# An Investigation into Factors that Cause Inhibition of the Growth of Animal Cells *In Vitro*.

by

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*For Gearoid, Rebecca, Amy and baby Hannah.*

**I hereby certify that this material, which I now submit for the assessment of the programme of study leading to the award of M.Sc is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work .**

**Signed:** Angela O'Toole **I.D. number:** 87700808.

**Date:** 5/2/97

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# ABSTRACT

In the last two decades, much work has focused on factors causing growth limitations of animal cells in large scale culture systems and on strategies to overcome these limitations. It is agreed that growth is limited by a combination of environmental factors and by growth inhibitory molecules produced by the cells themselves. Identification of cell lines which may be resistant to some or all of any potentially growth limiting factors would be of considerable theoretical and industrial use.

The aim of the work undertaken in Section 3.1 of this thesis was to identify and characterise growth inhibitors produced by animal cells when growing *in vitro*. It was found that MSV-3T3 cells produce and secrete factors which either inhibit the growth or have a cytotoxic effect on epithelial carcinoma cells *in vitro*. Either one factor or an aggregate of factors may be responsible for the cytotoxic activity observed. This activity was variable and was seen in some molecular weight fractions and not in others suggesting perhaps that the factors responsible may exist as:- an inhibitory molecule and stabiliser entity, a precursor and protease entity or in a TGFα/TGFβ complement existence. The results suggest that the molecules responsible for the cytotoxic activity are unusually stable entities with high heat and pH resistance.

As well as endogenous growth inhibitors, environmental factors such as agitation intensity, osmotic pressure, temperature, oxygen levels and pH may also limit the growth of animal cells *in vitro*. The build up of inhibitory metabolic waste products such as ammonia and lactate should also be considered. Section 3.2 of this thesis presents investigations on environmental factors which may cause inhibition of animal cell growth in scaled up *in vitro* systems. A decrease in apparent growth rates was noted with extremes of agitation, osmotic pressure, lactate and ammonia. Of particular interest in this work was the observation that while high agitation rates caused a decrease in the growth of CHO-K1 cells, no such growth limitation was seen in their multi-drug resistant (MDR) counterparts, CHRC5, at high agitation rates. This observed agitation resistance in the MDR cells (CHRC5) was consistently noted in a number of different experiments. An increased agitation resistance was also noted in the MDR cell lines, Hep-2A when compared to its MDR sensitive parent, Hep-2 and DLKPA (MDR) when compared to DLKP. Investigations on the plasma membrane fluidity of CHRC5 and DLKPA suggests that there is a correlation between increased agitation resistance and decreased plasma membrane fluidity in the two MDR cell lines studied. If it is considered that MDR may be conferred on cells by over-expression of membrane proteins, (for example, p-glycoprotein in CHRC5 cells), then an increased protein content in the cell membrane may lead to an increased packing density giving a more rigid structure with decreased plasma membrane fluidity and so resulting in an increased shear/agitation resistance being exhibited by the cells. In additon, lactate sensitivity was noted in the two MDR cell lines, CHRC5 and Hep-2A when compared to their parent cells.

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# Abbreviations

aFGF - acidic fibroblast growth factor
ATCC - American Type Tissue Collection
ATP -
bFGF - basic fibroblast growth factor
cm - conditioned medium
D.O. - dissolved oxygen
$D_i$ = impeller diameter
DME - Dulbecco's Modified Eagles Medium
DMSO - dimethyl sulphoxide
DTT - dithiothreitol
EDTA -
EGF - epidermal growth factor
ELISA - Enzyme Linked ImmunoSorbent Assay
FCS - foetal calf serum
FGF - fibroblast growth factor
HEPES - (4-(2-HydroxyEthyl)-Piperazine EthaneSulphonic) acid
IGF - insulin-like growth factor
IMS - industrial methylated spirits
LAK - lymphocyte-activated killer
MEM - Minimal Eagles Medium
MEM NEAA - MEM non-essential amino acids.
MW - molecular weight
MWCO - molecular weight cut off
n.d. - not determined
$NaHCO_3$ - sodium bicarbonate.
NBCS - new born calf serum
PBS - phosphate buffered saline
PDGF - platelet derived growth factor
PMF - plasma membrane fluidity.
RT-PCR - reverse transcriptase polymerase chain reaction
TGF-$\alpha$ - transforming growth factor alpha
TGF-$\beta$ - transforming growth factor beta
TMA-DPH - 1-[4-trimethyl-amino phenyl] -6-phenylhexa-1, 3, 5-triene
TNF - tumour necrosis factor
uncm - unconditioned medium, ie.medium control

1000R - retentate produced when cm passed through a 1000 molecular weight membrane during ultrafiltration.

# CHAPTER ONE
# INTRODUCTION

## 1.1: General Introduction

The mass culturing of animal cells *in vitro* in order to produce important diagnostic and therapeutic biologicals has become an integral part of the Biotechnology Industry. Products derived from animal cells currently account for half the profits generated by the 'new' Biotechnology industries. The growing need for correct post-translational modification of proteins will inevitably consolidate this position for the forseeable future. As the limitations of exclusively utilising microbial cultures to produce biological products for human use have been realised, the last ten years have seen a definite trend towards licensing of products such as monoclonal antibodies, viral vaccines, hormones, immunological regulators and recombinant proteins, all of which are produced from animal cells. However the cultivation of animal cells may be difficult given that the cells are larger, more fragile and more complex than bacteria. The cytoplasmic membrane is not covered by a tough cell wall and the cells are more susceptible to damage by osmotic pressure, shear and other cytotoxic effects. The cell nutritional requirements are more stringent and although still not fully optimized , demand a complex low molecular weight medium, which often must be supplemented with serum. The cells grow more slowly, cell productivites are low and the cultures are more liable to contamination by faster growing bacterial or fungal cells. The high sensitivity of the cells demands strict control of fermentation conditions: temperature, pH, dissolved oxygen and the supply of nutrients combined with the adequate removal of waste products.

Maintaining homeostasis in the culturing environment is important for promoting cell productivity, thus suspension cultures in stirred tank reactors are emerging as the preferred large scale cultivation technique. Much of the sterilisation, automation and inoculation technology developed for microbial suspension cultures has been directly applied to suspension culture bioreactors for growth of animal cells. Stirred bioreactors allow ease of scale up, homogeneous reactor conditions and ease of control and monitoring of reactor conditions. However maximum cell densities comparable with those attained by cells in their natural physiological environment of the tissue are not being achieved in stirred reactors, although great improvements in cell numbers are observed when the bioreactor is operated in a continuous perfusion mode.

A considerable body of research in the last two decades has consequently focused on what may be causing growth limitations in large scale animal cell culture and on strategies to overcome these limitations. Many questions have been answered. For example, it is known that animal cells are extremely shear sensitive due mainly to their lack of a protective cell wall. Consequently cultivation of cells in a high shear hydrodynamic environment is undesirable. Build up of metabolic waste products (e.g. ammonia and lactate) in batch culture has been shown to greatly inhibit growth, thus various strategies such as perfusion culture have been developed to try to reduce this problem. Optimisation of other potentially growth limiting factors such as pH, temperature, dissolved oxygen levels, etc. has also been investigated. However, even if the external environment is 'ideal', growth limitation may still occur. The importance, therefore, of endogenously produced growth inhibitors (small growth factor like proteins which have a negative effect on cell growth, for example TGF-β ) should not be underestimated. In conclusion, it may be suggested that the identification of cells which may be resistant to some or all of these potentially growth limiting factors would be desirable. In particular, cells more resistant to hydrodynamic damage would simplify the design, operation and productivity of most bioreactors.

## 1.2: A Review of Negative Regulators of Cell Proliferation

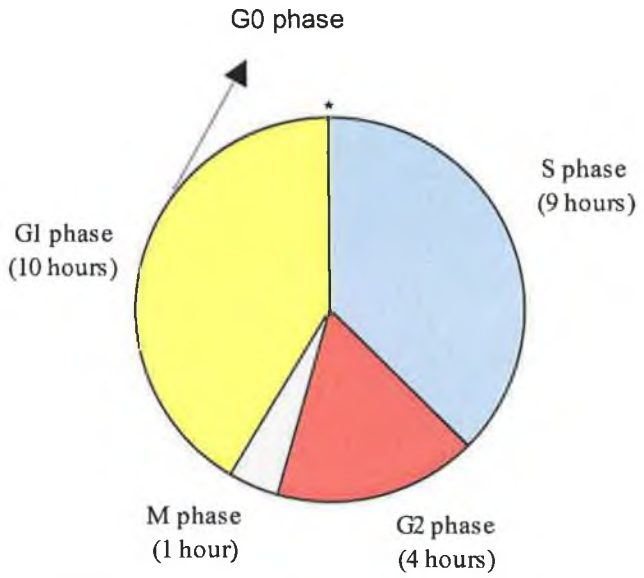
Animal cell growth and differentiation appears to be initiated, promoted, maintained and regulated by a multiplicity of stimulatory, inhibitory and synergistic factors and hormones (Miyazaki and Horio, 1989). Such regulatory molecules interact with specific cell surface receptors to mediate a cellular 'perception' of the external environment and to facilitate cell-cell communication (James and Bradshaw, 1984). The actions of these molecular signals in regulating cell cycling is of paramount importance to all multicellular organisms since the alteration/breakdown of cellular homeostasis is a fundamental cause of growth related diseases, the most important of these being cancer. However an understanding of these growth regulatory signals is vital to virtually all areas of biological sciences, including immunology and infectious disesases, embryogenesis and developmental biology, wound healing, programmed cell senescence and apoptosis (Johnson, 1994). Although significant progress has been made in defining the chemical/physical nature and the mode of action of growth factors there has been a paucity of information concerning similar features of inhibitory molecules that provide a negative signal of cell division.In this review it is hoped to provide a basic overview of cell growth regulation, to outline the role played by mitogenic/growth factors in that regulation and to concentrate in particular on the diversity and action of negative growth factors.

### 1.2.1: Cellular Homeostasis

Multicellular organisms must maintain checks and balances to regulate cell cycle events both with one another and with the needs of the larger organism. The ability to regulate when cells will grow, divide and differentiate into specialised cells is critical to the health and ultimately the survival of mature organisms. Cellular homeostasis, the delicate balance between cell production and cell removal in various tissues, depends to a large extent on two alternatives modes of existence, the quiescent and the proliferative, which in

turn depends on what stage of the cell cycle the cells occupy. Figure 1.1 is a schematic representation of the cell cycle (adapted from Alberts, 1994).

**Figure 1.1: Cell Cycle**




Where:

* = start of the cycle

S phase = DNA synthesis

G0 phase = quiescent synthesis

G1 phase = pre-DNA synthesis

G2 phase = post-DNA synthesis

M phase = mitosis

The rate of increase of a population of cells is primarily dependent on the fraction of cells that are in cycle or G1 as contrasted to those in G0. Cells can remain healthy and viable in a quiescent stage for a long time, both *in vivo* and *in vitro*. A critical regulatory event occurs at a restriction or start point in the G1 phase (represented as * in Figure 1.1). Extracellular factors will determine whether a quiescent cell (in G0) will begin to proliferate and whether a normal proliferating cell in G1 will continue to cycle or will revert to quiescence. Cell cycle events become largely independent of extracellular factors after cells enter into S phase, where they will go on to divide and produce two daughter cells. These latter processes such as mitosis depend on intracellularly triggered controls (Hartwell and Weinert, 1989).

The transition from G1 to G0 requires a mitogenic stimulus. Polypeptide growth factors are considered powerful mitogens capable of inducing quiescent animal cells *in vitro* to re-enter the cell cycle to proliferate. However both positive signals (mitogens and tumour promoters) and negative signals (inhibitors and tumour suppressors) can interact at this point thus influencing the ultimate decision of the cell to divide or not to divide. Movement from G1 to S, through * is also controlled in part by growth factors, in addition to nutrients and hormones. These agents act by controlling the accumulation of cyclin prior to * which forms a complex directly with a protein cdc-2. This complex directly and indirectly initiates all events of mitosis (Murray and Kirschner, 1991).

Two approaches to study the growth regulation of cells, based on the model described above for the cell cycle, have been proposed. One claims that cells are naturally quiescent, requiring a stimulatory encounter with growth factors for induction of cell division. The other considers cellular multiplication as the natural steady state; cessation of multiplication is thus a restriction imposed on the system. In the latter case emphasis is mainly on the signals involved in arrest of multiplication. In the following sections positive and negative growth regulators will be discussed.

### 1.2.2: Growth Factors

Growth factors are polypeptides which interact with specific cellular receptors, evoking pleiotropic biological responses including modulation of the differentiated phenotype, changes in cell motility and cytoskeletal structure and altered proliferation rate (Pusztai *et al* 1993). Most mammalian cells secrete a range of growth factors and respond to a variety of these molecules in an autocrine (where cells synthesize and respond to their own specific growth signal) and paracrine (where the growth factor diffuses a short range through intracellular spaces and acts locally) manner. Growth factors may be grouped into distinct families based on sequence homology and

structural similarities of the peptides. Examples of growth factor families are given in Table 1.1.

Table 1.1 Growth Factor Families (Adapted from Pusztai *et al.* 1993)

| **Growth Factor Family** | **Source** | **Target Cells** | **Major Effect** |
|---|---|---|---|
| *Epidermal growth factor family* | | | |
| EGF (Gill et al, 1987) | epithelial cells | epithelial, mesenchymal cells | mitogen |
| TGF-α (Brachmann et al, 1989) | tumour-derived, transformed cells | epithelial, mesenchymal cells | mitogen, transforming for fibroblasts |
| Amphiregulin (AP) (Plowman et al, 1990) | carcinoma cells | epithelial, mesenchymal cells | mitogen for fibroblasts, inhibits breast carcinoma cell growth |
| *Heparin binding growth factor family* (Burgess and Maciag1989) | | | |
| aFGF | ubiquitous | ubiquitous | mitogen, angiogenesis, neurotrophic |
| bFGF | ubiquitous | ubiquitous | mitogen, motility factor |
| int-2 oncoprotein (FGF-3) | embryonic cells | fibroblasts | mitogen |
| hst oncoprotein (FGF-4) | embryonic cells | fibroblasts | mitogen |
| FGF-5 | fibroblasts | fibroblasts | mitogen |
| Keratinocyte growth factor (KGF) | stromal cells | keratinocytes | mitogen, angiogenesis |
| vascular endothelial cell growth factor (VEGF) | monocytes/ macrophages | vascular endothelial cells | mitogen, angiogenesis, vascular |

| | | | |
|---|---|---|---|
| *Platelet derived growth factor family* (Heldin and Westermark, 1989) | | | |
| PDGF | mesenchymal cells, platelets | mesenchymal cells | mitogen |
| v-sis-viral oncoprotein | Simian sarcoma virus | fibroblasts | mitogen |
| Insulin-like growth factor family (Cullen, Yee and Rosen, 1991) | | | |
| IGF-I | ubiquitous | ubiquitous | mediates growth hormone activity, insulin-like effects |
| IGF-II | ubiquitous | ubiquitous | insulin-like effects |
| *Transforming growth factor-β family* (Massague, 1990) | | | |
| TGF-$\beta_1$ - $\beta_2$ - $\beta_3$ - $\beta_4$ | ubiquitous | ubiquitous | mitogen for fibroblasts, inhibits proliferation of many cell types, angiogenesis |
| *Growth factors not classified into the above families* | | | |
| Hepatocyte growth factor (HGF) (Nakamura et al, 1989) | many cell types | hepatocytes, epithelial cells | mitogen |
| Platelet -derived endothelial growth factor (PDEGF) (Ishikawa et al, 1989) | platelets | endothelial cells | angiogenesis, mitogen |
| Endothelin-1 (Shichiri et al, 1991) | many cell types | endothelial, mesenchymal cells | vasoconstriction, mitogen |

It has become evident that many growth factors have a much wider range of action than the biological activity for which they were originally named. In addition the range of cell types that respond to the growth factors is much wider than originally determined. Significant progress has been made in defining the chemical/physical nature and the mode of action of growth factors (Pusztai *et al*, 1993). Growth factors exert most of their effects on target cells via interaction with a family of specific cell surface receptors. The biological response to any given growth factor depends on the cell type and differentiation stage of the cell, as well as on other stimuli simultaneously acting on the cell. Two factors together may have a different effect to either one alone. Furthermore it has been found that most growth factors receptors interact with a whole family of growth factors with members of the same receptor family evoking distinct biological responses.

The mechanisms of cell proliferation regulation by growth factors are currently being explored. However a simplified model of growth regulation by Pusztai *et al* (1993) is as follows. Growth factor binding to a receptor activates the receptors through dimerisation and conformational changes which trigger an array of biochemical events including protein phosphorylation, diacyl glycerol, inositol phosphate, prostaglandin and cyclic nucleotide generation and alterations in intracellular $Ca^{2+}$, $Na^{+}$ and $H^{+}$ ion concentrations. These events bring about cytoskeletal and morphological changes, alterations in the metabolic pathways of glucose and protein synthesis, which eventually culminate in cell cycle progression. Recent research has found that some viral oncogenes have cellular counterparts, proto-oncogenes, which are expressed as growth factors or components of growth factor receptors (Bishop, 1991).

### 1.2.3: Negative Regulation of Growth

Much work has been carried out on the identification and characterisation of growth factors, however cell proliferation would appear to be restricted not only by limitations in the supply of these mitogenic factors or the activity of their receptors but also by the action of negative growth regulators. It would seem reasonable to assume that the exquisite control exerted on cell proliferation must, in part at least, involve negative or inhibitory mechanisms that slow down or inhibit cell growth. Thus the cell proliferation rate is likely to be determined by a balance of stimulatory and inhibitory signals with malignant cells perhaps having a diminished sensitivity to the inhibitory factors. To date a number of different classification of growth inhibitors have been suggested (Hsu and Wang, 1986; Miyasaki and Horio, 1989; Laiho *et al*, 1990) and these will be reviewed presently.

The negative regulation of cell growth appears to be complex process involving factors which may act in conjunction with one another or in isolation. In addition these factors may act only on a 'part time basis' as dictated by the cellular context (Massague and Weinberg, 1992). Consequently the task of identifying cellular components/factors that have a negative effect on growth is complicated. It is worth noting that most growth inhibition studies to date have been carried out *in vitro* however there is evidence to suggest that these results cannot be simply extrapolated to the *in vivo* situation. For example, the well documented inhibitory activity of TGF-β *in vitro* contrasts with its tumour promoting activties *in vivo* (Pusztai *et al*, 1993).

For the pupose of this review it is proposed to consider that growth inhibitors fall into two main classes:

1. Those factors that are products of genes acting at the nuclear level as negative signals to influence transcription of protein-protein and protein-

DNA interactions and play a regulatory role in cell proliferation, differentiation and related processes.

2. Those factors associated with the cell surface whose action depends on interacting via receptors with the cell to induce a negative signal. These include;

- polypeptides which can decrease the rate of cell transit through the proliferative cycle in a fully reversible manner (for example, TGF-β, interferons and oncostatin M)
- growth regulators that facilitate cell differentiation with permanent exit from the cell cycle (for example, nerve growth factor (NGF) or insulin-like factor (IGF))
- polypeptides which can induce cell death, for example, TNF-α.

The first class of factors mentioned here refer mainly to the tumour suppressor genes and associated products which have been extensively reviewed in the literature in recent years (Sager, 1989; Boyd and Barrett, 1990; Marshall, 1991; Weinberg, 1991; Massague and Weinberg, 1992). However, since the work on growth inhibitors described in this thesis is mainly concerned with factors in the second class it is proposed to concentrate most of this review on these factors and not on the tumour suppressor genes.

### 1.2.3.1: Molecular Mechanisms of Growth Inhibition and Tumour Suppressor Genes

The molecular mechanism of growth inhibition induced by growth inhibitors has yet to be elucidated. It is frequently coupled with down-regulation of oncogenes (*fos*, *myc* ) expression and the induction of new proteins absent in proliferating cells (Sorrentino, 1989). Differential hybridisation techniques applied to cDNA libraries generated from growth inhibited cells identified a number of genes associated with growth arrest (*gas*, quiescins and prohibitin) ( Schneider, King and Philipson, 1988). The function of these molecules is still obscure. This approach has been extended by the use of the functional

properties of genes encoded by cDNAs in a bioassay of growth arrest. Using this strategy where putative growth arrest cDNAs are microinjected into fibroblasts, Nuell *et al* have defined the gene product prohibitin to be a 272 amino acid protein expressed by resting but not proliferating normal fibroblastic and HeLa cells. There appears to be remarkable homology between this protein and Cc a *Drosophila* gene vital for development (Nuell *et al*, 1991).

Recent observations have indicated a possible link between growth inhibition and anti-oncogene (or tumour suppressor gene) activation. Over the past five years a large body of data has converged on the conclusion that cells often lose critical genetic information during their evolution from a normal to a malignant state. These discarded genes seem to act to constrain normal cell growth. Their loss liberates the cell from the growth constraints imposed by these genes, thus they have been called tumour suppressor genes. Several independent experimental strategies have demonstrated the existence of tumour suppressor genes. These include, the retinoblastoma gene, *Rb* (Friend *et al*, 1986), *p53*(Oren, 1985), a gene deleted in colon carcinoma (DCC) (Fearon *et al*, 1990) and a gene deleted in Wilms' tumour (Gessler *et al*, 1990).

Tumour suppressor genes may be linked to growth inhibition by the following model (Massague and Weinberg, 1992). Tumour suppressor proteins may act as transducers of signals that impinge on the cell and cause the cell to stop proliferating. The extracellular signals may be carried by a series of diffusable factors, including TGF-β. Tumour suppressor proteins would seem to be integral parts of the mechanisms that enable cells to acquire and process inhibitory signals. When a suppressor protein is deleted from the mechanism then the cell loses its ability to respond to such exogenous signals and continues active proliferation (as in the case of cancer cells). Laiho *et al* (1990) have demonstrated that TGF-β prevents phosphorylation of the retinoblastoma gene product and arrests cells in late G1 of the cell cycle,

indicating that these molecules participate in grwoht inhibitory cascades. Moreover, deletion of the *Rb* gene renders cells resistant to TGF-β inhibition. The DCC suppressor gene product is a transmembrane receptor. Loss of this protein accompanies and perhaps drives the conversion of many colonic adenomas into carcinomas (Fearon *et al*, 1990). These workers speculate that DCC acts as a signal transducing receptor that enables a normal colonic cell to sense and respond to growth inhibitory signals present in the extracellular matrix. Each of these tumour suppressor proteins seem to act as a critical link in a complex chain of anti-mitogenic signals. The identities of the remaining components of these signalling pathways are as yet unknown, however these may be resolved in the near future.

1.2.3.2: Growth Inhibitors

The negative control of mechanism of cell growth by growth inhibitors was first proposed by Weiss (Weiss and Kavanau, 1957). Bullough (1962) then reported that regenerative growth of mouse epidermal cells after skin injury might be caused by a major loss of a tissue-specific mitotic inhibitor , which was thought to be continuously produced and secreted from the epidermal cells to self-regulate their growth. The term **chalone** was given to this putative class of molecules that acted as inhibitory growth regulators. However, despite attempts to isolate chalones from skin, liver, lymphoid and other tissues, the problem of characterisation of these molecules using techniques available at that time proved too difficult (Patt and Houck, 1980). In recent years, the existence of negative regulators of cell growth has been proven with the identification and characterisation of numerous compounds involved in the inhibition of cell growth (Miyazaki and Horio, 1989). The proceeding sections will detail some of these inhibitors and their mode of actions beginning with the well known factors, TGF-β, IFN-γ and TNF.

1.2.3.3: Transforming Growth Factor-β

One of the most well documented growth inhibitors is TGF-β and members of the TGF-β family. TGF-β (now known as TGF-β1) was originally isolated and characterised for its ability to stimulate the growth of normal fibroblast cells in soft agar (Moses *et al*, 1981). However it was subsequently found to be the most potent growth inhibitor known for a wide range of cell types, both normal and transformed. These include, almost all epithelial, endothelial, neuronal and hematopoietic cells (Barnard, Lyons and Moses, 1990; Massague, 1990); lymphoid (B and T cells) (Sporn and Roberts, 1990) and some mesenchymal and myeloid cells. It has also been identified as hormonally regulated growth inhibitor of breast cancer cells (Knabbe *et al*, 1987). Existing as a family of several closely related polypeptides, TGF-β isolated from human, mouse, bovine and porcine species exhibits an unusually evolutionarily conserved amino acid sequence.Another family member, Mullerian inhibiting substance (MIS) induces regression of the female reproductive tract primordium in mammalian male embryos.

TGF-β is found in a wide variety of foetal and adult tissues and is isolated as a 25kDa polypeptide dimer. The inhibitory activity of TGF-β appears to be associated with its binding to either a 53kDa or 500-600kDa cell membrane high affinity receptor (Boyd and Massague, 1989).A third TGF-β binding component, the membrane proteoglycan betaglycan, may also be involved (Laiho *et al*, 1991). However, although TGF-β is one of the most intensively studied growth regulators the mechanism by which it mediates cell cycle arrest is complex and largely unresolved. As stated earlier (Laiho *et al*, 1990) , TGF-β may mediate its inhibitory activity in certain cells by an inhibition of several growth related genes. It has been proposed that the *Rb* protein is the necessary element in the pathway for the TGF-β suppression of *c-myc* expression in mouse kertinocytes (Murphy *et al*, 1991). This effect is significant because *c-myc* is required for the progression of keratinocytes into S phase.

1.2.3.4: Interferons

Interferons exert a wide range of biological effects. These include anti-viral activity, inhibition of protein synthesis, induction of gene expression and cell cycle arrest (Rubin and Gupta, 1980). Although all classes of the IFN family have been shown to bind high affinity cell surface receptors and to have antiprolifertive activity, ;most of the work investigating growth inhibition has been carried out on IFN-γ.

IFN-γ has been shown to abolish the mitogenic activity of endothelial cell growth factor on cultured human and murine endothelial cells (Friesel *et al*, 1987). Although IFN-γ did not directly compete with the growth factor cell surface receptors, the incubation of endothelial cells with IFN-γ downregulated the receptors by some unknown mechanism, thus presumbably decreasing the cellular response to the growth factor. IFN-γ has also been shown to block the mitogenic activity of PDGF, EGF and FGF (Olezak, 1988). The inhibition of cell proliferation seems to be cell cycle specific since IFN-γ was not effective after cells already had progressed into the S phase of the cell cycle.

The mechanisms by which IFNs mediate their inhibition of cell proliferation is still under study. Investigators have shown several possible sites of action the signal transduction level including:- events associated with the expression of the retinoblastoma (*Rb*) gene (Kumar and Atlas, 1992); proto-oncogene expression (Mundschau and Faller, 1992) and many others. The members of the IFN family would appear to exhibit cell growth regulatory activities that are consistent with the characteristics that serve to typify tumour suppressor gene product.

1.2.3.5: Tumour Necrosis Factor

Tumour necrosis factor (TNF) is a multifunctional cytokine produced mainly by activated macrophage, T cells, mast cells and some epithelial cells. TNF has a wide range of biological effects including haemorrhagic necrosis of normal cells, cytotoxicity, inflammation, immunoregulatory and antiviral responses. The characterisation and biological activity of TNF has been extensively reviewed in the literature (Camussi *et al*, 1991; Jaattela, 1991 ). TNF-α is characterised by the extremely pleiotropic nature of its actions This multiplicity of actions may be explained by the following;

- TNF receptors are present on virtually all cells examined
- TNF action leads to the activation of multiple signal transduction
- TNF action leads to the activation of an unusually large array of cellular genes (Vilcek and Lee, 1991).

There are two different receptors for TNF (TNF-R1 with molecular weight of 55kDa and TNF-R2 with molecular weight of 75kDa). These two receptors have been shown to mediate distinct cellular responses (Tartaglia *et al*, 1991). TNF-R1 appears to initiate signals for cytotoxicity and TNF-R2 initiates signals for thymocyte and cytotoxic T cell proliferation. The amount of each receptor expressed on different cell lines appears to vary and this may explain to a certain extent the range of activities displayed by TNF (Vilcek and Lee, 1991). It has been shown that at extreme concentrations of TNF the cytotoxic effect is decreased somewhat (Galloway *et al*, 1991).

1.2.3.6: Review of Known Growth Inhibitors Published in the Literature.

TGF-β, interferons and TNFα are the most documented of the known growth inhibitors. TGF-β, interferons being examples of the 'classical' reversible inhibitor mentioned in section 1.2.3 and TNFα an example of a growth inhibitor resulting in cell death. In the last 15 years a number of putative growth inhibitors have been identified and to a certain characterised, although

compared with the knowledge presently available concerning the structure and biological properties of growth factors research on growth inhibitory molecules is still in its infancy. Table 1.2 outlines some of the negative regulators identified in the literature and summarises the information currently known.

Table 1.2: Review of Known Growth Inhibitors

| **Growth Inhibitor** | **Comments** | **Reference** |
|---|---|---|
| Mammostatin | • 2 species, 47kDa and 65kDa fractions<br>• isolated from cm of normal human mammary cells<br>• tissue specific (chalone-like) only inhibits mammary derived cells<br>• heat labile, trypsin sensitive | Ervin *et al*, 1989 |
| Mammary derived growth inhibitor (MDGI) | • 13kDa protein<br>• isolated from bovine mammary tissue + ascitic fluid from Erlich mammary cells<br>• inhibits human mammary carcinoma cells<br>• heat labile, perhaps glycoprotein, membrane associated<br>• amino acid homology to *fibroblast growth inhibitor* | Lehmann *et al*, 1983<br>Bohmer *et al*, 1985<br>Brandt *et al*, 1988 |
| Glial maturation factor (GMF-β) | • 17kDa acidic protein (pI 4.6)<br>• found in astrocytes in brain<br>• reversible inhibitor for neuronal and non-neuronal cells<br>• competes with mitogenic activity of aFGF. | Lim *et al*, 1989<br>Lim *et al*, 1990 |

| | | |
|---|---|---|
| Contactinhibin | • 60-70kDa glycoprotein<br>• thought to play important role in density dependent growth inhibition<br>• isolated from confluent human fibroblasts<br>• reversible inhibitor against human and mouse fibroblasts<br>• thought to be integral membrane component - not heat labile or reduced by protein hydrolysis | Weiser and Oesch, 1986 Weiser, 1990. |
| Bovine brain cell surface glycopeptide | • 2 species, 10kDa and 45kDa fractions<br>• isolated from bovine cerebral cortex cells<br>• reversible non-toxic inhibitor of BHK and CHO cells<br>• arrests cycling in G2 phase of cell cycle | Kinders Rintoul and Johnson, 1982 Bascom *et al*, 1985. |
| Epithelins (1 and 2) | • 5-6kDa species<br>• isolated from rat kidney<br>• inhibit A431 cells and other carcinoma cells but may have mitogenic effects also<br>• may exist in an agonist/antagonist<br>• acid, base resistant; heat stable; protease sensitive | Shoyab *et al*, 1990 |

| | | |
|---|---|---|
| Colon carcinoma inhibitor | • 27kDa active fraction<br>• isolated from cm of colon carcinoma cell line, HT-29<br>• autoinhibitory effect<br>• significant homology to IGFBP (IGF binding protein) | Culouscou and Shoyab, 1991. |
| Tumour cytotoxic factor (F-TCF) | • 30-34kDa heterodimer<br>• isolated from cm of human embryonic lung fibroblasts<br>• cytotoxic against various tumour cells,<br>• bifunctional, ie appears to be mitogenic against some cells<br>• pH stable (6-9), heat labile (70°C)<br>• appears to be different from TNF, IFNβ and lymphotoxin<br>• may be similar to hepatocyte growth factor | Iwata *et al*, 1985 Higashio *et al*, 1990 |
| Melanoma inhibiting activity (MIA) | • 3 species, MIA2 most active - 14kDa<br>• isolated from human melanoma cells<br>• auto-inhibitory effect<br>• heat stable (100°C/3mins); protease labile<br>• exerts an irreversible, but not cytotoxic effect | Bogdahn *et al*, 1989 |
| Inhibitor of rat mesangial cell growth | • < 3kDa molecule<br>• isolated from cm of primary glomerular cultures<br>• inhibits rat mesangial cells, no affect on mouse fibroblasts<br>• reversible effect, protease stable | Groggel and Hughes, 1993 |

| | | |
|---|---|---|
| Lytic factor isolated from LAK cells | • isolated from enriched plasma membranes prepared from highly purified LAK cells<br>• effective against large range of cells<br>• trypsin labile; heat labile<br>• activity not blocked by antibodies to TNFα, lymphotoxin, IFNγ | Felgar and Hiserodt, 1992 |
| Fibroblast inhibitor from rat macrophages | • < 3kDa molecule<br>• isolated from rabbit macrophages<br>• not species specific, inhibits fibroblasts from human and rabbit source<br>• protease stable, heat stable | Concannon, M.J. *et al*, 1993 |
| Rabbit serum growth inhibitory proteins, GI-I and GI-II | • GI-I (18kDa)/ GI-II (36kDa)<br>• isolated from rabbit serum in latent form<br>• inhibit transformed rat cells<br>• cytostatic and reversible effect at low conc./ cytotoxic and irreversible effect at high conc.<br>• protease, acid, heat labile; stable to reducing agent | Kimura *et al*, 1992 |
| Epithelial cell growth inhibitor (PII) | • 15kDa molecule<br>• isolated from cultured intestinal epithelial cells<br>• non-cytotoxic, reversible effect against epithelial cells<br>• appears to cause cells to arrest in G1 phase of cell cycle | Lavagna *et al*, 1990 |

| | | |
|---|---|---|
| Growth inhibitor from macrophages | • 2.5-6.5kDa range<br>• isolated from cm of murine macrophages<br>• reversible inhibitory effect against 3T3 fibroblasts and porcine cells<br>• trypsin labile; heat stable | McMurray *et al*, 1993 |
| Growth inhibiting activity from tumourogenic Chinese hamster cells | • isolated in supernatants of neoplastic V79 Chinese hamster cells<br>• auto-inhibitory effect, also inhibits wide range of tumour cells but not normal fibroblasts<br>• non-species specific, reversible<br>• acid, heat, trypsin and DTT stable<br>• α-chymotrypsin labile | Ichinose *et al*, 1993 |

It can be seen that a large number of negative growth regulators have been identified. However only a few of these have been purified to homogeneity and more importantly very little is known about their mode of action. It is commonly agreed among the scientific community that it has been easier to characterise and purify stimulatory factors than it has been to do the same for inhibitory factors. There are many reasons for this, but the most important one is that there are many non-specific factors that inhibit growth and may modulate differentiation in cell culture. For example, Miyazaki *et al* (1990) have shown the inhibition of a human tumour cell line in their laboratory was not due to a true inhibitor but was caused by the depletion of arginine in the culture medium by the enzyme deiminase produced by mycoplasma infection. Consequently it made be stated that the search for specific growth stimulators has been much more successful than that for inhibitors. As more is learned about negative regulators it is reasonable to suspect that their impact in a biological sense will broaden. It is most likely that many of these factors eventually will also be shown to play a fundamental role in a wide array of biological processes including developmental and cell differentiation events, immune cell interactions, wound healing and tumourigenesis (Johnson, 1994).

## 1.3: A review of the effects of shear and other environmental factors on the growth of animal cells *in vitro.*

### 1.3.1: Introduction

*In vitro* cultures of animal cells are hosts for an increasing assortment of biological products. Efficient production of these biologicals requires an understanding of the influence that the culturing environment has on cell growth and product yield. Products include viral vaccines, insecticides, enzymes, hormones, monoclonal antibodies and immunological regulators (interleukins and lymphokines). Such products mostly represent low volume/high cost products, but in some cases the scale of operation can rise to several thousand litres. The importance of the culture environment is determined to a great extent by the type of bioreactor system used to grow animal cells on a large scale. Many different forms of bioreactor types are available (Griffiths, 1988). However, the choice of reactor depends on whether the cells exhibit anchorage dependent growth characteristics and must be attached to some form of solid matrix support or whether the cells can grow freely in suspended culture. Anchorage dependent cells are usually derived from 'normal' tissue with an untransformed phenotype, examples include diploid fibroblasts, primary cells and all the cell lines used over the last two decades for the production of veterinary vaccines. Anchorage independent (suspension) cells are usually derived from the haemopoeitic system or are cells of a malignant origin. The most commercially important anchorage independent cell type are hybridomas used for the production of monoclonal antibodies.

### 1.3.2: Animal Cell Bioreactor Design

The cultivation of microorganisms in bioreactors is well documented and in most cases their properties and characteristics are very similar. Possible exceptions include ; fungi where the hyphal structure produces either pellets or hyphal mats; cultures which produce high levels of extracellular polysaccharides (e.g. *Xanthomonas*) making the medium highly viscous; and algae which require light in order to grow. The broadly similar cultural characteristics of microorganisms mean that a single bioreactor design can, in general, be used to cultivate a wide range of microorganisms. However the bioreactor design and cultivation of cells from plants, insects and animals is more complicated due to their different characteristics. Table 1.3. highlights some of the cultural properties of microbial, plant and animal cell suspensions and the physical and metabolic differences between them.

Table 1.3: The Characteristics of Microbial, Plant and Animal Cultures.

| Characteristics | Microbial Cells | Plant Cells | Animal Cells |
|---|---|---|---|
| Size | 2-10μm | 10-200μm | 5-100μm |
| Individual Cells | often | aggregates up to 2 mm generally form | often, also many require a surface for growth |
| Growth Rate | rapid, doubling times of 1-2 hrs | slow, doubling time of 2-5 days | slow, doubling time 12-20 hrs |
| Inoculation Density | low | high, 10% | low |
| Shear Stress Sensitivity | not sensitive | sensitive and tolerant | sensitive |
| Aeration Requirements | high | low | low |

As can be seen plant and animal cell suspensions are generally slow growing, often require attachment to surfaces for growth, have specific nutrient requirements and may be sensitive to shear stress. Consequently for the cultivation of animal and plant cells a wide range of bioreactor designs have

been developed, this is particularly true of animal cell cultures where there is variety in both the bioreactor types and in the manner in which the various designs are employed.

Animal cell bioreactors may be operated in a batch, repeated fed-batch, chemostat or perfusion mode (Reuveny *et al*, 1986). In batch operation conditions in the medium change continuously with respect to time, owing to the depletion of substrates and the build up of waste products. This compares unfavourably with the living tissue, in which the cells are provided with a highly stable environment by an efficient circulation system. Batch operation provides the cells with a highly fluctuating and uncontrolled environment which can therefore never be optimal. Fed-batch and repeated fed-batch operation (Ingham *et al*, 1984) have the advantage that the supply of nutrients to the cells can be controlled to some extent by the feeding of essential nutrients during cultivation.

Given the extreme sensitivity of animal cells to their environment, however, more attention is being focused on the more constant and controllable conditions provided by continuous operation and perfusion. Continuous operation as in chemostat or turbidostat operation has many advantages (Feder and Tolbert 1985, Tolbert *et al* 1986). These include steady-state operation at controlled specific growth or cell density conditions. Specific nutrient limitations may be easily effected and deliberate manipulation of the cell physiology for optimal productivity can be obtained. Other advantages include reduced capital cost, increased media utilisation, reduced downtime and continuous removal of waste products from the reactor environment. Continuous operation however requires good control facilities and high standards of plant containment to avoid contamination.

Perfusion systems in which the cells are provided with a continuous supply of fresh medium and continuous withdrawal of waste products closely approaches the *in vivo* system and are particularly advantageous in cases in

which the kinetics of product formation are unrelated to growth. Frequently, perfusion is used in conjunction with an external recycle loop which recirculates medium to the cells at a controlled temperature, dissolved oxygen, pH, etc.

1.3.2.1: Anchorage dependent growth systems

As stated previously animal cell bioreactor design is dependent upon whether cells are anchorage dependent or anchorage independent. Attached systems for large scale growth of anchorage dependent cells require a design with a large surface area to volume ratio which will allow adequate nutrient supply, waste removal and ease of harvesting. The standard flat incubator T flask provides a specific surface area of up to 200 $cm^2/l$. Scaling up from flasks, roller bottles can provide between 600-1600 $cm^2/l$ surface area and are still used in industry for cultivation of cells (e.g. veterinary vaccine production) and as starting cultures for large scale suspension fermentations. Internal disks, coils, tubular and spherical inserts have been used to increase the internal surface area of roller bottles.

Various types of multi-tube unit have been developed, for example, multi-tray units, stacked plate units and plastic tube or film units. Multi-tray units (1500-2000$cm^2/l$ surface area) consist of sets of horizontal trays fixed vertically above one another and connected by channels for nutrient and gas flow. The main problem of unit scale-up is to maintain a uniform distribution of both cells and medium. Packed bed reactors can provide upto 18,000 $cm^2/l$ surface area and are a simple, reliable and easy to scale-up design. A wide variety of packing materials can be used and the packed beds can also be operated as a circulating perfusion system. However there may be uneven distribution of cells, difficulties in controlling cell environment and in monitoring cell growth processes, since direct measurement of cell concentration is very difficult. These disadvantages may apply to all the previously mentioned methods.

One of the best and most widely used attached systems is the hollow fibre bioreactor which allows very high surface area per unit volume ( 40,000$cm^2$/l) combined with very high cell densities (upto 2 x $10^8$ cells/ml). The cells normally grow externally on the tube surface and are fed by medium diffusing through the porous fibres. Products may be removed either by flushing the cells or by allowing product to diffuse back in the opposite direction to the nutrients. Separate fibre systems may be used to supply medium and oxygen to the cells. Hollow fibre units are relatively expensive and owing to the multi-layered nature of the cell growth, cells may suffer diffusional substrate limitation. Direct measurement of cell growth is not possible and instead substrate utilisation rates or metabolite production rates must be relied on for an indirect measure of growth. However hollow fibres units are to date the only bioreactor systems that allow cells to grow to cell densities approaching those found in the *in vivo* tissue environment.

Undoubtedly of all the types of surface growth bioreactors, the one with the easiest scale-up potential is the microcarrier system (Van Wezel, 1967). Microcarriers are small solid or semi-solid spheres of 50 to 200μm diameter whose surface provide a substratum to which cells may attach. The cells growing on the microcarriers may then be cultivated in a stirred tank bioreactor. The resulting culture condition thus resembles that of a standard stirred-tank suspended culture. A wide range of microcarriers is commercially available (for example, dextran beads, plastic beads) and these enable most cell types to be cultivated. The use of microcarriers offers advantages of high surface area per unit volume (25,000-70,000 $cm^2$/l), reliable scale-up, traditional stirred tank technology, ease of cell sampling/measurement (the only attached system to allow direct cell monitoring) and ease of removal of cells from the depleted medium. Disadvantages include the relatively high cost of microcarrier beads and the slow-stirring requirement for low shear damage which can then lead to possible oxygen supply limitation. Culturing in a perfusion mode, whilst enabling high cell concentrations, is advantageous in

this instance in that it permits a more economic use of the high cost microcarrier beads since the cells are easily retained within the reactor.

1.3.2.2: Anchorage Independent Growth Systems

Anchorage independent cells do not require a surface for attachment in order to grow and are easily cultivated in suspension cultures . Suspension systems have the advantages that they are relatively easy to scale up, the rate of cell growth may be measured directly and environmental conditions may be controlled. The traditional method of suspension culture used for low volumes (200ml - 20l) is the spinner flask which can have various types of agitator ranging from magnetic bar stirrers to propellers, the general requirement being to provide an adequate distribution of the cells through the medium under minimum shear conditions.

The stirred tank bioreactor is the next step in scaling up growth and is the most commonly used bioreactor type for growing animal cells. It is used mainly for suspended cells but it also supports anchorage dependent cells when grown on microcarriers. The conventional stirred tank bioreactor must be modified for animal cell cultivation. A round bottomed vessel is used with slow moving agitators and no baffles in order to minimise fluid shear. Under low cell density conditions, sufficient aeration may be obtained directly from the head space above the liquid surface as long as the aspect ratio is approximately 1:1. At higher cell densities additional oxygen transfer to the cells maybe provided by the direct sparging of air or oxygen into the vessel. Sparging of air however can lead to cell damage caused by bubble rupture (Handa *et al*, 1987). As an alternative to sparging, an additional supply of oxygen by means of lengths of silicone tubing immersed in the bioreactor may be utilised. The use of silicone tubing to aerate bioreactors was developed by Lehmann (Lehmann *et al*, 1987, 1988) to provide high gas transfer rates in a bubble free mode of operation. Airlift bioreactors may be used for animal cell cultivation since they lack any moving parts or mechanical seals and hence

involve a greatly reduced risk of contamination . The associated shear conditions are relatively gentle and airlift bioreactors have been used industrially to grow hybridomas for monoclonal antibody production (Birch *et al*, 1987).

### 1.3.3: Aim of Literature Review

The major biochemical engineering problem encountered in culturing animal cells on a large scale is the provision of the best environmental conditions (biological, chemical and physical) for both ample cell growth and optimal specific productivity of cells. To achieve this end the necessary inorganic nutrients and organic substrates must be supplied with the correct pH, ionic strength and below inhibitory concentrations. Build up of metabolic waste products must be minimised or removed from the culture environment. Temperature, osmotic pressure, dissolved oxygen, agitation intensity and other physical parameters must be optimised. Consequently it is a challenge to researchers and industry to design bioreactor systems and specify the optimal operating conditions for the requisite transient chemical and physical environment. The aim of this chapter is to review the literature with respect to some of these environmental factors that lead to loss of cell viability. Particular emphasis is placed on the shear sensitivity of animal cells and the consequent influence of agitation intensity in a bioreactor.

### 1.3.4: Shear Sensitivity In Animal Cell Culture

To achieve efficient cell propagation, the cell culturist needs to provide an *in vitro* environment as similar as possible to the natural cell environment. As the scale of cultivation increases, mixing of the culture medium is usually required to maintain uniform conditions and enhance the transport of limiting nutrients such as oxygen. Since mixing is accomplished by deformation of fluid elements, hydrodynamic stresses are transmitted to the cells (whether suspended or attached to a surface). The extent of cell damage by these forces depends on the magnitude of the shearing forces, the duration of forces, the cell type and to a certain extent the medium composition (Abu-Reesh and Kargi, 1989).In addition to information on the physical hydrodynamic environment, it is important to recognise the significance of cell structure and physiology in determining the shear sensitivity of the cells. Animal cells lack the protective cell wall found in prokaryotes, therefore they must rely solely on the cytoskeleton and plasma membrane to maintain their physical integrity. Consequently knowledge of the mechanism in which these cellular structures confer resistance to hydrodynamic injury would be useful for implementing methodologies tending to improve the native mechanical strength of a cell.

#### 1.3.4.1: Theory of Shear Forces in Bioreactors.

The forces acting on an organism suspended in a fluid are the result of the dissipation of kinetic energy, present in fluid in motion, to the external surface of the organism. The magnitude of these forces is a function of the fluid viscosity and the velocity gradient in the boundary layer surrounding a cell:

$$\tau = \mu \gamma = - \mu (dv / dy) \qquad (\textit{equation 1})$$

where $\tau$ is the shear stress, $\mu$ is the fluid viscosity and $\gamma$ is the rate of shear strain. The rate of shear strain (dv / dy)is the velocity gradient perpendicular to the surface of the organism. Two regimes may exist in a fluid, laminar in which the fluid flows in smooth layers and turbulent in which there is a vigorous interchange of small packets of fluid (eddies) between adjacent

layers of the fluid. The type of fluid flow field present in a given system is characterised by the dimensionless Reynolds number (Re);

$$Re = \rho v \ell / \mu \qquad \textit{(equation 2)}$$

where $\rho$, v and $\mu$ are the fluid density, velocity and viscosity respectively and $\ell$ is the characteristic length of the system. In an agitated reactor, typically, the characteristic length would be the impeller diameter. Most bioreactors are operated in the fully turbulent regime which is characterised by a Reynolds number of 1000 or greater (Nagata, 1975).

In laminar flow the shear stresses can be easily calculated from the fluid viscosity and the velocity profile of the system (equation 1 above). The situation is far more complex in the turbulent flow regime where a state exists such that the variations of velocity both in time and space are very irregular. A cell suspended in a laminar flow field will experience a mean shear stress that is independent of time, while a cell suspended in a turbulent flow field can experience bursts of shear stresses as well as dynamically oscillating forces. Evaluation of the Reynolds number will give a rough measure of the macro-scale turbulence, that is turbulence caused by eddies similar in size to the impeller blades. Micro-scale turbulence however, as will now be outlined, appears to be quite lethal and may cause the most cell damage.

An important feature of turbulence is the scale at which energy dissipation occurs. In a stirred tank bioreactor, kinetic energy is imparted to the fluid by the rotating impeller. Initially this energy is transported by turbulent eddies which are characterised by a length scale called the Kolmogorov microscale (Tennekes and Lumley, 1972; Cherry and Papoutsakis, 1988). In the turbulent flow of a stirred bioreactor, eddies range in size from the largest eddies with a scale of the order of the impeller diameter diameter to the smallest eddies whose scale is smaller than a microcarrier diameter. The impeller transfers energy to the largest eddies which in turn transfer their energy to smaller eddies. This energy cascade continues until the energy of the smallest eddies is lost as heat through viscous dissipation. The turbulence at the high

wavenumber range or small eddy size range is considered to be independent of the conditions of turbulence formation as well as locally isotropic. The inverse of the wavenumber, k, is taken to be equal to the eddy size.

Kolmogorov's theory of isotropic turbulence states that the properties of the smallest eddies are a function of energy dissipation rate per unit fluid mass, $\varepsilon$, and the kinematic viscosity,$\nu$ (see equation 3). It is proposed that cell damage from bulk-liquid turbulence can be correlated to the ratio of the Kolmogorov scale eddy size to bead diameter for microcarrier cultures (Cherry and Papoutsakis, 1986; Croughan *et al*, 1987) and to cell diameter for suspension cultures (Kunas and Papoutsakis, 1990; M$^{c}$Queen and Bailey, 1987). Damage initiates as the ratio approaches unity and intensifies at lower values. Thus eddies of the same size or smaller than cell particles (either microcarrier beads or suspended cells) cause high shear stresses on the cell surface, interparticle collisions and reactor-particle collisions (Cherry and Papoutsakis, 1986). For larger eddies, shearing and collisions are minimised as cell particles move in eddy streamlines. Kolmogorov's model for isotropic turbulence states that the order of magnitude of the smallest eddies is given by:

$$\eta = ( \nu^3 / \varepsilon )^{1/4} \qquad \textit{(equation 3)}$$

where $\eta$ is the size of the smallest eddy, $\nu$ is the kinematic viscosity and $\varepsilon$ is the energy dissipation rate per unit mass which may be defined as follows;

$$\varepsilon = ( N_p n^3 d_i^5 ) / V \qquad \textit{(equation 4)}$$

where n is the impeller speed, $d_i$ is the impeller diameter, $N_p$ is the dimensionless power number and V is the characteristic fluid volume. A more detailed consideration of the importance of Kolmogorov microscale eddies is undertaken in Section 1.3.4.4.1 when the effect of shear on microcarriers cultures is examined.

Quantitative studies of shear in stirred tank bioreactors is further complicated by the inhomogeneity of the flow fields in these vessels. In a stirred tank reactor there are regions of high shear stress and regions of low shear stress. The highest shear stresses are generated in the boundary layers surrounding

the impellers (van't Riet and Smith, 1975). The maximum time-averaged stresses occur in the trailing vortices in the wake of the blades generated as the impeller slashes through the liquid. In magnitude these stresses are directly proportional to the impeller Reynolds number. The mean shear stress in the bulk fluid drops off dramatically as a cell travels farther away from the impeller region. A further consideration in agitated reactor vessels is that suspended cells may experience cell-cell collisions and/or cell-impeller collisions. Damage will be a function of collision frequency and the amount of energy transmitted to the cells during the collision event (Cherry and Papoutsakis, 1986). The flow field will be modified by approaching collision partners such that suspended cells will be subjected to transiently increased shear stress levels. If the kinetic energy of the collision partners is sufficiently large a severe collision event may occur. Ways of evaluating collision severity will be discussed in a further section.

An integrated shear factor (ISF) has been defined to characterise hydrodynamic effects in a stirred reactor.

$$ISF = 2 \pi N D_i / ( D_t - D_i ) \qquad \textit{(equation 5)}$$

where N is the impeller rotation rate, $D_t$ is the tank diameter and $D_i$ is the impeller diameter. The integrated shear factor provides a measure of the strength of the shear field between the impeller and vessel wall. Hu (Hu, 1983) tested the criterion for different sizes of similar vessels and impellers and found that a decrease in cell density was associated with larger ISF values. However the ISF value is arbitrarily defined and lacks basic theory.

Conclusions

To understand the phenomena involved in hydrodynamic damage more fully, a fundamental description of fluid flow has to be adopted using results from the fluid-mechanical approach and basic equations of continuity, motion, energy balance, etc. It is not within the scope of this work to review some of the complex, computational fluid mechanical models that have been developed to describe the hydrodynamic environment. In summary it should

be noted that shear stress is a force per unit area acting on a surface in the tangential direction that arises from a variation in velocity. However it is often difficult to determine quantitatively the magnitude of shear stresses cells experience in many bioreactors because the shear stresses are not uniformly distributed. The flow field in a bioreactor is inherently transient, inhomogenous and anisotropic.

1.3.4.2: Inherent Fragility of Animal Cells

The extent of damage caused by shear is very much dependent on the magnitude of the shear forces and their duration. In section 1.3.4.1 a brief overview of what are shear forces and how they are generated in bioreactors was given. However it is important to realise at this point that the reason that consideration of shearing forces is so essential when scaling up the growth of animal cells is due to the basic structure of the cells themselves. Animal cells are inherently fragile due to the fact that they lack a protective cell wall and are of a relatively large size. Insight into why animal cells are sensitive to hydrodynamic shear stress can be obtained by examining four key aspects of cellular anatomy:-

1. The **cell membrane** which surrounds the cells and acts as the first line of defence against shearing forces.
2. The **cell cytoskeleton and the highly compartmentalised and viscous cytoplasm** which in addition to the outer cell boundary, absorbs some of the energy generated by turbulence.
3. The **cell size** which determines to what extent the kinetic energy present in turbulent flow will be adsorbed by the cell.
4. The presence of a mechanism to receive and amplify fluid mechanical stimuli.

The varying degree of sensitivity among different animal cell lines can be traced to fundamental differences in these criteria (Prokop and Bajpai, 1992).

Animal cells are surrounded by a lipid bilayer membrane, embedded with enzymes and structural proteins, which mediates communication between the cell and its environment. The lipid content of the membrane includes phospholipids, sphingolipids, glycolipids and sterols. The bilayer matrix is held together by a hydrophobic effect with the surrounding polar environment. As proposed by the fluid mosaic model of Singer and Nicolson (1972) the constituents of biological membranes are free to diffuse laterally, rotate about their major axes and oscillate about the normal to the membrane's plane. Traditionally these various motions as well as the degree of packing of the membane's components have been described collectively by the term '**plasma membrane fluidity (PMF)**' (Houslay and Stanley, 1982). The importance of PMF in determining the shear sensitivity of a cell line is considered in greater detail in Section 1.3.4.5.1. Turbulent eddies perform work on the cell membrane and expend their energy through the process of viscous dissipation. If the external cell boundary is deformable a portion of the work done on this boundary will be transmitted to the interior of the cells (Fischer, 1980). The cell membrane may experience microscopic deformation in which a significant portion of it may be affected.

The mechanical properties of the cytoplasm are largely determined by the cytoskeleton (Sato *et al*, 1984). The cytoskeleton is composed of several proteins which are capable of polymerising into long fibrous networks. The primary components are three interacting filament systems: the microtubules (tubulin polymers), the microfilaments (actin polymers) and the intermediate filaments. This multicomponent system mediates movement of the cell (Dunn and Heath, 1976) and of intracellular organelles and plays an important role in determining cell shape (Watt, 1986). The cytoskeleton is intimately associated with the cell membrane through specific protein interactions. The importance of the cytoskeleton to the mechanical strength of the cell is further considered inSection 1.3.4.6.

When the plasma membrane of certain cell types is acted on by mechanical stimuli, the cytoskeleton undergoes a rapid and dramatic reorganisation of its components (Franke *et al*, 1984; Prokop, 1991). Some cells possess receptors in the cell membrane which are sensitive to mechanical stress. The presence of such receptors implies that these cells have evolved specific regulatory responses to mechanical stimuli (Guharay and Sachs, 1984, 1985; Stockbridge and French, 1988). Stretch-sensitive receptors are actually believed to be ion channels which pass specific ions when mechanically stimulated (Guharay and Sachs, 1984). Localised membrane tension causes a subpopulation of ion channels to open. The signal is rapidly transmitted throughout the entire cell by ion channel networking via cytoskeletal elements. The flood of ions into or out of the cell is believed to be one of the first events in a signal amplification cascade.

1.3.4.3: Methods of Assessing Shear Sensitivity

There are two basic approaches to the study of shear sensitivity in animal cell cultures. The first involves investigating the effects of the shear stress to which a cell is exposed in the bioreactor of interest. The advantage of this method is that long term biological responses such as the specific growth rate, production rate and product yield can be measured as a function of the hydrodynamic environment. The problem with this approach is that most bioreactors are operated in the turbulent flow regime. Therefore, due to the chaotic nature of turbulent flow one can only obtain empirical information about the sensitivity of the cell that is specific to that reactor type and geometry. The complexity of the flow patterns in bioreactors and the difficulty of quantifying the magnitude of shear stress on these cells under agitation makes it difficult to ascertain in detail how flow affects cell growth and metabolism.

A second approach to assessing shear sensitivity is to expose cells to a well defined shear field. A number of flow devices producing known and

homogenous shear stresses have been investigated in order to correlate morphological or physiological changes in cells to a certain shear rate or stress. A summary of those flow devices that have been well documented in the literature is given in Table 1.4.

Table 1.4: Methods used to assess shear sensitivity.

| FLOW DEVICE | REFERENCE |
|---|---|
| Parallel Plates | Diamond et al, 1989. |
| | Eskin et al 1984 |
| | Frangos et al, 1985, 1988. |
| | Grimm et al, 1989 |
| | Ives et al, 1983 |
| | Kruegar et al, 1971 |
| | Levesque and Nerem, 1985 |
| Cylindrical Tubes | Augenstein et al, 1971 |
| | Goldsmith and Mason,1975 |
| | M$^{c}$Queen et al, 1987, 1989. |
| Concentric Cylinders | Abu-Reesh and Kargi, 1989 |
| | Chien et al, 1992 |
| | Chittur et al, 1988 |
| | Drasler et al, 1987 |
| | Peterson et al, 1988 |
| | Smith et al, 1987 |
| | Tramper et al, 1986 |
| | Tran-Son-Troy et al, 1986. |
| Cone and Plate | Brooks, 1984 |
| | Bussolari et al, 1982 |
| | Dewey, 1984 |
| | Francke et al, 1984 |
| | Goldblum et al, 1990 |
| | Tran-Son-Troy et al, 1984, 1987. |

All the devices mentioned produce well-defined flows useful for characterisation of the response (deformation, injury and lysis) to cells to various levels of stress. The main geometry's which have been used are the cone and plate and the concentric cylinders both of which were developed for

rheological measurements. The cone and plate devices maintain a constant value of the shear rate in the entire volume. In concentric cylinders the shear rate changes in the gap as a function of the radius, however the gap must be kept small in order to assure laminar flow. For a rotating inner cylinder and a stationary outer cylinder, the Taylor number can be defined as;

$$Ta = ((U\ h)\ /\nu)\ (h\ /\ R)^{1/2} \qquad \textit{(equation 6)}$$

where:U is the peripheral velocity of inner cylinder (cm/s), h is the gap width (cm), $\nu$ is the kinematic viscosity ($cm^2/s$) and R is the radius of inner cylinder (cm). If the Taylor number is less than 41.3 then laminar flow prevails in the gap. If Ta is between 41.3 and 400 then Taylor vortices (internal eddies in gap) will exist and if Ta is greater than 400 then turbulent conditions exist.

In a series of defined shear experiments, Dewey *et al*, (1984), investigated shear stresses experienced by endothelial (anchorage dependent) cells. Using vascular endothelial cells in a rotating cone and plate viscometer it was observed that a laminar shear stress of 5 to 10 dynes/$cm^2$ caused a time-dependent change in the cell shape from polygonal to ellipsoidal and that certain endothelial cell functions including fluid endocytosis, cytoskeletal assembly and non-thrombogenic surface properties were sensitive to shear stress. Levesque and Nerem (1985) subjected endothelial cells cultured on plastic coverslips to uniform laminar shear stresses of 10, 30 and 85 dynes/$cm^2$ in a parallel flow chamber. They reported that endothelial cells became more elongated under higher shear stresses or after longer exposure to shear. Augenstein *et al* (1971) utilized a capillary system to investigate the shear tolerance of mouse and human tumour cells. The cells were pumped through various lengths and diameters of capillaries and the viability of the cells correlated with either the average wall shear stress or the power dissipation within the capillary. M$^c$Queen *et al* (1987) used a similar system to study the effects of flow and turbulence on the viability of suspended mouse myeloma cells that were subjected intermittently to sudden contraction and then turbulence. It was found that cell lysis began when the Kolmogorov eddy

size was less than the average cell size (10 μm). Stathoupoulos and Hellums (1985) examined the influence of shear on human embryonic kidney epithelial cells that were attached to a flat solid substrate in a laminar flow chamber. The changes in orientation and morphology of epithelial cells resembled those found on endothelial cells, that is, the morphological and alignment changes increased with increasing shear and exposure time.

Smith *et al*, (1987) furthered these studies by investigating the shear sensitivity of suspension cells. Mouse hybridoma cells were sheared at constant shear rates in a Couette viscometer over a period of 15 hours (cell doubling time). Viability cell counts and LDH release were monitored to characterise cell damage. It was found that shear rates of 870 $s^{-1}$ (6.7 dynes/$cm^2$ ) caused extensive cell damage but no decrease in cell growth was found at shear rates of 420 $s^{-1}$ (3.3 dynes/$cm^2$). Peterson *et al* (1988) conducted laminar Couette flow experiments to expose hybridoma cells to higher levels of shear stresses for shorter periods of time. They measured cell viability and LDH activity for increasing levels of shear from 0 to 50 dynes/$cm^2$ for 10 minutes and for a shear level of 50 dynes/$cm^2$ for 0 to 10 minutes. Harmful effects were apparent for both increased duration and intensity. Another study using hybridoma cells was conducted by Schurch *et al* (1988) in a rotating viscometer under laminar conditions. It was found that cell death began at a shear stress less than 60 dynes/$cm^2$ and increased with increasing stress. However it was concluded that cells surviving a shear stress proliferated and metabolised at the same rate as cells not sheared.

In the above work, shear stresses in the laminar regime only were studied. Abu-Reesh and Kargi (1989) examined the effects of both laminar and turbulent shear on cells in a Searle viscometer. An important observation made was that the respiration activity of the cells was adversely affected before loss of membrane integrity and cell lysis. Therefore it was suggested that shear stresses should be kept low to keep the cells functional noting that viability might be lost at shear stresses below those necessary to damage the

cell membrane. Higher shear stress levels were necessary in the laminar regime to cause the same degree of cellular damage as observed in turbulent flow for equal exposure times.

All of the above work provided the means to measure shear rates in defined shear fields in order to examine the shear sensitivity of different cell types. Thus the complex flow patterns found in bioreactors were avoided and conclusive data on the effect of shear forces was obtained. However the long term biological responses of the cells to shear cannot be elucidated in these controlled shear devices. Consequently although these studies are invaluable in terms of providing information on shear sensitivity of particular cells, in order to study the effect of shear of cells in large scale production vessels it is necessary to examine the growth and productivity of cells in agitated bioreactors where the complex fluid mechanical environment elicits a large number of responses in cells.

1.3.4.4: The Effect of Shear in Agitated Bioreactors

Mixing in animal cell bioreactors is essential for cell growth. It is needed to distribute the nutrients throughout the culture and to keep the cells in suspension in a homogenous environment. Traditionally animal cell bioreactors tend to use impellers within a cylindrical vessel with a hemispherical bottom to provide efficient mixing. It has been shown that the agitation required to keep the cells well suspended as well as to provide a molecularly well mixed environment for the cells is in the turbulent regime (Cherry and Papoutsakis, 1986; Kunas and Papoutsakis, 1990). Consequently the turbulent stresses present in the bioreactor can cause damage to the cells. The effect of shear in bioreactors has been extensively documented in the literature and a summary of relevant studies is presented in Table 1.5.

Table 1.5: Literature reports on shear in animal cell bioreactors

| **Small Scale Agitated and/or Sparged Bioreactors** |
|---|
| **Literature Citations** |
| Abu-Reesh and Kargi, 1991 |
| Cherry and Papoutsakis, 1986, 1988, 1989. |
| Croughan et al, 1987, 1988, 1989 |
| Dodge and Hu, 1986 |
| Gardner et al, 1990 |
| Handa-Corrigan et al, 1987, 1989 |
| Jobses et al, 1991 |
| Kunas and Papoutsakis, 1989, 1990 |
| Lakhotia and Papoutsakis, 1992 |
| Lee et al, 1988 |
| Oh et al, 1989, 1992 |
| Passini and Goochee 1989 |
| Peterson et al, 1988 |
| Smith and Greenfield, 1992 |
| Tramper et al, 1988 |
| Van der Pol et al, 1990 |

The effect of the fluid environment on cell growth very much depends on whether the cells are anchorage dependent or are growing freely in suspension. Therefore the following sections will examine, separately, the effect of shear (referring in this context to all mechanical forces existing in bioreactors) on microcarrier cultures and on suspension cultures. It is proposed to review anchorage dependent cells in terms of their cultivation in microcarrier bioreactors as this method is by far the most popular used by industry. Although hollow fibres and encapsulation systems have been developed for animal cells they have not gained widespread acceptance due to special start up procedures and nutritional limitation problems). In addition much work has been reported recently on attempts to define the hydrodynamic field in bioreactors.

*1.3.4.4.1: Microcarrier cultures*

Anchorage dependent cells in general are more shear sensitive than suspended cells because of their inability to translate or rotate, thus requiring them to absorb the hydrodynamic shock. One of the most popular methods of cultivating anchorage dependent cells is on microcarriers beads. The beads usually composed of polymer or glass are nearly neutrally buoyant and are available in a variety of sizes and configurations. However the cells are not protected from the fluid environment when growing on microcarrier beads thus they may experience significant and in many cases, lethal levels of hydrodynamic forces. A large body of work has been reported in the literature which has studied the hydrodynamic environment in microcarrier bioreactor cultures (Sinskey *et al*, 1981; Hu *et al*, 1985; Croughan *et al*, 1987, 1988, 1989; Cherry and Papoutsakis, 1988, 1989). These studies have been either phenomenological, correlational or semi-empirical in nature. However, they have provided some working rules for scale up as well as some approximate criteria for design.

Sinskey *et al* (1981) and Hu *et al* (1985) were the first to correlate cell damage in microcarriers with agitation conditions. Sinskey *et al* correlated cell death based on an empirical quantity called the Integral Shear Factor (ISF) (defined in section 3.4.1) similar to the shear rate. They observed for Chick embryo fibroblasts grown on Cephadex beads that cell density dropped when the ISF exceeded a critical value. Hu *et al* extended this approach and studied the effects of microcarrier concentration on cell density. They also studied the optimal inoculum requirement for the growth of FS-4 human foreskin fibroblast cells.

Croughan *et al* (1987, 1988, 1989) used a slightly different approach and applied hydrodynamic theory, based on particles in turbulent flow, to re-evaluate previously published shear sensitivity data for microcarriers. They suggested that cell survival was dependent on impeller tip speed or maximum

shear rate but on turbulent eddy size. Cherry and Papoutsakis (1986, 1988 , 1989) also used this same approach of applying Kolmogorov's theory of isotropic turbulence to explain the effects of turbulent mixing. They showed that turbulent eddies of similar sizes to microcarriers caused cell damage.

Cherry and Papoutsakis (1988) proposed three mechanisms of cell damage for cells growing on microcarriers:-

1. Interaction between turbulent eddies and microcarriers.
2. Collisions of a microcarrier with other beads.
3. Collisions with parts of the bioreactors (primarily the impeller).

The first and second mechanisms were thought to be most important and models to support this were proposed, based on the theory of isotropic turbulence. To characterise the interaction between turbulent eddies and microcarriers it was suggested (Cherry and Papoutsakis, 1986, 1988; Croughan, 1987) that the important parameter was the Kolmogorov eddy length to bead diameter ratio;

$$\text{ratio} = \eta / d \qquad \textit{(equation 7)}$$

where $\eta$ is the Kolmogorov length scale (characteristic length for the smallest turbulent eddies, defined in section 1.3.4.1, equation 3) and d is the microcarrier bead diameter.

It was found that the apparent growth rate decreased with decreasing ($\eta$ / d) values, suggesting that small sized eddies are responsible for cell damage.Small eddies cause shear stresses on the surface of the microcarriers. Based on average power dissipation rates, the authors found that detrimental effects on cells were observed when the average Kolmogorov length scale dropped below approximately 100μm, corresponding to a energy dissipation rate ($\varepsilon$) of 100 $cm^2/s^3$. This scale is about half of the average bead diameter of 185 μm. Larger eddies (of a size greater than the microcarriers) surround the microcarriers completely and may rotate or translate them without generating an excessive amount of surface stresses. In order to validate these results which suggest that turbulent eddies are very important in causing cell damage

in microcarriers Croughan *et al* (1988) performed experiments with dilute cultures in order to reduce the possible effect of the other important cell damage mechanism, bead-bead interaction. It was found that again intense agitation reduced the apparent growth rate, and these injurious effects became apparent when the Kolmogorov eddy scale fell below about 130μm or about two thirds of the bead diameter of 185μm. Croughan *et al* (1987, 1989) considered eddy-microcarrier and microcarrier-microcarrier interactions and theoretically predicted and experimentally confirmed that the apparent growth rate was proportional to the eddy concentration and that this concentration was proportional to $(\varepsilon / \nu)^{3/4}$. Cell removal from microcarriers because of hydrodynamic forces is another harmful effect attributed to intense agitation in microcarriers cultures (Croughan *et al*, 1989). The removal of cells increases with the level of agitation and removal would appear to be irreversible and lethal for anchorage dependent cells in suspension that are incapable of reattachment.

Cherry and Papoutsakis (1988) examined microcarrier-microcarrier collisions and microcarrier-impeller collisions. While their experiments suggested that microcarrier-impeller collisions did not contribute to cell damage, the importance of bead-bead interactions was noted. The authors proposed the term, turbulent collision severity (TCS) which represents collision energy per bead per time, to describe these interactions;

TCS per bead = (kinetic energy) (collision frequency/volume).

Experimentally they found that the apparent growth rate decreased with increasing TCS, that is the more collisions between beads, the more damage to cells growing on the beads. In a subsequent paper (Cherry and Papoutsakis, 1989) it was reported that smaller beads were associated with a higher cell growth rate. It was also suggested that the formation of bead aggregates by cellular bridging reduced the growth rate because the clumps were in essence large microcarriers with irregular shapes.

Recently the bead collision mechanism proposed by Cherry and Papoutsakis has been integrated with the eddy size correlation by considering the total energy in all turbulent eddies equal to or smaller in size than the microcarriers. A spectrum of eddies is considered in the proposed model rather than the Kolmogorov scale eddy size alone (Lakhotia and Papoutsakis, 1992). The effect of medium viscosity on the growth of bovine embryo kidney cells was examined since increasing viscosity will increase the Kolmogorov eddy scale ( $\eta$ is directly proportional to $\nu$, see equation 3). Also increasing viscosity was shown previously to decrease agitation induced cell damage (Cherry and Papoutsakis, 1989; Croughan *et al*, 1989). This model (Lakhotia and Papoutsakis, 1992) predicts that the rate of cell death resulting from agitation should decrease with increasing medium viscosity and that the protective effect of increased viscosity is more significant at greater agitation intensities. The predictions of this theoretical model were experimentally verified.

Venkat *et al*, (1993) further investigated the effect of the hydrodynamic environment in microcarrier cultures. These authors also highlighted the importance of the larger scales of turbulence in causing cell damage. They suggest that although a large percentage of the energy is dissipated at the Kolmogorov scales, these eddies are very weak. On the other hand, the larger eddies possess greater energy and the energy dissipation in the impeller region due to shear flows is probably much more intensive. This extensive energy dissipation may result in extensive cell damage. Oh *et al* (1992) in their study of agitated suspended cell reactors also point out the importance of larger energy eddy scales.

Recent work by Garcia-Briones and Chalmers (1994) states that in an ideal situation, cell damage should be predicted by knowing the actual shear stresses that the cell experiences and from intrinsic cell mechanical properties the resulting cell deformation predicted. To achieve this goal, it is important to identify the relevant parameters of the flow field governing cell injury and their relationships with the equipment geometry (reactor design) and

operation. Of the flow parameters studied to date (including, shear stress and shear rate in laminar flow devices, impeller tip speed, ISF, power dissipation rates (ε) and Kolmogorov eddy length (η) ) while they are relevant for each system studied they do not allow the correlation of cell damage in one system to predict cell damage in a different system. These authors suggest that relevant flow parameters that govern hydrodynamic injury in any given flow must be identified. These parameters must be general ( i.e. not from geometry but from intrinsic characteristic of flow itself) and local ( not an average value since external stresses cause local intensity and average values prevent identification of these regions in the flow). The authors suggest two possible relevant flow parameters;

- state of stress - proportional to the actual stress that cells experience and equal to the stress tensor.
- Flow classification criteria, $R_D$ which is related to the possibility of stress relaxation by rotation.

However this work must be extended and validated with experimental data.

*1.3.4.4.2: Suspension Cultures*

Cells grown in suspension behave very differently from anchorage dependent cells, indeed some suspension cells are quite tolerant of normal to suprisingly high agitation rates. Many experiments have been carried out using viscometers to impose well defined laminar shear stresses on the cells for short periods of minutes to several hours (see section 3.4.3) ( M$^c$Queen and Bailey, 1989; Goldblum *et al*, 1990; Ramirez and Mutharasan, 1990; Peterson *et al*, 1990; Michaels *et al*, 1991). The results are usually reported as fractional survival of the cells. Shear rates of the order of $10^3 s^{-1}$, corresponding to fluid shear stresses of 1-10 $N/m^2$, typically yield 20-80% cell death after $10^1$ minutes exposure. These short term conditions are representative of what a cell would experience in process operations such as passing through a pump or a microfiltration unit.

Long term experiments study the effects of different agitation conditions on cell growth rates in fairly typical culture conditions over several days in a bioreactor. It has been reported (Fazekas de Groth, 1983) that the growth rate of hybridoma cells was 15% lower in a stirred vessel in comparison to a stationary culture. Dodge and Hu (1986) examined the growth of hybridoma cells and confirmed that rapid agitation of batch cultures in spinner flasks decreases the growth rate during exponential growth, however no adverse effects on cell growth were observed at the minimum agitation rate necessary to suspend the cells. Lee *et al* (1988) noted that agitation increased the decline in cell concentration during death phase. On a larger scale the use of a 23 cm diameter marine propeller in a 150 litre bioreactor caused significant cell damage at an agitation rate of 170 rpm (Backer *et al*, 1988).

Papoutsakis and Kunas (1989) studied hydrodynamic effects on cultured hybridoma cells in both spinner flasks and in a 2 litre bioreactor. High agitation intensities caused a substantial reduction of cell growth rate or even complete cell death. Severe damage of cells was observed at 200 rpm. An interesting observation was that unlike microcarrier cultures, an increased medium viscosity had a negative effect on cell viability at higher agitation rates. Oh *et al* (1989) performed a series of experiments with hybridoma cells to determine the response of the cells to agitation in stirred bioreactors; the apparent growth rate was not altered by agitation forces up to 450 rpm. The calculated Kolmogorov eddy size at 450 rpm was 57 μm, considerably larger that the cell diameter of 10 -20 μm. In further studies, this group reported hybridoma growth at even higher agitation speeds (700 rpm) however this was only possible by allowing no gas sparging and rigourous exclusion of headspace air to prevent entrainment into the impeller by a surface vortex. The influence of the air-liquid interface has important consequences for the susceptibility of a cell to shear damage and will be discussed presently.

Kunas and Papoutsakis (1990) provided further information on shearing forces in suspension cell cultures supporting the findings of Oh *et al.*(1989). Noting

the importance of bubble formation and entrainment in causing cell damage at relatively low agitation rates (190 rpm), they performed experiments in 2 litre bioreactors where no bubble formation or headspace air was present. Upto 700 rpm no noticeable cell damage was observed. Above this value cell damage was attributed to stresses in the bulk turbulent liquid phase associated with interaction between hybridomas and eddies. Using a reasonable range of power numbers and performing calculations on the basis of both the total reactor volume and the volume in the vicinity of the impeller ($D_i^3$), they estimated the Kolmogorov scale eddy size at an agitation rate of 800 rpm and reported that the Kolmogorov length scale was between 12.6 and 22.2 μm. That is, in the absence of surface vortex and bubble entrainment, cell damage was only observed at high agitation rates and was associated with dissipative eddy size being similar to or smaller that the cell size. This proposal is similar to the reported damage mechanisms in microcarrier cultures except that cell damage is observed at much lower agitation rates in microcarrier systems. O'Connor and Papoutsakis (1992) supported previous work by investigating the growth of CHO cells in microcarrier and in suspension systems. The importance of Kolmogorov eddies as a correlaing factor for cell damage are highlighted in Table 1.6

Table 1.6: Reynolds number, Power number, Energy dissipation Rates and Kolmogorov length for CHO Cells in Microcarrier and Suspension Culture (from O'Connor and Papoutsakis, 1992).

| | **CHO Microcarrier Culture (rpm)** | | | **CHO Suspension Culture (rpm)** | | | |
|---|---|---|---|---|---|---|---|
| | **60** | **100** | **150** | **100** | **270** | **340** | **400** |
| **Re** | 2000 | 3400 | 5100 | 4200 | 1100 | 14000 | 17000 |
| **$N_p$** | 0.7 | 0.6 | 0.5 | 0.95 | 0.7 | 0.65 | 0.6 |
| **ε ($cm^2/s^3$)** | 14 | 57 | 160 | 110 | 1600 | 3000 | 4500 |
| **η (μm)** | 160 | 120 | 90 | 100 | 50 | 45 | 40 |

It was found that as $\eta$ decreased below 175 $\mu$m (microcarrier diameter), cell damage increased. In contrast for suspension cellls, $\eta$ did not fall below the cell diameter even at 340 and 400 rpm in the suspension cultures even when cell damage was observed. Consequently the authors propose that the dominant factor causing cell damage in the microcarrier culture is bulk fluid turbulence which produces cell-destroying micro-eddies and in the suspension culture the gas/liquid interface is causing vortex formation and bubble entrainment.

Importance of Gas/Liquid Interface in Suspension Cultures

The work described thus far has only focused on shear effects in the bulk liquid but this is not necessarily the site of most cell death in suspension cultures (Hua *et al*, 1993). Cells can be killed in great numbers in gas bubbles rupturing at the liquid surface. The deleterious effects of forces originating at gas-liquid interfaces was first studied in airlift reactors or bubble columns. Tramper *et al* (1986) proposed that bubbles rising through the liquid and bubbles bursting at the surface of the suspension were potential sources of cell damage in airlift reactors. In subsequent work, (Tramper *et al*, 1987, 1988), the authors concluded that most damage to the cells occurred at the point of air injection with the death rate of the cells much faster when small air bubbles were generated.

Handa *et al* (1987) examined gas-liquid interfacial effects on hybridomas in a bubble column. A bubble column is convenient to study bubbles effects as it may be divided into three major zones with respect to bubble influence: air distributor zone, bubble rise zone and bubble disengagement zone. These workers found that frequent bubble formation and small bubble size reduced cellular viability. Long time exposure of cells to bubbles resulted in irreversible damage to the cells. At high gas flow rates the cells were sheared considerably in the medium, but the region of highest shear and turbulence was at the liquid surface where cells were subjected to very strong forces because of the energy released by bubble disengagement. Some cells were

entrained in bubble films at the surface. In further work (Handa-Corrigan *et al*, 1989) experiments varying column length confirmed the damaging effect of the disengagement zone. Two mechanisms of cell damage were proposed;- damage due to rapid oscillations caused by bursting bubbles and damage due to shearing in draining liquid films (lamellae) in foams.

As well as air bubbles being potentially lethal to cells, the gas/liquid interface is also important in surface aerated suspension cultures. In conjuction to their work with suspension cells in the absence of bubbles or vortexing from the headspace as mentioned, Kunas and Papoutsakis (1990) studied the effect of bubble entrainment on suspended hybridoma cells in agitated bioreactors with surface aeration. It was observed that a large vortex was present around the impeller shaft in the centre of the gas-liquid interface formed between the gas headspace and culture medium. At the bottom of vortex the interface between the headspace gas and liquid medium is unstable and rapidly moving. Bubbles detach from the surface and are entrained in the turbulent liquid. As bubbles become entrained or burst at the liquid surface, shear as well as pressure fluctuations are generated in the media. Likewise as a bubble moves relative to the liquid surrounding it areas of shear can be created. Using photography to visualise bubbles, the authors observed bubbles (1-3 mm) detach from the vortex and become entrained in the medium at agitation rates greater than 140 rpm. Apparent growth rates decreased above this agitation rate with a rapid decrease in cell growth seen in agitated cultures at 190-250 rpm. Overall it was concluded that the region near a freely moving gas-liquid interface (the vortex) with entrained bubbles was harmful to cells.

Oh *et al* (1989, 1992) used a baffled bioreactor to prevent vortex formation and thus eliminate the shearing effect caused by bubble entrainment in order to study hybridoma growth in an agitated and sparged bioreactor. When continuous air sparging was used, cell growth and antibody production were reduced and these effects were observed to increase with agitation speed. It was later noted that cell damage was also reduced with decreasing sparging

rate. The authors proposed that bubbles bursting in clusters and bubbles of size less than 5 mm were most dangerous to the cultured cells and the extent of cell damage increased with decreased bubble size. Thus agitation resulted in an increase in the number of smaller bubbles and hence increased cell damage.

Chalmers and co-workers have contributed important work to this area by investigating the interactions between insect cells and the gas-liquid interface using microscopic, high speed video techniques (Bavarian *et al*, 1991; Chalmers and Bavarian, 1991). These workers looked at rising bubbles, medium surface, foam layer, bubble films and bursting bubbles for their effect on cells. They suggested that in aerated bioreactors the shear stress associated with rising bubbles may be damaging and moreover, that cells trapped in the foam layer may experience inhibitory environmental conditions such as dehydration and nutrient depletion. Using a mechanistic approach they showed that bubble rupture destroys cells. Accounting for the small number of cells that can be entrained into the liquid film of a bubble gives a good prediction of the observed death rates in a sparged cell suspension. (Cherry and Hulle, 1992). Although the exact mechanism is still being studied these authors hypothesise that death is caused by the release of the bubble's surface energy into a very small volume of liquid when the bubble bursts.

Overall the work carried out to date on the influence of the gas-liquid interface on animal cells suggests that it is the interaction between agitation and sparging that causes cell death under mild agitation. This combination of agitation and sparging leads to bubble break-up and coalescence which probably causes the observed loss of cellular viability.

*1.3.4.4.3: Summary of Shear Effects in Agitated Bioreactors for Microcarrier and Suspension Cultures*

Over the last ten years explanations for cell damage in large scale bioreactors has evolved from vague presumptions of excessive 'shear sensitivity' in animal cells to recognition of the main factors contributing to cell damage in the bioreactor environment. For microcarrier cell cultures the important cell damage mechanisms are suggested to be as follows;

1. The direct interaction of microcarriers with turbulent eddies that are usually characterised using Kolmogorov's theories of isotropic turbulence with cell damage only occurring when the dissipative eddies are smaller than the microcarriers.
2. Collisions between microcarriers.
3. Collisions of microcarriers with the impeller or other stationary surfaces.

In suspension cultures deleterious effects of agitation on cells may originate from the turbulent eddies. However, under normal operating conditions agitation alone is frequently insufficient to cause cell damage. Consequently the effect of the gas/liquid interface and bubbles play an important part in determining the shear environment, especially in an airlift or bubble reactor. The dynamic forces associated with bubble formation, bubble break-up/coalescence and bubble bursting appear to be the major sources of cell damage. In non sparged cultures, vortex formation from the headspace seems to be important because bubbles can rapidly detach from the bottom of the fluctuating vortex, become entrained and swirl throughout liquid volume, rise and disengage at the surface often causing irreparable cell damage. When there is no air sparging and no surface aeration in an agitated culture, shear related cell death is only observed to occur at high agitation rates (700-800 rpm).

1.3.4.5 Shear Protective Agents Used in Animal Cell Culture

Serum, an undefined blood fraction which is added to culture medium to support active cell growth, has been shown to alleviate the destructive effects of hydrodynamic shear (Hulscher and Onken, 1988; M$^{c}$Queen and Bailey, 1989; Kunas and Papoutsakis, 1989, 1990; van der Pol *et al*, 1992). Other polymers, such as Pluronic F-68, methylcellulose have also been used to diminish the detrimental effects of shear forces ( Goldblum *et al*, 1990; Murhammer and Goochee, 1990; Handa-Corrigan *et al*, 1989). However there is still little evidence as to how these shear protective agents work.

*1.3.4.5.1: Serum as a shear protective agent.*

In the case of serum both a physical and nutritional role has been proposed. Kilburn and Webb (1968) were the first workers to observe the protective effect of serum when they noted the increased growth of suspension cells in sparged bioreactors upon an increase in serum concentration from 2% to 10%. These authors suggested that a highly condensed interfacial structure at the cell-liquid interface, formed by adsorbed serum molecules may prevent the forces created in the vicinity of a bubble from acting directly on the cell surface. Hulscher and Onken (1988) observed that serum protected myeloma cells from shear in a capillary tube at concentrations above 5%.

Kunas and Papoutsakis (1990) proposed two mechanisms for serum protection, one physical and one nutritional. The physical mechanism involves the serum changing the hydrodynamic environment only (not the cell itself) either macroscopically or in the immediate vicinity of the cell and would occur within minutes of the serum being added to the agitated culture. If the protection mechanism is nutritional then serum components would be taken up and utilised in some way by the cell, perhaps changing properties of the cell cytoskeleton, membrane or other organelles, and this effect would take time, in the order of hours, to achieve. Based on these hypotheses it was

suggested by these workers that the effect appears physical since addition of 10% serum to hybridoma cells which were increased in agitation rate from 60 to 220 rpm resulted in an increase in growth when compared to a 1% serum addition, within one hour of serum addition. This almost immediate action of serum on cell growth suggests the effect is at least in part a physical one. An additional protective effect of a nutritional nature cannot be ruled out however. This work also ruled out an increase in viscosity caused by serum addition as a mechanism of shear protection. Although increasing the serum concentration from 1 to 10% does increase medium viscosity by 13%, an increase in viscosity of the medium to 50 or 100% by the addition of high molecular weight dextran was shown previously to have no effect on cell growth at high (220 rpm) agitation rates (Papoutsakis and Kunas, 1989). However in contrast a subsequent paper by Lakhotia and Papoutsakis (1992) did report that increasing medium viscosity suppressed cell death rates in an agitation intensity dependent fashion in microcarrier cultures. Since serum addition does not cause a large increase in medium viscosity it is unlikely that the shear protection mechanism of serum is viscosity related.

Other researchers have presented evidence for both a physical and nutritional role (Ramirez and Mutharasan, 1990; van der Pol *et al*, 1992). Michaels *et al* (1991) extended the description of these two possible protection mechanisms. It is suggested that the physical effect does not change the cells' resistance to shear but factors affecting the level or frequency of transmitted shear forces to the cell may be changed thus allowing cell damage. If the protective effect is biological, then it may be fast acting ( alterations quick and without metabolic events) or metabolic ( metabolic events likely e.g. cell cycle and may need longer exposure for effect). Thus a fast acting biological effect could include modulation of the cell membrane and the results observed by Kunas and Papoutsakis above might be better explained by a combination of a physical and a fast acting biological event. Recently Ramirez and Mutharasan (1990, 1992) have presented some very interesting work relating the protective effect of serum to plasma membrane fluidity (PMF) which is a collective description

of the various motions of the components of a biological membrane. A brief overview of this work will be presented in section 1.3.4.5.1.

*1.3.4.5.2: Other shear protective agents.*

Pluronic F-68 is a non-ionic surfactant first used by Kilburn and Webb (1968) to alleviate the effects of sparging on cells. These authors suggested that the Pluronic F-68 molecules surround the cells and thus protect them. This proposal was supported by Mizrahi (1975) who also suggested that surface tension reduction caused by Pluronic F-68, might facilitate nutrient transport. The work by Handa-Corrigan *et al* (1987, 1989) investigating the influence of bubbles on the shear environment also looked at the role played by both serum and Pluronic F-68 . They found that these agents stabilised the gas/liquid interface and allowed the formation of a stable foam layer that the cells did not penetrate. Thus the cells avoided the damaging forces attributed to film drainage and/or bubble bursting. Murhammer and Goochee (1988) studied the effect of Pluronic F-68 on insect cells and concluded that it caused a relatively stable foam layer on the medium surface in an airlift reactor. Interaction between Pluronic F-68 with the cell membrane was examined and it was proposed that the surfactant protects by incorporation into and stabilising of the cell membrane. Work by Jobses *et al* (1991) agreed with the previous work and concluded that the protection resulted from the direct interaction of Pluronic F-68 with the cells rather than its influence on gas-liquid interfacial properties. Goldblum *et al* (1990) noted that other polymers besides Pluronic F-68 might have shear protective effects, for example methylcellulose and dextran. They found that 0.5% methylcellulose and 4.5% dextran protected insect cells from laminar shear stress by factors up to 76 and 28 respectively and suggest that these polymers keep the cells from shear damage through adsorbing to cell membranes to form protective layers on the cells. Murakami *et al* (1983) showed that the addition of a soybean phospholipid fraction to serum free medium allowed the growth of a myeloma cell line in spinner flasks. Overall however the role of serum and other additives mentioned needs

further investigation most importantly in order to elucidate a mechanism by which they exert this protection.

*1.3.4.5.3: Plasma Membrane Fluidity and the Role of Shear Protective Agents*

In section 3.4.6 to follow, sublethal effects of shear on a cell will be examined. One of the most important of these sublethal effects is the change in plasma membrane fluidity (PMF) which results when cells are under shear stress. However it is proposed to review the significance of plasma membrane fluidity in determining cellular shear sensitivity in this section on shear protective agents because PMF is known to be very much affected by the addition of such agents and consequently this work has suggested a mechanism by which shear protective agents might exert their effect.

As stated earlier (Section 1.3.4.2) animal cells are surrounded by a bilayer membrane composed of a variety of lipids and proteins. The degree of packing of the constituents in the membrane as well as their movement is referred to collectively as the PMF. PMF may be detected by a number of techniques including fluorescence polarisation, Raman spectroscopy and electron spin resonance. In the work by Ramirez and Mutharasan (1990, 1992) which is the only study to date to have examined the importance of PMF in shear sensitivity determinations, fluorescence polarisation was used because of its high sensitivity, reproducibility and ease of operation and data interpretation.

Steady state fluorescence polarisation involves labeling the cellular membrane with a fluorescent probe which is excited with a polarised monochromatic light. According to the movement of the probe (which depends on the PMF) the emitted light will be partially depolarised with respect to the plane of polarisation of the excitation light. This partial depolarisation may be quantitatively expressed as;-

$$r_s = \frac{I_{0°0°} - I_{0°90°}}{I_{0°0°} - 2I_{0°90°}}$$

where; $r_s$ is the fluorescence anisotropy and $I_0{}^{o}{}_0{}^{o}$, $I_0{}^{o}{}_{90}{}^{o}$ are the emission intensities determined with the emission polariser oriented parallel and perpendicular to the direction of polarisation of the excitation light respectively. The anisotropy of the fluorescence emitted by the probes is inversely related to the fluidity of the lipid surroundings of the membrane.

Quantitation of PMF involves the assessment of two parameters;

1. the local microviscosity which is a dynamic factor related to the rotational rate of a membrane component.
2. the molecular order, a static factor related to the degree to which the rotations of such a component are restricted.

In the steady state fluorescence polarisation method the second parameter is mostly being measured. A change in molecular order is associated with the reciprocal of PMF. Thus an increase in the $r_s$ value indicates a decrease in PMF. It is important to note that biological membranes are highly heterogeneous therefore fluidity may vary within regions of the membrane. Consequently measurements made by this technique are an average value of PMF only. The probe used to label the membranes is 1-[4-trimethyl-amino phenyl] -6-phenylhexa-1, 3, 5-triene (TMA-DPH) which rapidly partitions in the bilayer matrix due to its nonpolar nature. Previous work has shown that the probe is incorporated into the membrane in approximately thirty seconds (Illinger *et al*, 1990). The positively charged head of TMA-DPH prevents the probe from penetrating further into the cytosol and binding with the

membranes of internal organelles. Thus the measurements achieved with this probe are specific to the external plasma membrane.

Initially Ramirez and Mutharasan (1990) investigated the effect of a number of agents for their effect on PMF. They found that benzyl alcohol and high temperatures increased PMF and resulted in a more fragile cell when exposed to laminar shear stress in a Couette flow device. Cholesterol, Pluronic F-68 and serum all decreased PMF implying that all these compounds interact directly with the membrane to change the PMF. A subsequent decrease in shear sensitivity was noted with these agents. The authors concluded that the fluid state of the plasma membrane is important in determining the integrity of a cell when exposed to lethal shear levels. They stated that increasing membrane fluidity correlated with increasing shear sensitivity.

In their second publication, Ramirez and Mutharasan (1992) investigated how serum might act as a shear protective agent. Noting that cellular growth rate depends on the initial serum concentration in a Monod fashion, these authors disagreed with the suggestion by Kunas and Papoutsakis (1990) that the protective effect is entirely of a physical nature. Instead they suggest a fast acting biological action while acknowledging that a physical role may also be important. Their work showed that increasing the serum concentration from 0 to 9% serum resulted in a reduction in PMF and an accompanying reduction in shear sensitivity. Since the earlier work had indicated that increasing the cholesterol content of membranes decreased the PMF, the importance of cholesterol in modulating PMF was then evaluated through direct cholesterol modulation and lipoprotein-mediated cholesterol modulation. The main mechanism by which cells control cholesterol is through the bi-directional flux of cholesterol between extracellular lipoproteins and the plasma membrane (Phillips *et al*, 1987). Lipoproteins have been shown to constitute an important component of serum (Barnes, 1987). The results presented by Ramirez and Mutharasan (1992) showed that increased cholesterol levels by both direct and indirect methods resulted in high $r_s$ values when cells were

exposed to turbulent shear in spinner flasks, that is indicating a decreased PMF and shear sensitivity. Also the time scale in which changes in PMF were observed (2 to 18 hours) coincides with those reported for the transfer of cholesterol between cells and lipoproteins (Phillips *et al*, 1987). On the basis of these results it was suggested that the protective action of serum is mainly related to the modulation of the PMF through the transfer of cholesterol or analogous compounds from the culture medium into the plasma membrane. Serum appears to have a condensing effect on PMF. However to unequivocally confirm this mechanism it would be necessary to directly measure changes in the cholesterol and lipid composition of the plasma membrane as a function of serum concentration.

While the above results suggest a biological role for shear protection by serum, a physical role may also be involved since in their previous work, Ramirez and Mutharasan (1992) had shown that a supplement of 0.5% Pluronic F-68 to cells raised the $r_s$ values (decreased the PMF). It was proposed that since Pluronic F-68 is an amphipathic agent it can intercalate easily into the membrane. Thus its effect on PMF might be related to either direct combination with the membrane or through a possible interaction with serum lipoproteins inducing a higher cholesterol flux into the cell membrane.

1.3.4.6: Sublethal Effects of Shear

In recent years the it has been acknowledged that as well as causing cell death in bioreactors, shear forces can affect cellular physiological function in a non-lethal manner. For example, at low levels of shear stress, enzyme activity, and cytoskeletal organisation change in a highly regulated manner (Frangos *et al*, 1985; Stathopoulos and Hellums, 1985; Chasis and Mohandas, 1986). Cellular volume and metabolism may also be affected (Chittur *et al*, 1988; Diamond *et al*, 1989; Al-Rubeai *et al*, 1990; Frangos *et al*, 1988). It is extremely important to recognise these effects, which usually occur at shear stresses much lower than those required to physically break the cell membranes, and thus realise

their implication in bioprocessing. While some nonlethal responses may actually be beneficial for example the stimulation of cellular metabolism by shear stress may be an advantage when the target products are metabolites (Al-Rubeai *et al*, 1990; Frangos *et al*, 1988) not all responses are desirable. A review of sublethal effects on cells will be presented including a section on the effect of shear on the cellular cytoskeleton which is vitally important in determining the shear sensitivity of a cell.

Initial observations that shear stresses might affect the physiological condition of the cell was observed in endothelial cell cultures where researchers in order to simulate the hydrodynamic forces caused by blood flow have elicited responses from cells by exposing them to well-defined shear. It was noticed that endothelial cells are able to modify their morphology, growth rate and metabolism in response to changes in fluid flow. Exposure of human endothelial cells to well defined shear stresses resulted in stimulation of arachidonic acid metabolism (M$^{c}$Intire *et al*, 1987); activation of a $K^{+}$-ionic current (Oleson *et al*, 1988); increased production of tissue plasminogen activator (Diamond *et al*, 1989). In studies with bovine aortic endothelial cells , shear stress induces changes in cell shape, cell mechanical properties and the rate of cell proliferation (Levesque *et al*, 1989).

Recent work has shown that flow-induced production of platelet-derived growth factor mRNA and prostacyclin appears to follow similar signal transduction pathways. Fluid flow results in activation of G proteins which in turn activate phospholipases (Hsieh and Frangos, 1992; Berthiaume and Frangos, 1992). These phospholipases then cleave phospholipids, including phosphatidylinositol, phosphatidylethanolamine and phosphatidic acid (Bhagyalakshmi *et al*, 1992). It appears that the products of the phospholipid cleavage then act as secondary messengers which activate the cell. The response of the cell to the secondary messenger is not a non-specific activation but a specific and coordinated metabolic change. For example, flow induces endothelial cells to produce an endothelium-derived relaxing factor, a

vasodilator while subsequently inhibiting the production of endothlelin-1 a powerful vasoconstrictor (Kuchan and Frangos, 1993). Since the components of the signal transduction pathway such as G proteins and phospholipases are found not only in endothelial cells but in all cells, it is not surprising that there are reports in the literature that osteoblasts and fibroblasts (Reich *et al*, 1990) and kidney tubule cells (Kawahara and Matsuzaki, 1992) are also stimulated by flow.

Suspension cells also exhibit a variety of responses to hydrodynamic forces. Effects of fluid mechanical forces on red blood cells, platelets and leukocytes have been widely reported (O'Rear *et al*, 1982; Hellums and Hardwick, 1981; M$^{c}$Intire and Martin, 1981). Exposure to sublytic shear stresses has been shown to result in alterations to the proliferative response of human T cells (Chittur *et al*, 1988). Several workers have shown that under higher agitation conditions, glucose consumption rates and mitochondrial activity increase (Abu-Reesh and Kargi, 1989, 1991; Al-Rubeai *et al*, 1990; Oh *et al*, 1992) especially in the absence of serum (Smith and Greenfield, 1992).

Recent work with Chinese hamster ovary cells (Lakhotia *et al,* 1992) has shown that although increased agitation intensity decreases cell viability, increased agitation induces the viable cells to proliferate more rapidly compared to low agitation control cultures. The increased proliferation rate was demonstrated by flow cytometry which revealed an increase in the rate of DNA synthesis and an increased fraction of viable cells in S phase. The proliferation rate remained elevated for at least six hours after the agitation rate was returned to control levels as demonstrated by a continued elevated rate of DNA synthesis and intrinsic cellular growth.

In further work by Lakhotia and coworkers (1993) the effect of shear on the expression of surface proteins in the HL60 cell line was examined. The importance of this research should not be underestimated since a great variety of cellular functions, such as, signal transduction and recognition, transport

processes, enzymatic catalysis of surface mediated reactions and cell-cell recognition, are mediated by membrane bound proteins. Since critical levels of fluid mechanical forces may damage the plasma membrane then consequently membrane proteins (whether embedded in or attached to the membrane) will also be affected. Shear forces may affect activity of such proteins by altering the extracellular domain and/or altering their surface concentration. Other workers have observed membrane damage when cells were exposed to fluid mechanical forces. For example, Dewitz *et al* (1979) noted that shear forces induced clublike cytoplasmic protusions from polymorphonuclear leukocytes indicating that cytoplasm was extruding through punctures in the membrane. Frangos and Berthiaume (1990) observed that after exposure to fluid flow the amount of merocyanine dye (intercalates between phospholipids of the upper membrane) bound by endothelial cells increased, implying that phospholipids had become more loosely packed.

In this work, (Lakhotia *et al*, 1993) the effect of hydrodynamic stress (turbulent flow in a bioreactor agitated at 270 rpm) on the quantity of two surface proteins, CD13 and CD33 was examined. Both proteins are transmembrane glycoproteins spanning the membrane once and fluorimetric antibody staining was used to label the epitopes of the proteins. The fluorescence of the cells which was measured by a flow cytometer, is proportional to the number of epitopes recognised by the labeling monoclonal antibodies. Fluid mechanical forces can alter the number of epitopes recognised by the antibodies by damaging the three dimensional structure of the epitope or by altering the number of proteins. The results showed that an increase in agitation from 80 to 400 rpm reduced the apparent growth rate and the average CD13 and CD33 content per HL60 cell. The effects on the two surface proteins were observed within thirty to sixty minutes following the increase in the agitation and preceded the observed effects on cell growth by at least ten hours. Most importantly the CD13 and CD33 content was reduced even at agitation rates that did not affect cell proliferation. In both cases the

quantity of each membrane protein recovered to control levels (80 rpm) if agitation was reduced.

This work reiterates the other findings sited here, that is that agitation intensities not causing apparent cell damage can still affect cell physiology or other cell processes. The implications of this research for the growth of cells in agitated bioreactors cannot be ignored. In essence any cellular process which may be affected by alterations of the functionality or concentration of surface proteins will be affected by the hydrodynamic environment of the bioreactor where the cells are grown. For example, nutrient transport and hormonal regulation are two vitally important processes which may be changed by a reduction in the concentration of membrane bound proteins.

Hydrodynamic effects on surface proteins may have more serious implications in processes where a specific membrane protein plays a critical role in either the production phase or the therapeutic phase. Examples include;

- *in vitro* viral infection for the production of vaccines - specific receptor proteins on a host cell membrane are required for the initiation of viral infection. Sinskey *et al* (1981) showed that in the microcarrier cultures of chick embryo fibroblasts, the productivity of Sindbis virus is reduced by agitation even when there was no effect on the apparent growth rate.
- the production of recombinant proteins in insect cells using the baculovirus expression system - production rates decreased by 50% in bioreactors with intense mixing and sparging when compared to static or low agitation cultures (Murhammer and Goochee, 1988).
- the use of cells in cellular therapies - gene and other cellular therapies such as adoptive immunotherapy for cancer using tumour infiltrating lymphocytes (TIL) or lymphocyte activated killer (LAK) cells are based on the ability of *in vitro* grown cells (TIL or LAK) which when given to back to the patient, will recognise through specific receptor proteins, the targeted autologous tumour cells. Thus the culture environment of the TIL or LAK cells should have no detrimental effect on the critical receptor proteins.

Muul *et al* (1986) have reported that LAK cells grown in agitated spinner cultures have a significantly reduced cytotoxicity against tumour cells compared to cells grown in static cultures and these workers suggested that changes in the receptor surface proteins may have been responsible for these effects.

The plasma membrane contains many ion pumps and channels which transport small water soluble molecules through the highly impermeable hydrophobic lipid membrane. In SF-9 insect cells high levels of shear stress trigger rises in intracellular calcium, an important second messenger (Cherry and Aloi, 1992; Aloi and Cherry , 1993). The rise is seen even in the absence of external free calcium ions implying that the effect is more than simply perforation of the cell membrane. Fluid forces appear to be triggering the release of calcium ions from internal stores. Perhaps hydrodynamic forces are affecting calcium channels in the membrane which regulate calcium entry and export. Previous work on endothelial cells, revealed the presence of stretch-activated calcium ion channels in the membrane (Lansman *et al*, 1987). It was speculated that these ion channels may flood the cell with free calcium ions which activate enzymes responsible for prostacyclin synthesis. Providing further evidence to support this model are the observations by Ando *et al* (1988) that laminar stresses cause an almost instantaneous three fold rise in the intracellular free calcium level. The results from Ando *et al* and Aloi and Cherry appear very similar, that is shear forces cause a rise in intracellular calcium levels. Christensen (1987) observed stretch-activated calcium ion channels in epithelial cells residing next to calcium and voltage activated potassium channels. As mentioned in section 3.4.2. stretch sensitive receptors (thought to be ion channels which pass specific ions when mechanically stimulated) have been identified in a number of cell types and are believed to indicate that cells have evolved specific regulatory responses to mechanical stimuli (Guharay and Sachs, 1984, 1985; Stockbridge and French, 1988).

The cytoskeleton lies under the plasma membrane and is connected to it by various proteins (Luna and Hill, 1992). Researchers are now examining the role of the cytoskeleton in the mechanical stress response of cells. The cytoskeleton is composed of two major proteins polymerised into filamentous structures in a highly organised network. Microtubules are polymerised tubulin monomers and microfilaments are polymerised actin and actin binding proteins. The organisation of the cytoskeleton is dynamic with polymerisation and depolymerisation occurring constantly involving a high energy requirement. The assembly and disassembly of the filaments in the cytoskeleton may be affected by temperature changes, calcium concentration and certain reagents. Papoutsakis *et al* (1991) exploited this knowledge by using drugs in experiments to selectively inhibit specific components of the cytoskeleton. Colchicine inhibits addition of tubulin monomers to microtubules causing depolymerisation of the microtubules. Cytochalasins work in a similar way and depolymerise actin microfilaments. Results showed that while colchicine addition has no effect on cells when exposed to laminar shear stress, cytochalasin addition caused 20% more cell death. The workers concluded that microtubules would appear to play no role in the shear response of the cells, whereas microfilaments or other structures of polymerised actin are important for cell protection from shear. Other work has also emphasised the importance of actin in the cytoskeleton in determining shear resistance (Mazur and Williamson, 1977). Microfilaments in platelets were shown to be important mediators of structural and contractile properties in plasma clots while microtubules had no effect (Jen and M$^{c}$Intire, 1982). Franke *et* al (1984) have shown that shear stress induces actin stress fibres in endothelial cells. 3T3 cells grown on microcarriers display an altered distribution of actin stress fibres (du Laney *et al*, 1992). Microfilaments are also known to be important to cell shape and macromolecular metabolism ( Fulton *et al*, 1980; Cervera *et al*, 1981).

*1.3.4.6.1: Conclusions on Sub-Lethal Effects of Shear*

Cell death is not the only response a cell can show after being subjected to external physical forces. Hydrodynamic forces may result in increased energy metabolism, disturbed levels of second messengers, changes in gene expression and an altered cytoskeletal structure, amongst others. Although animal cells are now being grown successfully in a number of large scale industrial applications without a detailed understanding of these sublethal effects, the normal research and development process would have selected those products and cell types in which these sublethal responses were not a problem. Future production of other products, for example, highly glycosylated proteins, may require more stringent control of the physiological state of the cells to obtain fully functional products. In recent years there has been a trend towards growing a wider variety of cell types for research and therapeutic purposes and in these cases it may be critical to know and control these sublethal alterations in order for a successful outcome. Consequently an appreciation of agitation in a bioreactor as not only being capable of causing cell death but also of producing more subtle changes in animal cells is essential.

### 1.3.5: Role Of Growth Limiting Metabolites In Animal Cell Culture.

1.3.5.1: Introduction

In batch suspension cultures, cells show a rapid decline in cell viability after reaching a maximum cell density. This may be due to nutrient deficiency, however feeding glucose, amino acids and vitamins in late exponential phase has been shown to prolong viability but does not increase cell number significantly (Schumpp and Schaelger, 1990). Similarly fed-batch cultures prolong exponential phase but do not eliminate cells entering decline phase. Thus the importance of the accumulation of toxic metabolic waste products in animal cell cultures was realised. Lactate and ammonia are the primary products of animal cell metabolism and their accumulation has been shown to greatly inhibit cell growth ( Butler and Spier, 1984; Reuveny *et al*, 1986; Miller *et* al, 1988; Glacken *et al*, 1989(a)(b); Newland *et al*, 1990). Lactate and ammonia are produced from the catabolism of glucose and glutamine, essential components of animal cell growth media. However there is evidence that even by using methods to decrease lactate and ammonia accumulation there is still not a resultant increase in cell density (Imamura *et al*, 1982; Glacken *et al*, 1986; Hassell and Butler, 1990). Furthermore, work with lactate and ammonia double resistant clones demonstrates that cell proliferation stops at the same density as non-resistant control cultures (Schumpp and Schlaeger, 1992). Thus, it may be concluded that other more complex factors must be responsible for growth inhibition. Some evidence for this supposition has been reported in the literature (Holley *et al*, 1978; Merten *et al* 1985; Emery *et al*, 1987; Dodge and Hu, 1987; Thorpe *et al*, 1987).

The accumulation of lactate and ammonia in cell cultures and the possible mechanisms of toxicity will be discussed in the following sections. The importance of other toxic cellular inhibitors will also be considered.

1.3.5.2: Lactate Accumulation and Toxicity

Glucose is the most abundant sub-unit found in animal cell culture and is usually present at 5-20mM. It is the main carbohydrate utilised by animal cells for energy metabolism. Glucose is metabolised by cells to pyruvate via the glycolytic pathway for energy production. Pyruvate may be reduced anaerobically to lactate which has been found to be the most important waste product of glucose metabolism because of the oxygen limiting conditions in culture (Glacken, 1986). 80% of glucose in HeLa cells is converted into lactate with 8% metabolised via the pentose phosphate pathway and 5% or less shunted into the TCA cycle (Newland *et al*, 1990). This represents an inefficient use of glucose , lactate formation results in only 2 ATP molecules being produced as opposed to 36 ATP from TCA cycle metabolism. Lactate may also be produced in small amounts from glutamine metabolism.

The concentration of lactate which may accumulate in cell culture varies depending on glucose concentration, reactor operation and cellular activity. 35mM lactate after 5 days batch growth is commonly quoted in the literature as the average value (Ozturk *et al*, 1992). Lactate tolerance differs considerably from one cell line to another and with experimental conditions. For example, CRL-1606 cells are 50% growth inhibited at 40mM lactate (Glacken *et al*, 1986) as opposed to 55mM for a mouse hybridoma cell line (Ozturk *et al*, 1992). Other hybridomas show no inhibition up to concentrations of 40mM (Miller *et al*, 1988). Lactate was first noted to inhibit cell growth in medium without pH control and this inhibition was attributed to medium acidification (Eagle, 1973). However lactate inhibition was also observed in cultures grown at constant pH. It is thought that lactate may inhibit cell growth by a number of different mechanisms, for example:

- Excessive accumulation of lactate can decrease pH thus inhibiting growth (Imamura *et al*, 1982).
- Lactate may cause pH perturbation of electrochemical gradients thus affecting all membrane transport (Dean *et al*, 1984; Glacken *et al*, 1988).

- Lactate may act as a potent calcium chelator (Glacken *et al*, 1988).
- Lactate causes an increase in medium osmolarity (Ozturk *et al*, 1992).

This last mechanism is probably the most important, as it can be clearly shown that increasing lactate concentrations cause medium osmolarity values to increase. Lactate at concentrations of 23, 46 and 69mM results in osmolarity values of 0.33, 0.37 and 0.42 osm/kg respectively. Animal cells grow within a very defined osmolarity range (0.28-0.32 osm/kg) and it has been shown that increasing medium osmolarity results in decreasing growth rates (Ozturk and Palsson, 1991). It is believed that increased medium osmolarity results in growth inhibition due to cytoplasmic alkalinisation. The importance of osmotic pressure in animal cell cultures is further discussed in Section 1.3.6.

1.3.5.3: Ammonia Accumulation and Toxicity.

Glucose is not the only major energy source in animal cell medium. Glutamine is present in relatively high concentrations (2-5mM) and glutamine oxidation is responsible for 40% of energy production (Newland *et al*, 1990). Ammonium is produced from the chemical decomposition of glutamine. Catabolism of glutamine results in the formation of the ammonium ion, $NH_4^+$ and glutamate. Conversion of glutamate produces more $NH_4^+$, α-ketoglutarate and alanine.

Ammonia (sum of ammonium ion, $NH_4^+$, and ammonia, $NH_3$, species) accumulation in cell cultures depends on the bioreactor mode, cell and glutamine concentration and cellular activity. Ammonia accumulates in culture media to concentrations between 2-5mM and is particularly toxic to cellular growth and/or end product formation. Ozturk *et al* (1992) reports that 4mM ammonia is required to inhibit the growth of a hybridoma by 50% while Reuveney *et* al (1986) observed growth inhibition at 2mM ammonia in a mouse hybridoma cell line and a cytotoxic effect at 4mM. 8mM ammonia is

required to decrease the viability of MDCK cells by 50% (Glacken '86) while it has been reported that 40mM ammonia does not inhibit the growth of mouse ascites tumour.

The exact mechanism of ammonia toxicity is not known, however a number of proposals have been suggested in the literature:

- Ammonia can inhibit release of iron from transferrin inside acidic vesicles (Karin and Mintz, 1981).
- Non-ionised ammonia can diffuse readily through plasma membrane and inner membrane organelles causing pH elevation of acidic intracellular compartments (Glacken *et al* ,1988: Kovacevic and M$^{c}$Given, 1983).
- Ammonia may cause a decrease in intracellular pH due to the increase of the weak acid, $NH_4^+$ into the cytoplasm or uptake of the weak base, $NH_3$ into lysosomes can increase the pH of intracellular organelles (M$^{c}$Queen and Bailey, 1990, 1991).
- Ammonia may cause depletion of TCA cycle intermediates (Newland *et al*, 1994).

It is thought that the toxic effect of ammonia may be due to a combinations of mechanisms. A recent publication by Martinelle and Haggstrom (1993) develops some of the theories suggested in earlier work. These authors postulate that one important toxic effect of the ammonium ion is to cause an increased demand for maintenance energy caused by the need to maintain the ion gradient over the cytoplasmic membrane. The $NH_4^+$ ion is thought to compete with the $K^+$ ion for transport into the cell and may thus decrease the intracellular $K^+$ concentration, which is an important factor for many enzymes and for the potassium gradient over the cytoplasmic membrane. Free ammonia may also cause an increase in the intracellular pH when it diffuses through the membrane by associating with $H^+$ ions, subsequently disrupting intracellular enzymes, especially lysosomal enzymes which require an acidic pH for optimum activity.

While the exact mechanism of ammonia inhibition is not certain, the toxic effect that excessive accumulation may have on cells is unequivocal. Both lactate and ammonia inhibit growth in batch cultures, with the inhibition concentration of lactate at least one order of magnitude higher than that for ammonia. Lactate appears to inhibit cell growth by no more complicated a mechanism than causing a rise in medium osmolarity. The mechanism of ammonia toxicity is not fully elucidated but appears to be related to changes in intracellular pH.

1.3.5.4:Other Growth Limiting Molecules in Animal Cell Culture

Cells growing in suspension show a rapid decline in cell viability after reaching a maximum cell density. Cessation in cell growth may in principle be due to exhaustion of specific nutrients or growth factors, degradation of a critical medium component, accumulation of toxic metabolites (lactate and ammonia) or be in response to other more complex factors e.g. accumulation of cytotoxic products in the culture broth. The weight of evidence tends to favour the latter (Visk *et al* , 1972; Holley *et al* , 1978) and the question must then be asked as to what are the cytotoxic/growth inhibitory components ? Work in cancer research and cell biology has led to the identification of growth inhibitors, usually growth factor-like proteins or peptides which have a negative effect on cell growth (Miyasaki and Horio, 1989). Small molecular weight cytotoxic proteins such as Tumour Necrosis Factor have also been discovered (Hori *et al*, 1988). Section 1.2 of this thesis gives a short review of growth inhibitors with particular emphasis on the more recently identified factors. Thus the importance of growth inhibitory molecules in cell proliferation and differentiation has been realised by researchers in specialised cell control studies. However it is only comparatively recently that the significance of growth inhibitory and/or cytotoxic molecules produced by cells growing in large scale systems has been investigated.

Merten *et al* (1985) studied monoclonal antibody kinetics with high and low producing hybridomas. What appeared to be feedback inhibition was observed when spent culture supernatant was included with fresh medium. Emery *et al* (1987) reported that investigations of spent culture medium fractions were being carried out in an attempt to identify cytotoxic components. Dodge *et al* (1987) identified a cytotoxic factor(s) in the conditioned medium (cm) of late exponential and decline hybridoma cultures. This factor appeared to be of low molecular weight since it was dialysable through 10,000MW tubing. Dilution of the cm resulted in a decrease in cell death again supporting the theory of a cytotoxic factor as the causative agent as nutrient depletion would cause an increase in cell death upon dilution. This factor which was identified in a mouse hybridoma cell line also affected another hybridoma cell line. These workers suggest that as yet unidentified inhibitory substances exist and propose that process optimisation will require removal of such components from the culture medium. Ronning *et al* (1991) showed that it was not possible to restore growth supporting property of spent medium by supplying additional serum, glutamine or glucose (amino acids, ammonia and lactate were not at growth limiting levels). Two cytotoxic molecules were identified one with an approximate molecular weight of 5000 daltons and the other of approximately 67,000 daltons which the authors suggest may be bound to a carrier such as albumin. However growth promoting properties of the medium may be returned by dialysis of the medium.

To date the growth inhibitory molecules identified in large scale cultures appear to be of relatively low molecular weight. This observation might go some way to explain the success of dialysis based bioreactors such as hollow fibres and microencapsulation reactors. By far the highest cell densities may be attained in these type of reactors. As a result, novel dialysis bioreactors have been designed allowing the achievement of high cell numbers (Comer *et al*, 1990; Sjorgren-Janssen and Jeansson, 1985).

### 1.3.6: Effect Of Osmotic Pressure On Animal Cell Growth

Animal cells are sensitive to changes in medium ionic strength and osmolality. The osmolality of serum is 0.29 osm/kg and thus the ideal osmolality range for cell growth is 0.28-0.32 osm/kg. Extremes of osmolality have been shown to be detrimental to cells (Oyaas *et al* , 1989). In large scale culture, medium osmolality is a largely uncontrolled and ignored process parameter which is defined mainly by medium composition and allowed to vary freely during culture growth. Medium osmolality may change in the course of the growth cycle as a result of several factors; for example, accumulation of metabolic products or pH control with the addition of acid or base. Cell membranes are incapable of sustaining any sizable osmotic pressure difference and the osmotic pressure inside cells is therefore close to that of their surroundings. Through osmoprotective mechanisms, cells are protected against dehydration or swelling.

Work by a number of authors has highlighted the fact that increased osmotic pressure decreases cellular growth rates (Ozturk and Palsson, 1991(b); Oyaas *et al* 1989; Passini and Goochee, 1989(b)). Ozturk and Palsson (1991(b)) increased osmolarity using both ionic compounds (NaCl and PBS) and by non-ionic agents (sucrose). Decreased apparent growth rates were noted above 0.38 osm/kg. The mechanisms of osmolarity sensitivity are not completly known but several investigations have been carried out. Studies with Erlich ascites mouse tumour cells shows that exposure to hypo-osmotic media involves selective changes in the surface membrane permeability to $Na^+$, $K^+$, $Cl^-$ and amino acids (Hoffman, 1980). Exposure of renal cells to hyperosmolarity resulted in higher concentrations of myo-inositol, glycerophosphorylcholine and glycine betaine which have been identified as cellular osmoprotectants (Nakanishi *et al*, 1988). Recent work by Oyaas *et al* (1994) demonstrates that a range of osmoprotective compounds (including sugars, polyhydric alcohols, free amino acids, methylamines and urea)

accumulate inside cells exposed to high osmotic pressure in order to balance the increased extracellular osmolarity.

Interesting work by Oh *et al* (1995) suggests that osmolarity may be used in process design to enhance antibody production. These workers observed substantial overproduction of antibodies by applying osmotic pressure using NaCl to suppress cell growth. It is proposed that increases in osmolarity may enhance transport of nutrients, in particular amino acids into cells since an increased uptake via $Na^+$-dependent transport systems was noted. These amino acids act as osmoprotectants and precursors for cell metabolism and antibody synthesis. Increased osmotic pressure decreased DNA synthesis and increased glucose consumption, glutamine consumption and metabolic rates. Thus an improved medium for antibody production may be designed by simply increasing medium osmolarity.

## 1.4: Aims for the Experimental Work in this Thesis

The aim of the work presented here is to investigate factors, both endogenously produced and external, that inhibit the growth of animal cells in *in vitro* large scale culture systems. The first section of Chapter Three of this thesis (Section 3.1) presents investigations into growth inhibitory factors produced by a number of cell lines *in vitro*. Most of the work involves an inhibitory/cytotoxic factor produced by a mouse transformed cell line (MSV-3T3) that inhibits the growth of a number of carcinoma cell lines. Efforts to characterise the inhibitory/cytotoxic factor(s) in medium conditioned by MSV-3T3 cells are made using ultrafiltration fractionation and by comparing the activity of the MSV-3T3 factors to the two most well documented inhibitors/cytotoxic factors, TGF-β and TNFα.

Section 3.2 of this thesis examines the role of the external culturing environment and its potentially growth limiting effects.The effect of waste metabolite build up, osmotic pressure and in particular agitation are examined. In each case the effect of the external factor is compared between a multi-drug resistant (MDR) cell line and its drug sensitive parental cell line. Earlier work in this laboratory (Redmond, 1991) had suggested that MDR cells might be more physically robust than their drug sensitive counterparts. Thus the aim of this work is two-fold, that is, firstly to establish the negative effect on growth of factors such as metabolite build-up, osmotic pressure and agitation and secondly, to ascertain if there is a difference in response to these factors of MDR cells when compared to their parental cells. Ultimately, the identification of a more physically robust cell line would have both theoretical and industrial value.

Lactate and ammonia are the primary products of animal cell metabolism and their accumulation has been shown to greatly inhibit cell growth (Section 1.3.5.1). The tolerance of CHO-K1 (a Chinese hamster ovary fibroblastic cell line), its MDR counterpart, CHRC5, Hep-2 (a human carcinoma cell line) and

its MDR counterpart, Hep-2A to 'normal' and excessive lactate and ammonia levels is investigated. Extremes of osmotic pressure have been shown to be detrimental to cells (Section 1/3/5/2), the tolerance of CHO-K1, CHRC5, Hep-2 and Hep-2A cells to hyperosmotic and hypoosmotic pressure is investigated. Shear stresses are generated in bioreactors when the fluid is mixed. It is well documented (Section 1.3.4) that animal cells are inherently fragile due to their relatively large size and lack of cell wall. Thus particular emphasis is placed on the effects of agitation on the growth of cells in a small scale stirred bioreactor (spinner flask). The effect of agitation on CHRC5, CHO-K1, Hep-2 and Hep-2A cells is investigated. In order to explain the increased agitation resistance noted in the MDR cell lines, the influence of plasma membrane fluidity (PMF) is investigated. Increasing plasma membrane fluidity has been correlated with increasing shear sensitivity (Section 1.3.4.5.3). The effect of a high shear environment on CHO-K1, CHRC5, DLKP (a human lung carcinoma cell line which is noted for being particulary robust ) and DLKPA (MDR counterpart of DLKP) cell growth and on their resulting PMF is investigated. Finally an examination of the hydrodynamic environment of two different spinner vessels (one with a typical agitation system and one with a unique mixing system) is undertaken in order to estimate the shear levels to which cells are exposed during the agitation experiments.

## CHAPTER TWO

# MATERIALS AND EXPERIMENTAL METHODS

## 2.1: Media Preparation

### 2.1.1:Ultrapure Water

Ultrapure water is an absolute requirement for all medium and reagents used in animal cell culture. Tap water was purified using either a Millipore Milli-Q system or an Elga UMP system. The basic steps in producing ultrapure water were as follows:- tap water was passed through a Reverse Osmosis (Milli-Q) or double distillation system (Elga UMP). Two pre-filters were then used to remove ionic and non-ionic solutes. An ion-exchange filter followed by a carbon filter were then utilised. The purity of the final water was tested by an on-line conductivity meter. Only water of 12-18 mΩ- cm resistance was used for the work in this thesis.

### 2.1.2: Glassware

All glassware in contact was treated as follows. Bottles and lids were soaked in a 0.2% warm solution of a deproteinising agent, RBS (AGB Scientific) for 60-90 minutes. The glassware was then scrubbed and then rinsed in tap water before being washed in a industrial washing machine. The washing machine carried out a number of washing and rinsing cycles, including a neodisher cycle (this is an organic, phosphate based acid detergent); two rinsing cycles in distilled water and a final rinsing cycle in ultrapure water. Glassware was then sterilised by autoclaving at 121°C and 1 bar pressure for 15 minutes.

### 2.1.3: Sterilisation

All glassware, water for media preparation, some reagents and non-sterile plastics were autoclaved before use (121°C/15 psi/20 minutes; ). Unstable reagents were filter sterilised through sterile disposable 0.22μm filters (Millex-GV, SLGV025BS). Large batches of culture medium (5 litres) were peristaltically pumped through a Bell filter (Gelman, G.14238) into sterile 500ml glass bottles.

### 2.1.4: Media Preparation

All media was prepared according to standard laboratory practices. This basically involved, making up media in 5 litre batches from 10x liquid medium and depending on the media type adding various other components listed below in Table 2.1. The pH was adjusted with sterile 1.5N HCl (RDH, Cat. no. 37021) or NaOH (RDH, Cat. no. 30620). The media was sterilised through 0.22μm filters and stored in 500 ml aliquots at 4°C and in the dark to prevent the destruction of light sensitive vitamins (B and C groups) and to prevent the formation of free radicals. Sterility was checked using turbidity, pH, Columbia blood agar (Medlabs), Sabourand dextrose (Oxoid, CM143) and thioglycollate broth (Oxoid, CM173).

L-glutamine (2mM) (Gibco, Cat. no. 043-05030) was added to each media type before use. Antibiotics were not used in routine culture. In large scale cultures, medium was supplemented with 1% penicillin/streptomycin (Gibco, Cat. no. 043-05140).

Table 2.1: Media and Media Additives Used in This Work

| Additive | MEM (Gibco, Cat. no. 042-01430) | DMEM (Gibco, Cat. no. 042-02051) | Hams F12 (Gibco, Cat. no. 074-01700N) |
|---|---|---|---|
| ultrapure $H_2O$ | 4200 mls | 4300 mls | 4700 mls |
| 1M HEPES (Sigma, Cat. no. H9136) | 100 mls | 100 mls | 100 mls |
| 7.5% $NaHCO_3$ (Riedel-de-Haen, Cat. no. 31437) | 45 mls | 45 mls | 45 mls |
| MEM NEAA (Gibco, cat. no. 043-114OH) | 50 mls | — | — |

The abbreviations above are defined as; MEM - Minimal Eagles medium; DMEM - Dulbeccos' modified Eagles medium; HEPES - (4-(2-HydroxyEthyl)-PiperazineEthaneSulphonic) acid; $NaHCO_3$ - sodium bicarbonate; MEM NEAA - MEM non-essential amino acids.

## 2.2: Cell Lines Used In This Thesis

Table 2.2: Cell Lines Used in this Work

| Cell Line | Source | Medium |
|---|---|---|
| **NRK** - normal rat kidney fibroblasts | Ian Pragnell, Beatson Institute, Scotland | DME + 5% FCS |
| **CHRC5** - MDR variant of CHO cells | Victor Ling, Ontario Cancer Institute | MEMα + 5% FCS |
| **CHOK1** - Chinese Hamster Ovary | ATCC - CRL | Hams F12 + 5% FCS |
| **SCC-9** - squamous cell carcinoma | ATCC - CRL 1629 | ATCC medium (50% DME + 50% Hams F12) + 5% FCS |
| **MSV-3T3** - Molony MSV transformed mouse | ATCC - CRL 1568 | DME + 5% FCS |
| **RPMI** - 2650 - human nasal carcinoma | ATCC - CRL 1629 | MEM + 5% FCS |
| **BSC-1** - African green monkey | ATCC - CCL 26 | MEM + 5% FCS |
| **NRK-49F** - Clone of NRK | ATCC - CRL 1570 | DME + 5% FCS |
| **Hep-2** - human epithelial carcinoma of larynx | ATCC - CCL 23 | MEM + 5% FCS |

## 2.3: Routine Culture Of Cells

### 2.3.1: Cell Culturing

Cells were routinely maintained in their respective media described above (8-9mls of media in a 25cm$^2$ flask; 20mls in a 75cm$^2$ flask). Cells were incubated in the appropriate medium in tissue grade culture flasks (Cell Cult, Cat. no. 32025; Costar, Cat. no. 307S) at 37°C in a 5% $CO_2$ humidified atmosphere. The 5% $CO_2$ atmosphere allows the bicarbonate buffering system in the medium to operate to help control pH which becomes more acidic as cells grow. (Phenol red indicator in the medium changes from red to orange indicating that the pH has dropped to below pH 7.2). Cells were grown to confluency with feeding. All fresh media was incubated at 37°C before use. All manipulations involving cells were carried out using aseptic technique in a class 2 laminar flow. Every item entering the laminar flow was swabbed in 70% IMS (Lennox, Cat. no. 1170).

When dealing with several different cell lines a waiting period of 15-20 minutes was exercised to ensure that no cross contamination of cell lines occurred.

### 2.3.2: Subculture Of Cells

When cells were confluent (i.e. covering > 90% of available flask surface) they were trypsinised with a 0.25% trypsin versene (T.V.) solution. This trypsinisation procedure involved the following steps;

- waste medium was removed
- cells were washed in pre-warmed T.V. solution to remove any residual serum which contains enzymes to inhibit the action of serum
- 2ml of T.V. was added to a 25cm$^2$ flask (4mls to a 75cm$^2$ flask)
- the cells and T.V. were incubated at 37°C for 5-10 minutes until cells began to detach from surface

- when a single cell suspension was obtained, serum containing medium was added to inhibit further trypsin action
- the resulting solution was centrifuged at 1000rpm for 5 minutes
- the pellet was resuspended in fresh medium and cells were counted before being inoculated into new flasks.

2.3.2.1: Trypsin Versene Solution

50mls of porcine trypsin was sterilised by passing through a 0.22μm low protein binding filter (Millipore GV SLGV025BS ). This was added to 439mls of sterile PBS-A (Oxoid, Cat. no. BR12a) 11mls of 1% EDTA (Sigma, Cat. no. ED6758) which brought the final volume to 500mls. The resulting T.V. solution was dispensed into 20ml volumes in universals ( Sterilin, Cat. no. 128A) and stored at -20°C.

**2.3.3: Cell Counting**

A sample of single cell suspension (1ml) was mixed well with 200μl of trypan blue (Gibco, Cat. no. 043-05250) for 5 minutes. The cells were counted by loading a sample on a Waber haemocytometer (Improved Neubawer) slide using a Pasteur pipette. Each of the four large squares at each corner of the grid was counted and an average count obtained. Multiplication of this count by $1.2 \times 10^4$ (based on the volume of cell suspension under the coverslip) gave the the average cell count per ml of cell suspension. Cells taking up the blue dye were counted as non-viable, while non-stained cells were viable.

## 2.4: Long Term Cell Storage

Cells were routinely stored for indefinite periods at -196°C in liquid nitrogen. For each cell line, a master stock was kept in a separate culture collection. From the master stock, a working stock was obtained.

### 2.4.1: Cell Freezing

Cells were frozen in their exponential growth phase. They were screened to ensure mycoplasma infection was absent. Cells were trypsinised and resuspended in medium supplemented with 5% FCS. A viable count of at least 5 x $10^6$ cells/ml was required before freezing could take place.

The cryopreservant used was 10% DMSO/50% FCS in 40% medium. The freezing method was as follows;

- slowly in a dropwise manner, the DMSO (Sigma, Cat. no. D2650) solution was added to cells with a swirling motion. This action was very important because DMSO is toxic to the cells and by adding it slowly the cells have a period to adapt to its presence. Otherwise cell lysis may have occurred.
- the resulting suspension was aliquoted into sterile cryovials (Greiner, Cat. no. 122278)
- cells were slowly frozen in the vapour phase of liquid nitrogen for 3 hours and then stored in compartments in the liquid phase.

### 2.4.2: Cell Thawing

Cells were thawed quickly at 37°C. They were then transferred quickly to a sterile universal with serum supplemented medium and centrifuged at 1000rpm for 5 minutes to remove all traces of DMSO. Cells were then added to a 25$cm^2$ flask in the normal manner. After 24 hours cells were fed with fresh medium.

## 2.5: Contamination of Cultures

### 2.5.1: Microbial

Microbial contamination is easily detected in cell cultures. Gross contamination is often associated with cell detachment and acid production.

Lower levels of contamination may be detected by careful microscopic examination. Most cases of microbial contamination may be prevented by proper asceptic technique in a class II vertical laminar flow cabinet. All reagents and media should be sterility checked before use (section 2.1.4).

### 2.5.2: Mycoplasma

Mycoplasma contamination is not always obvious. Infected cells continue to grow but they appear granular and 'unhealthy'. During the course of this project cells were routinely checked for mycoplasma infection by Ursula Patterson, Una Gilvarry and Cathy Halligan. If a culture was found contaminated it was discarded immediately and fresh stocks thawed. The laminar flow cabinet was thoroughly cleaned and media and other reagents in contact with infected cells were not reused.

#### 2.5.2.1: Indirect Staining for Mycoplasma

An indirect staining technique was used to detect mycoplasma. Mycoplasma free stocks of NRK cells were grown overnight at 37°C on sterile coverslips in 1ml of medium at a concentration of 5 x $10^2$ cells/ml. The following day, 1ml of medium from a flask of cells that were known to be grown in antibiotic free medium was added (in duplicate). Control plates with added fresh medium were included. The cultures were incubated for 3-4 days at 37°C/5% $CO_2$. The slides were rinsed twice in PBS followed by a single rinse in a 1:1 dilution of Carnoys' reagent (acetic acid: methanol, 1:3) in PBS. The cells were fixed in Carnoys' reagent for 10 minutes. The coverslips were removed and rinsed in ultrapure water. Hoescht 33258 (Sigma, Cat. no. B2883) at 0.5mg/ml in PBS was added to the cells (2mls) and left for 10 minutes at room temperature. Due to the light sensitivity of Hoescht these manipulations were carried out in petri dishes covered in tinfoil. Excess stain was removed by rinsing in ultrapure water and the slides were then mounted in glycerol. The cells were observed under oil immersion using 405nm light. Extranuclear fluorescence indicated the presence of mycoplasma contamination.

2.5.2.2: Direct Culture of Mycoplasma

In this method the conditioned medium from the test cells was inoculated into broths or agars designed specifically to enrich mycoplasma growth. Mycoplasma Agar (CM401) and Broth (CM403) base are supplied by Oxoid. To 9mls of sterile agar or broth base, the following supplements were added: 2ml Horse serum, 1ml fresh yeast extract (0.25g.ml), 25µl penicillin and 130µl of 0.2% DNA (BDH Cat. no. 42026). The test sample was then inoculated onto plates and into broth and allowed to incubate at 37°C for 3 weeks. The plates were incubated in sealed $CO_2$ jars to prevent contamination. Broths and plates were then checked regularly under a microscope for growth.

**2.5.3: Cell Line**

If proper procedures are adhered to, cell line cross contamination should not be a problem. The precautions taken against this type of contamination included:

- Working with only one cell line in the laminar flow cabinet at any one time. Allowing at least 15 minutes between cell lines. Swabbing all surfaces well with 70% IMS before and after use.
- Using separate media bottles, sera, trypsin, L-glutamine and waste bottles for each cell line, ensuring that they were labeled clearly.
- Ensuring all flasks and cryovials were clearly labeled and documented.

## 2.6: Large Scale Culture Of Cells

If greater volumes of cells than could be provided by 25 or 75cm$^2$ flasks were required then growth could be scaled up using either roller bottles (anchorage dependent cells) or spinner flasks (anchorage independent cells).

### 2.6.1: Roller Bottle Cultures

A pre-sterilised glass (Bellco - 670cm$^2$ internal surface area) or plastic (Costar) roller bottle was incubated with a few mls of fresh medium at 37°C. Cells were trypinised and counted. The medium was removed from the roller bottle and cells in a volume of 100mls of medium were added (the inoculem size varied from cell line to cell line). The roller bottle was incubated at 37°C on specialised roller bottle appartatus (Bellco) which slowly rotated the bottle thus alternatively exposing the cells to medium and oxygen in the headspace. To encourage attachment of cells, roller bottles were rotated at 0.25rpm for 24 hours. The speed was increased to 0.75rpm after this time as cell clumping would occur if left at the slower speed.

Cells were examined microscopically every day to observe growth. When near confluency was obtained, cells were trypanised using 20 mls of trypsin. Roller bottles were incubated on the roller apparatus during trypanisation to ensure all cells were removed.

### 2.6.2: Spinner Flask Cultures

250ml and 500ml glass spinner flasks from Techne (Cambridge, U.K.) were used for the majority of this project. 500ml spinner flasks from Bellco (New Jersey, USA) were used towards the end of the project.Before glass spinner flasks can be used for cell cultures the glass must first be treated to prevent cells adhering to the glass. This was accomplished using a siliconisation procedure.

#### 2.6.2.1: Siliconisation of Spinner Flasks

A small amount of 2% trimethyldichlorosilane in 1,1,1 trichloroethane (BDH 33164) was added to the flasks which were rotated by hand to ensure even coating of all surfaces (including the agitator). This procedure was carried out in a fume hood. The flasks were allowed to dry for approximately an hour.

The flasks were then rinsed three times in distilled water followed by a final rinse in ultrapure water. A small amount of ultrapure water was left in the flask and the two side arm caps left a little open to allow steam ventilation during autoclaving. Flasks were covered in tinfoil and sterilised at 121°C/1 bar absolute pressure for 15 minutes.

2.6.2.2: Culturing in Spinner Flasks

After sterilisation, flasks were rinsed in pre-warmed medium and incubated at 37°C ready for use. Cells were trypsinsed in the normal manner and counted. For spinner cultures it was important to try to have an actively growing culture to ensure that the lag phase in the spinner would be as short as possible. 100mls of medium containing cells was inoculated into the spinner and the cultures were then incubated at 37°C on a special spinner unit (Techne) at an average speed of 40rpm (depending on the experiment being undertaken).

2.6.2.3: Sampling from Spinner Flasks

Cells were counted every day, as per Section 2.3.3. To ensure a representative count while sampling in the laminar flow where the spinner unit could not be placed, it was imperative to keep the spinner unit rotating by hand while pipetting out a 2ml sample. If this was not done then cells would settle to the bottom and a representative count would not be obtained.

2.6.2.4: Feeding Spinner Flasks

Cultures could either be partially fed or totally fed. If the pH of the medium appeared to be becoming slightly acidic a partial feed was performed, if the medium appeared spent (i.e. an orangey colour indicating that the pH was below 7) a total feed was performed. In general spinners were either partially or totally fed every two days. If a partial feed only was required, then spinners were placed in the laminar flow and a cap on one of the side arms loosened. Cells were allowed to settle for 5 minutes. Medium was then decanted off the top of the culture. Fresh medium was then added to bring the final volume to the original value. If a total feed was required, cells and medium from the

spinner were poured into sterile universals and spun down at 1000rpm for 5 minutes. The resulting waste medium supernatant was discarded and the cell pellet resuspended in fresh medium. Cell counts were performed (section 2.3.3) and cells were then put back into the spinner with the fresh medium.

2.6.2.5: Gassing Spinner Flasks

Spinner flasks were incubated in an ordinary incubator not in a 5% $CO_2$ atmosphere as were flasks. As such the bicarbonate buffering system (used because of the presence of sodium bicarbonate in the medium) would not be used to control pH. Thus spinners were gassed every day with a 5% $CO_2$/ 95% air mixture to ensure this buffering system was in place. A tank of the gas mixture was attached to the laminar flow, tubing was attached to the cylinder head. Air filters (0.22μm) were autoclaved and attached to the cylinder tubing (swabbed with IMS before being put into the laminar flow). A side arm of the spinner was opened and the air filter held over the arm. The gas was turned on and sterile gas allowed to enter the headspace of the vessel for approximately 1 minute. This gassing system allowed the medium to provide adequate buffering in the spinners.

## 2.7: Collection of Conditioned Medium

### 2.7.1: Roller Bottle cm.

Roller bottle cultures were set up in the usual manner (Section 2.6.1). When cultures were approximately 60% confluent (2-3 days depending on cell line), medium was removed and the cells rinsed twice with PBS or medium without serum. 100mls of serum free medium was then added to the cells and incubated overnight. This medium was discarded and replaced with 100mls of fresh serum free medium, which was allowed to be conditioned by the cells for twenty four hours. This conditioned medium was designated cm1 and was stored at 4°C. This medium was collected and replaced with another 100mls

of serum free medium. This procedure was continued for 4-5 days depending on the condition of the cells, resulting in 4-5 batches of cm (cm1-cm5).

### 2.7.2: Spinner Flask cm

MSV-3T3 cells were grown in spinner flasks therefore the cm from these cells was collected in the spinners. Spinner flasks were set up as outlined in Section 2.6.2. After 2-3 days growth when cells were in exponential phase of growth (as determined by cell counting, Section 2.3.3) cells and medium were poured into universals and cells spun down at 1000rpm for 5 minutes. Waste medium was discarded and the cell pellet was rinsed in serum free medium and spun down again. Cells were resuspended in 100mls of fresh serum free medium and added back to the spinner. After 24 hours cells and medium were removed to universals, centrifuged, the medium was discarded, the cells resuspended in fresh serum free medium and added back to the spinner. After 24 hours the same procedure was followed except that the medium on the cells was not discarded and was designated cm1 and stored at 4°C. Approximately 4-5 batches of cm were collected from each spinner in this manner depending on the condition of the cells.

## 2.8: Processing of Conditioned Medium

### 2.8.1: Ultrafiltration

Ultrafiltration was used to concentrate and fractionate the cm collected. The stirred cell method was used (Amicon no. 8499 - 400ml capacity chamber), with the following MWCO membranes (Amicon):

| Amicon Membrane | MWCO | Retentate (R) and Filtrate (F) Generated |
|---|---|---|
| YM 100 | 100,000 | R100 and F100 |
| YM 30 | 30,000 | R30 and F30 |
| YM 10 | 10,000 | R10 and F10 |
| YM 5 | 5,000 | R5 and F5 |
| YM 2 | 2,000 | R1 and F1 |

In general, cm was concentrated in the range 5x to 20x by ultrafiltration. cm could be fractionated by concentrating the filtrate from one ultrafiltration membrane on a smaller one. For example, the R10-30 fraction was obtained by concentrating F30 through the YM10 membrane.

### 2.8.2: Dialysis

Concentrated cm samples and media controls were dialyzed against 50 volumes of PBS and 25 volumes of medium using either a low molecular weight, benzoylated 1,200 MWCO tubing (Sigma D7884) or a 10,000 MWCO visking tubing. Before using the low molecular weight tubing, the tubing was boiled for 5 minutes in 10mM EDTA. It was then washed well in distilled water and finally rinsed in ultrapure water. Dialysis was carried out at 4°C with the buffer changed twice daily for 2 days. Volume changes in the dialyzed samples were noted and corrections made to the concentration factor before the cm was assayed.

### 2.8.3: Lyophilisation

Lyophilisation of cm was carried out using a 4.5 litre Consol Freeze Dryer (Virtis). Samples were frozen to -30°C overnight. The freeze drying cycle was started and usually lasted for 36 hours or until samples were dry.

## 2.9: Stabililty of Conditioned Medium

### 2.9.2: Temperature Stability

Conditioned medium (10mls) concentrated by ultrafiltration (10x) was dispensed into clean 30ml glass universals and placed in a covered waterbath set at 65°C for the required length of time (1 hour). Samples for boiling were placed in a beaker of boiling water over a Bunsen burner with universal lids slightly open. When the temperature of the cm reached 98°C, the boiling was timed for 3 minutes. Each sample was then centrifuged at 3000rpm for 15

minutes to remove insoluable precipitates, and filter sterilised before being assayed for activity.

### 2.9.2: pH Stability

Conditioned medium samples were dispensed into universals and the pH of each adjusted with 5M HCl or 5M NaOH. The samples were incubated at 4°C for two hours before the pH was re-adjusted. Osmolarity was measured on the Osmomat 030 (Gonotec). Each sample was filter sterilised before assay.

### 2.9.3: Protease Stability

A concentrated cm sample (10x) was incubated for 2 hours at 37°C with 10μg/ml trypsin (Gibco). The reaction was stopped with 20μg/ml soybean trypsin inhibitor (Gibco).

## 2.10:Assay Systems Used

### 2.10.1: Growth Inhibitory Assay

Growth inhibitory assays were carried out on both cm samples for the growth inhibition work (Section 3.1) and on assays examining the inhibitory effects of reagents such as NaCl or lactate (Section 3.2).

Miniaturised assays were carried out either in 24 well or 96 well plates (Greiner). The results from 24 well plates were determined by image analysis or crystal violet dye elution. For 96 well plates, results were determined by acid phosphatase activity or crystal dye elution. The procedure for the growth inhibitory assay was similar despite the assay plate used and the end point determination used.

Cells were trypsinised and seeded at between $1 \times 10^5$ to $5 \times 10^5$ cells/ml (depending on cell type) in a $75cm^2$ flask, 48 hours before setting up the assay. This was to ensure the cells were in exponential phase before the assay

and to increase the reproducibility of the assay. Cells were trypsinised as in section 2.3.2. Cells were counted and set up at in 500µl (24 well plate) or 100µl (96 well plate) of serum with 2x the concentration of serum required (5% for SCC-9 cells, 2.5% for other cell lines used). Sample addition after 24 hours was in serum free medium, hence the 2x concentration of serum on day 1. Cells were plated at the following concentrations depending on the cell type;

| Cell Type | Cell Concentration per well |
|---|---|
| SCC-9 | $5 \times 10^5$ cells per well (24 well plate) |
| Hep-2 | $2.5 \times 10^3$ cells per well (24 well plate) |
| Hep-2 | $5 \times 10^2$ cells per well (96 well plate) |
| CHO-K1 | $5 \times 10^3$ cells per well (96 well plate) |
| CHRC5 | $5 \times 10^3$ cells per well (96 well plate) |

Plates were shaken gently in all directions to ensure even attachment of cells on the well surface. Plates were incubated at 37°C/5% $CO_2$ for 24 hours. 500µl of cm or reagent test sample was then added to the appropriate wells. Conditioned medium was collected in serum free medium as stated earlier, concentrated cm samples were at 2x the desired concentration since when added to the assay they were diluted 1 in 2. Likewise the reagent samples were made up at 2x the desired final concentration. Samples were added in triplicate in 24 well plates and in 8 replicates in 96 well plates. A medium control was added to every plate in the assay to ensure reproducibility between plates. After 5 days for 24 well plates and 3 days for 96 well plates, growth inhibition was determined by one of the following methods.

2.10.1.1: Image Analysis

Image analysis was used to measure cell area on 24 well plates. Crystal violet (BDH, 42555) was used to stain the cells. Crystal violet was made up as a 0.25% solution in ultrapure $H_2O$ and filtered before use. This is a non-specific binding dye. For this reason, sufficient washing steps before staining were carried out to remove serum proteins which could interfere with the assay.

Waste media was removed and the wells were washed 3 times in PBSA (approximately 0.5ml per well). 0.5ml of 0.25% crystal violet stain was addec to each well and left to develop for 5-10 minutes. The crystal violet was then decanted and the plates washed with tap water to remove any unbound crystal violet. The plates were left to dry before being analysed using an image analyser. Crystal violet dye could be filtered and resused.

2.10.1.2: Dye Elution

0.25% crystal violet was added to the cells as above. The dye was eluted from the wells by adding 250µl of 33% glacial acetic acid to each well. 100µl samples were aliquoted into a 96 well plate. The absorbance was read on an ELISA plate reader at dual wavelength of 570nm and 620nm; 570nm is the wavelength specific for maximum absorbance of the crystal violet dye and 620nm is the wavelength specific for absorbance from the plastic in the plates.

2.10.1.3: Acid Phosphatase Assay

The acid phosphatase assay is based on the activity of cellular acid phosphatases which can be related to cell number. For this procedure a para-nitro-phenyl phosphate buffer was required. The buffer was made up with 0.1% Triton X-100 (Sigma 119F0733) and 0.1M sodium acetate (Reidal-deHaen) at pH 5.5. This was stored at 4°C in the dark. The substrate for the reaction para-nitro-phenyl (PNP) (Sigma C104) was added just before use. The complete PNP buffer was stored in the dark as it was light sensitive. Waste medium was removed and the cells rinsed twice in 100µl PBS per well. 100µl of freshly prepared PNP buffer was added to each well. This buffer contained Triton X-100 which lysed the cells and exposed cellular phosphatases to the phosphatase substrate. The reaction was incubated in the dark at 37°C for 2 hours. The reaction was stopped by addition of 50µl of 1M NaOH to each well. The results were read on a Titertek plate reader at a dual wavelength of 405nm and 620nm; 405nm is the wavelength of maximum absorbance for the dye and 620nm for the plastic in the plates.

### 2.10.2: TNF Inhibitory Assay

TNF activity was measured as follows. 100µl of cell suspension was plated in 96 well plates at a concentration of 2 x $10^4$ cells/well for L929 cells and at 1 x $10^4$ cells/well for Hep-2 cells. After incubation of 24 hours to allow attachment of cells, 50µl of TNF at various dilutions in serum free medium was added. After 18 hours incubation , the cells were washed twice with 100µl PBS and growth analysed using the crystal violet dye elution method (2.10.1.2 above).

### 2.10.3: TNF ELISA

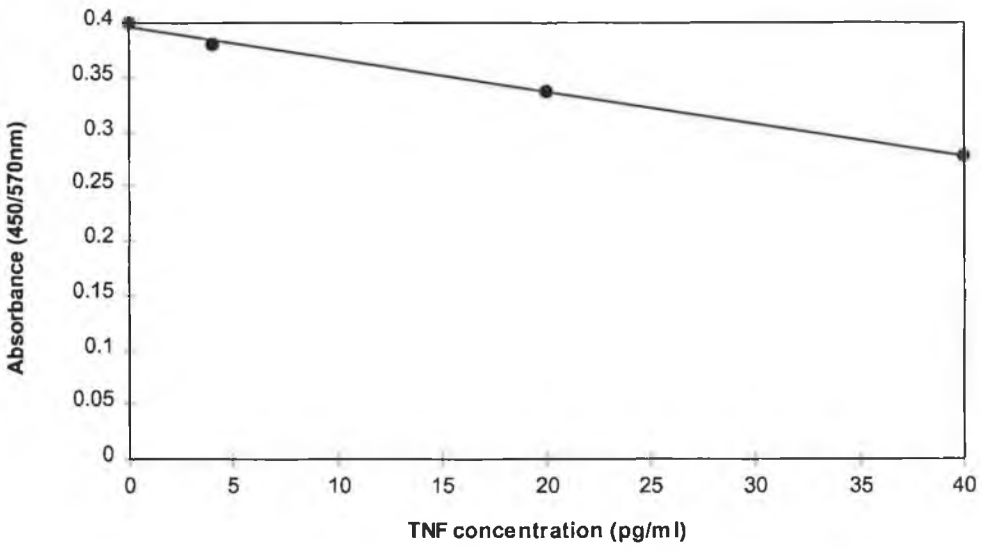
This assay employs the quantitative sandwich enzyme technique. A monoclonal antibody specific for TNF was coated ontothe microtitre plate (Quantikine). Samples were pipetted onto the wells and the TNF if present bound by the immobilised antibody. After washing away any unbound sample proteins, an enzyme linked polyclonal antibody specific for TNF was added to the wells and allowed to bind to any TNF which was bound during the first incubation. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells and colour developed in proportion to the amount of TNF in these standards. An exact assay protocol is as follows;

- A series of TNF dilutions were prepared using the TNF and diluent provided in the kit. All samples and standards were assayed in duplicate.
- 200µl of standard or sample was added per well. The wells were covered with the provided adhesive strip and incubated for 2 hours at room temperature.
- The wells were washed three times by filling each well with Wash buffer. Complete removal of liquid at each step was essential of ensure good performance.
- 200µl of TNF conjugate was added to each well. The wells were covered with a new adhesive strip and incubated for 2 hours at room temperature.
- The washing step was then repeated.

- 200µl of substrate solution (equal volumes of colour reagent A and B mixed together in equal volumes within 15 minutes of use) was added to each well and incubated for 20 minutes at room temperature.
- 50µl of stop solution was added to each well and mixed well.
- The absorbance at 450/570 nm of each well was determined within 30 minutes using the Titertek ELISA plate reader.

A standard curve for TNF is given below.

**Figure A1: Standard Curve for TNF Activity**




### 2.10. 4 : TGF-β Assay (NRK-49F Soft Agar Assay)

The presence of TGF-β biological activity was detemined by comparing cm fractions to a standard curve of TGF-β. A standard curve of TGF-β activity was set up as follows. TGF-β was dissolved in DME supplemented with 4mmol HCL/1ng/ml BSA. Two stock concentrations were made up (100ng/ml and 10ng/ml) with DME and diluted down to give TGF-β concentrations of 2, 1.5, 1, 0.5, 0.2, 0.1, 0.05 ng/ml. The appropriate controls containing HCL/BSA were assayed along with the prepared TGF-β standards.

2.10 4.1: Cell Pretreatment

NRK-49F cells were trypsinised three days prior to assay and fed at least three hours before trypsinisation.

2.10.4.2: Assay Protocol

The following protocol was followed;

- Weigh out 1.548g agar (Bacto Difco) and dissolve in 100mls of ultrapure water. Autoclave. Incubate at 44°C immediately.
- Calculate volume of agar medium (AgM) required by multiplying the number of samples by 7. Divide this figure by 2.58 to find the volume of DME2x required. Prepare the AgM in the following ratio and then equilibrate at 44°C;

| | |
|---|---|
| DME2x | 50mls |
| HEPES (1mM) | 2 mls |
| $NaHCO_3$ (7.5%) | 1ml |
| Pen.Strep. | 1ml |
| Growth medium (- FCS) | 14 mls |

- DME2x was prepared as follows:

| | |
|---|---|
| DME 10x | 20mls |
| ultrapure water | 76mls |
| HEPES | 4mls |
| $NaHCO_3$ | 2.2mls |
| adjust pH to 7.4 with 1M NaOH | |

- Place 35mm plates (Greiner 627160) on trays and label appropriately. Each sample is assayed in triplicate.
- Add the thermo-labile components to the AgM at the following concentrations, L-glutamine 1ml; FCS 10mls.
- Add agar to the AgM the same volume as DME2x. Mix well and quickly dispense 1.5mls onto each 35mm dish. The remaining AgM is returned to the water bath and the temperature reduced to 41°C. The plates are allowed to set at room temperature.

- Trypsinise cells and prepare a single cell suspension in medium without serum. The required concentration per ml to give the final concentration per dish is 1.3 x $10^5$ cells/ml.
- Add 0.5mls of cell suspension to each 2ml test sample and unconditioned medium control sample.
- Add 2.5 mls AgM (41°C) to each sample (containing cells) and immediately dispense out 1.5mls per plate (x3). After every five samples (maximum) re-equilibrate the Agm to 41°C.
- Cover the trays in tinfoil and incubate at 37°C/5% $CO_2$ for 10 days.

2.10.4.3: Colony Counting

Colonies were counted on an inverted microscope (Olympus) at 40x. All colonies greater than 50µm in diameter after 10 days in culture were counted. An eye piece graticule was graduated using a stage micrometer and was used to size colonies. All agar plates were viewed, superimposed on a transparent gridded disc with 4$mm^2$ grids. 25 grids were viewed for each 35mm dish. The average total number of colonies per plate was then estimated and the percentage colony forming efficiency (% CFE) determined;

$$\% \text{ CFE} = \frac{\text{number of colonies per plate}}{\text{number of cells plated}} \times 100$$

Each sample was set up in triplicate and a mean % CFE was determined for each sample and the standard error of the mean calculated by the following formula;

$$S.E.M. = \frac{\sqrt{\frac{\sum x^2 - \left(\frac{(\sum x)^2}{n}\right)}{n-1}}}{\sqrt{n}}$$

where $n$ = number of replicates per sample

$x$ = CFE of each replicate

$\sum$ = sum of the samples from 1 to $n$.

### 2.10.5: Assays for Lactate, Glucose and Ammonia

Biochemical assays for lactate, glucose and ammonia were all diagnostic kits, ammonia assay (Sigma, Cat. no. 170-A); lactate assay (Sigma Cat. no. 826-A); LDH assay (Sigma, Cat. no. DG1340-UV); glucose (Boehringer Mannheim, Cat.no. 166391). Assays were carried out according to manufacturers' protocols except for glucose assay where 0.6M (10%w/v) TCA was used as the deproteinising agent instead of URAC recommended by the manufacturer. Hams F12 media which contains 1802mg/L of glucose (Gibco catalogue) was used as the assay standard.

## 2.11: Toxicity Assays

### 2.11.1: Ammonia Tolerance Assay

Eight 25cm$^2$ flasks were set up at a concentration of 5 x $10^5$ cells/10ml of media. A 200mM $NH_4Cl$ (Sigma, ) stock solution was prepared and filter sterilised through a 0.22μm filter. Dilutions of this stock were made to give concentrations of 25, 50 and 100mM $NH_4Cl$. All $NH_4Cl$ solutions were made up in the appropriate medium with 5% FCS and 1% L-glutamine. 10ml of each $NH_4Cl$ concentration were added in duplicate to a 25cm$^2$ flask of cells after waste medium in the flasks had been removed. Cell number and viability counts were performed on day 3.

### 2.11.2: Lactate Tolerance Assay

Cells were plated into 96 well plates at a concentration of 1 x $10^3$ cells/well with 2x concentration of FCS and L-glutamine. Cells were incubated at 37°C/5% $CO_2$ overnight. A stock solution of lactate (Sigma) at 700mM was prepared in the appropriate medium and filter sterilised. Lactate concentrations ranging from 35 to 350mM were prepared from this stock. 100μl of each concentration (2x the final required concentration) were added to each well.On day 7 growth was assessed using the acid phosphatase method (Section 2.10.1.3),.

### 2.11.3: Sodium Chloride/Hyperosmotic Stress Assay

This assay was set up in the same manner as the lactate assay. A stock of 800mM NaCl was set up, filter sterilised and diluted to give NaCl concentrations ranging from 50-400mM. Osmolarity of the resulting solutions were read on a Osmomat 030 (Clandon) cryoscopic osmometer. 2 ml samples were put into the osmometer and the resulting osmolarity read from a digital readout. The osmometer was calibrated with a standard solution of osmolarity, 0.3osm/kg.

### 2.11.4: Adriamycin Toxicity Assay

Both CHRC5 and CHO-K1 cells were plated out at a concentration of $5 \times 10^3$ cells/well in 96 well plates. After 24 hours incubation, adriamycin drug concentrations ranging from 0.1 to 5µg/ml were prepared in serum free medium from a stock solution. 100µl of drug was added to each well. Growth was assessed after 4 days using the acid phosphatase method (Section 2.10.1.3).

**Note: All cytotoxic drug work was performed in a Cytoguard laminar air flow cabinet and special precautions were taken when handling the drug.**

## 2.12: Plasma Membrane Fluidity Measurement

Steady state fluorescence polarisation was used to determine the plasma membrane fluidity (PMF) of cells. This technique involves labeling the membrane with a fluorescent probe (TMA-DPH) and then exciting the membrane with polarised monochromatic light. The movement of the probe depends on the fluidity of the membrane.

A schematic of the optical system for the steady state fluorescence is given below:

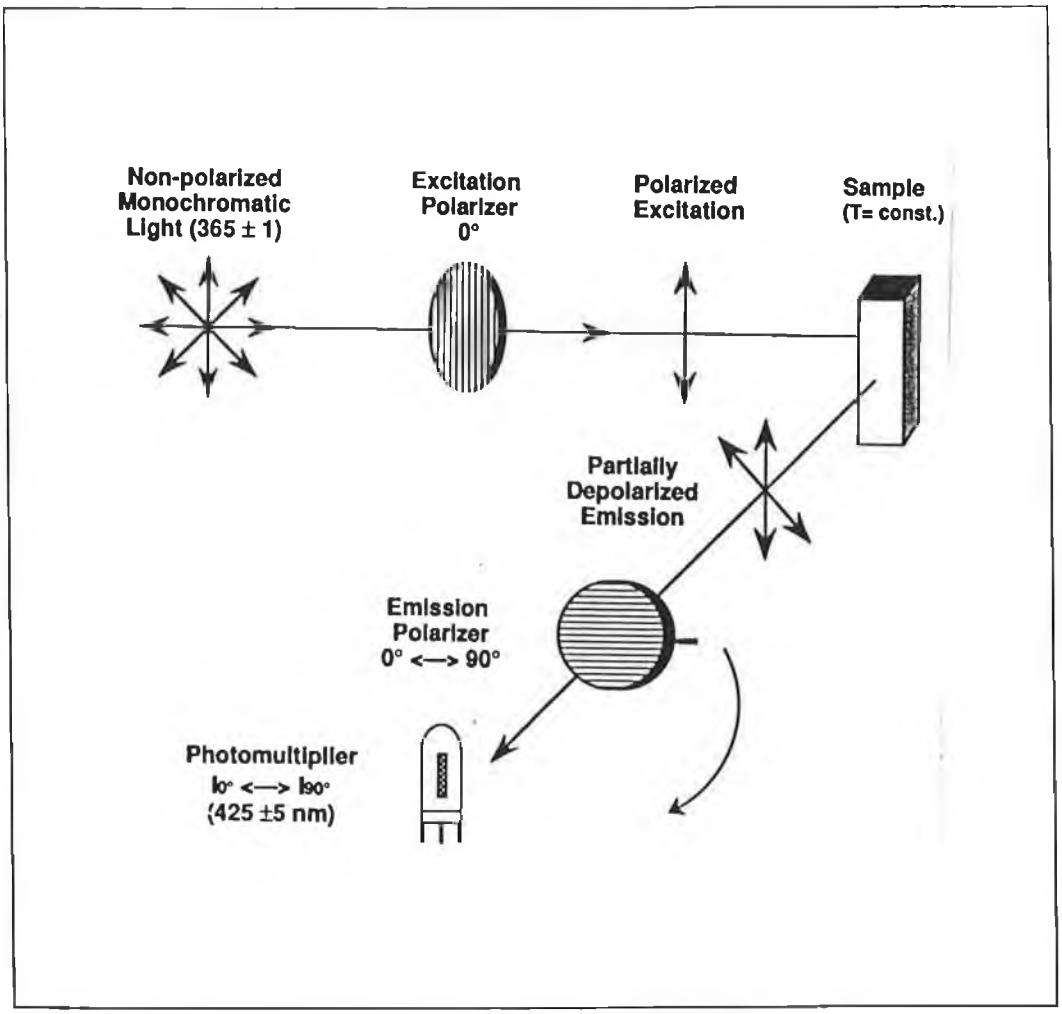



Figure 2.1: Steady State Fluorescence Polarisation: Schematic of optical system for steady state fluorescence anisotropy measurements. (from Ramirez and Mutharasan, 1990).

Non polarised light passes through the excitation polariser to produce plane polarised light. The sample is irradiated and fluorescence is detected at a right angle to the excitation light after passing through the emission polariser.

The method involves the following;

1. Cell monolayers/suspensions were washed three times in PBS.
2. Cells were trypsinised in 2.5% trypsin at 10°C for approx. 1 minute.
3. Cells were pelleted and resuspended in PBS. Viability was checked.
4. Cell density should be 1 x $10^6$ cells/ml.
5. To 1ml of cell suspension, 5µl of TMA-DPH solution was added to give a final concentration of probe of 1 x $10^{-6}$M.
6. The fluorescence anisotropy value of the cell suspension/probe mixture was immediately read on a Perkin-Elmer fluorimeter.

TMA-DPH stock Solution

A stock solution of 5 x $10^{-4}$M TMA-DPH was prepared by adding 1.15mgs of TMA-DPH (Sigma) to 5mls of dimethylformamide (DMF) (Sigma). This stock was stored in a dark bottle at 4°C for up to a month.

## 2.13: Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Cells from 40 and 120 rpm agitated Hep-2 and Hep-2A cultures were harvested on day 7. RT-PCR was carried out as outlined in *Cytotechnology*, **12**, 289-314, appendix 1. The target primers were directed towards the MDR-1 gene that codes for p-glycoprotein and the control primers were directed against β-actin. The end products of the RT-PCR reaction were separated on a 4% agarose gel run at 100 volts and the resulting bands analysed using a Densitometer (BioRad).

# CHAPTER THREE

# RESULTS AND DISCUSSION

## SECTION 3.1: INVESTIGATIONS INTO ENDOGENOUSLY PRODUCED GROWTH INHIBITORS.

As outlined in the Introduction (section 1.1), the aim of this thesis is to examine factors both external and endogenous that inhibit the growth of animal cells *in vitro*. The work in this section investigates the production of a number of endogenous growth inhibitory factors with the main emphasis placed on a growth inhibitory/cytotoxic activity produced by MSV-3T3 cells *in vitro*.

### 3.1.1:Investigation Of 3T3 And MSV-3T3 Conditioned Medium For Evidence Of Growth Inhibitory Activity

A possible growth inhibitory activity in 3T3 conditioned medium (cm) was identified in earlier work in this laboratory (M$^{c}$Donnell, 1987). SCC-9 was used as the indicator cell line in the *in vitro* assay. Hence the aim of initial experiments in this work was to concentrate on investigating further this inhibitory activity in 3T3 cells and in a virus transformed variant of 3T3 cells, MSV-3T3. The assay system used to detect growth inhibitory activity involved adding cm from 3T3 and MSV-3T3 cells to SCC-9 human carcinoma cells plated at high densities in low serum levels in a 24 well plate assay (Methods, section ). The assay was incubated for between 5-7 days and growth of SCC-9 assessed after this time by crystal violet staining of cell colonies and image analysis to calculate the area of the plate covered by the colonies. Conditioned medium from 3T3 and MSV-3T3 cells was fractionated

by ultrafiltration through a 1000 molecular weight (MW) membrane to a 10x concentrate, these were designated 1000R (retentate) samples.

Table 3.1 shows the results of the first assay undertaken using 3T3 and MSV-3T3 cm to investigate an effect on SCC-9 (squamous cell carcinoma of the tongue) cells.

Table 3.1: Effect of 3T3 and MSV-3T3 cm on the Growth of SCC-9 cells.

| Sample | Colony Area ($mm^2$) Assay 1 | Colony Area ($mm^2$) Assay 2 | Colony Area ($mm^2$) Assay 3 |
|---|---|---|---|
| MSV-3T3 1000R | 1.32 | 762.3 ± 131.4 | 13.6 ± 0.16 |
| $3T3^*$ 1000R cm 1 | 156.4 ± 59.4 | 20983.6 ± 602.6 | 737.4 ± 25.5 |
| $3T3^*$ 1000R cm 2 | 27.8 ± 6.3 | 19609 ± 114.5 | 734.9 ± 11.4 |
| $3T3^{\&}$ 1000R cm 1 | 1.12 ± 0.8 | n.d. | 178.4 ± 18.9 |
| $3T3^{\&}$ 1000R cm 2 | 43.2 | n.d. | n.d. |
| uncm (ATCC) control | 54.6 ± 21.8 | 22980 ± 2347 | 869.5 ± 36.5 |

Note:

- all retentates are 10x concentrates.
- the control in these assays was ATCC medium and not DME medium in which the cm was collected, however subsequent experiments showed that growth in these two media was similar.
- $3T3^*$ refers to 3T3 cm collected in M199 medium.
- $3T3^{\&}$ refers to 3T3 cm collected in DME medium.
- cm 1 and cm 2 refers to cm collected on day 1 and day 2 respectively.
- uncm refers to unconditioned medium, i.e. growth medium.
- n.d.- not determined.

This experiment was repeated three times as shown in Tables 3.1 and 3.2 and in each case the MSV-3T3 cm causes in excess of 96% growth inhibition of the control (see also Figure 3.1). However the 3T3 cm seems to give variable results and the level of inhibition is not as great as that in the MSV-3T3 cm.

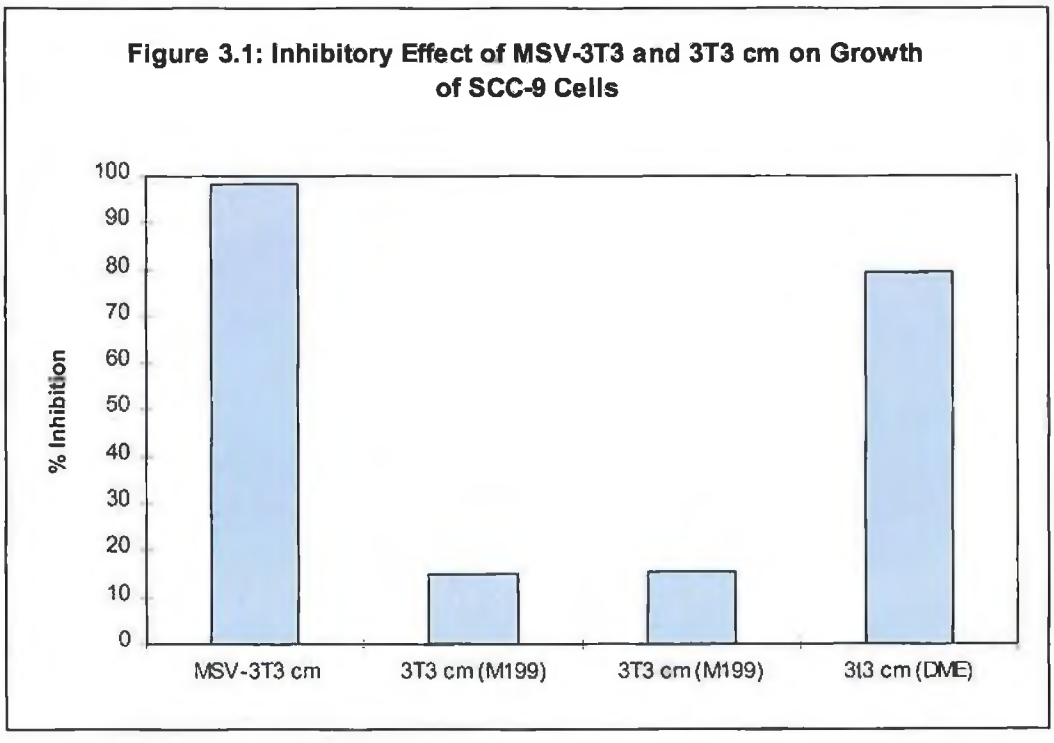
Table 3.2: Inhibitory effect of 3T3 and MSV-3T3 cm (calculated from results in Table 3.1)

| Sample | % inhibition Assay 1 | % inhibition Assay 2 | % inhibition Assay 3 |
|---|---|---|---|
| MSV-3T3 1000R | 97.6 | 96.7 | 98.4 |
| 3T3* 1000R cm 1 | stimulation | 9.0 | 15.2 |
| 3T3* 1000R cm 2 | 49.1 | 15.0 | 15.5 |
| 3T3& 1000R cm 1 | 97.9 | n.d. | 79.5 |
| 3T3& 1000R cm 2 | 21.0 | n.d. | n.d. |

**Note:**

**% inhibition refers to the ratio between growth in the treated wells to that in a control well where only growth medium was added;**

$$\% \text{ inhibition} = 1 - \frac{\text{treated wells}}{\text{control wells}} \times 100$$



In the assays presented in Table 3.1, MSV-3T3 cm was added at the same time as the SCC-9 indicator cells. Table 3.3 shows the results of the same cm added 24 hours after the SCC-9 cells were added, thus allowing the SCC-9 cells to attach before MSV-3T3 cm addition.

Table 3.3: Inhibitory effect of 3T3 and MSV-3T3 cm added to indicator cells after 24 hours

| Sample | Colony Area ($mm^2$) | % Inhibition |
|---|---|---|
| MSV-3T3 1000R | 30.73 ± 12.5 | 95.6 |
| 3T3* 1000R | 778.5 ± 27 | stimulation |
| 3T3* 1000R | 388.4 ± 62.8 | 44.5 |
| 3T3& 1000R | 473.8 ± 38.2 | 32.2 |

Again much greater inhibition seems to occur in the MSV-3T3 cm than in the 3T3 cm. Although there seems to be inhibitory activity in the 3T3 cm it does not seem to be consistent and is not of the same magnitude as that in the MSV-3T3 cm. The results in Table 3.3 are also interesting in that they seem to suggest that the inhibitory activity in the MSV-3T3 cm is active against both freshly inoculated SCC-9 cells and attached cells growing for 24 hours.

### 3.1.2: Further Investigations Into The Inhibitory Activity In MSV-3T3 Cm.

Due to the inconsistency of the inhibitory activity in the 3T3 cm it was decided to continue the work with MSV-3T3 cm only.

MSV-3T3 cm was dialyzed to exclude the possibility of low molecular weight metabolites (e.g. ammonia, lactate) causing the inhibition and the cm was diluted to see if the activity was dose dependent. Table 3.4 and Figure 3.2 outline the results from experiments to investigate the effect of dialysis and dilution on the inhibitory activity in MSV-3T3 cm.

Table 3.4: Effect of Dialysis on MSV-3T3 cm

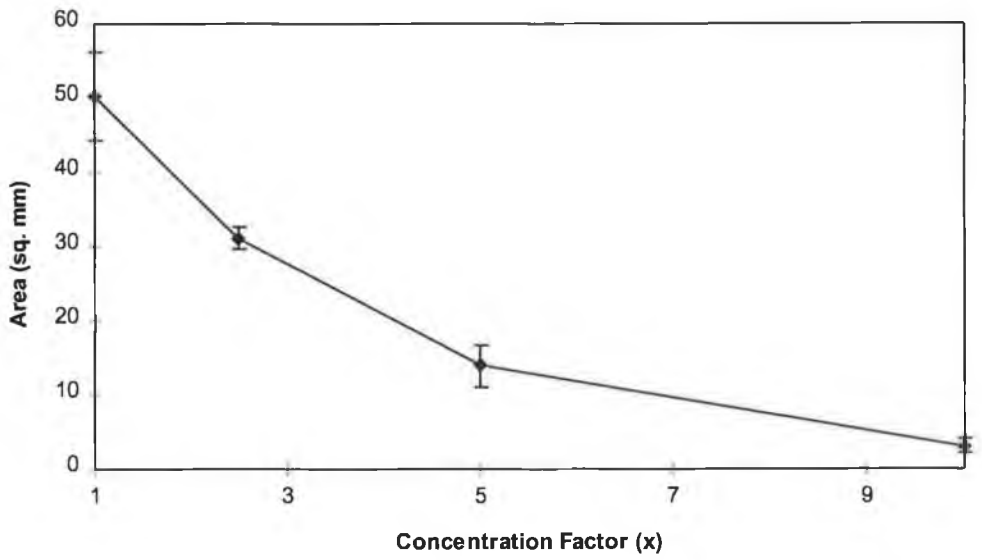
| Sample | Colony Area ($mm^2$) | % Inhibition |
|---|---|---|
| MSV-3T3 1000R (10x) 10,000 MW dialysate | 0.132 ± 0.16 | 99.7 |
| MSV-3T3 1000R (2.5x) 10,000 MW dialysate | 20.39 ± 1.53 | 56.8 |
| MSV-3T3 1000R (1x) 10,000 MW dialysate | 55.65 ± 1.6 | 0.0 |
| MSV-3T3 1000R (10x) 1,200 MW dialysate | 0.48 ± 0.77 | 99.0 |
| MSV-3T3 cm unconcentrated | 45.5 ± 3.37 | 3.5 |
| MSV-3T3 cm 1000 filtrate | 51.71 ± 3.87 | 0.0 |
| Uncm **CONTROL** | 47.16 ± 1.68 | 0.0 |

These results indicate that the inhibitory activity seems to be increased by dialysis through the 10,000 MW tubing and even more so through the 1200 MW tubing.

It was noticed by microscopic examination that the inhibitory activity present in the MSV-3T3 cm seemed to be not only inhibitory, but also toxic to the SCC-9 cells. Perhaps indicating that instead of there being a 'classical' growth inhibitor present in the cm (i.e.working in a reversible manner), there might be a Tumour Necrosis Factor (TNF) like cytotoxic factor present.

There seems to be an approximately linear dilution effect when the MSV-3T3 cm is diluted down from a concentration of 10x to 1x (see Figure 3.2).

**Figure 3.2: Dilution Curve for MSV-3T3 cm**




The inhibitory assay designed to detect activity in MSV-3T3 cm as stated previously used a low FCS concentration so as to allow SCC-9 attachment but not to mask growth inhibition. It was decided to optimise FCS levels in the assay. Results are presented in Table 3.5

Table 3.5: Effect of different background FCS levels on the MSV-3T3 inhibitory activity

| Sample | Area ($mm^2$) | % Inhibition |
|---|---|---|
| uncm + 5% FCS | 14.45 ± 0.22 | ** |
| MSV-3T3 1000R + 5% FCS | 0.805± 0.35 | 94.5 |
| uncm + 2.5% FCS | 14.25 ± 0.22 | ** |
| MSV-3T3 1000R + 2.5% FCS | 1.05 ± 0.21 | 92.5 |
| uncm + 1.25% FCS | 13.98 ± 0.99 | ** |
| MSV-3T3 1000R + 1.25% FCS | 2.47 ± 0.09 | 82 |
| MSV-3T3 cm (neat) | 12.72 ± 0.88 | 9 |

It is interesting to note that there seems to be increased inhibition by the MSV-3T3 1000R (10x) at increased FCS concentrations i.e. 94.5% inhibition in 5% FCS as opposed to 82% in 1.25% FCS. Perhaps factors present in FCS are potentiating the effect of the inhibitory factor , such 'competence' factors have been reported in literature (Kimura *et al*, 1992). For economical reasons it was decided to carry out subsequent assays at the reduced FCS level of 1.25% as growth inhibition is still clearly distinguishable at this FCS level.

### 3.1.3: Investigation Of TGF-β Activity In MSV-3T3 cm.

TGF-β is a growth factor with well documented growth inhibitory effects on a wide range of epithelial cells (Sporn and Roberts, 1988). Therefore it was important at this stage in the work to establish whether the inhibitory activity in the MSV-3T3 cm was TGF-β-like.

Table 3.6 shows the results from a TGF-β assay carried out on NRK-49F cells to test for the presence of TGF-β in MSV-3T3 cm (Methods 2.10.).

For growth stimulation in NRK-49F cells, TGF-β must be assayed for in the presence of EGF in order for TGF-β activity to be identified, therefore all the results presented in Table 3.6 were in the presence of EGF. Controls in the absence of EGF were included in the assay but the average number of colonies per plate never rose above 0.47 ± 0.007 % C.F.E. therefore these controls are not included here. This experiment was carried out twice and the same trends as are seen above were noted in both cases.

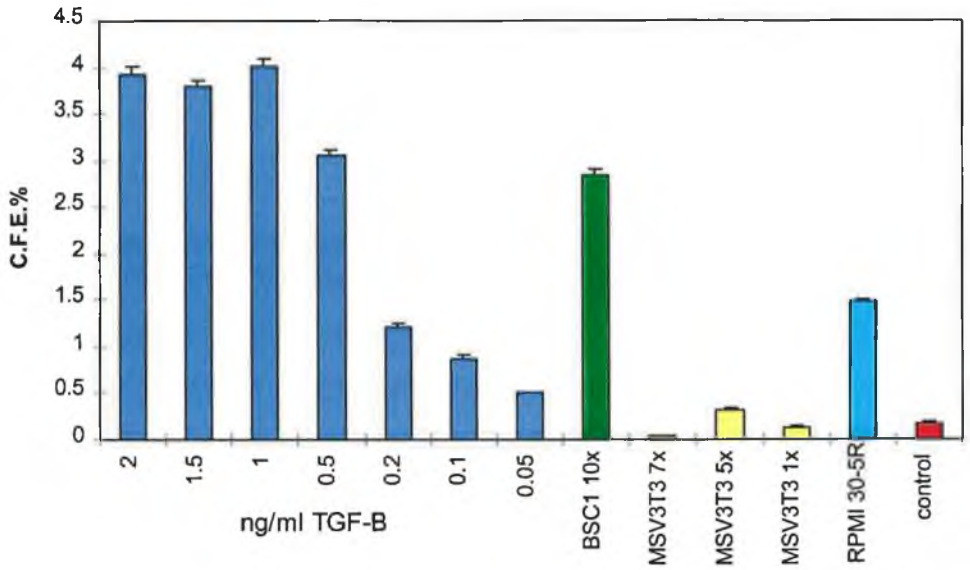
Table 3.6: Investigation for presence of TGF-β in MSV-3T3 cm.

| Sample | Average Number Colonies per plate | C.F.E. % | S.E.M. |
|---|---|---|---|
| 2 ng/ml TGF-β | 652.7 | 3.92 | 0.09 |
| 1.5 ng/ml TGF-β | 630.6 | 3.81 | 0.05 |
| 1 ng/ml TGF-β | 673.3 | 4.02 | 0.08 |
| 0.5 ng/ml TGF-β | 481.0 | 3.06 | 0.03 |
| 0.2 ng/ml TGF-β | 170.9 | 1.22 | 0.03 |
| 0.1 ng/ml TGF-β | 103.1 | 0.88 | 0.03 |
| 0.05 ng/ml TGF-β | 28 | 0.50 | 0.02 |
| BSC-1 10,000R (10x) | 570.2 | 2.85 | 0.059 |
| BSC-1 10,000R (1x) | 85.5 | 0.43 | 0.005 |
| BSC-1 10,000R (0.1x) | 85.5 | 0.43 | 0.007 |
| MSV-3T3 1000R (10x) | 7.4 | 0.037 | 0.003 |
| MSV-3T3 1000R (7x) | 81.8 | 0.41 | 0.03 |
| MSV-3T3 1000R (5x) | 64.8 | 0.32 | 0.015 |
| MSV-3T3 1000R (1x) | 26.5 | 0.13 | 0.004 |
| MSV-3T3 1000R (0.1x) | 26.5 | 0.13 | 0.002 |
| RPMI 30-5000R (10x) | 738.14 | 3.69 | 0.13 |
| RPMI 30,000R (10x) | 298.4 | 1.49 | 0.019 |
| uncm (DME) CONTROL | 61.88 | 0.31 | 0.01 |

Note:

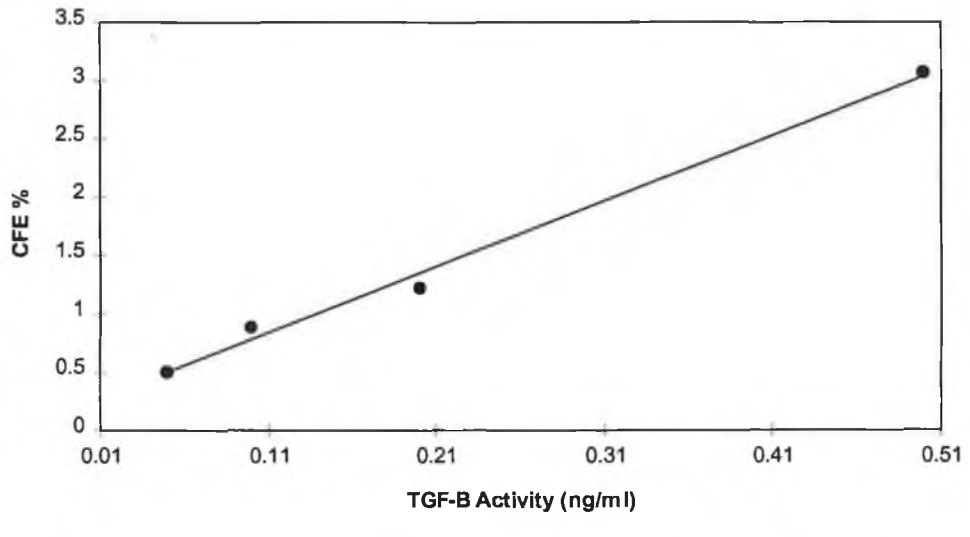
TGF-β was lyophilised upon arrival and was dissolved in BSA/HCl before dilution, therefore a control of the BSA/HCl concentration used was included in this assay and this value is subtracted from the TGF-β results in Table 3.6.

**Figure 3.3: Levels of TGF-B in MSV-3T3 cm and Other cm**




A standard curve of TGF-β concentration and the colony forming efficiency of NRK-49F cells in soft agar is given in Figure 3.4. It can be seen that the relationship is approximately linear. Comparison of the activity of the different cm samples against this standard curve gives an indication of the TGF-β like activity.

**Figure 3.4:Standard Curve for TGF-B Activity**



The calculated values are as follows;

| Conditioned Medium | TGF-β Like Activity (ng/ml) |
|---|---|
| MSV-3T3 | 0 |
| BSC-1 | 0.43 |
| RPMI (30-5R) | 0.20 |

This result indicates that there is minimal if any TGF-β activity in MSV-3T3 cm. In contrast there is a significant amount of TGF-β activity in RPMI cm, a human nasal carcinoma cell line being grown in the laboratory at that time. BSC-1 cm which contains a growth inhibitor reported to be TGF-β1 (Holley et al 1980) is also active in this assay.

However MSV-3T3 cm may still contain TGF-β which is either at a very low concentration and not detected in this assay or it may contain a latent form of TGF-β. It has been shown that TGF-β may cause inhibition in some cell lines when present at only picogram levels. Also TGF-β may need to be acid or protease activated in order for the active molecule to be released (Lawrence et al 1985; Lyons et al 1988). Therefore although this assay shows that MSV-3T3 cm contains very low levels (if any) of TGF-β when compared to the other cm (RPMI and BSC-1) it does not mean that TGF-β may be ruled out completely as the causative agent of inhibition in MSV-3T3 cm. However the fact that the inhibitory activity in MSV-3T3 cm seems to be cytotoxic rather than cytostatic may suggest that TGF-β, which functions as a reversible inhibitor, may not be the major growth inhibitory activity in MSV-3T3 cm.

### 3.1.4: Fractionation Of MSV-3T3 Cm By Ultrafiltration

MSV-3T3 cm was ultrafiltered through a number of different MW sized cut off membranes. The aim being to try and identify the molecular weight range within which the inhibitory activity might lie. Table 3.7 and Figure 3.5 show the results of one such experiment.

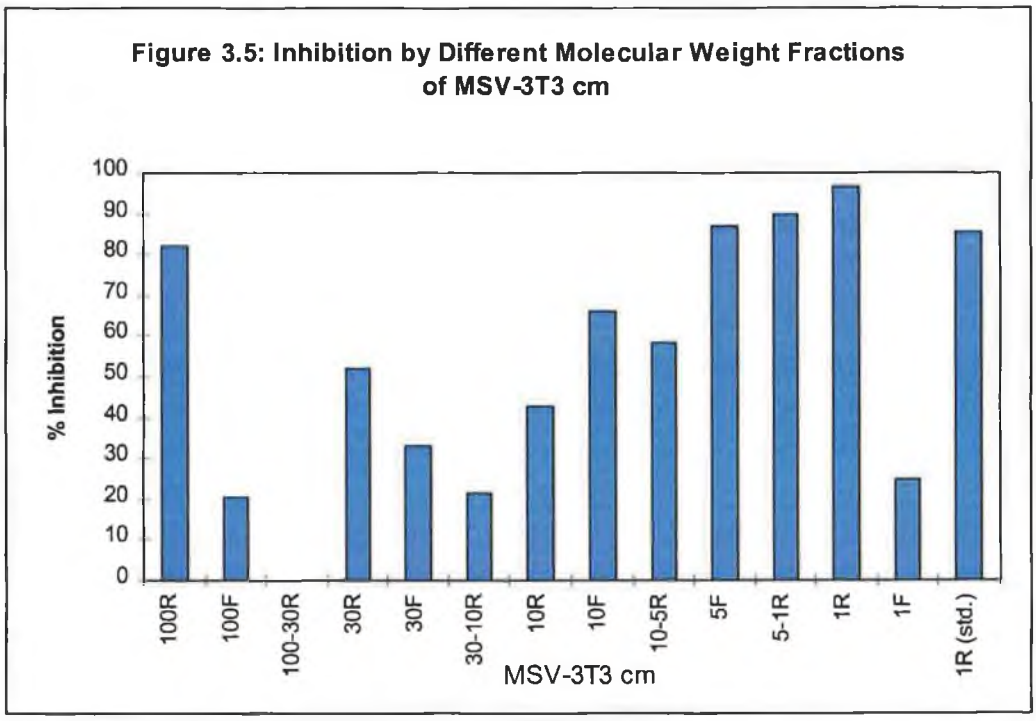
Table 3.7: Ultrafiltration of MSV-3T3 cm.

| Sample | Colony Area ($mm^2$) | % Inhibition |
|---|---|---|
| 100,000R (8x) | 6.34 ± 2.0 | 82 |
| 100,000 filtrate | 27.99 ± 1.5 | 20.3 |
| 100-30,000R (8x) | 35.76 ± 2.66 | 0 |
| 30,000R (8x) | 16.88 ± 4.18 | 52 |
| 30,000 filtrate | 23.85 ± 4.87 | 33 |
| 30-10,000R (8x) | 27.59 ±1.81 | 21.4 |
| 10,000R (8x) | 20.04 ± 6.83 | 42.9 |
| 10,000 filtrate | 11.93 ± 1.1 | 66 |
| 10-5,000R (8x) | 14.63 ± 5.31 | 58.3 |
| 5000 filtrate | 4.66 ± 0.42 | 86.7 |
| 5-1,000R (8x) | 3.6 ± 2.13 | 89.7 |
| 1,000R (8x) | 1.14 ± 0.62 | 96.7 |
| 1,000 filtrate | 26.41 ± 6.73 | 24.8 |
| uncm (DME) CONTROL | 35.12 ± 2.45 | 0 |
| 1,000R* (10x) | 5.06 ± 1.41 | 85.6 |

Note:

The MSV-3T3 cm samples fractionated in this experiment were not dialyzed before this assay, therefore small molecular compounds such as ammonia and lactate may be contributing to the inhibition seen here.

**Figure 3.5: Inhibition by Different Molecular Weight Fractions of MSV-3T3 cm**



Again, it was noticed at this point that the 'inhibition' of the growth of indicator cells was more of a cytotoxic nature than that of a 'true' growth inhibitor, i.e. the cells were being killed by the cm fraction rather than just growth retarded.

These results indicate that most fractions contain some inhibitory activity. From these results it might be proposed that MSV-3T3 cm contains an inhibitor greater than 100,000 MW, an inhibitor greater than 30,000, an inhibitor between 10 and 5000 MW, an inhibitor between 5 and 1000 MW and an inhibitor in the 10,000 retentate. Whether these are distinct inhibitors or simply aggregates of smaller MW inhibitors appearing in the larger MW fractions remains to be seen.This assay was repeated and results while not presented here are similar i.e. that most fractions of MSV-3T3 cm contain some inhibitory activity with the greatest activity seen in the 1000R (10x) fraction.

### 3.1.5: Effect Of MSV-3T3 Cm On A Number Of Different Indicator Cell Lines

At this point in the work, MSV-3T3 cm had only been tested for inhibitory effects on one cell line, SCC-9, a squamous cell carcinoma of the tongue. Therefore it was decided to test MSV-3T3 cm on a number of cell lines. The cell lines chosen were:

NRK - a normal rat kidney fibroblast cell line.

Hep2 - a human carcinoma of the larynx.

3.1.5. 1: Effect of MSV-3T3 cm on NRK cells

A range of molecular weight fractions were tested on NRK cells. The results are outlined in Table 3.8 and Figure 3.6;

**Figure 3.6: Effect of MSV-3T3 cm on Growth of NRK Cells**

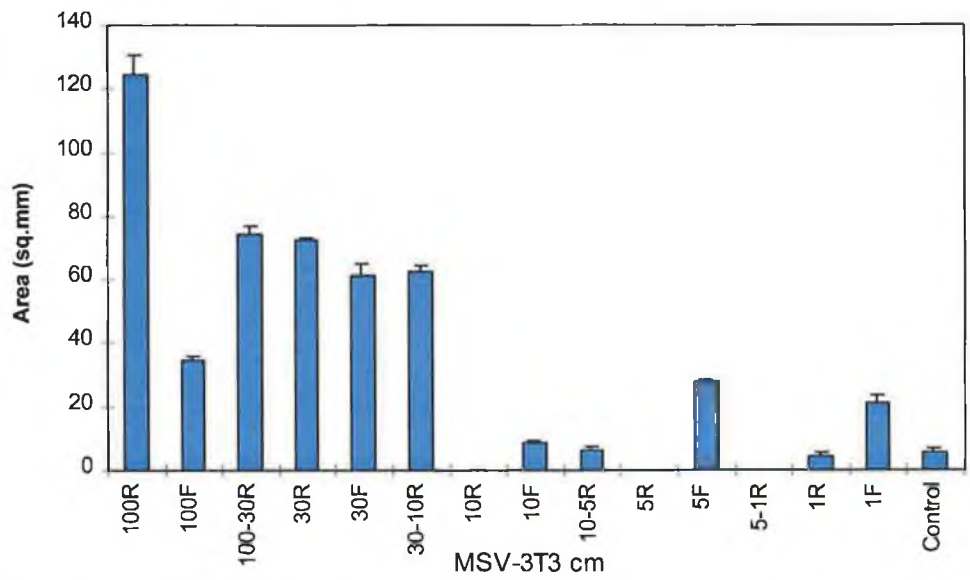



Table 3.8: Effect of MSV-3T3 cm on NRK cells

| Sample | Area ($mm^2$) | % Inhibition |
|---|---|---|
| 100,000R (8x) | 124.25 ± 6.23 | stimulatory |
| 100,000 filtrate | 34.66 ± 0.97 | stimulatory |
| 100-30,000R (8x) | 74.6 ± 2.39 | stimulatory |
| 30,000R (8x) | 72.72 ± 0.07 | stimulatory |
| 30,000 filtrate | 61.39 ± 3.64 | stimulatory |
| 30-10,000R (8x) | 62.84 ± 1.48 | stimulatory |
| 10,000R (8x) | 0.23 ± 0.32 | 96% inhibitory |
| 10,000 filtrate | 8.72 ± 0.56 | slightly stimulatory |
| 10-5,000R (8x) | 6.08 ± 1.36 | slightly stimulatory |
| 5,000R (8x) | 0.12 ± 0.14 | 98% inhibitory |
| 5,000 filtrate | 27.92 ± 0.38 | stimulatory |
| 5-1,000R (8x) | 0.07 ± 0.08 | 99% inhibitory |
| 1,000R (8x) | 4.19 ± 1.54 | 24% inhibitory |
| 1,000 filtrate | 21.13 ± 2.62 | stimulatory |
| Unconcentrated (1x) cm | 63.58 ± 6.72 | stimulatory |
| uncm (DME) CONTROL | 5.54 ± 1.31 | **** |

Note:This assay was repeated and results were similar except that the 1000 retentate fraction caused much greater inhibition in the repeat assay.

The results show that most MSV-3T3 fractions (especially the larger MW fractions) are stimulatory for NRK cells. It is worth noting that up to this MSV-3T3 cm has been inhibitory for the indicator SCC-9 cells which are of a carcinogenic origin, whereas MSV-3T3 cm seems to have the opposite effect on NRK cells which are normal, non-cancerous cells.

3.1.5. 2: Effect of MSV-3T3 cm on the growth of Hep-2 cells

The effect of MSV-3T3 cm was tested on another carcinoma derived cell line, Hep-2 which has proven easier to grow routinely than SCC-9 cells. The results are outlined in Figure 3.7 and. Table 3.9

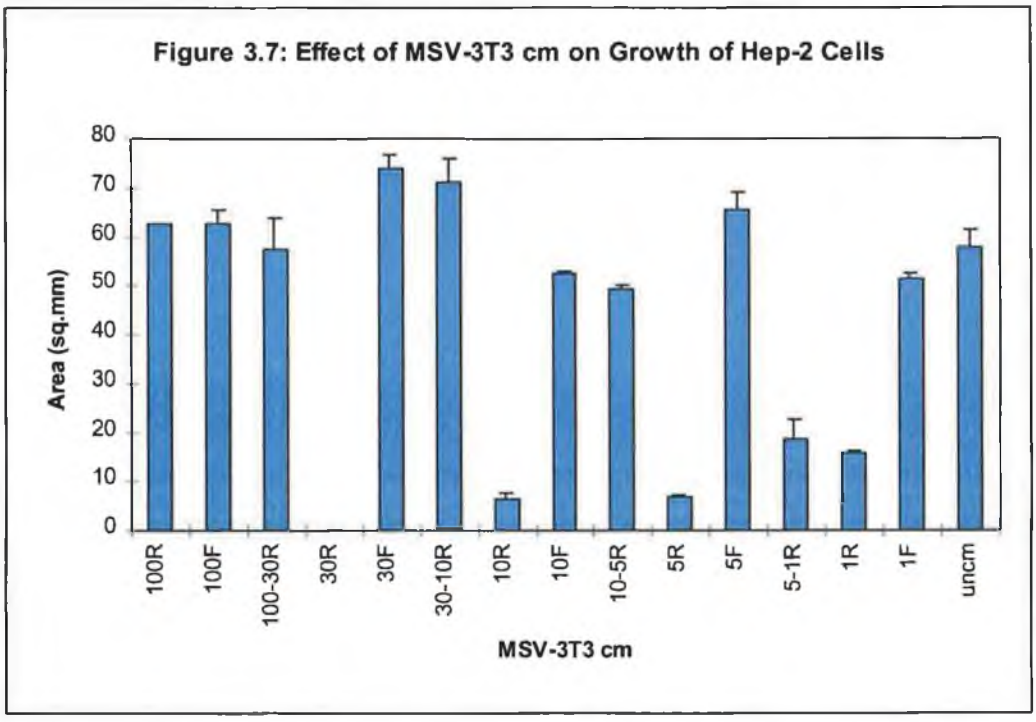


Table 3.9: Effect of MSV-3T3 cm on Hep-2 cells.

| Sample | Colony Area ($mm^2$) | Effect on Growth |
|---|---|---|
| 100,000R (8x) | 62.53 | slightly stimulatory |
| 100,000 filtrate | 62.73 ± 2.56 | slightly stimulatory |
| 100-30,0000R (8x) | 57.37± 6.46 | no effect |
| 30,000R (8x) | 0.11 ± 0.11 | 99% inhibitory |
| 30,000 filtrate | 73.76 ± 2.91 | stimulatory |
| 30-10,000R (8x) | 71.01 ± 5.14 | stimulatory |
| 10,000R (8x) | 6.27 ± 1.47 | 89% inhibitory |
| 10,000 filtrate | 52.36 ± 0.71 | no effect |
| 10-5,000R (8x) | 49.43 ± 0.68 | 14% inhibitory |
| 5,000R (8x) | 7.03 ± 0.16 | 88% inhibitory |
| 5,000 filtrate | 65.41 ± 3.6 | slightly stimulatory |
| 5-1,000R (8x) | 18.57 ± 4.2 | 68% inhibitory |
| 1,000R (8x) | 15.89 ± 0.14 | 72% inhibitory |
| 1,000 filtrate | 51.32 ± 1.14 | no effect |
| unconcentrated (1x) cm | 69.73 ± 3.86 | stimulatory |
| uncm (DME) CONTROL | 57.72 ± 5.45 | *** |

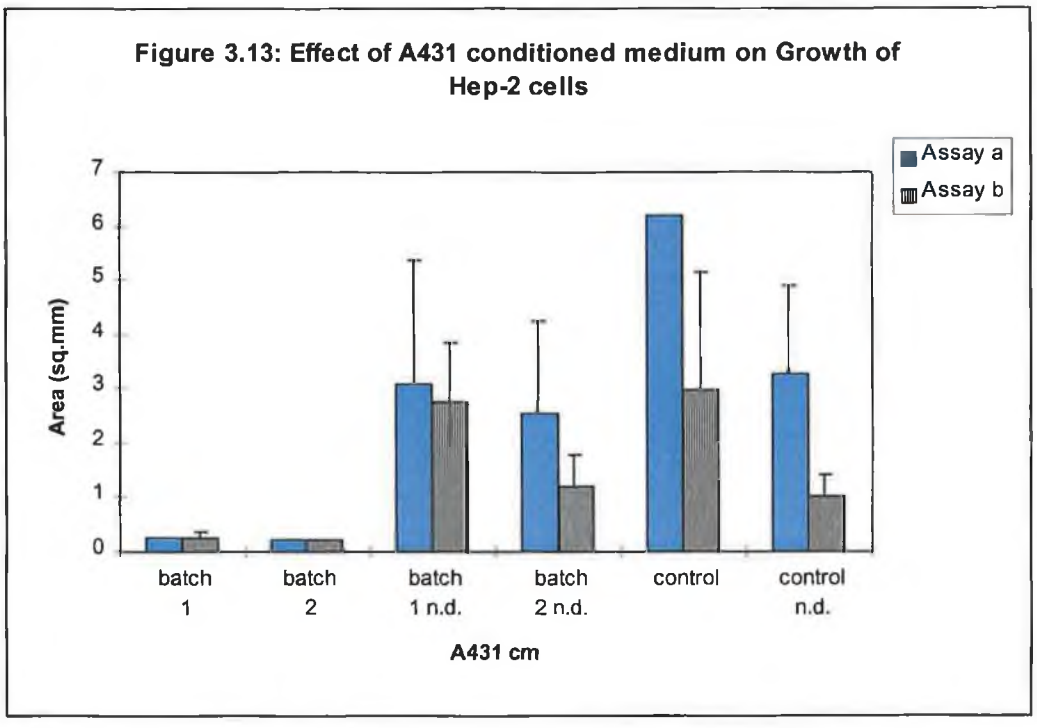
MSV-3T3 cm seems to have a more variable effect on Hep-2 cells than on SCC-9 cells. However MSV-3T3 cm seems to be generally inhibitory for Hep-2 cells. The following fractions are especially inhibitory: 1,000 retentate, 5-1,000 retentate, 10,000 retentate and the 30,000 retentate.

Conclusions

Experiments up to this point seemed to indicate that MSV-3T3 cells produce at least one and possibly more growth inhibitory/cytotoxic factors. These growth inhibitory substances were stable to dialysis and could be concentrated by ultrafiltration. A difference in effect on target cell type was also noted.

Table 3.20: Effect of A431 cm on growth of Hep-2 cells

| Sample | Colony Area ($mm^2$) Assay A | Colony Area ($mm^2$) Assay B |
|---|---|---|
| A431 batch 1 dialysed | 0.24 ± 0 | 0.26 ± 0.1 |
| A431 batch 2 dialysed | 0.0 ± 0 | 0.23 ± 0 |
| Control (MEM) dialysed | 6.22 ± 0 | 2.96 ± 2.2 |
| A431 batch 1 non-dialysed | 3.08 ± 2.3 | 2.74 ± 1.1 |
| A431 batch 2 non-dialysed | 2.53 ± 1.7 | 1.19 ± 0.6 |
| Control (MEM) non-dialysed | 3.28 ± 1.6 | 1.03 ± 0.4 |




Note: n.d. = non-dialysed.

Quite significant inhibition is seen in the dialysed cm fractions when compared to the dialysed MEM control which causes no inhibition of growth suggesting that dialysis is not having a toxic effect on the cells. The non-dialysed cm shows no real inhibition when compared to growth in control wells. Since dialysis itself does not, in this case, appear to be an inhibitory process, it would seem that dialysis may be in fact activating an inhibitor in A431 cm.

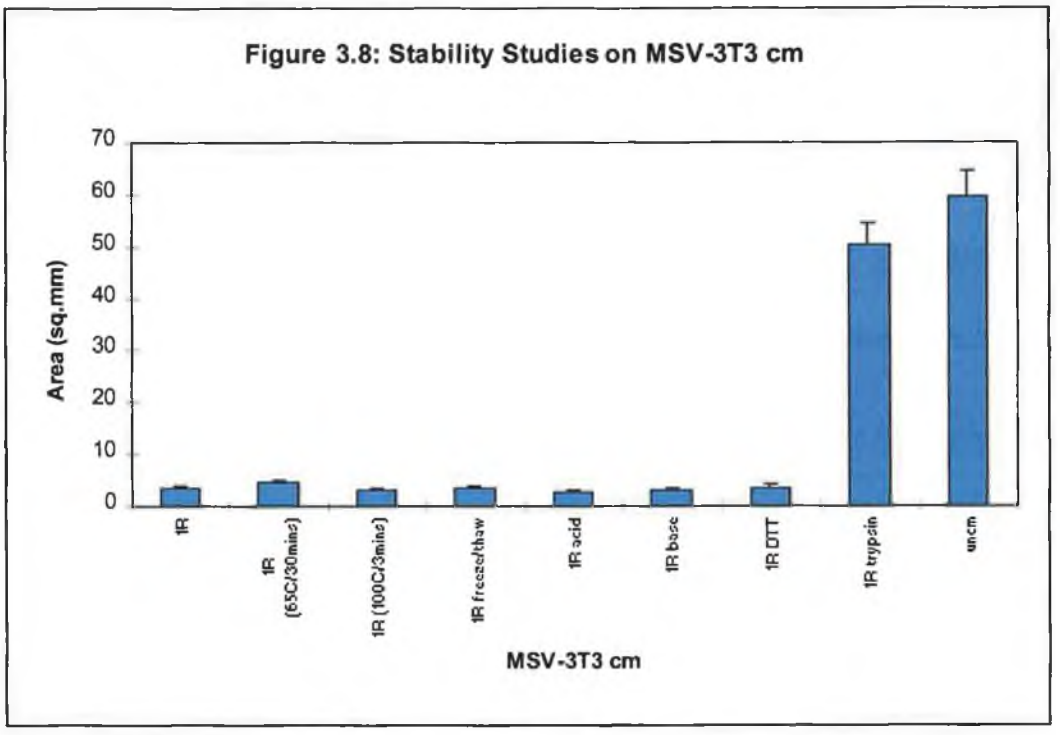
### 3.1.6: Mycoplasma Contamination Of MSV-3T3 cm

At this point in the project, MSV-3T3 cells were found to be contaminated by a mycoplasma positively identified as *Acholeplasma laidlawii*. This mycoplasma is of bovine origin and further investigation seemed to suggest that the MSV-3T3 cell line was contaminated when it came into this laboratory several years earlier. This would suggest that all cm collected from MSV-3T3 cells and assayed for growth inhibitory activity to date may have been mycoplasma infected.Therefore it was important to rule out at this stage whether the growth inhibition caused by the MSV-3T3 cm was due to the contamination of the cm by mycoplasma, especially since the growth inhibitory effect seems to be more cytotoxic than cytostatic in action.

Three possibilities existed at this point:

1. MSV-3T3 cells produce an endogenous TNF- like cytotoxic factor regardless of any mycoplasma infection.
2. Mycoplasma directly caused the cytotoxic effect observed in MSV-3T3 cm. A number of reports in the literature suggest that mycoplasma may have a cytotoxic effect, for example chromosomal damage can be caused by mycoplasmal mediated arginine depletion of culture medium or by mycoplasma inhibition of mammalian DNA synthesis (M$^{c}$Garrity, Vanaman and Saram,1984; Almayor, Yatsic and Kahane, 1983.).
3. Mycoplasma infection indirectly caused the cytotoxic effect by inducing the MSV-3T3 cells to produce a cytotoxic factor.

Since there were no mycoplasma-free MSV-3T3 cells available at this time, some experiments were designed to try to establish whether the cytotoxic effect of MSV-3T3 cm was due to mycoplasma or to a 'true' inhibitor. Results are presented in Figure 3.8.




A summary of results obtained, testing MSV-3T3 1000 retentate (8x) on Hep-2 cells, is as follows:

- MSV-3T3 cytotoxic/inhibitory activity seemed to be stable to high temperatures, i.e. 65°C for 30 minutes and 100°C for 3 minutes. Mycoplasmas (possessing no cell wall) are reported to be sensitive to temperatures above 45°C so it would seem that something other than mycoplasma infection might be responsible for the cytotoxic effect.
- MSV-3T3 cm was stable to extremes of acid and base, DTT treatment and repeated freeze/thawing. Again due to the fact that mycoplasmas have no cell wall it would be expected that they would be sensitive to freeze/thawing.
- MSV-3T3 cm seemed to be sensitive to trypsin, indicating a protein might be involved, however when this treatment was repeated the same result was not achieved.

Overall, it would seem that the entity causing inhibition/cytotoxicity is very stable. The high temperature resistance and pH stability would indicate that a protein may not be responsible. However it may be trypsin labile suggesting that perhaps a protein is in some way involved. The most important objective

at this point was to obtain mycoplasma free MSV-3T3 cells and repeat some of the previous work to elucidate if there is a genuine growth inhibitory effect caused by cm produced by MSV-3T3 cells.

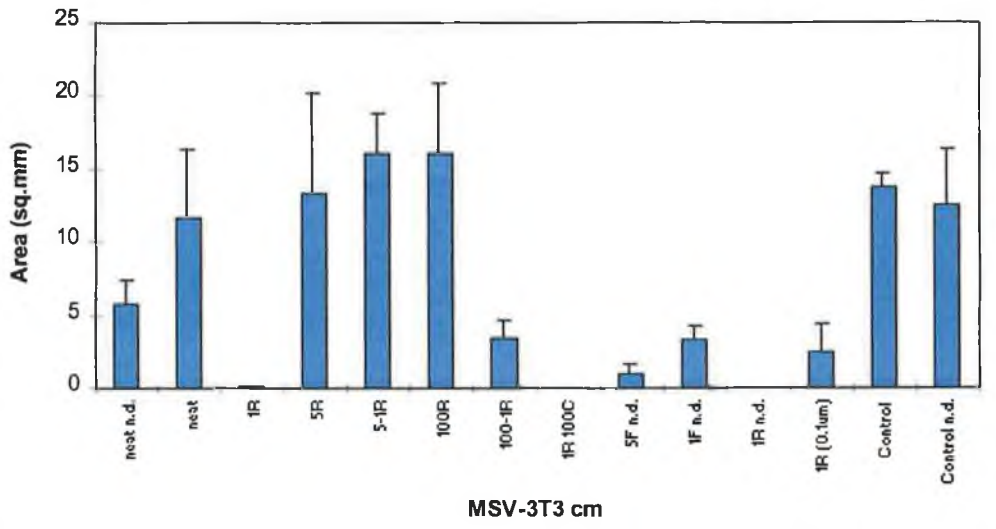
### 3.1.7: Collection Of Mycoplasma Free MSV-3T3 Cm And Its Effect On The Growth Of Hep-2 Cells.

A new stock of MSV-3T3 cells was obtained from the ATCC and further work performed to try to characterise the inhibitory/cytotoxic factor(s) in MSV-3T3 cm. Instead of using SCC-9 cells as the indicator cell line, it was decided to use Hep-2 cells because:

- SCC-9 cells are slow growing cells and quite difficult to grow routinely.
- Hep-2 cells grow at a faster rate than SCC-9 and therefore the growth inhibitor assay incubation period could be shortened to 5 instead of 7 days.
- MSV-3T3 cm seemed to have a similar growth inhibitory effect on Hep-2 cells as it has on SCC-9 cells (see Section 3.1.5).

Figure 3.9 and Table 3.10 presents results from investigations that test the effect of MSV-3T3 cm fractionated by ultrafiltration on Hep-2 cells. MSV-3T3 cm was confirmed to be mycoplasma free by both Hoescht staining and direct culturing (Methods 2.5.2). All samples were dialysed to eliminate the possibility that growth inhibition may be due to an accumulation of low molecular weight metabolic products such as ammonia and lactate. One sample was heated to 100°C for 3 minutes to eliminate the possibility that viral contamination may be a cause for inhibitory activity. Another sample was 0.1 micron filtered to remove mycoplasma contamination if present.

**Figure 3.9: Effect of Mycoplasma Free MSV-3T3 cm on Growth of Hep-2 Cells**




It would appear that the inhibitory/cytotoxic activity in the 1000 retentate from this mycoplasma free MSV-3T3 cm has been retained, implying that mycoplasma is not responsible for the activity. It can be seen that in the three assays inhibitory/cytotoxic activity has been retained by the 1000 MW membrane (99% inhibition of growth). This activity seems to be retained after boiling at 100°C for 3 minutes and after dialysis. However filtering through a 0.1 μm filter seems to affect the activity. It should be noted that the 0.1 μm filter used was not low protein binding therefore perhaps the inhibitory activity, if a protein, is being retained on the filter.

The 5000 retentate and the 5-1000 retentate show minimal or no inhibition of growth, which is in disagreement with the results in Table 3.9 which used mycoplasma contaminated MSV-3T3 cm.

Again the 100,000 retentate does not seem to adversely affect growth. However the 100-1,000 retentate gives very inconclusive results, perhaps indicating that a number of factors, inhibitors and stimulators lie in this molecular weight range.

Table 3.10: Effect of Mycoplasma Free MSV-3T3 cm on the Growth of Hep-2 Cells

| Sample | Area ($mm^2$) Assay A | Area ($mm^2$) Assay B | Area ($mm^2$) Assay C | % Inhibition Assay A | % Inhibition Assay B | % Inhibition Assay C |
|---|---|---|---|---|---|---|
| Neat cm (non-dia.) | 5.71 ± 1.77 | 188.6 ± 14.2 | 106.5 ± 16.5 | 54 | stimulation | 0 |
| Neat cm (dia.) | 11.62 ±4.71 | 212.7 ± 20.5 | 127.7 ± 1.45 | 16 | stimulation | slight stimulation |
| 1,000R (8x) (dia.) | 0.0865 | 0.63 ± 0.49 | 0.309 ± 0.01 | 99 | 99 | 99 |
| 5,000R (10x) (dia.) | 13.34 ± 6.83 | 338 ± 10.4 | 129.75 ± 0.6 | 3.2 | stimulation | slight stimulation |
| 5-1,000R (10x) (dia.) | 16.02 ± 2.74 | 186.5 ± 13.4 | 133.3 ± 16.5 | 0 | slight stimulation | slight stimulation |
| 100,000R (10x) (dia.) | 16.11 ± 4.81 | 236.7 ± 25.9 | 150.8 ± 24.7 | 0 | stimulation | stimulation |
| 100-1,000R (7x) (dia.) | 3.49 ± 1.14 | 226.1 ± 14.2 | 118.6 ± 24.7 | 75 | stimulation | 0 |
| 1,000R (8x) (dia.) 100°C | 0 | 3.096 | 88.9 ± 3.89 | 100 | 98 | 21 |
| 5,000 filtrate (non-dia.) | 1.03 ± 0.64 | 117.63 ± 5.5 | 82.32 ± 9.33 | 91.6 | 13 | 21 |
| 1,000 filtrate (non-dia.) | 3.26 ± 0.96 | 103.39 ± 8.1 | 79.88 ± 1.09 | 73.6 | 23 | 24 |
| 1,000R (non-dia) | 0.026 ± 0.02 | 0.79 ± 0.11 | 14.78 ± 2.42 | 99 | 99 | 86 |
| 1,000R (dia.) 0.1μm filtered | 2.499 ± 1.83 | 163.5 ± 4.02 | 110.4 ± 14.2 | 82 | 0 | 0 |
| uncm DME (dia)Control | 13.78 ± 0.97 | 159.64 ± 4.6 | 112.85 ± 11 | *** | *** | *** |
| uncm DME (non-dia.) control | 12.54 ± 3.8 | 135.79 ± 3.7 | 104.5 ± 11.0 | *** | *** | *** |

Overall the following may be concluded from these results:

1. A potent cytotoxic activity is present in the 1000 retentate concentrated. This result is the same as that always found with the previous mycoplasma contaminated MSV-3T3 cm.
2. The cytotoxic activity is stable to boiling at 100°C for 3 minutes, indicating that a very stable protein or a non-protein molecule is responsible.
3. The higher molecular weight retentates, greater than 5000 daltons, cause some stimulation. Perhaps there exists a stimulator greater than 1000 molecular weight whose effects are masked some what by the cytotoxic factor, also greater than 1000 daltons. It is unusual that such a dramatic cytotoxic effect is seen in the 1000 retentate but does not show in the 5000 and 100,000 retentates. It may suggest that the cytotoxic factor lies in the 1-5000 molecular weight range, however the 1-5000 retentate does not seem to be inhibitory in these experiments. One solution would be that two factors acting in cooperation are responsible for the activity. One factor lying in the 1-5000 MW range and a larger MW factor in the 1000 retentate. The 5000 and 10,000 MW retentates would lack the 1-5000 factor needed to cooperate with the larger MW factor to cause growth inhibition.
4. Previously mycoplasma contaminated MSV-3T3 cm showed inhibition in most MSV-3T3 MW retentates with the greatest cytotoxic effect seen in the 1000 MW retentate. These results with mycoplasma free cm indicate that the cytotoxic/inhibitory factor(s) in the 1000 retentate seems to be a genuine factor produced by the MSV-3T3 cells.

### 3.1.8: Investigation Into Effect Of 1000 MW Ultrafiltration Membrane On MSV-3T3 cm In A 96 Well Assay System

In order to confirm or disprove the hypothesis that cooperative factors may be responsible for the observed inhibition, fresh MSV-3T3 cm was collected and fractionated to generate a 5000 retentate and a 1-5000 retentate to see if, when added together, a similar response to the 1000 retentate could be obtained. In addition, since all the work at this stage suggested that the inhibitory/cytotoxic activity most consistantly lay in the 1000 retentate fraction, it was decided to investigate whether the ultrafiltration membrane itself was toxic, i.e. was any toxic material being leeched off the 1000 MW ultrafiltration membrane.

The following samples were put through the 1000 MWCO membrane:

1. MSV-3T3 cm
2. Unconditioned medium (DME) - CONTROL.
3. Acidic MSV-3T3 cm - pH 6.6, since often the cm when collected from the cells was slightly acidic and it was important to ascertain whether the lower pH might cause leeching from the membrane.
4. RPMI cm: previous work in this laboratory (Carey,B. 1992) had shown to be stimulatory in the 1000 retentate fraction, therefore if anything was leeching off the 1000 MWCO membrane it should also be in this cm fraction.

In an attempt to use a faster, automated assay system; a miniaturised 96 well plate assay was developed to assess the growth inhibitory effect of MSV-3T3 cm on Hep-2 cells. In this assay Hep-2 cells are plated on one day and cm samples added 24 hours later. Plates are read by dye elution with an ELISA plate reader (Methods, Section 2.10.1.2.). All previous work had used a 24 well plate system with crystal violet staining assessed by image analysis. Indicator cells and MSV-3T3 cm were added at the same time. Using the image analyser was quite time consuming and laborious since the actual reading of plates took a considerable time and calculating average cell areas

and standard deviations had to be carried out manually. The miniturised assay not only speeds up this process (the ELISA reader calculates these values) but also reduces the volume of test sample required. Results testing the effect of processing the cm via ultrafiltration are presented in Table 3.11;

Table 3.11: Investigation into effect of 1000 MWCO ultrafiltration membrane on MSV-3T3 cm in a 96 well assay system.

| Sample | $O.D._{570/620nm}$ Assay A | $O.D._{570/620nm}$ Assay B | $O.D._{570/620nm}$ Assay C |
|---|---|---|---|
| uncm(non-dialysed) | 0.453 ± 0.067 | n.d. | n.d. |
| uncm (dialysed) | 0.192 ± 0.06 | 0.28 ± 0.108 | 0.048 ± 0.009 |
| uncm 1000R | 0.287 ± 0.066 | 0.166 ± 0.058 | 0.046 ± 0.019 |
| 1000R | 0.393 ± 0.189 | 0.135 ± 0.109 | 0.005 ± 0.002 |
| 1000R pH 6.59 | 0.23 ± 0.082 | 0.171 ± 0.06 | 0.069 ± 0.028 |
| 1000 filtrate | 0.029 ± 0.011 | 0.023 ± 0.32 | 0.0065 ± 0.002 |
| 5000R | | 0.086 ± 0.059 | 0.0065 ± 0.002 |
| 5000 filtrate | 0.189 ± 0.028 | 0.015 ± 0.05 | 0.07 ± 0.016 |
| 5-1000R | | | 0.065 ± 0.01 |
| 5000R + (5-1000R) | 0.319 ± 0.079 | 0.246 ± 0.181 | n.d. |
| neat cm (1x) | 0.318 ± 0.029 | 0.279 ± 0.095 | 0.072 ± 0.002 |
| uncm (MEM) 1000R | 0.244 ± 0.062 | 0.128 ± 0.067 | 0.061 ± 0.001 |
| RPMI cm 1000R | 0.015 ± 0.004 | 0.013 ± 0.005 | 0.003 ± 0.001 |

Note:

- All samples were dialysed unless otherwise stated.
- All ultrafiltration retentates are concentrated to a 10x concentrate.
- The MEM medium sample is the control for the RPMI cm.

The 1000 MW retentate is inhibitory to Hep-2 cell growth in two of the three assays. However there does not seem to be the complete destruction of all cell growth (toxic effect) seen in previous assays. The 5000 MW retentate and the 1000 MW filtrate fractions are inhibitory.

The combination of the 5000 MW retentate and 5-1000 MW retentate fractions added in a 1:1 ratio does not appear to be particularly inhibitory. As before the neat MSV-3T3 cm does not cause growth inhibition.

The control medium, DME promotes growth. However after ultrafiltration (DME 10x) and especially after dialysis the growth promoting effect of the medium is reduced. This is an important observation because it would suggest that part of the growth inhibitory/cytotoxicity of the MSV-3T3 cm might in fact be as a consequence of processing the cm. Interestingly, cm from a nasal carcinoma cell line, RPMI , appears to contain a high level of growth inhibitory activity but its corresponding control medium, MEM, when concentrated to a 10x through a 1000 MW membrane by ultrafiltration is not inhibitory. Work previously published (Carey, 1992) has suggested that RPMI cells produce growth stimulatory molecules, however the assay system used to detect that stimulation was a soft agar, anchorage independent system. It might be suggested that the mode of cultivation of target cells (monolayer versus suspension) is important for the expression of growth stimulatory and growth inhibitory molecules.

Several other assays were performed on MSV-3T3 cm during this period (results not shown). In most assays some inhibitory activity was noted in the 1000 retentate, however this activity was not consistently present as had always been seen previously.

In summary, these results raise a number of questions:

1. Is the cytotoxic/inhibitory activity present in MSV-3T3 cm contained only in the 1000 retentate and if so why ? Previous work suggested activity was

consistently in the 1000 retentate, however these last experiments do not agree totally with this suggestion.

2. Combining the 5000 retentate and the 5-1000 retentate does not seem to mimic the response seen in the 1000 retentate, as had been suggested in the previous experiment (Table 3.10). Why should cytotoxic activity only be seen in a low MW fraction and not in higher fractions unless a combination of factors are acting together in a complementary manner to cause inhibition?
3. Is the processing of cm, ultrafiltration followed by dialysis, causing the medium to become slightly toxic ? In these experiments processing of the cm through the 1000 MW membrane seems to cause a decrease in the growth promoting ability of the control medium Perhaps there are toxic compounds leeching off the 1000MW ultrafiltration membrane or alternatively off the benzoylated dialysis tubing.The MEM medium concentrated through a 1000 MW membrane does not seem to be toxic in any way to the cells however this medium should be compared to a non-dialysed/ultrafiltrated control.

### 3.1.9: Investigations Into The Presence Of TNF-α in MSV-3T3 CM

TNF-α is a 17 kDa protein which has a cytotoxic effect on a number of epithelial cell lines (Palladino *et al*, 1987). Since results to date have seemed to suggest that MSV-3T3 cm contains cytotoxic factor(s), the aim of this portion of work was to establish if the cytotoxic effect of MSV-3T3 cm might be similar to or the same as TNF-α.

#### 3.1.9.1: Effect of TNF-α and TNF-α Neutralising Antibody on Growth of L929 cells

L929 cells are the commonly used target cells for TNF-α activity therefore these cells were used to assess the potency of the TNF-α being used. Results presented in Table 3.12 indicate that L929 cells have an approximately linear

response to increasing TNF-α concentrations. One unit of TNF-α activity was calculated from results in Table 3.12 to be approximately 500 pg/ml. This concentration of TNF-α consistently (in all assays using L929 cells) caused 50% cytolysis of the L929 cells. In order to ascertain that the cytotoxic effect observed was due to TNF-α alone, anti-TNF-α neutralising antibody was added to each TNF-α sample. The results for L929 cells area also presented in Table 3.12.

Table 3.12: Effect of TNF-α and anti-TNF-α on growth of L929 cells

| TNF-α Concentration (pg/ml) | $Absorbance_{570/620nm}$ **TNF-α only.** | $Absorbance_{570/620nm}$ **TNF-α + anti-TNF-α** |
|---|---|---|
| 0 | 0.400 | 0.362 ± 0.028 |
| 4 | 0.384 | 0.295 ± 0.061 |
| 20 | 0.337 | 0.329 ± 0.041 |
| 40 | 0.278 | 0.282 ± 0.063 |
| 200 | 0.252 | 0.293 ± 0.083 |
| 400 | 0.240 | 0.302 ± 0.053 |
| 2000 | 0.233 | 0.294 ± 0.039 |
| 4000 | 0.199 | 0.265 ± 0.05 |

It can be seen that anti-TNF-α antibody partially neutralised the TNF-α activity on L929 cells but growth was still not up to control levels. Perhaps a higher concentration of antibody is needed to totally neutralise the action of TNF-α.

3.1.9.2: Effect of TNF-α on Growth of Hep-2 cells

Since Hep-2 cells are the target cells used in the MSV-3T3 inhibitory assay, the response of these cells to TNF-α was of interest. In the same procedure as for L929 cells, Hep-2 cells were plated in 96 well plates on day 1 and TNF-α concentrations added on day 2. Cell growth was monitored (after 5 days ) by

crystal violet staining, elution and detection using a ELISA plate reader.Results are presented in Table 3.13.

Table 3.13 Effect of TNF-α and TNF-α Neutralising Antibody on Hep-2 Growth

| TNF-α Concentration (pg/ml) | $Absorbance_{570/620nm}$ **TNF-α only.** | $Absorbance_{570/620nm}$ **TNF-α + anti-TNF-α** |
|---|---|---|
| 0.0 | 0.283 | 0.271 ± 0.027 |
| 15.6 | 0.142 | 0.305 ± 0.021 |
| 31.2 | 0.154 | 0.174 ± 0.014 |
| 62.5 | 0.280 | 0.288 ± 0.033 |
| 125 | 0.289 | 0.226 ± 0.04 |
| 250 | 0.321 | 0.187 ± 0.019 |
| 500 | 0.340 | 0.279 ± 0.025 |
| 1000 | 0.316 | 0.215 ± 0.036 |

As can be seen in Table 3.13, an unusual cytotoxicity response, atypical of TNF-α, is obtained. It appears that Hep-2 cell growth is actually stimulated by this factor. This assay was repeated several times and the same trend obtained on each occasion. The results from the neutralising experiment show no clear trend emerging.

Although unusual, overall these results would seem to indicate that the action of TNF-α on Hep-2 cells is not similar to the activity of the cytotoxic factor(s) found in MSV-3T3 cm on the same cell line.

3.1.9.3: Investigation into the Presence of TNF-α in MSV-3T3 cm Using an ELISA Method.

Following the investigations looking for a similarity in response of Hep-2 cells to TNF-α and cytotoxic activity in MSV-3T3 cm (section 3.1.9.2), it was decided to examine for the presence of TNF-α in MSV-3T3 cm. An ELISA technique using TNF-α antibody was carried out on MSV-3T3 cm (Methods 2.10.3.). A positive TNF-α producing cell line, HL-60 (which was first endotoxin treated to ensure TNF-α production) was also tested to ensure that the technique was working properly. Results are tabulated in Table 3.14.

Table 3.14: Determination of TNF-α Levels in MSV-3T3 and HL-60 cm

| TNF-α (pg/ml) | $Absorbance_{450/570nm}$ |
|---|---|
| 0 | 0.00 |
| 125 | 0.1527 ± 0.023 |
| 250 | 0.3277 ± 0.042 |
| 500 | 0.6517 ± 0.10 |
| 1000 | 1.4117 ± 0.093 |
| HL-60 (neat) | 0.1637 ± 0.01 |
| HL-60 (1/10) | 0.0153 ± 0.01 |
| MSV-3T3 cm (neat) | 0.00 |

This ELISA technique is sensitive to 15 pg/ml of TNF-α, therefore the results above indicate that no detectable TNF-α activity is present in MSV-3T3 cm. However this assay was repeated with a greater range of lower TNF-α concentrations and including a MSV-3T3 30-10,000 retentate (since TNF-α has a MW of 17,000 daltons any TNF-α in the cm will lie in this MW range). Results are presented in Table 3.15.

Table .3.15: Determination of TNF-α in MSV-3T3 cm

| TNF-α (pg/ml) | $Absorbance_{450/570\ nm}$ |
|---|---|
| 0.0 | 0.030 ± 0.003 |
| 15.6 | 0.035 ± 0.011 |
| 31.2 | 0.045 ± 0.00 |
| 62.5 | 0.062 ± 0.002 |
| 125 | 0.091 ± 0.002 |
| 250 | 0.159 ± 0.013 |
| 500 | 0.269 ± 0.001 |
| 1000 | 0.491 ± 0.050 |
| 2000 | 0.905 ± 0.081 |
| MSV-3T3 cm (neat) | 0.029 ± 0.001 |
| MSV-3T3 / 30-10,000R | 0.031 ± 0.001 |

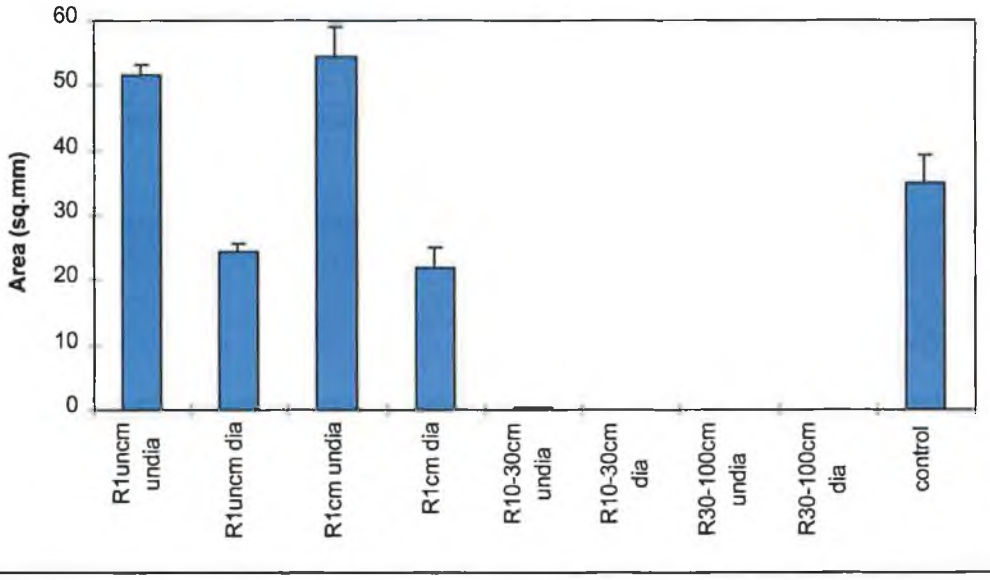
This assay confirms that there are no detectable levels of TNF-α in MSV-3T3 cm

### 3.1.10: Further Studies On MSV-3T3 cm

It was mentioned in Section 3.1.8 that inconsistencies were emerging in the appearance of the cytotoxic activity in the 1000MW retentate. It was found that in some assays (results not presented) that either no activity was detectable in the 1000 fraction or the level of inhibitory activity was not as high as previously noted (see Section 3.1.8). Up until this time (Sections 3.1.1 to 3.1.7) the 1000 fraction had always resulted in almost complete lysis of target cells. Inconsistencies also appeared in the other MW fractions, with inhibitory activity present in certain fractions in some assays and not in others.

An example of these developments can be seen in Figure 3.10 where several MSV-3T3 retentates were tested for growth inhibitory activity. Results are for both dialysed and non-dialysed fractions with uncm (DME medium) also fractionated through the 1000 MW membrane.

**Figure 3.10: Effect of MSV-3T3 Fractions on Growth of Hep-2 Cells**




It can be seen that very little inhibitory activity is present in the 1000 retentate fraction. High levels of activity is noted in both the 10-30,000 MW fraction and the 30-100,000 MW fraction. The processing of the cm seems to have an effect on the samples. The growth promoting properties of uncm (DME) concentrated through the 1000 MW ultrafiltration membrane and dialysed is reduced . This is in agreement with previous results (Table 3.11) where the uncm was slightly toxic after the 1000 MW ultafiltration. The high levels of inhibitory activity in the 10-30,000 fraction and the 30-100,000 fractions is surprising given that previously (Table 3.9) no activity was observed in these fractions. Uncm passed through the 10,000, 30,000 and 100,000 MW ultrafiltration membranes, unlike the 1000 MW membrane, was not affected by the concentration process.

Some further experiments with the 10-30,000 and 30-100,000 fractions were carried out. These were mainly stability studies to try to characterise the activites. Results are presented in Table 3.16.

Table 3.16: Stability Studies with MSV-3T3 10-30,000 and 30-100,000 MW Fractions

| Treatment | 10-30,000 MW Fraction Hep-2 Colony Area ($mm^2$) | 30-100,000 MW Fraction Hep-2 Colony Area ($mm^2$) |
|---|---|---|
| No trypsin treatment | 0.5644 ± 0.0627 | 0.7812 ± 0.2520 |
| Trypsin (10μg/ml), 3 hrs at 37°C; trypsin inhibitor (20μg/ml) | 22.85 ± 3.7 | 0.2156 ± 0.09 |
| No heat treatment | 3.3095 ± 0.109 | 5.379 ± 1.30 |
| 65°C / 30 mins | 3.725 ± 0.74 | 6.0725 ± 2.65 |
| 100°C / 3 mins | 14.239 ± 0.56 | 15.67 ± 2.65 |

The 10-30,000 MW fraction appears to be trypsin sensitive, resistant to 65°C but sensitive to 100°C. These results would indicate that a protein molecule(s) stable to relatively high temperatures (65°C) might be responsible for the inhibitory activity.The 30-100,000 MW fraction is not trypsin labile suggesting that a protein may not be responsible for the activity.

Conclusions

Overall it was decided at this stage of the work to investigate endogenous growth inhibitors produced by other cell lines since the MSV-3T3 system was proving inconsistent and elusive as is seen in the last few experiments. In conclusion it would appear that MSV-3T3 cells produce growth inhibitory/cytotoxic factor(s) which do not seem to be either TGF-β or TNF-α like. However isolation of these factors is difficult given that a negative result (which could be attributed to other factors besides genuine growth inhibitors) is being investigated. The inconsistencies in the results and the appearance of the growth inhibitory in several MW fractions makes the identification and characterisation of the factors difficult to achieve. Similar problems in work identifying growth inhibitors/cytotoxic factors have been reported in the literature (Emery *et al*, 1987).

### 3.1.11:Investigations Into Inhibitory Activity Produced By Other Cells *In Vitro*

The production of endogenous growth inhibitors by cell lines other than MSV-3T3 cm was investigated. Several cell lines that were available in the laboratory at the time were screened for growth inhibitory activity.

#### 3.1.11.1: Investigation of Other Cell Lines for the Production of Growth Inhibitors

A range of media from other cell lines was tested for growth inhibitory activity on Hep-2 cells. The following cell lines were investigated for the production of a growth inhibitor;

- RPMI (human nasal carcinoma)
- Vero (normal monkey)
- 1437A (primary human fibroblast)
- A431 (human vulval carcinoma)
- NCIH-128 (human lung carcinoma)
- SaOS-2 (human ovarian carcinoma)
- CHO-K1 (normal hamster ovary).

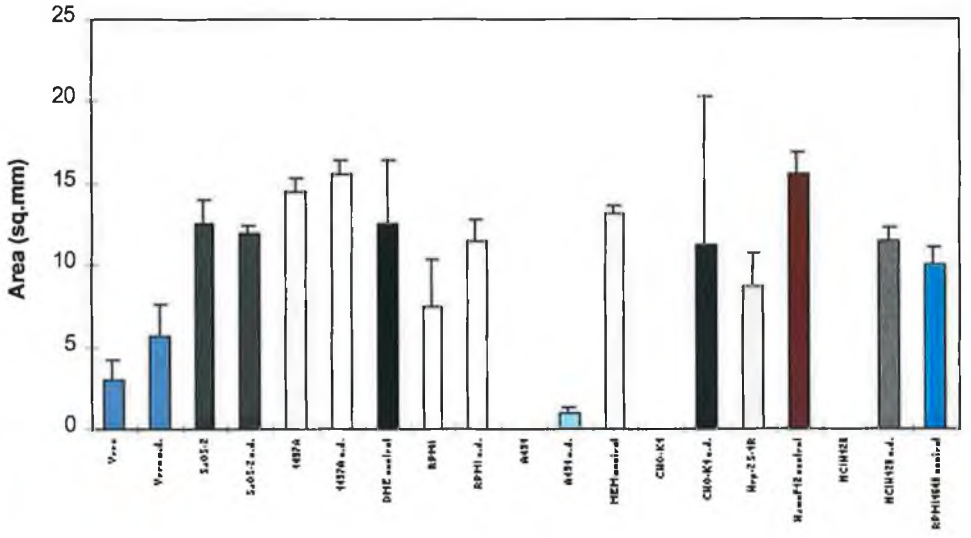
All samples used from these cell lines were 'waste' media, that is media taken directly from growing cultures in flasks and thus including FCS. The samples were not concentrated by ultrafiltration but were dialysed to remove low MW waste products. A 24 well plate assay system was used to assess the growth response for Hep-2 cells.

A Hep-2 , 1-5000 MW retentate was also tested in this assay, since this fraction has been shown to be inhibitory in a Hep-2 autocrine assay developed in this laboratory (Gregory, 1994). Results are presented in Table 3.17 and Figure 3.11 shows results from Assay A presented in Table 3.17.

Table 3.17 : Investigations of Media from Several different cell lines for effects on the growth of Hep-2 cells.

| Sample | Area ($mm^2$) Assay A | Area ($mm^2$) Assay B | Area ($mm^2$) Assay C |
|---|---|---|---|
| Medium control (DME) | 12.535 ± 3.84 | 135.79 ± 3.66 | 104.53 ± 11.05 |
| Vero cm dia. | 2.96 ± 1.22 | 52.52 ± 23.84 | 47.14 ± 5.64 |
| Vero cm non-dia. | 5.73 ± 1.87 | 134.27 ± 14.72 | 78.24 ± 15.99 |
| SaOS-2 cm dia. | 12.53 ± 1.52 | 182.18 ± 18.8 | 99.86 ± 6.18 |
| SaOS-2 cm non-dia | 11.914 ± 0.54 | 171.76 ± 14.27 | 109.91 ± 6.46 |
| 1437A cm dia. | 14.52 ± 0.76 | 156.13 ± 23.25 | 105.86 ± 15.79 |
| 1437A cm non-dia. | 15.64 ± 0.796 | 222.05± 12.46 | 121.11 ± 5.95 |
| Medium control (MEM) | 13.13 ± 0.55 | 189.7 ± 21.36 | 102.32 ± 17.03 |
| RPMI cm dia. | 7.51 ± 2.85 | 88.42 ± 3.87 | 98.37 ± 5.92 |
| RPMI cm non-dia. | 11.457 ± 1.29 | 186.52 ± 11.55 | 97.32 ± 6.21 |
| A431 cm dia. | 0 | 0.815 ± 0.54 | 72.36 ± 15.43 |
| A431 cm non-dia. | 0.9319 ± 0.4 | 46.34 ± 8.66 | 49.37 ± 2.05 |
| Medium control (Hams F12) | 15.58 ± 1.35 | 127.47 ± 7.33 | 83.37 ± 2.17 |
| CHO-K1 cm dia. | 0 | 0 | 0.08 ± 0.07 |
| CHO-K1 cm non-dia. | 11.23 ± 9.09 | 39.89 ± 26.4 | 73.83 ± 20.3 |
| Hep-2 cm 1-5000R dia | 8.65 ± 2.05 | 79.99 ± 10.0 | 62.7 ± 6.19 |
| Medium control (RPMI-1640) | 10.01 ± 1.14 | 118.42 ± 12.4 | 79.63 ± 10.65 |
| NCIH-128 cm dia. | 0 | 0.093 ± 0.01 | **** |
| NCIH-128 cm non-dia. | 11.44 ± 0.86 | 140.04 ± 7.71 | 85.88 ± 6.8 |

**Figure 3.11: Effect of Conditioned Media from Different Cell Lines on Growth of Hep-2 Cells**




A summary of results obtained is given below:

- **Vero cm** - both the dialysed and non-dialysed fractions displayed inhibitory activity with greater activity in the dialysed fraction.
- **SaOS-2 cm** - this cm seems to elicit no response from Hep-2 cells, either stimulatory or inhibitory.
- **1437A cm** - cm from this line promoted a slight increase in Hep-2 cell growth in both the dialysed and non-dialysed fractions.
- **RPMI cm** - non-dialysed fractions showed no effect on HEP-2 cells , the dialysed fraction displayed some inhibitory activity in 2 out of 3 assays.
- **A431 cm** - The non-dialysed fraction seems to contain some inhibitory activity while the dialysed fraction seems to cause complete destruction of Hep-2 cells in this 5 day assay.
- **CHO-K1 cm** - the non-dialysed fraction caused inhibition in 1 of the 3 assays , however the dialysed fraction had a cytotoxic effect on Hep-2 cells in all assays.
- **Hep-2 cm** - in each assay the Hep-2 1-5000 retentate inhibited the growth of the Hep-2 indicator cells (auto-inhibitory response). The level of inhibition is approximately 40% reduction in growth and does not seem to

be a cytotoxic efface as there was not complete detachment of the cells as with the MSV-3T3 1000 retentate.

- **NCIH-128 cm** - no real inhibitory effect is seen in the non-dialysed fractions however complete inhibition of Hep-2 cell growth is caused by the dialysed fractions.
- **MSV-3T3 cm** - The 1000 retentate was also tested in these assays and caused complete inhibition/destruction of Hep-2 cell growth.

It would appear that a number of cell lines produce growth inhibitory molecules, however the effect of dialysis on some of the cm cannot be ignored. In the RPMI, CHO-K1 and NCIH-128 cm dialysis seems to cause the medium to become inhibitory to the HEP-2 cells. Whether the inhibition is due to the dialysis process itself (e.g. leeching of toxic compounds from the benzoylated membrane) or to dialysis activating inhibitory molecules cannot be ascertained. Vero cm and A431 cm displayed inhibitory activity in both the dialysed and non-dialysed fractions however the activity was more pronounced in the dialyzed fractions. Since this assay was a preliminary screen and used cm from waste flasks to assess growth inhibitory potential, it was decided to collect cm from the following cell lines, Vero, A431 and CHO-K1 cells. Conditioned medium would be collected in serum free conditions, dialysed and assayed using a properly dialysed control.

*3.1.11.1.1: Effect of CHO-K1 cm on Growth of HEP-2 and CHO-K1 cells*

Two batches of CHO-K1 cm were collected under serum free conditions, one from a roller bottle and one from a flask culture. Both were tested for inhibitory activity and the results summarised in Table 3.18.

Table 3.18: Effect of CHO-K1 cm on growth of Hep-2 and CHO-K1 cells

| Sample | Effect on Hep-2 Growth | Effect on CHO-K1 Growth |
|---|---|---|
| CHO-K1 Batch 1<br>1200 MW dialysate | No growth | No growth |
| CHO-K1 Batch 2<br>1200 MW dialysate | No growth | No growth |
| CHO-K1 Batch 1<br>12,000 MW dialysate | No growth | No growth |
| CHO-K1 Batch 2<br>12,000 MW dialysate | No growth | No growth |
| CHO-K1 Batch 1<br>non-dialysed | Stimulation | Slight stimulation |
| CHO-K1 Batch 1<br>1000R non-dialysed | Slight inhibition | Slight inhibition |
| CHO-K1 Batch 1<br>1000 filtrate non-dialysed | Stimulation | No response |
| CHO-K1 Batch 2<br>non-dialysed | Stimulation | No response |
| Control Hams F12<br>dialysed | No growth | No growth |
| Control Hams F12<br>non-dialysed | Control | Control |

It can be seen that any medium dialysed, whether it is the CHO-K1 cm or the Hams F12 control medium results in complete inhibition of Both Hep-2 and CHO-K1 cell growth. Due to the possible toxic effects seen in other experiments using the benzoylated 1200 MW cut off tubing, another dialysis tubing, a 12,000 MW visking tubing was used here as well as the benzoylated

tubing. Dialysis consisted of three changes of ultrapure water over 1-2 days and a final dialysis against Hams F12 medium over night.

Several explanations may be offered as to why dialysis seems to be a toxic procedure to cells:

1. Perhaps the dialysis tubing contains a toxic factor which leaches into the cm during dialysis. Both tubing were pre-treated by boiling in 10mM EDTA for 10 minutes followed by washing in ultrapure water, however maybe this treatment is not rigorous enough especially for the benzoylated tubing. Subsequent work in this laboratory indicated that dialysis may indeed cause an inhibitory response and suggested an improved washing system for dialysis tubing (Gregory, 1994).
2. The final dialysis against Hams F12 medium so that essential amino acids or small MW compounds removed by the dialysis against water should be replaced. However perhaps one dialysis against medium is not enough.
3. Perhaps dialysis activates an inhibitory factor in Hams F12 medium.

In the non-dialysed CHO-K1 cm , a slightly inhibitory effect on both the HEP-2 and CHO-K1 cells is seen in the 1000 MW retentate only.

*3.1.11.1.2: Effect of Vero cm on Growth on Hep-2 and Vero cells*

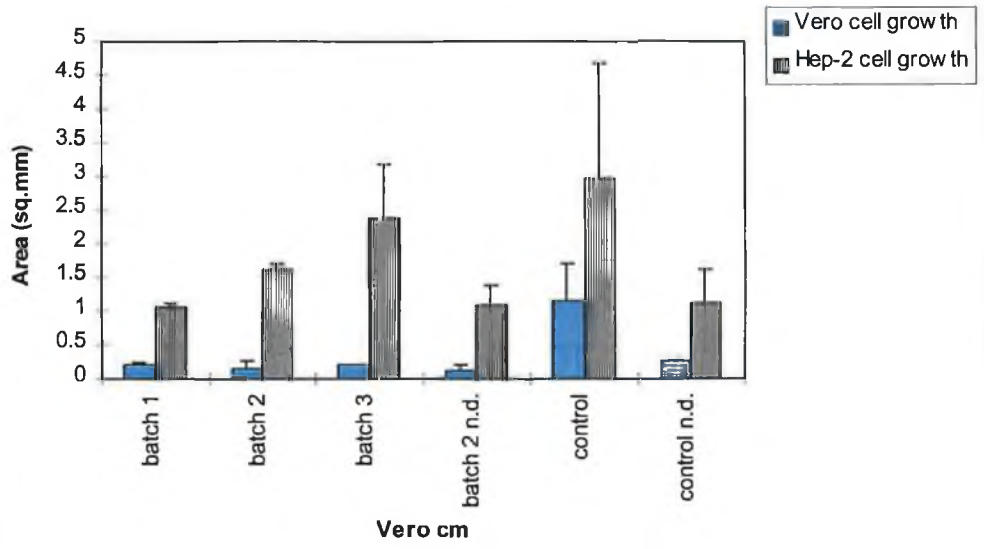
Vero cm was collected, dialysed and tested for both auto-inhibitory activity and inhibitory activity against Hep-2 cells. Vero cm samples were not ultrafiltrated as previous assays (Table 3.17) suggested inhibitory activity was present in the neat cm. Three separate batches of Vero cm were collected: Batch 1 from a roller bottle, batch 2 from a flask and batch 3 from a separate stock of cells in a sub-confluent flask. Results are presented in Table 3.19 and Figure 3.12

Table 3.19: Effect of Vero cm on growth of Vero and Hep-2 cells

| Sample | Effect on Vero Growth Colony Area ($mm^2$) | Effect on Hep-2 Growth Colony Area ($mm^2$) |
|---|---|---|
| Vero batch 1 dialysed | 0.215 ± 0.02 | 1.07 ± 0.06 |
| Vero batch 2 dialysed | 0.156 ± 0.11 | 1.61 ± 0.1 |
| Vero batch 3 dialysed | 0.2 ± 0.01 | 2.37 ± 0.8 |
| Control (DME) dialysed | 1.14 ± 0.57 | 2.97 ± 1.7 |
| Vero batch 2 non-dialysed | 0.105 ± 0.09 | 1.09 ± 0.3 |
| Control (DME) non-dialysed | 0.259 ± 0.03 | 1.11 ± 0.5 |

Each assay on both Vero and Hep-2 cells was repeated twice and similar trends as above obtained.

**Figure 3.12: Effect of Vero Conditioned Medium on Growth of Vero and Hep-2 Cells**




note: n.d. = non-dialysed.

Effect on Vero growth

Dialysis does not appear to be causing the growth medium (DME) to become inhibitory in this experiment as has been the case in some of the previous

work. The dialysed DME control supports a high level of Vero cell growth, indeed more than the non-dialysed control which is somewhat unusual. On the basis of this experiment it would seem that Vero cells do produce an auto-inhibitor, detectable unconcentrated in serum free medium and stable to dialysis. The nature of this inhibition would seem to more that of a classical inhibitor, i.e. it seems not to be cytotoxic in nature as opposed to the MSV-3T3 factor(s).

Effect on Hep-2 growth

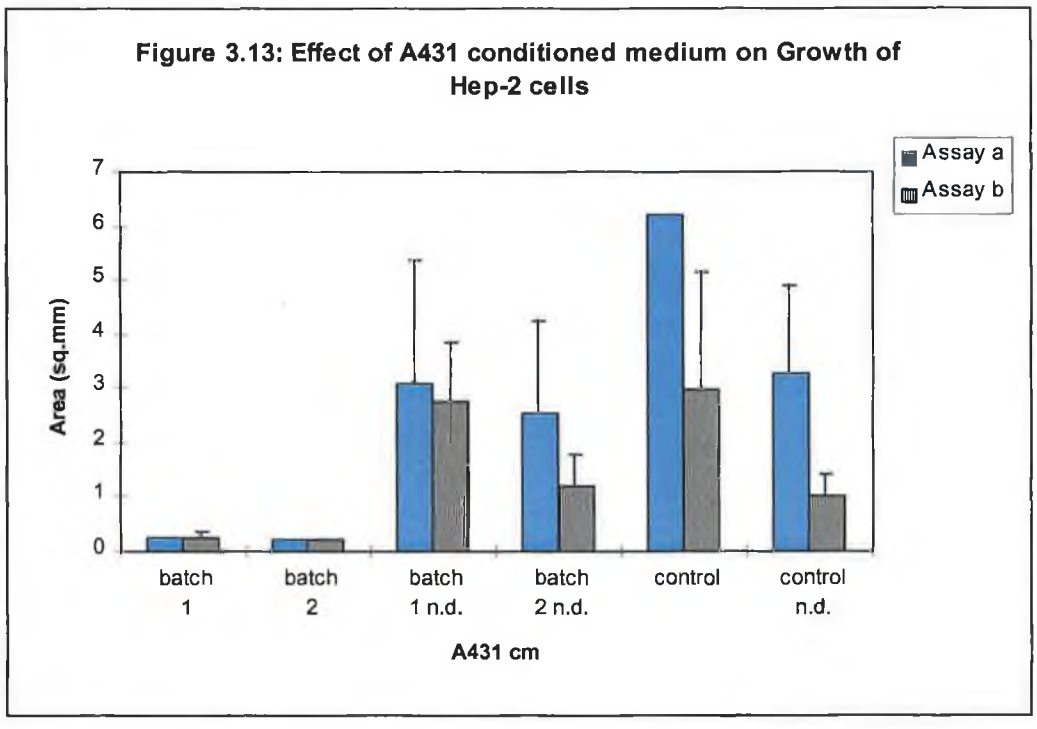
Again the dialyzed control does not seem to be inhibitory and supports good growth of HEP-2 cells, slightly more than the non-dialysed control. Vero cm would seem to contain some inhibitory activity for Hep-2 cells, although the magnitude of growth inhibition is not as great as that for Vero cells. An interesting observation noted both in this assay and in repeat assays (results not presented) is that there seems to be less inhibitory activity in the batch 3 cm than in batches 1 and 2. Batch 3 cm was collected from flasks that were less confluent than the flask/roller bottle from which batches 1 and 2 were collected. Perhaps Vero cells only produce growth inhibitors for Hep-2 cells in significant quantities when they reach a certain saturation density. However this result was not seen in the autoinhibitor (Vero cm on Vero cells) assay.

*3.1.11.1.3: Effect of A431 cm on Growth of Hep-2 Cells*

A431 cm was collected under serum free conditions, dialysed through 1200 MW cut off tubing and assayed for growth inhibitory activity on Hep-2 cells. Batch 1 cm refers to cm collected in roller bottle and batch 2 to cm collected in a flask. Results are illustrated in Table 3.20 and Figure 3.13.

Table 3.20: Effect of A431 cm on growth of Hep-2 cells

| Sample | Colony Area ($mm^2$) Assay A | Colony Area ($mm^2$) Assay B |
|---|---|---|
| A431 batch 1 dialysed | 0.24 ± 0 | 0.26 ± 0.1 |
| A431 batch 2 dialysed | 0.0 ± 0 | 0.23 ± 0 |
| Control (MEM) dialysed | 6.22 ± 0 | 2.96 ± 2.2 |
| A431 batch 1 non-dialysed | 3.08 ± 2.3 | 2.74 ± 1.1 |
| A431 batch 2 non-dialysed | 2.53 ± 1.7 | 1.19 ± 0.6 |
| Control (MEM) non-dialysed | 3.28 ± 1.6 | 1.03 ± 0.4 |




Note: n.d. = non-dialysed.

Quite significant inhibition is seen in the dialysed cm fractions when compared to the dialysed MEM control which causes no inhibition of growth suggesting that dialysis is not having a toxic effect on the cells. The non-dialysed cm shows no real inhibition when compared to growth in control wells. Since dialysis itself does not, in this case, appear to be an inhibitory process, it would seem that dialysis may be in fact activating an inhibitor in A431 cm.

### 3.1.12: FURTHER DISCUSSION OF RESULTS AND CONCLUSIONS

The complex process of development and adult homeostasis is apparently mediated and regulated by a multiplicity of positive and negative regulators of cellular proliferation, differentiation or both. The breakdown or alteration of the cellular homeostasis mechanism is a basic cause of cancer and other growth related illnesses. The aim of the work undertaken in this section was to identify and characterise growth inhibitors (negative growth regulators) produced by animal cells when growing *in vitro*.

#### 3.1.12.1: MSV-3T3 Conditioned Medium

Most of the work involves the mouse transformed cell line, MSV-3T3, since earlier work had indicated that 3T3 cells might produce a growth inhibitor (M$^{c}$Donnell, S: 1987). Initial results were very promising, with dramatic growth inhibition found in the 1000 MW retentate of MSV-3T3 cm against the squamous cell carcinoma cell line, SCC-9 (see Table 3.1). Less dramatic and more variable growth inhibitory activity was noticed in 3T3 cm. The growth inhibitory activity of MSV-3T3 cm seemed to be active against both freshly inoculated SCC-9 cells and attached (for 24 hours) cells (Table 3.3). A TGF-β assay (Table 3.6, Figure 3.3) showed that MSV-3T3 cm contains little or no active TGF-β (TGF-β being the most ubiquitous growth inhibitor identified to date).

Microscopic observation in subsequent assays suggested that the growth inhibitory activity of MSV-3T3 cm was of a more cytotoxic rather than cytostatic nature since SCC-9 cells seem to disintegrate after MSV-3T3 cm addition (Section 3.1.2). It was also noticed that dialysis causes the MSV-3T3 cm to become more active. Ultrafiltration studies show that most MW retentates of MSV-3T3 cm cause inhibition of SCC-9 growth, especially the lower MW retentates.

MSV-3T3 cm was also investigated for effects on cell lines other than SCC-9 and most MSV-3T3 cm ultrafiltration fractions seemed to be stimulatory for NRK cells (normal fibroblasts) and mainly inhibitory for Hep-2 cells (larynx carcinoma cells) (Section 3.1.5- Figures 3.6 and 3.7). These results are interesting since they imply that the inhibitory activity in MSV-3T3 cm is species specific (inhibitory for human cells) and/or multi-functional (stimulatory for fibroblasts, inhibitory for epithelial cells). TNF-α, the most well documented cytotoxic factor and interferon-γ have both been shown to stimulate fibroblast growth (Elias *et al*, 1988; Hori *et al*, 1988; Elias, Jiminez and Freundlich, 1987). In addition, these cytokines are of larger molecular weight (TNFα - 17kDa; IFNγ - 34kDa). Therefore the observation that the higher molecular weight fractions were less inhibitory than the lower molecular weight fractions for NRK cells (Figure 3.6) may be due to the action of these cytokines. Figures 3.5 and 3.7 show that in general the larger molecular weight fractions are less inhibitory than the lower molecular weight fractions. More than likely other growth stimulatory molecules such as EGF, PDGF etc. are present in the MSV-3T3 cm. The simultaneous presence of inhibiting and stimulating factors in cm is widely accepted (Coucke *et al,* 1991). As mentioned in section 1.2.1, cell growth appears to be regulated by a combination of positive and negative growth signals. In the higher molecular weight fractions the presence of stimulatory fractions may be masking the activity of cytotoxic/inhibitory activity. In lower molecular fractions, the stimulatory factors are excluded on the basis of their molecular weight, thus the effect of the toxic agent is more pronouced.

At this stage of the work it was discovered that the MSV-3T3 cells were mycoplasma contaminated and may have been since the work on the cm commenced. Mycoplasmas are prokaryotes without a cell wall which allows them to be resistant to many antibiotics, e.g. penicillin. They are small and flexible (no cell wall) so that they are not easily removed from cell cultures by 0.22μm filtration. Mycoplasmas create major problems in cell and tissue cultures and the true incidence of infection may be higher than published

figures. Mycoplasmas inhibit cell cultures by depleting the medium of essential nutrients. Miyasaki *et al* (1990) showed that the inhibition of human tumour cells in culture was due to the depletion of arginine in the medium by the enzyme deiminase produced by mycoplasma which had infected the cell line. It was found that the addition of excess L-arginine into the culture medium prevented inhibition. Certain mycoplasma infections may actually stimulate the growth of a cell culture. For example, Cole *et al* (1989) have reported that a mitogen derived from a mycoplasma stimulates mouse lymphocytes. Sasaki *et al* (1984 ) investigated the inhibition of growth of mammalian cell cultures caused by mycoplasma infection using cell-free extracts of 14 species of mycoplasma. In four cell lines tested, the growth of two, FM3A and MDCK, was inhibited by the extracts of arginine utilising mycoplasmas, whereas the other cell lines, Vero and LLC-MK2, were not inhibited by extracts of either arginine or glucsoe utilising mycoplasmas.

While waiting for new stocks of mycoplasma free MSV-3T3 cells, a number of stability studies conducted on MSV-3T3 cm indicated that the growth inhibitory activity maybe heat, acid, base and DTT resistant, but perhaps trypsin sensitive (Section 3.1.6). Stability studies provide an excellent means of characterising a putative growth regulator. Known growth factors/inhibitors have a well documented resistance or susceptibility to various agents. Comparison of the stability profile of a known factor against the putative novel factor facilitates characterisation of the factor. For example, TGF-β is acid and heat stable but trypsin sensitive. Therefore the factor in MSV-3T3 cm might be TGF-β like, however earlier work (Figure 3.3) has shown that there is very little TGF-β activity in MSV-3T3 cm. In addition, TGF-β acts in a reversible manner having a cytostatic effect. The inhibitor in MSV-3T3 cm is of a cytotoxic nature.

Mycoplasma free MSV-3T3 cm studies showed that the inhibition in the 1000 MW retentate is indeed a true effect and not an artifact of mycoplasma infection (Section 3.1.7). Further studies showed that the 1000 retentate was

stable to heating at 100°C for 3 minutes and that most of the inhibitory activity lies in the lower MW fractions, with little activity in the 30,000 and 100,000 MW retentates. Since the 1000 retentate fraction had consistently shown the highest level of growth inhibitory activity, an experiment was performed to assess the effect of the 1000 MW ultrafiltration membrane on control growth medium (DME) and on the MSV-3T3 cm (Section 3.1.8). It was found that both ultrafiltration through the 1000 MW membrane and dialysis affect the medium. However the level of inhibition caused by ultrafiltration and dialysis is not enough to account for that found in the 1000 retentate.

Further work on MSV-3T3 cm was undertaken to identify the nature of the active factors in the cm (Section 3.1.10). Since the growth inhibitory activity in MSV-3T3 cm seems to be of a cytotoxic nature it was important to investigate whether the activity might be due to TNF-α. L929 cells are commonly used as the indicator cell line for TNF-α activity in a TNF-α bioassay. It was found that these cells display a similar cytotoxic response to MSV-3T3 cm as to TNF-α. However Hep-2 cells which are used as the indicator cells for MSV-3T3 cm inhibitory activity show an unusual response to TNF-α, that is cell numbers actually increase with increasing TNF concentration. This is an unusual response to TNF-α and although TNF-α may have stimulatory effects for fibroblasts (Hori *et al*, 1988), stimulation of epithelial cells has not been reported before. A TNF-α ELISA technique revealed (Section 3.1.10.3)that MSV-3T3 cm contains no TNF-α upto a detection level of 15 pg/ml. It was found that TNF-α neutralising antibody did not quite abolish TNF-α activity on L929 cells.

Unfortunately, the appearance of the cytotoxic activity in the 1000R of MSV-3T3 cm studies became erratic at this point (Section 3.1.10). No cytotoxic activity was identified in the 1000 retentate (Figure 3.10) as had been the case up to this point. Cytotoxic/growth inhibitory activity was consistently seen in the 1000 MW fraction in all the preceding work. A number of MSV-3T3 cm

ultrafiltration fractions were tested for activity and the only fractions showing any real inhibition of growth are the 30-10,000 retentate and the 100-30,000 retentates. Subsequent stability studies undertaken with these fractions found that the 30-10,000 activity was trypsin sensitive and heat resistant to 65°C for short time periods (Table 3.16). The 100-30,000 activity seemed to be trypsin resistant, heat resistant to 65°C but sensitive to 100°C.

Similar problems with isolating growth inhibitors in cell culture systems have been encountered by other research groups. Emery *et al*, 1987, tried to characterise the cytotoxic components from culture medium at various stages of cultivation. In a similar manner to this work, they fractionated the medium by ultrafiltration using a range of membrane molecular weight cut-offs. However no consistent picture emerged. In one experiment, cytotoxic activity was found in the 100,000 MWCO fraction and in the 10,000 MWCO fraction. In another experiment, no activity was identified in these fractions but was found in molecular weight fractions in between 10,000 and 100,000 molecular weight.

There are a number of known growth inhibitors identified in the literature (Section 1.2.3 reviews some of these). Most of these are cytostatic molecules acting in a reversible manner (e.g. TGF-β, amphiregulin, mammary-derived growth inhibitor). Known cytotoxic molecules include, TNF-α, lymphotoxin, interferon-β and interleukin 2. This work seems to suggest that the growth inhibitory /cytotoxic activity in MSV-3T3 cm is not due to either TGF-β or TNF-α. However the use of neutralising antibodies to lymphotoxin, interferon-β or interleukin 2 would allow further elimination of which factors might be causing cytotoxicity in MSV-3T3 cm.

The identification of a number of new growth inhibitors in the literature is interesting because they appear to possess some of the characteristics of the cytotoxic activity in MSV-3T3 cm. These include the following.

1. Epithelins (Shoyab.M. *et al* 1990). are cytostatic molecules of low molecular weight (approximately 6000 daltons) which are heat and acid stable. They seem to be proteins since they appear to be trypsin sensitive. Epithelins inhibit the carcinoma cell line, A431 but display bifunctional characteristics in that they are inhibitory to tumour cells but mitogenic to fibroblasts. The activity in MSV-3T3 cm also appears to be heat and acid stable and have bifunctional properties in its affect on epithelial and fibroblasts cells. However the MSV-3T3 factor is not a cytostatic molecule.
2. Fibroblast Growth Inhibitory Factor (FGIF) (Sharma, C.P. and Gehring, H: 1986) is a cytostatic molecule of very small molecular weight (less than 2000 daltons), which appears to be protease resistant and acid/heat stable. This molecule is related to the density dependent inhibition of fibroblasts but the observed inhibition does not seem to be caused by ammonia or lactate accumulation. Again, the MSV-3T3 activity would appear to be similar to this molecule (acid/heat stable) however the mode of action is different, cytotoxicity as opposed to eliciting a cytostatic response.
3. Tumour Cytotoxic Factor (TCF) (Higashio, K: 1990) is a cytotoxic molecule which seems to be closely related to human Hepatocyte Growth Factor. It has bifunctional characteristics in that it seems to be cytotoxic to tumour cells tested and mitogenic to fibroblasts. Neutralising antibody studies indicated that molecule is not TNF-$\alpha$, lymphotoxin or interferon-$\beta$. The molecule seems to have molecular weight of 76-80,000 daltons, is stable to pH 6-9 and 60°C but labile to 70°C and reduction by mercaptoethanol. The MSV-3T3 cm is cytotoxic and mitogenic to fibroblasts, however its heat stability profile is different, since it appears to be stable to 100°C.
4. Fibroblast inhibitor from rat macrophages (Concannon, M.J. *et al*, 1993) is a small molecule (less than 3000 daltons) which inhibits fibroblasts from human and rabbits. It is a protease and heat stable molecule. It differs from the MSV-3T3 cm in its mode of action as it is a cytostatic agent which appears (thus far) to be only effective against fibroblasts.

5. 3T3 Inhibitors (Harel, Blat and Chatelain, 1985; Steck *et al*, 1982; Holley *et al*, 1980). A number of 3T3 autocrine factors have been identified. These include inhibiting factors (11, 13 and 45 kDa) and stimulating factors (10 and 35 kDa). Recent work (Siwek *et al* , 1991) has identified a cytotoxic factor in 3T3 cm with a molecular weight of between 1000 and 10,000 daltons. As well as being active against 3T3 cells this factor is also active against B16 melanoma cells. This cytotoxic activity is of particular interest since MSV-3T3 cells are a virus transformed variant of 3T3 cells. Therefore it is reasonable to presume that factors in 3T3 cm would also be found in MSV-3T3 cm, albeit in an altered form. Unfortunately, these workers have presented no more work on the 3T3 cytotoxic activity, so no further comparisons may be made.

It can be seen that a variety of growth inhibitors exist, some with quite unusual profiles similar to that of the MSV-3T3 cm (e.g. high heat stability). In order to identify molecules causing growth inhibition/cytotoxicity a number of fundamental questions must be answered. In the following sections some of these questions are asked of the MSV-3T3 cm.

- *Are there a number of molecules causing inhibition in MSV-3T3 cm ?*

For MSV-3T3 cm the answer would seem to be yes since although the most inhibitory fraction would seem to be the 1000 retentate, cytotoxic activity was also identified in the 10,000 retentate and subsequently the 30-10,000 and 100-30,000 fractions. Whether there are distinct separate molecules responsible for the activity or if the activity in the higher MW fractions is due to aggregates of lower MW inhibitors remains to be seen.

- *Is the molecule cytotoxic or cytostatic ?*

Both a cytotoxic and a cytostatic response by cells to factors in cm will result in a lower final cell density. However it is important to distinguish between the two responses. A cytotoxic response may be observed by microscopic evaluation (for cell detachment or disintegration) or by release of the intracellular enzyme, LDH. A cytostatic response may be monitored by a decreased specific growth rate, a decreased rate of DNA synthesis by serum stimulated cells or if the cytostatic factor is removed the target cells will re-grow. In the case of MSV-3T3 cm the growth inhibition is observed to be of cytotoxic nature since microscopic evaluation reveals the target cells to be disintegrating after the treatment period and in any particular well treated with the 1000 retentate there is complete wipeout of cell growth.

- *Is the molecule(s) a protein ?*

The best way to answer this question is to treat the cm with a protease such as trypsin. In the case of MSV-3T3 cm variable results are obtained, it would seem that the 1000 retentate may be trypsin sensitive but the 100-30,000 fraction seems to be trypsin resistant. More extensive protease studies using a number of proteases would be needed to categorically answer this question.

- *Is the molecule a carbohydrate or lipid ?*

In order to ascertain the chemical nature of the growth inhibitory molecule, treatment of cm with glycosidases or lipases would indicate whether oligosaccgarides or lipid moieties are present at the active site or needed for biological activity. Treatment with either of these enzymatic groups was not performed on MSV-3T3 cm.

- *Is the molecule a previously identified growth inhibitory molecule ?*

If neutralising antibodies or mRNA probes to known factors are available then these present the best means of identifying whether factors in cm are already identified. If antibodies are not available, then a comparison of the stability profile of factors in cm to the known factors is a useful investigation tool. In the case of MSV-3T3 cm, TGF-β and TNF-α studies suggest that the inhibitory activity is not caused by one of these factors. The stability profile is unusual , suggesting a very heat and pH stable molecule. However the literature does report the presence of growth inhibitory factors with similar stability profiles, e.g. FGIF, TCF and epithelins. The use of neutralising antibodies to interferon, interleukins and a more extensive stability profile on MSV-3T3 cm fractions would yield some useful information.

- *Are there artifacts in the in vitro growth inhibitory assay which lead to potentially misleading results ?*

A number of artifacts may exist in an *in vitro* based growth inhibitory assay system, these include:-

a) Mycoplasma contamination - mycoplasma and mycoplasma-derived arginine deiminase have been reported to be growth inhibitory for many cell types (Miyasaki *et al*, 1989). Steps should be taken to ensure that all cell culture work is mycoplasma free since contamination can cast doubt on results obtained. In the case of MSV-3T3 cm, cytotoxic activity in the 1000 retentate was present in both mycoplasma contaminated and mycoplasma free cm.

b) Proteases - proteases and their inhibitors may affect cell growth (Scott, 1992). When DNA synthesis of indicator cells is measured by incorporation of labeled thymidine, endogenous cellular thymidine and thymidine degrading enzymes may inhibit the incorporation of the labeled thymidine. MSV-3T3 cm seems to contain a relatively high level of cathepsin protease (results not

presented), whether this may be linked to the cytotoxic activity was not determined in this work.

c) Polyamines - when growth inhibitors are extracted from tissues the presence of polyamines such as spermine and spermidine in the tissue extracts is one of the major problems. Polyamine oxidase present in ruminant sera converts these polyamines to cytotoxic amine aldehydes.

d) Viruses - viral infection of host cells may be responsible for growth inhibitory activity, however in the case of MSV-3T3 cells, cytotoxic activity was still seen after heating at 100°C for 3 minutes, a treatment that would eradicate any viral contamination. Also no substantial growth inhibitory activity was seen in the 100,000 MW fraction (which should retain large virus particles) when assayed on Hep-2 cells.

e) Prions - prions have been implicated as the causative agents in a number of diseases such as scrapie in sheep, B.S.E. in cattle and even Alzeihmers Disease in humans. Prions seem to be very stable proteins with a high heat and pH stability. However they would seem to have a long replication period and may lay dormant for a number of years before becoming active. Since MSV-3T3 cm caused a cytotoxic effect on indicator cells in a 5-7 day period it would seem unlikely that a prion may be responsible for cytotoxic activity.

f) Experimental procedure - in growth factor assays a positive effect on cell growth is being monitored and the end result is usually relatively easy to interpret. In growth inhibitory assays a negative effect is being assessed and it is not always easy to interpret the end result. Factors such as target cell loading density, target cell condition, exposure time to factor,. serum batch variation, medium constituents, etc. may have a profound effect on the interpretation of results. Properly used controls are of extreme importance in growth inhibitory assays.

In conclusion, the following may be noted:

1. MSV-3T3 cells produce and secrete factors which either inhibit or have a cytotoxic effect on epithelial carcinoma cells.
2. Either one factor or an aggregate of factors may be responsible for the cytotoxic activity. Perhaps one of the following scenarios may explain why activity is seen in some fractions and not others. The factors may exist in any of the following relationships;-

   a) molecule and stabiliser

   b), precursor and protease

   c) a TGF-α and TGF-β complementary existance.
3. The cytotoxic activity would seem to be of a variable nature. The 1000 MW cutoff ultrafiltration retentate yielded mostly inhibition but not always (especially in later results).
4. Dialysis and ultrafiltration would seem to be potentially toxic steps in the processing of the cm.
5. The molecules responsible would seem to be very stable entities with high heat and pH resistance. TGF-β and TNF-α would not seem to be responsible for the inhibitory activity. The responsible molecules may be related to recently identified molecules such as TCF or FGIF.

Further work would be needed to extensively characterise the cytotoxic activity in MSV-3T3 cm.

3.1.12.2: Other Conditioned Medium

Growth inhibitory activity was also identified in the following cm:

- Vero cm - this cm inhibited both its own growth (Figure 3.12) and that of Hep-2 cells. Therefore these cells would appear to produce auto-inhibitory molecules as well as molecules effective against a cell line from another species. The inhibitory effect was observed microscopically to be non-cytotoxic. An interesting observation is the increase in inhibition observed when cm was produced from confluent Vero cultures. This result would imply that cells might produce the inhibitor or might produce more of the inhibitor when their own growth is being retarded due to lack of surface area for growth. There are a number of so-called density dependent inhibitors identified in the literature, particularly from 3T3 cells (Holley and Kieran, 1968; Holley *et al*, 1978; Harel *et al*, 1978). This work appeared to be quite promising, however it was not continued due to the demands of completing work on the next section of this thesis, the effect of external factors on the growth of animal cells *in vitro*.
- A431 - this cm appears inhibitory against Hep-2 cells.

## SECTION 3.2: INVESTIGATIONS INTO EXTERNAL FACTORS CAUSING GROWTH INHIBITION OF ANIMAL CELLS IN CULTURE

Section 3.1 describes work undertaken to identify and characterise endogenous growth inhibitors produced by animal cells growing in culture which have the overall effect of limiting the cell density achievable or in some cases actually killing the cells. As well as endogenous growth inhibitors, external factors such as agitation intensity, osmotic pressure, temperature, oxygen levels and serum concentration may also limit the growth of animal cells *in vitro*. The build up of metabolic waste products such as ammonia and lactate are also important factors to consider. While ammonia and lactate may be considered endogenous inhibitors, they differ from the protein like growth regulatory molecules considered in Section 3.1 in their mode of inhibition. For example, lactate would seem to inhibit in a physical manner by decreasing pH and increasing osmolarity of the culture medium (Ozturk and Palsson, 1991).

### 3.2.1: The Growth Of MSV-3T3 Cells: Influence Of Agitation And Inoculum Density On Cell Growth.

MSV-3T3 cells were being cultured in the laboratory at this time for the production of conditioned medium (cm) for growth inhibitor studies (see Section 3.1). The cm was collected from cells growing in suspension culture in spinner flasks since relatively large volumes were required for further processing of the growth inhibitory factor(s). As a consequence a preliminary investigation of the growth of MSV-3T3 cells in suspension culture was undertaken.

3.2.1.1: The Growth of MSV-3T3 Cells in Monolayer Versus Suspension Culture

MSV-3T3 cells are rountinely grown in monolayer culture but for collection of conditioned medium from the cells they were grown in suspension culture. Consequently, it was decided to examine the effect of culture mode on the apparent growth rate of MSV-3T3 cells grown in a monolayer flask culture and in a suspension spinner flask culture.

Figure 3.14 shows the total and viable growth curves obtained when MSV-3T3 cells are grown in monolayer for 10 days. When the viable cell number is plotted on a semi-logarithmic plot the apparent growth rate and doubling time may be calculated from the linear portion of the curve (see Figure 3.15). The apparent growth rate ($\mu_{app}$) has a value of 0.028 $hr^{-1}$ and the doubling time is 24.74 hrs. The cells are not in stationary phase for a significant amount of time. However it was noted that there was a substantial increase in cell detachment from the monolayer culture at the end of the batch cultivation phase.

**Figure 3.14: Growth of MSV-3T3 Cells in Monolayer Culture**

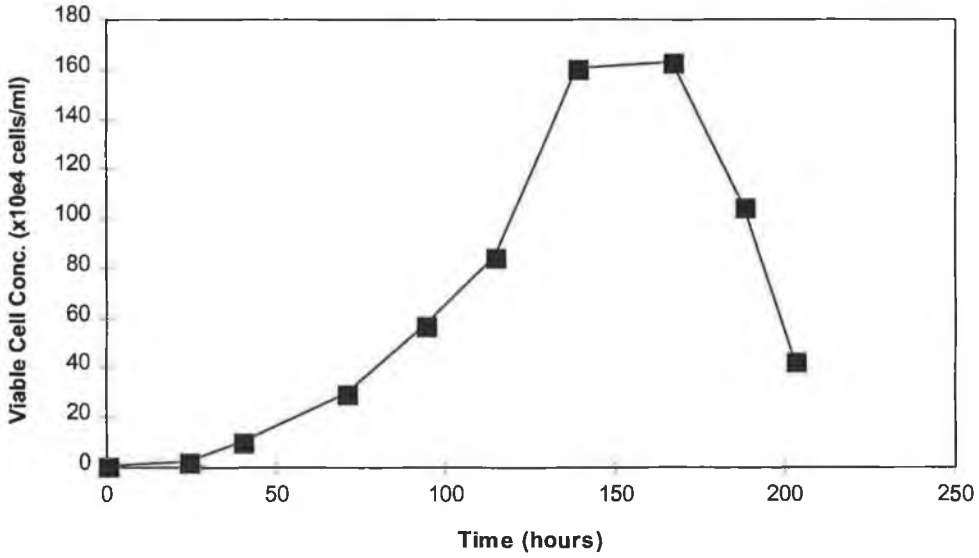


**Figure 3.15: Growth of MSV-3T3 Cells in Monolayer Culture: Calculation of Apparent Growth Rate**

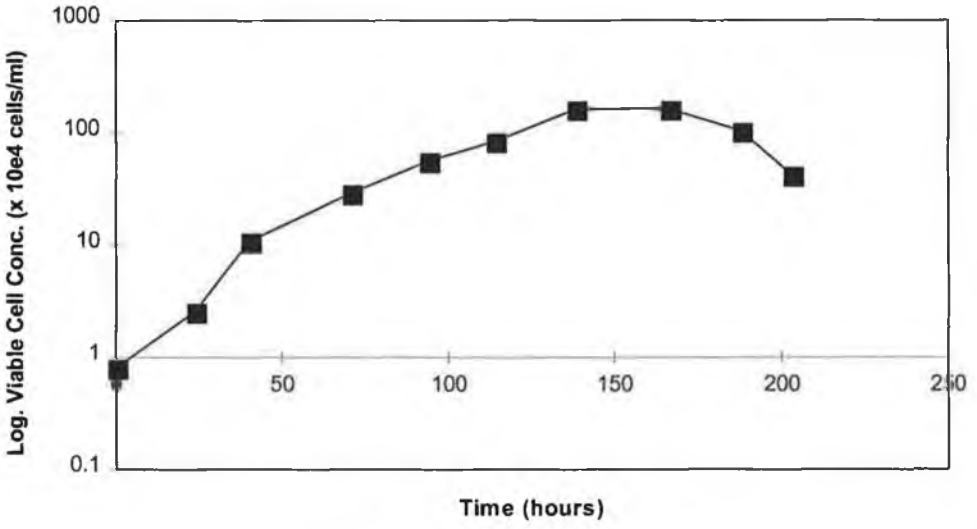



Figure 3.16 illustrates the results obtained from a suspension culture set up in a spinner flask at an agitation rate of 25 rpm for 24 hours and then 40 rpm for the remainder of the culture period. It can be seen from the growth curve that the cells remain in the lag phase for a number of days before entering logarithmic growth phase. In contrast no significant lag phase is observed in the stationary culture. The apparent growth rate for the cells in suspension is 0.014 $hr^{-1}$ and doubling time is 48.9 hours.

**Figure 3.16: Growth of MSV-3T3 Cells in Suspension Culture**

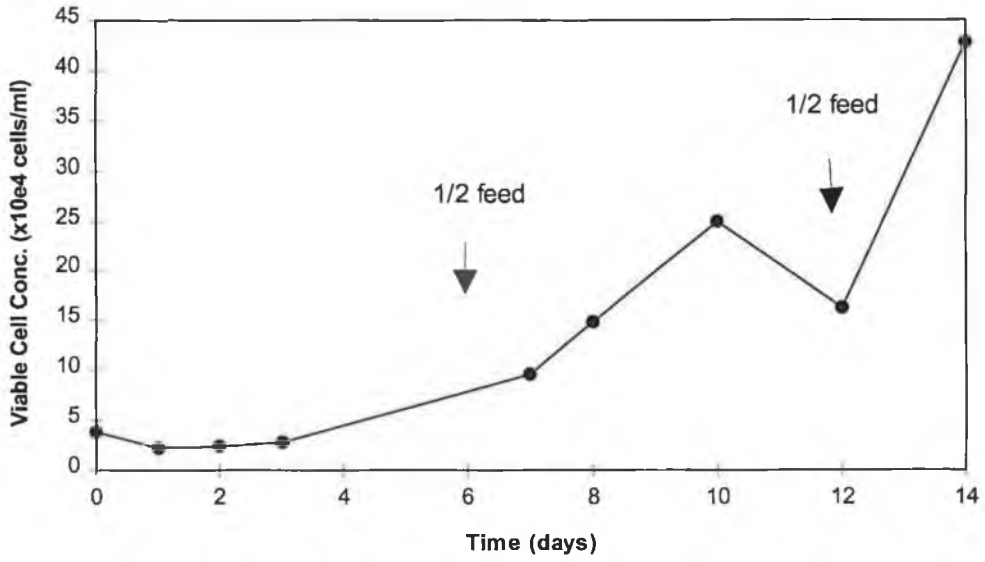



Table 3.21: Comparison of MSV-3T3 Growth in Monolayer and Suspension Culture

| Monolayer Culture | Suspension Culture |
|---|---|
| $\mu_{app} = 0.028\ hr^{-1}$ | $\mu_{app} = 0.014\ hr^{-1}$ |
| $t_d = 24.74$ hrs | $t_d = 48.9$ hrs |

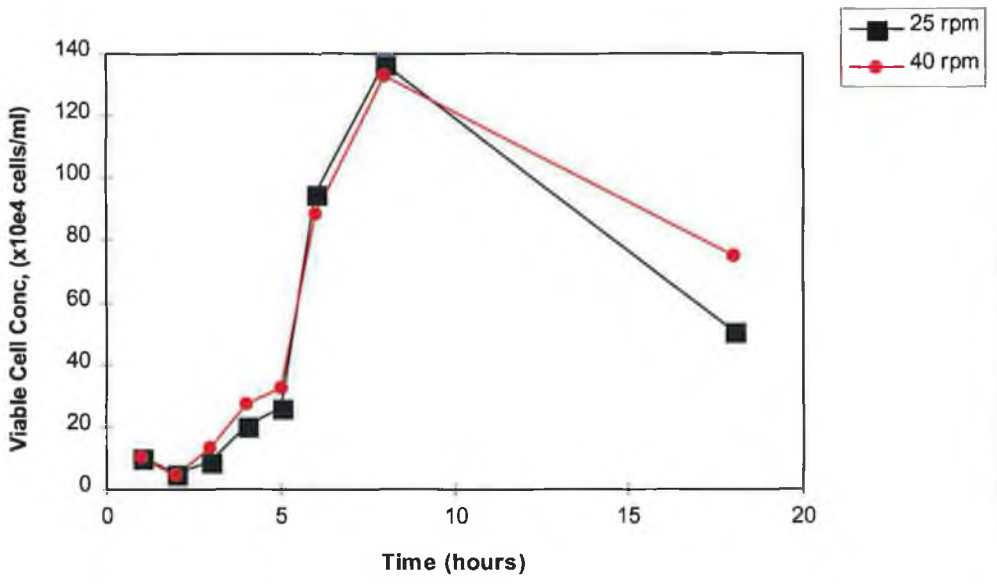
The following conclusions may be made:

- MSV-3T3 cells seem to grow twice as fast when grown in a monolayer system as opposed to a suspension system.
- MSV-3T3 cells adapt well to their environment in a monolayer culture with no appreciable lag phase. However in suspension culture a significant lag phase is observed as cells adjust from an attached mode of growth to a freely suspended mode.
- In the monolayer culture once the monolayer substratum is exhausted cells begin to die and the culture as a whole is observed to go into decline phase. In the suspension culture even after 14 days culture the cells have not become growth limited by their culture environment and continue to grow.

3.2.1.2: The Effect of Agitation Intensity on Growth of MSV-3T3 Cells

MSV-3T3 cells were set up at identical concentrations in two spinner flasks and agitated at 15 rpm for 24 hours. One culture was then spun at 25 rpm and the other at 40 rpm for the rest of the culture period. Growth was monitored for 18 days, the resulting growth curves are presented in Figure 3.17.

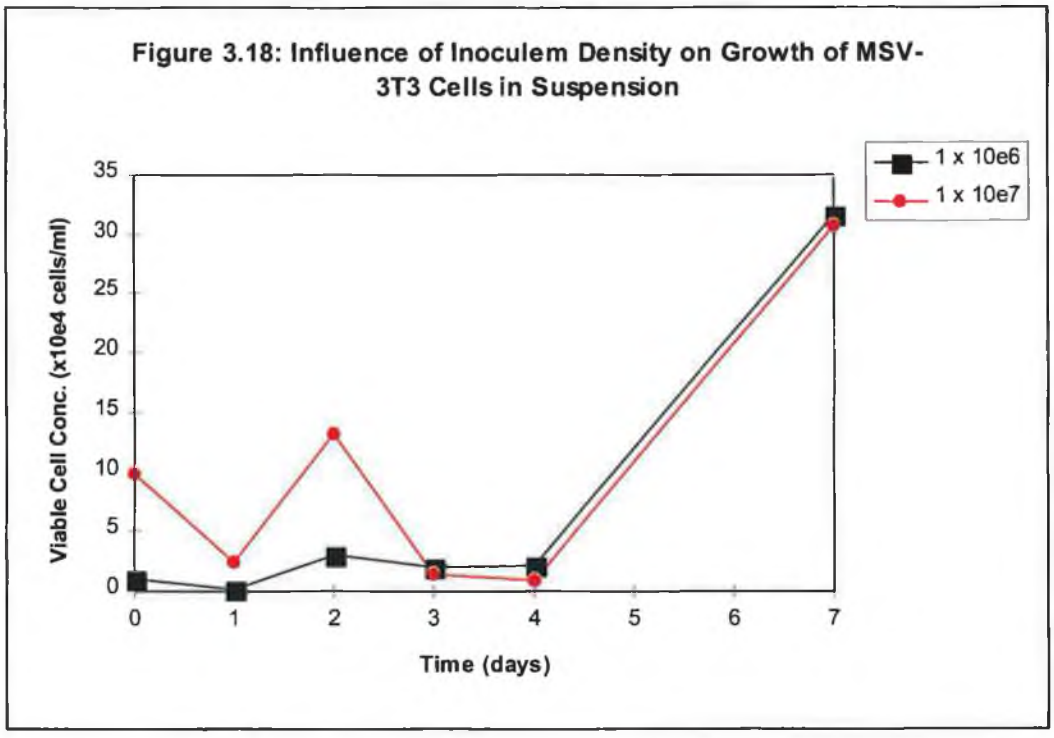
**Figure 3.17: Effect of Agitation on Growth of MSV-3T3 Cells**




A lag phase is again observed in these agitated cultures. Growth in both cultures is very similar over the culture period, with the 40 rpm culture giving a slightly higher final cell density than the 25 rpm culture. Therefore it would seem that moderate agitation speeds are well tolerated by MSV-3T3 cells and it might be necessary to go to much higher speeds to observe inhibition of growth.

3.2.1.3: Investigation of the Effect of Inoculum Density for MSV-3T3 Cells in Suspension Culture.

In order to elucidate the optimum inoculation density for MSV-3T3 cells growing in spinner cultures, cells were inoculated at two different concentrations, 1 x $10^6$and 1 x $10^7$ cells per ml. Growth was then monitored over a 7 day period and the results are illustrated in Figure 3.18.




Again the agitated cultures entered a lag phase during which quite considerable adjustment to the environment is evident. By day 4 it would seem that both cultures were adapted and beginning to enter logarithmic growth phase. Overall the final densities achieved were very similar and it would seem that the experiment was concluded too early as the cells were just entering logarithmic growth period.

In conclusion it would seem that inoculum density does not have a significant effect on cell densities acheived in suspension, indeed it would appear that after a period of adjustment all cultures approach a similar level of growth.

### 3.2.2: Effect Of Environmental Factors On The Growth And Viability Of CHO-K1 And CHRC5 Cells: A Multi-Drug Resistant Cell Line And Its Parental Counterpart.

Multi-drug resistant (MDR) cells are characterised by the fact that they have acquired (either by selection or by transfection) resistance to a broad range of structurally unrelated chemotherapeutic drugs. Previous work in this laboratory suggests that MDR cells may possess greater resistance to shearing forces than their non-MDR counterparts (Clynes, *et al* 1992; Redmond, *et al*, 1990). The shearing forces applied in these studies were sonicating forces and hypotonic treatment used to disrupt the cell membrane for Western Blotting investigations. This observation prompts the question, are MDR cells more physically robust than their non-MDR counterparts and if so why ?

It is not the aim of this thesis to give an unequivocal answer to this fundamental question. However it is within the scope of the work to gather evidence to confirm if there is a difference in the response of MDR cells to various environmental stresses when compared to their non-MDR cells and to propose reasons as to why this may occur. Given that some studies on the effects of the physical environment on cell growth had been previously examined (Section 3.2.1 preceding ) it was decided to investigate the effect of several physical parameters (osmolarity, ammonia and lactate), with the emphasis on shear stresses generated by **agitation**, on the growth of a MDR cell line (CHRC5) and its parental counterpart (CHO-K1) and observe any differences in their response.

The work described in this section investigates the effect of osmotic forces and the build up of toxic metabolites on cell growth.

3.2.2.1: Effect of NaCl (Hyper-osmotic Stress) on the Growth of CHO-K1 and CHRC5 Cells

Animal cell growth medium has a very defined osmolality range of 0.28-0.32 osm/kg (Freshney, 1990). Outside of this range cells will grow suboptimally or not at all. In order to investigate the physical robustness of MDR cells versus their parental cells it was decided to look at the effect on cell growth of various concentrations of NaCl (an ionic species), which induces a hypertonic environment for the cells.

*3.2.2.1.1:Effect of Hyperosmolality on Growth of CHO-K1 Cells*

CHO-K1 cells were plated at a density of 5 x $10^3$ cells per well and various concentrations of NaCl added, results are presented below in Figure 3.19 and Table 3.22.

**Figure 3.19: Effect of Hyperosmotic Stress on CHO-K1 Cells**

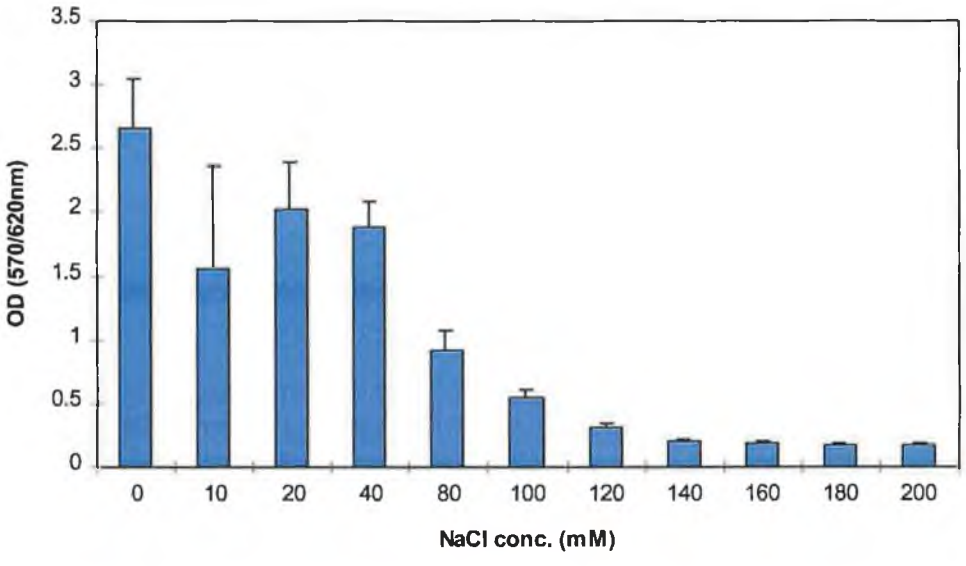


Table 3.22: Effect of Hyperosmolality on Growth of CHO-K1 Cells

| Sample | Osmolality (osm/kg) | $O.D._{570/620nm}$ Assay A | $O.D._{570/620nm}$ Assay B |
|---|---|---|---|
| Hams F12 | 0.300 | 2.66 ± 0.388 | 0.692 ± 0.157 |
| 10 mM NaCl | n.d. | 1.558 ± 0.804 | 0.341 ± 0.184 |
| 20 mM NaCl | 0.351 | 2.018 ± 0.373 | 0.357 ± 0.137 |
| 40 mM NaCl | 0.380 | 1.877 ± 0.213 | 0.419 ± 0.054 |
| 80 mM NaCl | n.d. | 0.917 ± 0.155 | 0.255 ± 0.033 |
| 100 mM NaCl | 0.470 | 0.537 ± 0.074 | 0.198 ± 0.016 |
| 120 mM NaCl | n.d. | 0.309 ± 0.037 | 0.141 ± 0.031 |
| 140 mM NaCl | n.d. | 0.195 ± 0.016 | 0.176 ± 0.01 |
| 160 mM NaCl | 0.573 | 0.182 ± 0.016 | 0.164 ± 0.011 |
| 180 mM NaCl | n.d. | 0.172 ± 0.018 | 0.17 ± 0.013 |
| 200 mM NaCl | 0.635 | 0.176 ± 0.017 | 0.155 ± 0.008 |

Note: Assay A and B refer to repeat assays of the same experiment, i.e. the addition of NaCl to CHO-K1. Figure 3.19 refers to the results from Assay A.

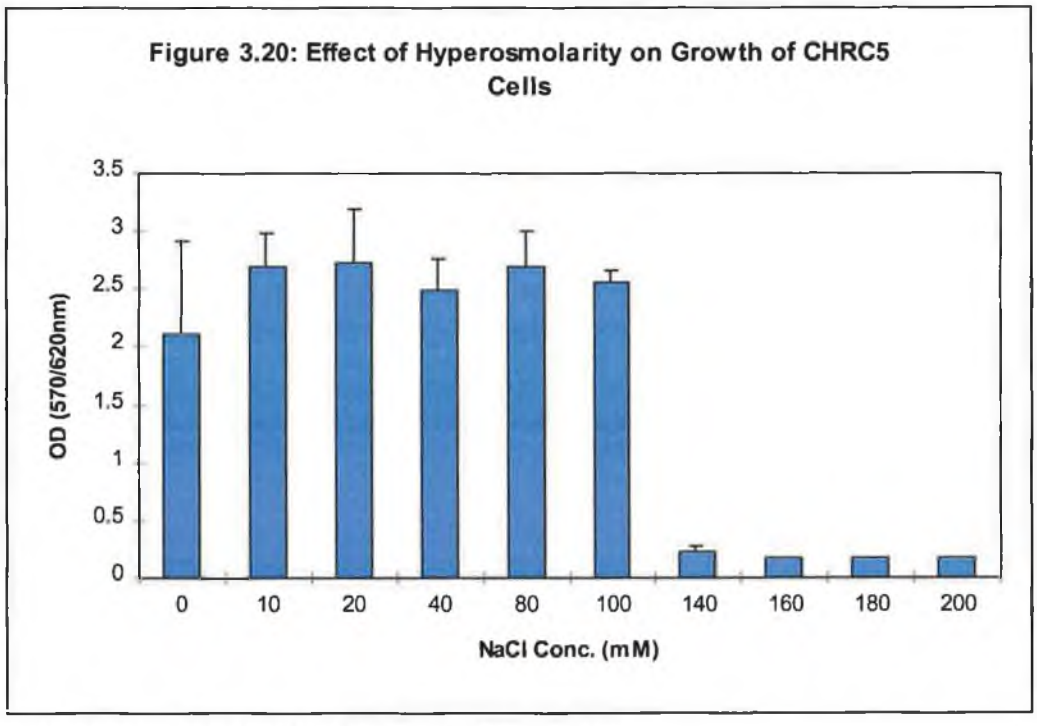
It can be seen that upto a concentration of 40mM NaCl, increased osmotic stress does not have a very significant effect on CHO-K1 cell growth (osmolality is in the range of 0.351-0.38 osm/kg upto 40 mM). However at 80 mM NaCl the osmolality has risen and growth inhibition may be observed. Above approximately 120 mM NaCl, growth is very much reduced. The osmolality value ranges from 0.470 osm/kg for 100 mM NaCl to 0.635 osm/kg for 200 mM NaCl. Thus CHO-K1 cells seem to be able to withstand an increase in osmotic stress upto approximately 0.38 osm/kg. Beyond this level the increase in osmotic pressure begins to cause growth inhibition.

*3.2.2.1.2: Effect of Hyperosmolality on Growth of CHRC5 Cells*

CHRC5 cells were plated at a density of 5 x $10^3$ cells per well and NaCl added 24 hours later. Results are presented below:

Table 3.23: Effect of NaCl on Growth of CHRC5 cells

| Sample | Osmolality (osm/kg) | O.D.$_{570/620nm}$ Assay A | O.D.$_{570/620nm}$ Assay B |
|---|---|---|---|
| MEMα | 0.270 | 2.115 ± 0.802 | 2.129 ± 0.114 |
| 10 mM NaCl | n.d. | 2.697 ± 0.289 | 1.393 ± 0.245 |
| 20 mM NaCl | 0.305 | 2.731 ± 0.457 | 1.396 ± 0.322 |
| 40 mM NaCl | 0.336 | 2.487 ± 0.271 | 1.769 ± 0.192 |
| 80 mM NaCl | 0.396 | 2.698 ± 0.298 | 1.713 ± 0.248 |
| 100 mM NaCl | 0.420 | 2.564 ± 0.094 | 1.483 ± 0.416 |
| 140 mM NaCl | n.d. | 0.23 ± 0.039 | 0.191 ± 0.016 |
| 160 mM NaCl | 0.530 | 0.166 ± 0.004 | 0.173 ± 0.005 |
| 180 mM NaCl | n.d. | 0.171 ± 0.007 | 0.175 ± 0.005 |
| 200 mM NaCl | 0.581 | 0.175 ± 0.005 | 0.171 ± 0.007 |




Note: The above graph refers to the results from Assay A.

CHRC5 cells tolerate an increase in salt concentration upto 100 mM NaCl corresponding to approximately 0.42 osm/kg,. At higher NaCl levels, 140-200 mM NaCl there is a dramatic decrease in cell growth, that is in the osmolality range of 0.5-0.581 osm/kg. This result seems to indicate that CHRC5 cells have increased resistance to higher osmotic pressure.

Conclusions

CHO-K1 and CHRC5 cells have a differing profile in their resistance to NaCl concentration/increased osmotic pressure, with CHRC5 showing an increased resistance. NaCl has little effect on CHO-K1 growth upto a concentration of 40 mM but for CHRC5 cells growth is not affected up to 100 mM.

3.2.2.2.: Effect of Hypo-osmolality Conditions on Growth of CHOK1 and CHRC5 Cells

Animal cells only grow within defined osmotic pressures, therefore this experiment investigated the tolerance of the two lines to low osmotic pressures. Medium was diluted and added to the cells and the resulting cell kill observed. Since low osmotic pressures should cause the cells to swell, perhaps to bursting point, cells were observed microscopically for detrimental effects.

*3.2.2.2.1: Effect of Hypo-osmotic Pressure on CHO-K1 Cells*

CHO-K1 cells were set up at a density of 5 x $10^5$ cells per 25 $cm^2$ flask on day 1. On day 2 Hams F12 medium, diluted to give low osmolarity readings, was added. Cells were observed microscopically after 1 hour. Cell counts were performed after 3 days to determine % survival.

Table 3.24: Effect of Hypo-osmotic pressure on CHO-K1 cells

| Sample | Total Cell Count (x $10^6$/ ml) | % Viability | Osmolality (osm/kg) |
|---|---|---|---|
| Hams F12 + 5% FCS (neat) | 1.836 | 79 | 0.3 |
| Hams F12 + 5% FCS (1/3) | 1.02 | 49 | 0.104 |
| Hams F12 + 5% FCS (1/5) | 0.612 | 33 | 0.068 |

Note: Within 10 minutes of adding the diluted medium, CHO-K1 cells began to swell noticeably.

*3.2.2.2.2: Effect of Hypo-osmotic Pressure on CHRC5*

CHRC5 cells were set up at a density of 8 x $10^5$ cells per 25 $cm^2$ flask on day 1. On day 2, MEMα medium was diluted to give low osmolality values and added to the cells. Cells were observed microscopically and cell counts performed after 3 days to assess % survival.

Table3.25: Effect of Hypo-osmolality on growth of CHRC5 cells.

| Sample | Total Cell Count (x $10^6$/ml) | % Viability | Osmolality (osm/kg) |
|---|---|---|---|
| MEMα + 5% FCS (neat) | 3.46 | 97 | 0.3 |
| MEMα + 5% FCS (1/3) | 0.279 | 96 | 0.105 |
| MEMα + 5% FCS (1/5) | 0.06 | 0 | 0.059 |

Again cells swelled up very quickly after the diluted medium addition. After 3 days, viability in the 1/5 diluted medium was zero. However cells appeared to

survive in the 1/3 diluted medium which corresponds to an osmolality value of 0.105 osm/kg.

This experiment would need to be repeated in order to obtain any definite conclusions. Viability counts should have been performed almost immediatley after the diluted medium addition.

3.2.2.3: Effect of Ammonia on CHO-K1 and CHRC5 Cells.

Ammonia is released by the chemical decomposition of glutamine and metabolic deamination of glutamine to α-ketoglutarate. Ammonia tolerance may vary from cell line to cell line and with experimental conditions, for example, cell concentration, glutamine concentration, mode of reactor operation and cellular activities (Ozturk and Palsson, 1992). The aim of these experiments was to establish the effect of ammonia over a range of concentrations on the growth and viability of the MDR cell line, CHRC5 and its non-MDR parent, CHO-K1. Ammonia is reported to be toxic at concentrations of approximately 4 mM in batch culture (Schumpp and Schlaeger, 1992).

The osmolality of the $NH_4Cl$ solutions were tested and results are presented in Table 3.26 for $NH_4Cl$ solutions made up in Hams F12 medium (CHO-K1 cells). Since Hams F12 and MEMα have quite similar osmolality values (see Table 3.30) it is assumed that the values for MEMα/$NH_4Cl$ solutions are very similar to those for Hams F12/$NH_4Cl$ solutions. It can be seen that only the highest concentration, 20mM $NH_4Cl$ is outside the optimum range (0.28-0.32osm/kg). pH is increased slightly but should not be inhibitory in this range.

Table 3.26: Osmolality and pH of $NH_4Cl$ solutions made up in Hams F12 medium

| Sample | Osmolarity (osm/kg) | pH |
|---|---|---|
| 20 mM $NH_4Cl$ | 0.341 | 7.55 |
| 10 mM $NH_4Cl$ | 0.322 | 7.65 |
| 4 mM $NH_4Cl$ | 0.309 | 7.74 |
| 2 mM $NH_4Cl$ | 0.308 | 7.64 |
| 1 mM $NH_4Cl$ | 0.303 | 7.67 |
| 0.4 mM $NH_4Cl$ | 0.247 | 7.7 |
| Hams F12 | 0.319 | 7.43 |

*3.2.2.3.1: Effect of Ammonium Chloride on Growth of CHO-K1 Cells*

Based on the assumption that in a 7 day batch culture approximately 4mM of ammonia will accumulate, CHO-K1 tolerance to $NH_4Cl$ up to five times this concentration is tested in this experiment.

Table 3.27: Effect of Ammonium Chloride on Growth of CHO-K1 cells

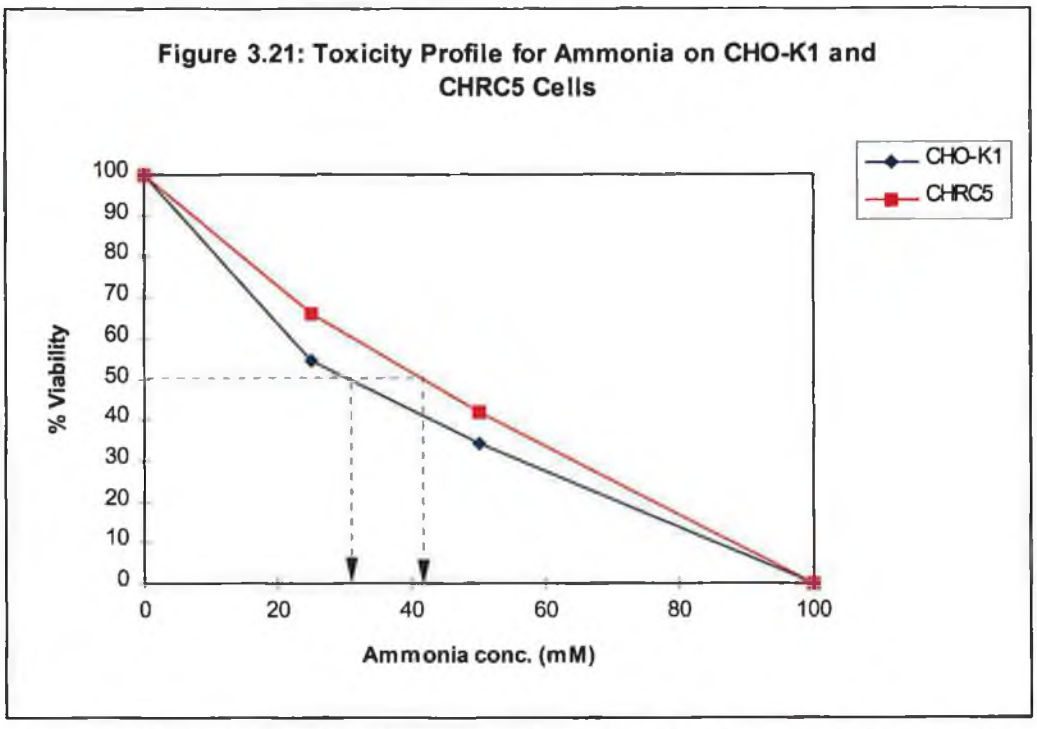
| Sample | $O.D._{570/620nm}$ | Std.Dev. | % C.V. |
|---|---|---|---|
| Control (Hams F12) | 1.138 | 0.105 | 9.24 |
| 0.4 mM $NH_4Cl$ | 1.455 | 0.045 | 3.08 |
| 0.5 mM $NH_4Cl$ | 1.466 | 0.034 | 2.34 |
| 1.0 mM $NH_4Cl$ | 1.49 | 0.065 | 4.34 |
| 1.5 mM $NH_4Cl$ | 1.441 | 0.041 | 2.86 |
| 2.0 mM $NH_4Cl$ | 1.505 | 0.036 | 2.41 |
| 5.0 mM $NH_4Cl$ | 1.77 | 0.059 | 3.33 |
| 10 mM $NH_4Cl$ | 2.237 | 0.077 | 3.31 |
| 15 mM $NH_4Cl$ | 2.177 | 0.47 | 2.15 |
| 20 mM $NH_4Cl$ | 2.31 | 0.84 | 3.64 |

As can be seen from the results, $NH_4Cl$ does not seem to have a very substantial effect on CHO-K1 cells. However ammonia is a volatile substance especially in the conditions used in this assay, i.e..warm temperature (37°C) and high humidity in the $CO_2$ incubator, perhaps the $NH_4Cl$ in the 96 well plates is dissociating into ammonia gas and spreading over the whole plate. Therefore it was decided to repeat the experiment in sealed 25 $cm^2$ flasks. Results are presented in Table 3.28.

Table 3.28: Effect of Ammonium Chloride on Growth of CHO-K1 cells in 25 $cm^2$ flasks

| Sample | Total Cell Count (x $10^4$/ml) | % Viability |
|---|---|---|
| Control (Hams F12) | 24.2 | 98.4 |
| 0.5 mM $NH_4Cl$ | 19.2 | 95.5 |
| 1 mM $NH_4Cl$ | 17.4 | 97.2 |
| 2 mM $NH_4Cl$ | 24.0 | 98.4 |
| 5 mM $NH_4Cl$ | 20.5 | 97.6 |
| 10 mM $NH_4Cl$ | 20.8 | 96.3 |
| 20mM $NH_4Cl$ | 21.3 | 95.9 |

Again it can be seen that $NH_4Cl$ is having very little effect on either the growth or viability of CHO-K1 cells at the concentrations used in this experiment. It must be assumed that these cells have an unusually high tolerance to ammonia, consequently an even higher range of $NH_4Cl$ concentrations were added to CHO-K1 cells growing in 25$cm^2$ flasks. Results are presented in Figure 3.21.




It can be seen that only at much higher concentrations than would be expected, is ammonia affecting the growth of CHO-K1 cells. .Based on Figure 3.21, the $IC_{50}$ ($NH_4Cl$ concentration at which 50% of cells are killed) of CHO-K1 cells to $NH_4Cl$ is approximately 30mM.

*3.2.2.3.2: Effect of ammonium chloride on growth of CHRC5 cells*

The effect of addition of $NH_4Cl$ to CHRC5 cells in 96 well plates was investigated.

Table 3.29: Effect of ammonium chloride on growth of CHRC5 cells in 96 well plates

| Sample | O.D.$_{570/620nm}$ | Std.Dev. | % C.V. |
|---|---|---|---|
| Control (MEMα) | 0.097 | 0.002 | 1.97 |
| 0.2 mM $NH_4Cl$ | 0.081 | 0.02 | 25.16 |
| 0.5 mM $NH_4Cl$ | 0.125 | 0.009 | 6.99 |
| 1 mM $NH_4Cl$ | 0.115 | 0.005 | 4.58 |
| 1.5 mM $NH_4Cl$ | 0.093 | 0.009 | 9.67 |
| 2 mM $NH_4Cl$ | 0.008 | 0.005 | 5.68 |
| 5 mM $NH_4Cl$ | 0.094 | 0.01 | 10.81 |
| 20 mM $NH_4Cl$ | 0.112 | 0.19 | 16.92 |

In a result similar to that of CHO-K1 cells, $NH_4Cl$ in the range of 0-20mM does not affect CHRC5 cell growth. Tolerance to higher levels of $NH_4Cl$ (0-100mM) was examined in $25cm^2$ flasks and results are given in Figure 3.21 (with CHO-K1 cells). From this toxicity curve the $IC_{50}$ of CHRC5 cells to $NH_4Cl$ is approximately 42 mM.

Table 3.30 gives osmolality and pH values for the higher $NH_4Cl$ solutions and upto 50 mM $NH_4Cl$. There is no significant increase in either osmolarity or pH. Consequently the inhibitory effect of $NH_4Cl$ is due to the toxic effect of ammonia on the cell alone and cannot be attributed to either of these physical factors.

Table 3.30: Osmolality and pH values for $NH_4Cl$ Solutions

| Conc. $NH_4Cl$ (mM) | Osmolality (osm/kg) | pH |
|---|---|---|
| **Hams F12 medium (CHO-K1 cells)** | | |
| 0 | 0.290 | 7.51 |
| 25 | 0.335 | 7.48 |
| 50 | 0.371 | 7.44 |
| 100 | 0.448 | 7.41 |
| **MEMα medium (CHRC5 cells)** | | |
| 0 | 0.270 | 7.54 |
| 25 | 0.297 | 7.48 |
| 50 | 0.323 | 7.46 |
| 100 | 0.389 | 7.36 |

Conclusions

Overall there appears to be a slight difference in the tolerance of the MDR cell line (CHRC5) to $NH_4Cl$ when compared to CHO-K1. The $IC_{50}$ value for CHRC5 is a little higher. However it is difficult to state whether this a significant difference. An interesting observation is the very high tolerance displayed by both cell lines to ammonia. Most cell lines are inhibited by ammonia concentrations of approximately 4 mM (Schumpp and Schlaeger, 1992), thus the $IC_{50}$ values seen here are unusually high.

3.2.2.4: Effect of Addition of Sodium Lactate on the Growth of CHO-K1 and CHRC5 Cells.

Lactate is a metabolic waste product which may accumulate due to anaerobic degradation of pyruvate metabolised via the glycolytic pathway from glucose. Lactate tolerance may vary from cell line to cell line however concentrations of greater than 60 mM are reported to be toxic to most cell lines (Schumpp and Schaegler, 1992). Lactate may inhibit cell growth by increasing the osmolality of cell culture medium or by decreasing the medium pH, therefore the osmolality and pH of medium in which the sodium lactate was dissolved was measured.

*3.2.2.4.1: Effect of Sodium Lactate on CHO-K1 cells.*

CHO-K1 cells were plated at a density of $8 \times 10^3$ cells per well in a 96 well plate assay. Various concentrations of sodium lactate were added 24 hours later. It has been widely reported that the inhibitory effect of lactate may be in fact due to increased osmolality value of the medium. The table below gives the osmolality and pH values of the Hams F12 medium after sodium lactate addition.

Table 3.31: Osmolality and pH of Sodium Lactate solutions made up in Hams F12 Medium

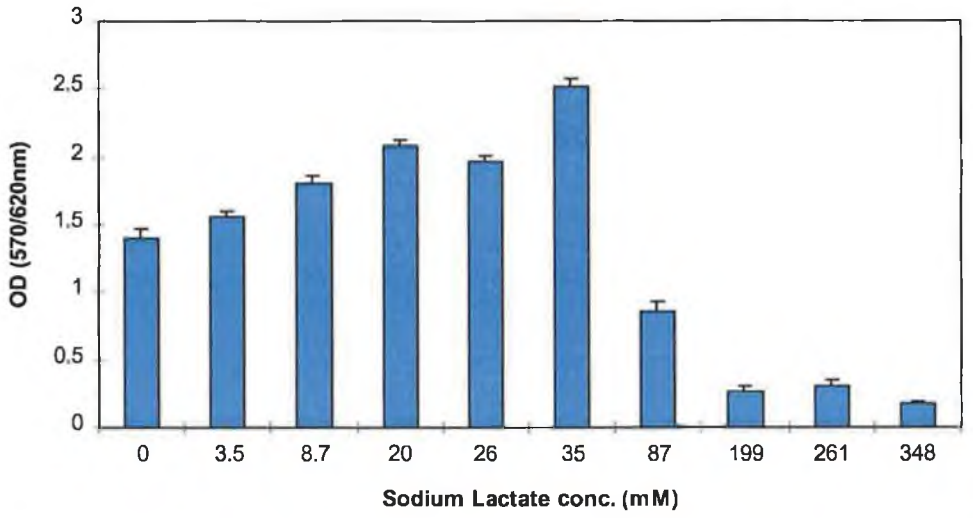
| Sample | Osmolality (osm/kg) | pH |
|---|---|---|
| Hams F12 medium | 0.319 | 7.43 |
| 398 mM Na lactate | 0.991 | 7.42 |
| 174 mM Na lactate | 0.588 | 7.59 |
| 70 mM Na lactate | 0.422 | 7.64 |
| 52 mM Na lactate | 0.355 | 7.63 |
| 40 mM Na lactate | 0.362 | 7.66 |
| 17 mM Na lactate | 0.332 | 7.66 |
| 7 mM Na lactate | 0.318 | 7.66 |

Results are given in Table 3.32 and Figure 3.22.

Table 3.32: Sodium Lactate Effects on CHO-K1 cells

| Sample | $O.D._{570/620nm}$ | Std. Dev. |
|---|---|---|
| Hams F12 | 1.393 | 0.083 |
| 348 mM Na lactate | 0.177 | 0.019 |
| 261 mM Na lactate | 0.310 | 0.037 |
| 199 mM Na lactate | 0.258 | 0.042 |
| 87 mM Na lactate | 0.854 | 0.078 |
| 35 mM Na lactate | 2.514 | 0.067 |
| 26 mM Na lactate | 1.959 | 0.052 |
| 20 mM Na lactate | 2.088 | 0.037 |
| 8.7 mM Na lactate | 1.813 | 0.054 |
| 3.5 mM Na lactate | 1.559 | 0.041 |

**Figure 3.22: Effect of Sodium Lactate on Growth of CHO-K1 Cells**



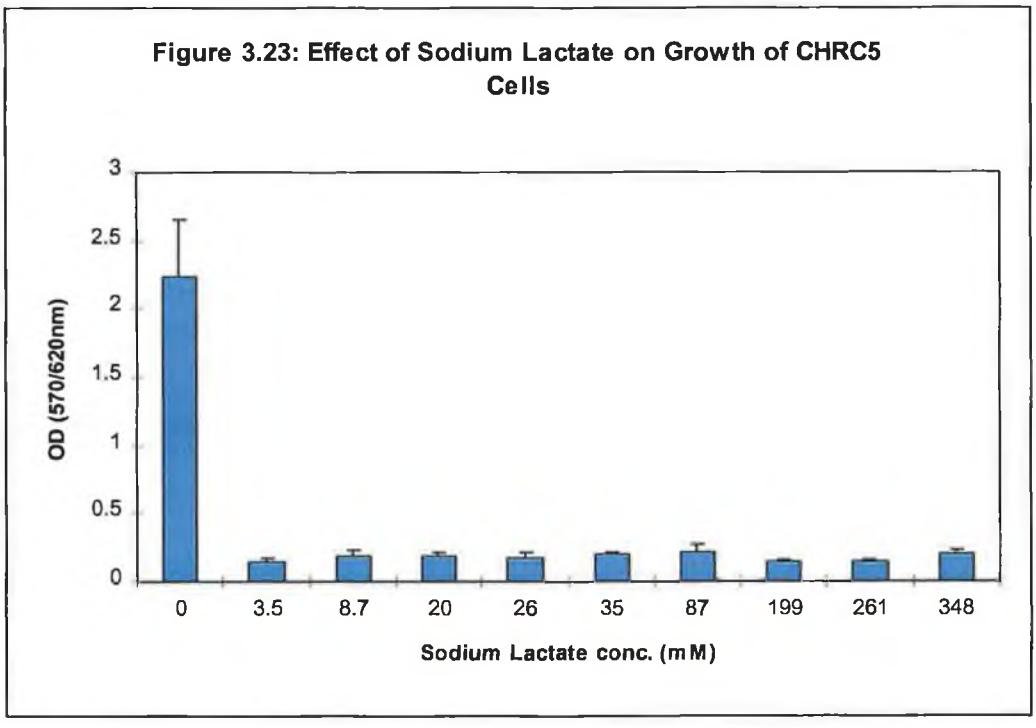

It can be seen that the CHO-K1 cells grow well at concentrations upto 35 mM sodium lactate. However at 87 mM growth has decreased (approximately 61% of control growth) and at 199 mM and above cell growth is very much inhibited. It has been reported (Ozturk, Riley and Palsson, 1992) that lactate may cause inhibition by no more complex a mechanism than that of increasing medium osmolality. In this experiment osmolality was measured and would seem to agree with this hypothesis. CHO-K1 cells grew well upto 35 mM sodium lactate and medium osmolality at 40 mM sodium lactate is 0.362 osm/kg. The optimum osmolality range for medium is 0.28-0.32 osm/kg (Freshney, 1990), so up to35 mM sodium lactate medium osmolality was within or just outside this optimum range (Table 3.31). However at 87 mM it would seem that medium osmolality has risen. For example, 70 mM has a osmolality of 0.422 osm/kg. At higher concentrations, e.g. 174 mM, osmolality was at 0.588 osm/kg. Therefore growth inhibition at higher concentrations of sodium lactate would seem to correspond to an increase in medium osmolality.

*3.2.2.4.2: Effect of Sodium Lactate on CHRC5*

CHRC5 cells were plated at a density of 5 x $10^3$ cells per well in a 96 well plate. sodium lactate concentrations were added 24 hours later. Results are presented below:

Table 3.33: Effect of Sodium lactate on CHRC5

| Sample | $O.D._{570/620nm}$ | Std. Dev. |
|---|---|---|
| MEMα | 2.241 | 0.411 |
| 348 mM Na lactate | 0.198 | 0.03 |
| 261 mM Na lactate | 0.144 | 0.02 |
| 199 mM Na lactate | 0.142 | 0.011 |
| 87 mM Na lactate | 0.210 | 0.058 |
| 35 mM Na lactate | 0.202 | 0.017 |
| 26 mM Na lactate | 0.179 | 0.033 |
| 20 mM Na lactate | 0.183 | 0.028 |
| 8.7 mM Na lactate | 0.192 | 0.045 |
| 3.5 mM Na lactate | 0.145 | 0.026 |



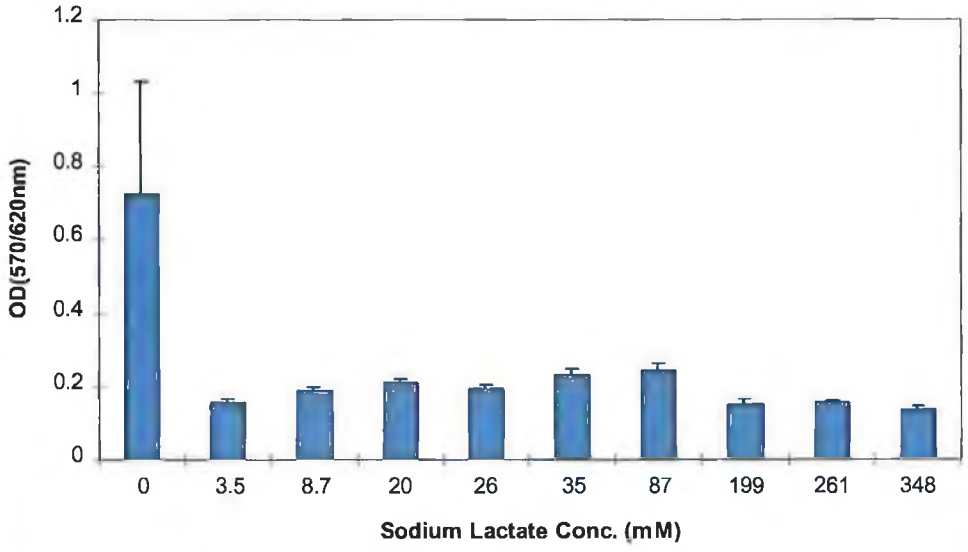
A dramatic decrease in cell growth is seen upon addition of sodium lactate to CHRC5 cells. It has been shown that MEMα and Hams F12 medium have similar osmolality values (Table 3.30), therefore the values of medium osmolality for sodium lactate in MEMα may be considered to be very similar to the medium osmolality of sodium lactate in Hams F12. Consequently upto a sodium lactate concentration of 20 mM, the medium osmolality should still be just within the optimum osmolality range, thus growth of CHRC5 should not be adversely affected if only increased medium osmolality is responsible for causing sodium lactate inhibition. At higher sodium lactate concentrations (87-348 mM), medium osmolality is increased beyond the optimum range therefore the observed growth inhibition at this stage may be caused by the high osmolality.

This experiment was repeated for CHRC5 cells and the results are presented in Table 3.34 and Figure 3.24

Table 3.34: Effect of Sodium Lactate on CHRC5 Cells

| Sample | $O.D._{570/620nm}$ | Std. Dev. |
|---|---|---|
| MEMα | 0.726 | 0.307 |
| 348 mM Na lactate | 0.136 | 0.009 |
| 261 mM Na lactate | 0.156 | 0.005 |
| 199 mM Na lactate | 0.151 | 0.017 |
| 87 mM Na lactate | 0.244 | 0.018 |
| 35 mM Na lactate | 0.230 | 0.019 |
| 26 mM Na lactate | 0.192 | 0.014 |
| 20 mM Na lactate | 0.209 | 0.012 |
| 8.7 mM Na lactate | 0.189 | 0.011 |
| 3.5 mM Na lactate | 0.158 | 0.008 |

**Figure 3.24: Effect of Lactate on Growth of CHRC5 Cells**




Again the addition of sodium lactate at all concentrations is responsible for a reduced level of growth in CHRC5 cells.

Conclusions

Overall these results suggest the following;

- Lactate inhibits CHO-K1 cells but only at concentrations where the lactate causes the medium osmolality to increase beyond the optimum range for cell growth.
- Lactate dramatically inhibits the growth of CHRC5 cells and this inhibition may not be linked to an increase in medium osmolality. Instead lactate may cause inhibition of CHRC5 cells by another mechanism.
- The parental, drug sensitive cells, CHO-K1 are more resistant to lactate than the MDR cells, CHRC5.

3.2.2.5: Toxicity Profile of CHO-K1 and CHRC5 Cells.

In order to ascertain that the cells being used in the preceding experiments were in actual fact drug sensitive (CHO-K1) or resistant (CHRC5), the level of both cell lines resistance to the chemotherapeutic drug, adriamycin was investigated. Previous work in this laboratory, (Redmond, 1991), had established that the $IC_{50}$ (drug concentration at which there is 50% cell kill), for these two cell lines in adriamycin is as follows:

$IC_{50}$ : CHO-K1 : 47.6 ± 5.1 nM adriamycin.
$IC_{50}$ : CHRC5 : 7252 ± 56.2 nM adriamycin.

*3.2.2.5.1: Addition of adriamycin to CHO-K1 and CHRC5 cells in flasks.*

Since the $IC_{50}$ of CHRC5 cells is 7252 nM (7.25μM) it was decided to add this concentration of adriamycin to each cell line and observe the subsequent cell survival. Cells were set up on day 1 in 25 $cm^2$ flasks and adriamycin added on day 2 at a concentration of 4 μg/ml (approx. 7.25μM). It was expected that CHO-K1 cells would be killed by this adriamycin concentration, with CHRC5 cells showing an approximate 50% survival rate.
Cells were observed microscopically for 4 days and it was found that CHO-K1 cells were indeed killed over this period, while CHRC5 cells showed some decrease in cell number but overall survived this adriamycin level.

This experiment was repeated and the results are presented in Table 3.35.

Table 3.35:Toxicity Profile of Adriamycin to CHO-K1 and CHRC5 Cells

| Adriamycin Conc. (μg/ml) | CHO-K1 Cell Number ($x10^4$/ml) | CHRC5 Cell Number ($x10^4$/ml) | % Viability - CHRC5 Cells |
|---|---|---|---|
| 0 | 27.9 | 14.5 | 100 |
| 2.5 | 0 | 9.45 | 65.17 |
| 3.75 | 0 | 4.41 | 30.14 |

The $IC_{50}$ for CHRC5 cells to adriamycin is approximately 3.9 μg/ml (Redmond, A: 1991). The toxicity profile obtained from these results seems to indicate that value may be slightly higher, perhaps between 8-9 μg/ml. However the important point to be concluded from these results is that CHRC5 cells obviously display MDR characteristics, showing a high level of resistance to adriamycin whereas CHO-K1 are drug sensitive.

3.2.2.6:Conclusions on the Effect of Environmental Factors on the Growth of CHO-K1 and CHRC5 Cells

Overall it may be stated that there are differences in the response of an MDR line, CHRC5 and its parental non-MDR cell line, CHO-K1, to external physical/chemical effects. There appears to be a difference in the response to:

- Osmotic pressure, CHRC5 being more resistant to hyperosmotic pressure.
- Ammonium levels, CHRC5 possessing a slightly higher $IC_{50}$ than CHO-K1.
- Lactate levels, CHRC5 showing a dramatic sensitivity to lactate when compared to CHO-K1.

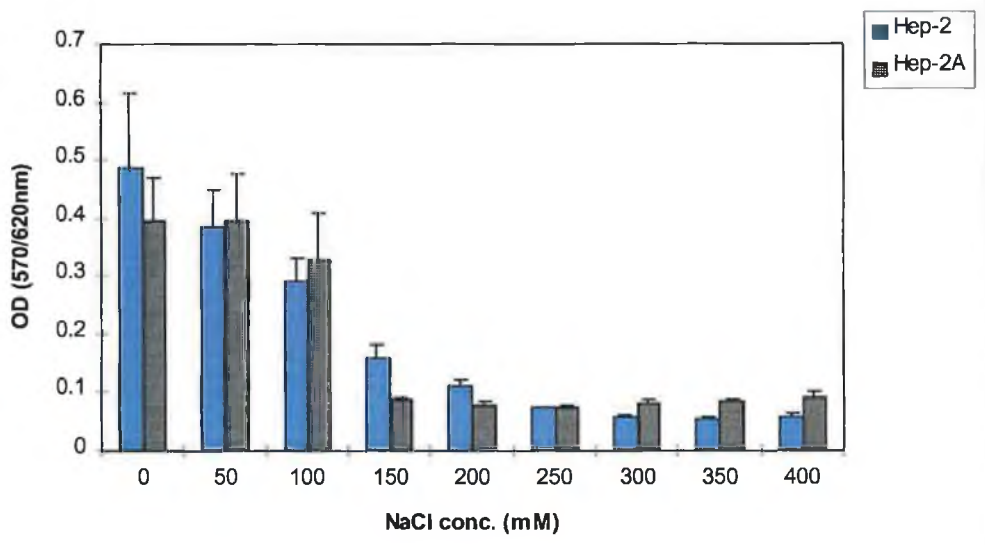
### 3.2.3: Effect Of Environmental Factors On The Growth And Viability Of Hep-2 And Hep-2a Cells

The preceding section outlines differences in the response to several physical and chemical factors of an MDR cell line (CHRC5) and its drug sensitive parent (CHO-K1). In order to ascertain whether the observed differences are unique to that cell system or whether they might apply to another MDR cell line and its parent, this section examines the effect of osmotic pressure, ammonia and lactate on Hep-2 cells (human larynx carcinoma) and its MDR counterpart, Hep-2A. Hep-2A was selected for its MDR properties by exposure to increasing concentrations of adriamycin, (Redmond *et al*, 1990).

3.2.3.1: Effect of Hyperosmotic Stress on Hep-2 and Hep-2A Cells

NaCl solutions were added to Hep-2 and Hep-2A cells at various concentrations in 96 well plates. Results were analysed by the acid phosphatase method (Methods, Section 2.10.1.3)and are presented in Figure 3.25.

**Figure 3.25: Effect of Hyperosmotic Stress on Growth of Hep-2 and Hep-2A Cells**




Both Hep-2 and Hep-2A grow well upto a NaCl concentration of 100mM, corresponding to an osmolality of 0.480 osm/kg (see Table 3.36). Growth at 150 mM (0.590 osm/kg) is reduced and above 200 mM very little cell growth occurs. Both cell lines display similar tolerance to hyperosmolality.

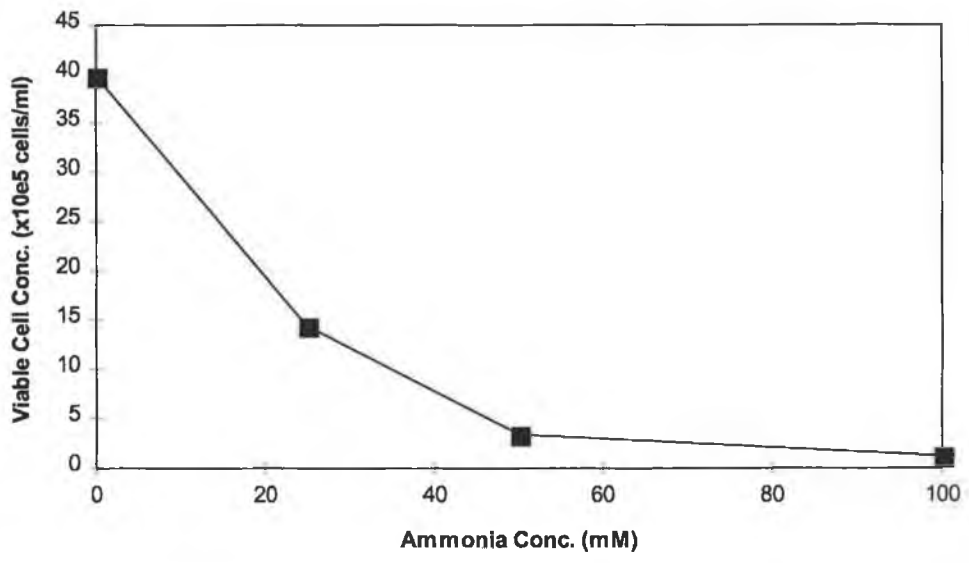
Table 3.36: Osmolality of NaCl Solutions Added to Hep-2 and Hep-2A Cells

| NaCl Conc. (mM) | Osmolality (osmol/kg) |
|---|---|
| 0 | 0.342 |
| 50 | 0.390 |
| 100 | 0.480 |
| 150 | 0.590 |
| 200 | 0.640 |

3.2.3.2: Ammonia Tolerance of Hep-2 and Hep-2A Cells

$NH_4Cl$ was added to cells in sealed 25 $cm^2$ flasks in the range of 0-100 mM. This range was chosen in light of the results from CHO-K1 and CHRC5 experiments where no affect on cell growth was observed upto 20mM $NH_4Cl$, even though 4mM $NH_4Cl$ is reported to be toxic (Schumpp and Schaegler, 1992). Results are presented in Figure 3.26 and Figure 3.27 for Hep-2 and Hep-2A respectively.

**Figure 3.26: Ammonia Toxicity Profile on Hep-2 Cells**



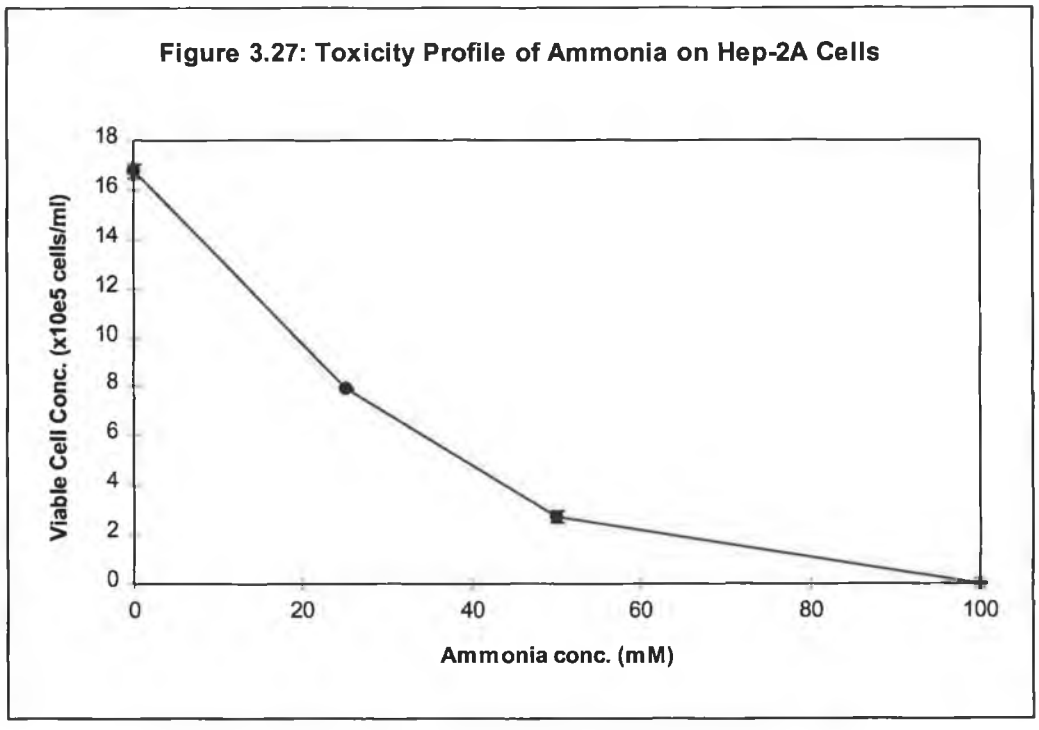



Table 3.37: % Viability for Hep-2 and Hep-2A Cells Exposed to Ammonia

| Hep-2 Cells | | Hep-2A Cells | |
|---|---|---|---|
| Ammonia Conc. (mM) | % Viability | Ammonia Conc. (mM) | % Viability |
| 0 | 99 | 0 | 99 |
| 25 | 93 | 25 | 97 |
| 50 | 98 | 50 | 96 |
| 100 | 87 | 100 | 50 |

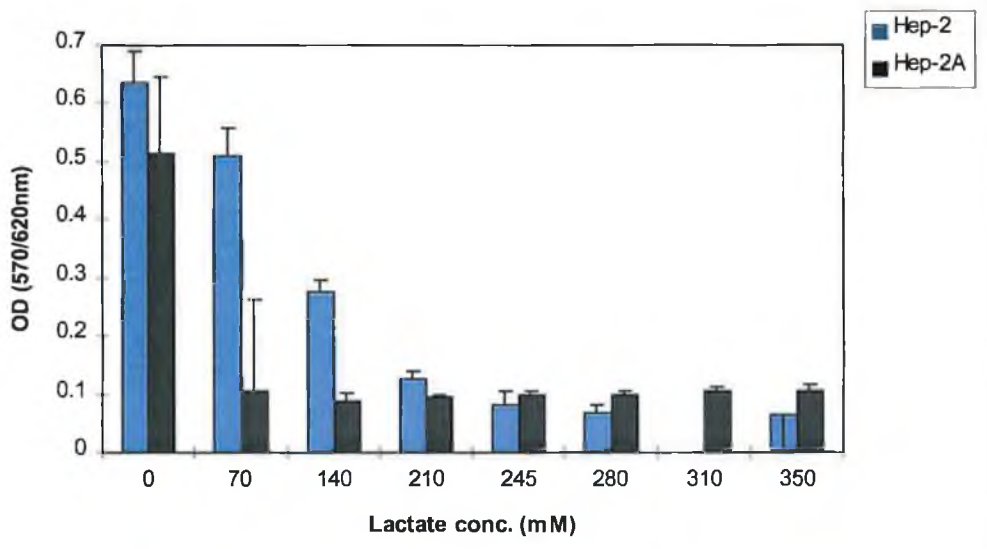
It can be seen that both cell lines are $NH_4Cl$ sensitive displaying almost first order death kinetics (when plotted on a semi-logarithmic scale, not shown). A large decrease in viable cell number is noted between 0 and 50 mM $NH_4Cl$ with a greater drop in number seen in Hep-2A cells. A less dramatic decrease is noted between 50 and 100mM $NH_4Cl$. However high cell viabilities are observed at the higher $NH_4Cl$ concentrations even when cell number is low. Greater than 88% viability remains in Hep-2 cells upto 100mM $NH_4Cl$ with slightly greater sensitivity in Hep-2A cells which are >90% viable upto 50mM

$NH_4Cl$ but then at 100mM viability drops to 50%. These results suggest that a subpopulation of both cell lines can adapt and survive in high ammonia levels.

3.2.3.3: Effect of Sodium Lactate on Hep-2 and Hep-2A Growth

Sodium lactate was added at various concentrations to cells in 96 well plates and results were analysed by the acid phosphatase method and plates read on the ELISA reader. Results are presented in Figure 3.28.

**Figure 3.28: Effect of Lactate on Growth of Hep-2 and Hep-2A Cells**




It can be observed that Hep-2 cells display a much greater tolerance of sodium lactate upto 140 mM than the MDR cell line, Hep-2A. At 140mM lactate 43% of Hep-2 cells are viable whereas only 17% of Hep-2A cells remain viable. As can be seen in Table 3.38 the osmolality of lactate above 35 mM is outside the range for optimum cell growth, however the Hep-2 cells grow well upto 140 mM when the osmolality is 0.520 osm/kg. Results in Section 3.2.3.1 show that both cell lines can tolerate and grow well at osmolalities of upto 0.480 osm/kg.

A similar result is obtained with CHO-K1 and CHRC5 cells (Section 3.2.2.4), i.e. the MDR cell line (CHRC5) displayed a greater sensitivity to lactate than the non-MDR cell line. In this experiment,Hep-2A appear to be more sensitive to lactate than Hep-2.

Table 3.38: Osmolality of Lactate Solutions Added to Hep-2 and Hep-2A Cells

| Lactate Conc. (mM) | Osmolarity (osm/kg) |
|---|---|
| 0 | 0.314 |
| 35 | 0.360 |
| 70 | 0.425 |
| 140 | 0.520 |
| 175 | 0.590 |
| 350 | 0.980 |

### 3.2.4: Investigations Into The Effect Of The Hydrodynamic Environment On The Growth Of CHRC5 And CHO-K1 Cells; A Multi-Drug Resistant Cell Line And Its Parental Counterpart

The effects of hydrodynamic forces on the growth and death of mammalian cells in suspension culture have been extensively studied (see Section 1.3 for review). Agitation generates shear stresses in bioreactors which can be detrimental to the growth, viability and metabolic functioning of cells. Recent work suggests that MDR cells appear to possess a greater resistance than non-MDR cells to some shearing forces (sonication and hypotonic stress). The work in this section presents investigations into the effect of agitation on and consequently the shear tolerance of, CHRC5 (MDR) cells and CHO-K1 (non-MDR) cells.

#### 3.2.4.1:Effect of Agitation on CHO-K1 Cell Growth

CHO-K1 cells were inoculated at a concentration of 1 x $10^5$ cells/ml into 100 mls of Hams F12 medium in spinner flasks. Six spinner flasks were set up at the following agitation rates;- 0, 15, 30, 40, 90 and 120 rpm. Spinners were counted in duplicate every day.

Tables 3.39and 3.40 and Figures 3.29 and 3.30 outline the results obtained.

**Figure 3.29: Effect of Agitation Rates (0, 15 and 30 rpm) on the Growth of CHO-K1 Cells**

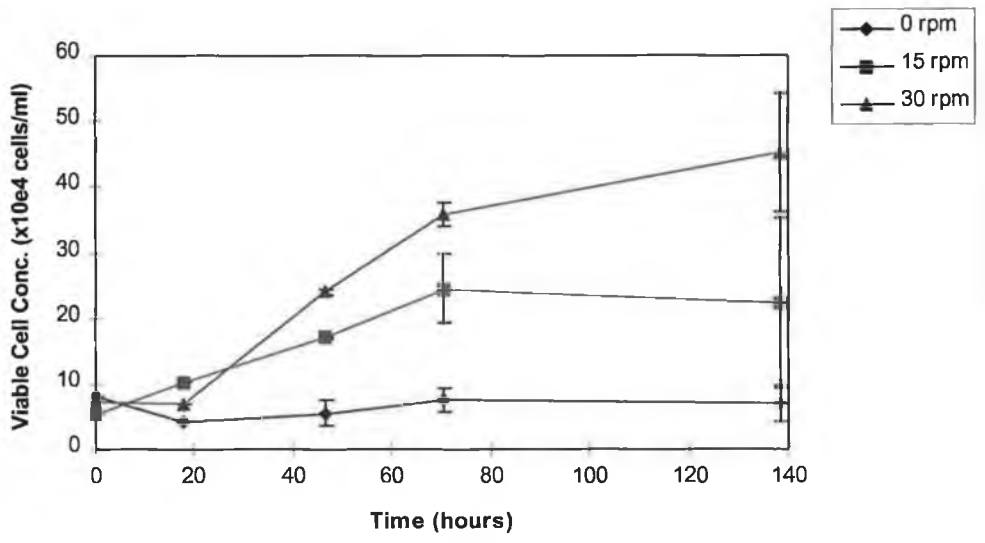



Table 3.39: Growth of CHO-K1 Cells at 0, 15, and 30 rpm.

| Agitation Rate (rpm) | Time (Hours) | Total Cell Count ($x10^4$/ml) | Std. Dev. ($x10^4$/ml) | % Viability |
|---|---|---|---|---|
| 0 | 0 | 8.4 | n.d. | 97 |
| 0 | 18 | 5.4 | n.d. | 80 |
| 0 | 46.5 | 5.8 | 1.9 | 95 |
| 0 | 70.5 | 7.9 | 1.9 | 95 |
| 0 | 138.5 | 7.5 | 2.5 | 91 |
| | | | | |
| 15 | 0 | 5.7 | n.d. | 95 |
| 15 | 18 | 11.8 | n.d. | 86 |
| 15 | 46.5 | 17.2] | 0.2] | 99 |
| 15 | 70.5 | 24.4] | 5.3 | 100 |
| 15 | 138.5 | 23.6 | 12.9 | 95 |
| | | | | |
| 30 | 0 | 7.2 | n.d. | 100 |
| 30 | 18 | 7.5 | n.d. | 92 |
| 30 | 46.5 | 24.3 | 0.4 | 99 |
| 30 | 70.5 | 36.6 | 1.7 | 98 |
| 30 | 138.5 | 47.5 | 9.1 | 91 |

**Figure 3.30: Effect of Agitation Rates (40, 90 and 120 rpm) on the Growth of CHO-K1 Cells**

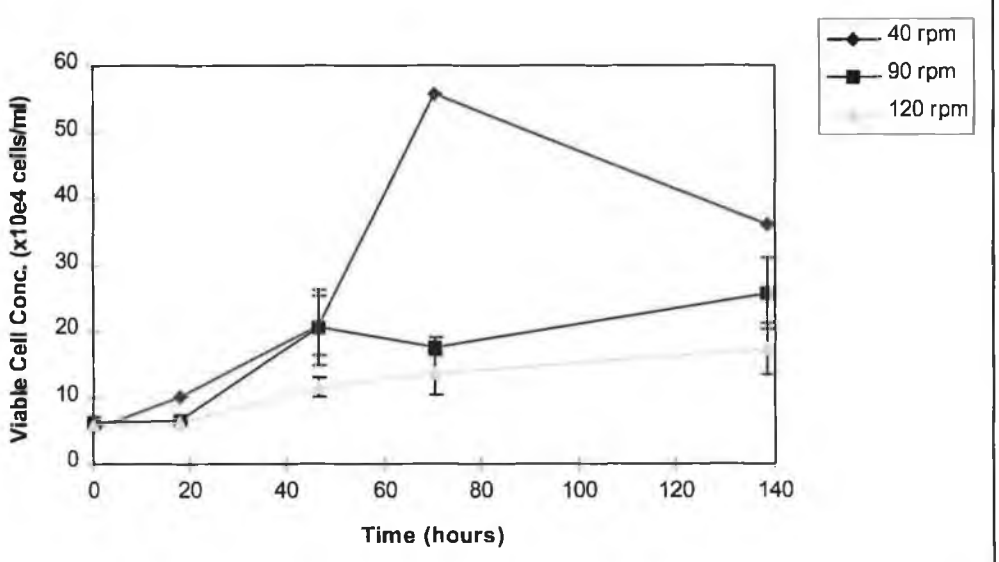
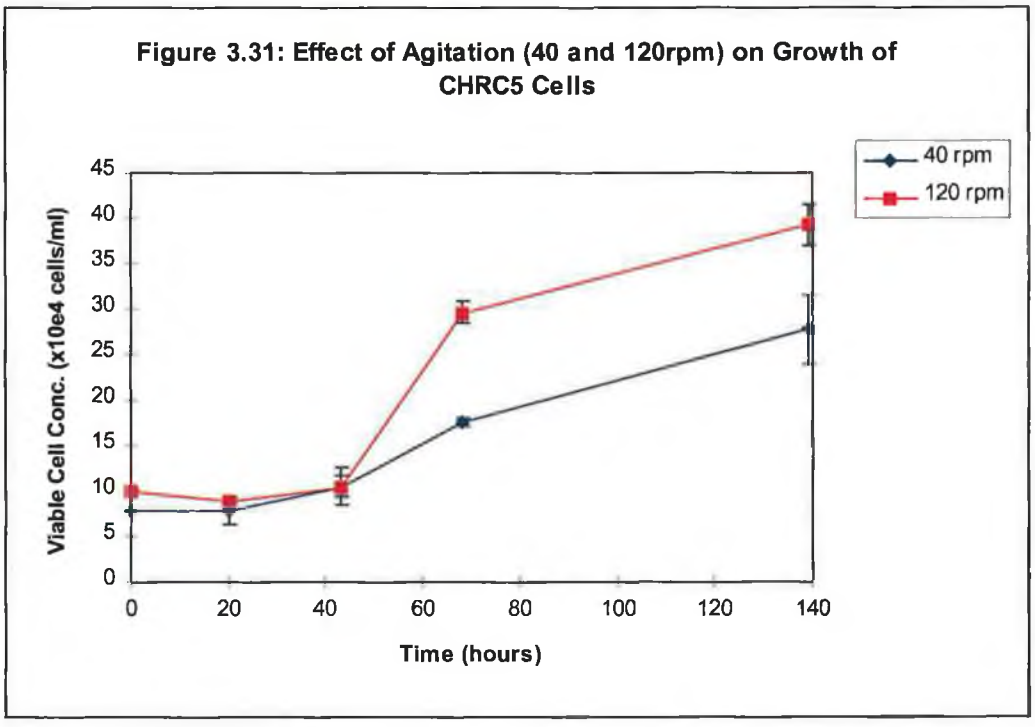



Table 3.40: Growth of CHO-K1 Cells at 40, 90 and 120 rpm

| Agitation Rate (rpm) | Time (Hours) | Total Cell Count ($x10^4$/ml) | Std. Dev. ($x10^4$/ml) | % Viability |
|---|---|---|---|---|
| 40 | 0 | 5.4 | n.d. | 100 |
| 40 | 18 | 10.2 | n.d. | 100 |
| 40 | 46.5 | 20.8 | 4.4 | 99.5 |
| 40 | 70.5 | 57.6 | n.d. | 97 |
| 40 | 138.5 | 40.1 | n.d. | 90 |
| | | | | |
| 90 | 0 | 6.6 | n.d. | 96 |
| 90 | 18 | 7.5 | n.d. | 88 |
| 90 | 46.5 | 20.8 | 5.7 | 99 |
| 90 | 70.5 | 18.3 | 1.3 | 97 |
| 90 | 138.5 | 27.3 | 5.5 | 94 |
| | | | | |
| 120 | 0 | 6 | n.d. | 100 |
| 120 | 18 | 6.6 | n.d. | 96 |
| 120 | 46.5 | 11.5 | 1.5 | 100 |
| 120 | 70.5 | 13.9 | 3.2 | 98 |
| 120 | 138.5 | 18 | 3.8 | 96 |

Table 3.41: Growth of CHRC5 at 40 and 120 rpm.

| Agitation Rate (rpm) | Time (hours) | Total Cell Count (x $10^4$/ml) | Std. Dev. (x $10^4$/ml) | % Viability |
|---|---|---|---|---|
| 40 | 0 | 9 | n.d. | 87 |
| 40 | 20 | 8.68 | 1.5 | 90 |
| 40 | 43 | 11.4 | 2.1 | 92 |
| 40 | 68 | 19.8 | 0.4 | 89 |
| 40 | 139 | 46.2 | 3.8 | 60 |
| | | | | |
| 120 | 0 | 11.21 | n.d. | 90 |
| 120 | 20 | 11.6 | 0.2 | 77 |
| 120 | 43 | 12.4 | 1.2 | 85 |
| 120 | 68 | 31.2 | 1.2 | 95 |
| 120 | 139 | 44.6 | 2.3 | 88 |



CHRC5 cells display a significantly different response to increased agitation rate. Unlike CHO-K1 cells which did not grow well at the higher agitation rate of 120 rpm, CHRC5 grow well with increased cell number and viability at 120 rpm. Indeed CHRC5 growth is greater at 120 rpm than 40 rpm. Overall viable cell number increased 4.5 fold in the 120 rpm culture, as opposed to approximately 2.5 fold in the 40 rpm culture.

These experiments suggest that CHRC5 cells grow better than CHO-K1 cells at higher agitation rates. Table 3. compares the apparent growth rates and doubling times, which are calculated from the linear portion of the growth curve plotted on a logarithmic scale (not shown), for the two cell lines at varying agitation rates.

Table 3.42: Comparison of Apparent Growth Rate and Doubling Time for CHO-K1 and CHRC5 at 40 and 120 rpm

| | CHO-K1 | | CHRC5 | |
|---|---|---|---|---|
| | 40 rpm | 120 rpm | 40 rpm | 120 rpm |
| $\mu_{app}$ ($hr^{-1}$) | 0.032 | 0.014 | 0.013 | 0.042 |
| $t_d$ (hrs) | 21.4 | 47.24 | 54.5 | 16.5 |

3.2.4.3: Further Investigations into the Effect of Agitation on CHO-K1 and CHRC5 Cell Growth

It was decided to repeat the experiments comparing the growth of CHO-K1 and CHRC5 at various agitation rates to confirm the findings in section 3.2.4.1 and 3.2.4.2.

In these experiments both cell lines were grown at 0, 40 and 120 rpm in spinner flasks using the same inoculum densities as previously. Again the CHRC5 spinner was gassed with a 95%air/5%$CO_2$ mixture in order to keep the medium buffered. Results are presented in Figures 3.32 and 3.33.

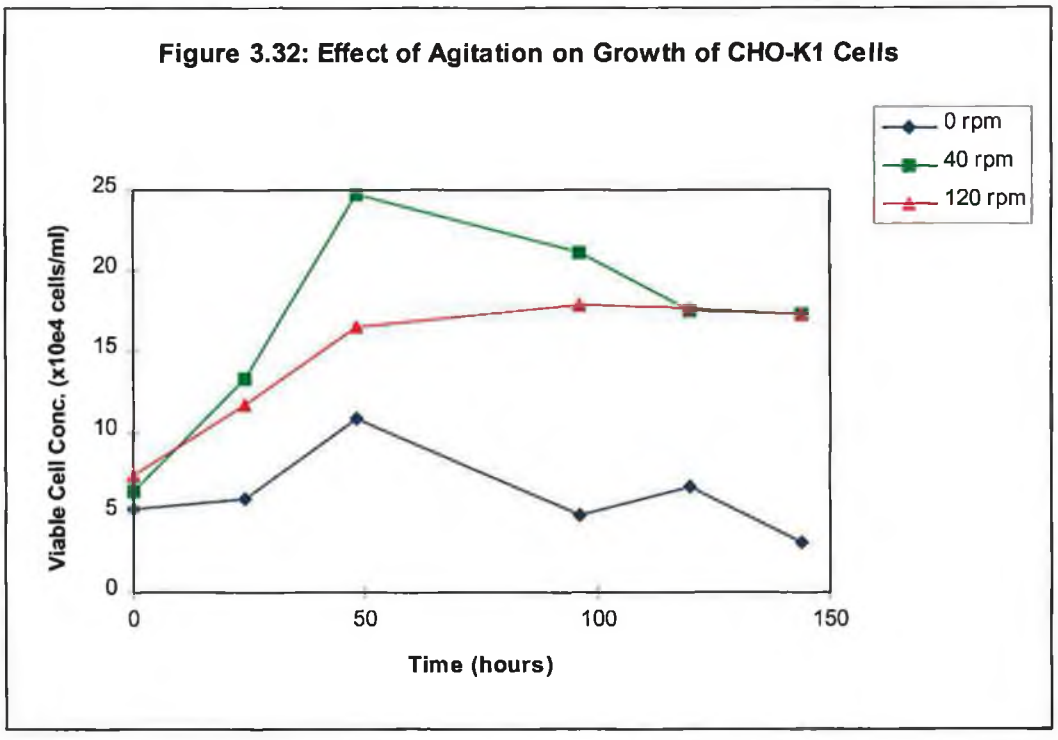


Figure 3.32: Effect of Agitation on Growth of CHO-K1 Cells
0 rpm
40 rpm
120 rpm
Viable Cell Conc. (x10e4 cells/ml)
25
20
15
10
5
0
0
50
100
150
Time (hours)

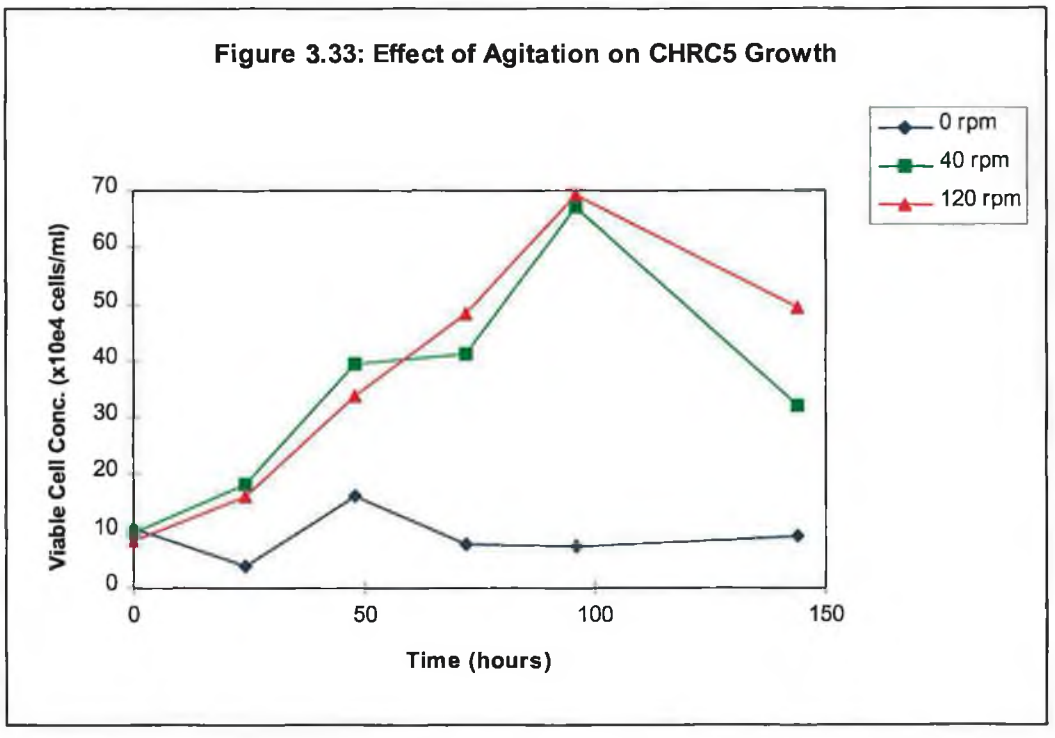


Figure 3.33: Effect of Agitation on CHRC5 Growth
0 rpm
40 rpm
120 rpm
Viable Cell Conc. (x10e4 cells/ml)
70
60
50
40
30
20
10
0
0
50
100
150
Time (hours)

Figures 3.32 and 3.33 confirm the earlier results, that is that CHRC5 cells grow better at higher agitation rates than their parental, non-MDR counterparts, CHO-K1. For both cell lines it can be seen that growth in the stationary spinner flask is low, however it was observed that cells began clumping after day 1 probably due to the lack of agitation and the unavailability of substratuum to which cells could adhere (spinners were siliconised to prevent cell attachment to the vesesl). Over the batch culture period it was observed that these cell clumps became bigger and the medium colour changed indicating a pH change.

Growth in the 40 rpm CHO-K1 culture is much better than in the 120 rpm culture initially, although towards the end of the run cell numbers become similar. However the 40 rpm culture became fungally contaminated after day 2 which might account for its premature entry into the decline phase. Apparent growth rate is higher for the 40 rpm culture than for the 120 rpm culture (Table 3.43). CHRC5 cells appear to grow well at both 40 rpm and 120 rpm despite the increased shear forces at the higher agitation rate. Cell viabilities remained high throughout the culture periods for both cell lines. At 40 and 120 rpm, viabilites did not decrease below 93% for the CHRC5 cells and below 88% for the CHO-K1 cells. Table 3.43 presents the apparent growth rates and doubling times for the 40 and 120 rpm cultures.

Table 3.43: Growth Characteristics for CHO-K1 and CHRC5 Cells

| | CHO-K1 | | CHRC5 | |
|---|---|---|---|---|
| | 40 rpm | 120 rpm | 40 rpm | 120 rpm |
| $\mu_{app}$ ($hr^{-1}$) | 0.026 | 0.017 | 0.028 | 0.029 |
| $t_d$ (hrs) | 26.93 | 41 | 24.3 | 24.1 |

3.2.4.4: The Role of Serum in Protecting CHO-K1 and CHRC5 Cells Against Hydrodynamic Damage

There have been many reports in the literature of serum not only having a growth promoting role in cell cultures, but in agitated bioreactors, a shear protective role (Kunas and Papoutsakis, 1990). How serum works as a protective agent is still not clear although recent reports suggest it may alter the plasma membrane fluidity of cells, rendering the cells less shear sensitive (Ramirez and Mutharasan, 1990, 1992). Since the work thus far had suggested that the MDR cells (CHRC5) might be more resistant to higher agitation intensities than CHO-K1, it was decided to investigate whether serum might affect this observed agitation resistance.

Cells were set up in spinner flasks as before and agitated at 40 and 120 rpm in low (1%) and normal (5%) serum levels. Results are presented in Figures 3.34 and Figure 3.35.

**Figure 3.34: Effect of Varying Serum Level on CHO-K1 Cells Agitated at Low and High Rates**

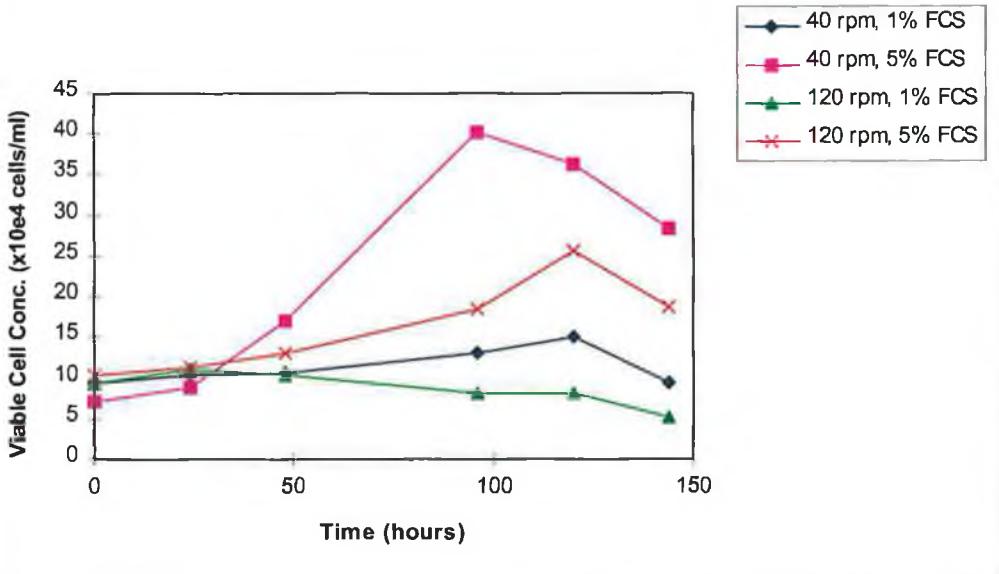
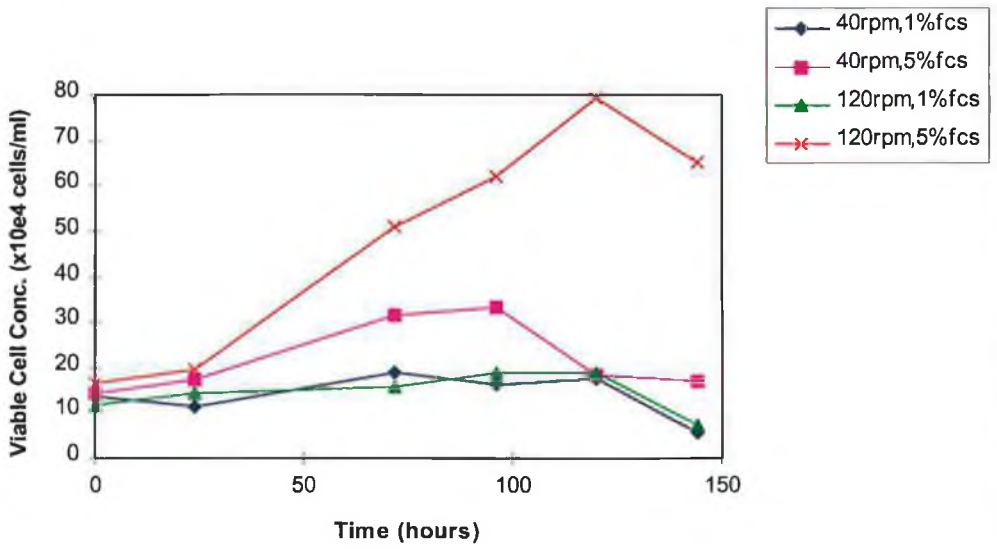


**Figure 3.35: Effect of Varying Serum Level on CHRC5 Cells Agitated at Low and High Rates**




These results show that serum has a profound effect on the ability of cells to grow under shear stressed conditons. At both the high and low agitation rates, the growth of the each cell line is very much reduced at 1% serum. At the higher serum level the growth profiles are similar to those previously observed, that is, CHRC5 grows well at 120 rpm, whereas CHO-K1 has a much lower apparent growth rate at this higher agitation rate.The optimum growth rate for CHO-K1 under these conditions is 40 rpm. The relatively poor growth of CHRC5 cells at 40 rpm (Figure 3.34) might be explained by the fact that the cells formed quite tight clumps at this agitation rate. These clumps were particularly noticeable from 96 hours onwards and might have led to mass transfer limitations. Formation of clumps of cells was also noted in the CHO-K1 cultures at 40 rpm, however at 120 rpm for both cell lines clumping was less of a problem and any clumps that did form were loosely held together.

Table 3.44: Growth Characteristics of Agitated, Serum Varied CHO-K1 and CHRC5 Cells

| | CHO-K1 | | | | CHRC5 | | | |
|---|---|---|---|---|---|---|---|---|
| | 1%,40 | 5%,40 | 1%, 120 | 5%,120 | 1%,40 | 5%,40 | 1%,120 | 5%,120 |
| $\mu_{app}$ ($hr^{-1}$) | 0.004 | 0.018 | n.d. | 0.0071 | n.d. | 0.012 | 0.004 | 0.0197 |
| $t_d$ (hours) | 163 | 38.3 | n.d. | 96.97 | n.d. | 56.3 | 185 | 35.04 |

3.2.4.5: Metabolic Consumption and Production by Agitated CHO-K1 and CHRC5 Cultures.

The shear sensitivity of cells may be affected by a number of factors, for example, ammonia accumulation and pH changes (Peterson *et al*, 1988). Also, shear forces can have other effects besides those on cell proliferation, Al-Rubeai *et al* (1990) observed that hybridomas had a modifed cellular metabolism upon exposure to shear stress. Therefore it is important to monitor any changes in ammonia and lactate (waste metabolites) production rates and in glucose consumption rates that may be caused by agitation.

Glucose, ammonia and lactate levels were measured for CHO-K1 and CHRC5 cells agitated at 0, 40 and 120 rpm in the batch culture runs of section 3.2.4.3. Results are presented in Figures 3.36-3.38.

*3.2.4.5.1:Glucose Consumption*

Animal cells *in vitro* utilise glucose as their main energy source with lactate produced as the primary waste metabolite. The initial glucose concentrations in Hams F12 medium (CHO-K1 cultures) is 10mM, while for MEMα medium (CHRC5 cultures) it is 5.5mM glucose.

**Figure 3.36: Glucose consumption by CHO-K1 Cells Agitated at Different Rates**

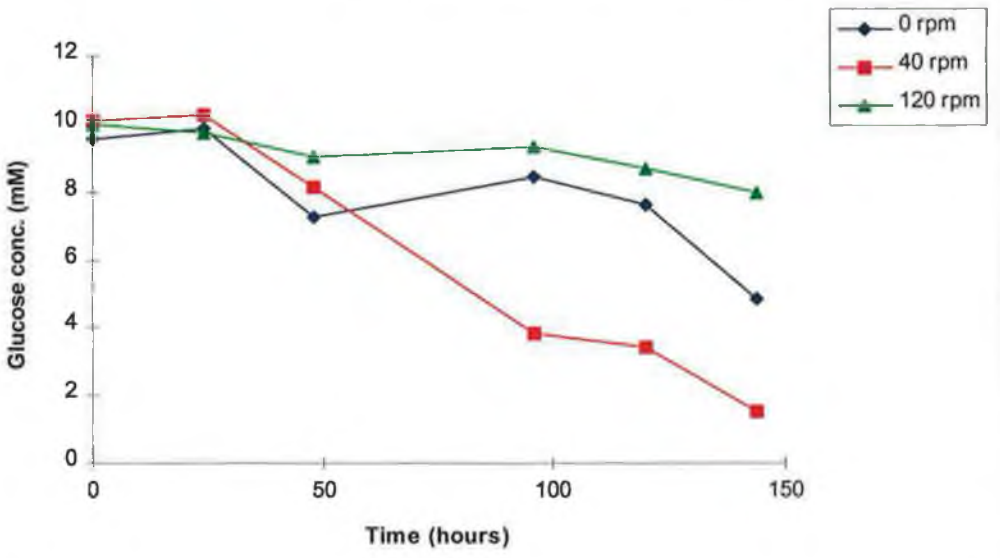
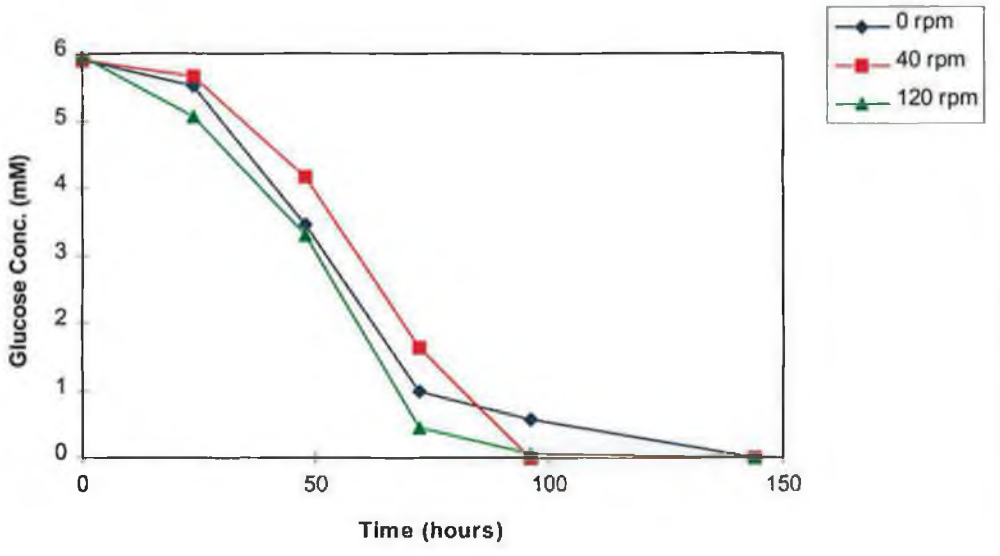



**Figure 3.37: Glucose Consumption by CHRC5 Cells Agitated at Different Rates**



For CHO-K1 cells glucose consumption is greatest for the 40 rpm culture which correspondingly grew at the fastest rate (Figure 3.32). In contrast glucose consumption by the cells agitated at 120 rpm is very low. At the end of the culture period 7.98 mM glucose remains which is very high given that 10 mM glucose is present initially in the medium. Although cell growth was greater at 40 rpm than at 120 rpm there was still substantial growth at the higher agitation rate thus this low usuage of glucose by the higher agitated CHO-K1 cells is somewhat unusual. Glucose consumption by the MDR cells is at a greater rate than for the non-MDR cells with both the 40 rpm and the 120 rpm culture consuming all the glucose available before the end of the culture period. For the 120 rpm culture, glucose levels were at zero by 72 hours, for the 40 rpm culture glucose was consumed by 96 hours. Thus the entry of both cultures into decline phase at 96 hours (Figure 3.33) is probably due to the depletion of glucose.

*3.2.4.5.2:Ammonia Production*

Ammonia is the major waste product from glutamine metabolism and may also be produced as a result of the spontaneous decomposition of glutamine.

**Figure 3.38: Ammonia Production in CHO-K1 Agitated Cultures**

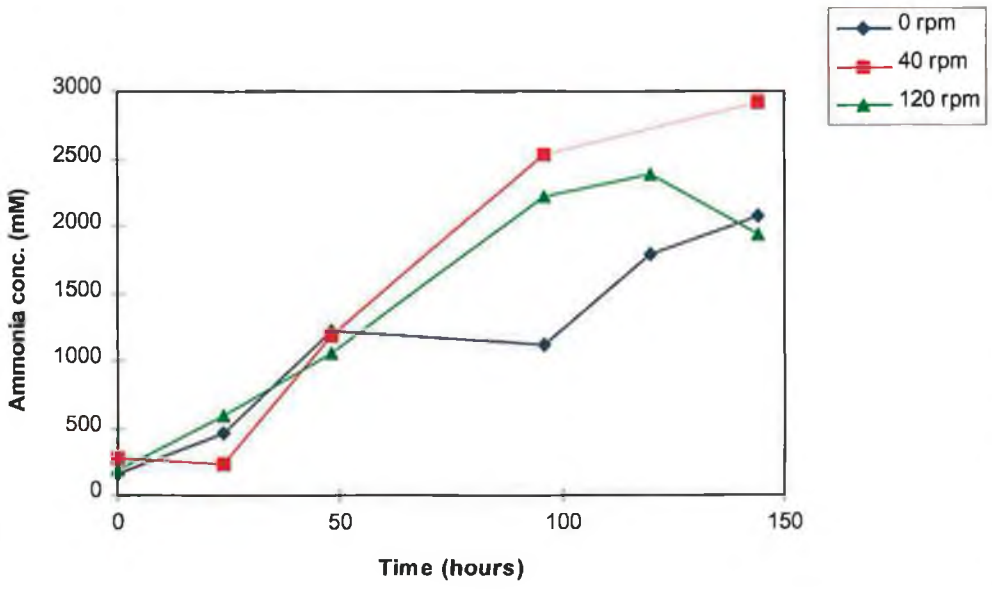
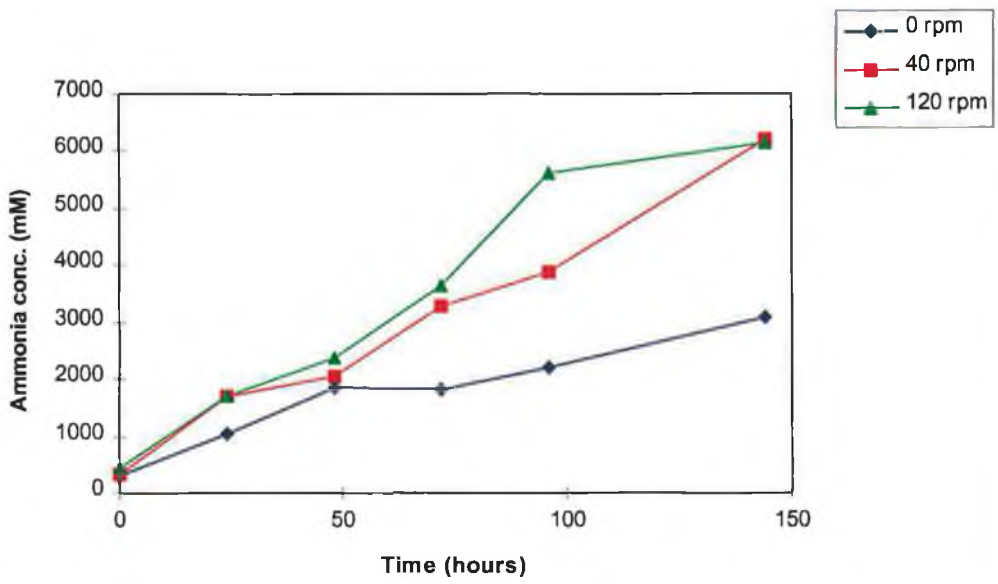


**Figure 3.39: Ammonia Production in CHRC5 Agitated Cultures**




These results clearly show that CHRC5 cells produce more ammonia during batch growth than their non-MDR counterparts, CHO-K1. Maximum ammonia accumulation for CHRC5 cells at 40 rpm is 6.2 mM and at 120 rpm is 6.1 mM at the end of the culture period. Corresponding values for CHO-K1 cells are 2.9 mM at 40 rpm and 2.4 mM at 120 rpm. Both cell lines had equal levels of glutamine in the medium at the start of the culture period.

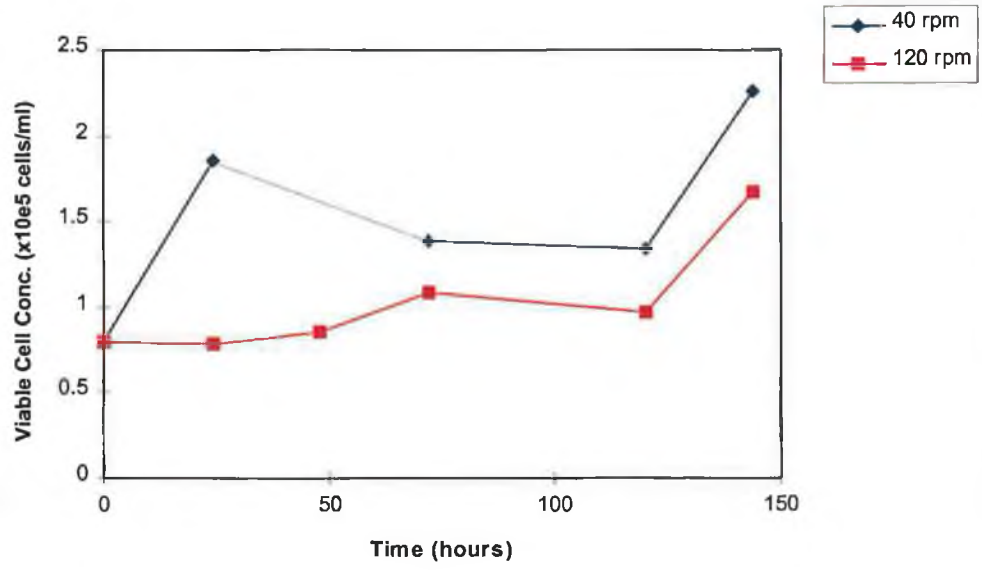
### 3.2.5: Effect Of The Hydrodynamic Environment On The Growth Of Hep-2 And Its Multi-Drug Resistant Counterpoint, Hep-2a

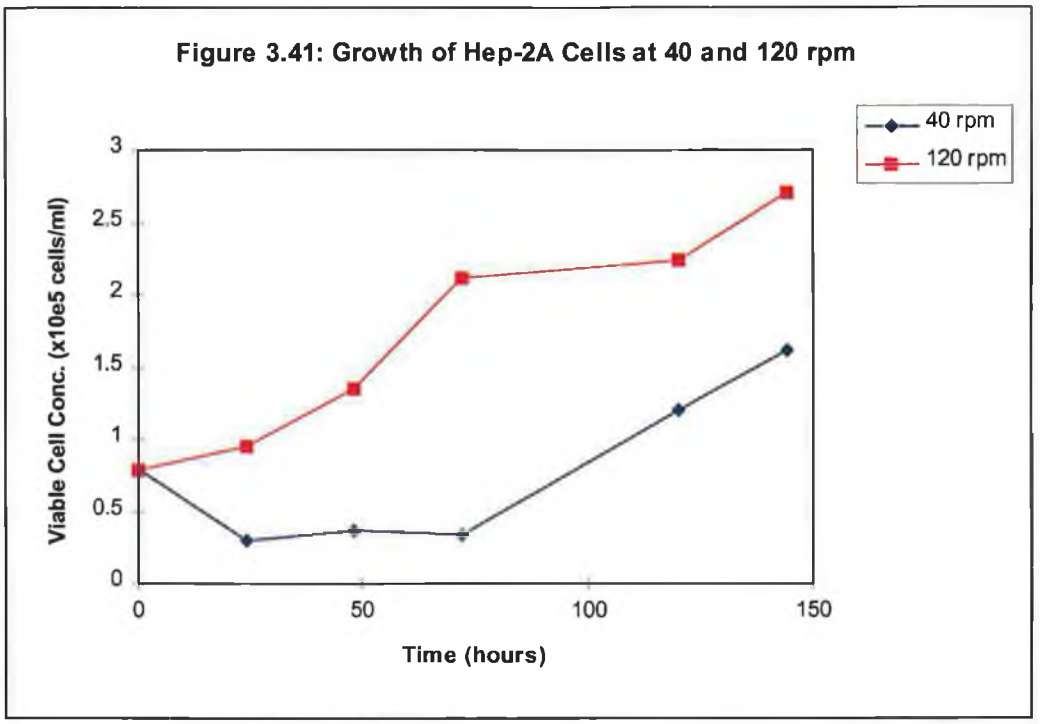
The observed differences in the response of CHO-K1 and CHRC5 to high agitation rates does not answer the general question posed, are MDR cells more physically robust than their parental counterparts? To gather further evidence to answer this question, this section of the work examines the effect of agitation on the growth of Hep-2 cells (human larynx carcinoma) and its MDR clone selected for its drug resistant properties in this laboratory, Hep-2A. It is important to determine if the observed, increased agitation resistance of CHRC5 (MDR) cells is unique to that cell system or if another MDR cell line will display the same increased agitation resistance when compared to its non-MDR parent.

3.2.5.1: Effect of Agitation on Hep-2 and Hep-2A Cells

Hep-2 cells were inoculated into 100 ml spinner flasks at a density of 8 x $10^4$ cells per ml. One ml samples were taken in duplicate every day to monitor cell growth. Cells were agitated at 40 and 120rpm. Results are presented in Figures 3.40 and 3.41.

**Figure 3.40: Growth of Hep-2 Cells at 40 and 120 rpm**





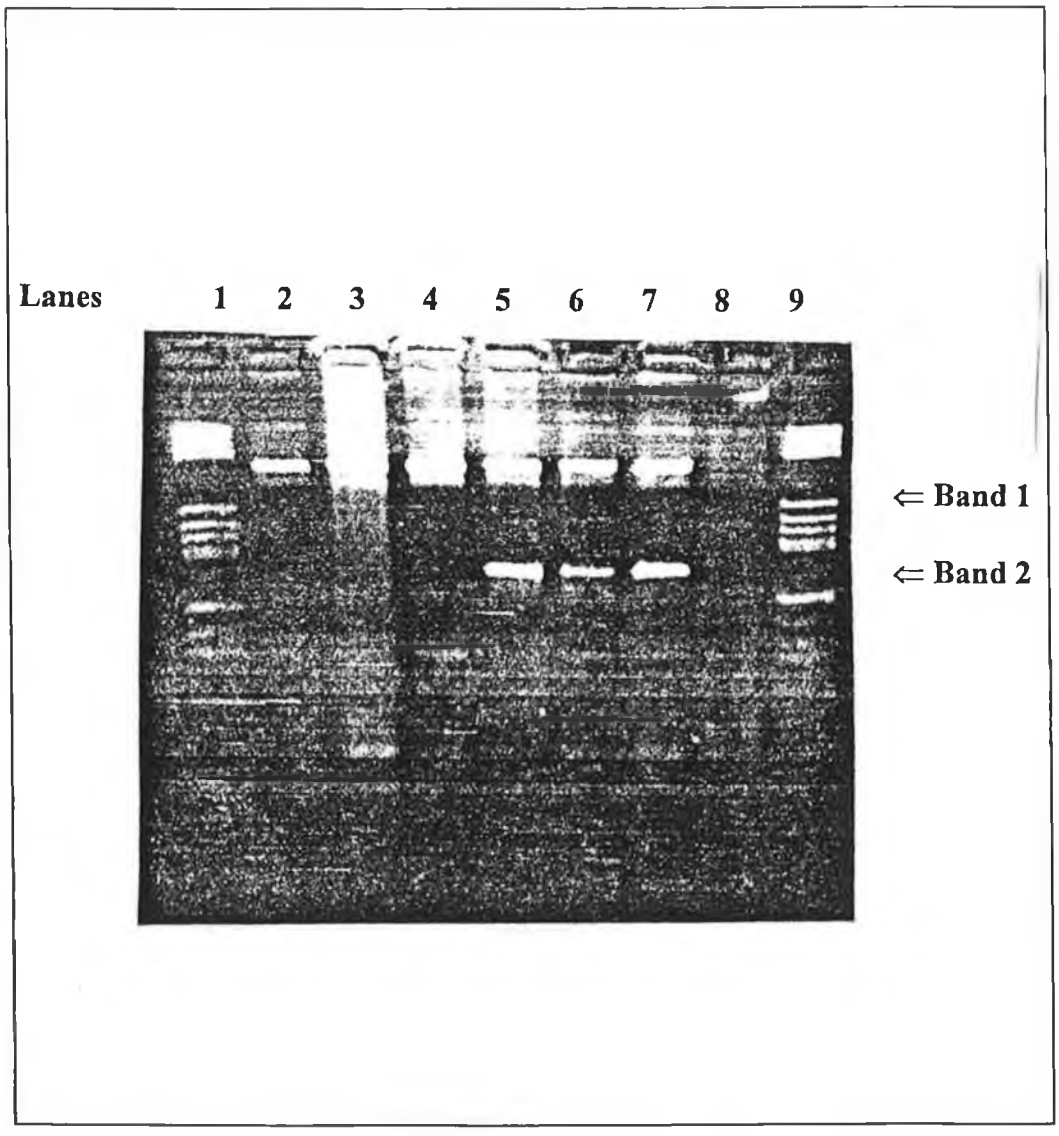

These results suggest that the MDR cell line, Hep-2A grows well at the higher agitation rate when compared to the growth of Hep-2 at the same rate. This result is very similar to the trend observed with CHRC5 cells and CHO-K1 cells, i.e. the MDR cells appear to be more agitation resistant. The growth at 40 rpm for both cell lines is slightly unusual, for the Hep-2 cells growth is very erratic but the overall trend is a small increase over the growth at 120 rpm. The Hep-2A cells appear to have a prolonged lag phase at 40 rpm and grow much better at 120 rpm. However it is important to note that cell clumping was again a problem at the lower agitation rates therefore cell counts may be slightly underestimated. Parallel cultures were carried out at 0 rpm (stationary spinner cultures) and results were similar to previous experiments, that is very much reduced cell growth probably caused by cell settling and clumping leading to nutrient diffusion problems.

3.2.5.2: Determination of MDR-1 Gene Expression in Hep-2 and Hep-2A Agitated Cultures Using RT-PCR

The primary mechanism of MDR is thought to be due to the overexpression of p-glycoprotein, a plasma membrane efflux pump with ATPase activity and a molecular weight of 170 kdaltons (Clynes, 1993). High levels of p-glycoprotein confers cross-resistance to a broad range of chemically and mechanistically unrelated drugs. The protein acts by promoting the efflux of drug resulting in a decrease in intracellular drug concentration. The gene coding for p-glycoprotein is MDR-1, a member of a small family of MDR genes. Overexpression of p-glycoprotein is frequently associated with amplification of the MDR-1 gene.

It will be shown in the following section (Section 3.2.6) that a decrease in plasma membrane fluidity (PMF) appears to correlate with decreasing shear sensitivity in CHRC5 and DLKPA cells. Loe and Sharom (1993) have suggested that p-glycoprotein induces physicochemical changes that results in tighter packing of the outer leaflet of the lipid bilayer. Thus the levels of MDR-1 gene which codes for p-glycoprotein was compared, using a reverse transcriptase- polymerase chain reaction (RT-PCR) technique, in Hep-2 and Hep-2A cells agitated at 40 and 120rpm.

The RT-PCR technique was carried out by Daragh Byrne, a 4th year Biotechnology student under my supervision. The following photograph overleaf shows the results obtained. Band 1 represents the control (β-actin) RT-PCR product and band 2 represents the MDR-1 gene.




*Legend:* *Lane 1 - Molecular weight marker; 2 - Hep-2 120rpm; 3 - Hep-2 40rpm; 4 - Hep-2 0rpm; 5 - Hep-2A 120rpm; 6 - Hep-2A 40rpm; 7 - Hep-2A 0rpm; 8 - Blank; 9 - Molecular weight marker. Band 1 β-actin: Band 2 MDR-1 gene*

It would appear that the MDR cells, Hep-2A, have a higher expression of the MDR-1 gene at the higher agitation rate of 120 rpm when compared to the 40rpm Hep-2A culture. Hep-2 cells do not appear to express the MDR-1 gene which is as expected given that these cells are not drug resistant. Quantification of the amount of MDR-1 gene in each agitated culture was carried out using a densitometer. It was found that Hep-2A cells agitated at

120 rpm expressed almost 2.5 times the amount of the MDR-1 gene when compared to the quantity of the MDR-1 gene produced by Hep-2A cells agitated at 40rpm ( 5.10 % band volume compared to 1.97 % band volume). Therefore these results confirm that the MDR cells, Hep-2A, express the MDR-1 gene and that the level of expression increases with an increase in agitation. This observed increase in levels of MDR-1 gene and thus most probably of p-glycoprotein in the Hep-2A cell membranes at higher agitation rates may be related to the decrease in PMF observed in two other MDR cell lines, CHRC5 and DLKPA (see Section 3.2.6).

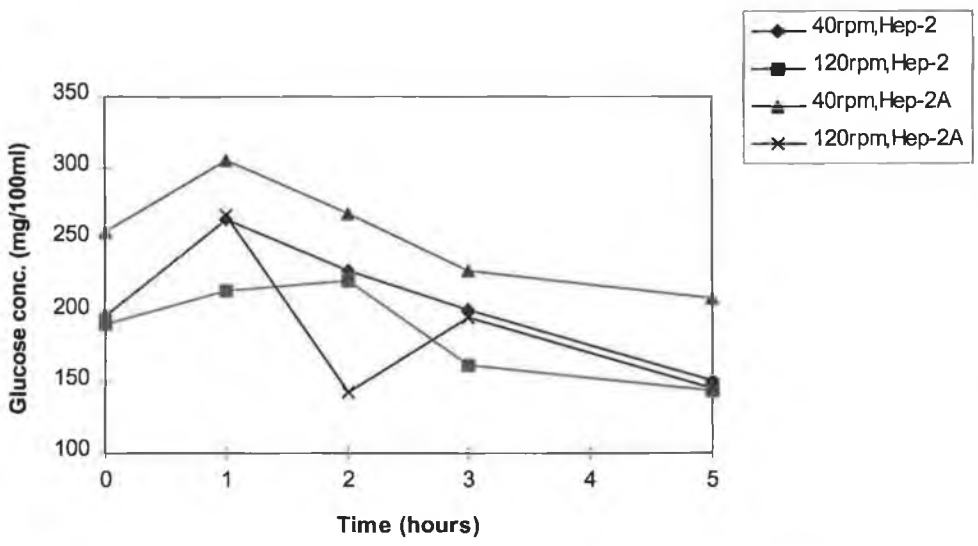
### 3.2.5.3: Metabolic Consumption and Accumulation by Agitated Hep-2 and Hep-2A Cultures

As stated in section 3.2.4.5 shear sensitivity of cells may be affected by physical factors such as pH, ammonia accumulation etc. The affect of shear may also alter the metabolism of cells. For these reasons, glucose consumption, ammonia and lactate accumulation in agitated Hep-2 and Hep-2A batch cultures was examined. The corresponding growth curves for these cultures are given in Figure 3.40 and Figure 3.41.

*3.2.5.3.1:Glucose consumption*

Glucose consumption was monitored over a 5 day batch culture for Hep-2 and Hep-2A cells agitated at 40 and 120 rpm. Results are presented in Figure 3.42.

**Figure 3.42: Glucose Consumption by Hep-2 and Hep-2A Cells Agitated at Low and High Rates**

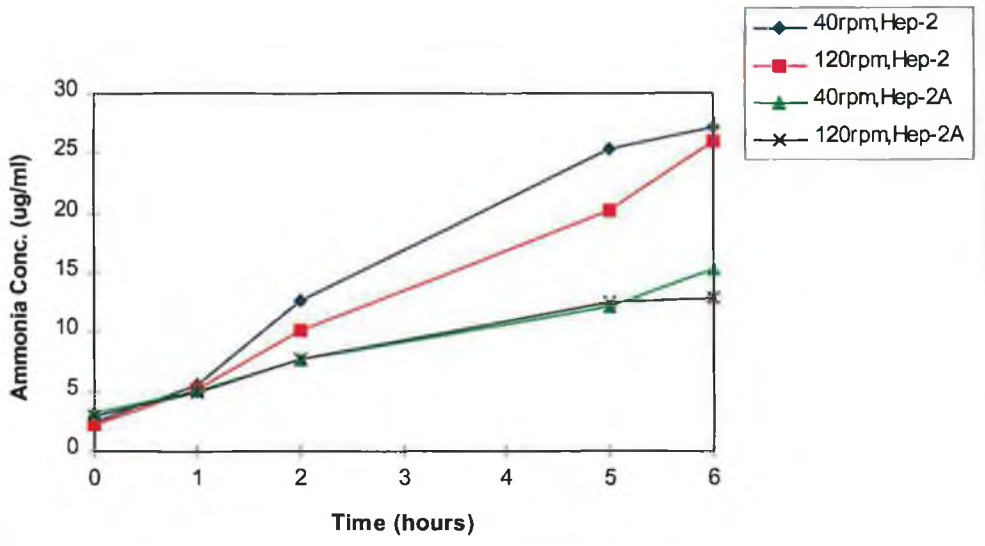



It can be seen that glucose consumption is quite similar for both cell lines at each agitation rate. The lowest glucose consumption is for Hep-2A cells growing at 40 rpm which corresponds to the growth curve in Figure 3.41 showing poor growth by these cells at this agitiation rate. The 120 rpm Hep-2 culture has marginally the greatest consumption rate even though Figure 3.41 indicates that growth at this agitation rate was not as great as at 40 rpm.

*3.2.5.3.2: Ammonia Accumulation*

Ammonia accumulation was monitored for both cell lines agitated at 40 and 120 rpm. Results are presented in Figure 3.43.

**Figure 3.43: Ammonia Production by Hep-2 and Hep-2A Cells at Varied Agitation Rates**




It would appear from these results that the non-MDR, Hep-2 cells accumulate much higher levels of ammonia and the accumulation rate is greater than for Hep-2A. This is in contrast to the CHO-K1/ CHRC5 system (Section 3.2.4.5.2) where CHRC5 cells, the MDR line, were shown to produce more ammonia. The faster growing 40 rpm, Hep-2 culture has the greatest accumulation rate.

### 3.2.6: Plasma Membrane Fluidity Measurements In CHO-K1 And CHRC5 Cells

Plasma membrane fluidity (PMF) is a collective description for the various motions of the components of a biological membrane. Recent work (Ramirez and Mutharasan, 1990, 1992) has shown that PMF is a critical factor in determining the shear sensitivity of cells. An increasing PMF has been associated with an increase in shear sensitivity. These authors have shown that modulation of PMF by different agents (e.g. serum/cholesterol) can alter the response of cells to shear stresses. Differences in PMF have also been noted in MDR cells and their parental cells (Callaghan *et al*, 1992 ). Consequently by investigating the PMF of CHRC5 cells (MDR cell line) and CHO-K1 cells (non-MDR cell line) it might be possible to determine whether a difference in PMF is responsible for the increased shear resistance of CHRC5 cells. CHRC5/CHO-K1 cells were chosen for further work since the difference in their shear tolerance was more pronounced than for the Hep-2/Hep-2A cell system.

#### 3.2.6.1: Determination of PMF for CHRC5 and CHO-K1 Cells Cultured in Flasks

PMF is measured in this work using steady state fluorescence polarisation (Methods, 2.12.). This involves labeling the cell membrane with a fluorescent probe (TMA-DPH) which is excited with a polarised monochromatic light. According to the movement of the probe (which depends on the PMF) the emitted light will be partially depolarised with respect to the plane of polarisation of the excitation light. The resultant fluorescence anisotropy is termed the $r_s$ value. A definition of this value is given in Section 1.3.4.5.3. Two parameters are being measured, the local microviscosity and the molecular order which is related to the degree of rotational restriction of the probe in the membrane. Using the steady state fluorescence polarisation method the second parameter is mostly being measured. A change in

molecular order is associated with the reciprocal of PMF. Thus an **increase in the $r_s$ value indicates a decrease in PMF**.

It was decided to determine the PMF of CHRC5 and CHO-K1 cells when the cells were growing in routine culture. That is growing in tissue flasks in an anchorage dependent mode with no shear stress present. Results are presented in Table 3.45.

Table 3.45: PMF Values for CHRC5 and CHO-K1 Cells Growing in Tissue Flasks.

| Cell Line | Fluorescence Anisotropy ($r_s$ value) |
|---|---|
| CHO-K1 | 0.219 |
| CHRC5 | 0.218 |
| CHO-K1 (serum free) | 0.194 |

Note: these values are the average of at least 5 readings.

It can be seen that there is no real difference in the $r_s$ value and thus in the PMF between the CHRC5 and CHO-K1 cells when growing in flasks. In order to ensure that the technique was capable of detecting differences in PMF, the $r_s$ value of a serum free variant of CHO-K1 was measured. These cells had been growing in serum free medium for 3 months. Serum is a well documented shear protective agent (refer to Section 1.3.4.5.1), consequently these cells would be expected to be more shear sensitive than their parental cells growing in serum. If increased PMF correlates with increased shear sensitivity then these cells should have an increased PMF and thus a decreased $r_s$ value. As can be seen this is the case, thus these cells would be expected to be more shear sensitive. Under non-stressful conditions there is no difference in the PMF of CHRC5 and CHO-K1 cells.

3.2.6.2: Determination of PMF for CHRC5 and CHO-K1 Cells Growing in Short Term, High Shear Cultures.

Since differences in shear sensitivities between CHRC5 and CHO-K1 cells can only be determined in agitated spinner cultures, it was decided to measure the PMF of the cells when agitated. Changes in PMF can be quite small in value , therefore it was decided to investigate any differences in short term, high shear experiments. If there are any differences in PMF between the cells then they should be detected under these more extreme conditions. All agitation experiments up to this point had examined the effects of low and moderate agitation and noted an effect on growth rate. The experiments proposed here are at very high agitation rates and no cell growth is expected, thus changes in the death rate are monitored.

Cells were inoculated at 2 x $10^5$ cells/ml into Bellco spinner flasks at time zero and agitated at 100 rpm. After one hour acclimatisation the agitation rate was increased to 500 rpm. Cell counts and $r_s$ values were monitored over the next 24 hours. The use of Bellco spinner flasks is emphasised here because up to this point all agitation work had been carried out in Techne spinners. Each spinner flask produces a completely different flow pattern and consequently produces different shear stresses to which cells are exposed. The Bellco spinner flasks were acquired because their geometry allows an estimation of the shearing forces present in the vessel. A comparison between the two spinner systems will be made further on in this work.

*3.2.6.2.1: Effect of High Shear on Viability of CHRC5 and CHO-K1 Cells*

Figure 3.44 presents the results of viability counts of both cell lines growing at 500 rpm for 24 hours. Results are presented in terms of normalised viability which refers to the ratio of viable cell number after shear to the viable cell number before shear.

Thus for these experiments;

$$\text{Normalised viability} = \frac{\text{Viable cell no. at } t = n}{\text{Viable cell number at } t = 0}$$

where n= time at which sample was taken.

**Figure 3.44: Death of CHO-K1 and CHRC5 Cells by High Shear Conditions**

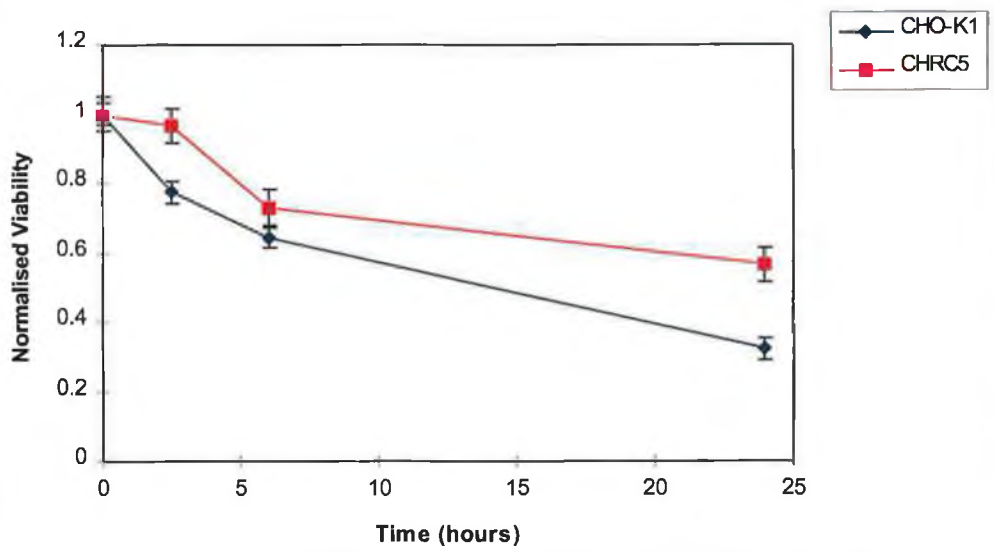
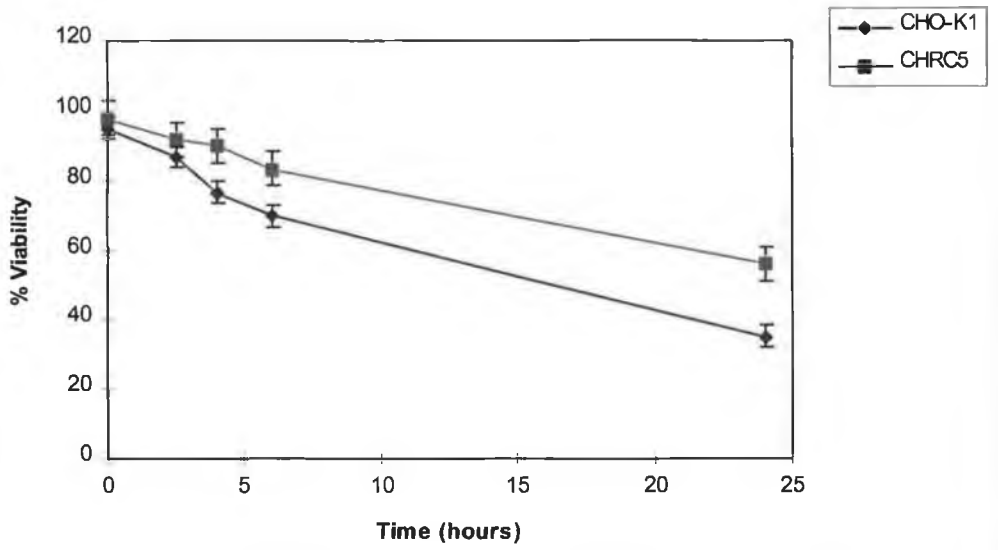


Figure 3.45 shows the results of the same experiment expressed in terms of % viability.

**Figure 3.45: Effect of High Shear on Viability of CHO-K1 and CHRC5 Cells**

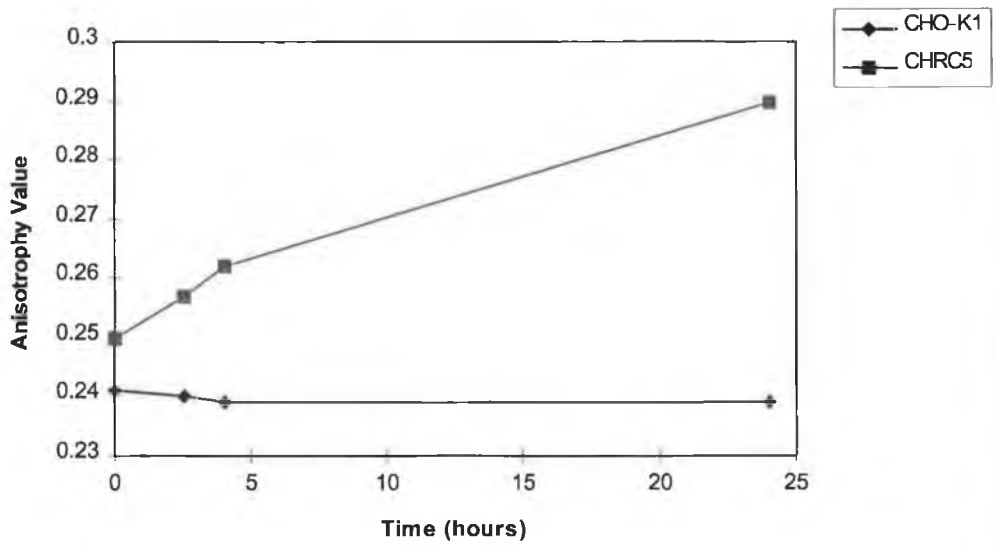



Figures 3.44 and 3.45 show that the agitation rate of 500 rpm is detrimental to both cell lines. However it can also be seen that CHRC5 cells do not lose viability as rapidly as CHO-K1 cells. If the plot of normalised viability versus time is converted to a semi-logarithmic plot, an approximately linear fit to the data is achieved (not shown). This indicates that the high agitation rate is killing the cells by first order death rate kinetics. Consequently a death rate value may be calculated for each cell line. For CHRC5 the death rate ($k_d$) is 0.014 $hr^{-1}$ and for CHO-K1 cells is 0.039 $hr^{-1}$. Thus these results confirm earlier work (Section 3.2.3), that is CHRC5 cells have increased resistance to shear stresses produced by agitation than CHO-K1 cells.

*3.2.6.2.2: PMF Measurements for CHO-K1 and CHRC5 Cells in High Shear Conditions*

Fluorescence anisotropy values were measured to evaluate the effect of the high agitation rate on the PMF of both cell lines. Results are presented in Figure 3.46.

**Figure 3.46 : Effect of High Shear on Fluorescence Anisotropy Values of CHRC5 and CHO-K1 Cells**




It can be seen that the $r_s$ value diverges significantly from the initial values for the two cell lines. The $r_s$ value for CHO-K1 cells decreases slightly in the first few hours and then reaches a steady state level. The $r_s$ value for CHRC5 cells increases dramatically over the culture period. The maximum $r_s$ value for CHRC5 cells is 0.29 whereas for CHO-K1 cells it is 0.24. Since an increase in $r_s$ signifies a decrease in PMF and a decrease in PMF implies a decrease in shear sensitivity, these results suggest that CHRC5 cells are less shear sensitive than CHO-K1 cells. As was seen in the normalised viability curve (Figure 3.44) this assumption is borne out in the death rate values.

In order to confirm these results the experiment was repeated. Viability curves from the second experiment are presented in Figures 3.47 and 3.48.

**Figure 3.47: Death of CHO-K1 and CHRC5 Cells Agiated in a High Shear Environment**

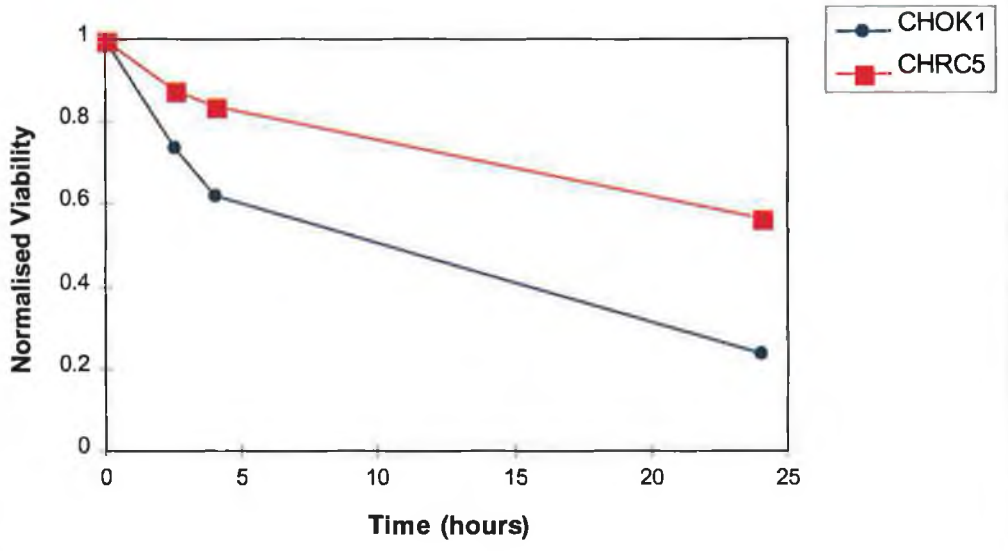
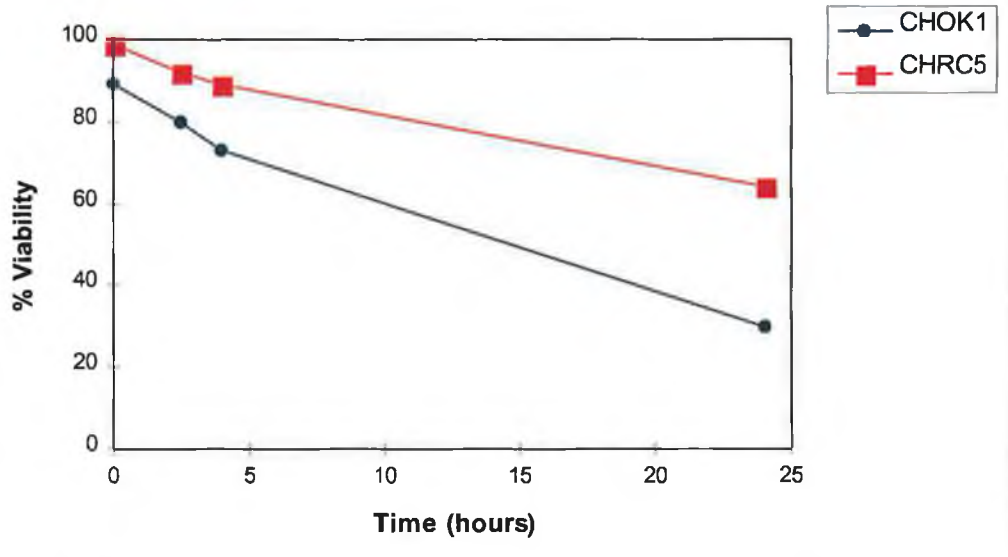


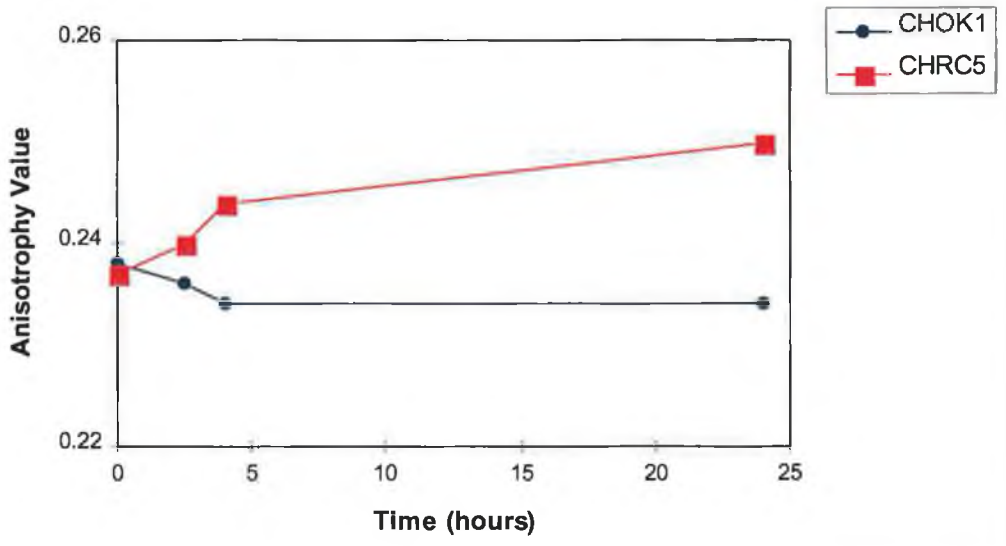

**Figure 3.48: Effect of High Shear on % Viability of CHO-K1 and CHRC5 Cells**



Again it may be seen that CHRC5 cells are more resistant to the higher agitation rate since these cells are not killed as fast as CHO-K1 cells. Death rates were calculated from the linear portion of a semi-logarithmic plot of normalised viability versus time (not shown) and are 0.08 $hr^{-1}$ for CHRC5 cells and 0.28 $hr^{-1}$ for CHO-K1 cells.

The effect on PMF was determined by measuring the $r_s$ value. Results are shown in Figure 6.54. That is the $r_s$ value of CHO-K1 cells is decreased indicating an increase in PMF after exposure to high shear, whereas the $r_s$ value for CHRC5 cells increased indicating a decrease in PMF upon exposure to high shear.

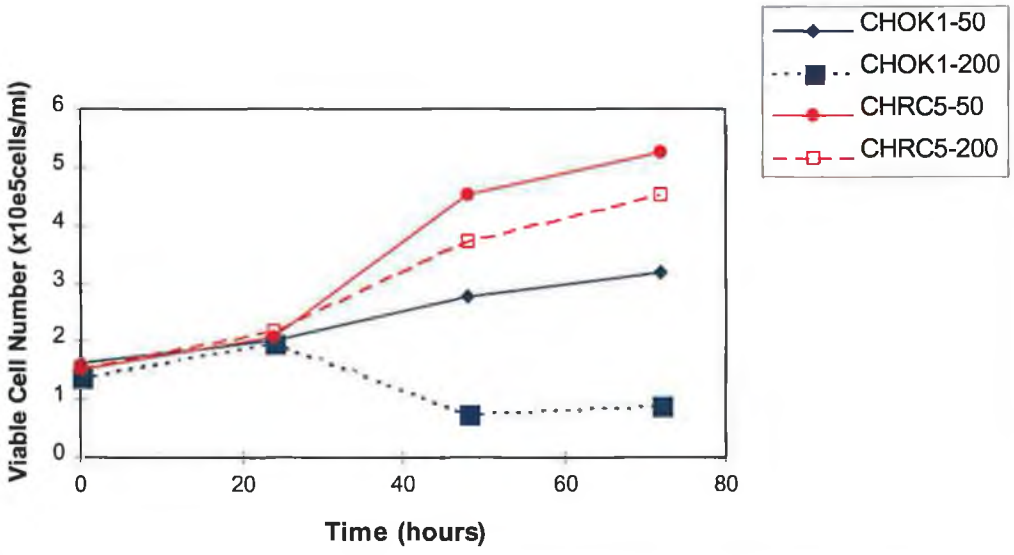
**Figure 3.49: Fluorescence Anisotrophy Measurements for CHOK1 and CHRC5 Cells Grown at High Shear**



3.2.6.3: Fluorescence Anisotropy Measurement at Low and Medium Shear Rates Over a Longer Culture Period

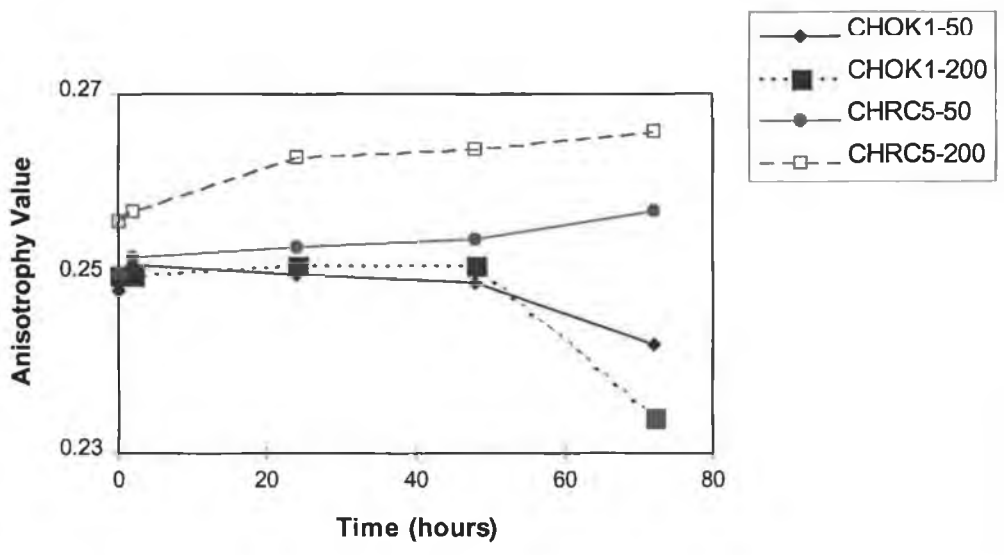
The preceding experiments have established that a high shear environment results in the MDR cells, CHRC5, displaying a greater shear tolerance than the non-MDR cells, CHO-K1. This appears to correlate with an associated decrease in PMF. It was important at this stage to ascertain whether differences in PMF between the two cell lines were also apparent at lower agitation rates and over longer cultivation times, i.e. under more usual culturing conditions. Cells were cultivated in Bellco spinner flasks at 50 and 200rpm over a 3 day period. Growth curves are presented in Figure 3.50.

**Figure 3.50: Viable Cell Number for CHOK1 and CHRC5 Cells Grown at Low and High Shear**



Again, CHRC5 cells grow well at the higher agitation rate of 200rpm. CHO-K1 growth is very much decreased at this agitation rate. It will be shown later (Section 3.2.7) that 200rpm in Bellco spinners results in cells being exposed to a turbulent flow field. At the lower agitation rate CHO-K1 growth is less than CHRC5 cells but is much greater than at 200rpm. Fluorescence anisotropy values were measured for the four cultures and results are presented in Figure 3.51.

**Figure 3.51: Fluorescence Anisotropy of CHOK1 and CHRC5 Cells Grown at 50 and 200rpm**

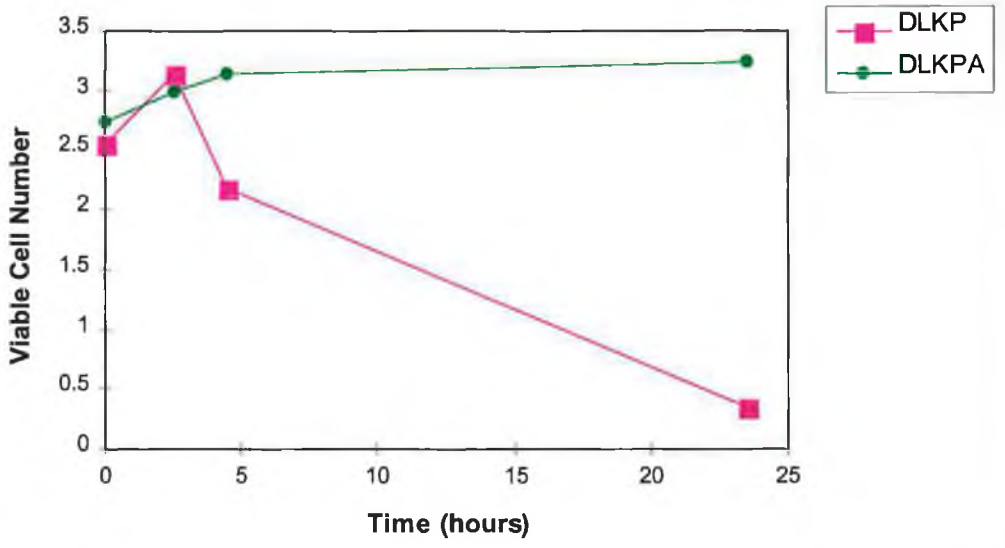


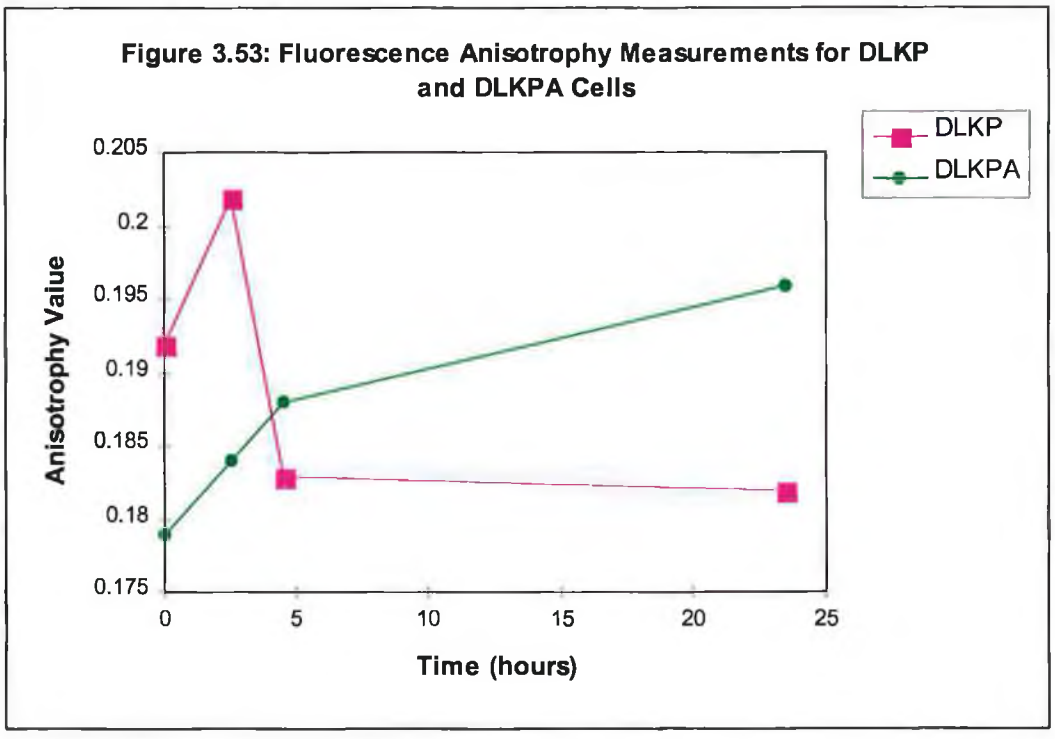

The $r_s$ value is seen to increase for CHRC5 cells at both 50 and 200rpm, most significantly at 200rpm. In contrast, the $r_s$ value for CHO-K1 decreases at 50rpm and more dramatically at 200rpm. Thus these results seem to confirm the conclusions from the high shear experiments. That is, PMF is decreased for CHRC5 cells in an agitated environment when compared to CHO-K1 cells. This decrease in PMF in CHRC5 cells is more evident at the higher agitation rate suggesting that the increased shear tolerance of these cells is due to the decreased PMF.

3.2.6.4: PMF Measurements for Another MDR Cell Line (DLKPA) in a High Shear Environment

In order to ascertain if the observed phenomenon of decreased PMF and increased shear tolerance noted in CHRC5 cells was a feature in other MDR cells, experiments were undertaken on DLKP and DLKPA cells. DLKP cells are derived from a human lung carcinoma and were established here in this laboratory (Grant, 1994). DLKPA are an MDR variant of DLKP selected for their drug resistance. The growth of these cells at 500 rpm over 24 hours was observed and the resultant PMF measured. Results are presented in Figures 3.52 and 3.53.

**Figure3.52: Viable Cell Growth of DLKP and DLKPA Cells at High Shear**






The experiment was repeated and the same trend was found. Results are present Figures 3.54 and 3.55.

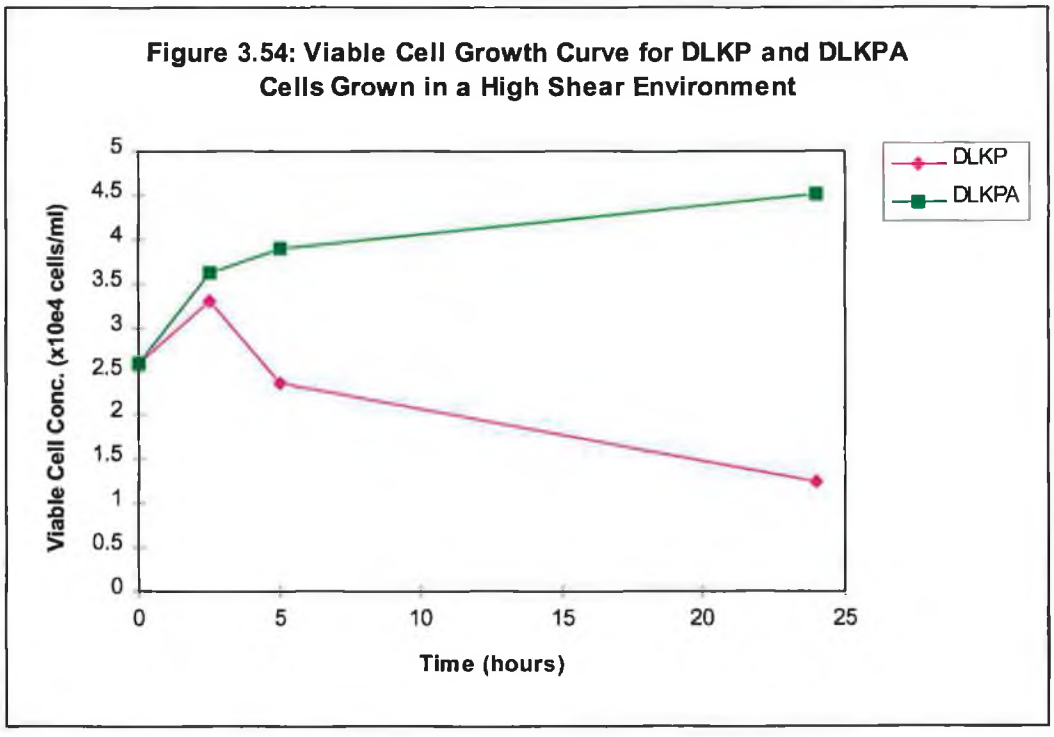
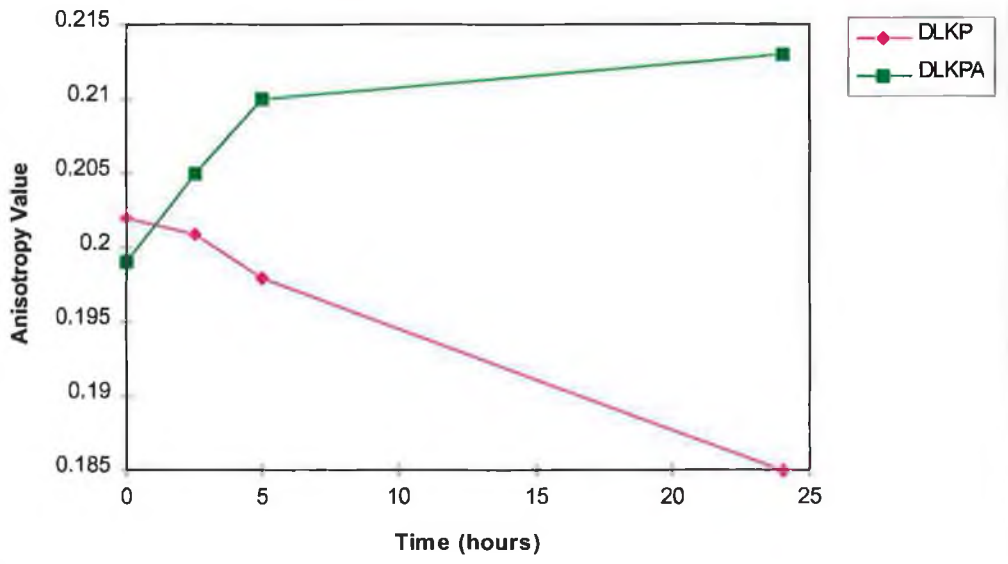


**Figure 3.55: Fluorescence Anisotropy Measurement for DLKP and DLKPA Cells Grown in a High Shear Environment**




It may be observed that DLKPA cells appear to be very shear tolerant. The growth curve experiments were carried out in a high shear environment (500rpm which as will be seen in the next section (3.2.7) corresponds to a very turbulent flow regime). CHRC5 cells although having an increased shear tolerance when compared to CHO-K1 cells were not able to grow at this agitation rate ( Section 3.2.6.2 ) and instead death rates were compared to assess agitation resistance. It can be seen in these experiments that DLKPA cells are not killed by this agitation rate and are even seen to replicate (Figures 3.52 and 3.54). In contrast the non-MDR, DLKP cells are sensitive to agitation at 500rpm and they die via approximately first order death rate kinetics. Thus it is very interesting to note that once more the MDR cells appear to have greater shear tolerance than their non-MDR counterparts. This is in agreement with the results from CHRC5 and Hep-2A multidrug resistant cells. PMF measurements show that the $r_s$ value increases for DLKPA cells and decreases for DLKP cells over the cultivation period at 500rpm. Consequently it may be stated that in a similar fashion to the CHRC5/CHO-K1 system, there is a strong correlation between decreased PMF and increased shear tolerance in DLKPA cells.

### 3.2.7: Defining The Hyrodynamic Shear Environment In Spinner Flasks

In all agitation experiments carried out prior to the work in this section, cells were grown in Techne spinner flasks. While these vessels provided efficient mixing of cultures and differences in agitation resistance between the MDR and non-MDR cells were observed using this spinner system there are a number of limitations to their use. These include the fact that the maximum agitation rate achievable is only 120 rpm which in terms of suspension cultures is only moderate agitation. Also these spinners do not have an impeller type agitator, instead a stirring rod with a bulbous end is used to mix cells. Consequently it is very difficult to quantify shearing forces present in the vessel since parameters which define shear stresses are based on impeller diameter .

The work to date has shown that CHRC5 cells have an increased resistance to relatively moderate agitation rates compared to CHO-K1 cells. Thus it was of interest in this work to examine the response of each cell line in terms of both viability and PMF to high shear rates. This work is presented in Sections 3.2.6.2 and 3.2.6.4 and is only possible by using Bellco spinners which have a maximum agitatation rate of 1200 rpm. Bellco spinners have a teflon coated agitator with a paddle impeller at the end of the shaft. The impeller is driven by a magnetic drive. Thus the use of Bellco spinners allows;

a) the response of CHRC5 and CHO-K1 cells to high agitation rates to be investigated
b) the calculation of important parameters which estimate the magnitude of shear stresses to which the cells may be exposed.

It is often difficult to determine quantitatively the magnitude of shear stresses cells experience in many bioreactors because the shear stresses are not uniformly distributed. The flow field in a bioreactor is inherently transient, inhomogenous and anisotropic. However estimations of Reynolds numbers and the Kolmogorov eddy length have been shown by previous work to correlate to cell damage from bulk liquid turbulence (Cherry and Papoutsakis, 1986; Croughan *et al*, 1987; Kunas and Papoutsakis, 1990).

Bellco spinners are glass vessels with side arms for sampling. The agitation system is composed of a fixed agitator shaft suspended from the top of the vessel with a teflon paddle type impeller (dimensions, diameter 4 cm and width 2 cm) at the end of the agitator. The impeller has a magnet at its centre and thus the vessel is placed on top of a magnetic drive motor to allow rotation of the impeller. Thus it is the rotation of the impeller alone which is mixing the fluid and not the whole stirring rod as in the Techne spinners. Given that impeller diameter is known, Reynolds number and Kolmogorov eddy length may be calculated for various agitation rates in the Bellco spinners. Table 6.31 in Section 6.8 of the Results section, presents data from work with the Bellco spinner flasks which calculates Reynolds number and Kolmogorov eddy length at various agitation rates.

In stirred vessels Reynolds number (Re) is defined as;

$$Re = \frac{D_i^2 N}{\nu}$$

where;

$D_i$ = impeller diameter (m)

$N$ = impeller speed ($s^{-1}$)

$\nu$ = kinematic viscosity

Reynolds number of 1000 or greater in a bioreactor generally characterise a turbulent flow regime (Nagata, 1975).

The Kolmogorov eddy length ($\eta$) is defined as;

$$\eta = ( \nu^3 / \varepsilon )^{1/4}$$

where;

$\varepsilon$ = the energy dissipation rate per unit mass ($cm^2/s^3$).

$$\varepsilon = \frac{N_p N^3 D_i^5}{V}$$

where;

$N_p$ = dimensionless power number (obtained from plots of $N_p$ versus impeller Reynolds number (Nagata, 1975) for unbaffled stirred tanks mixed with a two blade paddle. These plots are a function of the ratio of impeller diameter to vessel diameter (Di/D) and paddle width to impeller diameter ($B/D_i$)).

V = characteristic fluid volume ($cm^3$)

For the Bellco spinners used in these investigations the following applied;

$D_i$ = 4 cm

D = vessel diameter = 6 cm

B = paddle width = 2 cm.

$\nu$ = 0.01 $cm^2/s$ (water at 20°C)

Table 3.46 presents Reynolds number and the Kolmogorov eddy length calculated for different agitation rates in Bellco spinners.

Table 3.46: Reynolds Numbers and Kolmogorov Eddy Lengths for Various Agitation Rates in Bellco Spinners

| Agitation Rate (rpm) | Agitation Rate ($s^{-1}$) | Reynolds Number (Re) | Kolmogorov Eddy Length ($\eta$) | Energy Dissipation Rate ($\varepsilon$) | Power Number ($N_p$) |
|---|---|---|---|---|---|
| 40 | 0.67 | 1072 | - | | |
| 60 | 1 | 1600 | 173 | 11.2 | 0.7 |
| 80 | 1.33 | 2128 | | | |
| 100 | 1.67 | 2672 | 122 | 44.71 | 0.6 |
| 120 | 2 | 3200 | | | |
| 150 | 2.5 | 4000 | 95 | 125 | 0.5 |
| 200 | 3.33 | 5328 | | | |
| 500 | 8.5 | 13600 | 37-40 | 3930-4913 | 0.4-0.5 |

It can be seen that at 40 rpm Reynolds number is just above 1000 indicating that the fluid is just entering into the turbulent flow regime. At 60 rpm turbulent flow would still be in the transitionary phase and might not be fully developed. However for all agitation rates above 80 rpm it may be stated the spinners are operating in a fully turbulent regime. Thus all the hydrodynamic forces found in turbulent flow conditions may cause cell damage at agitation rates above 80 rpm. Cell size ranges from 10-20 μm, thus based on the calculations above, cell damage from Kolmogorov microeddies would not be relevant until agitation rates of 500 rpm and higher are used.

3.2.7.1: Comparison of Mixing Times for Techne and Bellco Spinner Flasks.

Reynolds numbers and Kolmogorov eddy lengths cannot be calculated for Techne spinners due to the agitation system used in these bioreactors. However most of the work in this thesis has been carried out in the Techne spinners. Given that the shear environment in the Bellco spinners has been quantitated to a certain extent it was decided to find some kind of correlation between the level of shear that cells would be exposed to in the Techne spinners to the levels they would be exposed to in Bellco spinners. The actual shear environment would be very different in each spinner since the agitation systems are quite distinct. However if the vessels are thought of as merely mixing vessels then a comparison of mixing times might allow some correlation between shear levels to be attempted. Figures 3.56 and 3.57 present results from experiments designed to determine mixing times in the Techne and Bellco spinners. Briefly, 100μl of an aqueous dye were injected through a very fine bore hypodermic needle into 100 ml of water. The time taken for complete dispersion of the dye was taken to be the mixing time at that agitation rate. While complete accuracy could not be guaranteed with this technique an approximate curve of time versus agitation rate was achieved.

**Figure 3.56: Mixing Times for an Aqueous Dye at Different Agitation Rates in a Bellco Spinner**

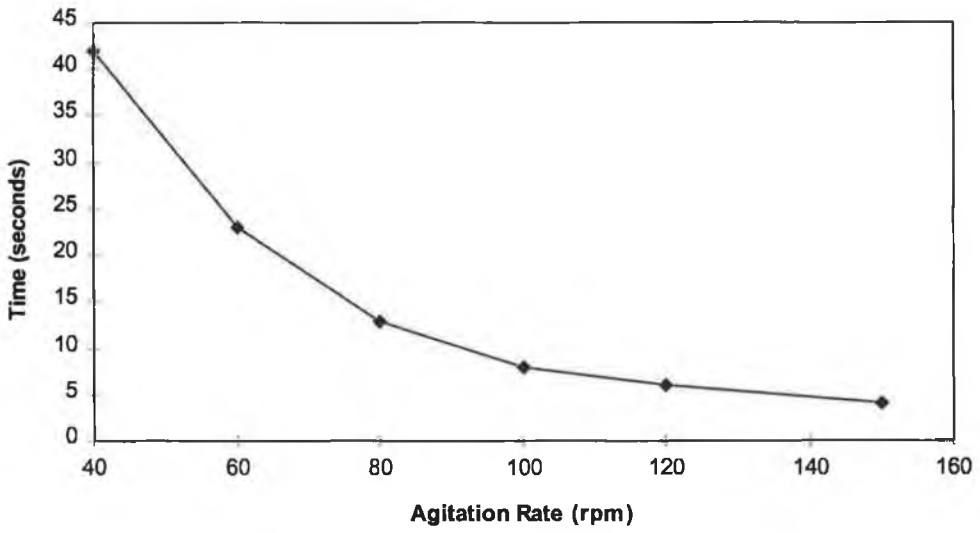
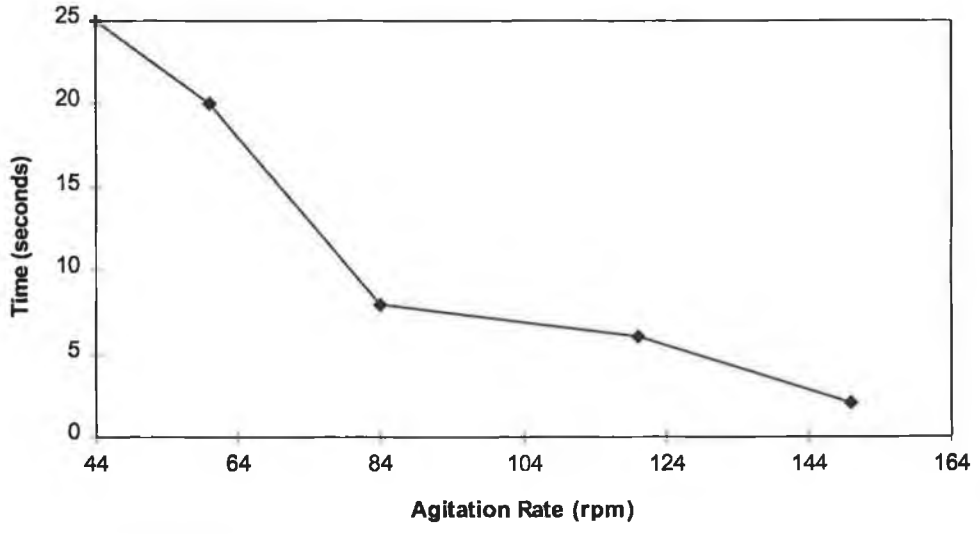



**Figure 3.57: Mixing Times for an Aqueous Dye at Different Agitation Rates in a Techne Spinner**



Based on these curves an approximate correlation between the time taken to mix 100µl of the dye in a Techne spinner and the time taken in a Bellco spinner can be made. Given that Reynolds number and Kolmogorov eddy length has been calculated for various agitation rates in the Bellco spinners then based on the mixing time correlation an estimate of these may be made for agitation rates in the Techne spinners. Table 3.47 presents these correlations.

Table 3.47: Correlation Between Mixing Times in Techne and Bellco Spinners - Relation to Reynolds Number and Kolmogorov Eddy Length

| Mixing Time (s) | Corresponding Agitation Rate in Techne Spinner | Corresponding Agitation Rate in Bellco Spinner | Reynolds Number (calculated in Bellco spinner) | Kolmogorov Eddy Length (calculated in Bellco spinner) |
|---|---|---|---|---|
| 25 | 44 rpm | 60 rpm | 1600 | 173 um |
| 8 | 84 rpm | 100 rpm | 2672 | 122 um |
| 6 | 120 rpm | 120 rpm | 3200 | n.d. |
| 2 | 150 rpm | 150 and above | 4000 | 95 um |

Note: Mixing times were taken from Figures 3.56 and 3.57.

It can be seen that the Techne spinners have a more efficient mixing systems at the lower agitation rates. A mixing time for 100µl of dye of 25 seconds is achieved at 44 rpm in the Techne spinners but the equivalent mixing time in the Bellco spinners is only achieved at the higher rate of 60 rpm. Similarly at 84 rpm the Techne spinners have a mixing time of 8 seconds whereas the Bellco spinners have to be agitated at 100 rpm to achieve the same mixing time. At 120 rpm both spinners have the same mixing time, above 150 rpm the mixing time becomes so short that it is not possible to record a value. Overall these results imply that mixing conditions are more intense in the Techne spinners at lower rpms, thus the shear stresses may also be greater.

## 3.2.8: FURTHER DISCUSSION OF RESULTS AND CONCLUSIONS

*In vitro* animal cell cultures are hosts for an array of commercially important biological products. Efficient production of these biologicals requires an understanding of the influence that the culturing environment has on cell growth and product yield. The general question posed by investigations into the growth of cells in large scale systems is, why do cells fail to grow beyond approximately $10^6$ cells per ml in batch culture (Newland *et al*, 1990). Studies are complicated by the interaction between energy substrates (glucose, glutamine), essential nutrients (amino acids), trace factors and inhibitory or toxic effects attributed to metabolic products (lactate, ammonia) generated chemically or resulting from environmental stress (shear, osmotic pressure, etc.). While there has been considerable effort at developing perfusion systems aimed at reducing some of these problems, regulatory and operational reasons still dictate batch or fed-batch operation be used in most industrial applications. The work described in this section focuses on the effect of environmental factors on the growth of cells in batch culture in small scale bioreactors. The influence of osmotic pressure and metabolite toxicity is investigated The effect of the hydrodynamic environment is examined for a number of cell lines, leading to the identification of two agitation resistant cell lines. The role that plasma membrane fluidity may play in determining the increased shear resistance of these cell lines is studied. Consequently this work aims to add to the general knowledge currently being accumulated in the literature on optimising the growth of cells in large scale culture.

### 3.2.8.1: Growth of MSV-3T3 Cells in Suspension Culture.

The growth of MSV-3T3 cells in suspension was investigated since earlier work (Section 3.1) had suggested that these cells produce a growth inhibitor (s) which needs to be concentrated (8x) before activity can be observed. Consequently large volumes of conditioned medium from the cells was required for processing of the product, indicating that growth of the cells had

to be scaled up from T flasks to a suspension, spinner flask system. Some basic experiments were undertaken to observe if the cells would grow in suspension and what would be the effect of agitation on cell growth. The initial work indicates that the cells do grow in suspension (Figures 3.14-3.16), however apparent growth rates are reduced with doubling times in the suspension culture half that in the monolayer culture. An appreciable lag phase is noted in the suspension culture, however cells in monolayer enter a decline phase after seven days with high levels of cell detachment whereas no decline phase is noted in the suspension culture after 14 days. Several authors have noted that cells can adapt to the shear generated in a agitated environment after an initial adjustment period (Peterson *et al*, 1988; Chittur *et al*, 1988). Overall it would seem that monolayer culture allows MSV-3T3 cells to grow well and with faster doubling times than suspension cultures. However for continued productivity of molecules of interest from MSV-3T3 cells (e.g. growth inhibitor) suspension cultures allow longer periods of sustained growth.

The effect of moderate agitation rates was investigated and very little difference in growth is observed between 25 and 40 rpm (Figure 3.17). Since it is now recognised in the literature that many suspension cells are in fact quite tolerant of normal to relatively high agitation levels (Cherry, 1993) it would appear that higher rates would have to be examined before a difference in growth might be observed. For the collection of conditioned medium this experiment confirmed that MSV-3T3 cells are capable of growing in suspension at moderate agitation intensities without affecting the either the growth of the cells or the production of the growth inhibitory factor of interest.

In the large scale culture of animal cells it is often noted that a decreased apparent growth rate is observed as initial cell density is decreased (Hu *et al*, 1985; Glacken *et al*, 1988). A simple experiment to investigate the effect of inoculum density size for MSV-3T3 cells in suspension was undertaken and

results indicate that a tenfold difference in inoculum appears to have no affect on the apparent growth rate (Figure 3.18). Again an appreciable period of adjustment to the agitated environment is apparent by both cultures ($1 \times 10^6$ inoculum and $1 \times 10^7$ inoculum), however the experiment was concluded too early and while inoculum density appears to have no effect on apparent growth rates in the exponential phase, it cannot be concluded whether it may affect the final cell density achievable.

A number of reports in the literature have indicated the importance of inoculum density on the growth and productivity of cells in culture (Ozturk and Palsson, 1990; Lauffenberger and Cozens, 1989). The question posed by the work asks are there limits on the inoculation size for cell growth in batch culture and are metabolic rates, growth and productivity affected by initial cell density ? Most of the work has linked initial growth rates with the serum concentration in the culture. The relationship between the initial growth rate and serum has been mathematically described by Monod kinetics (Dalili and Ollis, 1989; Glacken *et al*, 1989).

$$\mu = \mu_{max} \frac{S}{K_s + S}.$$

where S is the serum concentration. A more complicated model for inoculum cell density effects has been proposed by Lauffenberger and Cozens (1989) who link the initial growth rate to the presence of autocrine growth factors. The autocrine hypothesis was first suggested by Sporn and Todaro (1980) and describes autocrine growth factors as factors produced by a cell for which the cell itself has receptors. The overexpression of autocrine growth factors has been implicated in the onset of growth controlled diseases such as cancer. A mathematical model for the production, diffusive transport and binding of autocrine growth factors to cell surface receptors, coupled to a model for dependence of cell proliferation on growth factor receptor binding is proposed by Lauffenberger and Cozens to predict the initial cell growth rate as a

function of inoculation cell density. This model has been fitted to experimental data from Hu and co-workers (1985).

Overall this small section of work on MSV-3T3 confirmed that these cells could be cultured in suspension without adversely affecting growth or production of the growth inhibitory factor. This work also allowed an initial investigation into the effect of the external environment on cell growth in suspension cultures, thus providing a starting point for the work that was undertaken for the rest of the thesis.

3.2.8.2: Multi-Drug Resistance and the Investigation of MDR Cell Lines in this Thesis.

Multi-drug resistance (MDR) is a phenomenon whereby variants of cells become cross resistant to different sets of chemotherapeutic drugs, which are very different in structure and may act in the cell by a totally distinct mechanism. MDR arises by either the adaption of drug sensitive cells to progressively increasing concentrations of drug or by transfecting sensitive cells with the cDNA coding for the MDR-1 gene. MDR occurs *in vivo* during the treatment of some cancers where tumours initially responsive to chemotherapeutic drugs become increasingly resistant. Thus MDR present tremendous problems for the successful treatment of cancer by chemotherapy, consequently a huge body of work has been carried out in recent years to try to elucidate mechanisms of MDR and to develop methods of overcoming this phenomenon.

The primary mechanism of MDR is thought to be due to the overexpression of p-glycoprotein, a plasma membrane efflux pump with ATPase activity and a molecular weight of 170 kdaltons (Clynes *et al*, 1992). High levels of p-glycoprotein confers cross-resistance to a broad range of chemically and mechanistically unrelated drugs. The protein acts by promoting the efflux of drug resulting in a decrease in intracellular drug concentration. The gene

coding for p-glycoprotein is MDR-1, a member of a small family of MDR genes. Overexpression of p-glycoprotein is frequently but not always associated with amplification of the MDR-1 gene. The action of p-glycoprotein in effluxing drug from cells can be circumvented by certain drugs (many are calcium channel blockers, e.g. verapimil) which bind to the membrane and inhibit p-glycoprotein activity. Overexpression of p-glycoprotein is not the only mechanism of MDR, it has been found that even in cells overexpressing p-glycoprotein other mechanisms of resistance may co-exist within the same cells. Cytochrome P450 enzymes necessary for xenobiotic metabolism may play a role in MDR as can topoisomerases which are involved in the topological and conformational changes in DNA molecules required during DNA replication and transcription (Clynes *et al*, 1992).

Recent work in this laboratory has suggested that MDR cell lines show altered sensitivity to physio-chemical treatment, being more resistant to membrane rupture by sonication or hypotonic treatment but more sensitive to trypsinisation and cryopreservation techniques (Redmond *et al*, 1990; Clynes *et al*, 1992). The primary aim of this section of the thesis is to investigate the effects of the physical external environment on the growth of animal cells *in vitro*. The results presented thus far have consisted of a preliminary examination of the effects of the external environment. However given the interesting results suggesting that MDR cells might have an altered response to physical factors, it was decided to focus the work a little more by looking at the effect of the external environment on an MDR cell line and its parental cell line. By utilising this approach, the main aims of the thesis will be fulfilled and some clues may be gathered as to why MDR cells might behave differentally than their parent cells when exposed to physical forces. Basically the question being posed is how do physical forces and chemical levels affect the growth of an MDR cell line and its drug sensitive parent given that there is evidence to suggest that MDR cells respond differently to physio-chemical factors.

Agitation and osmotic pressure were the physical parameters chosen to be examined. Sonicating forces are basically shearing forces. A sonicator disrupts cells by creating vibrations which cause mechanical shearing of the cell wall/membrane. In order to have maximal shearing, it is often necessary to tune the sonicator to achieve maximal agitation (Bollag and Edelstein, 1991). Agitation in bioreactors generates shear stresses, thus given the observed resistance of MDR cells to sonication, the choice of agitation as a parameter to investigate is an obvious one. Osmotic pressure if increased causes hypertonic conditions causing the cell volume to decrease, hypotonic conditions can cause cell swelling. It had been already been shown that MDR cells are more resistant to hypotonic treatment therefore it would be of interest to investigate this phenomenon further.The chemical parameters to be investigated were chosen to be ammonia and lactate as these metabolic waste products may accumulate to quite toxic levels in batch cultures.Since the overall aim of this work is to investigate the growth of animal cells in large scale and in particular inhibitory effects caused by the culturing environment, identification of cell lines which may exhibit superior resistance to some of the growth limiting parameters (particularly agitation) would be of both academic and industrial interest.

### 3.2.8.3: Effect of Osmotic Pressure, Ammonia and Lactate on Growth of an MDR Cell Line (CHRC5) and its Drug Sensitive Parent Cell Line (CHO-K1).

CHO-K1 (drug sensitive) and CHRC5 (drug resistant) cells were chosen for this work. The Chinese Hamster Ovary (CHO) cell line is one of the most commercially important cell lines. Processes using these cells are licensed to produce important recombinant therapeutic proteins including Hepatitis B surface antigen and tissue plasminogen activator (Manos, 1988). CHO cells are extremely robust (Gottesman, 1985) and are adaptable from anchorage dependent to anchorage independent growth (Stanley *et al*, 1975). CHRC5

cells are an MDR variant of CHO which have acquired drug resistance by clonal selection after long term exposure to cytotoxic colchine. (Ling and Thompson, 1974). Thus work with these cell lines allows examination of an industrially relevant cell line (CHO-K1) and its MDR counterpart (CHRC5). Confirmation of the drug sensitivity of CHO-K1 and the drug resistance was undertaken. Results showed that CHO-K1 were sensitive to adriamycin whereas CHRC5 displayed resistance to the drug (Table 6.21). The $IC_{50}$ of CHRC5 to adriamycin was found to be between 8-9 μg/ml which is higher than the previously reported 3.9 μg/ml (Redmond, 1991).

*3.2.8.3.1 : Osmotic Pressure*

It is widely reported that animal cells are sensitive to changes in medium ionic strength and osmotic pressure (Oyaas *et al*, 1989,1994; Ozturk and Palsson, 1992; Oh *et al*, 1995). The osmolality of serum is 0.29 osm/kg and thus the ideal osmolality range for cell growth is 0.28-0.32 osm/kg. Extremes of osmolality have been shown to be detrimental to cells (Oyaas *et al* , 1989).The effect of both hyper-osmotic pressure and hypo-osmotic pressure on CHO-K1 and CHRC5 cell growth was examined by either adding sodium chloride (hyper-osmolality) to the cells or diluting the growth medium (hypo-osmolality) of cells, growing in a monolayer based assay system.

Addition of NaCl to CHO-K1 cells does not affect cell growth up to 40mM NaCl, corresponding to an osmolality of 0.38 osm/kg (Figure 3.19). 65% growth inhibition was noted at 80mM NaCl (0.445 osm/kg) and above 100mM NaCl, when osmolality values are above 0.47 osm/kg, very little cell growth is observed. In contrast, CHRC5 cells tolerate up to 100mM NaCl (osmolality, 0.42 osm/kg). Above 140mM greater than 89% growth inhibition is observed (Figure 3.20). CHO-K1 cells are grown in Hams F12 medium which has a osmolality value of 0.3 osm/kg whereas CHRC5 cells are grown in MEMα medium, with an osmolality value of 0.27 osm/kg. Hence NaCl solutions in Hams F12 medium result in slightly higher osmolality values than

the corresponding NaCl concentration in MEMα medium. However a significant difference in tolerance to higher osmotic pressure can still be noted between the MDR cells, CHRC5 and the non-MDR cells, CHO-K1. CHRC5 cells are resistant to approximately 0.42 osm/kg whereas CHO-K1 cells are resistant to 0.38 osm/kg.

Given that the optimum range for osmotic pressure is 0.28-0.32 osm/kg, the fact that the MDR (CHRC5) cells are resistant to an osmolality value (0.42 osm/kg), 0.1 osm/kg greater than the maximum ideal value is important. A number of workers have reported an osmolality of 0.38 osm/kg as the critical value above which the growth rate of cells is decreased. Ozturk and Palsson (1991) studied the effect of increased osmolality using both ionic compounds (NaCl and PBS) and non-ionic agents (sucrose) on the growth of hybridomas and noted decreased apparent growth rates at osmolality values above 0.38 osm/kg. Wentz and Schurgerl (1992) examined the effect of osmotic stress on BHK cells using NaCl to increase osmolality and also reported that osmolality values above 0.38 osm/kg were inhibitory. Thus based on the literature, CHO-K1 cells agree with previous results and are growth inhibited above values of 0.38 osm.kg. However CHRC5 cells display increased resistance and only become growth inhibited above 0.42 osmkg.

It is proposed that increases in external osmolality may enhance transport of nutrients, in particular amino acids, into cells since an increased uptake of amino acids via $Na^+$-dependent transport systems has been observed (Oh *et al*, 1993). These amino acids may act as osmoprotectants since it is suggested that animal cells protect themselves from high external osmotic pressure by seeking to increase their internal osmotic pressure to balance the external value. Osmoprotective agents such as sugars, polyhydric alcohols, free amino acids, methylamines and urea accumulate inside cells exposed to high osmotic pressure (Oyaas *et al*, 1994). The work presented here suggests that CHRC5 cells might be more efficient at regulating their internal osmotic pressure than the non-MDR cell line, CHO-K1. It is unclear why this should be so since if

p-glycoprotein is the causative agent of MDR CHRC5 cells might be expected to efflux osmoprotective agents at a higher rate than CHO-K1 and so be more sensitive to hyper-osmotic pressure. Indeed previous work has suggested that MDR cells are sensitive to changes in osmotic pressure (O'Riordan and Ling, 1985). Recent work has shown that p-glycoprotein is an essential component of a volume regulated chloride channel (Valverde *et al*, 1992). Perhaps it is due to the overexpression of p-glycoprotein in MDR cells that CHRC5 cells respond differently to hyper-osmotic pressure.

Hypo-osmolality in culture medium causes cells to swell and if severe enough to burst. The effects of hypo-osmotic pressure on CHO-K1 and CHRC5 cells was investigated by diluting the medium with water, measuring the subsequent osmolality value and observing the effect on cell viability over a short time period. Some difference in response was noted, CHO-K1 cells were only 50% viable in medium of 0.1 osm/kg after three days whereas CHRC5 cells were 96% viable, however cell number in the CHRC5 was only 8% of the control culture, as opposed to 34% for CHO-K1 cells (Tables 3.24 and 3.25). CHRC5 cells were completly killed by medium osmolality of 0.06 osm/kg whereas CHO-K1 cells remained 33% viable at 0.07 osm/kg. These results suggest that CHO-K1 cells can withstand quite dramatic drops in external osmolality whereas CHRC5 cells are completely killed at very low osmotic pressure. The low cell number but high viability of CHRC5 at 0.1 osm/kg suggests that most cells are destroyed at this osmolality but any cells surviving are adapting to the hypo-osmotic conditions. Work with Erlich ascites mouse tumour cells has shown that exposure to hypo-osmotic media changes the surface membrane permeability to $Na^+$, $K^+$, $Cl^-$ and amino acids (Hoffman, 1980).

Overall the results from the effect of hyper- and hypo-osmotic pressure on CHO-K1 and CHRC5 cells suggest that the MDR cells might be more resistant to increases in osmotic pressure and more sensitive to decreases in osmotic pressure than their non-MDR parents. However this work would need

to be repeated since both the nature of hyper-osmolality protection by cells and the mechanisms of MDR suggest that MDR cells should be quite sensitive to increases in osmolality.

*3.2.8.3.2: Ammonia*

Glutamine is an essential component of animal cell growth and is present at high concentrations. Chemical and metabolic breakdown of glutamine leads to the formation of ammonia, both in the form of $NH_3$ and as the ammonium ion, $NH_4^+$. At pH 7, approximately 97% of ammonia is present as $NH_4^+$. Ammonia accumulates in batch culture and has been found to be toxic at concentrations above 4mM although great variation in ammonia tolerance has been found in cells (Ozturk, Riley and Palsson, 1992). The effect of the ammonium ion (in the form of $NH_4^+Cl$) on the growth and viability of CHO-K1 and CHRC5 was examined.

Ammonium chloride up to 20 mM concentration was shown to have no effect on either CHO-K1 or CHRC5 cells (Tables 3.28 and 3.29). Thus higher concentrations were tested and the resultant $IC_{50}$ values for CHO-K1 and CHRC5 cells were estimated to be 30 mM and 42 mM ammonium chloride respectively (Figure 6.21). These values are exceptionally high since most cell lines are inhibited by ammonia concentrations in the range of 2 - 8 mM (Glacken *et al*, 1986; Ozturk, Riley and Palsson, 1992; Reuveny *et al*, 1986). However it has been found that HeLa cells can survive at a $NH_4Cl$ concentration of 30 mM (Glacken, 1983) and there is a report of mouse ascites tumour derived cells showing no growth inhibition at 40 mM $NH_4^+Cl$ (Hu, 1991). As can be seen, CHRC5 cells have a slightly greater resistance to ammonium than their parental cells, CHO-K1.

The toxic effect of ammonia is thought to be due to a combination of mechanisms (Karin and Mintz, 1981; Glacken *et al*, 1988; M$^c$Queen and Bailey, 1990, 1991; Newland *et al*, 1994). It is postulated by Martinelle and

Haggstrom (1993) that one important toxic effect of the ammonium ion is to cause an increased demand for maintenance energy due to the need to maintain the ion gradient over the cytoplasmic membrane. The $NH_4^+$ ion is thought to compete with the $K^+$ ion for transport into the cell and may thus decrease the intracellular $K^+$ concentration, which is an important factor for many enzymes and for the potassium gradient over the cytoplasmic membrane. Free ammonia may also cause an increase in the intracellular pH when it diffuses through the membrane by associating with $H^+$ ions, subsequently disrupting intracellular enzymes, especially lysosomal enzymes which require an acidic pH for optimum activity.

Perhaps the increased ammonia resistance of CHRC5 cells might be related to intracellular pH. It has recently been shown that some MDR cell lines have higher intracellular pH and an increased $Na^+/H^+$ antiport activity (Boscoboinik *et al*, 1990). These workers found that the intracellular pH of CHRC5 cells is 7.18 while that of Aux-B1 (CHO auxotropic variant) is 7.01. Because of the higher intracellular pH of CHRC5 cells (and other MDR cells) it could be speculated that less $H^+$ ions would be available to associate with the $NH_3$ and thus the rise in intracellular pH and subsequent metabolic disruption would not be as great for the MDR cell lines as for the parental cell lines. In addition MDR cells have been shown to contain lower lysosomal enzyme levels than their drug sensitive counterparts (Warren, 1991).

*3.2.8.3.3: Lactate*

Lactate is the primary waste metabolite produced from glucose metabolism due to the oxygen limiting conditions in culture (Glacken, 1986). It has been reported that 80% of glucose in HeLa cells is converted into lactate (Newland *et al*, 1990). Lactate may accumulate at concentrations in excess of 35 mM in batch culture (Ozturk, Riley and Palsson, 1992). The tolerance of cells to lactate, similarly to ammonia, varies from one cell line to another, however sensitivity to lactate in the range of 40 mM to 60 mM has been reported (Glacken *et al*, 1986; Miller *et al*, 1988; Ozturk, Riley and Palsson, 1992). The tolerance of CHO-K1 and CHRC5 to lactate was investigated in this work.

It was found that CHO-K1 cells are only growth inhibited at lactate concentrations which increase the osmolarity of the medium to beyond the tolerance level for CHO-K1. Thus at concentrations up to 35 mM lactate no growth inhibition is noted (Figure 3.22). The osmolality of the medium at 40 mM lactate is 0.362 osm/kg (Table 3.30) which has been shown previously to not cause growth inhibition in these cells (see Section 3.2.8.3.1). Growth inhibition of CHO-K1 cell growth is seen at 87 mM lactate, however the osmolality of 70 mM lactate is 0.422 osm/kg indicating that at 87 mM lactate and above the osmolality of the medium is too high to support cell growth in this cell line. The upper limit of osmolality tolerance for CHO-K1 cells is 0.38 osm/kg (Figure 3.19). Thus the results from this work with CHO-K1 cells supports the work of Ozturk, Riley and Palsson (1992) and Schumpp and Schlaeger (1992) which suggests that cells are sensitive to lactate concentrations in the range of 40 to 60 mM and that lactate toxicity is due to lactate increasing the osmolality of the medium beyond a tolerant level.

In contrast CHRC5 cells exhibit a very different response to increasing lactate concentration. Figure 3.23 indicates that every concentration of lactate tested (3.5 mM to 348 mM) causes a dramatic inhibition of CHRC5 cell growth. The

proposal that lactate toxicity is caused by increasing osmolality does not agree with the results from the lower lactate concentrations. Previous work has shown that CHRC5 cells have quite a high tolerance to increases in medium osmolality and growth inhibition due to excessive osmotic pressure is only observed at osmolality values above 0.42 osm/kg (Section 3.2.8.3.1). Thus for the lactate concentrations tested, growth inhibition due to increases in osmolality should only be seen at lactate concentrations above 87 mM. At 3.5 mM to 35 mM lactate, medium osmolality is within the tolerance level of CHRC5. However very little cell growth is seen at any lactate level.

Other mechanisms of lactate toxicity, besides osmolality increases, have been suggested. Dean *et al*, (1984) have proposed that lactate may cause pH perturbation of electrochemical gradients which would affect all membrane transport. Lactate may also act as a potent calcium chelator (Glacken *et al*, 1988) and this property might explain why the MDR cells here are affected so dramatically. As mentioned earlier overexpression of the efflux pump, p-glycoprotein has been determined to be the main mechanism of MDR in cells. However MDR in cells can be circumvented by the addition of certain drugs many of which are calcium channel blockers. For example, a calcium channel blocker, verapimil has been shown to bind to p-glycoprotein and inhibit its efflux activity (Clynes, 1992). Given that p-glycoprotein is a membrane pump and that its activity can be inhibited by calcium channel blocking agents, it is not unreasonable to assume that calcium may be essential for the p-glycoprotein activity. Lactate has potent calcium chelating properties thus when lactate is added to MDR cells it may remove calcium essential for the efflux activity of p-glycoprotein operation and thus render the cell sensitive to toxic agents (lactate in this case).

Another possible mechanism of lactate toxicity lies in the fact that p-glycoprotein is an ATP driven pump. The energy cost of ATPase activity is high, it can account for 50% of energy production in some cells (Newland *et al*, 1994). Thus in MDR cells high glycolytic rates are necessary to maintain

sufficient energy provision for ATPase activity. Addition of external lactate may inhibit glycolysis and thus inhibit growth of the MDR cells.

Overall the fact that very low concentrations of lactate seem to inhibit CHRC5 cells has several processing consequences. For example, cells should not be grown for prolonged periods in batch phase and medium should be changed frequently to avoid excessive lactate build up.

### 3.2.8.4: Effect of Osmotic Pressure, Ammonia and Lactate on Growth of Hep-2 and Hep-2A Cells

The investigations discussed in Section 3.2.8.3 above have yielded some interesting results. Overall there would appear to be some differences in the response of the MDR cell line CHRC5 to several physical/chemical factors when compared to its drug sensitive counterpart. The MDR cells appear to be more tolerant of higher osmotic pressure and ammonia levels but extremely sensitive to lactate. In order to ascertain whether these observations might apply to MDR cells in general or might be specific to the CHRC5/CHO-K1 system only it was decided to investigate the effect of the same parameters on the growth of another MDR cell line. Hep-2A cells are the MDR variant of Hep-2 cells, a human larynx carcinoma cell line. The Hep-2A cell line was established after long term exposure to increasing concentrations of the chemotherapeutic drug, adriamycin (Redmond, 1991). It is of relevance to study another MDR cell line and its parent which are quite different in origin to the CHO cells just studied. Hep-2A cells differ from CHRC5 cells in that they are of human origin and are relatively recently adapted to have drug resistant properties (three years at the time of the work as opposed to 20 years for CHRC5 cells).

*3.2.8.4.1: Osmotic Pressure*

The effect of increasing osmotic pressure on Hep-2 and Hep-2A cell growth yields similar growth profiles (Figure 3.25). Both cell lines are resistant to very high medium osmolality values, with no significant decrease in growth up to 0.480 osm/kg (100mM NaCl). Above 0.590 osm/kg (150mM NaCl) Hep-2 and Hep-2A are growth inhibited. The similarity in the growth response of the MDR and non-MDR cells to increasing osmotic pressure does not agree with the results found with CHRC5 and CHO-K1. In those cells, CHRC5 cells displayed a higher resistance to external osmolality with no effects on cell growth up to 0.42 osm/kg while for CHO-K1 this figure was 0.38 osm/kg. Most workers quote the osmolality value of 0.38 osm/kg as the maximum allowable for cell growth (Ozturk and Palsson, 1991; Wentz and Schugerl, 1992). Thus Hep-2 cells appear to have quite a high tolerance to osmotic pressure and there is no real difference between the MDR variant and the parental cell. The results from this work and the CHO-K1/CHRC5 cells would suggest that the increased tolerance of the CHRC5 cells is a characteristic of those cells only and not a general property of MDR cells.

*3.2.8.4.2: Ammonia*

The effect of ammonia, in the form of ammonium chloride, was examined for Hep-2 and Hep-2A cells. Although concentrations of ammonium greater than 4 mM are generally reported to be toxic (Schumpp and Schaelger, 1992) the tolerance of CHO-K1 and CHRC5 cells was shown to be much higher with $IC_{50}$ values of 30 and 42 mM $NH_4Cl$ respectively. Thus the effect of ammonium in the range of 0 to100 mM was investigated on Hep-2 cells. Figures 3.26 and 3.27 show that the results obtained are a little unusual. If viable cell number only is considered then an expected death rate curve, with almost first order kinetics is observed (when the growth curves are plotted on a semi-logarithmic scale a near linear response is noted) for both cell lines. If $IC_{50}$ values are extrapolated from the viable cell number curve and taken to be

the ammonium concentration at which there is a 50% reduction in cell number, then the $IC_{50}$ for Hep-2 is approximately 19 mM $NH_4Cl$ and 25 mM $NH_4Cl$ for Hep-2A cells. Thus a small difference in the resistance of the cells to ammonium exists with the MDR cells again exhibiting increased ammonium tolerance. Overall the tolerance of these cells to ammonium is not as great as for CHO-K1 and CHRC5 cells.

However, the interpretation of these results is not complete without considering the viability of the cells at each ammonium concentration. For Hep-2 cells, Figure 3.26 shows that while viable cell number decreases exponentially, % viability remains above 88%. Similarly Hep-2A viability remains above 96% up to 50 mM $NH_4Cl$ and falls to 50% at 100 mM $NH_4Cl$. Two suggestions may be proposed to explain these results. The high viability might mean that a subpopulation of each cell line is adapting to the high ammonia environment. Alternatively, perhaps these results indicate that dead cells are lysing very quickly after cell death occurs and are therefore not detected by the viability measurement method, trypan blue exclusion. This dye is excluded by viable cells which remain white under microscopic examination, dead cells are classified as those with damaged membranes and thus dead cells cannot exclude the dye and stain blue. If complete cell lysis is occurring soon after cell death then trypan blue staining will not allow for these lysed cells to be enumerated and thus % viabilties will remain artificially high.

Overall these results seem to agree with those for the other MDR cell line and its parent, CHRC5 and CHO-K1, in that the MDR cells (Hep-2A) are slightly more resistant to ammonia than the non-MDR cells. Reasons as to MDR cells might have an increased ammonia resistance have already been discussed (Section 3.2.8.3.2). However given the unusually high viabilties obtained and the relatively small difference in ammonia tolerance found here it is not possible to conclude from these results whether increased ammonia tolerance

is as a consequence of MDR or is just a specific characteristic for the two cell systems studied in this work.

*3.2.8.4.3: Lactate*

The effect of lactate on Hep-2 and Hep-2A cell growth was examined and the results obtained are very interesting. Hep-2A cells are very sensitive to all concentrations of lactate tested (Figure 3.28). Growth of Hep-2A at 70 mM sodium lactate inhibits the cells by 80% when compared to growth in lactate free medium. Above 140 mM lactate very little Hep-2 growth is observed. In contrast, Hep-2 cells grow well at 70 mM lactate with no growth inhibition observed. At 140 mM cells are somewhat growth inhibited with 43% of control growth observed. Given that lactate appears to cause growth inhibition by increasing the osmolality of the medium ( Ozturk, Riley and Palsson, 1992) the osmolality of the lactate solutions was measured (Table 3.38). The osmolality of 70 mM lactate is 0.425 osm/kg which is outside the recommended osmolarity range of 0.28-0.32 osm/kg. However in work discussed earlier it was found that both Hep-2 and Hep-2A cells grow well at osmolalities up to 0.48 osm/kg (Section 3.2.3.1). It can be seen that Hep-2 cells grow well at 70 mM lactate and are not growth inhibited. The osmolality of 140 mM lactate is 0.520 osm/kg and Hep-2 cells grow less at this level. Thus at lactate concentrations above 140 mM it may be stated that the resulting osmolalities is beyond the range within which Hep-2 and Hep-2A cells can grow and thus high osmotic pressure is responsible for the observed growth inhibition. Below 140 mM lactate elevated osmolarity is not causing growth inhibition as can be seen from the Hep-2 cells. Thus the growth inhibition observed in Hep-2A cells at 70 mM lactate must be due to some other mechanism. This result is interesting since the same trend was found in CHRC5 cells, that is the MDR cells (both Hep-2A and CHRC5) are extrememly sensitive to lactate at any concentration. As discussed earlier this phenomenon may be due to the calcium chelating properties of lactate (see Section 3.2.8.3.3).

*3.2.8.4.4: Conclusions*

Overall this work investigating the effects of external factors on the growth of Hep-2 and Hep-2A suggests that general statements concerning the physical properties of MDR cells cannot be made from studies on just one MDR cell system. The work with CHRC5 and CHO-K1 suggested that the MDR cells are more resistant to osmotic pressure and ammonia, while much more sensitive to lactate. This work shows that there is no difference between Hep-2 and Hep-2A in their response to osmotic pressure. Hep-2A appear to be slightly more resistant to ammonia than Hep-2. However the finding that Hep-2A are very sensitive to lactate, while Hep-2 cells are not, might indicate that lactate sensitivity might be a property of MDR cells. In conclusion this work highlights that the external environment of animal cells growing *in vitro* must be considered when attempting to optimise the growth of cells since both physical and chemical factors may inhibit growth.

3.2.8.5: The Effect of Agitation Intensity on the Growth of CHO-K1 and CHRC5 Cells.

The main aim of this thesis is to examine factors that limit the growth of animal cells *in vitro*. One of the main limiting factors in large scale culture is the detrimental effect of shear on cell growth. Shear is generated in suspension cultures by agitation of the culture which causes deformation of fluid elements resulting in hydrodynamic shear stresses being transmitted to cells. Animal cells lack a protective cell wall and are particularly sensitive to shear stresses. The effect of agitation intensity is examined in this thesis. Given that the work preceding this section has considered the effect of environmental factors on cell growth with respect to a 'normal' cell line and a drug resistant (MDR) cell line, the same strategy is adopted for the investigations on shear sensitivity. The reasoning for comparing an MDR cell line and its drug sensitive parent

for shear sensitivity is a valid one since previous work has suggested differences in the physical robustness of MDR cell lines and their parent cells. Higher sonicating forces are required to lyse MDR membranes than non-MDR cells (Redmond *et al*, 1990). Sonication lyses cells by creating vibrations which cause mechanical shearing of the cell wall/membrane. The results from studies investigating the effect of agitation intensity on the growth, viability and metabolic rates of CHO-K1 (non-MDR) and CHRC5 (MDR) are discussed in this section.

*3.2.8.5.1: Effect of Agitation on CHO-K1 and CHRC5 Cells*

CHO-K1 cells were grown at a variety of agitation rates, with the best growth achieved in the 40 rpm culture (Figure 3.30). While cell number increased ten fold in the 40 rpm over the five day culture period, only a three fold increase in cell number is seen in the 120 rpm culture. In contrast, the CHRC5 cells grow well at 120 rpm with growth comparable to and even better than at 40 rpm (Figure 3.31). Table 3.42 compares apparent growth rates and doubling times for both cell lines at 40 and 120 rpm. It can be seen that $\mu_{app}$ for CHRC5 cells at 120 rpm is 0.42 $hr^{-1}$ as opposed to 0.014 $hr^{-1}$ for CHO-K1 cells. Thus the initial conclusions from this work is that the MDR cells appear to have an increased resistance to shear stresses produced by agitation in the spinner flasks used in these experiments.

When these experiments were repeated similar results were obtained. CHO-K1 cells were grown at 0, 40 and 120 rpm with maximum cell number and the highest apparent growth rate in the 40 rpm culture (Figure 3.32, Table 3.43). CHRC5 cells again grew very well at 120 rpm even though shear forces resulting from agitation are increased at this rate (Figure 3.33). Both cell lines grew poorly at 0 rpm the stationary spinner culture. However cell clumping was observed from day 2 onwards with clump size increasing throughout the culture period. Clumping probably occurred due to the lack of mixing in the spinner vessel. Also, given that CHO cells will grow preferentially in an

anchorage dependent mode, if there is no attachment substratum then cells will attach to each other thus forming clumps. Renner *et al* (1993) have reported that cell clumping can lead to problems of nutrient limitation for cells in the inside of the clumps. It was observed that the culture medium of the 0 rpm culture for both cell lines changed colour during the culture period indicating a decrease in pH. This is probably due to oxygen limitation problems for cells growing in the inside of the clumps leading to the formation of lactate through anearobic glycolysis. Results for lactate accumulation from these cultures confirmed that high levels of lactate were produced (results not presented). The release of DNA from lysed cells is reported to increase the likelihood of clump formation due to the 'sticky' nature of DNA promoting the binding of cells together. The addition of DNAse to CHO cultures has been shown to reduce clump formation and greatly increase the viabilty of the cultures (Renner *et al*, 1993). Thus for future work where cell clumping is a problem it would be suggested to include DNAse into the culture medium.

The growth of CHRC5 cells at 120 rpm is much better for these cells with an apparent growth rate of 0.029 $hr^{-1}$ ($t_d$ = 24 hours), than for CHO-K1 cells at the same agitation rate when $\mu_{app}$ is 0.017 $hr^{-1}$ ($t_d$ = 41 hours). Cell viabilites for CHRC5 cells remained above 93% for both the 40 rpm and 120 rpm culture. Thus these experiments suggest that CHRC5 cells have increased resistance to higher agitation intensities. In addition these results lend weight to the proposal that MDR cells might be more physically robust than their non-MDR parents.

*3.2.8.5.2: Effect of Serum on Growth of CHO-K1 and CHRC5 Agitated Cultures*

Serum is an undefined blood fraction necessary for the growth of animal cells *in vitro*. In recent years it has been recognised that serum plays an important role as a shear protective agent in agitated cultures (Papoutsakis and Kunas,

1989; Kunas and Papoutsakis, 1990; Michaels *et al*, 1991). How serum protects cells from shear is as yet unclear however it is thought that it may have both a physical and biological role (Michaels *et al*, 1991). In order to examine further the observed increased agitation resistance of CHRC5 cells the effect of high and low agitation on both cell lines in medium with 5% serum and 1% serum was examined. CHO-K1 cells grew optimally at 40 rpm, 5% serum with an apparent growth rate of 0.018 $hr^{-1}$ (Figure 3.34). The growth rate of the 120 rpm, 5% serum culture is lower at 0.007 $hr^{-1}$ while growth rates at 1% serum for both agitation rates are very much reduced. CHRC5 cell growth at 120 rpm, 5% serum was again found to be greater than for CHO-K1 cells in these conditions (Figure 3.35). CHRC5 growth at 40 rpm, 5% serum is unusually low, however quite high levels of cell clumping was observed in this culture, especially from day 4 onwards. Thus cell counts might not have been accurate for this culture and cell number was probably underestimated. As mentioned earlier, any future work might require DNAse additon to avoid problems of cell clumping. While growth at 1% serum is very much reduced for both cell lines when compared to growth at 5% serum, some interesting observations may be made. Overall growth of CHO-K1 in the lower serum level is less than that of CHRC5 cells at both 40 and 120 rpm. Maximum cell densities for CHO-K1 at 40 rpm, 1% serum is 15 x $10^5$ cells/ml and is 18 x $10^5$ for CHRC5 cells under the same conditions. Even more interesting is the result for CHO-K1 at 120 rpm, 1% serum when maximum cell density obtained is 8.3 x $10^5$ compared to 19.1 x $10^5$ cells/ml for CHRC5 at the same conditions. Thus the increased agitation resistance of CHRC5 cells may be observed even under the stressful conditions of low serum levels. It is difficult to ascertain from these results whether serum is protecting cells from agitation induced shear or if the poor growth observed in the 1% serum cultures is due solely to nutritional effects. That is 1% serum might be too low a level of serum to support optimum cell growth regardless of whether cultures are agitated or not. However this work does confirm once again the increased agitation resistance of CHRC5 cells.

3.2.8.6: Effect of Agitation Intensity on the Growth of Hep-2 and Hep-2A Cells

As with the earlier work examining the effect of environmental factors on cell growth, a difference in response of one MDR cell line to a physical parameter cannot be taken to be a general characteristic of all MDR cell lines. However if another MDR cell line shows a similar response then this would suggest that the observed response might be apply to some if not all MDR cells. Consequently the investigations suggesting that CHRC5 cells are more resistant to agitation intensity than their parental cells were repeated on Hep-2 and Hep-2A to see if these human cells responded in a similar manner to increased agitation.

The results for the growth of Hep-2 and Hep-2A cells at 40 and 120 rpm are not as clearly obvious as for CHRC5 and CHO-K1 cells (Figures 3.40 and 3.41). However the overall trend suggests that the MDR cells (Hep-2A) grow better than the non-MDR cells (Hep-2) at the higher agitation rate. Growth of Hep-2 cells at 40 rpm is somewhat erratic with cells exhibiting a prolonged lag phase before appearing to enter exponential growth towards the end of the culture period (Figure 3.40). Growth at 120 rpm is not as great as at 40 rpm for Hep-2 cells. Maximum cell density at 40 rpm is $2.26 \times 10^5$ cells/ml and at 120 rpm is $1.68 \times 10^5$ cells/ml. Hep-2A cells grow better at 120 rpm with the maximum cell density achieved, $2.71 \times 10^5$ cell/ml (Figure 3.41). Both cell lines exhibit surprisingly low growth at 40 rpm which perhaps masks the actual difference in tolerance between the cells to the higher agitation rate. The poor growth at 40 rpm can again be attributed to possible inaccuracies in cell counts due to cell clumping which was observed to be very pronouced at the lower agitation rate. Even though care was taken to trypsinise these clumps before counting it was often difficult to break up clumps into single cell suspensions and so counts may not have been entirely representative. At the higher agitation rate clumping was not so great a problem. Overall the important conclusion to be taken away from this experiment is that again, in a

similar manner to the CHRC5/CHO-K1 system, the MDR cells display a greater resistance to high agitation intensity than their non-MDR counterparts.

In addition to the difference in agitation tolerance noted between Hep-2 and Hep-2A cells it was found that Hep-2A cells agitated at 120 rpm expressed greater levels of the MDR-1 gene than when agitated at 40rpm (Section 3.2.5.2). This result suggests that Hep-2A cells may have greater agitation resistance due to the increased expression of p-glycoprotein induced at the higher agitation rate. Since it has been suggested that p-glycoprotein may decrease the degree of packing in cell membranes (Loe and Sharom, 1993), perhaps the increased p-glycoprotein levels in Hep-2A cells agitated at 120rpm may cause a more tightly packed membrane, i.e. with decreased plasma membrane fluidity. The implications of this result for the CHRC5/CHO-K1 cell system is discussed more fully in Section 3.2.8.8.4 following.

3.2.8.7: Aim of Remainder of Discussion

The results presented thus far have identified two MDR cell lines with increased resistance to agitation when compared to their parental cells. While conclusions about the physical resistance of MDR cell lines in general cannot be made based on these results it is important to attempt to explain why the two MDR cell lines studied are more shear resistant. In order to do this it is necessary to understand that cell injury caused by agitation has two components.

1. Biological - what biological processes determine a cells' shear sensitivity and what cellular processes are affected and how by fluid forces ?
2. Fluid-Mechanical - the aim is to identify the shear stresses existing in the bioreactor which cause the cell damage (Papoutsakis *et al*, 1991).

It is the aim of the remainder of this discussion to cover all aspects of these components that determine the extent of cell injury caused by agitation in

bioreactors. The main biological determinants of shear sensitivity are generally agreed to be the cellular cytoskeleton and the plasma membrane. Both of these will be discussed here with the emphasis on plasma membrane fluidity (PMF) which was investigated for the CHRC5/CHO-K1 cell system to determine whether PMF might be contributing to the increased agitation resistance of CHRC5 cells. Changes in the physical properties of the plasma membrane have been discovered in some MDR cells thus this work will be discussed with respect to how it might be related to the increased agitation resistance of CHRC5. The effect of shear on other biological processes will be discussed based on the results of the metabolic rates of both cell systems during agitated cultures. Finally an attempt to characterise the fluid mechanical environment existing in the Techne spinner bioreactors used for the majority of the agitation experiments and in the Bellco spinners used for the PMF work will be undertaken.

3.2.8.8: Biological Factors Determining Shear Sensitivity

Animal cells lack the protective cell wall found in prokaryotes, thus they must rely solely on the cytoskeleton and plasma membrane to maintain their physical integrity. Therefore knowledge of the mechanisms by which these cellular structures confer resistance to hydrodynamic injury would be very useful for implementing strategies to improve the native mechanical strength of a cell. Investigations on the viscoelasticity (flexability, 'springiness') of erythrocytes in relation to the cytoskeleton and plasma membrane are well documented in the literature (Chasis and Mohandas, 1986; More and Thurston, 1987). The effect of shear stress on the structure and function of vascular endothelial cells has described changes in deformability, rigidity, cell lysis and physiological responses (Francke *et al,* 1985; Frangos *et al*, 1986; Ando *et al*, 1988; Levesque *et al*, 1989; ). Surprisingly little information is available on the cellular aspects that determine at a molecular level the fragility of commercially important cells. Petersen and co-workers (Petersen *et al*, 1989; Papoutsakis *et al*, 1991) are among few workers to date that have

investigated the importance of the cytoskeleton in determining the shear sensitivity of cells other than blood or endothelial cells. The cytoskeleton is composed of two major proteins (microtubules and microfilaments) polymerised into filamentous structures in a highly organised network. Using specific cytoskeletal inhibiting agents these workers identified that microfilaments and/or other structures of polymerised actin are important for hybridoma cell protection from shear.

A number of workers have attempted to understand the effect of shear on the cytoskeleton and plasma membrane by examining the mechanical properties of the cell. By applying known forces to an individual cell and observing its deformation and/or lysis, it is possible to measure the mechanical properties of the cell (Gooch and Frangos, 1993). One method of determining cortical tension and apparent viscosity of the cell is to aspirate the cell into a micropipette with a diameter less than that of the cell and observe its rate of deformation. This method was utilised by Needham *et al* (1991) to characterise a hybridoma cell line. These authors found that the mechanical propeties of hybridomas unlike erythrocytes cannot be characterised by a single value for a given parameter, but instead exhibit a wide range of values that are dependent on the growth stage of the cell. Another method of measuring a cells physical properties is to compress it between two surfaces and measure the force required for deformation and lysis. Utilising this method, Zhang *et al* (1991, 1992) showed that the bursting strength of a cell is dependent on its size, while the mean compressibility modules, bursting membrane tension and relative increase of cell area at bursting all vary with the age of the culture but are independent of cell size.

When examing a cells response to shear it is important to determine how shearing forces applied to the cells surface are transmitted internally (Cherry, 1993). Does the cytoplasm respond as a homogenous viscoelastic fluid or do structural elements of the cytoskeleton carry all the load ? The rheological behaviour of cytoskeletal elements has been studied in some detail and

suggests that cells can only support quite low fixed shear stresses of about 0.1-0.2 $N/m^2$ (comparable to shear stresses present in bioreactors) above which they fail and flow quite readily (Jamney, 1992). In a bioreactor it may be considered that the surrounding fluid does mechanical work on a cell which triggers the observed biochemical responses. The significant part of this mechanical work might be any of ;

1. the total stress on the cytoskeleton or cytoplasm
2. a local effect on an individual membrane protein such as a hydrodynamically forced conformation change in a receptor ion channel
3. enhanced convective motion of membrane proteins in the cell membrane (i.e. changes in PMF).

The ultimate question to be answered by all the current research is how is the physical stimulus of shear stress is transduced to a biochemical signal ? If the pathway of the response is known it is conceivable that different branches of it could be regulated to alter the cells'response.

*3.2.8.8.1: Plasma Membrane Fluidity and Shear Sensitivity*

Animal cells are surrounded by a bilayer membrane composed of a variety of lipids and proteins.The membrane is fluid and thus constituents may move around in the bilayer. The degree of packing of the constituents in the membrane as well as their movement is referred to as the plasma membrane fluidity (PMF). Recently work by Ramirez and Mutharasan (1990, 1992) has found that changes in the PMF of a cell can alter that cells' shear sensitivity. Specifically, it has been found that a decrease in PMF results in a decrease in shear sensitivity. The mechanism for the change in shear sensitivity produced by changed membrane fluidity is unknown, however it is possible that a stronger membrane offers more resistance to shear forces acting on the cell surface. Ramirez and Mutharasan (1990) investigated a number of agents for their effect on PMF. Cholesterol, Pluronic F-68 and serum all decreased PMF implying that these compounds interact directly with the membrane to change the PMF. A subsequent increase in shear resistance was noted with these

agents. The authors concluded that the fluid state of the plasma membrane is important in determining the integrity of a cell when exposed to lethal shear levels. They stated that increasing membrane fluidity correlated with increasing shear sensitivity. Further work (Ramirez and Mutharasan, 1992) investigated the effect of serum on PMF in more depth. It was suggested that serum has a condensing effect on PMF. Given that the time scale in which changes in PMF were observed (2-18 hours) it was concluded that serum decreases PMF by the transfer of cholesterol or analogous compounds from the culture medium to the membrane. High shear stresses resulting in an increased cholesterol flux. Tomeczkowski *et al* (1993) have found similar effects on PMF by cholesterol in anchorage dependent cells and suggest that decreased PMF might be due to the rigid structure of cholesterol. If more cholesterol molecules are embedded into the membrane then they will decrease the membrane fluidity and permeability. Cholesterol also impedes the movement of fatty acid chains thus rendering the membrane less fluid.

*3.2.8.8.2: Measurement of PMF in CHRC5 and CHO-K1 Cells*

Given that decreases in PMF correlates with a decrease in shear sensitivity it was decided to examine if the difference in shear sensitivities of CHRC5 and CHO-K1 cells might be related to differences in the PMF of these cells. The work to date investigating PMF and shear sensitivity has only looked at how changing PMF, by the addition of various agents, can alter shear sensitivity (Ramirez and Mutharasan, 1990, 1992; Tomeczkowski *et al*, 1993). In the work in this thesis, differences in PMF are examined in agitated cultures ( containing growth medium with no additions) to investigate if there is an inherent difference in PMF between the MDR cell line, CHRC5 and its non-MDR parent, CHO-K1 which might explain the increased shear resistance of CHRC5 cells.

PMF was measured using a fluorescence polarisation technique which measures changes in the molecular order of a component in the membrane.

This is a static factor (termed $r_s$, the fluorescence anisotropy) which is related to the degree to which the rotations of a component are restricted. A change in molecular order is associated with the reciprocal of PMF. Thus an **increase in the $r_s$ value indicates a decrease in PMF**. The fluorescent probe used to label the membranes is 1-[4-trimethyl-amino phenyl] -6-phenylhexa-1, 3, 5-triene (TMA-DPH) which rapidly partitions in the bilayer matrix due to its nonpolar nature. TMA-DPH 's positively charged head prevents the probe from penetrating further into the cytosol and binding with the membranes of internal organelles, thus the measurements achieved with this probe are specific for the external plasma membrane.

Table 3.45 gives the $r_s$ values for CHRC5 and CHO-K1 cells grown in monolayer culture and thus not exposed to shear stress. As can be seen there is little difference between the values indicating that under 'normal' non-stressed conditions CHRC5 cells do not have a decreased PMF. The $r_s$ value of a CHO-K1 variant which had been growing in serum free medium for 3 months was also determined. Its value is lower than for serum supplemented CHO-K1 cells, thus the PMF of the serum free cells is higher and cells would be more shear sensitive. This result agree with the work of Ramirez and Mutharasan (1992) who have suggested that serum protects cells from shear due to the incorporation of cholesterol in serum into the plasma membrane. Cholesterol has a condensing effect on the membrane and renders the cell more resistant to shear. These authors also report that serum free cells have a more fluid membrane due to the absence of cholesterol in the medium.

Thus there appears to be no inherent difference in the PMF of the MDR and non-MDR cells when cells are not exposed to stressful conditions. The PMF of the cells was then examined under agitated conditions.It was decided to carry out these experiments under different agitation conditions then previously used. Another spinner type (Bellco) was used since higher agitation rates could be achieved with these spinners and an estimation of the fluid hydrodynamic environment in the spinner could be made (see Section 3.2.8.9

to follow). Also instead of culturing cells in batch culture over a period of 5-7 days at low and moderately high agitation rates, it was decided to investigate the effect of high shear (agitation at 500 rpm) over a short time (24 hours) on CHRC5 and CHO-K1 cells. When long term cultures are carried out the effect of environmental factors such as metabolite concentration have to be taken into account since they might affect shear sensitivity. Petersen *et al* (1988) has shown that higher ammonium concentrations and lower pH can increase the shear sensitvity of hybridomas in batch culture. Also it was important to determine whether CHRC5 cells respond to increased agitation by increasing their growth rate only (as has been observed in all results in long term culture) or by also having a decreased death rate. In these experiments investigating PMF changes, the cultures were agitated at 500 rpm for 24 hours and this allowed the determination of death rates for the cells in conditions where growth rates were negligible.

Figures 3.44 and 3.45 indicate that both cell lines are damaged by the shearing forces present in the spinner at 500 rpm. First order death rate kinetics are observed allowing the calculation of apparent death rates. CHRC5 cells have a lower apparent death rate (0.014 $hr^{-1}$) compared to CHO-K1 cells (0.039 $hr^{-1}$). Thus CHRC5 cells are more resistant to high agitation rates being killed less fast than CHO-K1. At moderately high agitation it has already been observed that CHRC5 cells grow faster than CHO-K1 cells. This experiment was repeated (Figures 3.47 and 3.48) and the same result was found, that is CHRC5 cells have a lower death rate (0.05 $hr^{-1}$ in this case) than CHO-K1 cells (0.07 $hr^{-1}$). At 500 rpm in the Bellco spinners used here it can be seen (Table 3.46) that turbulent conditions exist and a substantial vortex is present which greatly increases the shear forces experienced by the cells. Thus these experiments show that CHRC5 cells are more resistant to turbulent shear and respond to shear stress by decreasing their death rate as well as increasing their growth rate (as previously shown in Section 3.2.4).

The corresponding plasma membrane fluidity results from these experiments are very interesting (Figures 3.46 and 3.49). It can be seen that the $r_s$ values for the CHO-K1 cell line decreases slightly over the 24 hours exposure to high shear. However the $r_s$ values for CHRC5 cells increase substantially over the same time period. As stated earlier, an increase in $r_s$ indicates a decrease in PMF and a decrease in PMF as shown by Ramirez and Mutharasan indicates a lowered shear sensitivity. Therefore these results show conclusively that the MDR cell line, CHRC5, has a decreased PMF in response to high shear conditons and this lower PMF probably results in a more condensed plasma membrane rendering the cells less sensitive to shear. However the possibilty that other mechanisms might also be contributed to the observed agitation resisitance of CHRC5 cells should not be discounted.

These experiments show that CHRC5 cells are more shear tolerant than CHO-K1 cells at high agitation and there is a correlation between this observed shear tolerance and decreased PMF in these cells. Experiments were then undertaken to ascertain if this trend was observed when cells were grown at agitation rates at which suspension cells would normally be grown. Figure 3.50 show that CHRC5 cells have an increased growth rate at 50 and 200rpm when compared to CHO-K1 cells. At 50rpm the shear environment in the Bellco spinner is in transition between laminar and turbulent flow (see Table 3.46). At this agitation rate, CHO-K1 cells grow quite well, however CHRC5 cells have an increased growth rate. At 200rpm turbulent flow exists within the spinner (Table 3.46), CHO-K1 growth is very much decreased whereas CHRC5 growth is only decreased somewhat from that at 50rpm. PMF results (Figure 3.51) confirms the results of the high shear experiments. That is there is a correlation between increased shear tolerance in CHRC5 cells and decreased PMF. The $r_s$ value for CHRC5 cells at 50 and 200rpm is increasing corresponding to a decreasing PMF. The $r_s$ value for CHO-K1 cells is decreasing, most especially at 200rpm, corresponding to a increasing PMF. Thus these results present strong evidence that CHRC5 cells are more shear

resistant than CHO-K1 cells and this increased resistance is due to a decreased PMF.

Increased agitation resistance was observed in the MDR cells, Hep-2A as well as in CHRC5 cells. In order to ascertain whether increased agitation resistance is a feature of MDR cells, high shear experiments and PMF measurements were performed on another MDR cell line, DLKPA and its drug sensitive parent, DLKP. Figures 3.52 and 3.54 show once again the MDR cells are more shear tolerant. Indeed, DLKPA cells appear to be especially shear resistant, growing at agitation rates of 500rpm which as stated previously results in very turbulent conditions within the spinner flask. PMF measurements (Figures 3.53 and 3.55 ) show that PMF is decreasing for DLKPA cells and increasing for DLKP cells. Thus once again, there appears to be a correlation between shear tolerance and decreased PMF in the MDR cells. This result is interesting also since Alon *et al* (1991) has stated that changes in membrane physical properties, in particular PMF, is not a feature of all MDR cells

Ramirez and Mutharasan (1992) showed that PMF may be altered by the addition of certain agents. More specifically they speculated that it was cholesterol in serum, which having a condensing effect on the cell membrane, decreased the PMF of cells. However there are no reports in biochemical engineering literature of the PMF of cells changing without addition of specific agents (e.g. serum, pluronic F68) being responsible for the observed change in PMF. The results presented here however suggest that the PMF of CHRC5 and DLKPA cells is decreased in response to a physical stimulus rather than chemical modification of the medium. The results show that there is little difference in PMF between CHRC5 and CHO-K1 cells when the cells are growing in an anchorage dependent mode in flasks. However upon exposure to turbulent shear CHRC5 cells respond by decreasing their PMF and are thus noted to be more shear resistant than CHO-K1 cells whose shear sensitivity is highlighted by a corresponding slight increase in PMF. Since the

medium is not chemically modulated to change PMF then it is reasonable to assume that the difference noted in PMF and thus shear sensitivity between CHRC5 and CHO-K1 cells may be due to the MDR nature of CHRC5 cells. PMF is a collective description for the various motions of the components of a biological membrane. Consequently any differences in the plasma membrane of MDR cells and non-MDR cells might explain the decreased PMF response of CHRC5 cells to shear stress. The following section describes recent work investigating the physical properties of the plasma membrane of MDR cells.

*3.2.8.8.3: Physical Properties of the Plasma Membrane in MDR Cells*

Since the transfer of solutes across the membrane is influenced by the physical state of the lipid bilayer then it may be assumed that MDR cells (characterised by decreased drug accumulation) may have alterated membranes. Thus the lipid composition and physical properties of membranes from parental and MDR cell lines have been compared in several studies. In the study by Alon *et al* (1991) on CHRC5 and Aux-B1 (CHO auxotropic variant) cell lines, no gross differences in lipid composition have been noted overall. In particular there was no difference in two major determinants of membrane fluidity, that is the degree of fatty acid unsaturation and the cholesterol /phospholipid ratio.The degree of fatty acid unsaturation influences the order of the lipid phase and thus transport through it. There are some reports of significant differences between MDR and parental cells in a number of minor lipid components (Ramu *et al*, 1991). Since there appears to be no major differences in membrane lipid composition between MDR and parental cells, differences in membrane physical properties have been examined.

Callaghan *et al* (1992). have compared the physical properties and membrane composition of CHRC5 and Aux-B1 cell lines. They found that there was no difference in total phospholipid or fatty acid composition between CHRC5 cells and Aux-B1, making it less likely that there are differences in membrane

lipids. However a difference was noted in membrane physical properties between the two cell lines. CHRC5 cells were observed to have:

- A decreased surface hydrophobicity. When cells are clonally selected for MDR there is amplification of several other gene products besides p-glycoprotein, for example, the EGF receptor, in the membrane. A combination of these overexpressed gene products may be responsible for the decreased hydrophobicity.
- A decreased PMF, the $r_s$ value for Aux-B1 cells is 0.185 and for CHRC5 cells is 0.211. However decreased PMF may not be a characteristic of all MDR cell lines. Montaudon *et al* (1986) has reported that rodent tumour cells, Mdr C6, have an increased PMF and the authors attributed this to an increased level of unsaturated fatty acids found in the membranes of these cells. It has been speculated that increased PMF, if due to increased levels of unsaturated fatty acids, could help to decrease the intracellular drug concentration by preventing diffusion of the drugs through the membrane bilayer (Alon *et al*, 1991).
- An increased plasma membrane recycling. Beck *et al* (1987) have speculated that increased membrane recycling may be a drug resistance mechanism. Cytotoxic agents may be concentrated in the cytoplasmic vacuoles which subsequently fuse to the membrane and extrude their contents. Membrane recycling is controlled by a wide variety of factors including membrane composition and protein-lipid interactions. Boscoboinik and Epand (1989) suggest that resistant cells might have less stable membranes resulting in increased membrane recycling. Higher membrane recycling through endosomes has been noted in a number of MDR cell lines (Sehested *et al*, 1987).

Since the results presented by Callaghan *et al* (1992) indicate that there is no real difference in membrane lipid composition then the authors suggest that the differences in physical properties between the MDR cells and their parents might be due to differences in membrane proteins and more specifically to the

overexpression of p-glycoprotein. However it still cannot be ruled out that the MDR cells have a slightly changed lipid environment . Differences in the distribution of fatty acid chains on the glycerol backbone and among lipid types were not determined and these could influence the physical properties of the membrane.

Deliberate modification of the lipid composition of MDR cell membranes in order to observe changes in physical properties and in the abililty of the cells to accumulate drugs ( decreased accumulation of drug is the basic mechanism of MDR) has been investigated by Callaghan *et al* (1993). It was found that CHRC5 cells accumulated increased levels of drug when their membrane properties were perturbed by lipid addition. Heptadecanoic acid and stearic acid when added to culture medium was found to increase drug accumulation. Also the ratio of saturated to polyunsaturated fatty acids in lipids was increased resulting in a decreased PMF. Cholesterol based amphiphiles also were found to increase drug uptake and decrease PMF. The fact that cholesterol analogues were found to decrease PMF is in agreement with the work of Ramirez and Mutharasan (1992) who looked at cholesterol addition with the aim of decreasing shear sensitivity and found that cholesterol decreased PMF. Linoleic acid was found to increase drug accumulation but in contrast an increased PMF was observed. The authors concluded that several amphiphiles, particularly those perturbing PMF (causing either an increase or decrease), increase drug accumulation. They suggest that biological membranes adjust their physical properties for the optimal functioning of membrane proteins and any deviations will decrease activity of membrane proteins, thus p-glycoprotein levels may be decreased by changes in the membrane environment.

Further evidence relating physical perturbation of the membrane and p-glycoprotein has been provided by Loe and Sharom (1993). These authors noted an increased sensitivity by MDR cells to membrane active agents including non-ionic detergents, local anesthetics and stearoids. Experiments

were also carried out using a merocyanine fluroescent probe (MC540), a negatively charged dye which is confined to the outer leaflet of the membrane and does not penentrate the interior. It displays differential partitioning into the membrane depending on the lipid packing density in the outer leaflet and is used to monitor the molecular packing of the phospholipid monolayers (Yu and Hui, 1992). Using this probe it was concluded that overexpression of p-glycoprotein induces physicochemical changes that results in tighter packing of the outer leaflet of the lipid bilayer. This result is interesting given that Frangos and Berthiaume (1990) observed that after exposure to fluid flow the amount of merocyanine dye bound by endothelial cells increased, implying that phospholipids had become more loosely packed.

Other workers have observed changes in membrane permeability (Riordan and Ling, 1985), differences in ultrastructure (Arsenault *et al*, 1988), addition of verapimil causing increases in the level of phosphotidlycholine (Ramu *et al*, 1991) and other more general differences in the physical properties of the membrane bilayer ( Wheeler *et al*, 1982; Kessel 1988). All of these observations suggest that the overexpression of p-glycoprotein might lead to perturbations in membrane structure and function, perhaps arising in part from the insertion of a large hydrophobic protein into the membrane.

A number of membrane physical properties have been considered, however PMF is a particularly important physical property to consider for MDR cells. The organisation of lipids and proteins in membranes is intrinsically dynamic (Singer and Nicholson, 1972). Since the major route of amphiphilic drugs into cells seems to by diffusion across the bilayer then changes in the rotational and lateral mobility (PMF) of the bilayer might influence entry of a drug. As mentioned by Callaghan *et al* (1992) decreased PMF is a characteristic of some but not all MDR cells. Ling *et al* (1977) has speculated that decreased PMF might be responsible for a decreased influx of drugs into MDR cells, Montaudon *et al* (1986) has an opposing view stating that increased PMF might decrease drug accumulation by preventing diffusion of drugs through

the bilayer. Steady state arrangement of lipids and proteins may be visualised by freeze fracture electron microscopy which allows direct visualisation of the organisation of the membrane bilayer at the internal surfaces of the outer (exoplasmic) and inner (protoplasmic) leaflets of the bilayer. This technique was carried out by Arsenault *et al* (1988) for a CHO resistant cell line and its parent. An increase of intramembranous particles in both number and size was noted in the MDR (CHRC5) cells on the protoplasmic face of the membrane. While the molecular constituent of these particles is not known the authors reported a rough correlation between their number, the degree of drug resistance and the level of p-glycoprotein present. Thus it appears that overexpression of p-glycoprotein causes structural changes in the membrane of MDR cells.

Ramu *et al* (1983) have performed electron spin resonance studies on MDR cells and found that the membranes of these cells have a more rigid lipid environment with an increased membrane order than their parental cells. These authors also reported that decreased PMF increased the lipid structural order and subsequently an increase in drug resistance was noted. Increasing drug resistance with decreasing PMF is in agreement with the results of Callaghan *et al* (1992) and others. However there is conflicting evidence about the effect of PMF on drug resistance. Rintoul and Center (1984) found that increasing PMF results in increased resistance of lung cells and concluded that changes in lipid structural order/membrane fluidity is not invariably linked to the MDR phenotype. As mentioned Montaudon *et al* (1986) working with glioblastomas also found that increased PMF resulted in increased resistance to doxorubicin. These workers correlated this increased PMF with a three fold increase in polyunsaturated acly groups in the membrane lipids. It was then shown that when sensitive cells were cultured in excess polyunsaturated fatty acids PMF increased but there was no concomittant increase in drug resistance. Thus these workers argue that changes in membrane fluidity or lipid structural order alone are not sufficient for expression of MDR and these physical changes may be inevitable secondary processes in MDR rather than

essential determinants for the expression of the MDR phenotype. Indeed it is not surprising that there are changes in the physical properties of MDR membranes given that these cells have to cope with the overexpression of p-glycoprotein. This may be quite a demanding burden to bear , for example, CHO B30 cells are MDR variants of Aux-B1 cells and it has been shown that p-glycoprotein accounts for 20% of the total protein content of the cell membrane.

*3.2.8.8.4:Conclusions*

A decrease in PMF has been strongly associated with decreasing shear sensitivity, however this phenomenon has only been noted when the medium has been modulated with shear protective agents (Ramirez and Mutharasan, 1990, 1992). The results presented here differ, CHRC5 cells have been shown to be shear resistant and have a decreased PMF upon exposure to high agitation intensity in medium without additives. Also these cells appear to have no inherent difference in PMF from their shear sensitive parent cells when not exposed to a shear environment. Thus it appears that decreased PMF is induced in the MDR cells when exposed to shear and that as a result of the cells having a less fluid membrane they can withstand higher levels of shear stress. MDR cells seem to have no difference in the gross lipid composition of their membranes (Alon *et al*, 1991). There are, however, a number of differences between MDR cells and their parents in terms of membrane physical properties. These include decreased surface hydrophobicity, increased membrane recycling and variation in PMF. Differences in the PMF of MDR cells are a little confusing, with some reports of decreased PMF in MDR cells possibly resulting a decreased influx of drugs into MDR cells (Ling *et al*, 1977; Callaghan *et al*, 1992) . Other reports report increased PMF in MDR cells and suggest that a more fluid membrane might prevent diffusion of drugs into cells (Montaudan *et al*, 1986). It may be concluded that the overexpression of p-glycoprotein might lead to perturbations in membrane

structure and function, including changes in PMF, which are caused by the insertion of a large hydrophobic protein into the membrane.

In summary these results strongly suggest that the mechanism of increased shear resistance displayed by CHRC5 cells may well be due to a decrease in PMF, thus rendering the membrane of these cells more rigid and thus stronger to resist shear forces acting at the cell membrane. PMF in these cells is not being decreased by the addition of shear protective agents. PMF is not decreased in CHRC5 cells when the cells are not exposed to shear stress. PMF may be important in determining drug resistance however there is no proof that a decreased PMF is necessary for drug resistance in these cells. However even if there is no relation between PMF and drug resistance the fact that CHRC5 cells are multidrug resistance cells is probably responsible for the decreased PMF and thus the increased shear resistance. Possible mechanisms as to why MDR cells might have a decreased PMF upon exposure to shear stress are discussed below.

P-glycoprotein overexpression results in higher protein levels being incorporated into the membrane and this may cause tighter packing of the cell membrane resulting in a less fluid entity. Loe and Sharom (1993) have suggested that p-glycoprotein induces physicochemical changes that results in tighter packing of the outer leaflet of the lipid bilayer. Since fluid flow in endothelial cells has been shown to cause a loosening of phospholipids in the bilayer, then the overexpression of p-glycoprotein in MDR cells might counteract any decrease in phospholipid packing caused by shear stress. An increased number of intramembranous particles, which correlate in size and number to p-glycoprotein, have been found on MDR cells indicating that these cells have a membrane that is structurally different from parental cells (Arsenault *et al*, 1988). Other gene products may be overexpressed with p-glycoprotein, for example the EGF receptor (Callaghan *et al* , 1992) and these may also be incorporating into the membrane and thus increasing the packing density. In addition, p-glycoprotein is the gene product of the pgp1 gene in

hamster cells which is equivalent to the human MDR-1 gene. However there are a family of MDR genes expressed in hamster cells, pgp1, pgp2 and pgp3 (Ng *et al*, 1989). There is evidence that pgp3 codes for a distinct efflux pump (Herweijer *et al*, 1990). The gene product of the pgp2 gene is poorly characterised but is believed to be a p-glycoprotein-like product which can contribute to MDR (Hsu *et al*, 1989). Thus overexpression of products from these genes may also contribute to the increased structural order and decreased PMF seen in the CHRC5 MDR cell line.

Thus the conclusion that may be reached is that MDR leads to increased levels of proteins (p-glycoproteins and others) incorporating into the cell membrane which in turns leads to an increased packing density (decreased PMF) of the membrane. From the results though, it can be seen that no inherent difference between the PMF of CHRC5 and CHO-K1 cells was measured when the cells were growing in non-stressed conditions in flasks (Table 3.45). Differences in PMF between the MDR and non-MDR cells were only observed when cells were agitated. However it may be suggested that agitation and the accompanying increase in shear is causing the MDR cells to become stressed and this stressful environment may be inducing increased levels of MDR expression which may in turn be causing changes in the membrane of the CHRC5 cells. For example, increased levels of p-glycoprotein and other MDR proteins may be expressed under stressful conditions and the incorporation of these into the membrane may be causing the observed increase in PMF. It can be seen from these results that Hep-2A cells agitated at 120 rpm express over 2.5 times the level of the MDR-1 gene than cells agitated at 40 rpm (Section 3.2.5.2 using an RT-PCR technique). Obviously this technique should be repeated for CHRC5 cells and validated using another technique where the expression and thus quantity of the MDR-1 gene product, p-glycoprotein, could be verified (e.g. Western blotting). However it is an interesting observation that could explain the decreased PMF of CHRC5 cells when agitated. Several authors have shown that drug resistance (and thus increased levels of efflux pumps in the membrane) can be induced by extreme

conditions. Chin *et al* (1990) report that the expression of MDR-1 mRNA can be induced by heat shock or treatment with cadmium or arsenite. Sakata *et al* (1991) have shown that hypoxic conditions can induce drug resistance. Confluence dependent resistance has been reported by Dimanche-Biotrel *et al* (1992). Another possible explanation for the decreased PMF of CHRC5 cells is that stressful conditions at high agitation might induce increased production of lipids in the MDR cells which may then be incorporated into the membrane and increase the rigidity of the membrane.

It should be considered that decreased PMF might only be partially contributing to the observed increased shear resistance of CHRC5 MDR cells. MDR cells have been reported to have increased repair rates (Harris and Hochhauser, 1992). Since exposure to high shear causes cell damage, then cells with a more efficient repair mechanism would have a growth advantage over cells without such a mechanism. High levels of shear stress have been shown to trigger rises in intracellular calcium, (Cherry and Aloi, 1992; Aloi and Cherry , 1993). The rise is seen even in the absence of external free calcium ions implying that the effect is more than simply perforation of the cell membrane. Fluid forces appear to be triggering the release of calcium ions from internal stores. Previous work on endothelial cells, discovered the presence of stretch-activated calcium ion channels in the membrane (Lansman *et al*, 1987). It was speculated that these ion channels may flood the cell with free calcium ions which activate enzymes responsible for prostacyclin synthesis. Providing further evidence to support this model are the observations by Ando *et al* (1988) that laminar stresses cause an almost instantaneous three fold rise in the intracellular free calcium level. The results from Ando *et al* and Aloi and Cherry appear very similar, that is shear forces cause a rise in intracellular calcium levels. Christensen (1987) observed stretch-activated calcium ion channels in epithelial cells residing next to calcium and voltage activated potassium channels. Stretch sensitive receptors (thought to be ion channels which pass specific ions when mechanically stimulated) have been identified in a number of cell types (Guharay and

Sachs, 1984, 1985; Stockbridge and French, 1988). MDR cells (including CHRC5 cells) overexpress p-glycoprotein which is a membrane efflux pump. This pump may also be a calcium channel (since calcium channel blockers can circumvent p-glycoprotein action). The work discussed here emphasises the importance of intracellular calcium concentration and the role of calcium channels as possible shear/stretch sensitive receptors. Thus it may be possible that in MDR cells the p-glycoprotein pump (and other MDR related pumps) may be serving the same function as these stretch sensitive receptors thus rendering MDR cells less sensitive to shear. However much more work in the whole area of determining the biochemical response of cells to mechanical stimuli such as shear is needed before any definite conclusions may be made.

### 3.2.8.9: Effect of Shear on the Metabolic Rates of Cells

Most of the work discussed this far has detailed the effect of shear and other environmental factors on the growth and viability of cells. However other cellular processes may be affected by shear. The importance of sub-lethal levels of shear is now being realised. Since damage caused by shear is determined by both the magnitude and the duration of the shearing event then the effect of agitation over a long term period has to be assessed. Sub-lethal levels of shear may cause subtle, transitionary shifts in metabolism. Enzyme activity and gene expression may be influenced by relatively innocuous stress conditions. As the shear stress and/or exposure time increase, dramatic and irreversible changes in metabolism may occur. Al-Rubeai *et al* (1990) observed that hybridomas had a modifed cellular metabolism upon exposure to shear stress. Several workers have shown that under higher agitation conditions, glucose consumption rates and mitochondrial activity increases (Abu-Reesh and Kargi, 1989, 1991; Al-Rubeai *et al*, 1990; Oh *et al*, 1992) especially in the absence of serum (Smith and Greenfield, 1992).

As well as shear affecting cell metabolism, it is also known that the sensitivity of suspension cells to hydrodynamic stress may be increased by for example ammonia accumulation and pH changes (Peterson *et al* 1988). Thus in addition to monitoring cell growth rates and viabilities, the effect of low and moderate agitation rates on the metabolic rates of CHO-K1/CHRC5 and Hep-2/Hep-2A cells was determined. It was hoped to observe any effects of the agitation on glucose consumption, lactate and ammonia accumulation rates and to detemine if the MDR cell line responded differently than the non-MDR cells in terms of metabolic rates.

*3.2.8.9.1: Effect of Shear on Metabolic Rates for CHO-K1 and CHRC5 Cells.*

Glucose consumption was measured for CHO-K1 and CHRC5 cells growing at 0, 40 and 120 rpm. Figures 3.36 and 3.37 outline the results obtained. For CHO-K1 cells the culture at 40 rpm utilised the greatest amount of glucose, correspondingly this culture grew the best. Glucose consumption by the 120 rpm CHO-K1 culture is low. The MDR cells, CHRC5 at both 40 and 120 rpm appear to have overall increased consumption rates if compared to CHO-K1 cells.All available glucose is consumed in 72 hours for the 120 rpm culture and in 96 hours for the 40 rpm culture, which is not the case for the CHO-K1 cultures. However it should be noted that since the cell lines grow in different media the initial levels of glucose differ. Hams F12 medium used for CHO-K1 cells has almost twice as much glucose present as MEMα (10 mM as opposed to 5.5 mM) the medium for CHRC5 cells. Thus the CHRC5 cells probably used up all the available glucose quickly in comparison to CHO-K1 cells simply because less glucose was available. In order to gain a more representative picture of the effect of agitation on glucose consumption it is necessary to compare specific glucose consumption rates (μmoles/$10^6$ cells • hr) for both cell lines. To do this glucose concentration (converted into μmoles) was plotted against cell number for cells in exponential phase (not shown). An approximately linear response was obtained and the slope of this curve when multiplied by the apparent growth rate gives the consumption rate.

Cellular yield defined as the apparent number of cells produced per mole of glucose consumed (Kunas and Papoutsakis, 1990) can also be obtained from the curve of glucose concentration versus cell number. Values of specific glucose consumption rates and cellular yields are presented in Table 6.32 for CHO-K1 and CHRC5 cells at 40 and 120 rpm.

Table 3.48: Specific Glucose Consumption Rates and Cellular Yields for CHO-K1 and CHRC5 Cells at Different Agitation Rates

| Cell Line/Agitation Rate | Specific Glucose Consumption Rate ($\mu$moles/$10^6$ cells • hr) | Cellular Yield ($10^6$ cells/$\mu$mole glucose) |
|---|---|---|
| CHO-K1 - 40 rpm | 0.35 | 81.27 |
| CHO-K1 - 120 rpm | 0.17 | 99.46 |
| CHRC5 - 40 rpm | 0.27 | 73.24 |
| CHRC5 - 120 rpm | 0.33 | 72.41 |

It can be seen that when specific consumption rates are compared there is very little difference between either cell line except for the CHO-K1 cells at 120 rpm. The value for this culture is very low and although the growth rate was not as high at this higher agitation rate as at 40 rpm there is still substantial growth over the batch period (Figure 3.32). The cellular yield value for this culture is the highest indicating that a relatively high number of cells are produced given the low amount of glucose consumed.

Several authors have reported increased metabolic rates by cells experiencing stressful conditions (Abu-Reesh and Kargi, 1989, 1991; Al-Rubeai *et al*, 1990; Oh *et al*, 1992). Al-Rubeai *et al* (1990) using the MTT assay noted increased specific metabolic activity in hybridomas exposed to shear in spinner flasks. They hypothesised that shear invoked or upgraded the synthesis of enzymes involved in energy metabolism and others not involved in cell growth but with damage repair. Specific glucose consumption also

increased indicating, according to the authors, high maintenance requirements. Since it has been shown that CHO-K1 cells are sensitive to the increased agitation of 120 rpm it might be expected that in a similar manner to work cited in the literature, its glucose consumption rate (and other metabolic rates) might increase in order to help cope with the stressful conditions. Instead the opposite is observed to happen, specific consumption of glucose at 120 rpm is 0.17 μmoles/$10^6$ hr as opposed to 0.35 μmoles/$10^6$ hr at 40 rpm. However there has been reports that metabolic rates are not always increased by shear. Schurch *et al* (1988) found that hybridoma cells exposed to laminar shear stress in a viscometer did not have altered glucose consumption or lactate production rates.

Cellular yield values for CHRC5 cells are similar at both 40 and 120 rpm. The culture at 120 rpm has a slightly increased glucose consumption rate, this may be due to increased energy metabolism in response to the more stressful environment as has been previously reported (Al-Rubeai *et al*, 1990). Overall increasing agitation appears to decrease glucose consumption in CHO-K1 cells and slightly increase it in CHRC5 cells.

Ammonia production by CHO-K1 and CHRC5 cells yields an interesting result (Figures 3.38 and 3.39). It can be seen that CHRC5 cells produce almost three times the levels of ammonia during the batch culture than CHO-K1 cells. Perhaps CHRC5 cells metabolise glutamine to a greater extent than CHO-K1 cells. Lower concentrations of glucose are present in MEMα and as mentioned earlier most of the glucose was consumed by the cells by Day 5. Thus the CHRC5 cells may have used glutamine as its energy source resulting in the production of increasing levels of ammonia. Zielke *et al* (1978) found that 30% of the energy requirements for human diploid fibroblasts is supplied by glutamine and this energy contribution would be expected to increase as the cells deplete the media of glucose. Miller *et al* (1988) found that glucose concentration decreased at maximal cell concentrations while glutamine consumption continued during the stationary phase of growth as well as in the

decline phase. Doyle and Butler (1990) concluded that the greatest amountof ammonia production occurs when cell division is at its lowest. For both cell lines the level of ammonia accumulation is not really affected by increasing agitation.

*3.2.8.9.2: Effect of Shear on Metabolic Rates of Hep-2 and Hep-2A Cells.*

In a similar manner to the work on CHO-K1 and CHRC5 cells, the effect of shear on glucose consumption and ammonia production was examined for Hep-2 and Hep-2A cells. Figure 3.42 shows the results of glucose consumption for both cell lines. In contrast to the results from CHO-K1 and CHRC5 cells, no major differences may be noted. Hep-2A cells at 40 rpm have the lowest glucose consumption however this corresponds to the growth profile of these cells (Figure 3.41) which showed an unusually prolonged lag phase when compared to the other cultures. As cell enter exponential growth at approximately 72 hours, glucose begins to be consumed more rapidly. Hep-2 cells at 120 rpm have the highest glucose consumption which may be because the cells are under stress at this agitation rate, thus glucose consumption rates are increased to provide the energy necessary for cell repair mechansims. This hypothesis would be agreement with some published results (Al-Rubeai *et al*, 1990; Oh *et al*, 1992) but differs from the results of CHO-K1 cells at this agitation rate where glucose consumption was decreased.

Ammonia accumulation yielded a pronounced difference between the MDR and non-MDR cells in the level of ammonia accumulated. Hep-2 cells at both agitation rates produce almost twice as much ammonia as Hep-2A cells. The accumulation rate (μg/ml hr) is also faster for Hep-2 cells than Hep-2A cells. This difference may be linked to energy requirement. As stated earlier when cells are under stress they require more metabolic energy for their repair mechanisms (Al-Rubeai *et al*, 1990). Hep-2A are more resistant to the shear stresses caused by agitation therefore may not require the same amounts of energy as Hep-2 cells. Since glutamine oxidation may be responsible for 40%

of energy production (Newland *et al*, 1990) Hep-2 cells may metabolise glutamine to a greater extent in order to supplement their energy requirements thus resulting in greater production of ammonia. This result is again in contrast to the results from CHO-K1 and CHRC5 cells where the non-MDR cells showed much greater production of ammonia. However a reasonable explanation was suggested, relating increased ammonia accumulation to depletion of all available glucose thus resulting in cells using glutamine as its main energy source.This does not apply here since Figure 3.42 shows that glucose was not depleted at the end of the culture period for either cell line.

*3.2.8.9.3: Conclusions*

Death is not the only response a cell can show after being subjected to external physical forces. Agitation can affect other cell processes and recognition of the possible consequences of these effects is important both for the growth and productivity of large scale animal cell cultures. Increased energy metabolism by cells experiencing shear stress has been noted by a number of literature reports. In this work the effect of agitation on the metabolic rates of CHO-K1/CHRC5 cells and Hep-2/Hep-2A cells and differences in response (if any) of the MDR cells compared to the non-MDR cells was investigated. In general no definite trends emerged. For the CHO-K1/CHRC5 cell system, glucose consumption generally increased as the growth rate increased. CHO-K1 cells at 120 rpm appeared to have lower glucose consumption even though most literature reports have found increased metabolic activity, due to an increased maintenance requirement, in shear stressed cultures. Hep-2 cells at 120 rpm did appear to have greater energy metabolism, with glucose consumption and lactate accumulation rates increased when compared to the non-shear stressed culture. Ammonia accumulation was higher for the CHRC5, MDR cells but lower for the Hep-2A MDR cells when both cell lines were compared to their non-MDR counterparts. The specific metabolic activity for these cultures may have been measured using the MTT assay which measures the activity of mitochondrial dehydrogenase. A number of

authors base their conclusions of increased energy metabolism on an increased activity from the MTT assay (Al-Rubeai *et al*, 1990; Abu-Reesh and Kargi, 1991) since mitochondrial dehydrogenase activity indicates that cells have an intact electron transport chain.

3.2.8.10: Hydrodynamic Environment of Spinner Flasks Used in Agitation Experiments.

Any bioreactor with a liquid volume greater than about 25 ml requires that the fluid be mechanically mixed. This mixing usually creates turbulence in the liquid which in combination with the cells leads to an extremely complex fluid dynamic environment. While the fluid dynamics in agitated vessels have been studied in great detail for standard chemical engineering applications this information has not been extensively applied to the problems of animal cell bioreactors. As mentioned previously in Section 1.3, many different types of animal cell bioreactors are available. Most of the work in the literature investigating the shear sensitivity of animal cells have grown cells in suspension bioreactors based on the traditional microbial fermenter. However the capital and running cost of even a small agitated bioreactor can be prohibitive for a research group. Thus many research groups wishing to scale up the growth of cells in suspension do so by growing cells in spinner flasks. Though they cannot be considered as scaled down versions of large scale and medium scale bioreactors they certainly qualify as the most simple stirred tank configuration of agitated vessels available. Various designs of spinner flasks are available but the common features of all types are as follows;

- a glass vessel with a working volume ranging from 50 ml to 2 litres
- sampling ports present as side arms at the top of the vessel
- a magnetically driven agitator with a variety of impeller configurations
- vessels capable of repeated sterilisations and due to their small size the vessels fit easily into laboratory autoclaves

- most vessels have no ports for pH, oxygen, temperature probes and thus cells grow in an uncontrolled environment with temperature controlled by placing the vessels into incubator units.

Spinner flasks are in essence small scale agitated bioreactors. They provide an excellent means for scaling up the growth of cells and for studying the susceptibility of cells to shear. They were utilised for the work in this thesis because they allowed the effect of agitation intensity to be observed for a variety of cell lines; cell growth could be easily monitored in the vessels and they were relatively inexpensive to run. However the hydrodynamic environment in a spinner flask, in the same manner as for larger bioreactors, is often difficult to determine quantitatively. Due to the chaotic nature of turbulent flow one can only obtain empirical information about the sensitivity of the cell that is specific to that reactor type and geometry. The complexity of the flow patterns in bioreactors and the difficulty of quantifying the magnitude of shear stress on these cells under agitation makes it difficult to ascertain in detail how flow affects cell growth and metabolism.

However based on work by Cherry and Papoutsakis (1986, 1988, 1989), Croughan *et al* (1987, 1988, 1989) and others estimations of shear stresses that may be causing cell damage can be attempted. Reynolds number defines the type of flow regime present in the vessel at a particular agitation rate. In laminar flow, fluid flows in smooth layers, in turbulent flow there is a vigorous interchange of small packets of fluid (eddies) between adjacent layers of the fluid. Thus estimation of the Reynolds number allows the prevailing fluid regime in the bioreactor to be assesed. Most bioreactors are operated at agitation rates such that there is turbulent conditions in the vessel. Inspection of a turbulent fluid reveals eddies and whorls of a range of sizes. The largest eddies are of the scale of the system and are formed in some way related to the input of kinetic energy to the fluid. The passage of an impeller blade through the liquid generates eddies of roughly the size of the blade height. These large eddies then pass their kinetic energy on to smaller eddies

which pass it on to even smaller ones through a cascade of eddiess. At the smallest scales viscosity becomes important and dissipates the kinetic energy of the eddies as heat. Thus the Reynolds number will give a rough measure of the macro-scale turbulence, that is turbulence caused by eddies similar in size to the impeller blades.

Micro-scale turbulence however appears to be quite lethal and may cause the most cell damage. The Kolmogorov eddy length microscale is commonly used to approximate the size of turbulent eddies within the fluid and thus the possibility of the cells' exposure to high shear regimes caused by microeddies can be estimated. Cell damage from bulk liquid turbulence has been correlated to the ratio of Kolmogorov scale eddy size to cell diameter in suspension cultures (Kunas and Papoutsakis, 1990; M$^{c}$Queen and Bailey, 1987). Damage initiates as the ratio approaches unity and intensifies at lower values. It has been proposed that eddies of the same size or smaller than cell particles cause high shear stresses on the cell surface. For larger eddies shearing and collisions are minimised as cell particles move in eddy streamlines. It should be noted that there are limitations with the Kolmogorov model for microscale turbulence. It is assumed that the turbulence in the bioreactor is homogenous (that is the same everywhere in the system) and isotropic (meaning that at each point the statistics of the fluctuating properties are the same in all three coordinate directions). However this is not entirely the case for fluid mixing in a bioreactor. The largest eddies are predominantly anisotropic and the flow field is inherently transient, inhomogenous and anisotropic. Cells are exposed to a turbulent field characterised by time average and time fluctuating velocity and pressure components. Reynolds stresses (representing extra momentum transfer in the fluid associated with the fluctuating velocity components), elongational forces and collisions with the impeller or other components of the reactor.

In summary, there is as yet no completely satisfactory theory of turbulent flow, but a great deal has been achieved based on the theory of isotropic

turbulence of Kolmogorov. The same conclusion may be made for turbulent flow in a bioreactor. In an ideal situation cell damage should be predicted by knowing the actual stresses that the cell experiences and from intrinsic cell mechanical properties the resulting cell deformation. However there are correlations between the extent of cell damage and microscale turbulence based on Kolmogorovs' theory of isotropic turbulence (Cherry and Papoutsakis, 1986, 1988,. 1989; Croughan *et al* 1987, 1988, 1989; Kunas and Papoutsakis, 1990). Thus it is reasonable for the work presented in this thesis to base estimations of shear in the spinner flasks used on macroscale turbulence as quantified by Reynolds number and microscale turbulence quantified by the Kolmogorov eddy length.

*3.2.8.10.1: Hydrodynamic Environment in Techne Spinner Flasks*

Most of the work in this thesis was carried out in Techne spinner flasks. These spinners are similar to all spinners in that they are glass vessels with two side arms and an agitator suspended from the lid of the vessel. However the agitator design is slightly different to conventional spinners (e.g. Bellco spinners which will be described shortly). The agitator in Techne spinners is a bulbous glass rod attached to the inside of a screw cap by a short length of silicone rubber. Agitation is due to a magnet which is located in the bulb end of the rod and is attracted to one pole of a magnet rotated by an electric motor. The mixing environment in this spinner has been described by du Bruyne and Morgan (1981) as 'teaspoon mixing' (Einstein, 1954). The bulbous rod induces mixing of cells by secondary motion. Secondary motion in a tea cup is depicted in Figure 3.58.

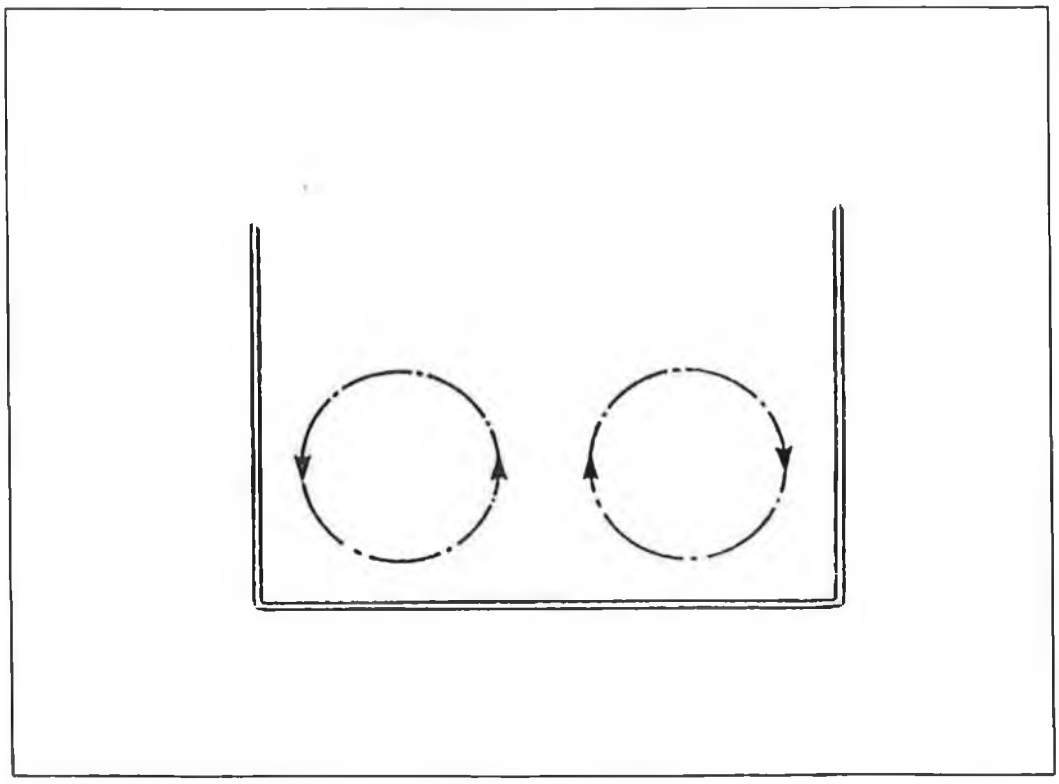


**Figure 3.58: Einstein's concept of the secondary flow in a stirred tea cup. It is this secondary flow that brings all the tea leaves to the centre of the bottom of the cup when the stirring spoon is removed.**

In a liquid made to rotate in a stationary flask there is a drop in rotational velocity and dynamic pressure due to viscous drag between the liquid and the inside of the glass. Particles at the top of the surface of the liquid will be thrown outwards but as they approach the side of the flask they are slowed down and descend to the bottom, in a helicoidal motion, where they move in to the centre and spiral up to the top surface. In a Techne spinner flask, this secondary motion has been maximised by the use of a stirring rod suspended on a flexible connection at the top with a bulb at the bottom which orbits in a circular trough formed by the rounded base periphery and an indented centre.(Figure 3.59).

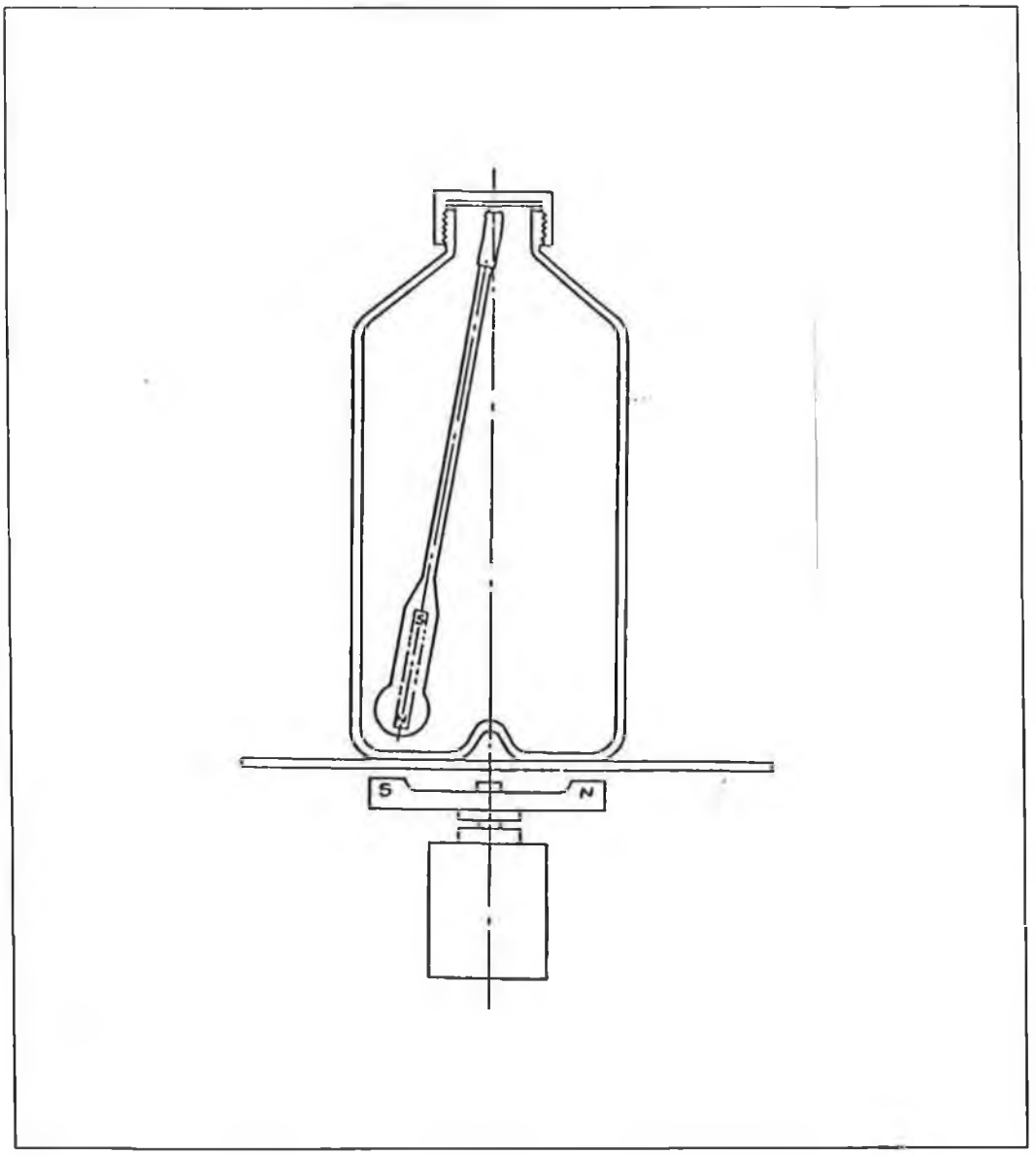



**Figure 3.59: The stirrer has a stirrer rod suspended at the top: the bulb at the bottom contains a bar magnet which is pulled round by a master magnet inside the platform.**

Cells at the top of the medium are thrown out by centrifugal forces to the periphery. Viscous drag then reduces the dynamic pressure so that the cells fall to the bottom in a helical path. On the bottom surface there is a steady decrease in the viscous drag along a radius, this causes cells to move horizantally inwards to the centre of the bottom surface from which they will ascend to the free surface at the top.Thus a continuous spiral of cells circulating upwards and downwards should exist in the spinner in the agitation

range of 10 to 80 rpm. Figure 3.60 is a photograph of an ink trace left by a drop of ink after 90 seconds of stirring in a 0.4% aqueous polyacrylamide solution. The picture is somewhat flattering to the stirrer becasue polyacrylamide suppresses eddies from being formed.

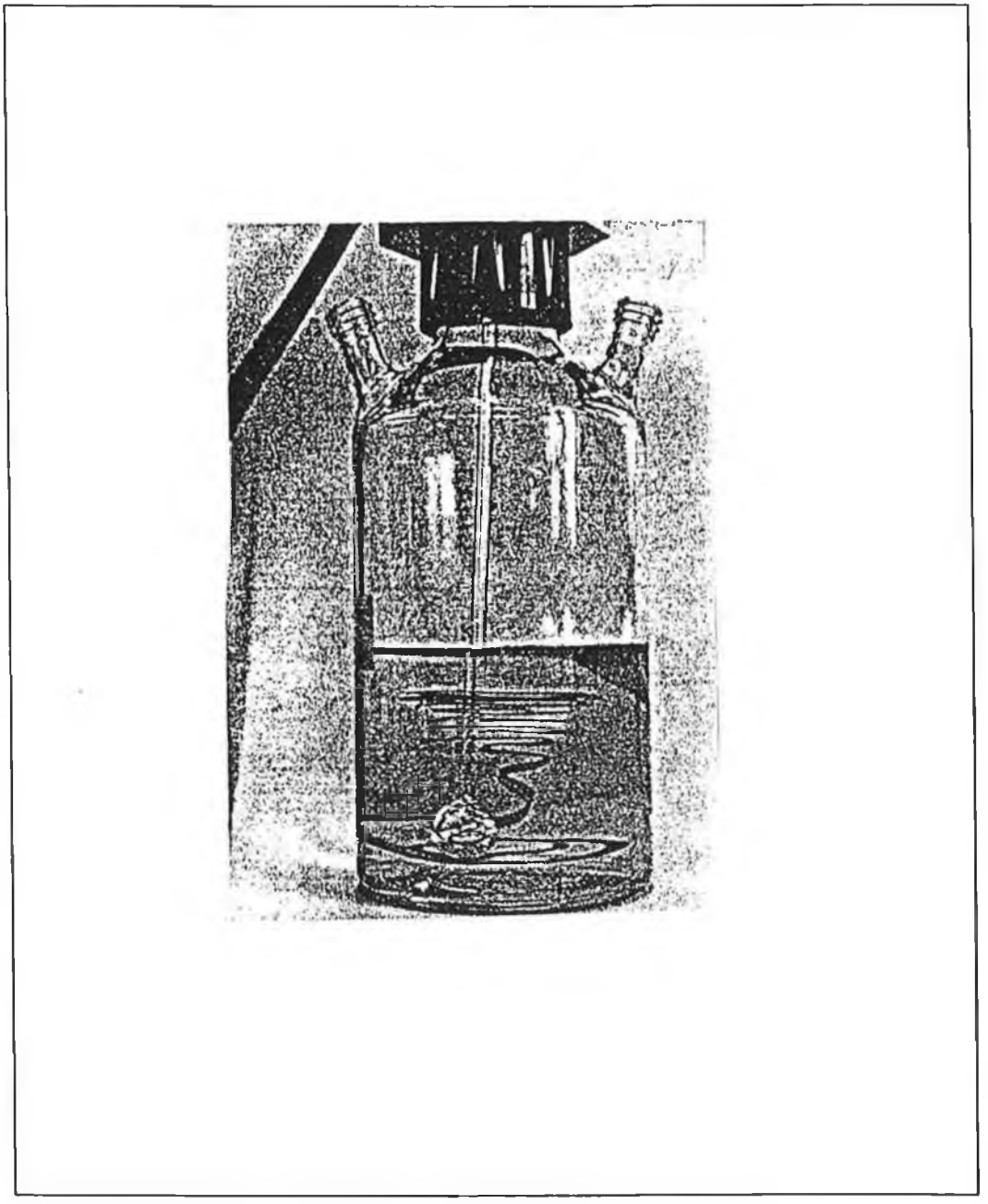


**Figure 3.60: This shows the trace of an ink drop after 90 minutes of stirring water with 0.4% polyacrylamide. It descended from the surface in a helix close to the side and is now ascending from the centre of the base in a coil of increasing radius.**

While there is no doubt that these spinners provide a very well mixed culture system there are drawbacks associated with their use. The agitator system is in essence a stirring rod similar to those used in chemical laboratories. There is no impeller type agitator where the whole assembly is suspended from the top of the vessel and is stationary, the only part moving being the actual impeller at the bottom at the end of the rod which actually mixes the fluid.

In the Techne spinners, the whole stirring rod assembly is rotating from the top of the vessel and this 'swinging' out motion of the rod creates the forces mentioned above which induces the mixing pattern described. Since no impeller as such is used then calculations of Reynolds number and Kolmogorov eddy length cannot be made since these quantities are both based on calculating shear stresses in the immediate vicinity of the impeller where most of the energy dissipation is occurring. Thus impeller diameter is an important quantity to be measured for shear calculations. The Techne spinners are also limited because according to the manufacturers the maximum agitation rate achievable is 80 rpm. Most of the literature quotes values of 150rpm upwards as being detrimental to cell growth. However most of the work carried out by other groups has been in a more traditional spinner system. Due to the different flow fields that would be present in another spinner system it cannot be stated that an agitation rate in one spinner type would cause the same level of cell damage in another spinner agitated a the same rate. The work in this thesis has shown that cells may be damaged by agitation rates of 120 rpm in the Techne spinners. This higher than recommended agitation rate was only possible due to the faulty operation of the magnetic drive motor which allowed higher agitation rates to be achieved in the spinner. While this 'fault' was very fortunate as it allowed the differentiation of shear sensitive cell lines (CHO-K and Hep-2) from more shear resistant cells (CHRC5 and Hep-2A) it cannot be stated that the mixing pattern at this higher rate is as described by duBruynes and Morgan (1981). Thus while the Techne spinners are useful tools for scaling up the growth of cells they are limited in that for agitation sensitivity studies their fluid mixing

regime and thus the nature of shear stresses exerted on cells, cannot be quantified.

*3.2.8.10.2: Hydrodynamic Environment in Bellco Spinner Flasks*

The identification of agitation resistance in a number of cell lines was carried out in Techne spinner flasks. However these spinners are limited and thus Bellco spinners were purchased in order to allow some quantification of the shear stresses to which cells were exposed. Also shear resistance up to 120 rpm only had been investigated for CHRC5 cells thus it was of interest to examine in greater detail the resistance of these cells. In the results presented, all studies on the plasma membrane fluidity of cells and cell growth at 500 rpm are carried out in the Bellco spinners (Section 3.2.6).

From Table 3.46 it can be seen that Reynolds number determines that fluid in the spinner will only be in fully developed turbulent flow at agitation rates above 80 rpm. Thus cells agitated at this rate and above will be exposed to the combined effects of all hydrodynamic forces found in TF conditions. An estimate of the extent of microscale turbulence shows that the Kolmogorov eddy length only becomes a dominant damage mechanism at agitation rates of 500 rpm and above. This conclusion is based on the work by Cherry and Papoutsakis (1988) which states that damage initiates as the ratio of Kolmogorov eddy length to cell/microcarrier diameter approaches unity. Thus for cells in the size range of 10-20 μm, eddies of 37-40 μm generated at 500 rpm would probably cause cell damage. At agitation rates below 500 rpm it can be seen that the eddy length is too large to cause much cell damage. However if cells were grown on microcarriers where the average diameter is 175μm it can be seen that even agitation rates of 60 rpm in Bellco spinners might cause cell damage.

In the work investigating cell viability and PMF changes for CHRC5 and CHO-K1 cells at 500 rpm (Section 3.2.6) it can be seen that both cell lines are

susceptible to the shear forces being generated at this agitation rate (with decreased susceptibility by the CHRC5 cells). It has just been shown above that microscale turbulence would be contributing to cell damage as eddy length at this rate is similar to cell diameter. However another mechanism of cell damage caused by the fluid environment if probably also making a significant contribution. It has been shown by Kunas and Papoutsakis (1990) that vortex formation at high agitation rates can severely affect cell viability. These authors noted vortex formation at agitation rates greater than 140 rpm in Bellco spinners. A vortex forms around the agitator shaft in the centre of the gas-liquid interface. At the bottom of the vortex , bubbles detach from the vortex and become entrained or burst at the liquid surface, causing shear as well as pressure fluctuations in the medium. Shear is also generated from the motion of the bubble moving relative to the liquid surrounding it . The combination of all these effects can be irreparable cell damage. In the experiments presented here, a vortex with a maximum length approximately 1/5th of the liquid height was noted at 500 rpm. Bubble formation and entrainment was also observed at this agitiation rate. Thus the mechanism causing CHRC5 and CHO-K1 cell damage at 500 rpm is probably a combination of microscale turbulence and high shear forces produced by vortex formation, as well as other undefined shear stresses that might be present.

*3.2.8.10.3: Comparison of Mixing Times for Techne and Bellco Spinner Flasks*

Reynolds number and Kolmogorov eddy lengths cannot be elucidated for Techne spinner flasks in which the majority of the agitation work in this thesis was undertaken. Since the shear environment in the Bellco spinners could be quantified somewhat it was decided to find a mechanism which would allow a correlation between the hydrodynamic environment in the Techne and Bellco spinners. This was undertaken by observing mixing times for a tracer dye in the two spinner systems. Results given in Table 3.47 show the resulting

correlation. It would appear that Techne spinners have a more efficient mixing system at lower agitation rates. A mixing time of 25 seconds was observed at 44rpm in Techne spinnners, however Bellco spinners only achieve an equivalent mixing time at the higher agitation rate of 60rpm. This observation is in line with a comparison of stirring systems presented by Hirtenstein, Clark and Gebb (1981). These authors found that the Techne spinner resulted in the most effective mixing of cells at low stirrer speeds when compared to conventional stirrers.

More importantly, these results allow an approximation of the shear environment in the Techne spinners at 40 and 120 rpm, the agitation rates used for most of the experimental work with both the CHRC5/CHO-K1 and the Hep-2/Hep-2A cell systems, to be made. An agitation rate of 44 rpm in the Techne spinners correlates to a Reynolds number of 1600 and a Kolmogorov eddy scale length of 173 μm. An agitation rate of 120 rpm correlates to a Reynolds number of 3200. Thus cells agitated at 40 rpm in Techne spinners are in a transitional turbulent flow regime and the microscale turbulence is not at a small enough scale to cause cell damage. At 120 rpm in the Techne spinners (when shear sensitivity was seen in the non-MDR cell lines) fully developed turbulent flow conditions prevail and cells may thus be damaged by all the shear stresses associated with turbulence. The Kolmogorov eddy length was not calculated at this rate but at 150 rpm is approximately 95 μm thus at 120 rpm microeddies would not seem to contributing to cell death in the Techne spinners.Vortex formation was noted in these spinners at 120 rpm but no bubble entrainment was noted on any occasion thus cell damage due to bubbles detaching from the vortex must be discounted. It must be cautioned that these description of shear in the Techne spinners are based on the mixing times correlation only and as such are not entirely valid. However they allow some quantification of the shear stresses occurring in the Techne spinners. This might explain why cells are generally resistant to agitation rates of 40 rpm (turbulent flow not fully developed) in these spinners, but at 120 rpm (turbulent flow) shear sensitivity is seen in the non-MDR cells.

## CHAPTER FOUR

# CONCLUSIONS AND DIRECTIONS FOR FUTURE RESEARCH

### 4.1:Conclusions

The aim of this thesis was to investigate factors, both endogenously produced and external, that inhibit the growth of animal cells *in vitro*. Section 3.1 presents results and discussions on investigations into growth inhibitory factors produced by animal cells. Section 3.2 presents results and discussions on the effect of external factors on cell growth and viability. The main conclusions that may be drawn from this work are as follows:

- MSV-3T3 cells produce and secrete factors which either inhibit or have a cytotoxic effect on epithelial carcinoma cells. Either one factor or an aggregate of factors may be responsible for the cytotoxic activity. The molecules responsible seem to be very stable entities with high pH and heat resistance. The appearance of the cytotoxic activity in MSV-3T3 cm however, seems to be variable.
- Vero cells may produce an auto-inhibitor and/or an inhibitor for Hep-2 cells. The inhibitory activity may be produced by Vero cells in response to attachment area limitations (density dependent inhibition).
- CHRC5 and Hep-2A cells both appear very sensitive to lactate build up in their culture environment. Since both of these cell lines are multidrug resistant (MDR) and their drug sensitive variants are not sensitive to lactate, this observed lactate sensitivity may be a property of MDR cells in general. The fact that lactate is a potent calcium chelator may be related to the lactate sensitivity of these MDR cell lines.

- CHRC5, DLKPA and Hep-2A cells all appear to have greater agitation resistance than their non-MDR variants. This thesis has shown that these cells have an increased growth rate and/or decreased death rate at agitation rates that inhibit the growth of their parental cells. If this is a trend of MDR cells in general, then MDR cells may potentially be of industrial value since they would withstand higher shear levels and could be agitated at higher agitation rates, allowing more efficient mixing of oxygen and thus leading to possible increased productivity rates.
- A strong correlation between the observed increased agitation resistance of the MDR cell lines (CHRC5, DLKPA and Hep-2A) and a decreased plasma membrane fluidity is observed. It is recognised in the literature (Ramirez and Mutharasan, 1992) that decreased PMF results in a more shear resistant cell. Thus the MDR cell lines would appear to be more agitation resistant due to their decreased PMF.
- The hydrodynamic environment of the Techne spinner flasks used in this work is correlated with the defined shear environment of a Bellco spinner flask so that predictions about the fluid regime in the Techne spinner can be made. A correlation based on mixing times is determined.

## 4.2: Directions for Future Work

- Investigate Vero cm with the aim of identifying and characterising the growth inhibitory factor(s) that may be present.
- Examine whether the lactate sensitivity of CHRC5 and Hep-2A cells is a property of all MDR cells. Try to elucidate mechanisms for this sensitivity.

Most of any future work however should should focus on the observed shear resistance of the two MDR cell lines and should aim to;

- Investigate whether increased shear resistance is a general property of all MDR cell lines.
- Confirm if decreased PMF is the sole mechanism of shear resistance or if other resistance mechanisms might be important.
- If decreased PMF is the main mechanism then reasons as to how MDR cells might decrease their PMF upon exposure to agitation should be investigated.

More specifically the following experimental areas are proposed;

1. Examination of a diverse range of MDR cells and their non-MDR parents to investigate differences in shear resistance.
2. Utilising a Western blotting or immunocytochemistry technique to quantify p-glycoprotein levels in MDR cells before and after agitation. Thus any increase in p-glycoprotein levels that might occur in MDR cells exposed to high shear conditions would be observed. Previous work has examined MDR-1 gene expression only, therefore it is relevant to examine p-glycoprotein levels also.
3. Direct measurement of lipid and cholesterol levels in the cell membranes of MDR cells after exposure to shear to observe if the stressful conditions has induced changes in the membrane which in turn may decrease PMF.

4. Monitor any changes in drug accumulation for cells before and after exposure to shear. Callaghan *et al* (1993) has shown that addition of lipids to MDR cells can perturbate the membrane PMF which in turn was shown to result in decreased rhodamine accumulation. Since agitation also decreases PMF for some MDR cells lines then the effect of agitation on the MDR cells ability to accumulate drug should also be investigated.
5. Apply a microscopic technique to physically examine the cell membrane of MDR and non-MDR cells for damage caused by shear. Electron microscopy has shown that MDR cells might have structural differences in their membranes (Arsenault *et al*, 1986). Also, Dewitz *et al* (1979) noted that shear forces induced clublike cytoplasmic protusions from polymorphonuclear leukocytes indicating that cytoplasm was extruding through punctures in the membrane
6. Examine the cytoskeleton in MDR cell lines and determine with the use of cytoskeleton inhibiting agents (cytochalasins and colchicine) the importance of the cytoskeleton in the observed shear resistance in MDR cell lines.
7. Monitor differences in PMF when MDR cells and parental cells are exposed to moderate agitation conditions over longer time periods, previous experiments only examined differences under high shear conditions.Consequently it would be important to determine whether decreased PMF was also responsible for the increased shear resistance of the MDR cells (CHRC5) in the initial experiments in Techne spinners agitated at 120 rpm.
8. Determine DNA repair rates of MDR cells compared to their parental cells when both cells have been exposed to high agitation. Differences in rates of repair of damaged DNA between MDR and parental cells may contribute to the observed shear resistance of the MDR cells.
9. Examine differences in the response between the MDR cells and parents to defined levels of shear stress in a Couette flow viscometer. Quantification of shear in bioreactors is only an estimated value, defined laminar flow

experiments would allow the actual difference in shear sensitivity between cells to be quantified.

10. Investigate whether the addition of lipids or additives such as Pluronic F68 might affect the shear resistance and PMF of the MDR cells
11. Use MC540 dye to examine differences in the packing density of the MDR cells and parents upon exposure to shear. Frangos and Berthiaume (1990) observed that after exposure to fluid flow the amount of merocyanine dye (intercalates between phospholipids of the upper membrane) bound by endothelial cells increased, implying that phospholipids had become more loosely packed.
12. Examine the difference in membrane recycling between MDR cells and non-MDR cells during agitation to observe if a difference in the rate of internalisation might influence agitation resistance. MDR cells have been shown to have an increased rate of membrane recycling (Callaghan *et al*, 1992).
13. Investigate what effect if any verapamil (a p-glycoprotein circumvention agent) might have on the observed agitation resistance of the MDR cells.
14. To prove whether MDR is responsible for the agitation resistance, an agitation resistant variant of CHO-K1 cells could be selected over time and its adriamycin resistance investigation. Similarly an adriamycin variant of CHO-K1 cells could be selected and its subsequent agitation resistance investigated.
15. The mechanical strength of the cell membrane in MDR cells could be examined using the micromanipulation techniques developed by Zhang *et al* (1991, 1992). These workers have proposed a model for assessing cell damage in animal cells based on three parameters which can be measured by micromanipulation. The parameters are the bursting membrane tension, the elastic area compressibility modulus and the cell diameter. These parameters give a measure of the strength of the cell membrane and it would be interesting to investigate whether there is an inherent difference between the membrane strength of MDR cells compared to their parental cells.

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