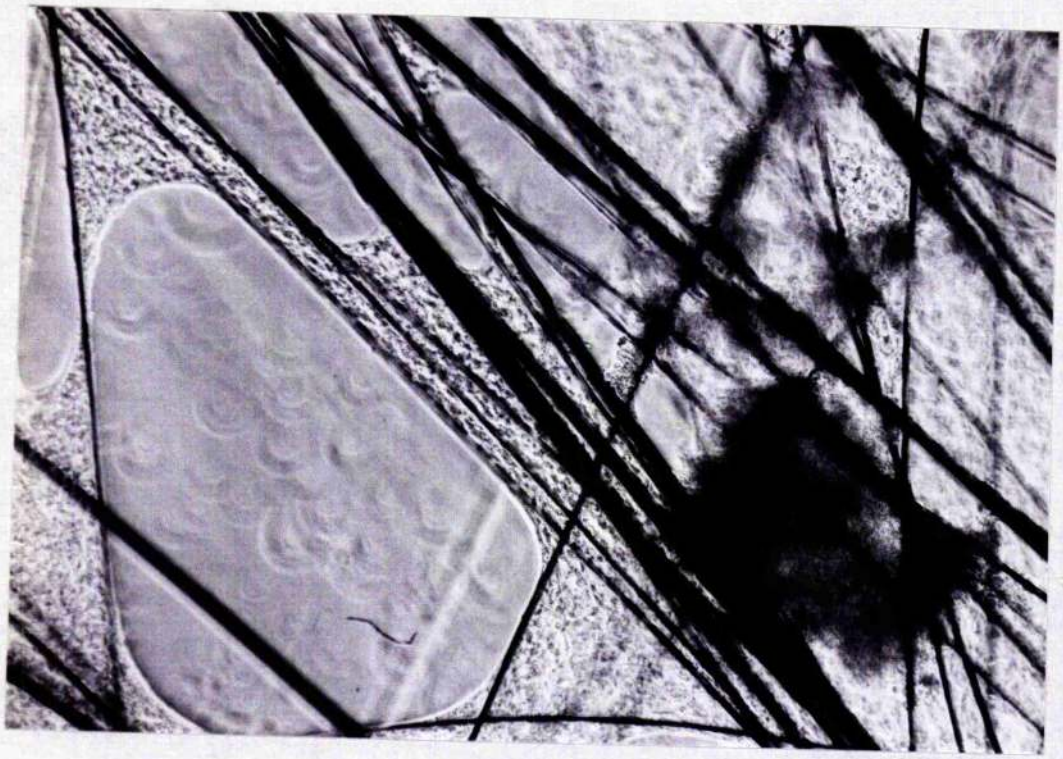


25 μm

PLATE 4.

(a) The plate shows an unstained sail-sheet culture (7 days age) of Chick heart fibroblasts on Nitex mesh under low power. Optics phase contrast. Mesh size 400 X 400 μm^2.

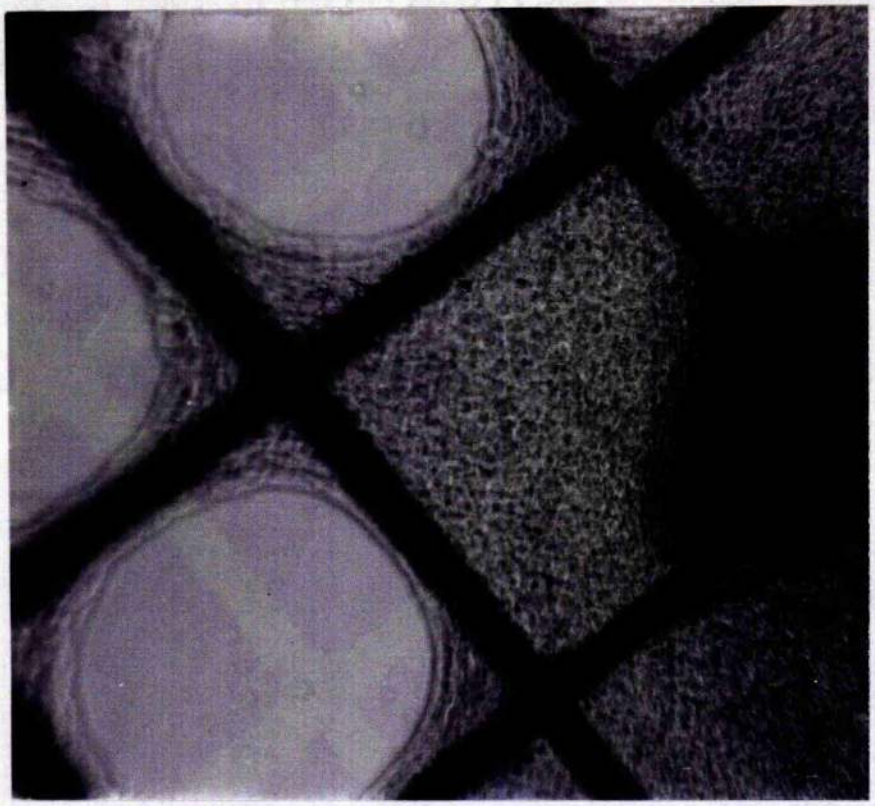
(b) same as above under higher magnification.



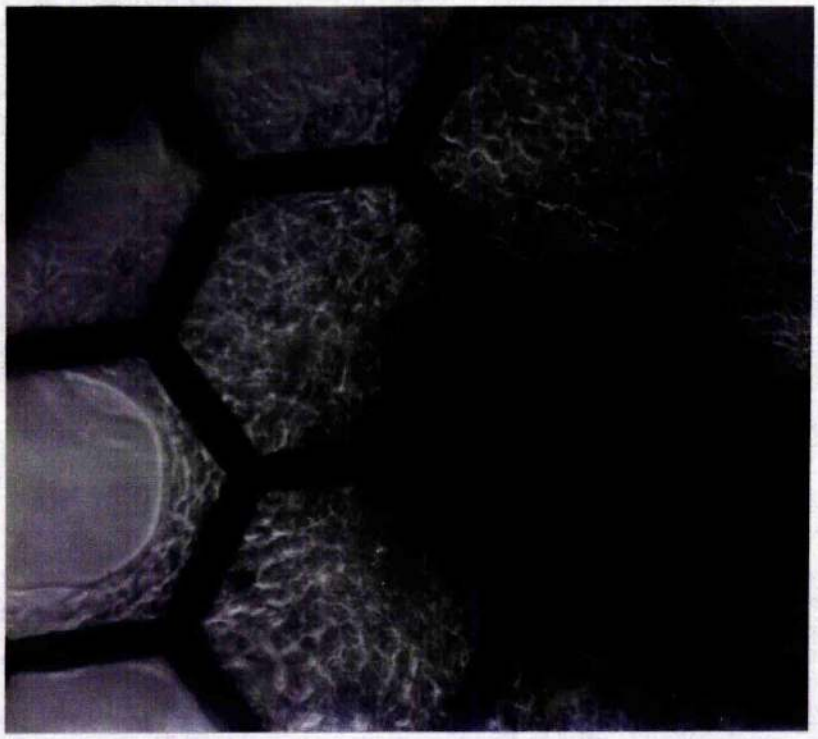
0·3 mm.

PLATE 5.

An unstained sail-sheet culture (7 days age) of Chick heart fibroblasts on an irregular array of carbon fibres. Phase contrast optics.



125 μm.

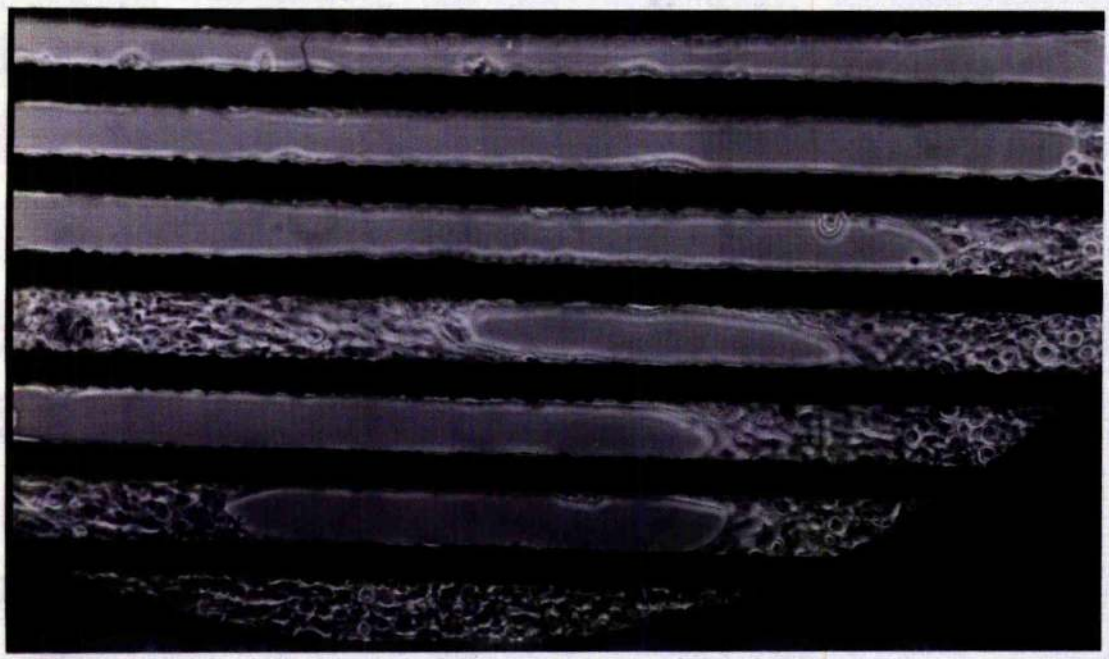


57 μm.

PLATE 6.

(a) shows an unstained sail-sheet culture (7 days age) of Chick heart fibroblasts on a gold grid with square hole shape. Mesh size 400 X 400 μm^2. Phase contrast optics.

(b) shows an unstained sail-sheet culture (7 days age) of Pigmented Retinal Epithelia (PRE) from chick embryo on a gold grid with hexagonal hole shape. Cell sheets from dispersed cells. Mesh size 200 X 200 μm^2. Phase contrast optics.



60 µm

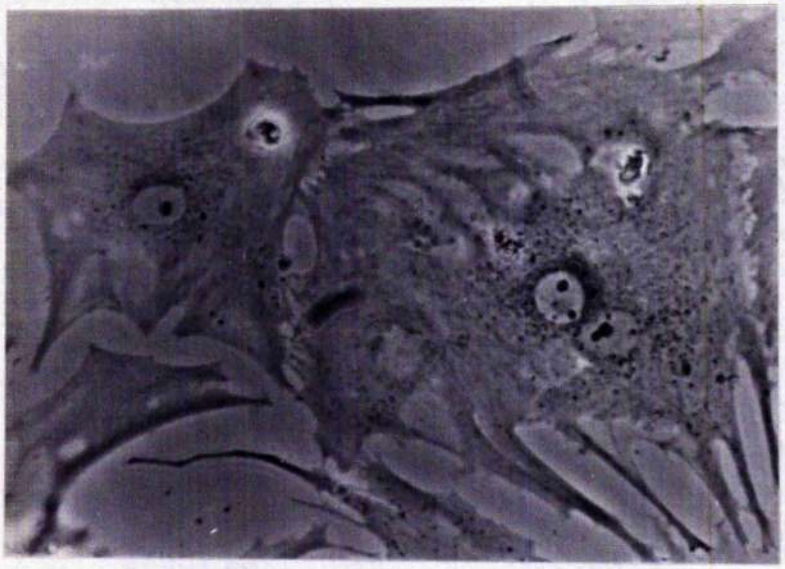
PLATE 7.

An unstained sail-sheet culture (3 days age) of glial cells from a Chick embryo on a gold grid with slotted hole shape. Grid spaces not completely filled with cells. Cell sheets from dispersed cells. Mesh size R 200. Phase contrast optics.

(a)

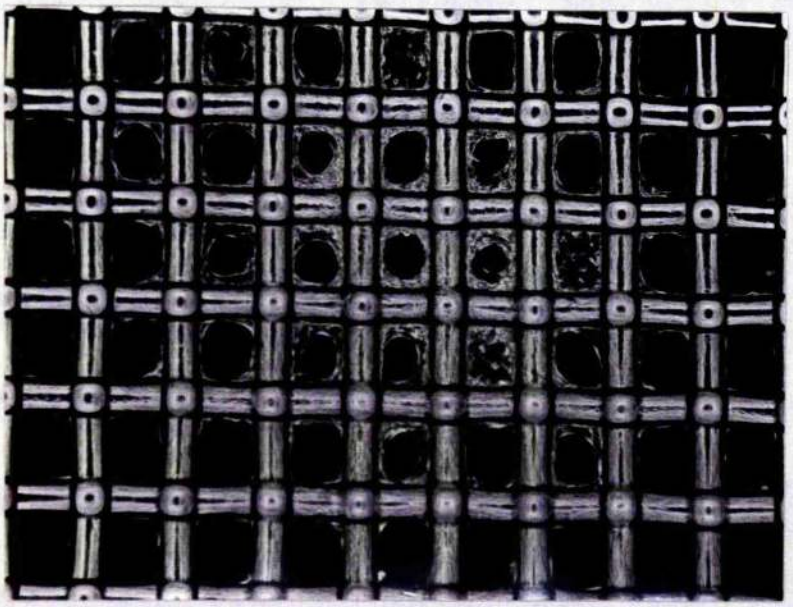
95 μm

(b)



30 μm

(c)



119 μm

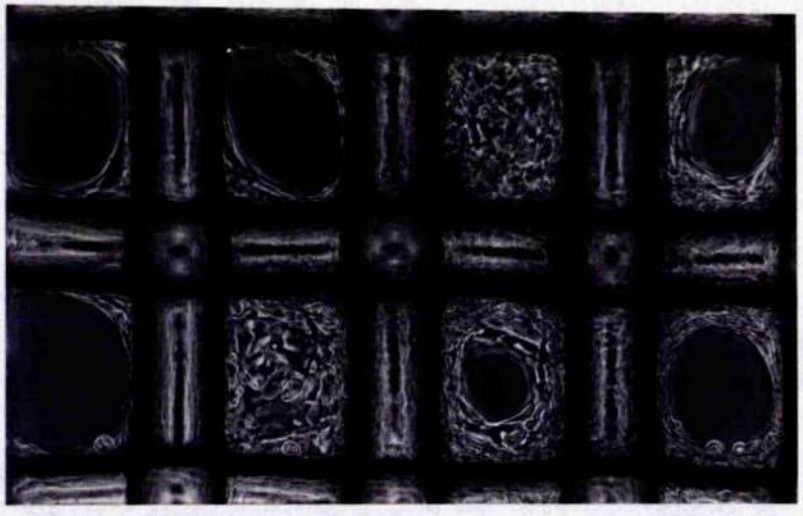
PLATE 8.

Cultures grown using endothelial cells from pig aorta. Phase contrast optics.

(a) A low power view of the conventional culture (5 days age) on a plastic tissue-culture petri dish.

(b) A high power view of the same as (a).

(c) A low power view of the sail-sheet culture (7 days age) on Nitex mesh. Mesh size 185 X 166.5 μm^2.



49μm

PLATE 9.

Low power view of a sail-sheet culture (7 days age) grown on Nitex mesh using endothelial cells from pig aorta . Mesh size 185 X 166.5 μm^2 . Phase contrast optics.

of the layer of cells facing upwards and the lower side of the layer of cells facing downwards are in direct contact with growth medium.

Different cell types from several animals (chick, mouse, pig) have been cultured as sail-sheets. But, all tend to look morphologically similar as judged by phase microscopy (plates 4-7, 8 c, 9) in contrast to differences generally observed in conventional cultures.

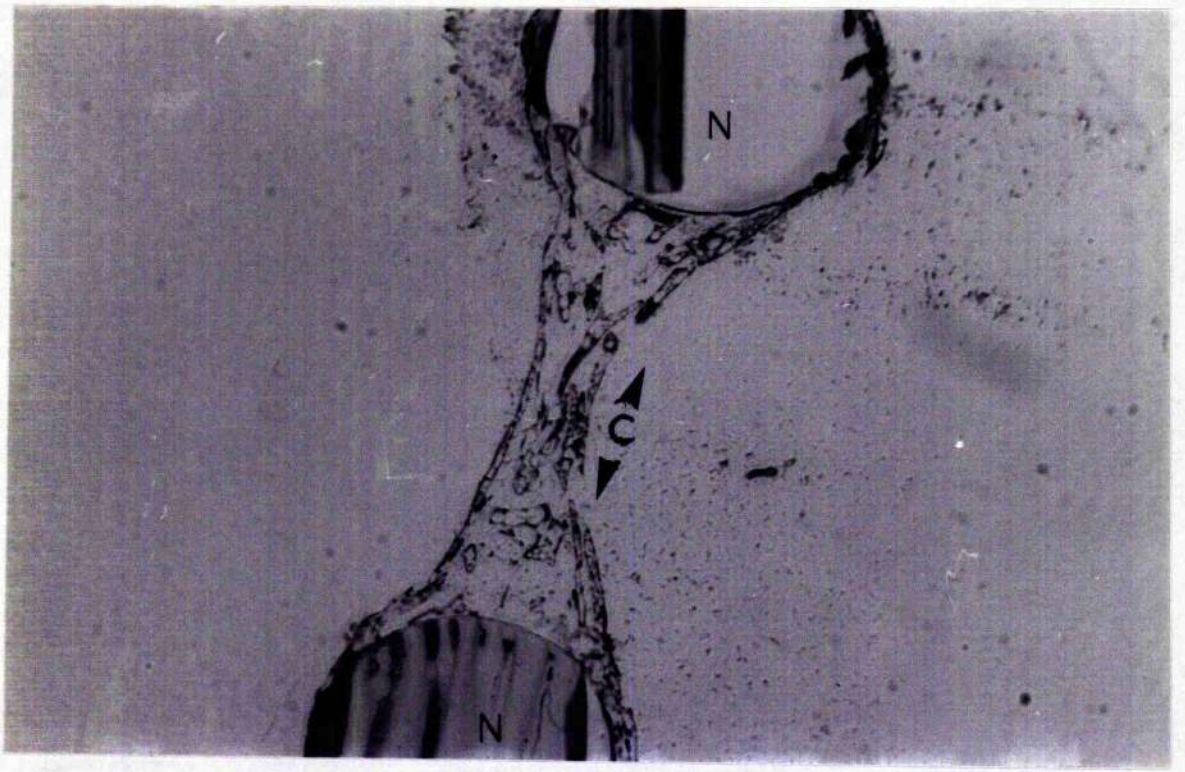
A new method has been devised to grow a wide variety of cell types on a number of different substrata relatively rapidly. This involves use of conventional cultures as starting material, preparing a cell suspension of 5×10^6 cells/ml from these and growing sail-sheets on metal meshes such as gold grids (page 43, plates 1, 6, 7) and carbon fibres (plate 5). Growing cells on the carbon mesh is of interest because of its use in tendon replacement therapy (Jenkins et al., 1977).

(b) Histological Studies :

(i) Optical Microscopy.

Cells in sail-sheets grow within the mesh holes and also on the mesh itself. The cells on the mesh grow as individuals or in small groups in monolayers.

Semi-thin sectioning and staining with Azure-II (plate 10) of a sail-sheet culture of chick heart fibroblasts grown on Nitex mesh, after filling the mesh holes, shows that the cells in this culture system form closely packed layers one cell thick at both sides with a few cells sandwiched between these two layers forming a multilayered structure.



50μm.

PLATE 10.

The plate shows a semi-thin (1.2 µm. thickness) vertical section of a sail-sheet culture (7 days age) of Chick heart fibroblasts growing between the fibres of the Nitex mesh. The cells have been transversely sectioned and appear to align parallel to each other and also, to the mesh fibre.

N = Nitex mesh

C = Cellular area

The mesh holes which are not completely filled with cells leave 'free space' at the centre which can be seen by looking at the cultures under light microscopy. Time-lapse filming shows that the cell sheets near the 'free space' may be one cell thick. These 'free spaces' can also be visualised histologically by sectioning the cultures transversely. But, sectioning through such areas was difficult because the sections tore and no good preparations were obtained.

Because the cells in sail-sheets are attached to each other only at the edges and since the sheets do not sag under gravity, it is possible that the cells are under tension.

Most cells in sail-sheets are supported by other cells at the edges only and, due to this, some gaps are left between them and also between the bilayer. Some of these gaps may be occupied by extra cellular matrices. The occurrence of gaps between the cells in cultures means that individual cells can find space to spread when sail-sheets are stretched using a piezo-electric ceramic probe. It is not known whether this affects their behaviour and the cell cycle.

When sail-sheet cultures are examined under a phase contrast microscope, some mesh holes are seen with only a few cells at the corners, others with some cells along the edges directed towards the corners and the remainder have enough cells at the corners and edges to give a roughly circular outline at the inner edge of the mesh holes. These three situations may represent some of the intermediate steps in the complete closure of mesh holes.

Optical Microscopy has also been used to study the behaviour and movement of CHFs and the effects of mechanical tension on the duration

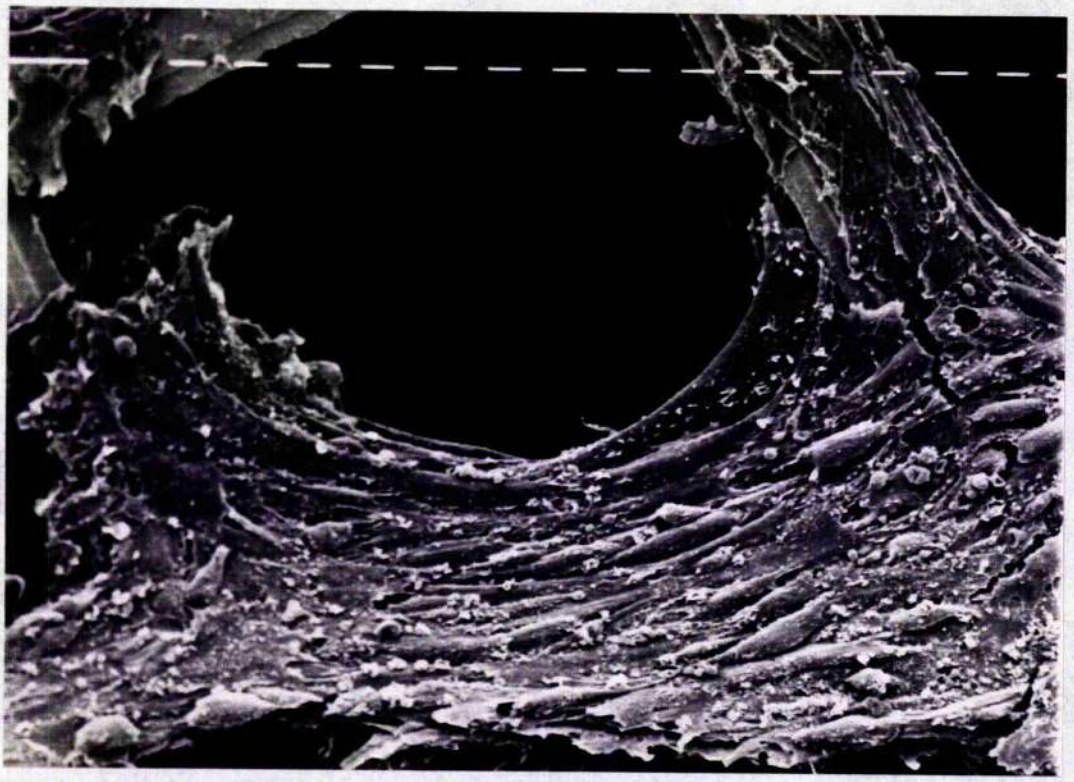
of the cell cycle. Results from these studies will be described in seperate sections (pages 65 - 75).

(ii) Electron Microscopy.

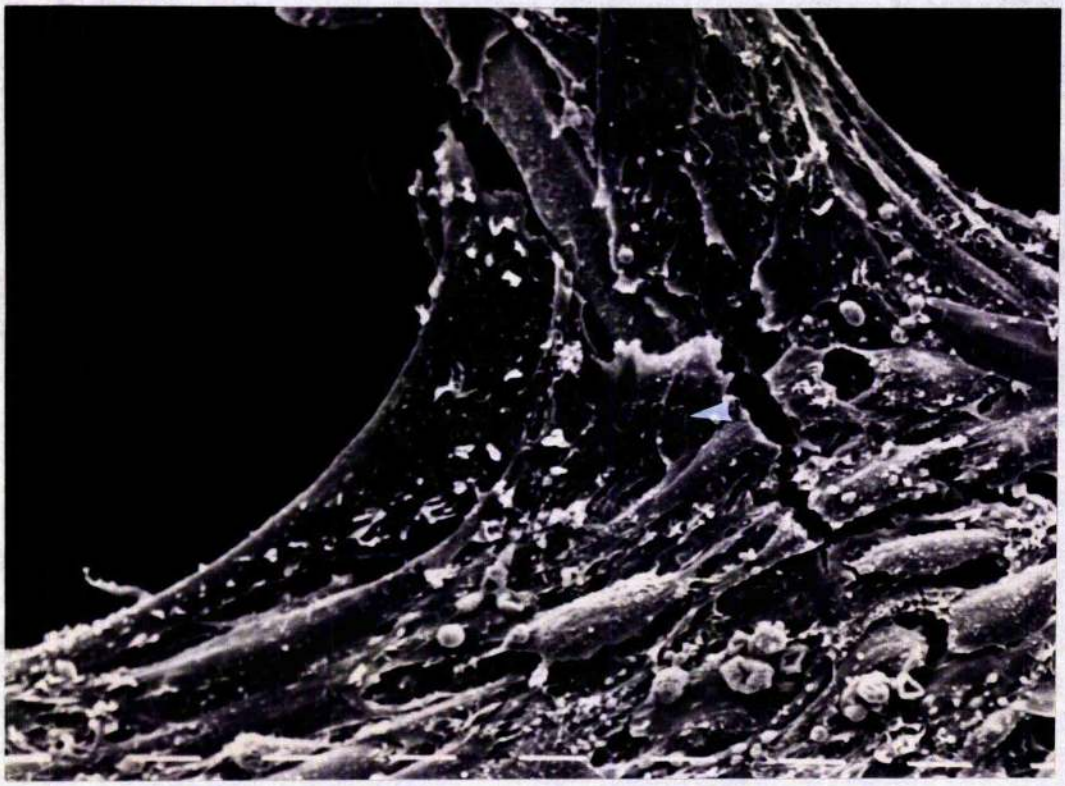
Scanning Electron Microscopic (SEM) studies of the primary cultures of Chick heart fibroblasts in sail-sheets show that most cells appear to be bipolar or spindle shaped (plates 11 a, 11 b). They are flattened and taper at both ends (plates 11 a, 11 b) and vary in their length (30-45 μm) and breadth (4-10 μm). Often, a few fan (f) shaped cells spanning the grid itself can be seen (plate 11 b).

Like optical microscopy, SEM studies carried out on the Chick heart fibroblasts grown in the form of sail-sheets on both gold grids (plates 11 a, 11 b, 12) as well as on Nitex meshes (plate 13) also show cells growing within the holes as well as on the perimeter of grids and meshes. In addition to this, in both types of cultures, cells exhibit a parallel alignment (parallel to the edge of the rectangular grids) within the grid (plates 11 a, 11 b, 12) and mesh (plate 13) holes. Some cells form bridges (plate 12) from one grid edge to another. At higher magnification, cells have been seen to have microprocesses (m) - see plates 11 b and 12.

Transmission Electron Microscopic (TEM) studies show the morphological appearance of Chick heart fibroblasts in sail-sheet cultures grown on Nitex mesh (plate 14). These cells show microprocess-like structures (m) emerging out from the cells (plate 14). Two characteristic features of fibroblasts can be noted : there seems to be high nuclear to cytoplasmic ratio and an abundance of granular endoplasmic reticulum (ger). This is based upon qualitative observation



10 μm



10 μm

PLATE 11.

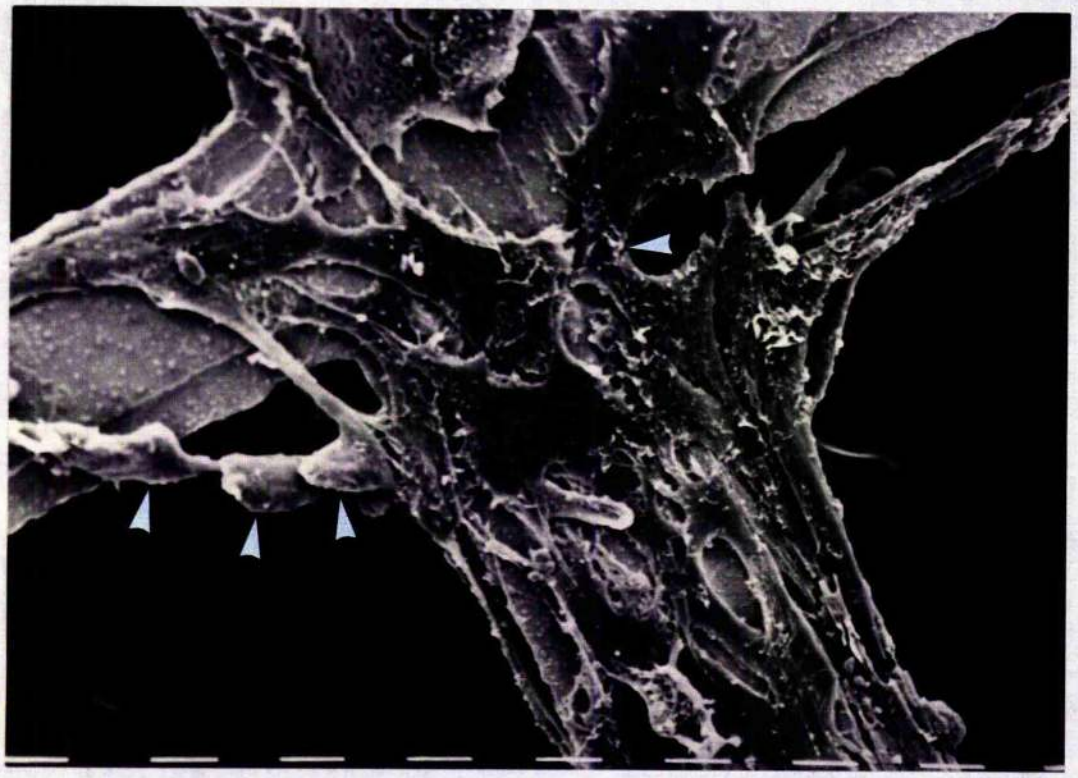
Scanning electron micrographs showing a sail sheet culture (7 days age) of chick heart fibroblasts on a gold grid with square hole shape. Most cells appear to be bipolar (spindle shaped) except a few which are fan shaped. Cells are present at the periphery of the grid as well as within grid holes.

f = a fan shaped cell.

m = microprocesses. Mesh size 200 X 200 μm^2.

(a) shows low power view showing infilling of the cells within a grid hole. The parallel alignment (parallel to the edge of the grid) of cells within the grid hole can be noted.

(b) A higher power view of the same (as above) showing infilling of the grid hole and elongation of cells showing clear microprocesses. One large fan shaped cell is seen spanning a corner of the grid.



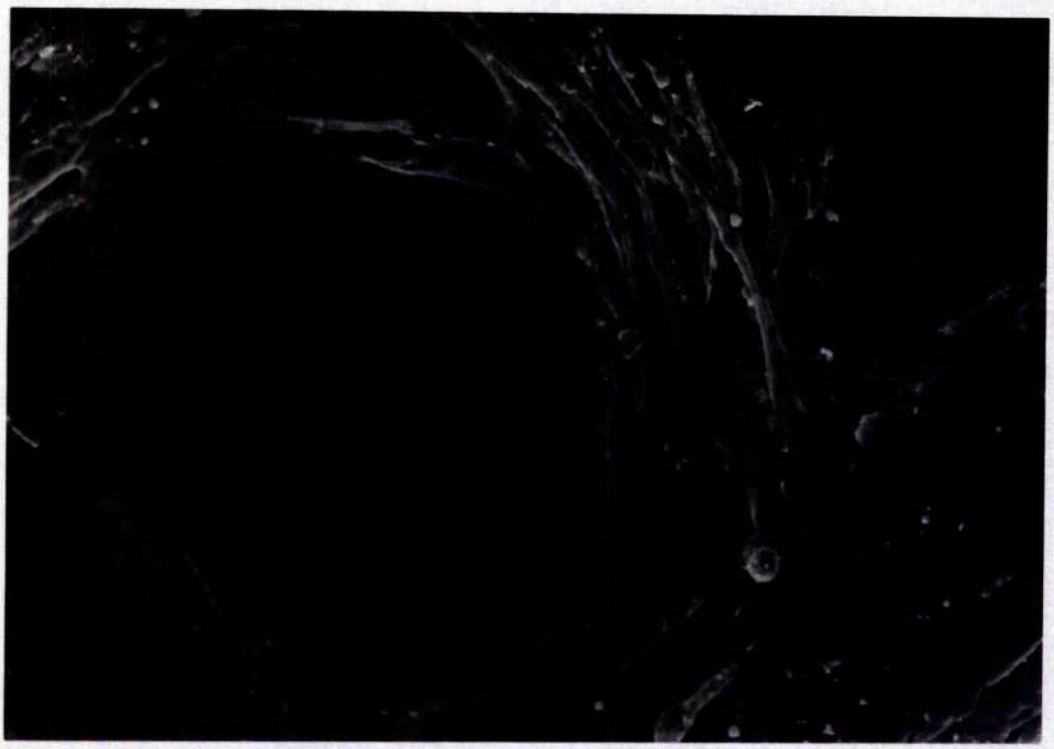
10 μm

PLATE 12.

Scanning electron micrograph showing high power view of a sail-sheet culture (7 days age) of Chick heart fibroblasts growing on a gold grid and beginning to fill the corners. Pointing white arrows are cells showing bridging effect.

m = microprocesses

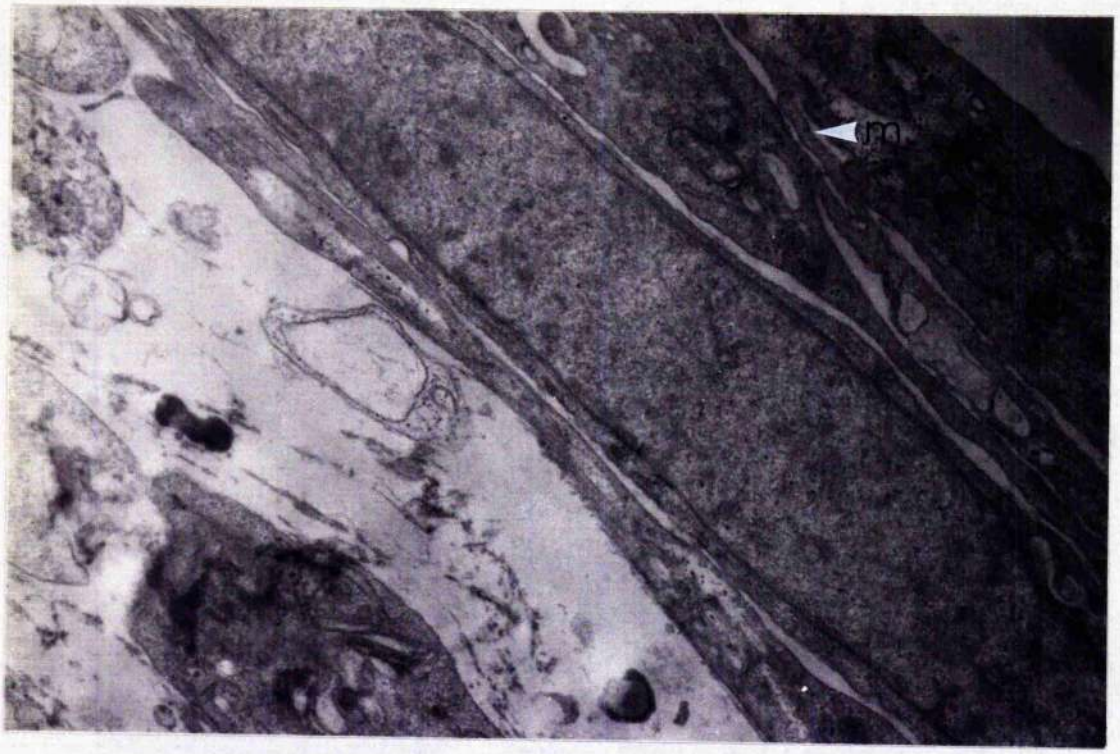
Mesh size 200 X 200 μm^2 .



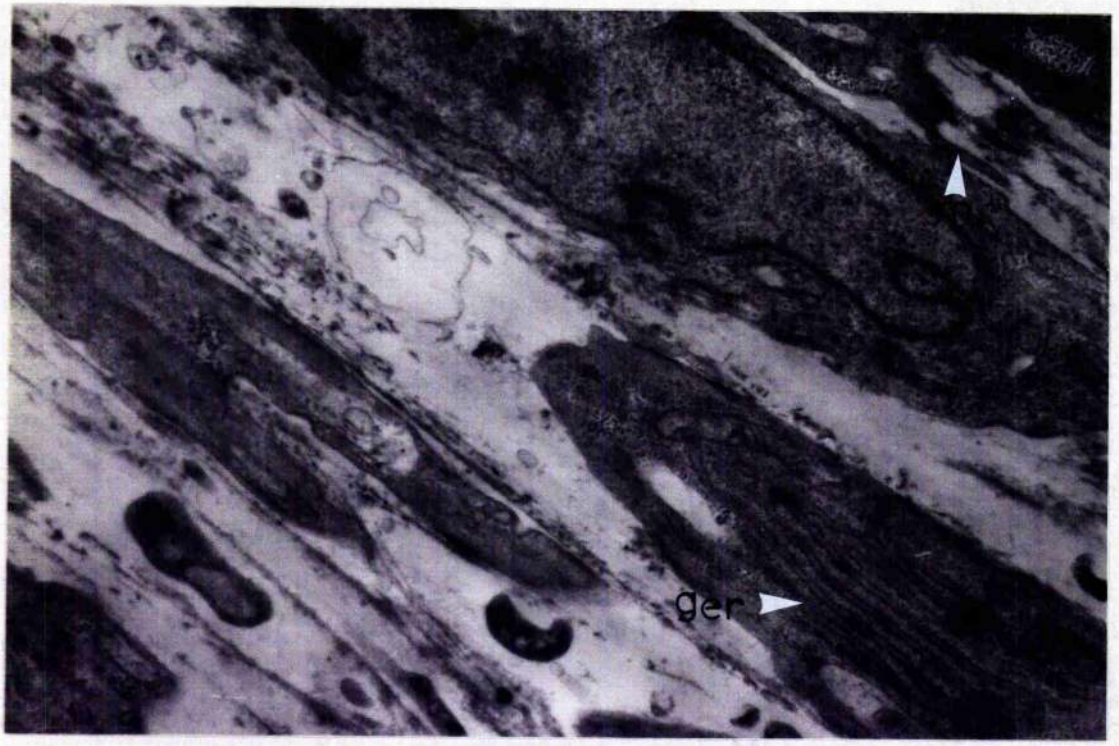
10 μm.

PLATE 13.

Scanning electron micrograph of a sail-sheet culture (7 days age) from Chick heart fibroblasts grown on Nitex mesh showing cells within the mesh hole and also on the perimeter of the mesh. Mesh size 185 X 166.5 μm^{-2}.



1 µm.



1 µm.

PLATE 14.

Transmission electron micrographs of sail-sheet cultures (7 days age) of Chick heart fibroblasts grown on Nitex mesh. Cells showing microprocesses in both the micrographs (a and b). Mesh size 200 X 200 μm^2.

m = microprocesses ger = granular endoplasmic reticulum.

(a) Cells showing high nuclear to cytoplasmic ratio, a characteristic feature of fibroblasts.

(b) Cells showing abundance of granular endoplasmic reticulum another feature representative of fibroblasts.

only. More work is essential in order to get quantitative data. Collagen fibres (C) in transverse and longitudinal sections can be seen between the cellular layers of the bi- or multilayered cultures (plate 15). These sections were made between the edges where the cells are bi- or multilayered. More TEM work is needed to find out whether the collagen is formed at the edge of the outgrowth and when is the collagen actually formed between the cells in sail-sheets.

(c) Effects of Tension on Cellular Morphology.

Results from SEM studies carried out on tensed (for 12 hours using 1.0 Hz. frequency) and control (untensed) sail-sheet cultures of Chick heart fibroblasts (plate 16) appear to show that tension may reduce the size and number of microprocesses (table 1).

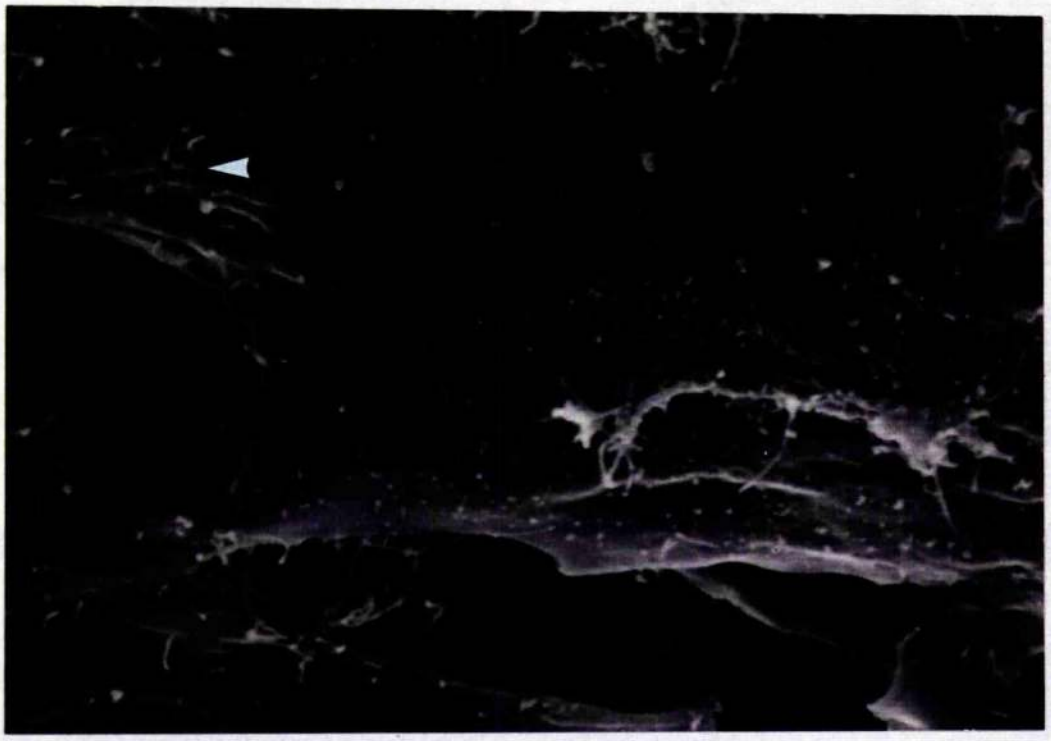
The mechanical stretching of sail-sheets by the probe deforms the Nitex mesh itself and this deformation is transmitted to the cell sheets. The linear deformation of a sheet is in the range of 3.3-13.3 μm. (average deflection = 9.4 μm. ; standard deviation ± 3.3 μm. ; mesh size = 185 X 166.5 μm^2). The percentage deformation of a sheet lengthwise is in the range of 1.8% - 7.2% and breadthwise 2.0% - 7.9% (average = 5.6%), deformation per unit (10000 μm^2) area being 3.1 μm^2. These values were measured with a light microscope using a graticule.

1 μm.

PLATE 15.

The plate shows a transmission electron micrograph of 7 days old sail-sheet culture (of Chick heart fibroblasts grown on Nitex mesh) overlying collagen. Mesh Size 200 X 200 μm^2.

C = Collagen fibres in longitudinal and transverse sections.



10μm.

10μm.

PLATE 16.

Scanning electron micrographs showing an experimental (tensed) and a control (untensed) sail-sheet culture (7 days age) from Chick heart fibroblasts grown on Nitex mesh.
Mesh size 185 X 166.5 μm^2.

(a) Tensed- showing membranes, smooth in appearance and with microprocesses which appear to be shorter and fewer in number. Tensed for 12 hours (1.0 Hz.).

(b) Control (untensed)- showing membranes with microprocesses which appear to be larger and more in number (than tensed cultures).

TABLE 1.

EFFECTS OF REPETITIVE TENSING (FREQUENCY = 1.0 HZ. ; DURATION = 12 HOURS) OF SAIL-SHEETS ON SIZE AND NUMBER OF MICROPROCESSES IN CHICK HEART FIBROBLASTS.

Type of Culture	Size of Microprocesses* Mean Length µms.	(S.D.)	No. of Microprocesses Per Unit ($10000\mu m^2$.) Cellular Area	Repetitions
Tensed (Exptl.)	5.9	(3.0)	47	2
Untensed (Control)	9.5	(6.2)	89	2

Tensed cultures compared with untensed cultures by t tests :

$t = 5.4$ $p < 0.001$

* Total number of microprocesses measured : 96 for tensed (experimental) and 178 for untensed (control) cultures.

[B] CELL BEHAVIOUR AND MOVEMENT

This sub-section describes some features of the behaviour of fibroblasts in sail-sheets. A comparison of the features of movement of fibroblasts in sail-sheets and conventional cultures will be addressed.

The conventional cultures used for this study were monolayers. The sail-sheets were one cell thick when mesh holes were not completely filled with cells. The sail-sheets tend to become multilayered once the mesh holes are filled with cells (see page 61). Conventional cultures are significantly denser than sail-sheets (table 2) but the difference is not large.

This sub-section can be divided in to following parts :

(a) Behaviour of Fibroblasts in Sail-Sheets.

(b) Movement of Fibroblasts in Sail-Sheets and Conventional Cultures.

(a) Behaviour of Fibroblasts in Sail-Sheets.

Cells in sail-sheet cultures move as individuals (plate 17 a-d) and as groups (plate 18), there being a general centripetal closing of the mesh hole from the edges inwards (figure 2). The main features of movement are :

(a) Cells from all sides of the explant move along the fibres towards the mesh holes ; movement along the fibres proceeds to a considerable distance before cells begin to span the corners of the mesh hole. (figure 2 a, 2 b).

(b) Once there is a sufficient number of cells at the corners, cells move from the fibres along the edges of cells already in the corners

TABLE 2.

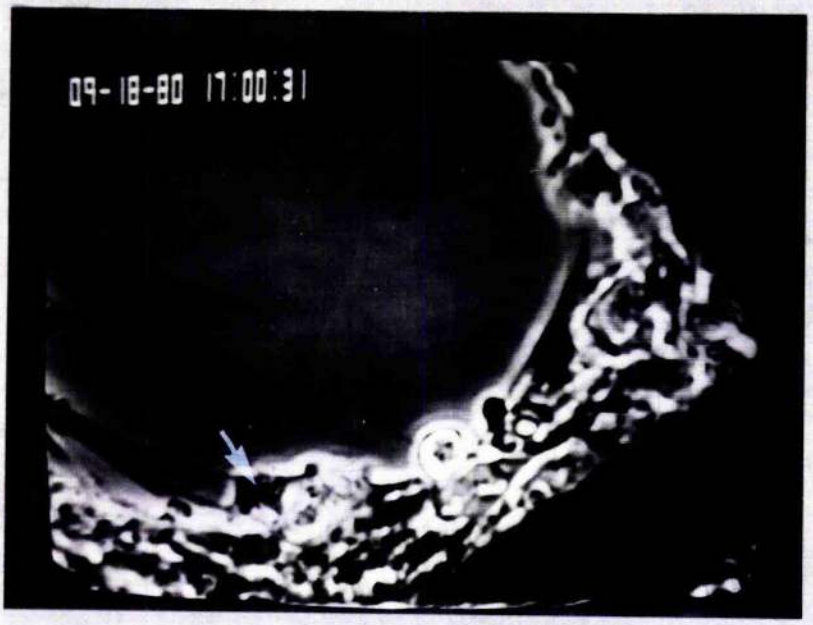
DENSITY OF CHICK HEART FIBROBLASTS IN CONVENTIONAL AND SAIL-SHEET CULTURES.

Culture Type	Cell Density per 10000 μm^2 Area		Repetitions
	Number of Cells	(S.D.)	
Conventional	19.0	(1.7)	10
Sail-sheets	16.5	(1.4)	11

t test :

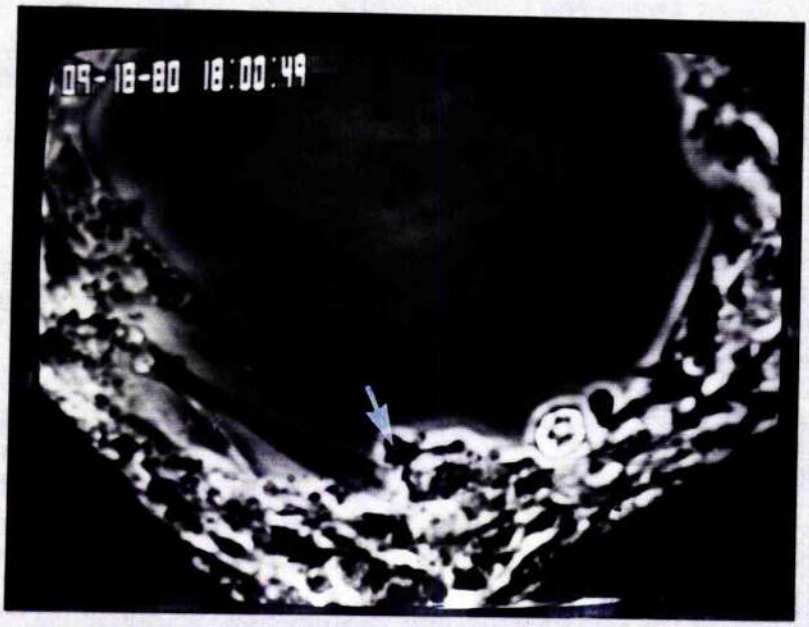
(Conventional cultures compared with sail-sheets)

t value	probability
3.7	p = 0.003



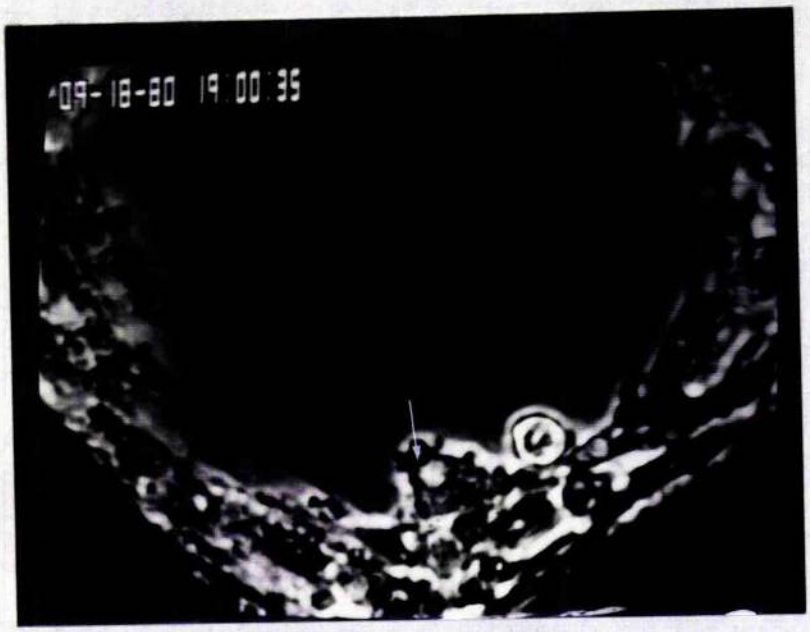
a

22 μm



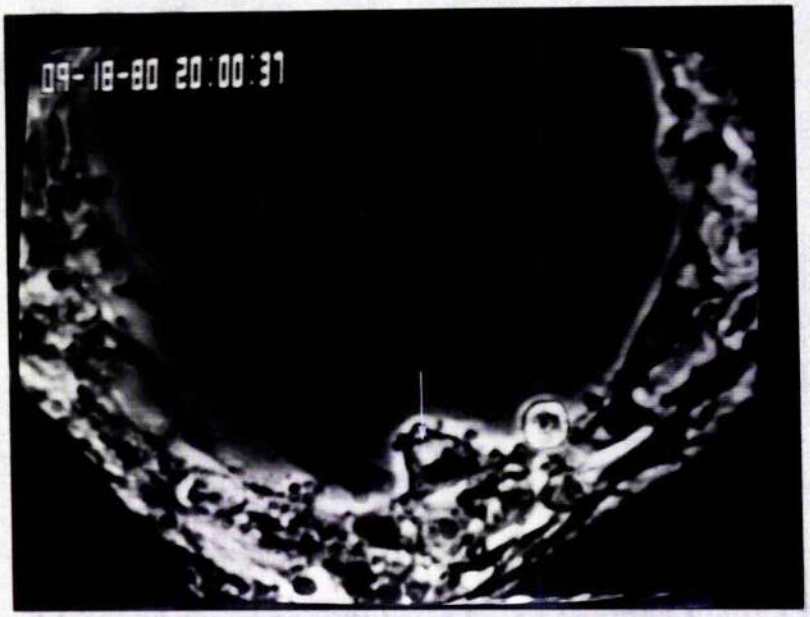
b

22 μm



c

22 μm



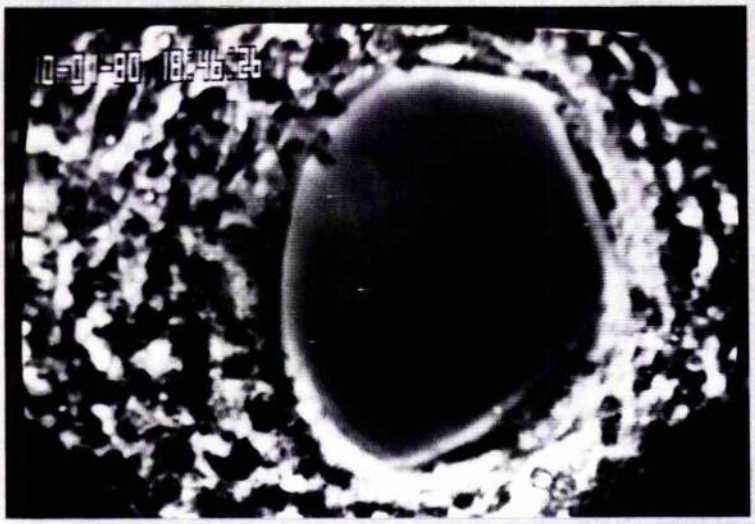
d

22 μm

PLATE 17.

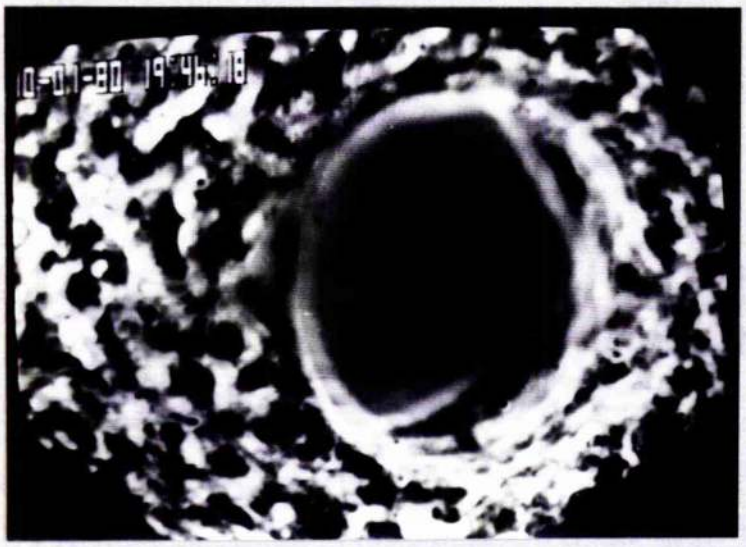
A series of views taken from a time-lapse video of a sail-sheet culture (7 days age) of Chick heart fibroblasts grown on a gold grid.

The photomicrographs from a - d show movement of an individual cell as pointed out by a white arrow mark. These pictures are taken directly from time-lapse screen. Time interval from a - d : 3 hours. Mesh size $200 \times 200\ \mu m^2$. Distance travelled by this cell/hour = 18.3 µm.



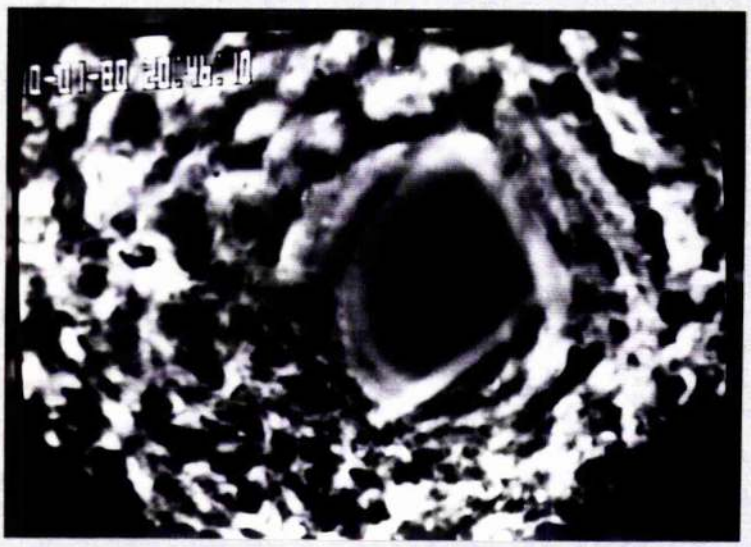
a

22μm.



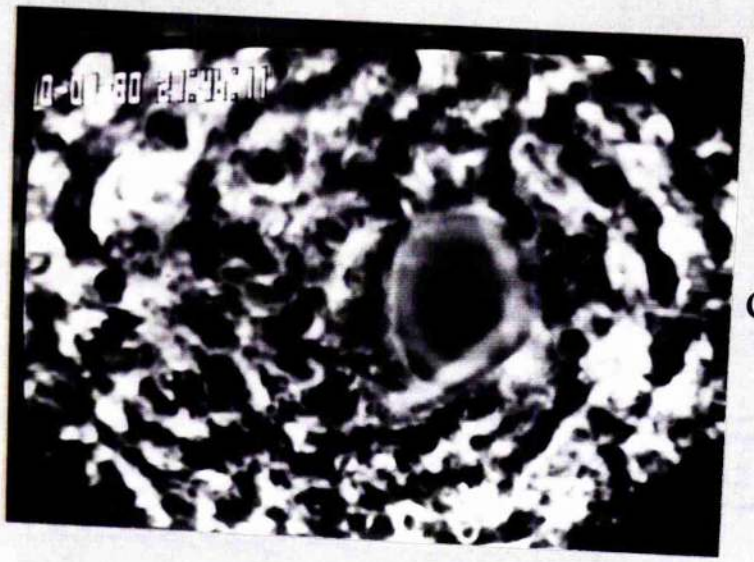
b

22μm.



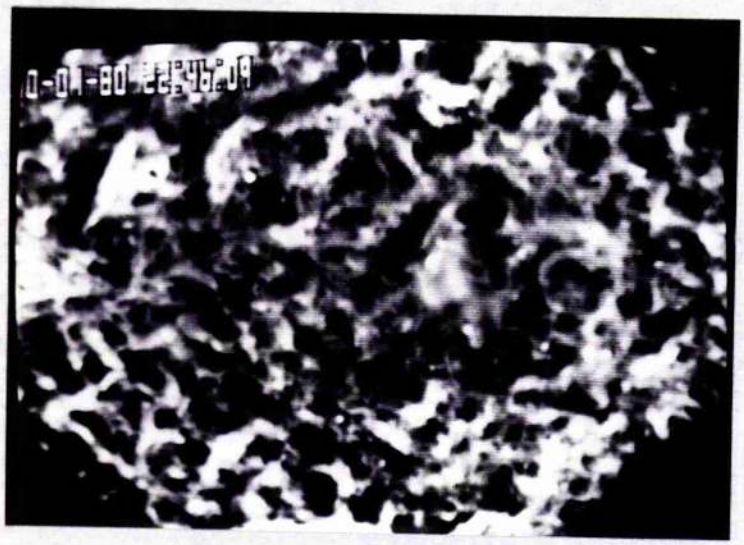
c

22μm.



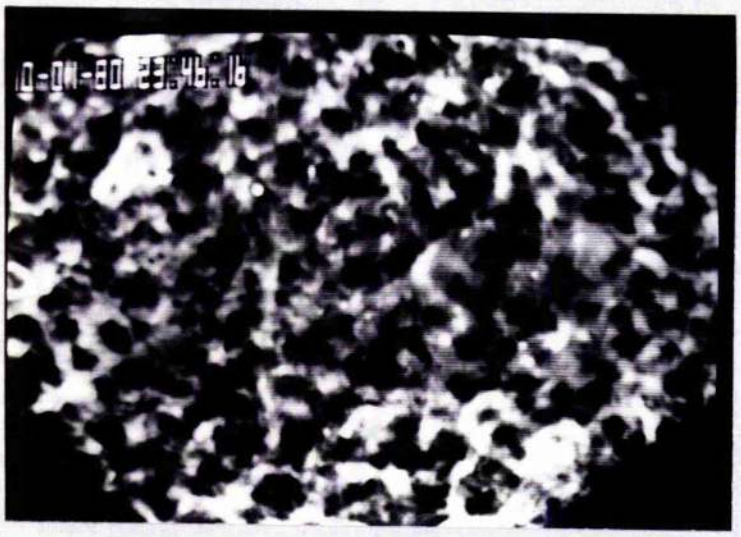
d

22 µm.



e

22µm.



f

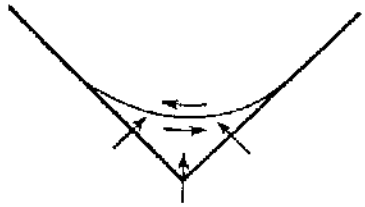
22µm.

PLATE 18.

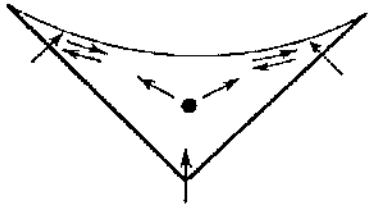
A series of views taken from a time-lapse video of a sail-sheet culture (7 days age) of Chick heart fibroblasts grown on a Nitex mesh. The photomicrographs show movement of whole sheet leading to the closure of the mesh hole. These pictures are taken directly from time-lapse screen. Time interval from a - e = 5 hours. Mesh size 185 X 166.5 μm^2.

Rate of closure of mesh hole area = 326.9 μm^2./hour.

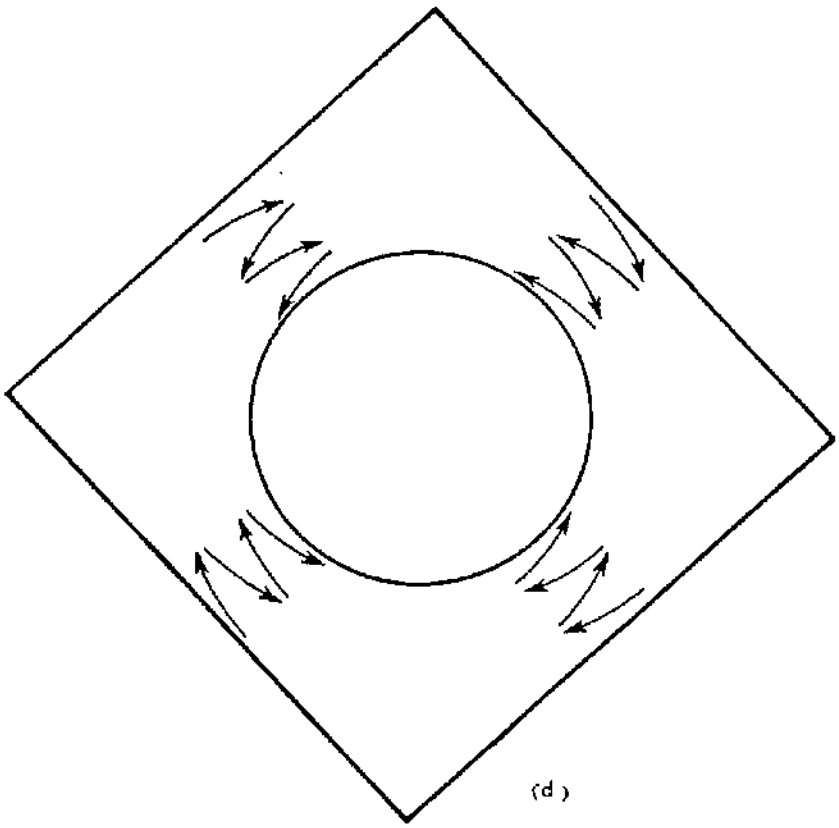
(a)



(b)



(c)



(d)

FIGURE 2.

The figure summarises observations on the movement of chick heart fiboblasts (7 days old culture) into sheets and in the sheets:

(a) Cells move from explant to the corners of the mesh holes and accumulate there.

(b) Cells are continuously added to the corners, their movement being restricted there.

(c) Once a sufficient number of cells has accumulated at the centre, they move from the corners along the edges. Some cells divide (as shown by a black circle) before moving.

(d) Due to continuous addition of cells, inner free edge of the mesh hole takes up a circular shape. Cells now show shuttling movement.

i.e. from densely populated areas towards the free edge (figure 2 c) while other cells move off the fibres into the corners. Cells coming from opposite directions along fibres often collide.

(c) Some cells divide, after which they tend to move towards the free edge. Cells from corners and inner edges of the grid hole continue their movement towards the free edge (figure 2 c).

(d) Once enough cells have accumulated at the corners, they begin to move towards the free edge equally. This results in the formation of a circular empty free space in the centre (figure 2 d ; plate 4) and the cells begin to show (figure 2 d) 'shuttling' (see page 67).

In sail-sheet cultures, cells move from all sides (i.e. corners and edges) towards the centre to bring about the closure of the mesh holes. The results show (table 3) that in both the sail-sheet culture types i.e. those grown on gold grids and on Nitex mesh, there is no significant difference (at 2.5% level of significance) in the speed of closure of mesh holes before and after the free inner edge of these cultures attains a circular shape. On closure of the hole, there is still considerable cellular activity (ruffling) for some time which decreases gradually. Thus, cells in sail-sheet cultures appear to behave differently to those in conventional cultures where, once a confluent monolayer is formed, substantial inhibition of the cellular activity (upper surface) takes place.

There is no significant difference (at 2.5% level of significance) between the two culture types in their speed of closure of mesh holes before the free inner edge attains a circular shape (table 3). Once the free inner edge of the cultures takes up a circular shape, the rate of closure of mesh holes becomes significantly higher (at 2.5% level of

TABLE 3.

DATA ON THE CLOSURE OF MESH HOLES IN SAIL-SHEET CULTURES OF CHICK HEART FIBROBLASTS.

Mesh Type	Rate of movement of the free inner edge of the sheet before and after attaining circular shape by the - cellular area within the mesh holes					
	(a) before			(b) after		
	Mean Rate of Change in Area μm^2 ./hr.	(S.D.)	Repetitions	Mean Rate of Change in area μm^2 ./hr.	(S.D.)	Repetitions
Gold grid	221.7	(56.8)	4	323.3	(65.7)	4
Nitex mesh	582.2	(307.3)	4	645.1	(191.0)	4

t tests

	t value	probability	
1. (a) compared with (b) :			
Gold grid	2.4	p = 0.05	*
Nitex mesh	0.4	p > 0.5	*
2. Gold grid cultures- compared with Nitex mesh :			
(a) before	2.4	p = 0.05	*
(b) after	3.4	p = 0.015	

* Non-significant at 2.5 % level of significance.

significance) in Nitex mesh cultures than that in gold grid cultures.

The cells in sail-sheets show 'shuttling' (lateral movement) rather than inhibition of individual cell movement. However, not all the cells shuttle simultaneously. 'Shuttling' may be partly responsible for the tearing of sheets. The tearing produced by whole sheets appears to support the idea that cells in sail-sheets may be under tension. In almost all cases studied by time-lapse filming, tearing appears to be associated with individual cell movement. Quite often, tearing of the sheets can be associated with collision of cells moving in opposite direction. Sometimes, while both its lateral ends trying to move in opposite direction, an individual cell breaks itself in to two different parts and these parts move along opposite edges of the mesh. A few cells in the culture round up, detach from the sail-sheets and fall off the outgrowth in to the culture dish. Quite frequently, a rapid movement of these spherical cells can be seen prior to their detachment. These may be dividing cells.

(b) Movement of Fibroblasts in Sail-Sheets and Conventional Cultures.

Chick heart fibroblasts (CHFs) in sail-sheets move as individuals at varying speeds in irregular paths (figure 3). The whole sheets move centripetally to close the mesh holes. A comparative account of some features of movement of CHFs in sail-sheets and conventional cultures is presented in table 4. This table shows that the values of net displacement (speed of movement), fluctuation (number of times the leading edge changes its advancement forwards and backwards with a period of standstill over a period of one hour), length of formation and withdrawal of pseudopodial protrusions, % of time cells are in

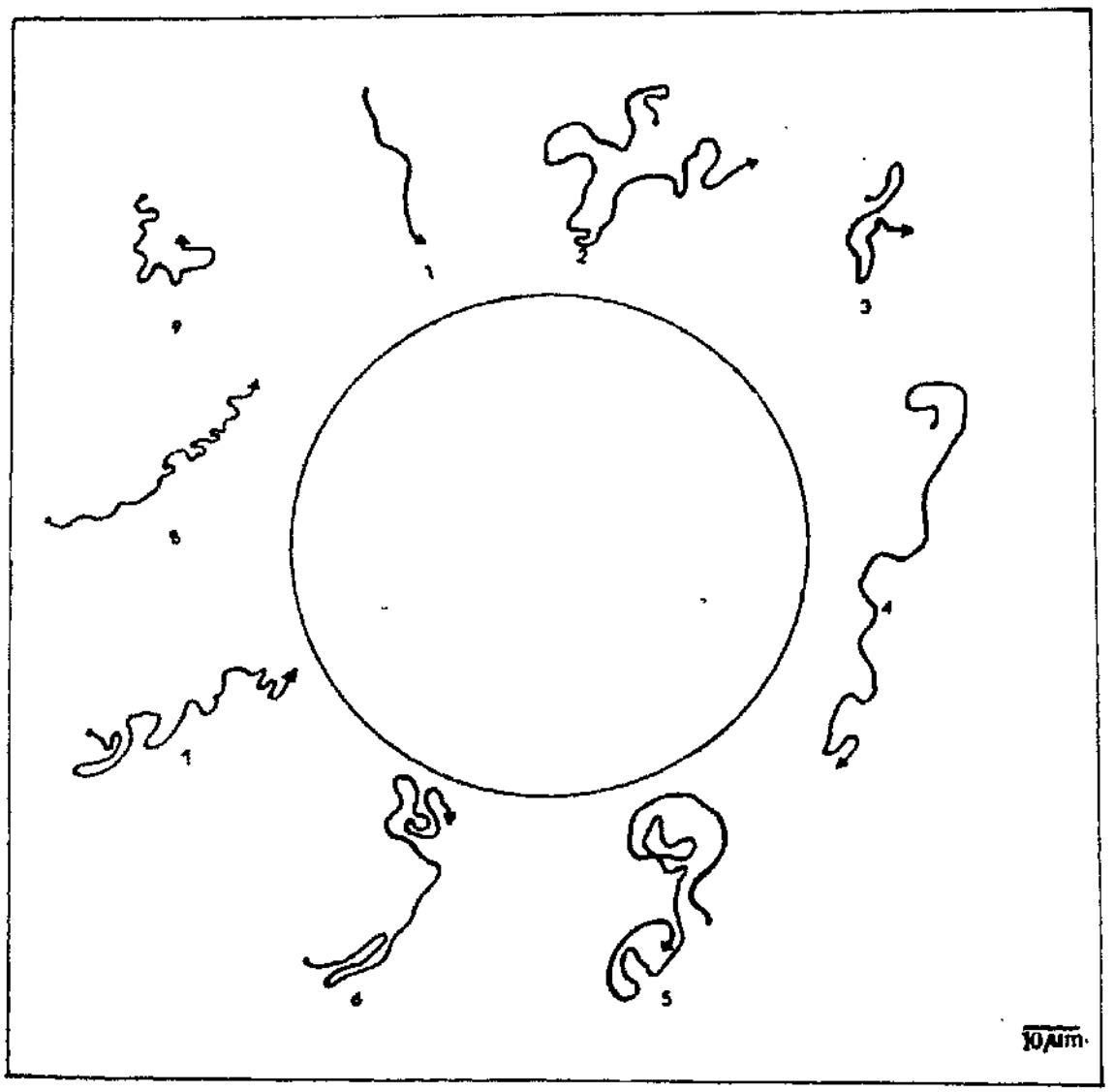

1
2
3
4
5
6
7
8
9
10 µm

FIGURE 3.

The figure shows movement of individual chick heart fibroblasts within one hole of a gold grid (size 200 X 200 μm^2) in 22 hours. Sail-sheet culture of 7 days age.

Diagram No.	Distance (µm.)	Time (Mins.)	Speed (µm/hour)
1	30.3	170	10.7
2	151.5	855	10.6
3	60.6	264	13.8
4	130.3	645	12.1
5	157.6	77	122.8 *
6	106.1	756	8.4
7	130.3	243	32.2
8	60.6	1003	3.6 **
9	50.0	728	4.1

---

* Fastest moving cell in the field.

** Slowest moving cell in the field.

TABLE 4.

MEASUREMENTS ON THE MOVEMENT OF CHICK HEART FIBROBLASTS IN 'SAIL-SHEET' AND 'CONVENTIONAL CULTURES.

Features of cell Movement	Sail-sheet Culture (a) ** Mean (S.D.)	Conventional Culture (b) Mean (S.D.)	Data for (a) Compared with (b) t value	p
1.Net displacement (µm./hr.)				
a) Individual Cells	15.2 (8.5)***	30.4 (17.2)	5.0	p < 0.001
b) Whole sheet	5.0 (3.9)			
2.Fluctuations/hr.	18.0 (6.0)	17.0 (5.0)	0.4	p > 0.5 *
3.Protrusion Distance (µms.)	4.1 (1.8)	7.5 (1.9)	4.1	p < 0.001
4.Withdrawal Distance (µms.)	3.9 (1.7)	5.0 (1.1)	1.7	p = 0.1 *
5.% of Time Cells Stationary	33.0 (11.3)	34.7 (12.1)	0.3	p > 0.5 *
6.% of Time Cells Moving Forward	35.6 (16.8)	42.2 (12.5)	1.0	p = 0.3 *
7.% of Time Cells Moving backward	31.4 (10.1)	23.1 (13.2)	1.6	p = 0.18*
8.Speed While Moving, Protrusion (µm./min.)	1.2 (0.5)	1.8 (0.6)	2.4	p = 0.025
9.Speed While Moving, Withdrawal (µm./min.)	1.0 (0.4)	1.8 (0.6)	3.5	p = 0.003

* Non-significant at 2.5% level of significance. ** Number of observations : 10 each for both sail-sheet and conventional cultures except in case of feature 1 (a) where the number of observations is - sail-sheet = 33 cells, conventional = 275 cells while in 1 (b) the number of frames examined = 101. *** This data excludes one single observation in which an individual cell moved at a speed of 122.8 µm./hour.

stationary and motile (both forward and backward movements) phases and the speed of formation of pseudopodial protrusions and their withdrawals during cell movement, obtained from all measurements taken on the movement of CHFs in sail-sheet cultures are generally lower than those on conventional cultures. Significant differences in values between sail-sheet and conventional cultures have been measured for speed of cell movement (net displacement), speed of formation of pseudopodial protrusions and their withdrawals during cell movement and length of pseudopodial protrusions.

The mean speed of cell movement (= net displacement) in sail-sheet cultures has been found to be significantly lower than in conventional cultures (table 4). The rate of movement of the free inner edge of the sheet is lower than that of the individual cells. The average protrusion and withdrawal speeds in the mobile phase of cells in sail-sheets is significantly lower than that of conventional cultures. The mean length of pseudopodial protrusions is also significantly lower in sail-sheets than in conventional cultures. However, data on the means of % time spent by cells in sail-sheet cultures in stationary and mobile phases (for both the forward as well as backward movements) is not significantly different from that in conventional cultures. Similarly, data obtained for withdrawal distance from cells in sail-sheets is also not significantly different from that in conventional cultures although data for protrusion distance shows significant difference between the sail-sheet and conventional cultures. The average protrusion distance covered by the cells is significantly shorter in sail-sheets than that in conventional cultures.

[C] THE CELL CYCLE

This sub-section describes results on the measurement of duration of the cell cycle of chick heart fibroblasts in sail-sheets and the effects of mechanical tension on the cell cycle.

In order to investigate the effect of mechanical tension on the cell cycle, the sail-sheets of fibroblasts were labelled with [^{3}H]-thymidine for the first and the final hour during the actual periods of tensing (3-12 hours). The cultures were labelled for the first and the final hour of tensing in order to find out how long a period of tensing is necessary before labelling for effects to appear.

This sub-section can be divided in to following parts :

(a) Duration of Cell Cycle of Fibroblasts in Sail-Sheets.

(b) Effect of Mechanical Tension on the Proportion of Labelled Cells.

(c) Effect of Mechanical Tension on the Positional Localisation of the Proportion of Labelled Cells :

  (i) Comparision between the Experimental (Tensed) and Control (Untensed) Cultures.

  (ii) Comparision between the Various Individual Positions Within Experimental (Tensed) and Control (Untensed) Cultures.

(a) Duration of Cell Cycle of Fibroblasts in Sail-Sheets.

The duration of the cell cycle for chick heart fibroblasts, grown as sail-sheets, was determined using the method described on pages 49-51. In essence, this method consisted of measuring total DNA from cell

samples at various time intervals. In order to measure the number of cells, cultures were [^{3}H]-thymidine labelled at the start of the experiment and the ratio DNA/[^{3}H]-thymidine incorporation was used as a measure of replication. As DNA doubles due to cells passing through the cycle, the ratio rises. Results from these studies show (figure 4) an eight-fold increment in the DNA/[^{3}H]-thymidine incorporation counts ratio in 33 hours time. Therefore, the time taken in a two-fold increase in this ratio (being equal to about one DNA doubling) would be 11 hours and hence, the duration of the cell cycle of Chick heart fibroblasts grown as sail-sheets may be about 11 hours. Data on the proportion of labelled cells (after labelling cells for the first hour, giving a cold chase for one hour and continuing up to 12 hours including the period of label and cold chase) shows (table 5) a significant difference between 3 and 12 hour cultures (at 2.5 % level of significance) and about two-fold increase during this time interval suggesting thereby that the duration from S phase to early G_1 in these cultures is less than 12 hours. This suggests that G_1 is short.

(b) Effect of Mechanical Tension on the Proportion of Labelled Cells.

Autoradiographic studies (tables 6-11 ; figure 5) show that the application of 0.01 Hz tension increases the proportion of labelled cells in all cases. Using 0.1 Hz tension, the proportion of labelled cells is increased in all cases except in 12 hours tensed and first hour labelled cultures. 1.0 Hz tension increases the proportion of labelled cells in most cases except 3 hours tensed and final hour labelled cultures.

Data repeating Curtis and Seehar's (1978) experiments are presented

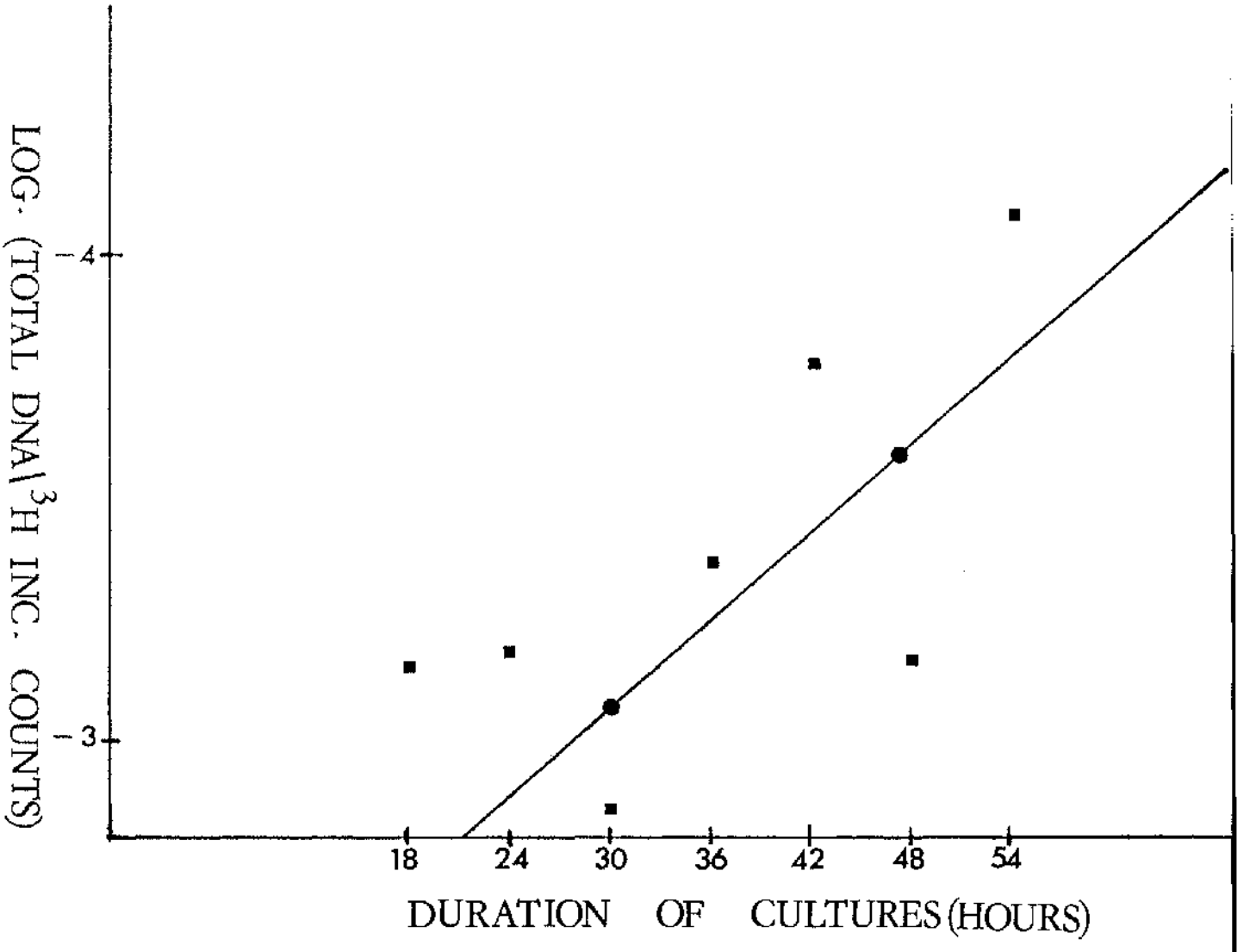

LOG. (TOTAL DNA\$^3$H INC. COUNTS)
4
3
18
24
30
36
42
48
54
DURATION OF CULTURES (HOURS)

FIGURE 4.

Graph showing the total DNA per unit [^{3}H] thymidine incorporation and the duration (hours) of culturing sail-sheet cultures (7 days age). Curve fitted by linear regression analysis (Regression Co-efficient 0.026). Data tested for the significance of regression co-efficient (being different from zero).

TABLE 5.

DISTRIBUTION OF % FREQUENCY OF LABELLED CELLS IN THE SAIL-SHEET CULTURES OF CHICK HEART FIBROBLASTS AFTER 1 HOUR PULSE LABELLING AND CULTURING FOR 3-12 HOURS. **

Duration of Culturing (Hours)	% Frequency of Labelled Cells Mean	(S.D.)	Repetitions
3	13.8	(1.8)	15
6	15.3	(3.6)	15
12	24.2	(6.2)	15

Comparisions (t tests)	t values	Probability
3 Hrs. with 6 Hrs.	1.6	$p = 0.25$ *
6 Hrs. with 12 Hrs.	4.8	$p < 0.001$
3 Hrs. with 12 Hrs.	6.2	$p < 0.001$

* Non-significant at 2.5 % level of significance.

** Data reproduced from the controls in tables 6, 8 and 10.

TABLE 6.

EFFECTS OF REPETITIVE TENSING OF SAIL-SHEETS ON % FREQUENCY OF LABELLED CELLS.

(i) Duration of tension 3 hours with first hour of labelling.

Frequency (Hz.)	Mean % Labelled Cells (S.D.)	t-value compared with Control (Probability, p) *	Repetitions
Experiment			
0.01	22.0 (2.3)	10.7 ($p < 0.001$)	14
0.1	28.3 (6.0)	9.0 ($p < 0.001$)	16
1.0	24.8 (4.0)	9.6 ($p < 0.001$)	13
Control			
0.0	13.8 (1.8)		15
1 Curtis & Seehar (1978)			
Experiment			
0.25	21.4 (5.1)		
0.50	22.5 (5.6)		
1.00	15.3 (2.5)		
Control			
0.00	14.7 (2.2) **		
0.00	14.3 (2.2) ***		

1. Curtis & Seehar (1978) pulse labelled and tensed their cultures for 1 hr. using 2µCi ml^{-1} [^{3}H] thymidine. * See table 12 for difference in effects of different frequencies. ** Probe not inserted. *** Probe inserted.

TABLE 7.

EFFECTS OF REPETITIVE TENSING OF SAIL-SHEETS ON % FREQUENCY OF LABELLED CELLS.

(ii) Duration of tension 3 hours with final hour labelling.

Frequency (Hz.)	Mean % Labelled Cells (S.D.)	t-value compared with Control (Probability, p) *	Repetitions
Experiment			
0.01	21.1 (5.6)	5.0 ($p < 0.001$)	15
0.1	26.5 (7.0)	5.1 ($p < 0.001$)	16
1.0	20.0 (4.1)	2.6 ($p = 0.017$)	15
Control			
0.0	16.1 (4.3)		16

* See table 12 for difference in effects of different frequencies.

TABLE 8.

EFFECTS OF REPETITIVE TENSING OF SAIL-SHEETS ON % FREQUENCY OF LABELLED CELLS.

(iii) Duration of tension 6 hours with first hour of labelling.

Frequency (Hz.)	Mean % Labelled Cells (S.D.)	t-value compared with Control (Probability, p) *	Repetitions
Experiment			
0.01	21.7 (4.7)	4.1 ($p < 0.001$)	13
0.1	21.1 (2.3)	5.3 ($p < 0.001$)	13
1.0	16.7 (3.3)	1.1 ($p = 0.3$) **	13
Control			
0.0	15.3 (3.6)		15

* See table 12 for difference in effects of different frequencies.

** Non-significant at 2.5 % level.

TABLE 9.

EFFECTS OF REPETITIVE TENSING OF SAIL-SHEETS ON % FREQUENCY OF LABELLED CELLS.

Duration of tension 6 hours with final hour labelling.

Frequency (Hz.)	Mean % Labelled Cells (S.D.)	t-value compared with Control (Probability, p) *	Repetitions
Experiment			
0.01	31.4 (7.2)	7.3 ($p < 0.001$)	16
0.1	24.5 (5.3)	5.0 ($p < 0.001$)	16
1.0	30.1 (6.3)	7.4 ($p < 0.001$)	16
Control			
0.0	16.1 (4.2)		16

* See table 12 for difference in effects of different frequencies.

TABLE 10.

EFFECTS OF REPETITIVE TENSING OF SAIL-SHEETS ON % FREQUENCY OF LABELLED CELLS.

(v) Duration of tension 12 hours with first hour of labelling.

Frequency (Hz.)	Mean % Labelled Cells (S.D.)	t-value compared with Control (Probability, p) *	Repetitions
Experiment			
0.01	40.4 (21.7)	2.8 (p = 0.01)	16
0.1	34.0 (16.5)	2.2 (p = 0.041) **	16
1.0	32.8 (16.8)	1.9 (p = 0.075) **	16
Control			
0.0	24.2 (6.2)		15

* See table 12 for difference in effects of different frequencies.

** Non-significant at 2.5 % level.

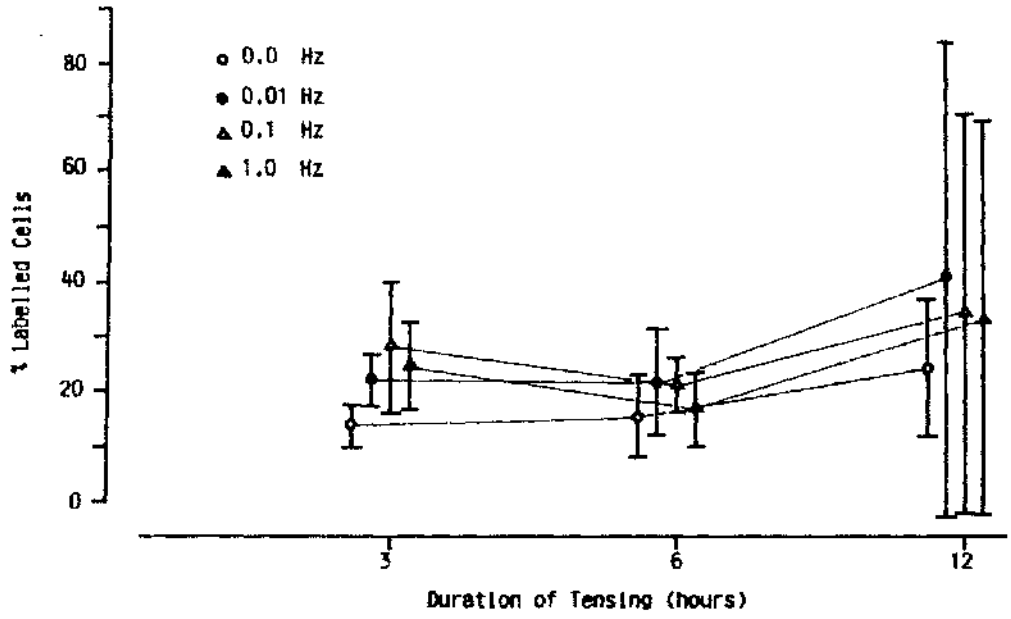
TABLE 11.

EFFECTS OF REPETITIVE TENSING OF SAIL-SHEETS ON % FREQUENCY OF LABELLED CELLS.

(vi) Duration of tension 12 hours with final hour labelling.

Frequency (Hz.)	Mean % Labelled Cells (S.D.)	t-value compared with Control (Probability, p) *	Repetitions
Experiment			
0.01	28.3 (6.3)	6.2 ($p < 0.001$)	16
0.1	36.6 (8.0)	8.9 ($p < 0.001$)	16
1.0	30.4 (6.4)	7.2 ($p < 0.001$)	16
Control			
0.0	16.3 (4.5)		16

* See table 12 for difference in effects of different frequencies.


o 0.0 Hz
● 0.01 Hz
▲ 0.1 Hz
▲ 1.0 Hz
% Labelled Cells
0
20
40
60
80
3
6
12
Duration of Tensing (hours)

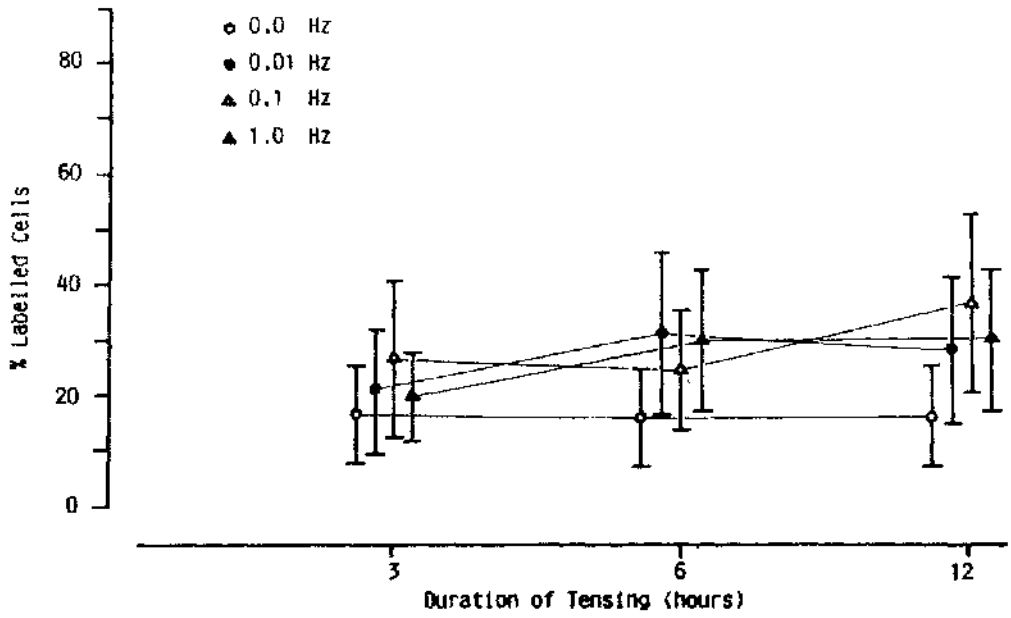

o 0.0 Hz
● 0.01 Hz
▲ 0.1 Hz
▲ 1.0 Hz
% Labelled Cells
0
20
40
60
80
3
6
12
Duration of Tensing (hours)

FIGURE 5.

(a) The figure shows how different frequencies of tension affect the proportion of [^{3}H]-thymidine labelled chick heart fibroblasts as the duration of tensing (with first hour labelling) is increased from 3 to 12 hours.

Bars represent ± 2 standard deviations.

(b) The figure shows how different frequencies of tension affect the proportion of [^{3}H]-thymidine labelled chick heart fibroblasts as the duration of tensing (with final hour labelling) is increased from 3 to 12 hours.

Bars represent ± 2 standard deviations.

in the table 6. Curtis and Seehar (1978) found an increment in the percentage frequency of labelled cells in their experimental (tensed) series (using 0.25, 0.5 and 1.0 Hz. frequencies and tensing for one hour with simultaneous labelling) as compared with their controls. Results from my experiments presented in the same table support their results.

The relative effects of various frequencies of stretching on the proportion of labelled cells was tested at 2.5% level of significance (table 12). The proportion of labelled cells increased in the following order :

Duration of Tension	First Hour Labelled Cultures	Final Hour Labelled Cultures
3 Hours	0.01 < 1.0 < 0.1	1.0 < 0.01 < 0.1 Hz
6 Hours	1.0 < 0.1 < 0.01	0.1 < 1.0 < 0.01 Hz
12 Hours	1.0 < 0.1 < 0.01	0.01 < 1.0 < 0.1 Hz

Although these results are not all significantly different from the controls (untensed cultures) at 2.5% level of significance, the highest proportion of labelled cells is always significantly different from the lowest proportion of labelled cells (obtained from the various frequencies of tension applied for a particular duration) except in the case of the 12 hours tensed and first hour labelled cultures. In this case, the standard deviations are much higher (see table 10). Though there is not a fully consistent story to be derived from these results, the highest proportion of labelled cells is always found at the two lower frequencies of tensing and overall there is a trend such that there is higher proportion of labelled cells at lower frequencies.

TABLE 12.

COMPARISION OF THE EFFECTS OF VARIOUS FREQUENCIES OF TENSING (USING t TEST) ON THE MEAN % LABELLED CELLS (DATA FROM TABLES 6-11).

Details of Comparision (Duration of Tensing)	Mean % Labelled Cells: First Hour Label t-value, Probability		Final Hour Label t-value, Probability	
0.01 Hz. with 0.1 Hz.				
(3 Hours)	3.7	$p < 0.001$	2.4	$p = 0.025$
(6 Hours)	0.4	$p > 0.5$ *	3.1	$p = 0.004$
(12 Hours)	0.9	$p = 0.36$ *	3.3	$p = 0.004$
0.1 Hz. with 1.0 Hz.				
(3 Hours)	1.8	$p = 0.083$*	3.1	$p = 0.004$
(6 Hours)	4.2	$p < 0.001$	2.7	$p = 0.011$
(12 Hours)	0.2	$p > 0.5$ *	2.4	$p = 0.022$
0.01 Hz. with 1.0 Hz.				
(3 Hours)	2.3	$p = 0.035$*	0.6	$p > 0.5$ *
(6 Hours)	3.1	$p = 0.004$	0.5	$p > 0.5$ *
(12 Hours)	1.1	$p = 0.278$*	0.9	$p > 0.5$ *

* Non-significant at 2.5 % level of significance.

Whether labelling for first or final hour is important is not clear.

(c) Effect of Tension on the Positional Localisation of the Proportion of Labelled Cells :

Simple engineering considerations (Peterson, 1974) suggest that stress gradients should be concentrated in the corners of the cell sheets. Thus, if tension affects the cell cycle, I would expect to find more cells being affected at the corners than at the edge or centre.

A method of investigating effects of mechanical tensions on cells in sail-sheets was developed by obtaining data on the distribution of the proportion of labelled cells at different parts in the culture for experimental (tensed) as well as control (untensed) cultures (see page 58-59). In a nutshell, this method consisted of recording of the proportion of labelled cells at various sites in sail-sheets in mesh holes (i.e. corner, edge and centre) and analysing the effects of various treatments on the positional localisation of the proportion of labelled cells by analysis of variance (tables 13-15 ; appendices 1-2, 4-5, 7-8) and testing the significance of data at 95 % confidence interval (appendices 3, 6, 9). These tension effects may be described as follows :

(i) Comparison between the Experimental (Tensed) and Control (Untensed) Cultures.

Results from analysis of variance (tables 13-15) on the distribution of the proportion of labelled cells at various parts (corner, edge and centre) in the experimental (tensed) cultures showed significant differences from those in controls (untensed cultures). The

TABLE 13.

ANALYSIS OF VARIANCE OF DATA FROM APPENDICES 1 & 2 ON EFFECTS OF REPETITIVE TENSING OF SAIL-SHEETS ON THE POSITIONAL LOCALISATION OF PROPORTION OF LABELLED CELLS (DURATION OF TENSING 3 HOURS).

Source of Comparision	Degree of freedom, df		SS	Mean Square	F
Blocks	(r-1) =	9	385.8	42.9	0.3 *
A = frequency of tension	a-1 =	3	9152.1	3050.7	19.9
B = positional localisation of proportion of labelled cells	b-1 =	2	7432.5	3716.3	24.2
C = First and final hour of labelling	c-1 =	1	400.4	400.4	2.6 *
AB	(a-1) (b-1) =	6	5604.2	934.0	6.1
AC	(a-1) (c-1) =	3	483.8	161.3	1.1 *
BC	(b-1) (c-1) =	2	490.8	245.4	1.6 *
ABC	(a-1) (b-1) (c-1) =	6	952.5	158.8	1.0 *
Error	(r-1) (abc-1) =	207	31794.2	153.6	
Total	abcr-1 =	239			

* Non-significant at 2.5 % level of significance.

TABLE 14.

ANALYSIS OF VARIANCE OF DATA FROM APPENDICES 4 & 5 ON EFFECTS OF REPETITIVE TENSING OF SAIL-SHEETS ON THE POSITIONAL LOCALISATION OF PROPORTION OF LABELLED CELLS (DURATION OF TENSING 6 HOURS).

Source of Comparision	Degree of freedom, df		SS	Mean square	F
Blocks	(r-1) =	9	509.3	56.6	0.5 *
A = frequency of tension	a-1 =	3	8392.8	2797.6	26.3
B = positional localisation of proportion of labelled cells	b-1 =	2	15911.9	7955.9	74.9
C = first and final hour of labelling	c-1 =	1	6562.6	6562.6	61.8
AB	(a-1) (b-1) =	6	1155.6	192.6	1.8 *
AC	(a-1) (c-1) =	3	4805.3	1601.8	15.1
BC	(b-1) (c-1) =	2	1804.0	902.0	8.5
ABC	(a-1) (b-1) (c-1) =	6	1681.9	280.3	2.6
Error	(r-1) (abc-1) =	207	21993.2	106.3	
Total	abcr-1 =	239			

* Non-significant at 2.5 % level of significance.

TABLE 15.

ANALYSIS OF VARIANCE OF DATA FROM APPENDICES 7 & 8 ON EFFECTS OF REPETITIVE TENSING OF SAIL-SHEETS ON THE POSITIONAL LOCALISATION OF PROPORTION OF LABELLED CELLS (DURATION OF TENSING 12 HOURS).

Source of comparision	Degree of freedom, df		SS	Mean square	F
Blocks	(r-1) =	9	1321.3	146.8	0.6 *
A = frequency of tension	a-1 =	3	26577.9	8859.3	38.7
B = positional localisation of proportion of labelled cells	b-1 =	2	21527.5	10763.8	47.1
C = First and final hour of labelling	c-1 =	1	6826.7	6826.7	29.8
AB	(a-1) (b-1) =	6	4464.2	744.0	3.3
AC	(a-1) (c-1) =	3	1523.3	507.8	2.2 *
BC	(b-1) (c-1) =	2	9785.8	4892.9	21.4
ABC	(a-1) (b-1) (c-1) =	6	1015.8	169.3	0.7 *
Error	(r-1) (abc-1) =	207	47353.8	228.8	
Total	abcr-1 =	239			

* Non-significant at 2.5 % level of significance.

effects due to various frequencies of tension were studied on the first and the final hour labelled cultures of various durations (3-12 hours). It was found that different periods of tensing affected the positional distribution of the proportion of labelled cells differently, e.g., for a tensing period of 3 hours, (table 13) though the various frequencies of tension affected the positional distribution of labelled cells, whether these cultures were labelled for the first or final hour did not. For 6 hours tensing period (table 14), the first or final hour of labelling affected the positional distribution of the proportion of labelled cells. For the tensing period of 12 hours (table 15), various frequencies of tension and also the first or final hour of labelling affected the positional distribution of the labelled cells.

(ii) Comparison between the Various Individual Positions within Experimental (Tensed) and Control (Untensed) Cultures.

Application of mechanical tensions of various frequencies together with the first or final hour of labelling affected the distribution of the proportion of labelled cells at various parts in the culture (i.e. at corner, edge and centre) ; see appendices 3, 6 and 9.

On the whole, tension appears to result in a relatively higher proportion of labelled cells at the corners (than at the edge or centre) in most experimental situations as compared with controls (untensed cultures) though in case of 3 and 12 hours tensed (0.1 Hz.), first hour labelled cultures, the proportion of labelled cells is not significantly higher at the corner. A detailed analysis of these results is as follows :

For 3 hours tensing period (appendix 3) with first hour of labelling, untensed (control) cultures showed a higher proportion of labelled cells at the corner (than at edge or centre). Among the tensed cultures, 0.01 Hz showed significantly higher proportion of labelled cells at corner and edge (than at the centre), 0.1 Hz showed no significant differences while, 1.0 Hz showed significantly higher proportion of labelled cells at the corner. For 3 hours tensing with final hour of labelling, untensed (control) cultures showed a significantly higher proportion of labelled cells at the edge (than at the corner or centre). Results obtained from tensed cultures are as follows : 0.01 Hz showed a significanly higher proportion of labelled cells at the corner (than at the centre ; data on the proportion of labelled cells at the edge being not significantly different from that of centre), 0.1 Hz showed no significant differences among the three positions and 1.0 Hz showed a higher proportion of labelled cells at the corner (than at the edge or centre).

In case of 6 hours tensed (appendix 6) and first hour labelled cultures, all experimental (tensed) cultures showed a significantly higher proportion of labelled cells at the corner (than at the edge or centre). Control cultures showed no such effect. Among 6 hours tensed and final hour labelled cultures, the experimental (tensed) cultures which were tensed with 0.01 Hz showed a significantly higher proportion of labelled cells at the corner but, in the cultures which were tensed using 0.1 and 1.0 Hz frequencies, no significant difference was seen in the distribution of the proportion of labelled cells at various positions (corner, edge and centre).

For the cultures which were tensed for 12 hours (appendix 9) and

labelled for the first hour, controls (untensed cultures) did not show any positional localisation of the proportion of labelled cells while the experimental (tensed) cultures showed position effects as follows : 0.01 Hz showed a higher proportion of labelled cells at the corner (than at the edge or centre), 0.1 Hz at the edge (than centre ; data for corner showing no significant differences from edge or centre) and 1.0 Hz at the corner (than at the edge or centre). Cultures which were tensed for 12 hours with final hour of labelling did not show significant differences in the positional distribution (at corner, edge and centre) of labelled cells for controls (untensed cultures) as well as experimental (tensed) cultures.

Clearly, complex effects due to stress frequency and probably to stress gradients are being observed.

[D] THE MICROFILAMENT SYSTEM

Earlier work by Curtis and Seehar (1978) has shown that tension shortens the cell cycle. To explain this observation, one of several possible hypotheses is as follows : tension orients microfilaments and/or changes their number and/or form (from F to G actin or vice-versa) and these changes affect the nuclear activity of the cells directly or indirectly.

This sub- section describes a possible role of actin in controlling the cell cycle and, can be divided in to following parts :

(a) Fluorescence Microscopic Studies.

(b) DNAase-I Inhibition Studies.

(a) Fluorescence Microscopic Studies.

Fluorescent labelled phallacidin indicated the presence of microfilamentous bundles in conventional cultures (plate 19) but in sail-sheet cultures or gold grids, only bright ringed nuclei are seen. This may be due to some actin component near the nuclear membrane. In sail-sheet cultures on Nitex mesh, very few microfilament bundles can be seen but there seems to be a low diffuse fluorescence which may indicate microfilament turnover (Mautner & Hynes, 1977). It is hardly possible that the cells which are grown in the form of sheets may lack actin but G-actin levels might be much higher in these cultures than F-actin. The gold grids show slight autofluorescence and thus, cause difficulty in locating actin.

10μm

PLATE 19.

High power view of Chick heart fibroblasts grown as conventional culture (5 days age) on a glass coverslip stained with labelled Nitrobenzoxadiazole-phallacidin (NBD-Ph.) for detection of actin. Microfilament bundles can be clearly seen.

NBD-Ph. has an excitation maximum between 460 and 470 nm and emits over the range 510-650 nm. Blue light excitation filter and green light pass filter used.

(b) DNAase-I Inhibition Studies.

If the above hypothesis is correct, it might be expected that tension would affect F/G actin amounts or ratio. The measurements of G- and F-actin levels in the cells have been carried out using the method described by Blikstad et al.(1978). This method is based on the inhibition of the enzymic activity of DNAase-I by actin. In a mixture of monomeric (G) and filamentous (F) actin, only the monomeric form can be measured as a DNAase-I inhibitor. The filamentous form of actin can be measured after depolymerising it with 1.5 M guanidine hydrochloride (see pages 55-56).

Effects of tension on actin content of the cells : The results from DNAase-I inhibition experiments show (table 16, appendix 10) that the untensed (control) sail-sheet cultures have more F-actin per unit total cellular protein than conventional or tensed sail-sheet cultures. There is some evidence that short term application of mechanical strain diminishes total actin.

TABLE 16.

EFFECTS OF REPETITIVE TENSING (FREQUENCY = 1.0 HZ.) OF SAIL-SHEETS ON G, F AND TOTAL (G+F) ACTIN CONTENT/100 MICROGRAMS PROTEIN.

Culture Type	G-Actin µgms. Mean (S.D.)	F-Actin µgms. Mean (S.D.)	G+F Actin µgms. Mean (S.D.)	Repetitions
Experiments				
Sail-Sheets tensed for :				
(i) 3 Hours	7.9 (4.5)	9.2 (3.8)	15.6 (2.2)	4
(ii) 12 Hours	27.7 (1.4)	5.3 (2.2)	33.0 (1.9)	4
Controls				
Sail-Sheet	21.5 (3.1)	16.4 (0.3)	37.9 (2.8)	2
Conventional	25.1 (1.6)	5.3 (0.7)	30.4 (2.3)	2

D I S C U S S I O N

Cells can be grown in the form of sail-sheets, a culture system in which most cells adhere to one another, and mainly at their edges, rather than to a non-living substrate such as plastic, although cells at the outer edge of the cultures adhere to the substratum. The cells in sail-sheet cultures are often multilayered and confluent and confluence does not result in the cessation of cell movement or division.

Sail-sheets of fibroblasts have been grown from explants in earlier studies (Harrison, 1914 ; Curtis & Varde, 1964 ; Curtis & Seehar, 1978) and also in the present study. In addition to this, sail-sheets have also been grown from dispersed cells in the present study. Using this technique, cells can be grown relatively rapidly compared to conventional cultures which may be significant in an earlier healing of wounds. But, it is not known whether the technique of growing sail-sheets from dispersed cells could be developed further so that a mixture of various cell types can be grown.

The cells in sail-sheets seem to form monolayers at the edges of the cultures, cells between the edges being bilayered or often multilayered. The multilayers may be formed because of presence of the extra-cellular matrix (e.g., collagen ; see page 64) between the cells.

The closure of the free inner edge in sail-sheet cultures can be compared with the phenomenon of healing of wounds. Abercrombie et al. (1960) proposed the following hypotheses to explain possible mechanisms by which free edges of a skin wound might be moved inwards in order to

diminish the size of the defect : (1) They may be pushed in by extension of the surrounding skin. (2) They may by their own growth push themselves inwards, pushing against the surrounding skin. (3) There may be a sphincter-like action of contractile material at the wound edge. (4) The wound edges may pull themselves inwards, pulling on the material within the margins of the defect. (5) They may be pulled inwards by the material within the margins of the defect. (6) Cells actively migrate into the wound and then pull on surrounding cells to close the wound.

Abercrombie et al. (1960) showed that when wound contraction in rabbit is obstructed by gluing a splint to the surrounding skin, a considerable tension builds up in the granulation tissue filling the wound. Their data supported hypotheses (5) and (6) mentioned above, suggesting thereby that 'granulous contraction' may be responsible for wound healing. In my sail-sheet cultures, the rectangular mesh holes made up of nylon fibres may resemble the glued splints of Abercrombie et al. (1960). I have found that in the sail-sheet cultures, collagen is present between the cells but it is not known whether it is present at the free inner edge of the culture. Collagen, if present at the free inner edge, may provide support for cell migration in to the mesh holes. If this is so, then the hypotheses (5) and (6) proposed by Abercrombie et al. (1960) listed above might explain the closing of the mesh holes in sail-sheet cultures. This would presume that collagen protruded beyond the cells.

The closure of the free inner edge of the mesh holes may be similar to some events in early embryogenesis. Vakaet (1962) suggested that the closure of chick blastocoel is caused due to cell movement as well as

to cell division. During the gastrula stage in the early development of the chicken embryo, the blastoderm is separated in to two distinct layers : the epiblast and the hypoblast. Then, one end of the blastodisc is thickened by movement of lateral cells towards the centre. Cells growing within mesh holes in sail-sheet cultures also show movement of cells towards the centre, though the cells are not separated in to two morphologically distinct cellular layers.

Abercrombie et al. (1957) showed that when sarcoma cells and fibroblasts are caused to meet by juxtaposing two explants, sarcoma cells tend to move towards the fibroblast explant, a possible model of metastasis. Unlike Abercrombie et al. (1957)'s cultures, cells in sail-sheets migrate up the collagen planes, hence, sail-sheet cultures may provide a better model of metastasis.

A number of hypotheses can be suggested to explain how the mesh holes are filled with cells : (i) The mesh holes may be filled by cells because of their pseudopodial activity. The formation of pseudopodia might move cells towards the free space available at the centre. (ii) The continuous addition of cells from outer edges might push the cells at the inner edge inwards towards the available free space at the centre. (iii) Self tension within the cells at corner and also at the outer edge may cause an inward movement of cells towards the available free space. (iv) Cells may move towards the free space preferentially rather than forming multilayers (Abercrombie & Gitlin, 1965).

Sail-sheets growing within the mesh holes are filled by migration of cells from the explant/s along the mesh fibre to the corners from where they move towards the edge of the mesh and free inner edge in the

centre. This process may result in complete closure of mesh holes with cells. I found a decline in the rate of addition of cells per unit area within the mesh holes as the area of the holes decreases (figure 6). This is contrary to the expectation had the rate of division of cells would been uniform. This implies either that division plays no part or no large part in the closure of the sheet.

I found a 63.4% increase in the cellular area within the mesh holes over a culture period of 21 hours using time-lapse filming method. If the duration of the cell cycle of chick heart fibroblasts grown as sail-sheets is about 11 hours, as measured in the present study, then, the cell population within the mesh holes would be expected to double during 21 hours of culturing and filming period and concurrently, an increase in the cellular area by 100% would result. It is possible that the less than expected increase in the cellular area might, at least partly, be due to the multilayering of the cells. More work will be required to determine the actual cell number at various time-intervals during the closure of the mesh holes. As a matter of fact, measurements in terms of cell number rather than cellular area would be comparable with the duration of the cell cycle more precisely and easily. This may probably be due to cellular area being a parameter which may be affected by the factors such as spreading of cells, collagen synthesis between the cells and multilayering etc.

The results show that the cells on the fibres migrate quickly which may be because their migration does not seem to be hindered by the topography of the substratum (unlike at the corners where they are supported at either side of the mesh fibres).

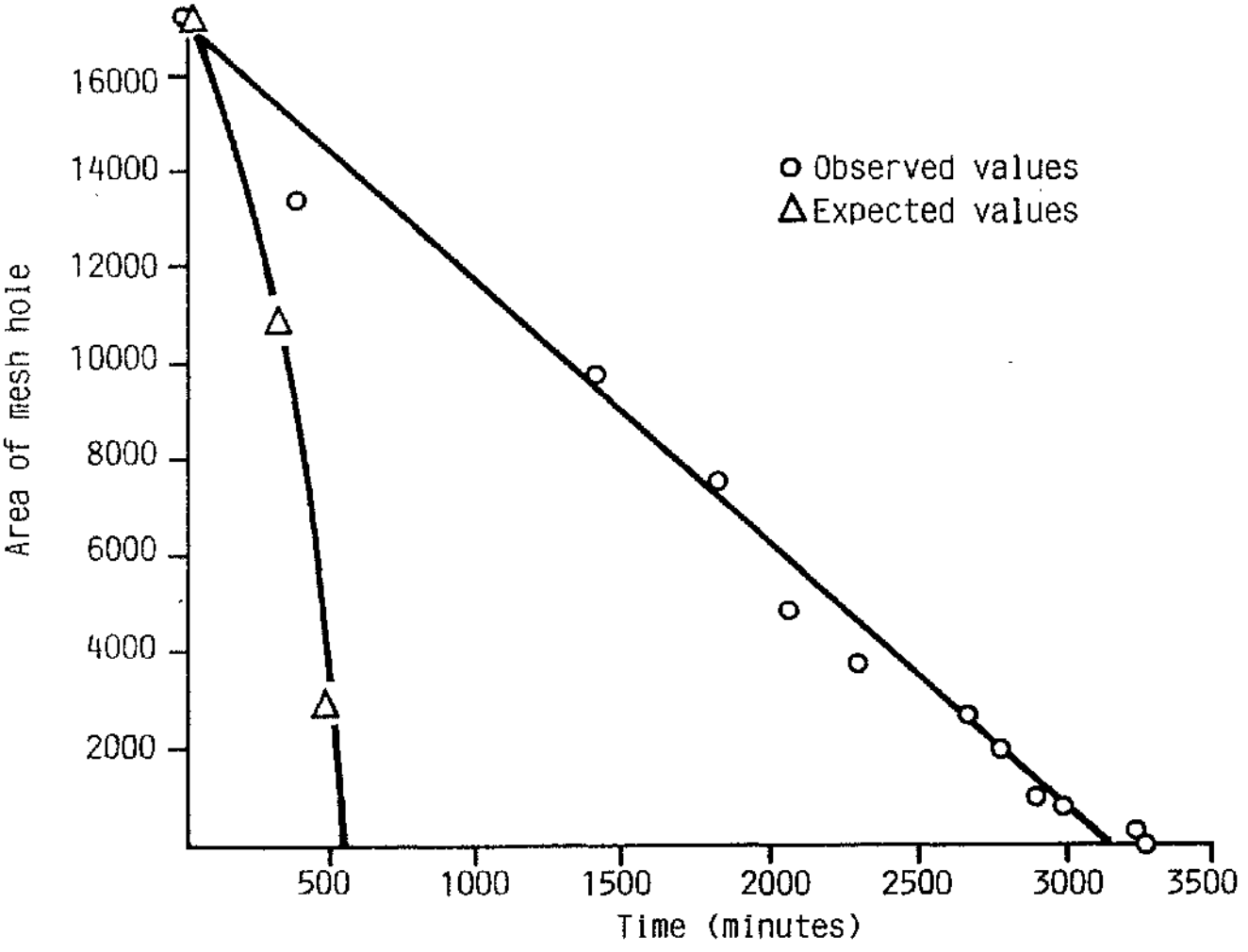

Area of mesh hole
16000
14000
12000
10000
8000
6000
4000
2000
500
1000
1500
2000
2500
3000
3500
Time (minutes)
Observed values
Expected values

FIGURE 6.

The figure shows how the observed values on the rate of closure of mesh holes (area) decrease with time contrary to that which would result from a constant rate of cell division. Data for observed values obtained from a sail-sheet culture of chick heart fibroblasts (7 days age). Size of the mesh = 185 X 166.5 μm^2.

I have found that fibroblasts growing on the mesh in the sail-sheet cultures do not continue their fast movement along the fibres only. In fact, they adhere on the mesh fibres, flatten and spread and then, migrate to the corners. The migration of fibroblasts from the mesh fibres towards the corners of the mesh holes may probably be due to the fact that at the corners, they are provided with support from the fibres joining at the corners while on the fibres, they may be able to adhere themselves only temporarily. The accumulation of cells at the corners may be because cells are possibly trapped due to the formation of a right angle (90°) between the mesh fibres.

I have found that fibroblasts grown as sail-sheets exhibit most of the features of cell movement shown in conventional cultures (see table 4 & Abercrombie et al., 1970 a). It may be that the cells in sail-sheets behave like those in conventional cultures and show features of cell behaviour in vivo. However, in sail-sheets, the rate of different events of cell movement is slower than that in conventional cultures which may be due to the absence of a non-living substratum and less available space for spreading in sail-sheet cultures. Unfortunately, some differences observed between the two culture systems may be artifacts of the optical system though care was taken to avoid such artifacts.

The presence of collagen between cells in conventional cultures was reported by McKinney (1930) after the cultures were 4 days old while in sail-sheets, it was established after 7 days of culturing by Seehar (1978) and the present study. It seems likely that certain features of cells in sail-sheets might be a response due to the presence of collagen as suggested by Elsdale and Bard (1972). Cells in sail-sheets

are nearly monolayered at the inner edges of a closing cell-sheet though they are bi- or multilayered between the edges. It would have been useful to know whether collagen is present in those parts of the cultures where they are just one cell thick at the edge of the sheet outgrowth or if it is formed while multilayering takes place or after multilayering (Elsdale & Bard, 1972). Multilayering might also result from the great thickness of fibres. If the collagen is absent when the sail-sheets are one layer thick could it be present when they are multilayered. It may be that collagen is responsible for multilayering of cells. In order to answer the question of when is the collagen formed in sail-sheets, more TEM work on the sail-sheet cultures of various ages is necessary. My time-lapse studies appeared to show that in sail-sheet cultures, contact with neighbouring cells may not affect cell movement. It may be because cells may insinuate on the sides of other cells as suggested by Boyde et al. (1969). Also, collagen may aid cell movement.

When the dispersed cells are seeded to grow a conventional culture, cells settle on the substratum, extend their cell perimeters and form microspikes and broader flattened leading lamella (also known as lamellipodia). As adhesive contacts form with the substratum, cells flatten and spread on the available space. In sail-sheets, cells are bipolar or spindle shaped and possibly due to this, cells at the edge of the mesh fibre can spread lengthwise only while cells at the corners may be slightly better off in finding support from and space between the two mesh fibres. Another reason why cells in sail-sheets do not spread as much as cells in conventional cultures do may be due to the fact that the actin in the cells in sail-sheets is probably mainly in

the form of meshworks contrary to the conventional cultures where cells have actin in the form of bundles. It may be that the cells in sail-sheet cultures have bundles which cannot be seen due to the formation of sheets.

The leading lamella (lamellipodia) of a fibroblast in a conventional culture is usually a broader, flattened end protruding from the cell towards the direction of the prospective movement. In sail-sheets, often both ends of the cells look alike due to cells being bipolar in shape hence, it becomes difficult to actually pinpoint any leading lamella. However, it may be possible to study the position of a leading lamella in sail-sheets if 'monolayers' could be grown as sail-sheets within the holes of the Nitex mesh. This may be possible if the Nitex mesh could be made using thinner fibres.

Fibroblasts in conventional cultures have been reported (Abercrombie & Gitlin, 1965 ; Vasiliev et al., 1970) to show polarity i.e. an ability of movement in the direction of the region of maximum spreading over the substratum. It may be due to the involvement of microtubules that fibroblasts are capable of maintaining this feature of movement (Vasiliev et al., 1970). Osborn and Weber (1976) found that the distribution of microtubules in the cytoplasm is appropriate to ensure their unidirectional assembly thus, ensuring polarity. Since the movement of cell sheets within the mesh holes is directed centripetally, it may be that individual fibroblasts in these cultures may show polarity. However, more experimental work needs to be done to find out whether cells in sail-sheets have microtubular organising structures similar to those found in conventional cultures (Osborn & Weber, 1976)).

Reichhart and Thiebold (1977) reported that mechanical factors may be involved in controlling proliferation of mesothelial cells in the mullerian ducts of chick embryos. Curtis and Seehar (1978) suggested that cell division may be controlled by mechanical factors (tension). The tension experiments described by them made use of a piezo-electric ceramic element which oscillated with a movement of 60 µm when a very low frequency strain (0.25-1.0 Hz., point-to-point) was applied. They did appropriate controls and showed that the stirring effects due to movement of the probe did not produce any increase in cell division. There could be an objection that the stirring in control and experimental series were not identical because in experimental series, the stirring is more effective due to the fact that the whole Nitex mesh moves. However, Seehar (1978) countered this with the assumption that the mechanical effects of a probe with a tip of 100 µm. diameter moving through a distance of 60 µm. at a frequency of 0.25 to 1.0 Hz. are much less than those of a Riddle pump used by Stoker (1973) that ejected medium at a rate of 1 to 10 cm/sec. in a jet 1 mm. wide. Hence, there appears to be almost no likelihood of effective stirring in Seehar's (1978) and my experiments. I extended the range of frequencies and still found results similar to those of Seehar (1978), which also showed that lowering the frequency of agitation shows a decline in the duration of the cell cycle. This would have been the other way round had the decrease in the cell cycle duration been caused by the stirring of the medium. It is interesting to speculate that the effectiveness of these very low frequencies of stimulation may represent an effect of duration of tension in one direction rather than a cyclical activity.

My SEM studies showed that fibroblasts, in control (untensed) sail-

sheet cultures, possess microprocess-like structures. This has been confirmed by my TEM studies. However, it is not known whether these structures play a role in cellular activity or whether they are a pathological feature. Results from the experiments carried out on the effects of mechanical tension (1.0 Hz, applied for 12 hours) on cell morphology suggest that tension can reduce the size and number of microprocesses. More work needs to be done in order to establish tension effects on cellular morphology and alignment (with other cells and also with Nitex fibre).

Results obtained with the DNA/[^{3}H]-thymidine incorporation method showed that the duration of one complete cell cycle in CHFs grown as sail-sheets is about 11 hours. By scoring the proportion of labelled cells, I found that the duration from S phase to G_1 phase of the cell cycle in these cultures is about 12 hours. The duration of the cell cycle of fibroblasts or fibroblast-like cells in conventional cultures can be found out by indirect (Balk et al., 1971, 1982) and direct (Mak, 1965 ; Macieira-Coelho et al., 1966 ; Bartholomew et al., 1975) methods. Balk et al. (1971) reported that the duration of one cell cycle in normal chicken fibroblasts grown in 0.005 mg/L folate or 5% serum is about 16.8 hours for the first doubling of cells while cells take longer in completing their subsequent cycles. Balk et al. (1982) found that one average population doubling period in normal chicken heart mesenchymal cells in the presence of epidermal growth factor at 1 µg/ml lasts 24 hours. Among the earlier workers, Mak (1965) working on strain-L of mouse fibroblasts in cultures found that the duration of G_1+S+G_2 is 18 hours. Macieira-Coelho et al. (1966) found doubling period for early passage human fibroblasts (strain WI.38) of fetal lung

origin as being 24 hours. Bartholomew et al. (1975) measured cell cycle duration of mouse fibroblasts (cell line Balb 3T3 A31 HYF) in 10% serum as 16 hours. These findings suggest that the duration of the cell cycle of fibroblasts in sail-sheets is shorter than that in conventional cultures and the precise reason for such a difference is not known. One possibility is that the cells in sail-sheets are under more tension and that their self tension might have speeded up the cell cycle.

Curtis and Seehar (1978) tensed their cultures only for an hour and reported an increase in the proportion of labelled cells. It might be possible that extending the range of frequency and tensing for longer durations could have produced a different result (see page 86) and, therefore, it seemed necessary for me to determine whether the range of frequency (0.01-1.0 Hz) and the duration (3-12 hours) of tensing affects the cell cycle. I found a higher proportion of labelled cells in most experimental (tensed) cultures (in comparison to their respective controls) for all periods of tensing (with the application of 0.01 Hz, tension increased the proportion of labelled cells during 3-12 hours of tensing ; with 0.1 Hz, the proportion of labelled cells is increased in all cases except for 12 hours tensed and first hour labelled cultures ; with 1.0 Hz, the proportion of labelled cells is increased in most cases except for 3 hours tensed and final hour labelled cultures), suggesting that tension may control the cell cycle irrespective of a wide range of durations for which it is applied. Curtis and Seehar (1978) stated that decrease in the frequency of tension was followed by an increased division rate. Although these authors did not use tension frequencies lower than 0.25 Hz, it might have been possible that the frequencies lower than this could have

produced different results. In present study, a further increase in the proportion of labelled cells has been found using frequencies as small as 0.1 and 0.01 Hz and duration of tensing as long as 6 and 12 hours. However, tensing for much longer duration may be desirable in order to compare this situation with that in the body where natural tension may last for several days, e.g., during the closing of neural plate in embryogenesis.

The results show that the linear deformation of cell-sheet caused due to the effect of mechanical stretching is quite low (in the range of 3.3 - 13.3 µm.) as compared with the actual size of the mesh (185 X 166.5 µm.). The percentage deformation of a sheet is also quite low (lengthwise in the range of 1.8% - 7.2% and breadthwise 2.0% - 7.9%).

Engineering studies on stress concentration (Peterson, 1974) suggest that the effect of stress should be more concentrated at the corners (than at the edge or centre) of a rectangular hole. Hence, the effect of mechanical stretching of sail-sheets growing within the rectangular mesh holes should also be localised. In my present studies, I have found a significantly higher proportion of labelled cells at the corner (than at the edge or the centre) part of the cultures in most experimental situations (as compared with their respective controls). This suggests that the effects of tension may be localised at the corners. If the cell cycle is limited by diffusion (as suggested by Stoker, 1973) then, one would expect to find a uniform distribution of the proportion of labelled cells throughout the cultures in the controls (untensed) as well as tensed cultures, or more cells in S phase in the centre of the mesh holes arise from diffusion blocking by the nylon. This obviously has not been the case in my present study.

Therefore, it seems likely that the cell cycle may be determined by some factor/s other than diffusion and that tension may be (one) such factor since the effect of tension may concentrate more at the corners than at the edge or the centre.

Curtis and Seehar (1978) postulated that since extension of cells and the generation of tension within them is dependent upon the microfilament system, the microfilament system might be involved in the control of the cell cycle. One can ask two questions : (i) does tension orient microfilaments and/or affect their number and content ? (ii) do microfilaments control the nuclear activity (i.e. mitosis) of cells ?

A fluorescence assay (phallacidin staining) was tried to locate F-actin in sail-sheet cultures of CHFs. However, F-actin could not be visualised well by this assay because of several reasons viz. (i) F-actin in the sail-sheets of fibroblasts may not be in the form of bundles and due to this, only low and diffuse fluorescence can be seen. This situation appears to be like that of transformed cells in which F-actin is present mainly in the form of meshworks (Mautner & Hynes, 1977). (ii) The orientation of F-actin in cells in sail-sheets may be different from that of conventional cultures and hence, their localisation becomes difficult. (iii) The Nitex mesh and gold grid show autofluorescence and because of this, there were difficulties in interpreting the results, (iv) There were experimental problems because of poor optics due to multilayering of cells.

The DNAase-I inhibition method was also used for these studies. It showed that sail-sheet cultures of chick heart fibroblasts have more actin content per unit cellular protein than conventional cultures. The

precise reason for this difference is not known although it is possible that cells in sail-sheets , due to continuous shuttling, may require more actin than those in conventional cultures. Further, I found more F-actin per unit cellular protein in the sail-sheet cultures than in conventional cultures. This result seems to be consistent with the idea that cells in sail-sheets may produce more F-actin which will be required for 'shuttling'. Although, there is more F-actin content per unit cellular protein present in the fibroblasts grown as sail-sheets, it cannot be seen in the fluorescence (phallacidin) assay because F-actin may be present in cells in the form of meshworks which cannot be visualised well by this assay.

There is evidence to show (see page 30-31) that tension affects the orientation of microfilaments (Fleischer & Wohlfarth-Botterman, 1975 ; Abercrombie, 1977 ; Vasiliev, 1982). However, it is not known whether the effects of tension on cell division are due to their effect on the microfilament system. Pickett-Heaps *et al.* (1982) suggested that filamentous actin was not involved in mitosis. Results from the experiments carried out in the present study showed an apparent decline in the total actin content/unit cellular protein in experimental (tensed for 3 and 12 hours using 1.0 Hz frequency) series of sail-sheet cultures in comparison to their respective controls. However, whether the direction of the displacement of probe is significant is not known. It can be seen that increasing the period of tensing is accompanied by an increase in the G-actin content indicating a possibility of some conversion from F-actin to G-actin. Such F-actin to G-actin conversion may affect cell movement which might lead to the rounding up of cells which alone might affect the duration of the cell cycle. The results

presented in this thesis suggest that the microfilaments (including both F-actin and G-actin) may be involved with the cell cycle. However, in these experiments, I have measured actin content in relation to total cellular protein only and therefore, differences in data between the tensed and untensed cultures may well have been due to an alteration (slowing down or speeding up) in the rate of total protein synthesis. This problem can be overcome (for future experiments) by measuring actin content per cell.

The work carried out in this project suggests that chick heart fibroblasts grown as sail-sheets may show locomotory features similar to those shown by these cells in conventional cultures. The mechanical stretching of sail-sheets may decrease the duration of the cell cycle and the effects of mechanical stress may be concentrated more at the corners of the rectangular cell sheets. Whether the effect of mechanical stress on the cell cycle is due to its effect on the microfilaments remains obscure.

I found that dispersed cells can be grown as sail-sheets. Sail-sheets grown from chicken heart explants were often multilayered. Efforts should be made to grow monolayered sail-sheets using meshes made from relatively thinner fibres. The monolayered sail-sheets, if achieved, may be more suitable for the study of cell behaviour in sail-sheets. Attempts should be made to grow a mixture of different cell types as sail-sheets. This may have an implication on an earlier healing of wounds. I used optical microscopy for the study of the cellular alignments and the thickness of sail-sheets and, electron microscopy for visualising the effects of tension on cellular morphology. SEM study showed that tension may affect microprocess-like structures being

present on the cells. My TEM work was, however, limited to the study of untensed (control) sail-sheet cultures only but, the study of experimental (tensed) sail-sheets is required. TEM study carried out on untensed (control) sail-sheet cultures showed the presence of collagen fibres between the cells. More TEM work would be required to investigate whether collagen plays a role in multilayering of sail-sheets. More TEM work would also be needed for studying the effect of tension on microfilament system. I studied some features of behaviour and movement of fibroblasts in sail-sheets by time-lapse filming but the effect of tension on the locomotory behaviour of individual cells and cells in sheets is yet to be investigated. Also, the distribution of microfilaments in the cells in sail-sheets during the resting and mobile phases should be compared in order to determine whether tension affects the locomotory machinery of the cells in sail-sheets. Experiments should be done in order to locate the microtubule organising structures in the cells in sail-sheets. This might give information about the polarity of these cells. Whether addition of cell suspension of fibroblasts, collagen and fibronectin etc can affect the rate of closure of mesh holes should also be examined because this may be significant in the phenomenon such as healing of wounds. My studies on cell behaviour of fibroblasts in sail-sheets were carried out using the grids with square and rectangular mesh holes only. Grids with circular holes should be used in order to see whether topography of grid holes plays a role in cell behaviour. I measured the duration of the cell cycle of chick heart fibroblasts grown as sail-sheets and studied the effects of mechanical tension on the cell cycle, but, the effect of tension was studied using a probe oscillating the cultures to and fro in one direction only. More work would be required to see whether a

change in the oscillation in two or three dimensions would affect the cell cycle differently.

B I B L I O G R A P H Y

Abercrombie, M. (1970 a). Contact inhibition in tissue culture. In Vitro. 6 128-142.

Abercrombie, M. (1970 b). Control mechanisms in cancer. Europ. J. Cancer 6 7-13.

Abercrombie, M. (1980). The crawling movement of metazoan cells. Proc. Royal Soc. (London). B. 207 129-147.

Abercrombie, M. & Ambrose, E.J. (1958). Interference microscopic studies of cell contacts in tissue culture. Expl. Cell Res. 15 332-345.

Abercrombie, M., Dunn, G.A. & Heath, J.P. (1976). Locomotion and contraction in non-muscle cells. In Contractile Systems in Non-muscle Tissues (eds. Perry, S.V., Margreth, A. & Adelstein, R.S.). pp 3-11. North-Holland Publishing Co., Amsterdam.

Abercrombie, M., Dunn, G.A. & Heath, J.P. (1977). The shape and movement of fibroblasts in culture. In Cell and Tissue Interactions (eds. Lash, J.W. & Burger, M.M.) pp 57-70. Raven Press, New York.

Abercrombie, M., Flint, M.H. & James, D.W. (1954). Collagen formation and wound contraction during repair of small excised wounds in the skin of rats. J. Embryol. Expl. Morphol. 2 264-274.

Abercrombie, M. & Gitlin, G. (1965). The locomotory behaviour of small group of fibroblasts. Proc. Royal Soc. B 162 289-302.

Abercrombie, M. & Heaysman, J.E.M. (1953). Observations on the social behaviour of cells in tissue culture. I. Speed of movement of chick

heart fibroblasts in relation to their mutual contacts. Expl. Cell Res. 5 111-131.

Abercrombie, M. & Heaysman, J.E.M. (1954). Observations on the social behaviour of cells in tissue culture. II. 'monolayering' of fibroblasts. Expl. Cell Res. 6 293-306.

Abercrombie, M., Heaysman, J.E.M. & Karthauser, H.M. (1957). Social behaviour of cells in tissue culture. III. Mutual influence of sarcoma cells and fibroblasts. Expl. Cell Res. 13 276-291.

Abercrombie, M., Heaysman, J.E.M. & Pegrum, S.M. (1970 a). The locomotion of fibroblasts in culture. I. Movement of the leading edge. Expl. Cell Res. 59 393-398.

Abercrombie, M., Heaysman, J.E.M. & Pegrum, S.M. (1970 b). The locomotion of fibroblasts in culture. II. 'Ruffling'. Expl. Cell Res. 60 437-444.

Abercrombie, M., Heaysman, J.E.M. & Pegrum, S.M. (1970 c). The locomotion of fibroblasts in culture. III. Movement of particles on the dorsal surface of the leading lamella. Expl. Cell Res. 62 389-398.

Abercrombie, M., Heaysman, J.E.M. & Pegrum, S.M. (1971). The locomotion of fibroblasts in culture. IV. Electron microscopy of the leading lamella. Expl. Cell Res. 67 359-367.

Abercrombie, M., Heaysman, J.E.M. & Pegrum, S.M. (1972). Locomotion of fibroblasts in culture. V. Surface marking with concanavalin A. Expl. Cell Res. 73 536-539.

Abercrombie, M., James, D.W. & Newcombe, J.F. (1960). Wound contraction

in rabbit skin, studied by splinting the wound margins. J. Anat. 94 170-182.

Albertini, D.F. & Anderson, E. (1977). Microtubule and microfilament rearrangements during capping of Concanavalin A receptors on cultured ovarian granulosa cells. J. Cell Biol. 73 111-127.

Albrecht-Buehler, G. (1976). Filopodia of spreading 3T3 cells. J. Cell Biol. 69 275-286.

Allen, J.C. & Smith, Ch.J. (1979). Chalones: a reappraisal. Biochem. Soc. Trans. 7 584-592.

Ash, J.F., Louvard, D. & Singer, S.J. (1977). Antibody-induced linkages of plasma membrane proteins to intracellular actomyosin-containing filaments in cultured fibroblasts. 74 5584-5588.

Ash, J.F. & Singer, S.J. (1976). Concanavalin-A-induced transmembrane linkage of Concanavalin-A surface receptors to intracellular myosin-containing filaments. 73 4575-4579.

Badley, R.A., Lloyd, C.W., Woods, A., Carruthers, L., Allcock, C. & Rees, D.A. (1978). Mechanism of cellular adhesion. III. Preparation and preliminary characterisation of adhesions. Exptl. Cell Res. 117 231-244.

Badley, R.A., Woods, A., Smith, C.G. & Rees, D.A. (1980). Actomyosin relationships with surface features in fibroblast adhesion. Exptl. Cell Res. 126 263-272.

Balk, S.D. (1971). Stimulation of the proliferation of chicken fibroblasts by folic acid or a serum factor (s) in a plasma-containing

medium. Proc. Natn. Acad. Sci. U.S.A. 68 1689-1692.

Balk, S.D., Shiu, R.P.C., LaFleur, M.M. & Young, L.L. (1982). Epidermal growth factor and insulin cause normal chicken heart mesenchymal cells to proliferate like their Rous sarcoma virus-infected counterparts. Proc. Natn. Acad. Sci. U.S.A. 79 1154-1157.

Bard, J.B. & Hay, E.D. (1975). The behaviour of fibroblasts from the developing avian cornea. Morphology and movement in situ and in vitro. J. Cell Biol. 67 400-418.

Barranco, S.C. & Haenelt, B.R. (1977). Cell cycle time determinations based on liquid scientillation counting of ^{3}H - TdR labelled mitotic cells. Cell Tissue Kinet. 10 335-340.

Bartholomew, J.C., Yokata, H. & Ross, P. (1975). Effect of serum on the growth of Balb 3T3 A31 mouse fibroblasts and an SV40-transformed derivative. J. Cell. Physiol. 88 277-286.

Baserga, R. (1965). The relationship of the cell cycle to tumour growth and control of cell division. a review. Cancer Res. 25 581-595.

Baserga, R. & Nemeroff, K. (1962). Two-emulsion radioautography. J. Histochem. Cytochem. 10 628-635.

Berlin, R.D., Caron, J.M. & Oliver, J.M. (1979). In "Microtubules" (eds. Roberts, K. & Hyams, J.S.). pp 443-485. Acad. Press, New York.

Berridge, M.J. (1976). Calcium, cyclic nucleotides and cell division. In Calcium in Biological Systems, Symposia for Experimental Biology 30 (ed. Duncan, C.J.) pp 219-231 Cambridge Univ. Press, London, New York, Melbourne.

Bertch, S., Csontos, K., Schweizer, J. & Marks, F. (1976). Effect of mechanical stimulation on cell proliferation in mouse epidermis and on growth regulation by endogenous factors (chalones). Cell Tissue Kinet. 9 445-457.

Bhargava, P.M. (1977). Regulation of cell division and malignant transformation : A new model for control by uptake of nutrients. J. Theoret. Biol. 68 101-137.

Billingham, R.E. & Medawar, P.B. (1955). Contractile and intussusceptive growth in the healing of extensive wounds in mammalian skin. J. Anat. 89 114-123.

Blikstad, I., Markey, F., Carlsson, L., Persson, T. & Lindberg, U. (1978). Selective assay of monomeric and filamentous actin in cell extracts, using inhibition of deoxyribonuclease-I. Cell 15 935-943.

Boyde, A., Grainger, F. & James, D.W. (1969). Scanning electron microscopic observations of chick embryo fibroblasts in vitro, with particular reference to the movement of cells under others. Zeitschrift fur Zellforschung und mikroskopische Anat. 94 46-55.

Bradford, M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72 248-254.

Bray, D. (1979). Mechanical tension produced by nerve cells in tissue culture. J. Cell Sci. 37 391-410.

Bretscher, M.S. (1976). Directed lipid flow in cell membranes. Nature (London) 260 21-23.

Brown, S., Levinson, W. & Spudich, J.A. (1976). Cytoskeltal elements of Chick embryo fibroblasts revealed by detergent action. J. Supramol. Struct. 5 119-130.

Buckley, I.K. & Porter, K.R. (1967). Cytoplasmic fibrils in living cultured cells. Protoplasma 64 349-380.

Bullough, W.S. (1962). The control of mitotic activity in adult mammalian tissues. Biol. Rev. 37 307-342.

Burridge, K. & Phillips, J.H. (1975). Association of actin and myosin with secretory granule membranes. Nature (Lond.) 254 526-529.

Burton, K. (1968). Determination of DNA concentration with diphenylamine. In Methods in Enzymology (eds. Colowick, S.P. & Kaplan, N.O.) 12 Nucleic Acids Part B (eds. of vol. 12: Grossman, L. & Moldave, K.) pp 163-166. Academic Press.

Buultjens, T.E.J. & Edwards, J.G. (1977). Adhesive selectivity is exhibited in vitro by cells from adjacent tissues of the embryonic chick retina. J. Cell Sci. 23 101- 116.

Campisi, J. & Medrano, E.E. (1983). Cell cycle perturbations in normal and transformed fibroblasts caused by detachment from the substratum. J. Cell. Physiol. 114 53-60.

Chambers, R. & Fell, H.R. (1931). Micro-operations on cells in tissue culture. Proc. Roy. Soc. (London) .B. 109 380-403.

Clarke, G.D., Stoker, M.G.P., Ludlow, A. & Thornton, M. (1970). Requirements of serum for DNA synthesis in BHK21 cells: Effects of density, suspension and virus transformation. Nature (London) 227 798-

801.

Cleaver, J.E. (1967). In Thymidine Metabolism and Cell Kinetics. North Holland, Amsterdam.

Cloney, R.A. (1966). Cytoplasmic filaments and cell movements: Epidermal cells during ascidian metamorphosis. J. Ultrastruct. Res. 14 300-328.

Cloney, R.A. (1969). Cytoplasmic filaments and morphogenesis: The role of the notochord in ascidian metamorphosis. Zeitschrift Zellforsch 100 31-53.

Curtis, A.S.G. (1964). The mechanism of adhesion of cells to glass. A study by interference reflection microscopy. J. Cell Biol. 20 199-215.

Curtis, A.S.G. & Varde, M. (1964). Control of cell behaviour: topographical factors. J. Nat. Cancer Inst. 33 15-26.

Curtis, A.S.G. & Seehar, G.M. (1978). The control of cell division by tension or diffusion. Nature (London) 274 52-53.

DeRosier, D.J. & Tilney, L.G. (1982). How actin filaments pack into bundles. Cold Spring Harbor Symposia on Quantitative Biolgy. 46 part B, pp 525-540 Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Dische, Z. (1930). Mikrochemie 8 4.

Dulbecco, R. (1970). Topoinhibition and serum requirement of transformed and untransformed cells. Nature (London) 227 802-806.

Dulbecco, R. & Elkington, J. (1973). Conditions limiting multiplication

of fibroblastic and epithelial cells in dense cultures. Nature (London) 246 197-199.

Dulbecco, R. & Stoker, M.G.P. (1970). Conditions determining initiation of DNA synthesis in 3T3 cells. Proc. Natn. Acad. Sci. U.S.A. 66 204-210.

Dunn, G.A. (1971). Mutual contact inhibition of extension of Chick sensory nerve fibres in vitro. J. Comp. Neurol. 143 491-508.

Dunn, G.A. (1980). Mechanism of fibroblast locomotion. In Cell Adhesion and Motility, 3rd Symposium British Soc. Cell Biol. (eds. Curtis, A.S.G. & Pitts, J.D.) pp 409-423. Cambridge Univ. Press.

Dunn, G.A. & Ebendal, T. (1978). Contact guidance on oriented collagen gels. Expl. Cell Res. 111 475-479.

Dunn, G.A. & Heath, J.P. (1976). A new hypothesis of contact guidance in tissue cells. Expl. Cell Res. 101 1-14.

Ehrmann, R.L. & Gey, G.O. (1956). The growth of cells on a transparent gel of reconstituted rat tail collagen. J. Natn. Cancer Inst. 16 1375-1404.

Elgjo, K., Laerum, O.D. & Edgehill, W. (1971). Growth regulation of mouse epidermis. I. G_2 inhibitor present in the basal cell layer. Virchows Archiv (Cell Path.) 8 277-283.

Elsdale, T. & Bard, J. (1972). Cellular interactions in mass cultures of human diploid fibroblasts. Nature (Lond.) 236 152-155.

Elgjo, K., Laerum, O.D. & Edgehill, W. (1972). Growth regulation of

mouse epidermis. II. G_1 inhibitor present in the differentiating cell layer. Virchows Archiv (Cell Path.) 10 229-236.

Fitchen, J.H. & Cline, M.J. (1981). Inhibition of hematopoietic cell proliferation. In Tissue Growth Factors (ed. Baserga, R.), Handbook of Experimental Pharmacology 57 pp 385-404. Springer-Verlag Berlin, Heidelberg, New York.

Flanagan, J. & Koch, G.L.E. (1978). Cross-linked surface Ig attaches to actin. Nature (Lond.) 273 278-281.

Fleischer, M. & Wohlfarth-Bottermann, K.E. (1975). Correlation between tension force generation, fibrillogenesis and ultra structure of cytoplasmic actomyosin during isometric and isotonic concentrations of protoplasmic strands. Cytobiologie 10 339-365.

Folkman, J. & Moscona, A. (1978). Role of cell shape in growth control. Nature (London) 273 345-349.

Forer, A. & Behnke, O. (1972). An actin-like component in spermatocytes of a Crane fly (Nephrostoma suturalis Loew). II. The cell cortex. Chromosoma 39 175-190.

Froehlich, J.E. & Anastassides, T.P. (1975). Possible limitation of growth in human fibroblast cultures by diffusion. J. Cell Physiol. 86 567-580.

Glucksmann, A. (1942). The role of mechanical stresses in bone formation in vitro. J. Anat. (Lond.) 76 231-239.

Goldman, R.D., Lazarides, E., Pollack, R. & Weber, K. (1975). The distribution of actin in non-muscle cells. Expl. Cell Res. 90 333-344.

Goldman, R.D., Schloss, J.A., and Starger, J.M. (1976). Organizational changes of actin in microfilaments during animal cell movement. In Cell Motility, Book A: Motility, Muscle and Non-muscle Cells (eds. Goldman, R., Pollard, T., Rosenbaum, J.) Cold Spring Harbor Conf. Cell Proliferation 3 pp 217-245 Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Gray, J.W., Carver, J.H., George, Y.S. & Mendelsohn, M.L. (1977). Rapid cell cycle analysis by measurement of the radioactivity per cell in a narrow window in S-phase (RCSi). Cell Tissue Kinet. 10 97-109.

Gruenstein, E., Rich, A. & Weihing, R.R. (1975). Actin associated with membranes from 3T3 mouse fibroblast and HeLa cells. J. Cell Biol. 64 223-234.

Gustafson, T. & Wolpert, L. (1967). Cellular movement and contact in sea urchin morphogenesis. Biological Rev. Cambridge Phil. Soc. 42 442-498.

Hanks, J.H. & Wallace, R.E. (1949). Relation of oxygen and temperature in the preservation of tissues by refrigeration. Proc. Soc. Exp. Biol. Med. 71 196-200.

Harris, A. (1974). Contact inhibition of cell locomotion. In Cell Communication (ed. Cox, R.P.), pp. 147-185. John Wiley & Sons, New York.

Harris, A.K. (1973 a). Cell surface movements related to cell locomotion. In Locomotion of Tissue Cells, Ciba Foundation Symposium 14 3-20. Churchill Livingstone, Edinburgh & London.

Harris, A.K. (1973 b). Location of cellular adhesions to solid substrata. Develop. Biol. 35 97-114.

Harris, A.K. (1973 c). Behaviour of cultured cells on substrata of variable adhesiveness. Expl. Cell Res. 77 285-297.

Harris, A.K. (1976). Recycling of dissolved plasma membrane components as an explanation of the capping phenomenon. Nature (London) 263 781-783.

Harrison, R.G. (1912). The cultivation of tissues in extraneous media as a method of morphogenetic study. Anatomical Record. 6 181-193.

Harrison, R.G. (1914). The reaction of embryonic cells to solid structure. J. Expl. Zool. 17 521-544.

Heath, J.P. & Dunn, G.A. (1978). Cell-to-substratum contacts of Chick fibroblasts and their relation to the microfilament system. A correlated interference-reflexion and high-voltage electron microscopic study. J. Cell Sci. 29 197-212.

Heaysman, J.E.M. (1978). Contact inhibition of locomotion: a reappraisal. Internatl. Rev. Cytol. 55 49-66.

Heaysman, J.E.M. & Pegrum, S.M. (1973). Early contacts between fibroblasts: An ultrastructural study. Expl. Cell Res. 78 71-78.

Heck, H.D'A., McReynolds, J.H. & Anbar, M. (1977). A stable isotope method for measurement of thymidine incorporation into DNA. Cell Tissue Kinet. 10 111-119.

Herman, I.M., Crisona, N.J. & Pollard, T.D. (1981). Relation between

cell activity and distribution of cytoplasmic actin and myosin. J. Cell Biol. 90 84-91.

Herman, S.P., Golde, D.W. & Cline, M.J. (1978). Neutrophil products that inhibit cell proliferation: Relation to granulocyte chalone. Blood 51 207-219.

Hilscher, W. & Maurer, W. (1962). Autoradiographische bestimmung der dauer der DNS-Verdopplung und ihres zeitlichen verlaufs bei spermatogonien der ratte durch doppelmarkierung mit C-14 und H-3 thymidin. Naturwiss. 49 352*

Hochhauser, S.J., Stein, J.L. & Stein, G.S. (1981). Gene expression and cell cycle regulation. Internatl. Rev. Cytol. 71 95-243.

Howard, A. & Pelc, S.R. (1951 a). Nuclear incorporation of P^{32} as demonstrated by autoradiographs. Expl. Cell Res. 2 178-187.

Howard, A. & Pelc, S.R. (1951 b). Synthesis of deoxyribose nucleic acid and nuclear incorporation of S^{35} as shown by autoradiographs. Isotopes in Biochemistry, CIBA Foundation Conference, J. & A. Churchill, London.

Howard, A. & Pelc, S.R. (1953). Synthesis of deoxyribonucleic acid in normal and irradiated cells and its relation to chromosome breakage. Heredity (London) 6 (Suppl.) 261-273.

Ingram, V.N. (1969). A side view of moving fibroblasts. Nature (Lond.) 222 641-644.

Isenberg, G., Rathke, P.C., Hulsmann, N., Franke, N.W. & Wohlfarth Botterman, K.E. (1976). Cytoplasmic actomyosin fibrils in tissue culture cells. Direct proof of contractility by visualization of ATP-

induced contraction in fibrils isolataed by laser microbeam dissection. Cell and Tissue Res. 166 427-443.

Iversen, O.H. (1976). The history of chalones. In Chalones (ed. Houck, J.C.). pp 37-70 North Holland and American Elsevier, Amsterdam, Oxford, New York.

Iversen, O.H. (1981). The Chalones. In Handbook of Experimental Pharmacology (ed. Baserga, R.) 57 491-550.

Izzard, C.S. & Lochner, L.R. (1976). Cell-to-substrata contacts in living fibroblasts: An interference reflexion study with an evaluation of the technique. J. Cell Sci. 21 129-159.

Izzard, C.S. & Lochner, L.R. (1980). Formation of cell-to-substrate contacts during fibroblast motility : an interference-reflexion study. J. Cell Sci. 42 81-116.

Koch, G.L.E. & Smith, M.J. (1978). An association between actin and the major histocompatibility antigen H-2. Nature (Lond.). 273 274-278.

Kochhar, O.S. (1979). Changes in plasma membrane and microfilaments accompanying morphologic differentiation in chinese hamster ovary (CHO) cells. Expl. Cell Res. 118 191-203.

Kreis, T.E. & Birchmeier, W. (1980). Stress fiber sarcomeres of fibroblasts are contractile. Cell 22 555-561.

Lazarides, E. (1975). Tropomyosin antibody: the specific localization of tropomyosin in non-muscle cells. J. Cell Biol. 65 549-561.

Lazarides, E. (1976 a). Aspects of the structural organization of actin

filaments in tissue culture cells. In Cell Motility, Book-A, Muscle and Non-muscle Cells (eds. Goldman, R., Pollard, T. & Rosenbaum, J.). Cold Spring Harbor Conf. Cell Proliferation. 3 347-360 Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Lazarides, E. (1976 b). Two general classes of cytoplasmic actin filaments in tisue culture cells: the role of tropomyosin. J. Supramol. Struct. 5 531-563.

Lazarides, E. & Burridge, K. (1975). α- Actinin: immunofluorescent localisation of a muscle structural protein in non-muscle cells. Cell 6 289-298.

Lazarides, E. & Weber, K. (1974). Actin antibody: the specific visualisation of actin filaments in non-muscle cells. Proc. Natn. Acad. Sci. U.S.A. 71 2268-2272.

Le Blond, C.P., Kopriwa, B.M. & Messier, B. (1963). The application of autoradiography in the study of nucleic acid metabolism. Lab. Invest. 8 214-224.

Leighton, J. (1951). A sponge matrix method for tissue culture. Formation of organized aggregates of cells in vitro. J. Natn. Cancer Inst. 12 545-561.

Levy, O. (1904). Under die einfluss von zug auf die bildung faserigen bindegewebes. Arch. Entwmech. Org. 18 184-246.

Lochner, L.R. & Izzard, C.S. (1973). Dynamic aspects of cell-substrate contacts in fibroblast motility. J. Cell Biol. 59 199a*.

Loeb, L. & Fleisher, M.S. (1917). On the factors which determine the

movements of tissues in culture media. J. Med. Res. 37 75-99.

Macieira-Coelho, A., Ponten, J. & Philipson, L. (1966). The division cycle and RNA-synthesis in diploid human cells at different passage levels in vitro. Exp. Cell Res. 42 673-684.

Mak, S. (1965). Mammalian cell cycle analysis using microspectrophotometry combined with autoradiography. Exp. Cell Res. 39 286-289.

Maroudas, N.G. (1973 a). Growth of fibroblasts on linear and planar anchorages of limiting dimensions. Expl. Cell Res. 81 104-110.

Maroudas, N.G. (1973 b). Chemical and mechanical requirement for fibroblast adhesion. Nature 244 353-354.

Maroudas, N.G. (1974). Short-range diffusion gradient. Cell 3 217-219.

Maroudas, N.G. (1978). Diffusion-limitation of cell growth. Nature (Lond.) 274 722.***

Mautner, V. & Hynes, R.O. (1977). Surface distribution of LETS protein in relation to the cytoskeleton of normal and transformed cells. J. Cell Biol. 75 743-768.

Mazia, D. (1961). Mitosis and physiology of cell division. In "The Cell" (eds. Brachet, J. & Mirsky, A.E.). vol. 3 pp 77-412. Acad. Press, New York.

McGedey, J. & Reed, O. (1981). Differentiation of A- , B- and D- Cells of Guinea-pig pancreas using semi-thin epoxy-resin sections. Acta Diabetologica Latina 18 329-334.

McKinney, R.L. (1930). Studies on fibers in tissue culture. III. The

development of reticulum into collagenous fibers in cultures of adult rabbit lymph nodes. Archiv Fur Experimentelle Zelloforschung 9 14-35.

Middleton, C.A. (1972). Contact inhibition of locomotion in cultures of pigmented retinal epithelium. Expl. Cell Res. 70 91-96.

Miller, R.G. (1980). Estimation problems in microcolony autoradiography. In Biostatistics Case Book (eds. Miller, R.G.Jr., Efron, B., Brown, B.Wm.Jr. & Moses, L.E.). pp 220-238. John Wiley & Sons, New York.

Mitchison, J.M. (1971). The Biology of the Cell Cycle. Cambridge Univ. Press, Cambridge, England.

Mooseker, M.S. & Tilney, L.G. (1975). Organization of an actin filament-membrane complex. Filament polarity and membrane attachment in the microvilli of intestinal epithelial cells. J. Cell Biol. 67 725-743.

Osborn, M. & Weber, K. (1976). Cytoplasmic microtubules in tissue culture cells appear to grow from an organizing structure towards the plasma membrane. Proc. Natn. Acad. Sci. U.S.A. 73 867-871.

Osborn, M., Webster, R.E. & Weber, K. (1978). Individual microtubules viewed by immunofluorescence and electron microscopy in the same PtK2 cells. J. Cell Biol. 77 R27-34. **

Ostlund, R.E., Leung, J.T. & Kipnis, D.M. (1977). Muscle actin filaments bind pituitary secretory granules in vitro. J. Cell Biol. 73 78-87.

Paul, D., Henahan, M. & Walter, S. (1974). Changes in growth control

and growth requirements associated with neoplastic transformation *in vitro*. J. Natn. Cancer Inst. 53 1499-1503.

Perry, M.M., John, H.A. & Thomas, N.S.T. (1971). Actin-like filaments in the cleavage furrow of newt egg. Expl. Cell Res. 65 249-253.

Peterson, R.E. (1974). Stress Concentration Factors. John Wiley & Sons, New York, London, Sydney, Toronto.

Pickett-Heaps, J.D., Tippit, D.H. & Porter, K.R. (1982). Rethinking mitosis. Cell 29 729-744.

Pilgrim, Ch. & Maurer, W. (1962). Autoradiographische bestimmung der DNS-Verdopplungszeit verschiedener zellarten von maus und ratte bei doppelmarkierung mit H-3- und C-14-thymidin. Naturwiss 49 544-545.

Puck, T.T. & Steffen, J. (1963). Life cycle analysis of mammalian cells. I. A method of localising metabolic events within the cell cycle. Biophys.J. 3 379-397.

Puszkin, S., Kochwa, S. & Rosenfield, R.E. (1976). In "Cell Motility" (eds. Goldman, R.D., Pollard, T.D. & Rosenbaum, J.L.). Book B. pp 665-670. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Quastler, H. & Sherman, F.G. (1959). Cell population kinetics in the intestinal epithelium of the mouse. Expl. Cell Res. 17 420-438.

Rees, D.A., Badley, R.A. & Woods, A. (1980). Relationships between actomyosin stress fibres and some cell surface receptors in fibroblast adhesion. In Cell Adhesion and Motility (eds. Curtis, A.S.G. & Pitts, J.D.), British Soc. Cell Biology Symposium 3 pp 389-408. Cambridge Univ. Press, Cambridge, U.K.

Revel, J.P., Hoch, P. & Ho, D. (1974). Adhesion of cultured cells to their substratum. Expl. Cell Res. 84 207-218.

Reynolds, E.S. (1963). The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J. Cell Biol. 17 208-212.

Reynolds, S.R.M. (1949). Physiology of uterus, Paul B. Hoeber Publishers, New York.

Rieber, M. & Rieber, M.S. (1982). Novel correlation between greater cell adhesion to substratum and an increased association of cell surface proteins with polypeptides involved in actin polymerization. Biochem. Biophys. Res. Commun. 106 236-242.

Rogers, A.W. (1969). Techniques of autoradiography. Elsevier Publishing Co. Amsterdam, London, New York.

Rozengurt, E. (1983). Growth factors, cell proliferation and cancer : an overview. Mol. Biol. Med. 1 169-181.

Sanger, J.W. (1975). Changing patterns of actin localization during cell division. Proc. Natn. Acad. Sci. U.S.A. 72 1913-1916.

Schmidt, G. & Thannhauser, S.J. (1945). A method for determination of deoxyribonucleic acid, ribonucleic acid, and phosphoproteins in animal tissues. J. Biol. Chem. 161 83-89.

Schneider, W.C. (1946). Phosphorus compounds in animal tissues. III. A comparision of methods for the estimation of nucleic acids. J. Biol. Chem. 164 747-751.

Schneider, W.C., Hogenboom, G.H. & Ross, H.E. (1950). Intracellular

distribution of enzymes. VII. The distribution of nucleic acids and adenosine tri-phosphatase in normal mouse liver and mouse hepatoma. J. Natn. Cancer Inst. 10 977-982.

Schollmeyer, J.E., Furcht, L.T., Goll, D.E., Robson, R.U. & Stromer, M.H. (1976). Cell motility, localization of contractile proteins in smooth muscle cells and in normal and transformed fibroblasts. In Cell Motility. Book-A. Motility, Muscle and Non-muscle Cells (eds. Goldman, R., Pollard, T. & Rosenbaum, J.). pp 361-388. Cold Spring Conf. Cell Proliferation. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Schroeder, T.E. (1968). Cytokinesis: Filaments in the cleavage furrow. Expl. Cell Res. 53 272-318.

Schroeder, T.E. (1969). Actin in dividing cells : contractile ring filaments bind heavy meromyosin. Proc. Natn. Acad. Sci. U.S.A. 70 1688-1692.

Schroeder, T.E. (1973). Actin in dividing cells : contractile ring filaments bind heavy meromyosin. Proc. Nat. Acad. Sci. U.S.A. 70 1688-1692.

Seehar, G.M. (1978). The Control of the Cell Cycle in Fibroblasts. Ph.D. thesis submitted to the University of Glasgow.

Shaffer, B.M. (1963). Behaviour of particles to amoebae of the slime mould Polysphondylium violaceum and the fate of the cell surface during locomotion. Expl. Cell Res. 32 603-606.

Shaffer, B.M. (1965). Mechanical control of the manufacture and

resorption of cell surface in collective amoebae. J. Theoret. Biol. 8 27-40.

Silverton, R.E. & Anderson, M.J. (1961). Handbook of Medical Laboratory Formulae. Butterworth & Co. (Publishers) Ltd. pp. 388-389.

Singer, S.J. (1971). The molecular organization of biological membranes. In "Structure and function of biological membranes" (ed. Rothfield, L.I.) pp 145-222. Acad. Press, New York.

Small, J.V., Isenberg, G. & Celis, J.E. (1978). Polarity of actin at the leading edge of cultured cells. Nature (London). 272 638-639.

Small, J.V., Riennerthaler, G. & Hinssen, H. (1982). Organization of actin meshworks in cultured cells: The leading edge. Cold Spring Harbor Symp. Quantitative Biol 46 pp 599-611. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Spooner, B.S., Yamada, K.M. & Wessels, N.K. (1971). Microfilaments and cell locomotion. J. Cell Biol. 49 595-613.

Steel, R.G.D. & Torrie, J.H. (1960). Principles and Procedures of Statistics. McGraw-Hill Book Co. Inc., N.Y., Toronto, London.

Stoker, M.G.P. (1973). Role of diffusion boundary layer in contact inhibition of growth. Nature (London). 246 200-203.

Stoker, M.G.P., O'Neill, C., Berryman, S. & Waxman, V. (1968). Anchorage and growth regulation in normal and virus transformed cells. Intn. J. Cancer. 3 683-693.

Stossel, T.P. & Hartwig, J.H. (1976). In "Cell Motility" (eds. Goldman,

R.D., Pollard, T.D. & Rosenbaum, J.). Book B. pp 529-544. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Szabo, G. (1972). Lipid bilayer membranes. In "Membrane Molecular Biology" (eds. Fox, C.F. & Keith, A.D.). pp 146-163. Sinauer Associates, Inc., Stamford, CT.

Tannenbaum, J. & Rich, A. (1979). An isoelectric focusing study of plasma actin. Anal. Biochem. 95 236-244.

Taylor, A.C. & Robbins, E. (1963). Observations on microextensions from the surface of isolated vertebrate cells. Develop. Biol. 7 660-673.

Taylor, D.G., Williams, V.M. & Crawford, N. (1976). Platelet membrane actin : solubility and binding studies with ^{125}I-labelled actin. Biochem. Soc. Trans. 4 156-160.

Tilney, L.G. (1975). Actin filaments in the acrosomal reaction of Limulus sperm: Motion generated by alterations in the packing of the filaments. J. Cell Biol. 64 289-310.

Tilney, L.G., Hatano, S., Ishikawa, H. & Mooserker, M. (1973). The polymerization of actin. Its role in generation of the acrosomal process of certain echinoderm sperms. J. Cell Biol. 59 109-126.

Todaro, G.J. Lazar, G. & Green, H. (1965). The initiation of cell division in a contact-inhibited mammalian cell line. J. Cell Comp. Physiol. 66 325-333.

Tyner, E.P., Heidelberger, C. & LePage, G.A. (1953). Intracellular distribution of radioactivity in nucleic acid nucleotides and proteins following simultaneous administration of ^{32}P and Glycine-2-^{14}C . Cancer

Res. 13 186-203.

Vakaet, L (1962). Some new data concerning the formation of the definitive endoblast in the chick embryo. J. Embryol. exp. Morph. 10 38-57.

Van Dilla, M.A., Trujillo, T.T., Mullaney, P.F. & Coulter, J.R. (1969). Cell microfluorometry: A method for rapid fluorescence measurement. Science. 163 1213-1214.

Vasiliev, J.M. (1982). Pseudopodial attachment reactions. In Cell Behaviour (eds. Bellairs, R., Curtis, A. & Dunn, G.). pp 135-138. Cambridge University Press, Cambridge, U.K.

Vasiliev, J.M., Gelfand, I.M., Domnina, L.V., Ivanova, O.Y., Komm, S.G. & Olshevskaja, L.V. (1970). Effect of colcemid on the locomotory behaviour of fibroblasts. J. Embryol. exp. Morph. 24 625-640.

Vesely, P. & Weiss, R.A. (1973). Cell locomotion and contact inhibition of normal and neoplastic rat cells. Intn. J. Cancer. 11 64-76.

Volkin, E. & Cohn, W.E. (1954). Estimation of nucleic acids. In Methods of Biochemical Analysis (ed. Glick, D.). 1 pp 287-305. Inter-science Publishers Inc., New York.

Wang, K., Ash, J.F. & Singer, S.J. (1975). Filamin, a new high molecular weight protein found in muscle and non-muscle cells. Proc. Natn. Acad. Sci. U.S.A. 72 4483-4486.

Weatherbee, J.A. (1981). Membranes and cell movement : Interactions of membranes with the proteins of the cytoskeleton. Int. Rev. Cytol. Suppl. 12 113-176.

Weber, K. (1976). Biochemical anatomy of microfilaments in cells in tissue culture using immunofluorescence microscopy. In "Contractile Systems in Non-muscle Tissues" (eds. Perry, S.V., Margreth, A. & Adelstein, R.S.). pp 51-66. North-Holland Publishing Co., Amsterdam.

Weber, K. & Groeschel-Stewart, U. (1974). Antibody to myosin: the specific visualisation of myosin containing filaments in non-muscle cells. Proc. Natn. Acad. Sci. U.S.A. 71 4561-4564.

Weiss, P. (1929). Erzwingung elementarer strukturverschiedenheiten am in vitro wachsenden gewebe. Archiv fur Entwicklungsmechanik der organismen 116 S 438-554.

Weiss, P. (1941). Nerve patterns: The mechanics of nerve growth. Growth (Suppl.) 5 163-203.

Weiss, P. (1945). Experiments on cell and axon orientation in vitro : The role of colloidal exudates in tissue organization. J. Exp. Zool. 100 353-386.

Weiss, P. (1958). Cell contact. Intn. Rev. Cytol. 7 391-423.

Wessels, N.K., Spooner, B.S., Ash, J.F., Bradley, M.O., Luduena, M.A., Wrenn, J.T. & Yamada, K.M. (1971). Microfilamnents in cellular and developmental processes. Science. 171 135-143.

Whitfield, J.F., Boynton, A.L., Macmanus, J.P., Sikorska, M. & Tsang, B.K. (1979). The regulation of cell proliferation by calcium and cyclic AMP. Mol. Cell Biochem. 27 155-179.

Whittenberg, B. & Glaser, L. (1978). Cell saturation density is not determined by a diffusion-limited process. Nature (London). 272 821-

823.

Wimber, D.E. & Quastler, H. (1963). A ^{14}C- and ^{3}H-thymidine double labelling technique in the study of cell proliferation in Tradescantia root tips. Expl. Cell Res. 30 8-22.

---

* Only abstracts published.

**R Rapid Communication.

*** Correspondence only.

APPENDIX 1.

DOES TENSION AFFECT THE PROPORTION OF CELLS UNDERGOING S-PHASE AT VARIOUS PA RTS IN CULTURE ?

(DURATION OF TENSING = 3 HOURS)

Treatments-	Proportion of Labelled Cells in each Repetition										Treatment Tota	
Position	I	II	III	IV	V	VI	VII	VIII	IX	X	Xi	$\sum_j X_{ij}^2$
0.0 Hz, First Hr. Label-												
Corner	15	10	05	20	10	15	10	05	10	15	115	1525
Edge	15	20	25	10	25	25	20	25	15	20	200	4250
Centre	00	00	00	00	00	00	00	00	00	00	000	0000
0.0 Hz, Final Hr. Label-												
Corner	05	15	15	20	10	15	10	15	05	20	130	1950
Edge	20	30	10	15	15	20	15	20	30	15	190	4000
Centre	00	00	00	00	00	00	20	00	00	20	40	800
0.01 Hz, First Hr. Label-												
Corner	35	30	40	35	30	20	45	35	25	40	335	11725
Edge	15	15	20	20	20	20	15	20	30	15	190	3800
Centre	00	00	00	00	00	00	00	00	00	00	000	0000
0.01 Hz, Final Hr. Label-												
Corner	40	20	15	20	25	25	10	30	15	20	220	5500
Edge	25	15	20	10	15	10	20	15	10	15	155	2625
Centre	00	00	20	20	00	00	20	00	00	00	60	1200
0.1 Hz, First Hr. Label-												
Corner	20	30	45	25	40	40	10	05	25	35	275	9125
Edge	25	10	10	30	05	45	45	55	40	40	305	12025
Centre	20	80	00	00	10	100	40	40	10	00	300	20200
0.1 Hz, Final Hr. Label-												
Corner	30	20	10	45	15	30	30	15	45	30	270	8600
Edge	15	20	30	35	20	35	40	20	30	15	260	7500
Centre	20	20	20	20	60	40	00	80	00	20	280	13600
1.0 Hz, First Hr. Label-												
Corner	25	20	15	30	35	50	20	30	30	40	295	9675
Edge	30	30	25	30	40	20	15	15	25	25	255	7025
Centre	20	20	40	00	00	00	40	00	00	00	120	4000
1.0 Hz, Final Hr. Label-												
Corner	30	30	35	25	25	30	30	25	40	20	290	8700
Edge	05	15	20	10	15	10	05	10	05	10	105	1325
Centre	20	20	00	00	00	00	20	00	00	20	80	800
Repetitions Total X_j	430	460	420	420	415	550	480	460	390	435	4470	
Block Total $\sum_i X_{ij}^2$	10750	15200	10800	11350	12025	24250	14150	17550	11500	11575		139950

APPENDIX 2.

TOTAL OF PROPORTION OF LABELLED CELLS AT THREE POSITIONS IN CONTROL (UNTENSED) AND EXPERIMENTAL(TENSED) SAIL-SHEET CULTURES (DURATION OF TENSING = 3 HOURS).

Frequency of Tension "A"	First/Final Hr. Label	Total of Proportion of Labelled Cells Localised at Various Parts of the Cultures "B"*			Total
		Corner b_1	Edge b_2	Centre b_3	$b_1 + b_2 + b_3$
0.0 Hz	First = C_1	115	200	000	315
	Final = C_2	130	190	40	360
	Total = $C_1 + C_2$	245	390	40	675
0.01 Hz	First = C_1	335	190	00	525
	Final = C_2	220	155	60	435
	Total = $C_1 + C_2$	555	345	60	960
0.1 Hz	First = C_1	275	305	300	880
	Final = C_2	270	260	280	810
	Total = $C_1 + C_2$	545	565	580	1690
1.0 Hz	First = C_1	295	255	120	670
	Final = C_2	290	105	80	475
	Total = $C_1 + C_2$	585	360	200	1145
Total	First = C_1	1020	950	420	2390
	Final = C_2	910	710	460	2080
	Total = $C_1 + C_2$	1930	1660	880	4470

* Total of proportion of labelled cells from 10 repetitions.

APPENDIX 3.

TEST OF SIGNIFICANCE OF DATA ON THE POSITIONAL LOCALISATION OF PROPORTION OF LABELLED CELLS AT 95% CONFIDENCE INTERVAL (DURATION OF TENSING = 3 HOURS).

Source of Comparision	Difference in Mean Proportion of Labelled Cells Between two Positions in the Cultures ± 14.9 **	
	With First Hr. Label	With Final Hr. Label
0.0 Hz (Control)		
a b - a b 1 1 1 2	- 8.5 ± 22.4, 6.4	- 6.0 ± -20.9, 8.9 *
a b - a b 1 2 1 3	20.0 ± 5.1, 34.9	15.0 ± 0.1, 29.9
a b - a b 1 1 1 3	11.5 ± - 3.4, 26.4 *	9.0 ± - 5.9, 23.9 *
0.01 Hz		
a b - a b 2 1 2 2	14.5 ± - 0.4, 29.4 *	6.5 ± - 8.4, 21.4 *
a b - a b 2 2 2 3	19.0 ± 4.1, 33.9	9.5 ± - 5.4, 24.4 *
a b - a b 2 1 2 3	33.5 ± 18.6, 48.4	16.0 ± 1.1, 30.9
0.1 Hz		
a b - a b 3 1 3 2	- 3.0 ± -17.9, 11.9 *	1.0 ± -13.9, 15.9 *
a b - a b 3 2 3 3	- 0.5 ± -15.4, 14.4 *	- 2.0 ± -16.9, 12.9 *
a b - a b 3 1 3 3	- 2.5 ± -17.4, 12.4 *	- 1.0 ± -15.9, 13.9 *
1.0 Hz		
a b - a b 4 1 4 2	4.0 ± -10.9, 18.9 *	18.5 ± 3.6, 33.4
a b - a b 4 2 4 3	13.5 ± - 1.4, 28.4 *	- 2.5 ± -17.4, 12.4 *
a b - a b 4 1 4 3	17.5 ± 2.6, 32.4	21.0 ± 6.1, 35.9 *

* Non-significant at 95% confidence interval.

** Estimated standard error for 95% confidence interval.

APPENDIX 4.

DOES TENSION AFFECT THE PROPORTION OF CELLS UNDERGOING S-PHASE AT VARIOUS PARTS IN CULTURE ?

(DURATION OF TENSING = 6 HOURS)

Treatments	Proportion of Labelled Cells in each Repetition										Treatment Totals	
Position	I	II	III	IV	V	VI	VII	VIII	IX	X	X_i	$\sum_j X_{ij}^2$
0.0 Hz, First Hr. Label-												
Corner	30	15	30	15	20	15	15	20	15	20	195	4125
Edge	15	10	10	20	25	15	15	10	15	20	155	2625
Centre	00	00	20	00	00	00	00	00	20	00	40	800
0.0 Hz, Final Hr. Label-												
Corner	15	20	10	20	15	10	15	10	20	15	150	2400
Edge	10	15	15	15	20	25	05	10	10	20	145	2425
Centre	00	00	00	00	00	00	20	00	00	00	20	400
0.01 Hz, First Hr. Label-												
Corner	30	35	30	35	35	25	30	40	10	30	300	9600
Edge	10	20	15	30	20	20	15	40	25	15	210	5100
Centre	00	00	00	00	00	20	00	00	00	00	20	400
0.01 Hz, Final Hr. Label-												
Corner	55	55	50	45	70	65	30	60	60	40	530	29400
Edge	25	25	15	40	20	25	45	25	40	40	300	9950
Centre	40	40	40	20	40	20	60	20	20	40	340	13200
0.1 Hz, First Hr. Label-												
Corner	40	25	45	40	40	30	35	30	40	40	365	13675
Edge	10	15	05	05	20	20	20	30	15	10	150	2800
Centre	00	00	00	00	00	20	00	00	00	00	20	400
0.1 Hz, Final Hr. Label-												
Corner	40	40	30	15	40	35	30	40	30	10	310	10650
Edge	05	45	30	30	20	40	10	40	15	15	250	8000
Centre	20	20	20	20	20	20	00	20	20	40	200	4800
1.0 Hz, First Hr. Label-												
Corner	35	25	40	20	35	20	15	30	25	30	275	8125
Edge	20	25	00	15	15	15	15	10	15	10	140	2350
Centre	00	00	00	00	00	00	00	00	00	00	000	000
1.0 Hz, Final Hr. Label-												
Corner	55	35	30	25	45	20	40	45	20	35	350	13450
Edge	50	35	35	20	20	30	30	25	30	15	290	9300
Centre	20	40	00	60	00	60	00	00	60	00	240	12800
Repetitions Total X_j	525	540	470	490	520	550	445	505	505	445	4995	
Block Total $\sum_i X_{ij}^2$	18975	18000	15150	15800	18450	18300	14325	17575	16775	13425		166775

APPENDIX 5.

TOTAL OF PROPORTION OF LABELLED CELLS AT THREE POSITIONS IN CONTROL (UNTENSED) AND EXPERIMENTAL (TENSED) SAIL-SHEET CULTURES (DURATION OF TENSING = 6 HOURS).

Frequency of Tension "A"	First/Final Hr. Label	Total of Proportion of Labelled Cells Localised at Various Parts of the Cultures "B"* Corner b_1	Edge b_2	Centre b_3	Total $b_1 + b_2 + b_3$
0.0 Hz	First = C_1	195	155	40	390
	Final = C_2	150	145	20	315
	Total = $C_1 + C_2$	345	300	60	705
0.01 Hz	First = C_1	300	210	20	530
	Final = C_2	530	300	340	1170
	Total = $C_1 + C_2$	830	510	360	1700
0.1 Hz	First = C_1	365	150	20	535
	Final = C_2	310	250	200	760
	Total = $C_1 + C_2$	675	400	220	1295
1.0 Hz	First = C_1	275	140	00	415
	Final = C_2	350	290	240	880
	Total = $C_1 + C_2$	625	430	240	1295
Total	First = C_1	1135	655	80	1870
	Final = C_2	1340	985	800	3125
	Total = $C_1 + C_2$	2475	1640	880	4995

* Total of proportion of labelled cells from 10 repetitions.

APPENDIX 6.

TEST OF SIGNIFICANCE OF DATA ON THE POSITIONAL LOCALISATION OF PROPORTION OF LABELLED CELLS AT 95% CONFIDENCE INTERVAL (DURATION OF TENSING = 6 HOURS).

Source of Comparision	Difference in Mean Proportion of Labelled Cells Between two Positions in the Cultures ± 17.5 **	
	With First Hr. Label	With Final Hr. Label
0.0 Hz (Control)		
a b - a b 1 1 1 2	4.0 ± -13.5, 21.5 *	0.5 ± -17.0, 18.5 *
a b - a b 1 2 1 3	11.5 ± - 6.0, 29.0 *	12.5 ± - 5.0, 30.0 *
a b - a b 1 1 1 3	15.5 ± - 2.0, 33.0 *	13.0 ± - 4.5, 30.5 *
0.01 Hz		
a b - a b 2 1 2 2	9.0 ± - 8.5, 26.5 *	23.0 ± 5.5, 40.5
a b - a b 2 2 2 3	19.0 ± 1.5, 36.5	- 4.0 ± -21.5, 13.5 *
a b - a b 2 1 2 3	28.0 ± 10.5, 45.5	19.0 ± 1.5, 36.5
0.1 Hz		
a b - a b 3 1 3 2	21.5 ± 4.0, 39.0	6.0 ± -11.5, 23.5 *
a b - a b 3 2 3 3	13.0 ± - 4.5, 30.5 *	5.0 ± -12.5, 22.5 *
a b - a b 3 1 3 3	34.5 ± 17.0, 52.0	11.0 ± - 6.5, 28.5 *
1.0 Hz		
a b - a b 4 1 4 2	13.5 ± - 4.0, 31.0 *	6.0 ± -11.5, 23.5 *
a b - a b 4 2 4 3	14.0 ± - 3.5, 31.5 *	5.0 ± -12.5, 22.5 *
a b - a b 4 1 4 3	27.5 ± 10.0, 45.0	11.0 ± - 6.5, 28.5 *

* Non-significant at 95% confidence interval.

** Estimated standard error for 95% confidence interval.

APPENDIX 7.

DOES TENSION AFFECT THE PROPORTION OF CELLS UNDERGOING S-PHASE AT VARIOUS PARTS IN CULTURE ?

(DURATION OF TENSING = 12 HOURS)

Treatments– Position	Proportion of Labelled Cells in each Repetition I	II	III	IV	V	VI	VII	VIII	IX	X	Treatment Total X_i	$\sum_j X_{ij}^2$
0.0 Hz, First Hr. Label–												
Corner	25	15	40	30	15	35	25	15	25	40	265	7875
Edge	25	25	20	30	40	10	35	20	20	25	250	6900
Centre	20	00	00	00	00	00	20	00	00	20	60	1200
0.0 Hz, Final Hr. Label–												
Corner	20	30	25	30	25	40	20	25	15	25	255	6925
Edge	15	10	00	10	05	05	10	10	30	05	100	1600
Centre	00	00	20	00	00	00	00	00	00	00	20	400
0.01 Hz, First Hr. Label–												
Corner	40	50	70	60	80	55	100	60	60	85	660	46450
Edge	50	80	75	50	90	60	95	65	35	80	680	49600
Centre	60	20	20	20	20	00	00	20	20	00	180	6000
0.01 Hz, Final Hr. Label–												
Corner	40	45	25	40	20	35	40	35	30	40	350	12800
Edge	20	35	30	30	40	50	75	20	10	20	280	9050
Centre	40	40	40	00	40	40	40	40	40	00	320	12800
0.1 Hz, First Hr. Label–												
Corner	60	65	35	60	45	65	65	35	50	40	520	28450
Edge	40	50	65	65	45	65	35	70	25	90	550	33650
Centre	20	40	00	20	00	40	40	40	40	40	280	10400
0.1 Hz, Final Hr. Label–												
Corner	45	35	40	40	50	50	45	15	50	30	400	17100
Edge	60	40	55	45	45	20	15	15	60	15	370	16950
Centre	80	40	40	40	40	20	00	40	80	00	380	21200
1.0 Hz, First Hr. Label–												
Corner	45	55	80	55	45	55	45	70	70	60	500	34950
Edge	40	60	25	50	35	65	50	70	60	35	490	26000
Centre	00	40	00	20	00	40	00	00	20	40	160	5600
1.0 Hz, Final Hr. Label–												
Corner	45	50	30	35	35	60	20	30	35	30	370	
Edge	15	35	65	20	40	30	30	25	25	25	310	11350
Centre	00	60	20	00	00	40	40	20	40	20	240	9600
Repetitions Total X_j	805	920	820	750	755	880	795	740	840	765	8070	
Block Total $\sum_i X_{ij}^2$	37075	44300	41300	32800	37225	43200	42325	34300	39550	39675		391750

APPENDIX 8.

TOTAL OF PROPORTION OF LABELLED CELLS AT THREE POSITIONS IN CONTROL (UNTENSED) AND EXPERIMENTAL (TENSED) SAIL-SHEET CULTURES (DURATION OF TENSING = 12 HOURS).

Frequency of Tension "A"	First/Final Hr. Label	Total of Proportion of Labelled Cells Localised at Various Parts of the Cultures "B"*			Total
		Corner b_1	Edge b_2	Centre b_3	$b_1 + b_2 + b_3$
0.0 Hz	First = C_1	265	250	60	575
	Final = C_2	255	100	20	375
	Total = $C_1 + C_2$	520	350	80	950
0.01 Hz	First = C_1	660	680	180	1520
	Final = C_2	350	280	320	950
	Total = $C_1 + C_2$	1010	960	500	2470
0.1 Hz	First = C_1	520	550	280	1350
	Final = C_2	400	370	380	1150
	Total = $C_1 + C_2$	920	920	660	2500
1.0 Hz	First = C_1	580	490	160	1230
	Final = C_2	370	310	240	920
	Total = $C_1 + C_2$	950	800	400	2150
Total	First = C_1	2025	1970	680	4675
	Final = C_2	1375	1060	960	3395
	Total = $C_1 + C_2$	3400	3030	1640	8070

* Total of proportion of labelled cells from 10 repetitions.

APPENDIX 9.

TEST OF SIGNIFICANCE OF DATA ON THE POSITIOINAL LOCALISATION OF PROPORTION OF LABELLED CELLS AT 95% CONFIDENCE INTERVAL (DURATION OF TENSING = 12 HOURS).

Source of Comparision	Difference in Mean Proportion of Labelled Cells Between two Positions in the Cultures ± 25.7 **	
	With First Hr. Label	With Final Hr. Label
0.0 Hz (Control)		
a b - a b 1 1 1 2	1.0 ± -24.7, 26.7 *	15.5 ± -10.2, 40.7 *
a b - a b 1 2 1 3	19.0 ± - 6.7, 44.7 *	8.0 ± -17.7, 33.7 *
a b - a b 1 1 1 3	20.0 ± - 5.7, 45.7 *	23.5 ± - 2.7, 49.2 *
0.01 Hz		
a b - a b 2 1 2 2	- 2.0 ± -27.7, 23.7 *	7.0 ± -18.7, 32.7 *
a b - a b 2 2 2 3	50.0 ± 24.3, 75.7	- 4.0 ± -29.7, 21.7 *
a b - a b 2 1 2 3	48.0 ± 22.3, 73.7	3.0 ± -22.7, 28.7 *
0.1 Hz		
a b - a b 3 1 3 2	- 3.0 ± -28.7, 22.7 *	3.0 ± -22.7, 28.7 *
a b - a b 3 2 3 3	27.0 ± 1.3, 52.7	- 1.0 ± -26.7, 24.7 *
a b - a b 3 1 3 3	24.0 ± - 1.7, 49.7 *	- 2.0 ± -27.7, 23.7 *
1.0 Hz		
a b - a b 4 1 4 2	9.0 ± -16.7, 34.7 *	6.0 ± -19.7, 31.7 *
a b - a b 4 2 4 3	33.0 ± 7.3, 58.7	- 7.0 ± -32.7, 18.7 *
a b - a b 4 1 4 3	42.0 ± 16.3, 67.7	13.0 ± -12.7, 38.7 *

* Non-significant at 95% confidence interval.

** Estimated standard error for 95% confidence interval.

Appendix 10.

% INHIBITION OF DNAse-I DUE TO G-ACTIN AND TOTAL (G+F) ACTIN/10 MICROGRAMS PROTEIN IN CONTROL AND TENSED CULTURES AT 260 nm.

Culture Type	% DNase-I inhibition due to G-actin Mean (S.D.)	% DNase-I inhibition due to total (G+F) actin Mean (S.D.)	Repetition
Experiments			
Sail-Sheets tensed for :			
(i) 3 Hours	17.6 (10.4)	35.1 (5.7)	4
(ii) 12 Hours	60.8 (3.0)	72.7 (4.2)	4
Controls			
Sail-Sheet	47.2 (6.6)	83.6 (0.3)	2
Conventional	55.1 (3.3)	66.9 (4.9)	2