STUDIES ON THE LARGE SCALE CULTIVATION OF ANIMAL CELLS: KINETICS AND BIOENGINEERING ASPECTS

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CHAPTER - 1

GENERAL INTRODUCTION

There has been a rapid development in the large scale cultivation of animal cells for the production of biologically important molecules. Traditionally products such as viral vaccines both human and veterinary (Table 1.1), interferon, and plasminogen activator have been produced using animal cells (Spier and Whiteside, 1976; van Wezel *et al.,* 1979; Giard *et al.,* 1981; van Wezel, 1984). Some animal cell proteins such as insulin and interferon have been successfully produced in bacteria using recombinant DNA technology. However, many of the biologically active proteins have complex tertiary and quaternary structures and require co- and post-translational modifications, and may not be properly expressed in bacteria due to the lack of the enzyme systems. These are produced using genetically engineered animal cells e.g., Hepatitis B virus surface antigen (Michel *et al.,* 1985), glycoprotein D of Herpes Simplex virus (Lasky *et al.,* 1984) and monoclonal antibodies synthesized by hybridoma cells. The potential use of animal cells for the production of various products is given in Table 1.2.

Some of the biological products mentioned above (such as vaccines) have been produced on a small scale using animals. The hybridoma cells are often grown as tumors in the peritoneal cavity of mice or other animals for the production of monoclonal antibodies. The antibody secreted by these cells is collected from the ascites fluid of the mice. Although, such cultivation systems are still in practice, they may not be suitable for those applications involving large quantities of antibodies e.g., *in vitro* diagnostics and large scale biological separations (Secher and Burke, 1980) or in its use in cancer therapy (Vitetta *et al.,* 1983). To meet the demands of large scale application would require the use of hundreds of mice. The difficulties in handling a large number of animals, purification from physiological fluids and variation in the product obtained from different animals

TABLE - 1.1

HUMAN AND VETERINARY VACCINES FROM CULTURED ANIMAL CELLS

Vaccine	Cells
Human	
Polio	Primary monkey kidney cells and Vero
Yellow Fever	Chick embryo culture
Influenza	Chick embryo culture and rCHO
Measles	Chick embryo culture
Mumps	Human diploid cells
Rubella	Human diploid cells
Hepatitis B	rCHO
Varicella	Human diploid cells
Japanese encephalitis	BHK21
Herpes simplex	Human diploid cells
HIV	rSF9 cells
Veterinary	
FMD	BHK
Rabies	BHK, Vero
Canine distemper	Dog kidney
New Castle disease	Pig kidney
Marek's disease	Chick embryo culture
Bovine diarrhoea	embryonic kidney cells
Infectious bronchitis	Cells carrying vaccinia vector

J.B. Griffiths (1985)

TABLE - 1.2

PRODUCTS OF ANIMAL CELLS

Whole cells	Used for assays, bone marrow, lymphocyte and skin grafts.
Cell constituents	Chromosomes, DNA, mRNA, organelles, tumour antigens.
Enzymes and proteins	t-plasminogen activator, urokinase, asparaginase, hyaluronidase, renin, pepsin, collagenase, Factor VII, VIII, X, Angiogenic factors.
Glycoprotein hormones	Chorionic hormone, HCG, erythropoietin, follicle stimulating hormone, thymopoientin, intestinal cell stimulating hormone.
Immuno regulators	Thymosin, interleukin, T cell growth factor, interferons, B-cell growth factor, serum thymic factor, colony inhibition stimulating, migration, macrophage cytotoxicity and T-cell replacing factor.

Spier and Honoud (1985)

limits the feasibility of this approach. The use of bioreactors for the cultivation of animal cells *in vitro* needs a systematic study leading to appropriate scale up considerations to meet the demands of the growing animal cell industry.

Although a large amount of information is available on biofermentation and scale up, it is restricted to fungal and bacterial fermentations. The animal cells differ from prokaryotic cells in their physiology and growth requirements and hence the existing technology has to be suitably modified in order to be used for animal cells. Some of the features of animal cells and the necessary modifications are shown in Table 1.3.

Animal cells can be broadly classified into two types depending on their growth characteristics *in vitro*. Some cell types can be grown as single cells in suspension or submerged liquid culture and are called as suspension cell cultures. The second type of cells require a solid substratum to which they adhere and spread before proliferating (Ben-Ze'ev *et al.,* 1980). These are called as anchorage dependent or adherent cells. These cells often exhibit a phenomenon of contact inhibition i.e., when they occupy all the available surface area growth ceases and they form a confluent monolayer of cells (Abercrombie and Heaysman, 1954). These then have to be harvested by trypsinization or by mechanical means, diluted suitably and reseeded into fresh tissue culture vessels. Here they again attach to the surface and growth resumes. This is often referred to as passaging or subculturing. In the case of most of the normal diploid cells, cell growth follows a typical pattern and the number of population doublings is finite. They then enter a period of poor growth called as **crisis** which is followed by death (Hayflick and Moorhead, 1961). In the transformed cells such as tumor cells this number of population doublings is infinite and hence they can be maintained for a number

TABLE : 1.3

SPECIAL FEATURES OF ANIMAL CELLS AND THE MODIFICATIONS REQUIRED FOR THEIR CULTIVATION

Character	Comments
Animal cells	The cells are irregularly shaped, lack cell walls, are larger than prokaryotes and are fragile. The cells are shear sensitive and demand special mixing devices.
Nutrients	The medium is nutrient rich and contains molecules of different molecular weights. Likely to get contaminated with bacteria and fungi, and causes problem in downstream processing.
Growth rate	The cells have a very slow growth rate and have very low final cell densities thus, requiring special monitoring systems.
Interaction	These show cell-cell and cell-substratum interaction and contact inhibition phenomena leading to difficulties in volumetric scale up and hindrances for adequate mass transfer.
Product	It is often produced continuously at low rate, labile, susceptible to degradation by enzymes present in the cells, and is present in dilute solution along with a large amount of other proteins. They are often glycosylated and are biologically or immunologically active. As the production time is extended it is necessary to maintain stringent aseptic conditions. It also leads to problems in downstream processing.

of years by subculturing e.g., HeLa cells. There are also cells of intermediate type which are able to grow as suspension cultures and as adherent monolayers, depending upon the medium and culture conditions for example BHK21- C13 cells (Panina, 1985).

A large variety of culture systems have been developed for the cultivation of animal cells. Several reviews have appeared in literature describing these systems (Glacken *et al.,* 1983; Hu and Dodge 1985; Griffiths 1988; Prokop and Rosenberg 1989; Hu and Peshwa 1991). No single cell culture system can be applied for all the cell types due to variations in the cell types (adherent and suspension), product expression kinetics (growth associated, cell associated or secretory) and process requirements for obtaining the products. Several reactor configurations are being used for the cultivation of animal cells. These are essentially the outcome of research in the areas of mixing, aeration methods, perfusion techniques and developments in the substrates for attachment. A brief review of these cultivation systems including the historical development of animal cell culture systems is presented here.

History

As early as in 1885 scientists were able to maintain chick embryo explants and frog leucocytes alive in warm saline or serum for several days. In 1907, the American Zoologist Harrison was able to achieve not only cell survival but also cell growth using a modification of the hanging drop technique. Rous and Jones (1916) were able to grow cells from explant or pieces of tissue using the plasma clot method. In this a small piece of explant or tissue was attached to a glass coverslip using plasma clot. These plasma clots or embryo extracts provide sufficient nutrients for the tissues to be maintained as well as to divide over

extended periods of time. This also allowed for the diffusion of waste products away from the explants. The explant inside its clot was inverted over a small chamber. The chamber environment provided the necessary oxygen for cell metabolism. Using this technique it was possible to keep cells alive and growing for long periods of time. When the cell mass became too large it could be removed, divided into smaller pieces and new cultures begun. This was thus the beginning of continuous *in vitro* cell culture. This chamber design is still in use (Rose, 1954).

Between 1907 and 1970 a number of important modifications and improvements in cell culture techniques occurred. In the 1970s came the major development of glass bottles or culture vessels introduced by Carrel, who also used his surgical procedures for the development of aseptic techniques which have led to the success of long term *in vitro* culture. All these culture vessels comprised explants or single cells which were bathed in a static layer of nutrients. The chamber atmosphere provided the oxygen. An improvement in this traditional method of cell culture was the introduction of **Roller** bottles. This has two advantages over the traditional culture vessel in that the movement of the medium keeps it mixed and prevents build up of diffusion gradients which exist in static layers. Hence the extracellular nutrient concentrations are not rapidly depleted nor does the toxic waste metabolite concentration build up as fast. Second is that the thin layer of medium that covers the cells when they are above the bulk of medium enables cells to grow at gas partial pressures closer to the bottles' atmosphere by minimizing the diffusion barrier (Jensen *et al.,* 1976). These bottles are being used for large scale work too, as they are practical upto sizes of 1500 cm^2 of culture surface area. Since 1959, a number of production units have been using roller bottles for virus cultivation for the production of veterinary and human vaccines, e.g., Salk polio and measles vaccine (Lederle Labs., Pearl River, New

York) and vaccines against foot and mouth disease, canine distemper, rabies, Mareks disease (USDA Facility, Plum Island, New York). The method is extremely expensive and requires manipulation of hundreds of vessels, involving high labor costs per batch, and increased risk of contamination due to handling and does not provide cells with optimum conditions for growth and product formation.

The first industrial processes were those used for the manufacture of polio vaccine from African Green Monkey kidney cells (Capstick *et al.*, 1962). These were adherent cells grown in simple tissue culture flasks either in stationary manner or by the use of roller bottles. The scale up of such a process was done by simply multiplying the number of basic units and hence did not require any additional skills or knowledge of the bioprocess. The ability to grow BHK21 cells as suspension cultures and the introduction of microcarrier cultivation opened up the possibilities of a single unit system for large scale animal cell culture.

Criteria for the Design of Animal Cell Culture Systems

The adherent cells must be provided with a surface to attach in order to enable them to function and divide in a normal manner. The surface should not have adverse effect on the cells or cell membranes. Ideally the culture surface should mimic the charge and surface interactions that the cells experience *in vivo*. The chemical environment of the cells should be maintained under optimum conditions by supplying nutrients and oxygen at a rate so as to satisfy their rate of uptake. The removal of waste products is also important to prevent the build up of toxic substances and to maintain physiological pH. These basic requirements affect design of cell culture systems.

For a large scale culture it is necessary to have a high ratio of surface area to the volume of the system. This leads to the presence of a large number of cells in a given volume. Therefore, an efficient supply of nutrients and removal of toxic metabolites are required to maintain the viability. This is critical as the cell density increases and attains levels of those present in tissues, e.g., in microencapsulated beads and in artificial capillaries. Hence, many culture designs incorporate medium perfusion.

The suspension culture is the preferred system as it offers a single homogeneous culture unit amenable to scale up. It also has the advantage of being supported by a large volume of fermentation data and equipment. Though the fermentation vessels used for microbial fermentations cannot be used directly for animal cell cultivation they can be adapted successfully to serve the purpose. The different reactors used for cultivation of suspension cells have been developed as a result of developments in the areas of mixing or agitation and aeration and hence vary mainly in the agitation and aeration systems used, which are modified to suit the large fragile shear sensitive animal cells. Most of these reactors can be operated in batch, fed batch or in continuous mode depending on the demands of the process.

Agitation

In order to reduce the damage due to mechanical stress nonturbulent streamline flow is required during cultivation of animal cells. The systems used on a small scale employ a magnetic bar which provides laminar mixing. Various designs of impellers have been used to allow better mixing with low shear at low impeller speed (Tolbert *et al.*, 1985; Griffiths, 1986). Turbine type impellers were found to be damaging to cells. Marine type impellers have been suitably

modified to provide mixing at low shear rates for animal cells. Mixing can also be achieved by vibromixers (Girard *et al.*, 1973), airlift pumps (Reuveny *et al.*, 1986a) or by the use of internal loops (Leist *et al.*, 1986).

Mixing or agitation can also be done by non-mechanical means as in airlift reactors (Katinger *et al.*, 1979; Katinger and Scheirer, 1982). The airlift reactor consists of a central hollow draught tube within the culture vessel (Fig. 1.1). The gas mixture introduced at the bottom of the tube establishes medium circulation due to density differences in the medium inside the draught tube (oxygenated) and the medium outside (unoxygenated). The dissolved oxygen and the pH of the culture medium can be controlled by varying the composition of the gas mixture and the gas flow rate. The advantage of the airlift system is that it provides gentle mixing with low shear and is easier to scale up. It has been widely used for cultivation of shear sensitive hybridomas and insect cells and has been employed on 100 L scale for the production of monoclonal antibodies. The productivity obtained in the airlift systems is similar to that obtained in conventional stirred tank systems. This can also be operated in batch, or in continuous modes. The cell density in the reactor may be increased by incorporating an external flow loop, wherein the cells are separated from the liquid and returned to the vessel. The advantages of this system include the absence of moving parts such as mechanical seals which thus reduces the risk of contamination. The same aspect also results in reduced power requirements.

Aeration

It is well known that sparging directly into the cell culture causes damage to animal cells due to bubble disengagement at the surface and bubble rupture. Supply of oxygen through soluble oxygen also has limitations because

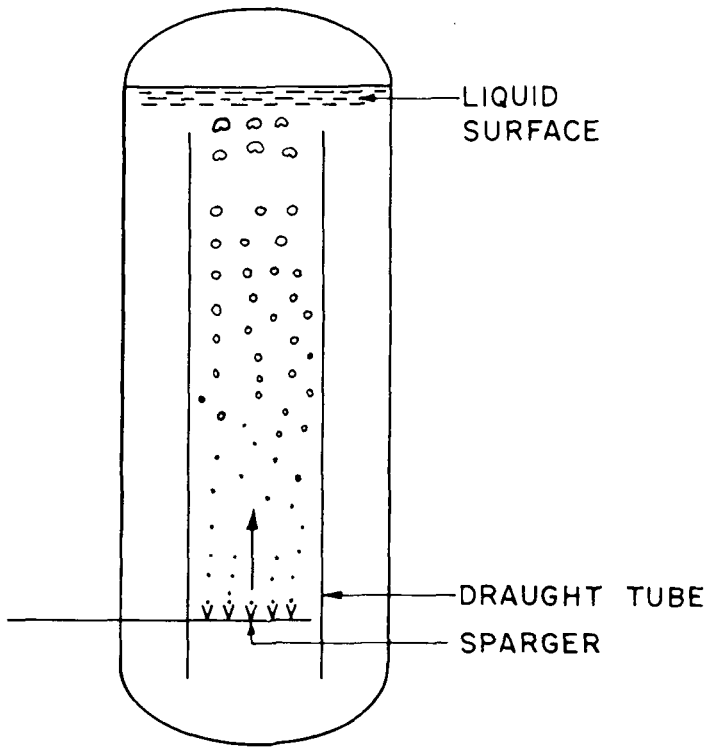


FIG.1·1 : SCHEMATIC DIAGRAM OF AIRLIFT BIOREACTOR
(REPRODUCED FROM RHODES et al., 1991)

of its low solubility (7.6 g/L) in water at 37°C. Sparging may only be done using large diameter bubbles (1-3 mm) at very low flow rates. Aeration can be achieved by many different devices which include surface aerators, air jets, air lifts and caged aerators. Another approach is to recirculate the medium in a separate compartment or oxygenator distinct from the cell compartment. This can be done within the cell culture system by means of mesh screens (Griffiths *et al.*, 1987a; 1987b) or caged aerators (Whiteside *et al.*, 1985). Bubble free aeration may also be done by diffusion through thin polyvinyl and other hydrophobic tubing. These may be introduced into the culture system, or may be included in the oxygenator compartment (Milenburger and David, 1980; Fleishaker and Sinskey, 1981).

In the case of airlift reactors it has been seen that damage to cells occurs mainly in the region of bubble disengagement at the liquid surface (Handa-Corrigan *et al.*, 1987). This may be overcome by reducing the zone of bubble disengagement by increasing the aspect ratio of the vessel. Certain shear protective additives such as Pluronic F-68 are also known to reduce damage due to bubbles. Suspension cultures in the batch mode can give about 2-3 x 10^6 cells mL^{-1}. In order to achieve higher cell yields which in turn would give higher productivity it is necessary to scale up the process either in terms of volume or in terms of intensity i.e. to obtain high density culture of cells. This can be done by using perfusion technology or cell immobilization.

Perfusion Culture Systems

Animal cells *in vivo* are perfused by the circulatory system in which there is a fresh supply of nutrients and removal of toxic waste metabolites. The perfusion system was introduced by Graff and McCarty (1957). They used a cytogenerator and achieved cell densities upto 1-2 x 10^7 cells mL^{-1}. A perfusion

system can be developed by incorporating a mechanism to separate the cells from the medium and retain them in the reactor while being able to remove the spent medium and replenish with fresh medium (Fig. 1.2). If this is done at a slow and constant rate it ensures that the cells are not exposed to drastic changes in their micro-environment. Perfusion is also a popular method to maintain the functionality of cells *in vitro*. The separation of cells from the medium can be done by different methods such as gravitational settling devices (Butler *et al.*, 1983; Feder and Tolbert, 1983), centrifugal separators (Sato *et al.*, 1983), static filters (van Hemert *et al.*, 1969) or spin filter devices (Thayer, 1973; Tolbert *et al.*, 1981). These filters vary from stainless steel meshes to microfibre filter tubes, ceramic and fused glass filters.

The cell densities obtained by perfusion systems are 10-50 fold higher than that obtained in non-perfused systems. In order to obtain higher cell densities of the order of that present in tissues ($1\text{-}2 \times 10^9$ cells mL^{-1}), it is necessary to have a critical control of the cell environment. This can be done by immobilization of cells in a matrix which is then perfused with medium. Various methods have been developed for this like microencapsulation, entrapment within porous matrices and physical entrapment in membranes or within hollow fiber devices.

Microencapsulation developed by Nilsson and Mosbach (1980) is currently used for the commercial production of monoclonal antibodies. The cells are trapped within sodium alginate gel beads coated with polylysine to form a semipermeable membrane. Then the sodium alginate is solubilized with sodium citrate. This results in cells, in free suspension encapsulated within the polylysine membrane. These beads can then be used in conventional stirred tank reactors.

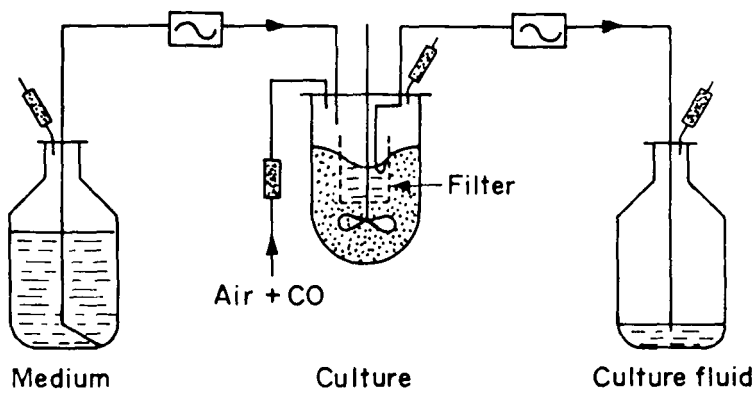


FIG.1·2: SCHEMATIC REPRESENTATION OF A CONTINUOUS PERFUSION SYSTEM
(REPRODUCED FROM VAN WEZEL, 1985)

Cell concentration within the bead can be as high as $5x10^8$ cells mL^{-1}. Materials other than alginate such as agarose, collagen fibers and fibrin have also been used (Nilsson *et al.,* 1983; Nilsson and Mosbach, 1987). The disadvantages of the system are that severe nutrient limitation can occur within the sphere. In order to obtain the product the beads have to be harvested and lysed hence, the system can be operated only in the batch mode.

Immobilization of cells can also be done by entrapping them in a textured or partitioned surface over which nutrient medium is allowed to flow. This can be done using ceramic surfaces (Katinger, 1987), polyurethane sponges (Lazar *et al.,* 1987) in the form of cubes, or on cellulose fibers (Larsson and Litwin, 1987). Several membrane devices are used for immobilizing cells, such as dialysis reactors, tubular film reactors (Jensen, 1981) or hollow fiber membrane devices. The hollow fiber membrane device was originally developed by Knazek *et al.,* (1972), who described the system as mimicking the *in vivo* capillary network system which perfuses cells and tissues in the body. It consists of a bundle of hollow fiber membranes which separates the reactor into two compartments, the lumen or intracapillary space through which medium is perfused and the shell or extracapillary space where the cells are entrapped. The capillaries are made up of semipermeable membrane. The product secreted by the cells is retained in the extracapillary space (due to the molecular weight cut off) and can be harvested in a concentrated form. The cells can be immobilized and perfused with the medium, which is recirculated several times in order to get maximum utilization of nutrients (Chapters 2 and 4).

Other configurations of reactors used for the cultivation of adherent cells include plate reactors and packed bed reactors. Plate reactors employing

glass plates (Litwin, 1976; Robinson *et al.,* 1979), stainless steel, treated polystyrene and titanium are also used. The plates are either static or in motion. The multitray units function like a set of bottles with the depth of liquid similar to that in bottles (5mm). These plates can also be designed as a parallel stack which is held in a cage like arrangement (Weiss and Scleicher, 1968). These have provisions for the addition and removal of medium using a tilting mechanism. Packed bed reactors are usually operated as perfusion systems. The cells are attached to a solid substratum in a cell growth vessel while the bulk of the medium in the reservoir is recirculated through the culture vessel. Evans and Earle (1947) described such a system using perforated cellophane. Other packing materials like Raschig rings, glass spheres (Spier and Whiteside, 1976), diatomaceous earth (Telling and Radlett, 1971) and stainless steel springs (Merck, 1982) have also been used. These systems are employed on an industrial scale as they can be scaled up.

Microcarriers

The advantages of suspension systems of cultivation was extended to anchorage dependent cells by the development of microcarrier cultures. The growth of anchorage dependent cells on microcarriers introduced by van Wezel (1967) provides an attractive alternative to the roller bottles hitherto used for the cultivation of such cells. This enabled anchorage dependent cells to be cultivated in stirred tanks in a manner similar to suspension cells. The early studies with microcarriers mainly involved the use of dextran as substratum for the growth of adherent cells. Levine *et al.,* (1977), showed that the surface charge density of DEAE-Sephadex had to be optimized to allow cell growth on these microcarriers (Fig. 1.3). This led to the development of a wide range of

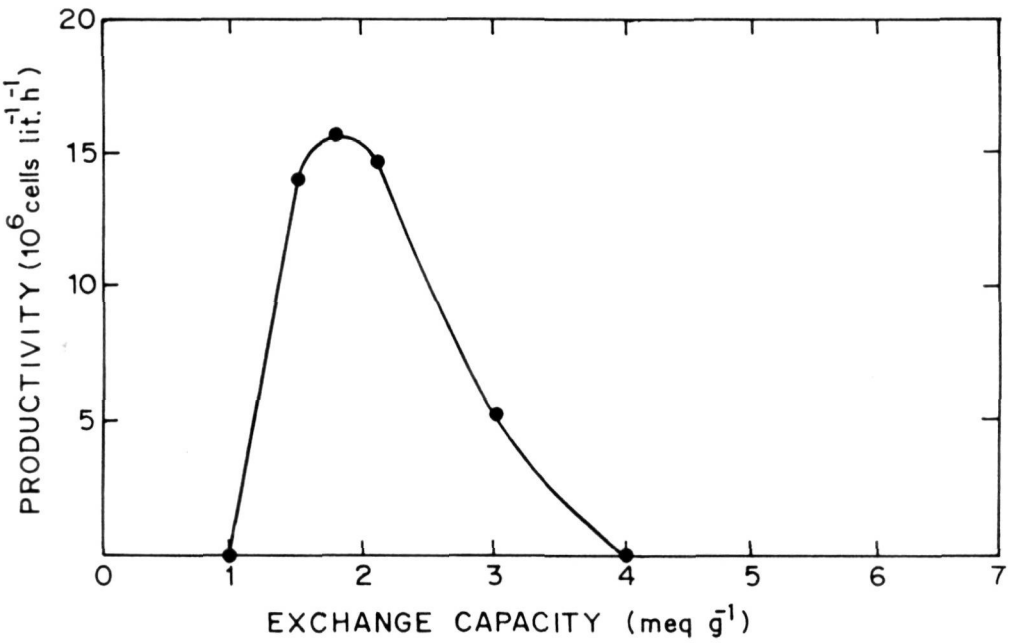


FIG.1·3: THE EFFECT OF MICROCARRIER CHARGE DENSITY ON GROWTH OF CELLS
(REPRODUCED FROM BUTLER, 1988)

microcarriers suitable for cultivation of animal cells. The interaction between cells and the surface involves a combination of electrostatic attraction and van der Waal's forces. The traditionally used surfaces such as glass and plastic are negatively charged. The outer membrane of cells is also negatively charged hence, positively charged molecules like divalent cations and basic proteins which are included in the culture medium, are required to be present on the substratum prior to cell adhesion. The cell-substratum interaction involves a wide range of nonspecific proteins. Fibronectin present in the surface of many cells is commonly involved in cell adhesion, especially if collagen is coated on the substratum.

The surface area per unit volume of culture is increased by selecting the smallest possible dimensions of the microcarriers. The size of the beads is controlled by two factors: The bead should be of sufficient size to support significant growth and multiplicity of the initial cell inoculum and should have a minimum diameter of 50μm (Maroudas, 1972) as the cells show preference for the substratum with the least curvature, which may be due to the rigidity of cytoplasmic microfilaments (Grinnel, 1978). Most microcarriers have a diameter of 100-200μm. In order to provide a homogeneous environment for growth, cultures are stirred at a low speed to prevent damage by shearing. The microcarriers have to be light in weight and should have densities ranging from 1.03-1.05 g cm^{-3}. A wide variety of microcarriers have been developed, which vary in the basic matrix and in the nature of the surface coating material used.

Dextran: One of the most widely used microcarriers, is a polymer of glucose and is treated with epichlorohydrin to obtain a stable non-rigid and non-toxic matrix

suitable for cultivation of animal cells. Dextran has been used for chromatographic purposes and is easily available commercially. These are available as Superbeads, Microdex or Cytodex (Pharmacia, 1981).

The dextran beads used for microcarrier culture are of 150μm diameter. They can be easily suspended in liquid media and provide a large surface area for growth. These are routinely used in vaccine production. A major limiting factor is that bead to bead cell transfer occurs only in the case of a few cell types (Spier, 1980). In most cases the attachment to the bead is too strong to allow the transfer of cells to the unoccupied beads. This causes problems in cell recovery and requires harvesting of cells from the beads by trypsinzation before being used as inoculum for larger vessels. The procedure of trypsinization generally results in low yields of viable cells.

Plastic: Sulphonated polystyrene beads with negatively charged surfaces have been used for animal cell cultivation (Maroudas, 1977). Polystyrene modified using glycine (Kuo *et al.*, 1981) and polyacrylamide derivatized with DEAE or dimethyl aminopropyl (DMAP) groups have also been used (Hirtenstein *et al.*, 1979). Some of the plastic microcarriers used are agarose coated polyacrolein (Lazar *et al.*, 1985) and DEAE- polyacrolein beads. The advantage of plastic beads is that they are shape stable, non-swelling and resistant to breakage. These materials being non-porous with low surface charges do not adsorb medium components. Various cell lines, including primary cell cultures of chick and human endothelial cells have been cultivated on these (Davies, 1981). The problems associated with these beads relate to the detachment of cells from the beads when confluency is reached (Morandi *et al.*, 1982) and the lack of translucency of the beads, which hampers microscopic visualization.

Gelatin: These microcarriers have been employed for the growth of different cells e.g., Vero cells and bovine embryo kidney cells (Gebb *et al.*, 1984). Gelatin which is denatured collagen, is used as a cell culture substratum for a wide range of cells, since the specificity of interaction is decreased due to denaturation. The cells are harvested from these microcarriers conveniently by proteolytic digestion (Paris *et al.*, 1983) and cell yields with 95% viability can be obtained. Macroporous gelatin beads which are made by allowing gelatin spheres to form around droplets of toluene, which is later removed by washing, have also been employed for the cultivation of animal cells. Due to the porous nature of the beads a large surface area is provided for the growth of cells which are protected from shear forces in the liquid (Nilsson *et al.*, 1986).

Glass: These microcarrier beads made by coating glass with plastic having densities of 1.03- 1.04 g cm^{-3} are commercially available. It is easier to recover the cells from the glass surfaces by trypsinization. The growth of fibroblasts has been found to be better on glass than on DEAE dextran (Varani *et al.*, 1986).

Cellulose: The microgranular grade of DEAE-cellulose used as anion exchanger has also been used for growth of anchorage dependent cells and has been found to support the growth of a variety of cell types. The cell growth occurs along the length of the fibers and transfer of cells to newly added cellulose fibers takes place (Lazar *et al.*, 1985). The disadvantage is the large size distribution of the particles resulting in non-uniform distribution of cells and the aggregation of confluent fibers causing problems during stirring.

Liquid Microcarriers: They are prepared by forming fluorocarbon droplets in an aqueous medium. Agitation of such a mixture results in a stabilized emulsion (Keese and Giaever, 1983). This can be done in an alkaline polylysine solution

to obtain droplets of 100-500μm diameter with an outer coating of polylysine, which provides a suitable substratum for cell adhesion and spreading. The cells are harvested by centrifugation and phase separation, where the cells form an interphase. Though this is an extremely elegant method for the cultivation of adherent cells problems on a large scale exist, due to the large size distribution and instability of droplets. The centrifugation and phase separation steps also cause hindrances during the scale up of the system.

Microcarrier technology offers an efficient method of culturing adherent cells on a large scale with yields upto $2x\ 10^6$ cells mL^{-1} in batch cultivation. Further improvement in cell yields is possible by combining this technology with perfusion culture systems in order to obtain higher densities of cells and overcoming the damage to the cells due to shear forces.

The cultivation of animal cells on a large scale involves the interaction of biological and engineering principles in order to select a reactor system which is most suitable for the process under consideration with respect to the cell type, growth and production kinetics and nature of product. The major technological challenges are to reduce the cost of mammalian cell culture by reducing the requirements of substances such as serum, and overcoming the mass and oxygen transfer limitations on a large scale.

Present Study

The present investigation deals with different aspects of mass cultivation such as the nutritional requirements, growth in different operation modes (batch, fed batch and in hollow fiber reactor) and the effect of shear on cells. The nutritional requirements with respect to the major nutrients viz., glucose, glutamine

and foetal bovine serum have been studied for a murine hybridoma CC9C10. The kinetics of cell growth and production of metabolite i.e. lactic acid has been studied. The growth of another murine hybridoma AE9D6 was studied in batch and fed batch culture. This hybridoma was cultivated in a hollow fiber bioreactor and the growth was monitored by substrate utilization and metabolite production (Chapter 2). The flow patterns existing in the cell compartment of the hollow fiber bioreactor were characterized by carrying out tracer studies using a high molecular weight protein and analyzing the residence time distribution profiles obtained (Chapter 4).

The effect of turbulent shear stress was studied for a human lymphoblastoid cell line KG-1. The cell damage was assessed by monitoring the total and viable cell counts and by studying the proliferative capacity and the changes in the cytoskeletal structure of the shear treated cells. The turbulent shear stress existing in a stirred bioreactor was measured by using laser Doppler anemometric technique. The shear protective effect of serum was also studied and the mechanism of protection was examined by measuring the turbulent stresess in the presence and absence of serum. The effect of laminar shear stress was investigated for a fibroblast cell line WI 38 grown as adherent monolayers. The effect of laminar shear on the morphology, viability, cytoskeletal structure and proliferation of these cells was studied (Chapter 3).

CHAPTER - 2

NUTRITIONAL REQUIREMENTS AND THE GROWTH KINETICS OF MURINE HYBRIDOMA CELLS IN A BATCH, FED BATCH AND HOLLOW FIBER BIOREACTOR

2.0 INTRODUCTION

Kinetics of Hybridoma Cell Growth and Antibody Production

Monoclonal antibody production using hybridoma cells is one of the most important application of animal cell culture technology. Hybridomas or hybrid cells are made by the fusion of myeloma cells and spleen cells from the immunized donor. This results in a cell line which secretes a predefined antibody and can be maintained as a continuous tissue culture cell line (Kohler and Milstein, 1975). These cells can be grown *in vitro* on a large scale to obtain specific antibodies, which are used widely in diagnostics and therapy.

Hybridoma cells in batch culture follow the classical sigmoidal growth pattern exhibited by most microorganisms. The cell population exhibits a series of growth phases, an initial lag phase where growth rate is zero, which is followed by an exponential phase characterized by increasing growth rate. The growth rate then decreases and the population enters the stationary phase and finally undergoes decline and death. In a batch culture there is no addition of nutrients or removal of metabolic wastes or products. Hence the cessation of growth may occur due to the depletion of essential nutrients, accumulation of toxic metabolic inhibitors or due to both the above reasons. Under the conditions where all the nutrients are in excess, the cell concentration is given by

$$\frac{dx}{dt} = \mu_{max} X_o \qquad (1)$$

where x is biomass concentration at time t, X_o is concentration of biomass at time zero and t is the time. μ_{max} is the maximum specific growth considering that growth is limited by a single substrate S. The growth rate μ is derived on the basis of Monod's equation as

$$\mu = \frac{\mu_{max} S}{S + K_s} \qquad (2)$$

where S is the concentration of the limiting substrate and K_s is the Michaelis- Menten constant.

Continuous culture of mammalian cells has been reviewed by Tovey (1985). Most of the earlier studies were carried out by cultivating mouse L cells in chemically defined medium with glucose and choline limitation (Birch and Pirt, 1969; 1971; Blaker and Pirt, 1971) in chemostat culture. Though the steady state cell concentrations and dilution rates were in agreement at low dilutions, at higher dilutions considerable deviations were found to occur (Herbert *et al.*, 1956; Tovey and Boye 1976). These deviations could be due to the complex nutritional requirements of mammalian cell cultures. The Monod model is used for cell growth (Hu and Wang, 1986) with a single limiting substrate, which can be easily identified in the case of microorganisms cultivated in chemically defined growth media. In the case of mammalian cells it is difficult to identify the growth

limiting nutrient because the culture medium contains various amino acids, vitamins, fatty acids, and is often supplemented with serum, which is in itself a complex mixture of proteins and lipids, glycoproteins and other molecules.

A rational scale up of any animal cell process requires a prior knowledge of cellular physiology especially with respect to growth and production kinetics. Though antibody production is being carried out commercially there is a paucity of literature concerning the various aspects of growth and antibody production. This is mainly due to the large amount of research effort involved in collecting reliable data. Kinetic data may be obtained either in batch or in continuous culture. In the microbial systems this is done by taking samples at fixed time intervals and estimating the biomass and product. In this manner, over a differential time, the differential biomass and product can be calculated. From this the specific growth rate and product rate can be obtained. In microbial systems both the biomass and product can be measured accurately and with greater frequency. The viability of the biomass is high, generally above 95% and the product formed is stable. The analysis of the product is also often fairly simple involving commonly used analytical techniques.

The kinetic analysis of monoclonal antibody producing cell lines is made difficult due to the characteristics of the cells and the product. The hybridoma cell population comprises a percentage of cells which are non-viable and also a percentage of non secretory cells (Frame and Hu, 1991) resulting in problems for the estimation of growth and product formation rates. The analysis of product i.e. monoclonal antibody needs to be done using time consuming and expensive

techniques such as enzyme linked immunosorbent assay (ELISA), nephlometry or radioimmuno assay (RIA). Antibody degradation may also occur leading to further complications in kinetic analysis. The viable (X_v) and non viable (X_{nv}) cells as well as the degradation of the product has to be considered while carrying out the kinetic analysis of the experimental data obtained for these systems. The growth rate is expressed as

$$\mu = \frac{1}{X_v}\frac{dX_v}{dt} \tag{3}$$

as the non-viable population by definition would be unable to grow. If an antibody degradation rate (K_D) is defined, the product formation rate (Q_A) may be expressed as

$$Q_A = \frac{1}{X_v}\frac{dA}{dt} - \frac{K_D A}{X_v} \tag{4}$$

where it is assumed that the antibody is secreted by only the viable cells. It may also be expressed as

$$Q_A = \frac{1}{X_{ToT}}\frac{dA}{dt} - \frac{K_D A}{X_{ToT}} \tag{5}$$

and

$$X_{ToT} = X_v + X_{nv} \tag{6}$$

that is the non viable cells do not divide but secrete antibodies. Boraston *et al.*, (1984) and Velez *et al.*, (1986) showed that in batch culture a considerable amount of antibody production occurs after the growth phase is complete indicating that antibody production is non growth associated. Lavery *et al.*, (1985) suggested that antibody synthesis is growth associated. Low (1987) compared specific antibody production rates and specific growth rates in batch cultures and found that a proportion of the antibody was produced as the growth rate decreased and is hence at least partly non growth associated. These discrepancies in results may be obtained due to the different cell lines used, which differ in their kinetic parameters. The antibody synthesized during the earlier period of culture may also be released by cells as they die. Similar results were reported by Birch *et al.*, (1984) for hybridomas in batch and continuous cultures.

Growth of Hybridoma Cells and Antibody Production

The growth of hybridoma cells and production of antibody are affected by physical factors (temperature, pH, dissolved oxygen), chemical factors and nutritional aspects of the growth medium (Harbour *et al.*, 1988; Miller and Blanch, 1991).

Temperature: Temperature is an important variable in the cell culture process, which has to constantly monitored and controlled. It is generally known that though animal cells remain viable for longer periods at temperatures lower than 37°C the growth rate and consequently the product yield is lower (McAteer and Douglas, 1979; Reuveny *et al.*, 1986b; Sureshkumar and Mutharasan, 1991).

pH: The optimal pH range of 7.2-7.4 is commonly used for most of the mammalian cell cultures, but some cells also survive at pH 6.6- 7.8 (Rubin, 1971; Barton, 1971; Eagle; 1973). The pH of the medium can undergo fluctuations during the course of batch and fed batch cultivation which can affect final cell yields (Ceccarini and Eagle, 1971). During culture, pH of the medium can be controlled by using different buffering systems employing CO_2/ HCO_3, HEPES, acid /base or air/CO_2 combinations. The pH of the medium may also be controlled by restricting the amount of lactate produced. This can be done by maintaining low concentrations of glucose and glutamine in the medium by using a slow continuous feeding method or by substituting glucose with sugars which are not metabolized as rapidly as glucose.

Dissolved Oxygen: It is one of the most critical parameters in any cell culture process and has been identified as an important criterion in the scale up of animal cell culture processes. The supply of sufficient amount of oxygen to cells which are large and extremely shear sensitive poses a major challenge to the animal cell technologist. Some of the devices used for supplying oxygen in animal cell reactors have already been discussed earlier (Chap.1).

Oxygen can be considered to be one of the major nutrients for animal cells. Oxygen functions as the terminal electron acceptor in the electron transport chain and generates energy via oxidative phosphorylation. Its major role is to regenerate NADH formed in various catabolic pathways. It may also play a role in cell membrane synthesis and hence in maintaining cell viability thus influencing the shear sensitivity of animal cells. For a complete development of a process

and its control the oxygen demand of the cells should be established *a priori* (Fleischaker and Sinskey, 1981; Spier and Griffiths 1984). The oxygen uptake rate for a hybridoma cell line was found to be 0.21 pmol $cell^{-1}$ h^{-1} at a maximum specific growth rate of 0.045 h^{-1} and 30% of the oxygen was consumed for maintenance (Boraston *et al.*, 1984; Harbour *et al.*, 1988).

The solubility of oxygen in aqueous solution at 37°C is very low (0.2 $mmolL^{-1}$) and hence it is rapidly depleted from high cell density cultures and has to supplied continuously. The oxygen transfer rate (OTR) defined as the mmol of oxygen transferred per unit volume per unit time (mmol $O_2L^{-1}h^{-1}$) from the gas phase to the liquid phase in a vessel is expressed as

$$OTR = K_L a(C^* - C_i) \quad (7)$$

where K_L is the oxygen transfer coefficient, a is the area of the interface available where transfer of oxygen occurs, C* is the amount of oxygen dissolved under saturating conditions per unit volume and C_i is the amount of oxygen dissolved per unit volume at time t. Oxygen supply introduces a gaseous phase into a liquid phase, which gives rise to shear forces at the gas liquid interface. The effect of these shear forces on animal cells have to be considered while aerating animal cell cultures. Sparging air or oxygen into the liquid medium has been used for the cultivation of suspension cells (Acton and Lynn, 1977) but is not a general practice due to problems of foaming and the resulting cell damage. The supply

of oxygen to high densities of cells under low shear conditions has been an area of intense research effort leading to the development of different strategies for aeration of animal cell cultures (Chap. 1).

Nutritional Aspects of the Culture Medium: It has been shown that thirteen amino acids, eight vitamins, and six ions are essential for the growth and survival of a number of animal cell lines *in vitro* (Eagle, 1955a; 1955b). These essential components supplemented with glucose and serum can support the propagation of various cell lines. The non essential amino acids are usually added to most culture media. Glutamine was found to be an essential amino acid for *in vitro* cultivation of animal cells (Eagle, *et al.,* 1958).

Glucose is broken down primarily by the glycolytic pathway. The intermediates of glycolysis are further metabolized by five different pathways *viz.* pentose phosphate pathway, formation of lipids or lactate or amino acids or the tricarboxylic acid (TCA) cycle. Glucose was found to be essential for the cells in tissue explants and by ^{14}C labeled experiments was shown to be used for the synthesis of the nutritionally non essential amino acids, purines, pyrimidines, carbohydrates and lipids of the cells. Human fibroblast and HeLa cells could be grown in the absence of glucose when the medium was supplemented with ribonucleosides (Zeilke *et al.,* 1976; Wice *et al.,* 1981). This supports the view that glucose plays a major role as a precursor for biosynthesis of nucleotides, amino acids, lipids etc., and only has a secondary role as an energy source. At low concentrations (<25μM) most of the glucose is utilized for synthesis of nucleic acids, whereas at higher concentrations it is converted mainly to lactate (Imamura

et al., 1982; Miller *et al.*, 1989a; 1989b). The conversion of glucose to lactate is due to the inefficient use of glucose in glycolysis as compared to its complete oxidation by the TCA cycle which can be explained on the basis of the Crabtree effect, where the aerobic metabolism is inhibited at high concentration of sugars (Deken, 1966; Chico *et al.*, 1978). Glucose concentration in the medium is fairly high (between 5-25mM). The specific rate of glucose uptake varies with the pH, stage of growth of the cells and the cell line used (Gosalvez *et al.*, 1974; Birch and Edwards, 1980; Arathoon and Birch, 1986). In the case of hybridoma cell lines the glucose quotient in static batch culture is independent of the initial glucose concentration (Low *et al.*, 1984; Low and Harbour, 1985). The hybridoma cells utilize glucose more efficiently than MRC-5 or BHK cells (Birch and Pirt, 1971; Arathoon and Birch, 1986).

Glutamine has been shown to be a major energy source in cell culture (Reitzer *et al.*, 1979) and is also required for lipid biosynthesis (Reed *et al.*, 1981), for the synthesis of the amino acids proline, aspartic acid and asparagine (Levintow, 1957) and for the regulation of DNA synthesis (Zetterberg and Engstrom, 1981). Glutamine metabolism provides 30-65% of the energy requirements (Batt and Kompala, 1989), is the main source of nitrogen and also an additional source of carbon. The metabolic pathways for glutamine breakdown have been studied by Zeilke *et al.*, (1976; 1978; 1980). Glutamine is first oxidized partially to glutamate and then to pyruvate via the TCA cycle. Pyruvate is further used for the formation of lipids and lactate. This is often referred to as glutaminolysis in analogy to glycolysis. The concentration of glutamine in the medium is usually 0.7-5 mM. The

specific rate of utilization of glutamine is rapid and is depleted in batch cultures at the end of the growth period (Griffiths and Pirt, 1967; Butler and Thilly, 1982). The utilization of glucose and glutamine are interdependent in the case of hybridomas (Zeilke *et al.,* 1978; Reuveny *et al.,* 1986b). The breakdown of glucose and glutamine during metabolism results in production of lactate and ammonia which are toxic to the cells at higher concentrations. The metabolic pathway illustrating the breakdown of glucose and glutamine is shown in Fig.2.1.

Amino acids are normally added to the medium, however, they may also be present in significant amounts in media supplements such as tryptose phosphate broth, lactalbumin hydrolysate and serum. Amino acids act mainly as precursors for protein biosynthesis and also take part in energy metabolism (Mizrahi and Avihoo, 1976). These are divided into three groups based on their consumption during the culture of cells *in vitro* (Butler and Thilly 1982). The first group includes 8 amino acids which are rapidly depleted and consumed during growth of the cells, the second group includes 5 amino acids which show limited depletion and the third group of 2 amino acids i.e. alanine and glycine which are secreted into the medium. Similar observations have been made in the case of human diploid fibroblasts (Lambert and Pirt, 1975), mouse myeloma cells (Roberts, et al., 1976) and BHK cells (Arathoon and Telling, 1982). The depletion of the essential amino acids may cause limitations of final cell yields and hence a finer control of cell growth requires a complete study of the amino acid requirement of the cell line.

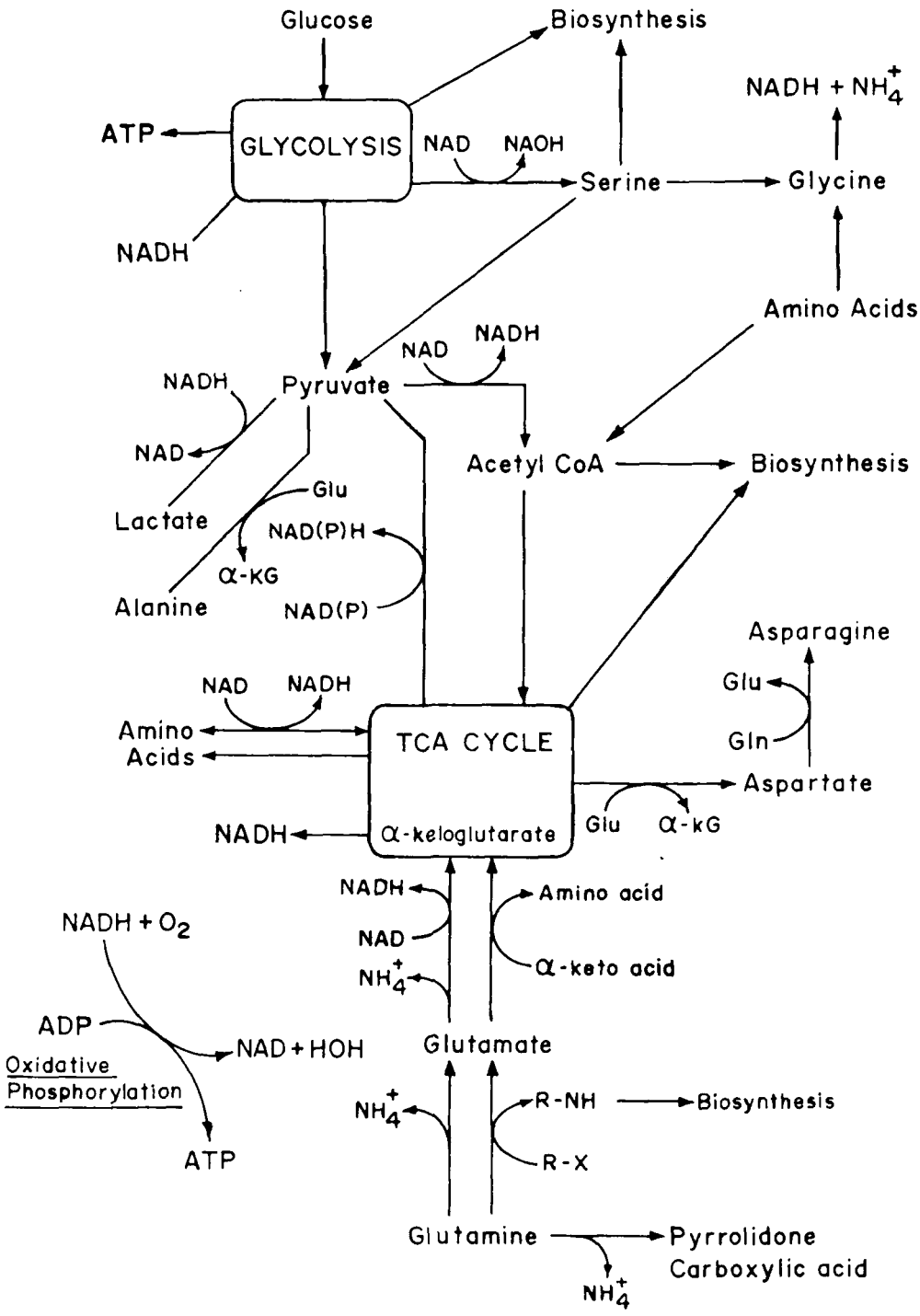


FIG. 2·1: SCHEMATIC DIAGRAM OF THE GLUCOSE AND GLUTAMINE METABOLIC PATHWAYS (REPRODUCED FROM MILLER et. al., 1988)

Serum is an essential requirement for the growth of most animal cells *in vitro*. It has a number of functions in the nutrition of cells, *viz*., it provides vitamins and trace elements, several protein factors required for the attachment and spreading of adherent cells, and also growth promoting and regulating factors and hormones. Serum also has a protective effect on cells. It has antitoxin and antioxidant activities and also is an inhibitor of proteases. Serum is also known to provide protection to cells from mechanical damage (Papoutsakis, 1991).

The addition of serum however, poses problems especially on a large scale, because it is a chemically undefined, complex mixture of proteins which creates difficulties in downstream processing. The composition of serum may vary from batch to batch which makes the reproducibility of culture conditions difficult. It is also the most expensive component of the medium, which makes it an important cost factor especially on scales greater than 1000 L. Besides serum being a nutritionally rich substrate is extremely susceptible to contamination, especially from viruses and mycoplasmas present in the donor animal.

A considerable extent of research has been done on the development of low and serum free media with a view to reduction in the cost of cell culture processes (Barnes and Sato, 1980; Mather, 1984; Butler, 1986). The serum requirement for cells is not constant during the total period of growth. It is required in larger amounts during the initial phase of high growth rate and thereafter the requirement is reduced (Horng and McLimans, 1975). Serum free media have to be supplemented with hormones and growth factors such as fibronectin, transferrin,

insulin, steroids, and polypeptide growth factors. Although these media have the advantages of being chemically defined with low protein concentrations, they are also significantly more expensive than serum supplements, as highly purified preparations of protein factors have to be used. Besides the requirement of these growth factors may vary from cell to cell and hence, the additional nutrients have to be standardized for each cell line used. On the other hand serum is a versatile substrate which can be used for the culture of different types of cells. Plasma fractions especially albumin has been shown to be a good alternative to the addition of serum (Birch, 1980).

Metabolic Inhibitors

The breakdown of glucose and glutamine leads to the production of waste substances which have a harmful effect on cell growth and production. The major metabolic waste substances produced by cells are ammonia and lactic acid. Ammonia is produced mainly due to deamination of glutamine and is inhibitory at concentrations above 4.0 mM (Glacken *et al.*, 1983). Ammonia at concentrations of 2-3 mM was found to be toxic to cell lines (Visek *et al.*, 1972; Holley *et al.*, 1978; Miller *et al.*, 1988). In the case of hybridomas, concentrations above 2 mM are known to cause a decrease in cell viability and lactate production (Reuveny *et al.*, 1986b). The accumulation of ammonia can be prevented by different methods such as perfusion, by the use of alternative carbon substrates or by removal of ammonia by dialysis.

Lactic acid is a product of glucose and glutamine breakdown (Zielke *et al.,* 1976; 1980). The amount produced depends upon the dissolved oxygen levels (Reuveny *et al.,* 1986b). An almost complete conversion of glucose to lactate has been reported (Boraston *et al.,* 1984; Low and Harbour, 1985). Lactate accumulation in the medium above 2.5 mm mL^{-1}, causes a reduction in pH which affects the cell growth. This accumulation can be controlled by various strategies such as substituting glucose with substrates which are utilized less rapidly e.g. galactose and fructose (Eagle *et al.,* 1958), supplying glucose at low levels by fed batch or continuous feeding of glucose (Glacken *et al.,* 1983) or by the addition of biotin in the media (Young and Nakano, 1980).

Mammalian cell growth is regulated by a complex physiology and besides being dependent on the concentration of the major nutrients present and the toxic metabolites produced, is also affected by certain protein factors which are synthesized by the cell themselves. This regulation is reflected in the well known requirement of serum for cell growth in culture. This is also evident in the case of anchorage dependent cells where the adhesion and proliferation factors are known to be present either in serum or secreted by the cells as soluble factors (Grinnel, 1978; Hay, 1981). Another manifestation of this regulatory mechanism is the inoculum cell density effect in *in vitro* cell culture, where the population growth rate is dependent on the initial inoculum cell density (Hu *et al.,* 1985).

In the present work, growth and antibody production of a murine hybridoma CC9C10, which produces monoclonal antibodies against insulin was

examined at different initial concentrations of glucose, glutamine and foetal calf serum. The effect of different inoculum cell densities on the cell growth rate has also been studied.

Another murine hybridoma AE9D6 which also produces anti insulin monoclonal antibodies was cultivated in batch culture. The kinetics of cell growth have been studied for this cell line in batch and fed batch culture. The hybridoma was also cultivated in a hollow fiber bioreactor. The growth in the reactor was monitored by measuring the concentration of the substrates glucose and glutamine and the metabolite produced i.e., lactic acid in the culture fluid.

2.1 MATERIALS AND METHODS

Cell Lines and Media

Two murine hybridoma cell lines CC9C10 and AE9D6 (NFATCC) were used in the study. Both the hybridomas produce IgG monoclonal antibodies against insulin. The cells were routinely maintained in static cultures in T flasks. The medium used was Dulbecco's Modified Eagle's Medium (DMEM, Gibco Laboratories) supplemented with 10% foetal bovine serum (FBS, Biologicals, Israel). No antibiotics were added to the medium. The cultures were incubated in a controlled 5% CO_2 atmosphere at 37°C.

The effect of different initial concentrations of glucose, glutamine and FBS was studied by cultivating cells in DMEM to which varying concentrations of the above nutrients were added. The experimental set up used for these studies was similar to that described by Truskey *et al.,* (1990). The experiments were carried out under batch operation by culturing cells in 24 well plates (Nunc, USA) and incubating them under the conditions described above. The seeding density of the cells was approximately 5-7 x 10^4/mL. The total volume in each well was 1 mL. The sampling was done at fixed intervals. The cells were enumerated to obtain the total and viable cell counts. The supernatants collected after microfuging the samples, were aliquoted and stored at -20°C. These were analyzed for the residual concentration of glucose, lactic acid and the antibody produced. The results presented are the mean of at least four independent samples.

Batch culture of the hybridoma AE9D6 was carried out in 75 cm^2 T flasks containing 30 mL culture volume using DMEM supplemented with 10% FBS and incubation conditions as described before. Samples were withdrawn at fixed time intervals and analyzed for the cell counts (total and viable), residual glucose and glutamine concentration and lactic acid produced. Fed batch cultivation of these cells was also carried out in a similar system using 125 cm^2 with an initial volume of 30 mL. This was fed with complete medium in a stepwise manner till a final volume of 120 mL. Samples were withdrawn and analyzed as in the earlier case.

Biological Analysis

The total and viable cell counts were estimated according to Phillips (1973) using trypan blue or Erythrosin B (Sigma Chemical Co., USA) at a final concentration of 0.4% (atleast 100 cells per sample were counted).

The residual glucose concentration was determined using the glucose oxidase-peroxidase enzymatic kit (Sigma Chemical Co., USA), according to the procedure described in the kit. The glutamine concentration in the culture supernatant were measured using an enzyme assay kit which included the use of asparaginase for the conversion of glutamine to glutamate (Boehringer Mannheim). The lactic acid concentration in the culture fluid was estimated according to Hohorst (1963) with some modifications. The assay mixture contained the following: 0.45 mL hydrazine glycine buffer (0.4M hydrazine- 1M glycine, pH 9.5), 0.05 mL NAD (40 mg/mL), 0.3 mL distilled water, 0.01 mL lactate dehydrogenase enzyme suspension (Sigma Chemical Co., USA) and 0.01 mL sample (suitably diluted).

The absorbance was read at 340 nm, at time zero and then incubated at room temperature for 60 min. The absorbance was read again at 340 nm and the concentrations of lactic acid determined from the difference in absorbance according to the procedure described by Hohorst (1963).

The indirect sandwich ELISA test was carried out for measuring the amount antibody present in the culture supernatants. The procedure followed is described briefly below. Bovine insulin (Boehringer Mannheim) at a concentration of 5µg/well was coated onto 96 well polystyrene plates (Nunc, USA). The nonspecific binding sites were blocked by coating with 1% BSA. Plates were washed with phosphate buffered saline (PBS)-tween (0.05%) thrice. The culture supernatant suitably diluted was then added to the wells. Plates were incubated at 37°C for 60 min. They were washed with PBS- tween (3X) and then anti-mouse IgG-HRP conjugate (polyvalent preparation from Sigma Chemical Co., USA., diluted 1:1000) was added to the wells and incubated at 37°C for 60 min. The amount of antibody present was estimated by using o-phenylene diamine substrate which is hydrolyzed by horse radish peroxidase enzyme to give a yellow coloured product. The reaction was stopped using H_2 SO_4(4N) and the colour developed read on an automatic ELISA reader (Dynatech, Labs). The values obtained were multiplied by a factor of 1000 to obtain an ELISA index indicative of the amount of antibody present in the culture fluid.

Cultivation of Murine Hybridoma (AE9D6) in a Hollow Fiber Bioreactor

The Acusyst-R hollow fiber bioreactor, Endotronics, Inc., USA) was used for the cultivation of a murine hybridoma AE9D6. The core of the system consists of a bundle of hollow fibers (23 cm x 2.0 cm dia). The fibers are potted at the ends with epoxy resin and are fixed in a polycarbonate casing. The reactor has two compartments, the intracapillary space (ICS) or the lumen and the extracapillary space (ECS) or the shell side. The cells are entrapped in the shell side (approximately 80 mL) and the nutrients are fed through the lumen side. The medium was recirculated several times through the lumen. The nutrients diffuse to the cells across the membrane and metabolites such as lactic acid diffuse back to the lumen from where they are carried away by the spent medium (Fig.2.2). The experimental set up of the reactor is shown schematically in Fig.2.3. The system has a device which alternates the direction of lumen flow in order to minimize the concentration gradients in the axial direction.

The hollow fiber cartridge is supplied as a presterilised module. The cultureware including the reservoir, the jet pump, the feed and outflow lines was assembled and autoclaved. The reactor was then attached under sterile conditions. The culture unit consists of a reservoir from which nutrient medium is supplied to the reactor via the recirculation pump. Fresh medium is fed into the reservoir after it is oxygenated during its passage through the jet pump where a gas mixture of air and 5% CO_2 is fed. The reactor assembly was placed in an incubator chamber at a temperature of 37°C.

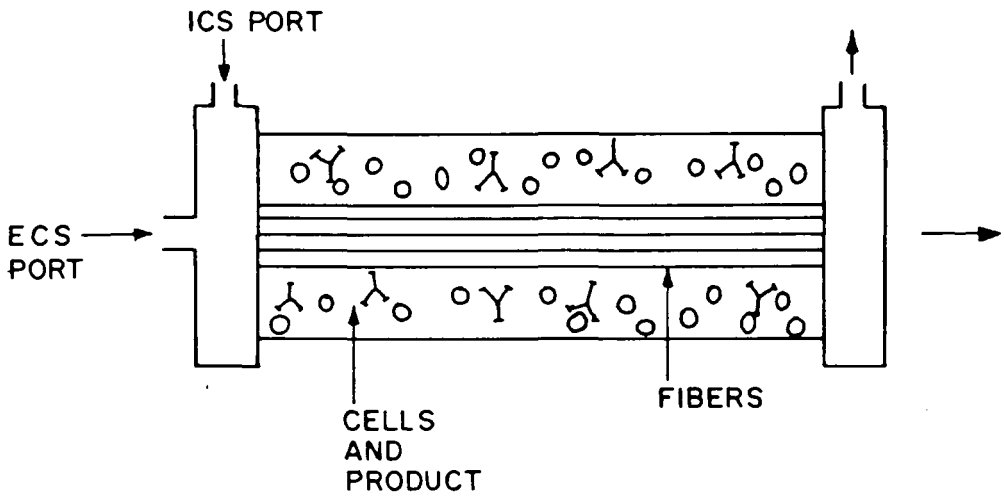
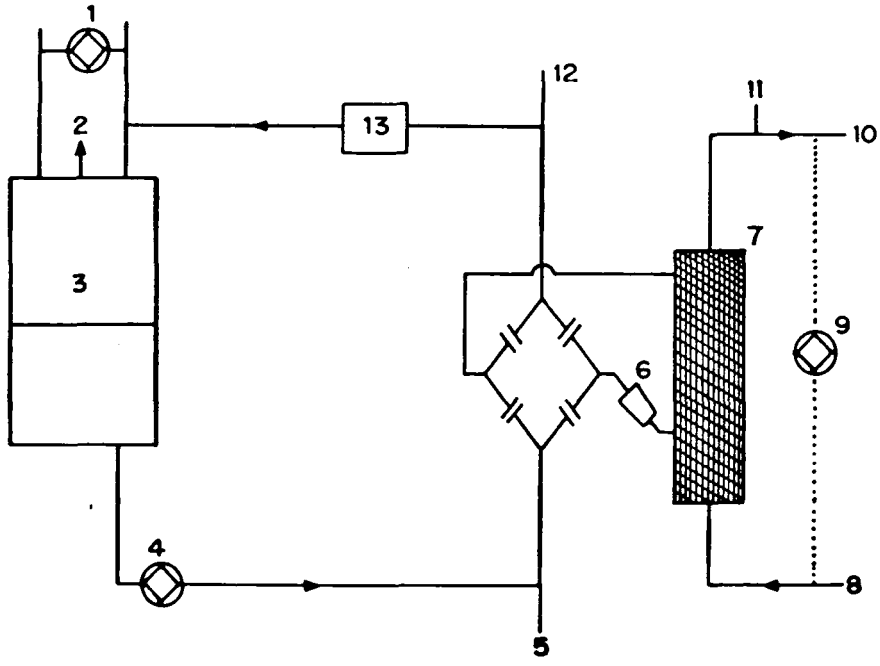


FIG.2·2: SCHEMATIC DIAGRAM OF GROWTH OF HYBRIDOMA CELLS IN THE HFBR



1) Media outflow pump ; 2) Vent ; 3) Reservoir ; 4) Circ. pump ;
5) Sample ; 6) Temp. probe ; 7) Bioreactor ; 8) Factor ;
9) Harvest factor pump ; 10) Harvest ; 11) Inoculation and sample ;
12) Sample ; 13) Gas jet

FIG. 2·3: SCHEMATIC REPRESENTATION OF THE FLOWPATH OF THE HFBR

The reactor was first flushed with medium (DMEM) and filled with fresh medium. During this filling procedure care was taken to fill the ECS first and then the ICS, in order to prevent the trapping of air inside the ECS. The sterility of the system was ascertained by incubating it for 3-4 days. The ECS was then conditioned by treating with DMEM containing 10% FBS. The cells cultivated in T-flasks were collected by centrifugation, washed with DMEM and resuspended in fresh medium containing serum. The cells were inoculated via the inoculation port, using a hypodermic syringe with a luer lock. The reactor was periodically inverted to allow the cells to distribute throughout the reactor. After allowing the cells to adhere to the fibers they were supplied with fresh medium containing serum. Nutrient medium was recirculated at the rate of 100 mL/min and medium replacement was done at the rate of 10mL/h. Samples were collected at fixed time intervals from the bioreactor as well as the reservoir via the sample ports. These were stored at -20°C and analyzed for residual concentrations of glucose and glutamine as well as the lactic acid produced. Samples of the cells could not be taken due to the construction of the system.

2.2 RESULTS

Nutritional Requirements of a Murine Hbridoma CC9C10:

The cell growth of a murine hybridoma with respect to the growth rate and the extent of growth was studied at different initial concentrations of glucose (5.5 mM - 22.0 mM) keeping the concentration of glutamine, serum and other nutrients constant. The pattern of growth of the cells was similar at all the concentrations studied (Fig.2.4A). The cell populations have an initial lag phase of 24 h after which they enter the exponential growth phase extending upto 6 days. An increase in the glucose concentration from 5.5 mM to 11.1 mM resulted in an increase in the growth rate by about 45%, from 0.019 h^{-1} at 5.5 mM to 0.027 h^{-1} at 11.1 mM (growth rates were calculated for the exponential phase). The final cell yields in terms of the total cell number was 42% less in the case of the lower glucose concentration. Increasing the initial glucose concentration from 11.1 mM to 22 mM did not have any effect on the growth rates or the final yield of cells. Similar observations have been made by Miller *et al.*, (1988) who reported that the growth rates are independent of glucose concentrations in the range 5.6- 27.8 mM.

The lactic acid production rates also exhibited a similar pattern with the rate at 5.5 mM glucose concentration about 40% (2.83 mmoles/day) less when compared to the rate at 11.1 mM (4.3 mmoles/day). There was no significant increase in the amount of lactic acid produced when the initial glucose concentration was increased to 22.2 mM (Fig.2.4B). The glucose consumption rate at 5.5 mM

was also 40% less than the consumption rate at 11.1 mM. The glucose consumption rate was independent of the initial glucose concentrations in the range 11.1 mM to 22.2 mM (Fig.2.4C). Further, it was observed that the amount of lactic acid produced is proportional to the number of cells present, and was lower at 5.5 mM glucose concentration, where the number of cells produced was also correspondingly reduced. The amount of antibody produced was also less at 5.5 mM glucose concentration and was independent of the initial glucose concentrations in the higher range (Fig.2.5).

The cell growth at different initial glutamine concentrations was studied with respect to the growth rates and total cell numbers. The growth rate and the cell yields were dependent on the initial glutamine concentration upto a concentration of 2.0 mM (Fig. 2.6). The antibody production was also found to increase with an increase in the initial glutamine concentration from 0 to 2.0 mM (Fig.2.7). The amount of antibody produced was dependent on the number of cells present. In another set of experiments the initial glutamine concentrations were increased beyond 2.0 mM and it was seen that the growth rates and cell yields increased with an increase in the initial glutamine concentration from 4.4 mM to 5.2 mM. A further increase in the glutamine concentration resulted in a decrease in the cell yields (results not shown). Glutamine was found to be the growth limiting nutrient and its consumption followed the Monod equation (Miller *et al.*, 1989b). The results observed in the present work are in agreement with the earlier observations.

Fig. 2.4 **Hybridoma cell growth at different initial glucose concentrations:** CC9C10 hybridoma cells were cultivated in DMEM + 10% FBS medium at different initial glucose concentrations (in mM -o- 5.5; -●- 11.0; -Δ- 16.5; -▲- 22.0) and the effect was studied by

A) Total cell count
B) Lactic acid produced
C) Glucose consumed by the cells.

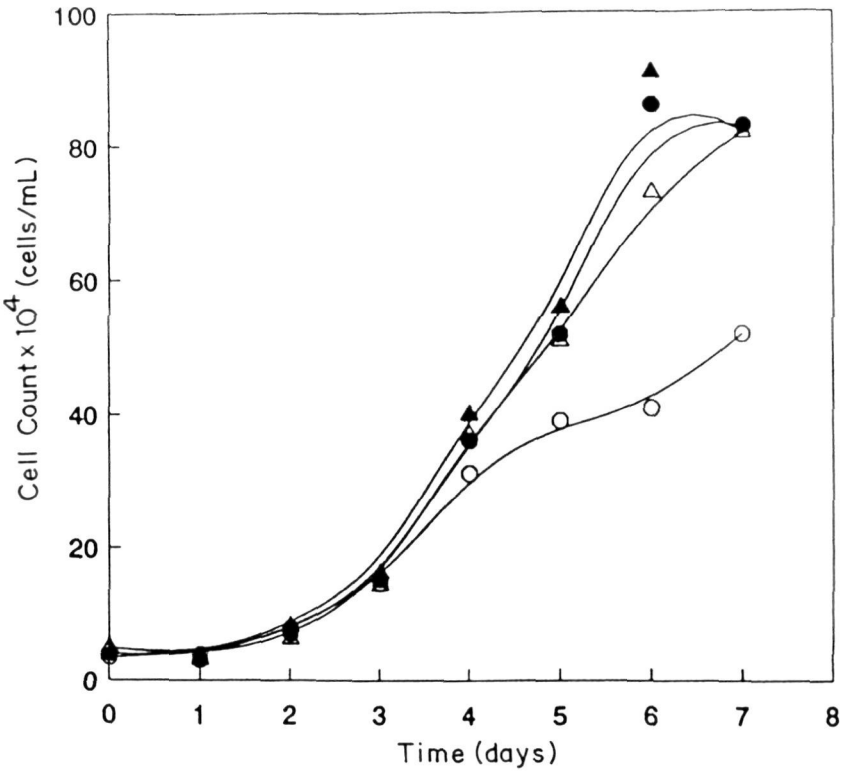


Fig.2·4A

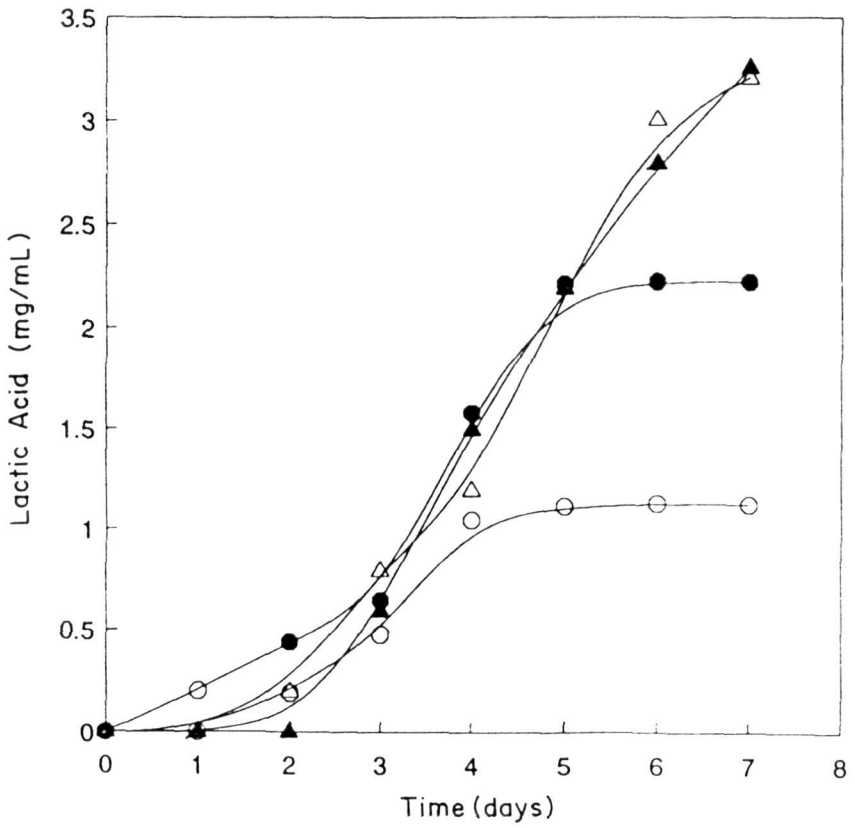


Fig.2·4B

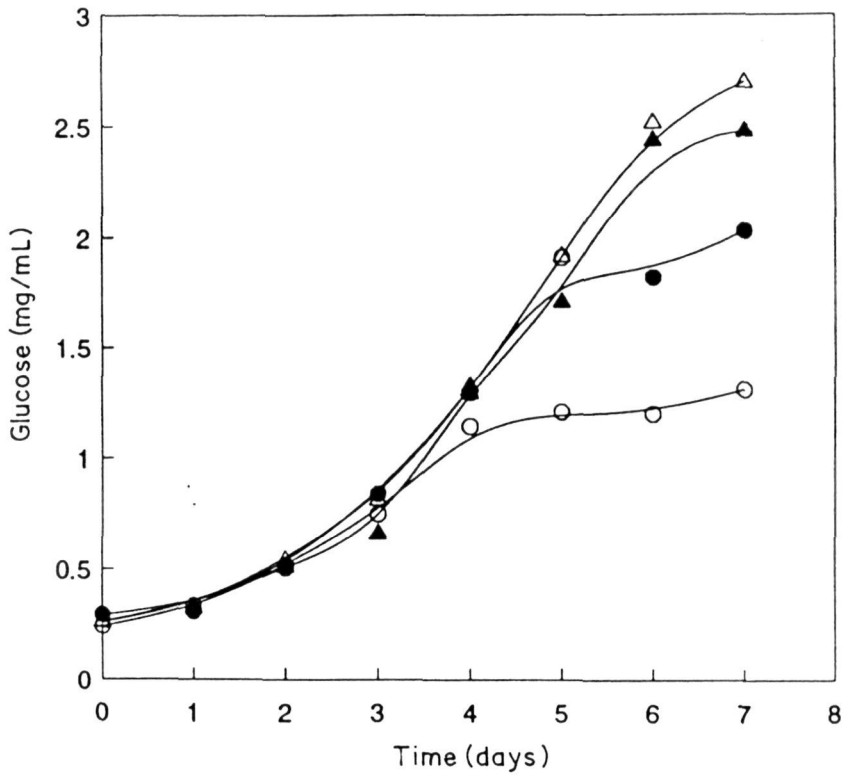


Fig. 2·4C

Fig. 2.5 **Effect of initial glucose concentration on production of monoclonal antibody:** CC9C10 cells were grown in complete medium (DMEM + 10% FBS) at different initial glucose concentrations. The culture supernatants were collected after 6 days of incubation and the amount of antibody was estimated by sandwich ELISA and the ELISA index obtained.

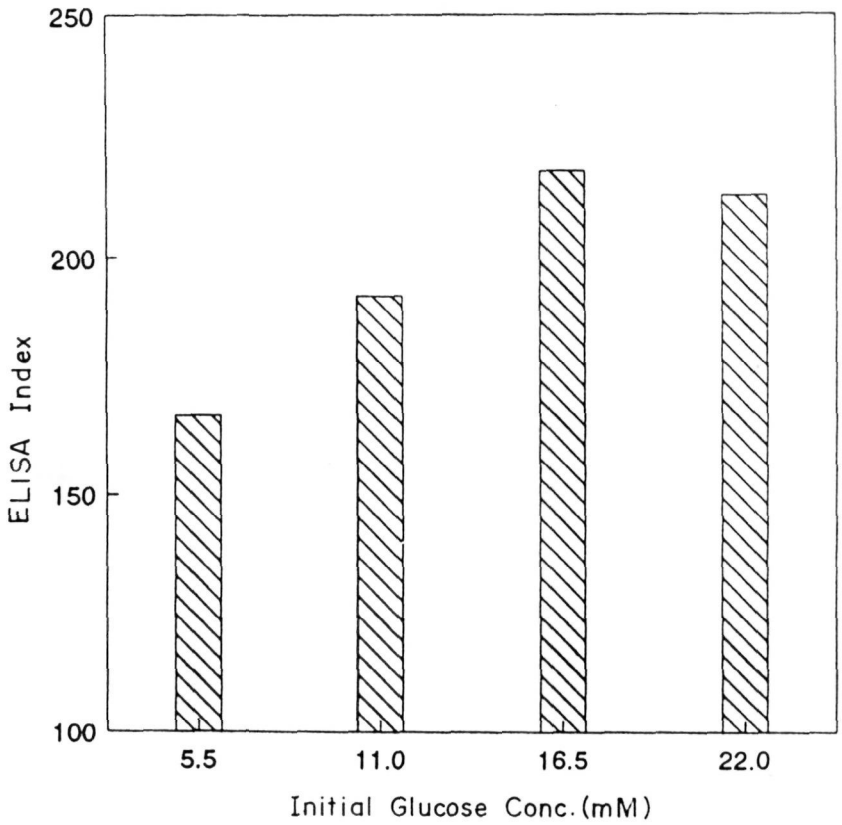

250
200
150
100
ELISA Index
5.5
11.0
16.5
22.0
Initial Glucose Conc.(mM)

Fig.2·5

Fig. 2.6 **Hybridoma cell growth at different initial glutamine concentrations:** CC9C10 cells were cultivated in complete medium (DMEM + 10% FBS) at different initial concentrations of glutamine (in mM -o- 0 ; -●- 0.5; -Δ- 1.0; -▲- 1.5; -□- 2.0) and the total cell counts were monitored.

Fig. 2.7 **Monoclonal antibody production by CC9C10 cells at different initial glutamine concentrations:** CC9C10 hybridoma cells were cultivated in complete medium (DMEM + 10% FBS) with varying initial glutamine concentration. The culture supernatants were collected after 6 days of incubation and sandwich ELISA was used to obtain ELISA Index indicating the amount of antibody produced.

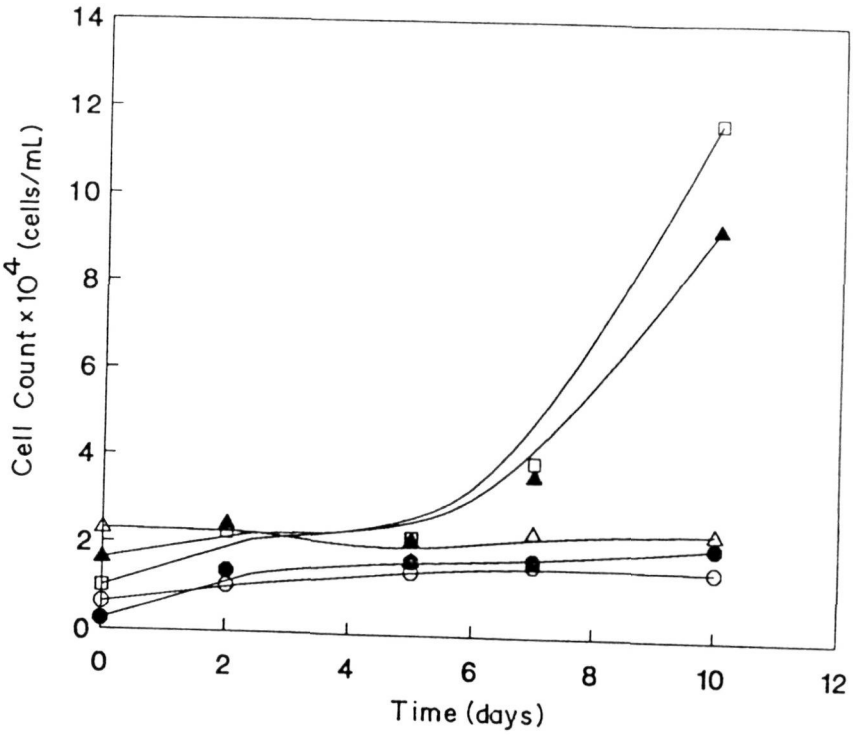

Cell Count x 10^4 (cells/mL)
14
12
10
8
6
4
2
0
0
2
4
6
8
10
12
Time (days)

Fig.2·6

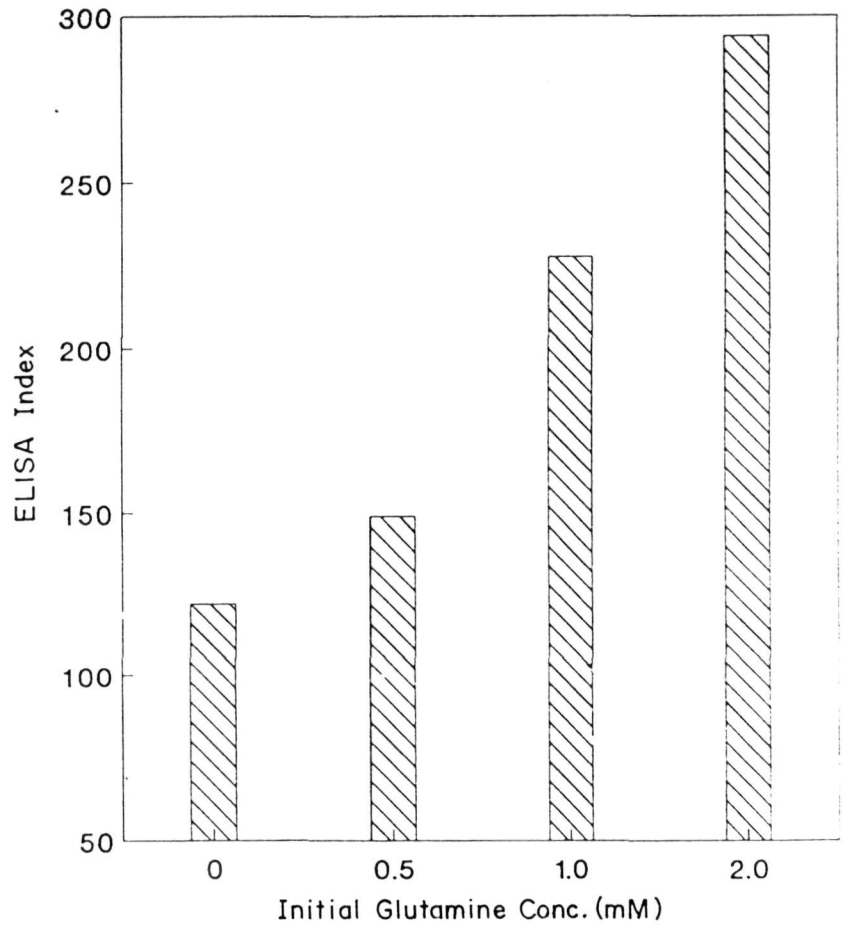

ELISA Index
300
250
200
150
100
50
0
0.5
1.0
2.0
Initial Glutamine Conc. (mM)

Fig. 2·7

The cells maintained in serum containing media can be grown for a few passages in low serum or serum free medium. The growth of cells in medium with varying concentrations of FBS (2.5% to 12.5%) was examined and it was observed that the growth rate (exponential phase) and the cell yields were independent of the initial serum concentrations (Fig.2.8A). However, there was a decrease in the amount of monoclonal antibody produced at 2.5% initial FBS concentration (Fig.2.8B). Similar observations have been made by Truskey *et al.*, (1990) for hybridoma cells.

The effect of the initial seeding density on the growth of hybridoma cells is shown in Fig. 2.9A. It is evident from the results that the average growth rates and the cell yield are reduced at lower initial inoculum cell densities. It was also observed that at low inoculum cell density the lag period is extended. The amount of antibody produced was also reduced by 32.5% at the initial cell density of 1 x 10^3 cells (Fig.2.9B).

Batch and Fed Batch Culture of a Murine Hybridoma AE9D6

A murine hybridoma AE9D6 was grown in T flasks under static conditions in a batch mode. Typical batch growth profiles were obtained for the cells over a period of 6-7 days. The cell population undergoes a critical initial lag period of about 2 days followed by an exponential phase and finally by a decline in the viable cell numbers (Fig.2.10A). Glucose and glutamine consumption and lactic acid production profiles are shown in Fig.2.10B. The apparent molar yield of lactate from glucose was 1.184 (59% of the theoretical maximum). The decline in

Fig. 2.8A **Effect of initial FBS concentration on the growth of hybridoma cells:** CC9C10 hybridoma cells were grown in DMEM containing different concentrations of FBS (in % -o- 2.5; -●- 5.0; -Δ 7.5; -▲- 10; -□- 12.5) and the effect on growth of cells was examined by monitoring the total cell counts.

Fig. 2.8B **Monoclonal antibody production at different initial FBS concentrations:** CC9C10 cells were cultivated in DMEM with different intial FBS concentration. The culture fluids obtained after 6 days of incubation were analyzed for the amount of monoclonal antibody secreted by sandwich ELISA technique.

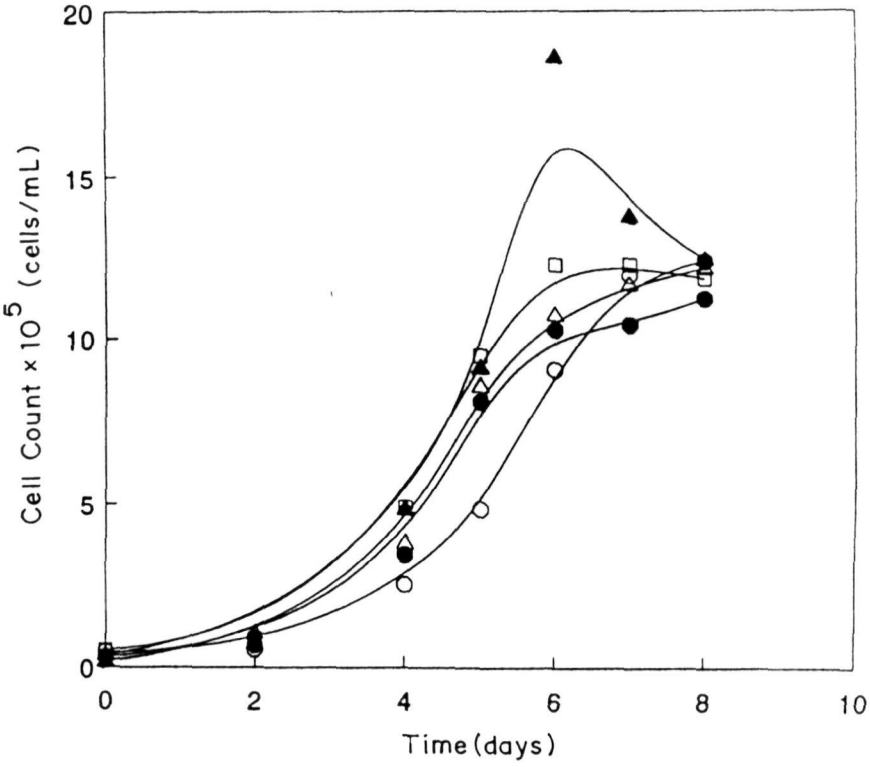


Fig.2·8A

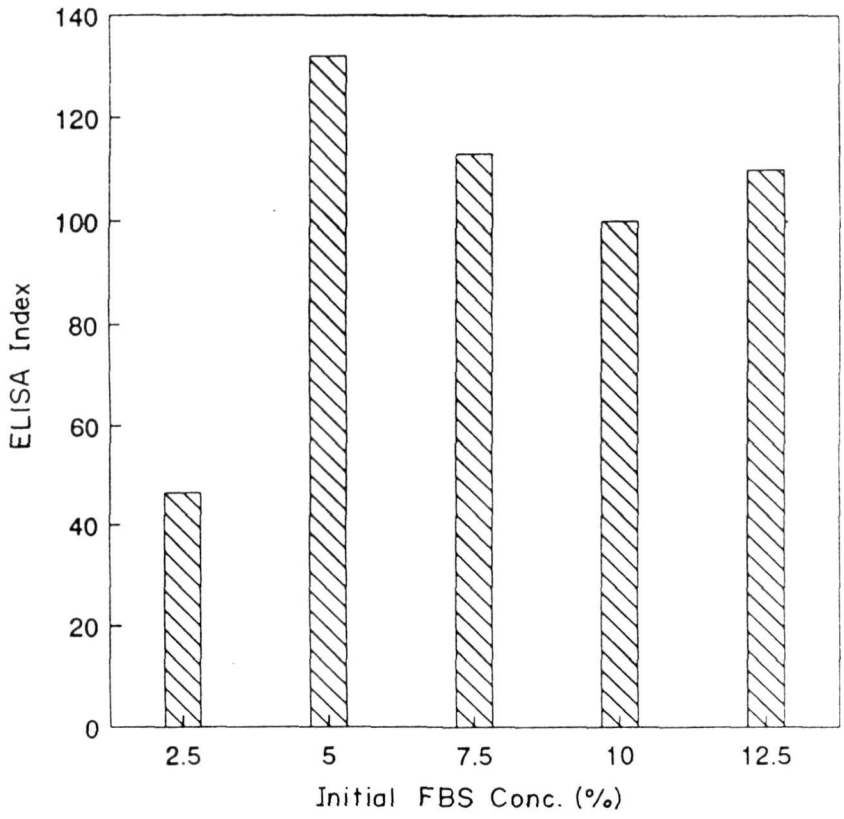


Fig. 2·8B

Fig. 2.9A Cell growth at different inoculum densities: CC9C10 hybridoma cells were plated in 24 well plates at varying initial cell concentrations (cells/mL, -Δ- 1.0 x 10^4 ; -▲- 6 x 10^3 ; -o- 3 x 10^3 ; -●- 1. x 10^3) and the effect of inoculum density was studied by monitoring the total cell count.

Fig. 2.9B Effect of inoculum density on antibody production: CC9C10 cells were grown in 24 well plates at different initial cell concentrations (cells/mL, A- 1.0 x 10^3 ; B - 3 x 10^3 ; C - 6 x 10^3 ; D - 1. x 10^4). Culture supernatants were harvested after 4 days incubation and the amount of antibody in the culture fluid was estimated by sandwich ELISA to obtain the ELISA index.

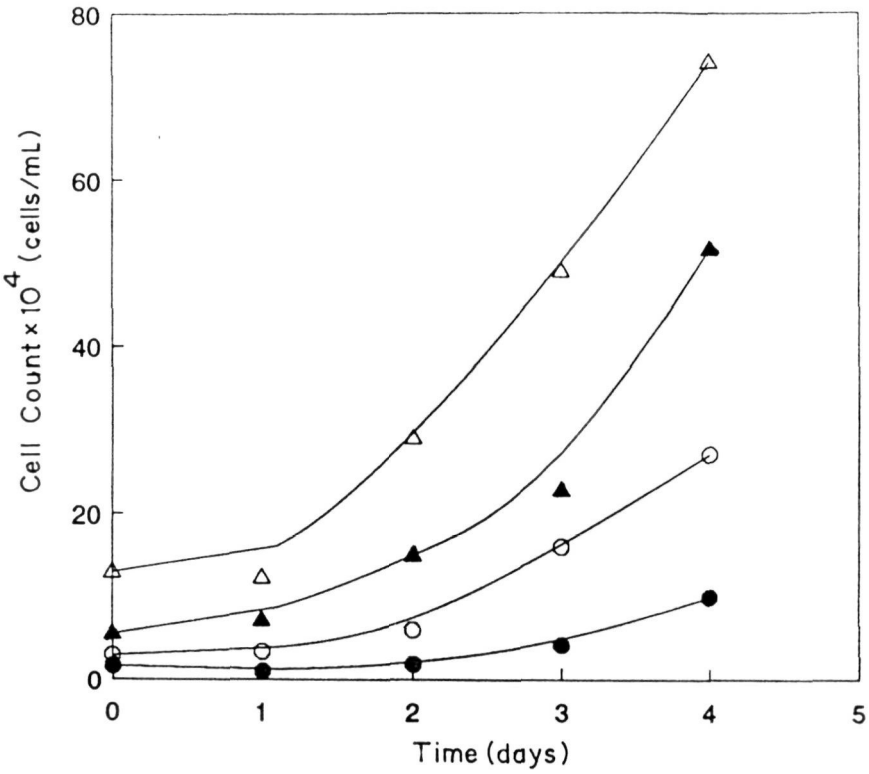


Fig. 2·9A

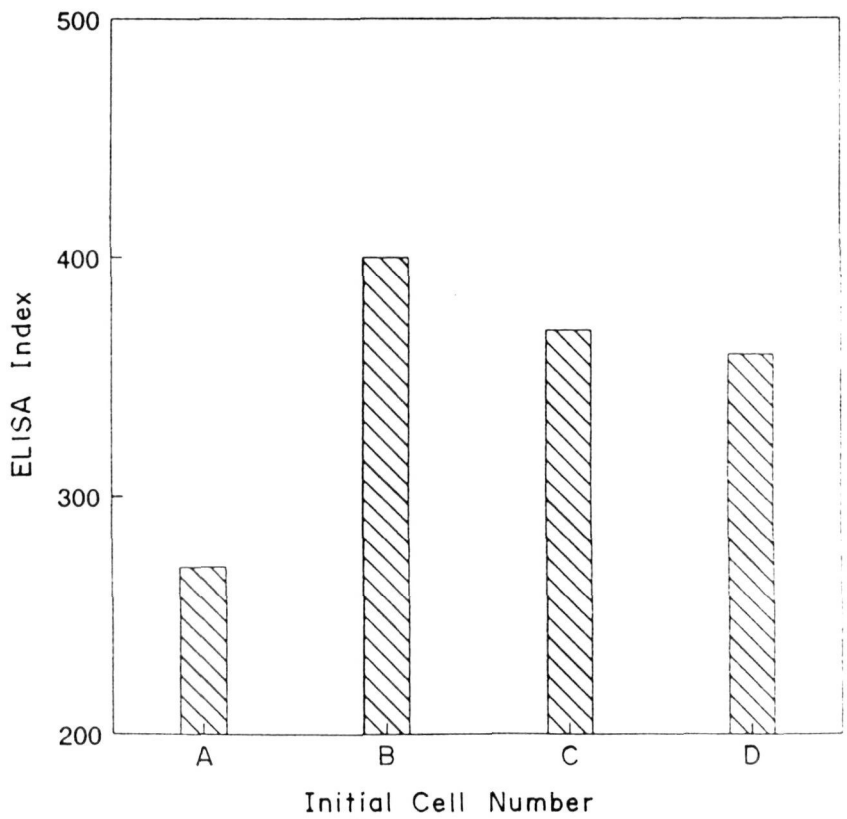


Fig. 2·9B

Fig. 2.10 **Batch cultivation of AE9D6 hybridoma cells:** AE9D6 hybridoma cells were cultivated in complete medium (DMEM + 10% FBS) in T-flasks under static conditions. The growth pattern of the cells was followed by

A) monitoring the total (-o-) and viable cell (-●-) counts

B) monitoring the consumption of glucose (-o-) and glutamine (-Δ-) and accumulation of lactate (-●-) in the medium.

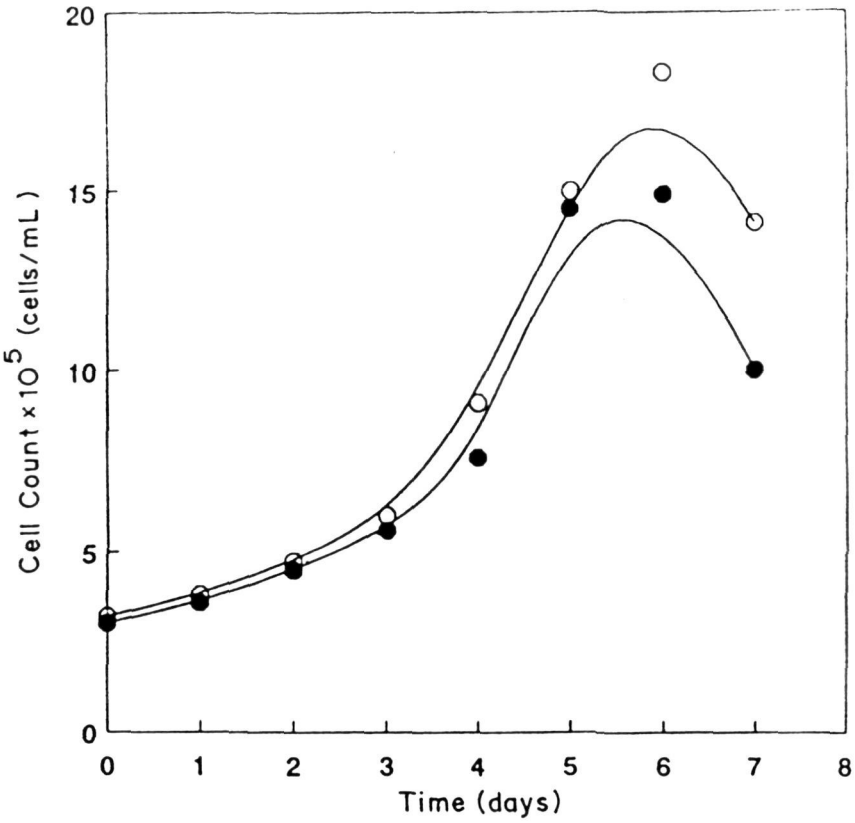


Fig. 2-10 A

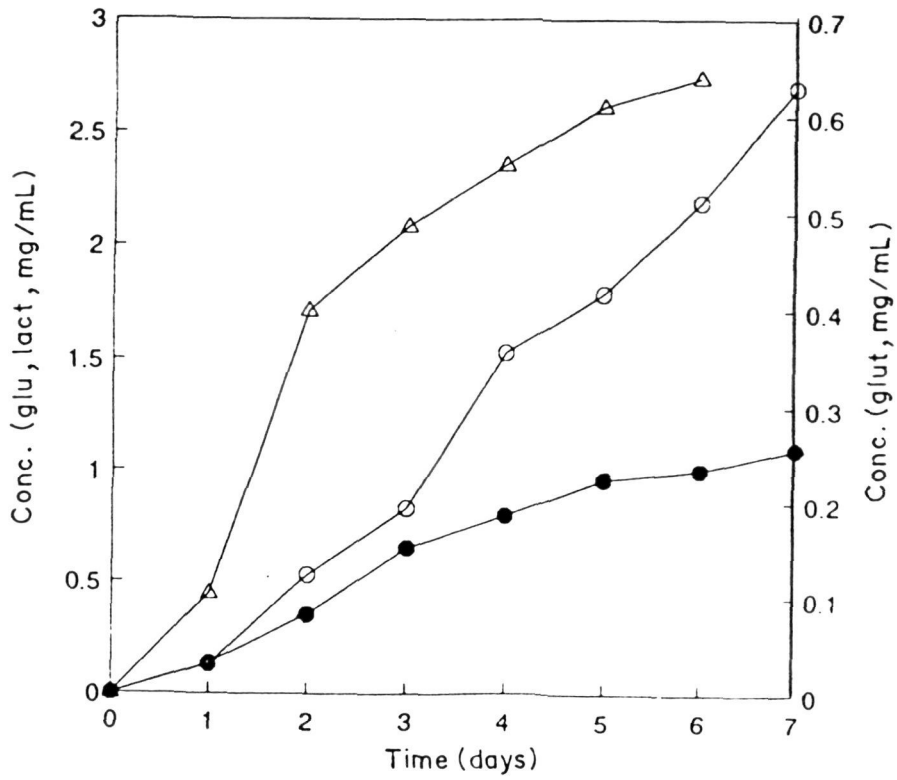


Fig. 2-10B

the viable cell number occurred at the time when the residual concentration of glutamine in the medium was below 1 mM. This indicates that the growth was limited by the depletion of glutamine. The lactic acid accumulated at the end of the batch period (18 mM) could also be the cause of growth inhibition. Nutrient consumption (q_s) in batch can be written as

$$q_s = \frac{\mu}{Y'_{x/s}} + m_s \qquad (8)$$

where $Y'_{x/s}$ is the yield coefficient of cells based on glucose, and m_s is the maintenance coefficient. $Y'_{x/s}$ was found to be 1.69 x 10^8 cells/mmol and m_s was 1.6 x 10^{-8} mmol/cell/day at $\mu = \mu_{max}$. These values are in agreement with the values reported for another murine hybridoma (Miller *et al.*, 1988). The production of lactic acid was found to be growth associated and can be expressed as

$$r_p = \alpha\mu X_v + \beta X_v \qquad (9)$$

where α = growth associated coefficient and β is a non growth associated parameter. Since lactic acid is growth associated β is assumed to be zero, and

$$r_p = \alpha\mu X_v \qquad (10)$$

For the batch culture, α was found to be 3.3 mmol/10^8 cells.

The hybridoma cells were also cultivated in a fed batch mode under static conditions. The cells were grown in T flasks and complete medium was fed to the cultures at fixed time intervals as single step additions. The period of

growth was extended upto 10 days and higher cell yields (as compared to the batch culture) were obtained (Fig.2.11A). The profiles of glucose, glutamine and lactic acid in the fed batch culture are shown in Fig.2.11B. The growth period can be extended by the addition of nutrients. At the end of the fed batch run, however, depletion of nutrients as well as accumulation of lactic acid occurred which led to a decrease in the viability of the population.

The kinetic parameters estimated from the batch experiments were used to calculate the viable cell numbers which could be expected in the batch and the fed batch culture under given experimental conditions. These were then compared to the values obtained in the experiments described above (Fig.2.12A and 2.12B). The results show that although the viable cell numbers calculated and observed are in agreement for the batch culture (since the parameters have been estimated from batch culture) the numbers in the fed batch system are overpredicted.

Cultivation of Hybridoma Cells in the HFBR

The AE9D6 hybridoma cells (1×10^9) were inoculated into the ECS space of the HFBR. The medium (DMEM) was passed through the ICS at a rate of 200 mL/min. Fresh medium was fed at the rate of 10mL/hr. Initially medium containing FBS (10%) was fed to the ECS at the rate of 10mL/h for about 12 h. Then the rate of serum addition was decreased to 2 mL/h. During the entire run 8.5 L of medium (DMEM) was consumed. The serum required was however, only 200 mL. After a period of about 8-10 days after inoculation the cell mass

9204

Fig. 2.11 Fed Batch cultivation of AE9D6 hybridoma cells: AE9D6 cells were cultivated in complete medium (DMEM + 10% FBS) in T-flasks under static conditions. The cultures were fed with complete medium at fixed time intervals as indicated by the arrow (↓). The growth pattern was followed by

A) monitoring the total (-o-) and viable (-●-) cell counts

B) monitoring the residual concentration of glucose (-o-) and glutamine (-Δ-) and the accumulation of lactate (-●-) in the medium.

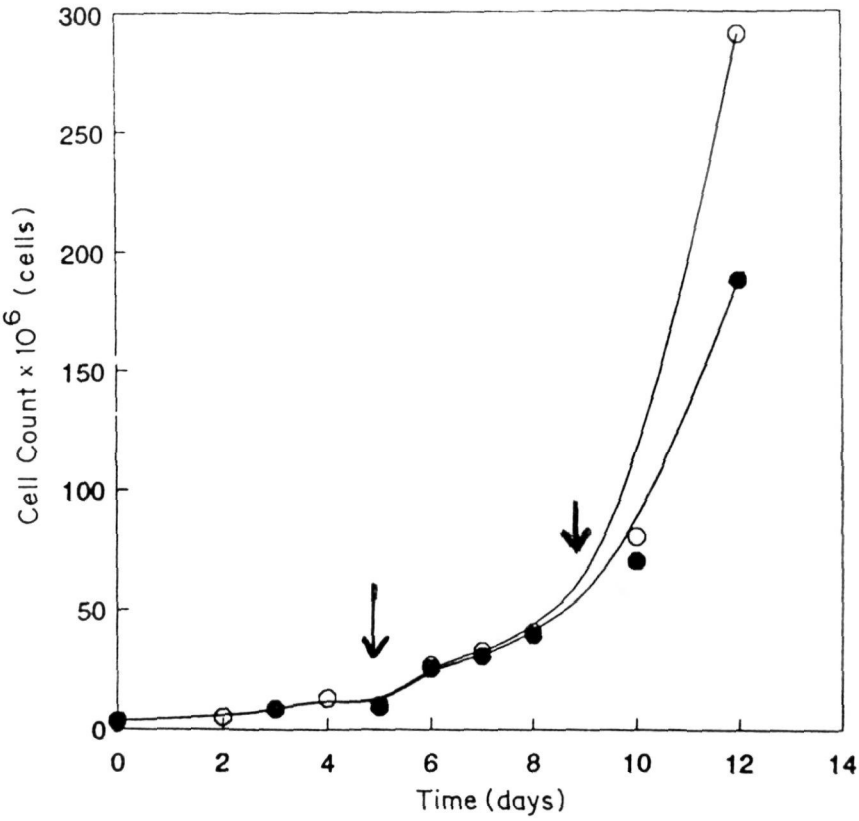


Fig. 2·11A

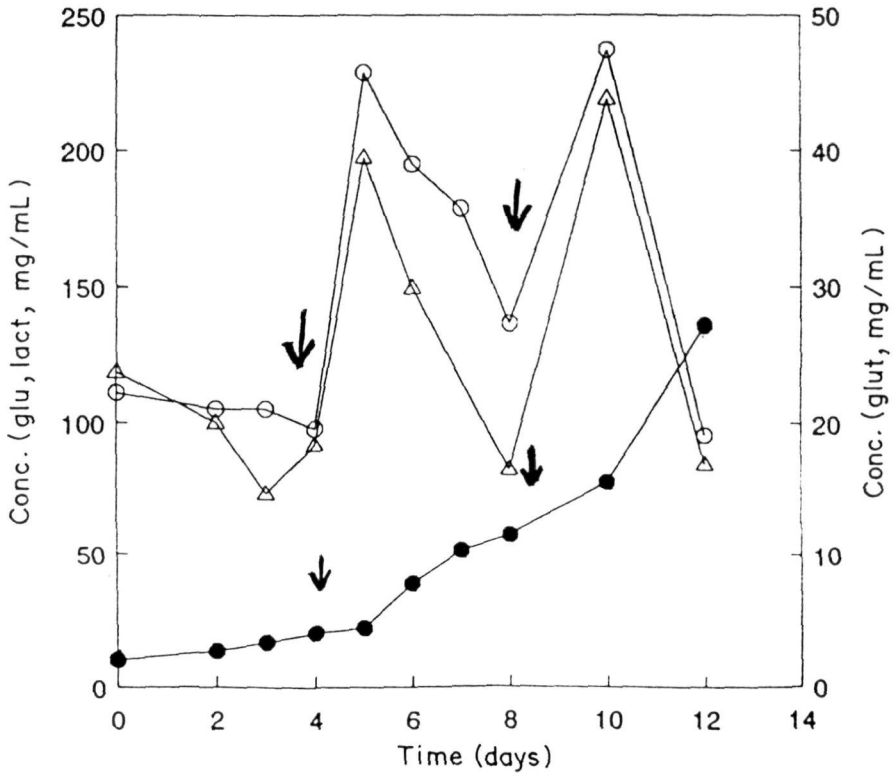


Fig. 2·11B

Fig. 2.12 Viable cell numbers predicted from batch kinetic data: The specific glucose consumption rate and yield coefficient obtained from batch culture were used to predict the viable cell numbers

A) comparison of predicted (-●-) and experimental (-o-) viable cell counts in batch

B) comparison of predicted (-●-) and experimental (-o-) viable cell counts in fed batch.

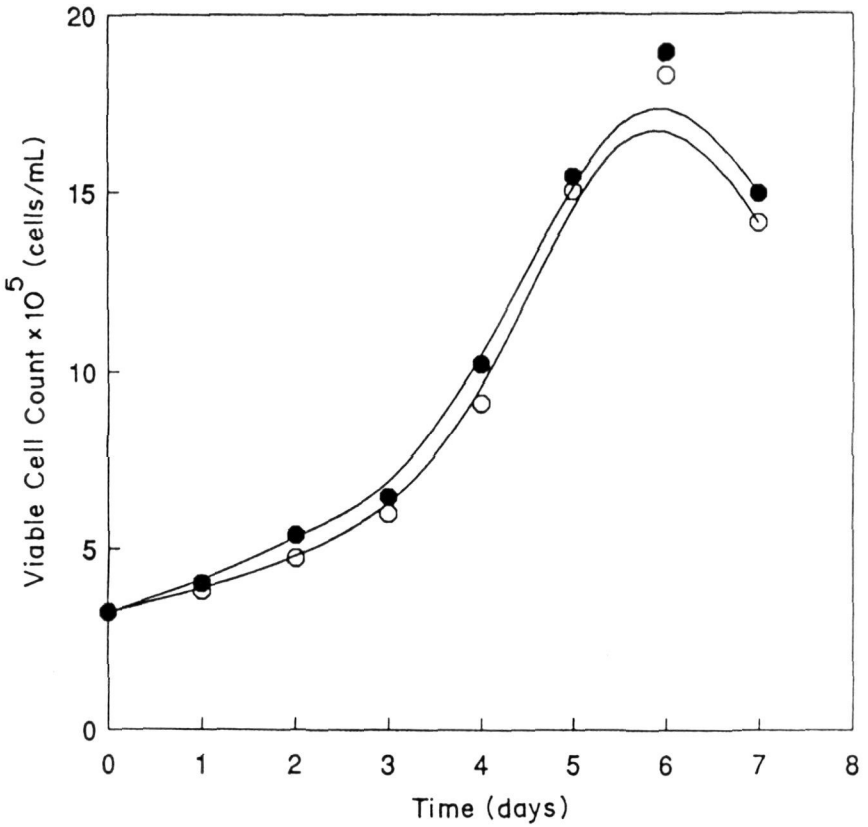

Viable Cell Count x 10^5 (cells/mL)
20
15
10
5
0
0
1
2
3
4
5
6
7
8
Time (days)

Fig. 2·12 A

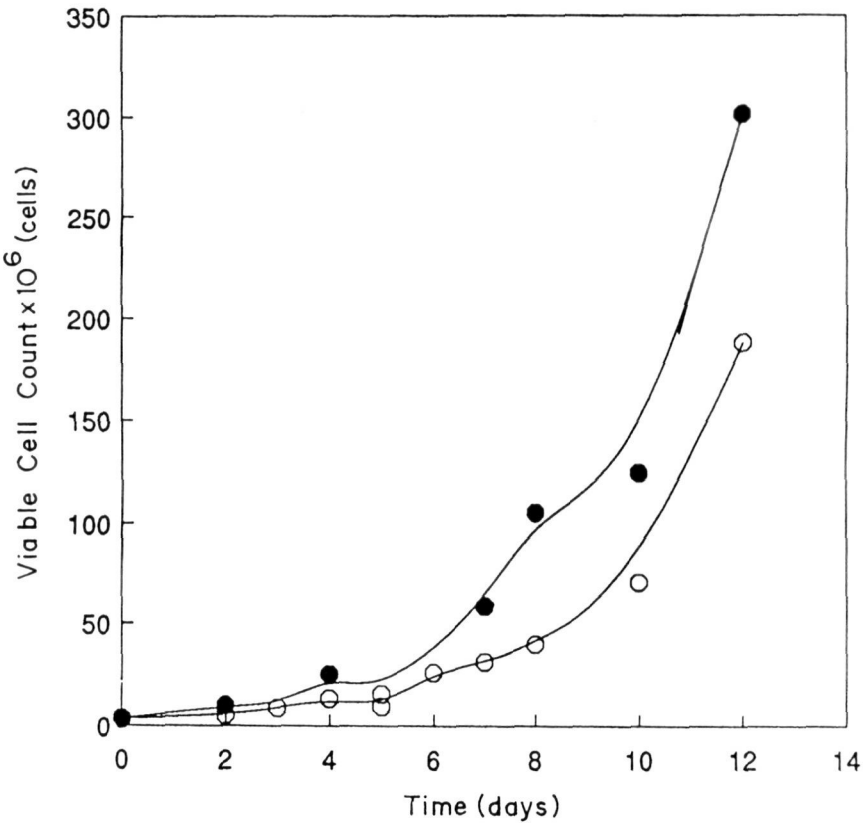

350
300
250
200
150
100
50
0
Via ble Cell Count x 10^6 (cells)
0
2
4
6
8
10
12
14
Time (days)

Fig. 2·12 B

could be observed visually (Fig.2.13). As the cell mass could be seen with the unaided eye, it indicates that the cells were present at very high density. An interesting observation was that the cells were not uniformly distributed throughout the reactor and grew at higher densities at the lower end of the reactor. The glucose and lactic acid profiles in the HFBR are shown in Fig.2.14. The glucose concentration was maintained at a fairly constant level during the first 15 days of culture. After that the perfusion rate (10 mL/h) was not sufficient to maintain the glucose level and depletion was evident. This period corresponds to the time taken for the development of large cell mass in the reactor. The lactic acid concentration during the initial period upto 15 days was at around 0.5 mg/mL, after which it increased and was maintained at about 1.5 mg/mL during the subsequent culture period. The glutamine utilization profile is similar to the glucose profile (Fig.2.15). These profiles indicate that both nutrient depletion and accumulation of lactic acid occur at higher cell densities at a given perfusion rate. From the results it is clear that the feed policy i.e., the rate of addition of fresh nutrients can be determined by monitoring the levels of glucose, glutamine and lactic acid in the bioreactor effluent. The requirement of serum for the growth of cells was greatly reduced. The cell numbers in the reactor can be estimated from the glucose consumption rates in a manner similar to the estimation of cell numbers in the fed batch system. However, this was not done in the present case because it has been reported that such estimations tend to overpredict the numbers and can be significantly erroneous (Chresand *et al.*, 1988).

Fig. 2.13 **AE9D6 hybridoma cell cultivation in the Acusyst-R, HFBR:** AE9D6 hybridoma cells were grown in the Acusyst-R HFBR. The cell growth could be visually observed after a period of 10 days. Non uniform cell distribution was observed with greater cell density at one end of the reactor (→).

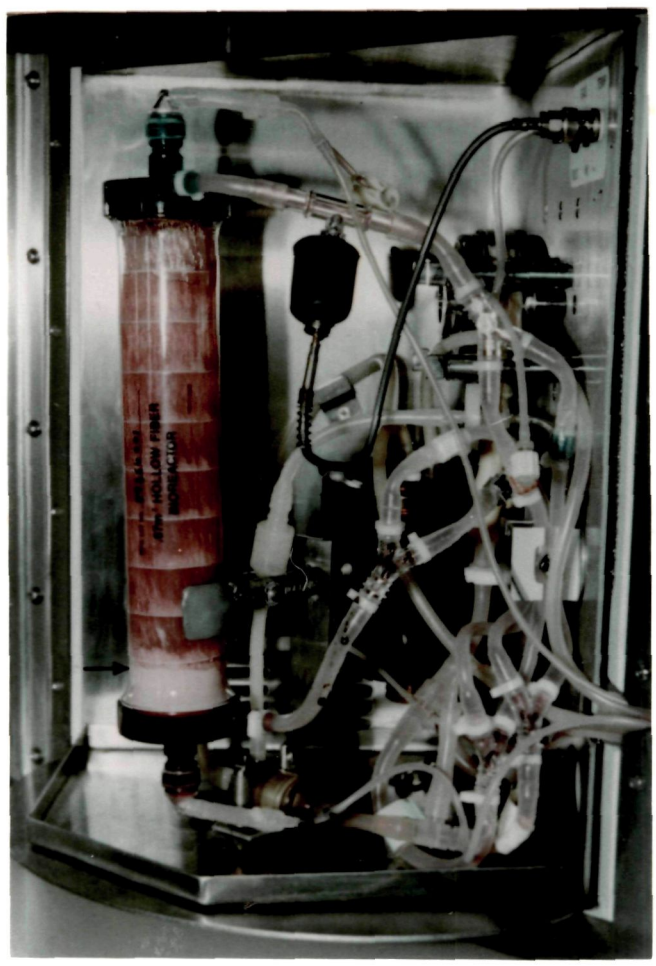


Fig. 2·13

Fig. 2.14 **Profiles of glucose and lactate in the HFBR during culture of AE9D6 hybridoma cells:** AE9D6 hybridoma cells were grown in the Acusyst-R HFBR. The growth of cells in the reactor was monitored by analyzing the concentrations of glucose (-o-) and lactate (-●-) in samples taken from the reservoir at fixed time intervals.

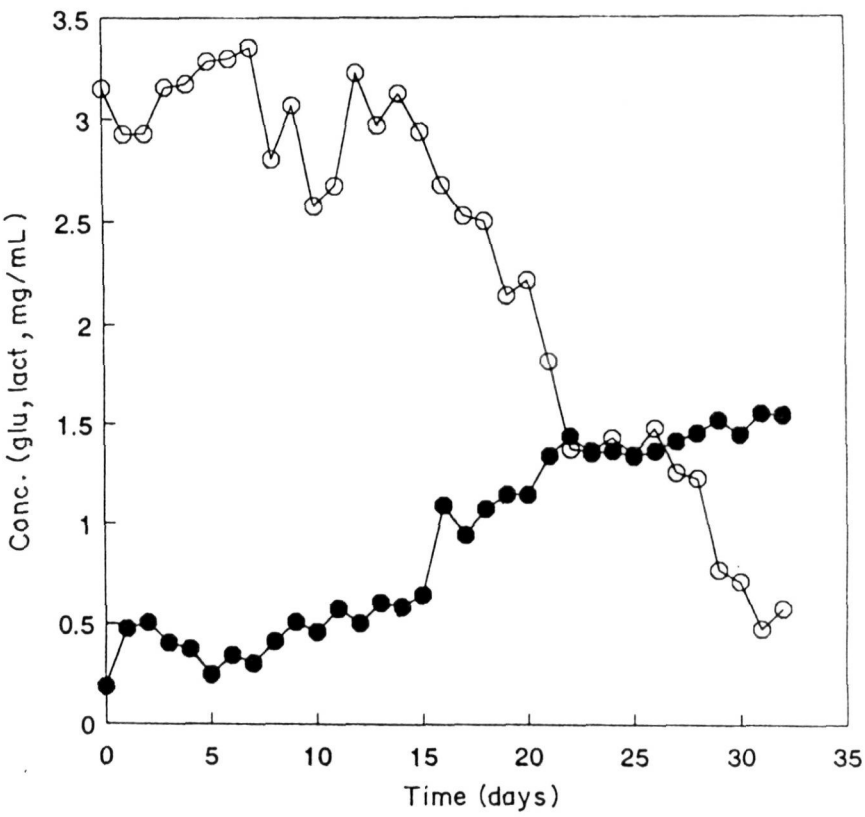


Fig. 2-14

Fig. 2.15 **Profile of glutamine in the HFBR during culture of AE9D6 hybridoma cells:** AE9D6 hybridoma cells were grown in the Acusyst-R HFBR. The consumption of glutamine by the cells in the HFBR was monitored by measuring the residual glutamine concentration in samples collected from the reservoir at fixed time intervals.

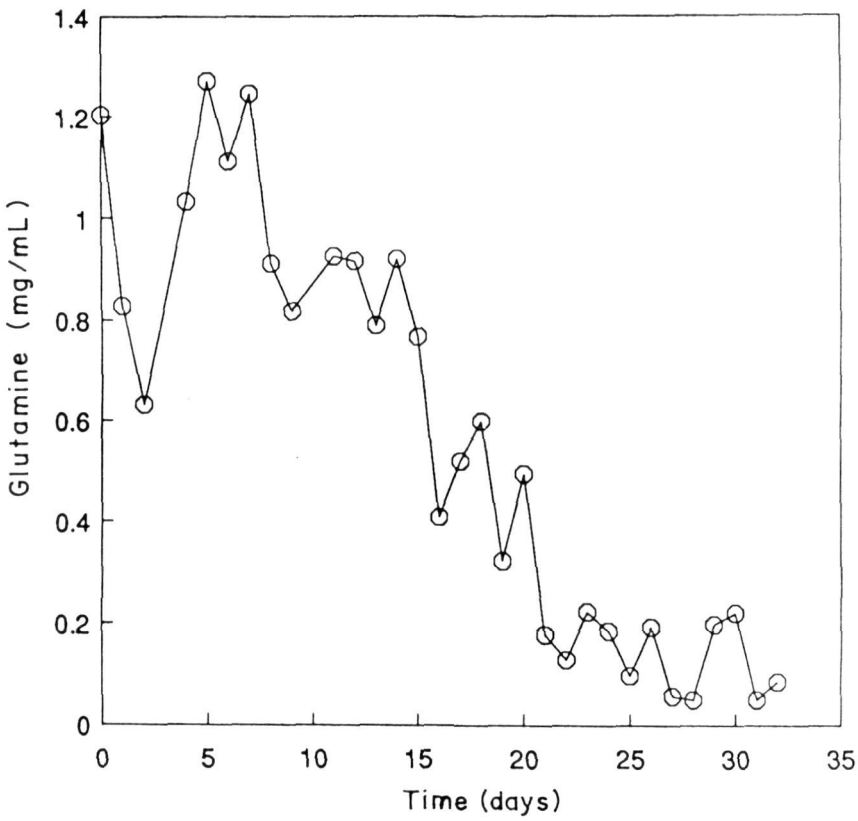


Fig.2·15

2.3 DISCUSSION

The nutritional environment influences the growth of hybridoma cells, as well as the secretion of antibody. The growth of cells is dependent upon oxygen, glucose, glutamine, and growth factors which are supplied mainly as serum supplements (Eagle, 1955a; and Zeilke *et al.*, 1984). An understanding of the role of nutrient concentrations in cell growth and product formation is a prerequisite for rational scale up of any cell culture process. One of the approaches towards this understanding is to determine the growth rates of the cells under different nutrient concentrations.

The experiments for examining the effect of different nutrient concentrations were carried out in the multiwell system. Using this system results under several different experimental conditions can be acquired simultaneously. The small volume of the system ensures that the nutrient and toxic metabolite concentrations do not vary significantly with time. Animal cells which exhibit density dependent growth effects can be conveniently grown in the multiwells (Truskey *et al.*, 1990).

The effect of varying the initial glucose concentrations was studied and it was seen that in the range used in most cell culture media the growth rate is insensitive to the concentration of glucose present. The initial growth rates at initial glucose concentrations above 5.5 mM fall on the same line indicating that the specific growth rates are not affected by the glucose concentrations in the range used. Miller *et al.*, (1988) have made similar observations for both

glucose and glutamine. Increasing the glucose concentration from 5.5 mM to 11 mM lead to an increase in the cell yield. At 5.5 mM glucose the cells may be limited by glucose as can be seen from the depletion of glucose in the medium at the end of the culture period. However, at higher glucose concentrations both glucose and glutamine may be limiting. The amount of lactate produced increased with an increase in the initial glucose concentration from 5.5 mM to 11 mM. Thereafter, the lactate production does not increase with increasing glucose concentration. This may be due to a change in the cellular metabolism from glucose to glutamine at higher glucose concentration. The amount of lactate and antibody produced depends upon the cell concentrations.

Glutamine is utilized faster than the other amino acids in culture. Glutamine was found to be limiting in the range 0.5-2.0mM. Truskey *et al.,* (1990) have also made similar observations for HuT-78 and for a hybridoma cell line. It has been reported that at glutamine concentrations higher than 2.0 mM the oxidation of glucose is decreased (Reitzer *et al.,* 1980). In the case of BHK21 cells it was observed that the breakdown of glutamine is inefficient at higher concentrations (Butler and Spier, 1984). The increase in the antibody production at higher glucose and glutamine concentrations is probably due to more number of days available for production rather than an increase in the rate of production.

Serum supplementation is widely practiced in cell culture in order to provide the cells with growth factors, vitamins and other, as yet undefined growth regulating substances (Sato, 1975). This is one of the most expensive components of any cell culture medium and a reduction in the amount of serum required is

an important cost factor for any process. The K_m value for serum has been reported for HuT-78 cells as 0.55% by Truskey *et al.*, (1990). The results obtained here indicate that though the cell growth rate is not significantly different at initial serum concentration ranging from 2.5%-12.5% the antibody production was decreased at the lower concentration. This implies that the effect of serum has to studied not only in terms of cell growth but also in terms of product formation. Serum has been also implicated in the protection of cells from mechanical damage especially when they are cultivated in stirred or airlift systems. This aspect has been described in greater detail in Chap.3.

The growth of mammalian cells is primarily controlled not by the nutrient environment but by growth regulating peptides which have highly specific interactions with the cell surface receptors (Holley, 1975; Kris *et al.*, 1985). These are manifested in the requirement of serum as discussed above and also in the inoculum cell density dependent growth effect seen for anchorage dependent and suspension cells. The results obtained also showed that the growth rates and the cell yield are influenced by the inoculum cell density. Lauffenburger and Cozens, (1989) described a mathematical model to explain the inoculum cell density effect observed by many workers (Hu *et al.*, 1985; Hu and Wang, 1987). They proposed a hypothesis that the interplay between the local concentrations of growth factors, the rate of production and transport of these factors and their binding to cell surface receptors controls the proliferation of cells.

Most of the earlier studies on hybridoma growth and antibody production showed that the specific antibody production is inversely proportional to the

growth rate of the cells (Fazekas de St Groth, 1983; Boraston *et al.*, 1984; Velez *et al.*, 1986) .The results obtained in the present work are in agreement with the earlier observations. Hence, in hybridoma cultivation for antibody production, the cell yield should be maximized as the product formed is directly dependent on the viable cell mass present.

The cell mass production was studied with respect to the murine hybridoma AE9D6. Batch cultivation was performed to understand the growth pattern of the cells. Batch culture experiments are easy to perform with the available laboratory cell culture vessels e.g. T-flasks, bottles etc. They can provide valuable information with regard to the initial growth rates, substrate uptake and metabolite production rates. The concentrations of the various nutrients can be conveniently monitored by sampling at fixed time intervals.

The data obtained from the batch experiments was used to estimate the kinetic parameters such as the glucose consumption rate and the metabolic/yield coefficient. These parameters can be used for the development of mathematical models for the control of cell mass production in large scale operation. However, the disadvantages of this system are that the conditions in the batch are constantly changing and hence the kinetic parameters obtained should be employed in further models only with caution. This is evident from the results obtained in the above work where the cell numbers obtained in the fed batch were compared to predictions from the simple model used. Though the cell numbers were in reasonable agreement in the initial period of growth, the numbers are overpredicted by more than 50% in the later half of the cultivation period. This could be due

to the fact that the accumulation of toxic metabolites and the availability of oxygen may become limiting at higher cell densities. Hence, kinetic parameters should be ideally obtained from continuous culture experiments where all the conditions except the limiting factor are maintained constant. However, these experiments are rather cumbersome to perform and achieving steady states with animal cells which have low growth rates is difficult and time consuming.

The initial information obtained from batch and fed batch experiments was used for the cultivation of hybridoma cells in the HFBR. The growth of cells within the reactor was not uniform and areas of high cell densities as well as spaces showing very little growth were seen. This has also been observed in earlier reports on cell cultivation in HFBRs (Piret and Cooney, 1990a; Sardonini and DiBiasio, 1993).

The concentration of the nutrients and lactic acid in the reservoir was a good indicator of cell growth in the reactor. However, predictions of cell numbers from the nutrient consumption rates was not attempted because it has been widely documented that such predictions are subject to large errors (Tharakan and Chau, 1986b; Chresand *et al.*, 1988; Sardonini and DiBiasio, 1993). This may be due to the non uniform packing of fibers in the reactor, non uniform cell distribution, maldistribution of flows in the cell compartment (ECS) and also because of nonuniform cell inoculation. The nutrient consumption rate of mammalian cells is related to their physiological status. The physiology of cells growing as high density cultures may differ from those growing in suspension

cultures as free cells. This also contributes to the inaccuracies in cell number prediction. The fluid flow patterns in the ECS which also play a great role in the distribution of cells and product in the HFBR have been discussed in Chap.4.

CHAPTER - 3

THE EFFECT OF HYDRODYNAMIC SHEAR STRESS ON ANIMAL CELLS *IN VITRO* AND MEASUREMENT OF THE TURBULENT SHEAR STRESS IN A STIRRED BIOREACTOR

3.0 INTRODUCTION

Hydrodynamic Shear in Bioreactors

The cultivation of animal cells on a large scale in bioreactors requires liquid agitation to maintain the cells or microcarriers in liquid suspension, to minimize the concentration gradient of nutrients and for mass transfer at the molecular level (gas-liquid, liquid-liquid, and solid-liquid). Liquid mixing is the result of velocity gradients within the agitated system (Bleim *et al.,* 1991). This means that there exist parallel streams of fluid, flowing with different velocities. This spatial gradient of velocities between two streams is called as the shear rate (γ) expressed as

$$\gamma = \frac{dv_x}{dy} \tag{1}$$

where v_x is the velocity in the x direction and y is perpendicular to x. Shear rate brings about convective transport as well as mass and heat transfer in the liquid (Merchuk, 1991). The shear rate is directly proportional to the force per unit area or shear stress (τ) given by

$$\tau = \mu\gamma \tag{2}$$

where μ is the viscosity of the fluid. The above equations are applicable to laminar flow and in the case of turbulent flows ε or turbulent viscosity is considered which depends on the liquid properties and on the flow patterns. Laminar flow

is characterized by viscous forces' dissipation with low energy input mixing and is attained in only few practical mixing devices, operating with an axial type of flow (Oldshue, 1983).

In almost all the commonly used bioreactors for animal cell culture, turbulent flow is prevalent and the laminar component is negligible, because a higher degree of mixing is required for suspension of the cells or microcarriers and for oxygen transfer. Thus, shear is one of the essential operational parameters for cell culture processes. However, it may also have a deleterious effect on animal cells which are large and fragile due to the absence of cell wall. In addition to oxygen transfer, shear sensitivity of the cells is also considered as an important aspect for the design and scale up of animal cell bioreactors. In stirred reactors the power input required to keep the cells from settling usually leads to high turbulent shear. In fluidized bed reactors the cells are generally protected within the macroporous microcarriers and in packed bed reactors the velocity of the bulk fluid is too low to cause any significant damage. Membrane reactors e.g., hollow fiber reactors are practically free from shear under the usual operating conditions (Bleim *et al.*, 1991).

Direct sparging of culture medium with air or oxygen has been found to be detrimental to the cells. Bubble associated cell damage depends on the cell type, bubble size and gas flow rate (Handa-Corrigan *et al.*, 1987). The shear stress in the case of sparged reactors e.g., airlift reactor is maximum in the zone of bubble disengagement at the liquid surface. Cell damage is also caused by coalescence and break up of bubbles (Bavarian *et al.*, 1991).

Effect of Shear on Animal Cells

The effect of laminar and turbulent hydrodynamic shear on different types of animal cells has been extensively reviewed in literature (Cherry and Papoutsakis, 1990; Papoutsakis, 1991; Merchuk, 1991; Croughan and Wang, 1991). The shear effects have been studied on suspension cells as well as adherent type of cells. Most of the studies have been carried out under controlled conditions of shear in viscometers (Petersen *et al.*, 1988; 1990). However, several other devices have been employed to generate shear forces in the laboratory. These include capillary flow devices (Augenstein *et al.*, 1971; McQueen *et al.*, 1987), laminar flow chambers (Levesque and Nerem, 1985; Viggers *et al.*, 1986; Kretzmer and Schugerl, 1991) and sparged cell suspensions (Cherry and Hull, 1992). Various parameters have been used as indicators of shear damage, such as changes in the morphology, viability as assessed by the cellular and metabolic functions, and proliferation of cells treated to shear.

Shear stress is known to have an effect on the morphology and mechanical properties of cells. Changes in the morphology are known to control the rate of DNA synthesis and hence the growth rate of cells (Folkman and Moscana, 1978). The mechanical properties of cells are attributed to the cytoskeletal structure of the cells. The cytoskeleton in eukaryotic cells comprises organized fibrous elements which form an integrated structural network. The major components of the cytoskeletal network are microtubules, microfilaments and intermediate filaments. The cytoskeleton has a wide array of functions in the cell which

includes providing skeletal support and maintenance of integrity, compartmentalization of the cytoplasm, cytoplasmic streaming, organelle movement, mitosis, endocytosis, secretion, intracellular transport and cell surface modulation.

The microtubular network is a dynamic structure which forms the major structural component of a large number of eukaryotic cellular structures such as mitotic spindles, flagella, cilia, nerve cell processes and cytoplasmic cytoskeletal elements. It is composed of the protein tubulin made up of two heterodimers which polymerize to form the hollow cylindrical microtubule. Its major functions are ciliary and flagellar movement, movement of chromosomes during cell division, movement of cellular organelles and cell receptors and maintenance of cellular architecture (Olmstead and Borisy, 1973).

The microfilaments are found in the cortical layer of the cells below the plasma membrane. It consists of a core of the protein F-actin and numerous other functionally important associated proteins such as myosin, tropomyosin, α-actin, filamin, vinculin and fibrin. Microfilaments are also found in bundles called as stress fibers, oriented parallel to the long axis of the cell mainly on the ventral side associated with the contact of the plasma membrane and substratum. Microfilaments are involved in various activities of the cell such as cell locomotion, membrane ruffling, phagocytosis, cytokinesis, cytoplasmic streaming, cell shape maintenance and cell adherence and spreading (Pollard and Cooper, 1986).

and are classified on the basis of the biochemical properties and distribution in tissues, as desmin filaments (skeletal, cardiac and smooth muscle), keratin filaments (epithelial cells), vimentin filaments (mesenchymal cells), neurofilaments (nerve cells) and glial fibrillary acidic proteins found in the glial cells. These play a structurally supportive role in anchoring the nucleus and other organelles. It is also closely associated with the microtubule structure (Steinert and Roop, 1988). In the cell all the three components of the cytoskeletal structure are associated with each other and this association is essential for the functionality of the cell (Luna and Hitt, 1992).

Dewey *et al.*, (1981) studied the effect of shear on cell shape and orientation (in a cone plate apparatus) of endothelial cells cultured in monolayers on glass slides. These cells *in vitro* have a closely packed polygonal arrangement. On prolonged exposure to shear they assume an ellipsoidal configuration with their major axis aligned in the direction of flow. The degree of confluency had an effect on this alignment indicating that cell-cell contact and the extracellular matrix influence shear induced changes in morphology. Similar observations have been made by Stathopolous and Hellums (1985) with human embryonic kidney (HEK) cells. Chittur *et al.*, (1988) observed an increase in the cell size of human T cells exposed to shear stress. Levesque and Nerem (1985) and Viggers *et al.*, (1986) examined the shear effect on bovine endothelial cells in a parallel plate and rectangular tube flow chamber respectively. The cultured monolayers exposed to shear stress showed elongation, orientation and alignment of cells in the direction of flow.

The changes in the morphology of cells is thought to be due to changes in the cytoskeletal structure. The alterations in the cytoskeletal structure on shear treatment was examined by Levesque *et al.,* (1989) who observed that the microfilament system was affected and an increase in stress fibers occurred as a result of shear exposure. The stress fibers were found to be aligned in the direction of flow. The function of the cytoskeleton in shear sensitivity was elucidated by Papoutsakis *et al.,* (1991) by treating the cells with cytoskeleton destabilizing drugs prior to exposure to shear. Treatment of the cells with cytochalasin E and B which destabilize the actin network resulted in an increase in the shear sensitivity of the hybridoma cells used in the study. On the other hand, treating cells with colchicine which disrupts the microtubule array did not affect the shear sensitivity of the cells. Thus, they inferred that it was actin which played a vital role in the ability of the cells to resist shear forces.

Studies on adherent cells further showed that the substrate on which the cells are grown also plays a role in their response to shear. This may be because different substrates induce cells to develop different cytoskeletal structures thus, resulting in varying mechanical properties. Though many workers have observed elongation and orientation of cells in response to shear, there are reports which describe other effects. The microvascular endothelial cells and mouse fibroblast 3T3 cells do not align in the direction of flow (Liu *et al.,* 1990). Adherent BHK cells when exposed to shear lost their longish form and became shorter and rounded (Ludwig *et al.,* 1992).

The effect of shear on cellular and metabolic functions is of great importance to processes involving animal cells. By suitably changing the hydrodynamic environment in the bioreactor an attempt can be made to enhance a specific cell function or to minimize its inhibition due to shear. Changes in the release of enzymes such as urokinase by HEK cells and in the production of prostaglandin I_2 (PGI_2) by human umbilical vein endothelial cells, (HUVEC) grown as adherent monolayers in response to shear exposure have been observed (Stathopolous and Hellums, 1985; Frangos *et al.,* 1985; 1988). The initial production of PGI_2 increased with increase in shear stress but the total production did not vary (Grabowski *et al.,* 1985). The HUVEC cultures exposed to shear produced 2-4 times more tissue plasminogen activator as compared to control cells (Diamond *et al.,* 1989) and increased levels of prostacyclin and inositol 1,4,5, triphosphate (Nollert *et al.,* 1991). The cellular functions of the cells such as endocytosis and pinocytosis also show changes in response to shear treatment. Confluent endothelial cultures exhibit increased rate of pinocytosis and platelet reactivity during exposure to shear (Dewey *et al.,* 1981). Receptor mediated endocytosis of low density lipoprotein (LDL) in the case of bovine aortic endothelial cells (BAEC) monolayers was enhanced due to shear exposure, however, the non-receptor mediated endocytosis was unaffected (Levesque *et al.,* 1989).

In the case of suspension cultures such as hybridoma cells, the rate of growth in agitated systems was slower than that of the static cultures. Antibody secretion was independent of agitation rate but depended on the viable cells present (Fazekas de St. Groth, 1983; Dodge and Hu, 1986 and Oh *et al.,* 1989).

The attachment and detachment of cells from surfaces is also affected by shear forces (Crouch *et al.*, 1985). The pH of the medium (Petersen *et al.*, 1988) and the serum concentration used (Kunas and Papoutsakis, 1989; Ozturk and Palsson, 1991) also play a role in the shear sensitivity of animal cells.

Single cell suspensions of human HeLa and mouse fibroblast cells subjected to shear by repeatedly passing through capillaries of different diameters resulted in a reduction in the viability of the cells as well as in the total count of the cells indicating the occurrence of lysis (Augenstein *et al.*, 1971; McQueen *et al.*, 1987). Insect suspension cell line subjected to shear in viscometers also showed a decrease in cell viability above a critical shear stress. This decrease was a function of time and was higher at higher shear rates (Tramper *et al.*, 1986). Studies on viability of these cells in bubble column reactors showed that cell death depends on the gas flow rate and is maximum in the region of bubble disengagement at the top of the reactor (Handa-Corrigan *et al.*, 1987). Further studies with hybridoma cells in viscometers have shown that cells are more sensitive to shear in the turbulent than in the laminar region (Abu-Reesh and Kargi, 1989; Petersen *et al.*, 1990). Hybridoma cells grown in stirred bioreactors and spinner flasks showed decreased viability and growth rates at higher agitation rates. The cells are more sensitive to shear in the lag and stationary phases of growth than in the exponential phase (Lee *et al.*, 1988; Petersen *et al.*, 1988; 1990).

Shear effects on microcarrier beads in stirred vessels indicate that cell growth is a function of a shear parameter referred to as **integrated shear factor (ISF)** which is based on a ratio between the impeller tip speed to tip to

wall clearance (Croughan *et al.,* 1987a; 1987b; Tyo and Wang, 1981). There are also several reviews dealing with the effect of shear on cells grown on microcarriers (Croughan and Wang, 1991).

The effect of shear has been overcome in many cases by the addition of certain shear protective substances such as Pluronic polyols, polyethylene glycol and foetal bovine serum. These additives have made possible the cultivation of animal cells on a large scale in stirred systems as well as in airlift reactors. There is a considerable amount of literature dealing with this aspect of shear sensitivity of animal cells (Croughan *et al.,* 1989; Murhammer and Goochee, 1990; Michaels *et al.,* 1991). Table 3.1 presents a brief summary of the different additives used and the mechanisms proposed to explain the protective effect. Two mechanisms have been proposed, one is the physical or nonspecific mechanism where the cells are protected from shear by the additives due to a reduction in the shear forces (van der Pol *et al.,* 1992; Cherry and Hull, 1992). The second is a biological mechanism indicating specific interaction of cells and additive (Ramirez and Mutharasan, 1990; 1992). However, the exact mechanism by which these additives afford protection to the cells is far from clear (Martens *et al.,* 1992).

From the foregoing discussion it is evident that animal cells respond to hydrodynamic shear and in most cases are damaged by it. The shear sensitivity of animal cells depends on various factors like cell type, substrate, pH, serum concentration, the growth phase of the cells, and their physiological status. The manner in which the cells have been grown prior to shear treatment also plays a role in the shear response of cells (Petersen, *et al.,* 1988; Schmid, *et al.,*

TABLE - 3.1

THE EFFECT OF SHEAR PROTECTIVE ADDITIVES ON THE SHEAR SENSITIVITY OF ANIMAL CELLS

Cell Type	Agitation system used	Shear protectant/ additives	Observations	References
Insect cells (Sf-9)	Bubble reactor	Pluronic F-68	Pluronic F-68 stabilized the foam from and decreased bubble breakup. This caused a reduction in the lysis of cells trapped in the liquid entrained in the bubbles.	Handa-Carrigan *et al., (1989).*
Insect cells (Sf-9)	Sparged bioreactor	Pluronic polyols	Two killing zones exist in the bioreactor, one in the vicinity of the sparger and the other in the region of bubble disengagement. Killing of cells occurs mainly due to interaction of cells with small turbulent eddies.	Murhammer and Goochee (1990).

Contd.....

Cell Type	Agitation system used	Shear protectant/ additives	Observations	References
			It is proposed that pluronic polyols interact with the cell membranes and bubbles and prevent damaging interactions.	
Murine hybridoma	**Couette viscometer**	Benzyl alcohol, cholesterol, FBS	Effect of shear on plasma membrane fluidity (PMF) was seen. The protectants modified the PMF.	Ramirez and Mutharasan (1990).
Murine hybridoma	Cells cultivated in stirred biorector and sheared in viscometer and the laminar flow conditions with no air liquid interfaces.	Pluronic F-68, Poly ethylene glycol, FBS	No effect of protectants was seen in viscometric studies. Specific death rate in the bioreactor decreased in the presence of shear protectants, which provided physical protection.	Michaels *et al.*, (1991).

Contd.....

Cell Type	Agitation system used	Shear protectant/ additives	Observations	References
Hybridoma	Bubble column reactor	FBS	Shear susceptibilty of cells can be reduced by adding extra serum before treatment to shear. Nonspecific physical protective effect.	van der Pol (1992).
Insect cells (SF-9)	Sparging and foam fractionation	Pluronic F-68	60% of cells in bulk are trapped in the liquid film and these are damaged when the thin films surrounding the bubbles rupture. Pluronic F-68 prevents the lysing of cells by reduction in the thin film bursting.	Cherry and Hull (1992).
Bovine embryonic kidney cells (BEK)	Microcarrier culture agitated in the spiner flask at speeds of 45-160 rpm	Dextran at different concentration (0-3%)	Increase in medium viscosity results in a decrease in death rate of cells at agitation speed > 100 rpm.	Lakhotia and Papoutsakis (1992).

1992). Inspite of the vast amount of literature existing, it is not possible to understand the relationship between the nature of shear forces and their effect on cells due to the varying nature of the cell lines and the devices used for subjecting cells to shear. Most of the studies have been carried out in viscometers where the shear stress applied is well defined. In the case of parallel flow chambers, which have been widely used to study shear effects on adherent cells, the geometry of the device enables calculation of the wall shear forces. However, in the case of stirred systems the shear forces are not quantitatively defined, and are characterized by time and position dependent velocity fluctuations. Though several reactor configurations have been developed, the stirred system still remains the reactor of choice due to its simplicity of operation, versatility (suspension as well as adherent cells on microcarriers can be cultivated) and amenability to scale up. Therefore, it is imperative to analyze the nature of shear forces in the stirred system.

Characterization of Shear Stress in a Stirred Vessel

For a vessel agitated with a rotating impeller the Reynolds number for the bulk flow is given by

$$Re = ND^2/\nu \qquad (3)$$

Here N is the rotational speed of the impeller, D is the diameter of the impeller and v is the kinematic viscosity of the fluid. In order to bring about complete suspension of the cells or microcarriers it is necessary to operate most stirred systems in the turbulent region.

The quantitative determination of shear rates in a stirred tank requires measurement of flow profiles in the vessel, from which the velocity gradient can be determined. The fluid velocity has to be measured at different points in the vessel so that the entire velocity profile is obtained. Different anemometric techniques (described briefly below) have been used for this purpose (Oldshue, 1983).

Photographic method: The agitated vessel is placed in a black box and a narrow light beam is projected through a slit. The flow velocity can be obtained from the length of trajectories and the time of exposure.

Hot film anemometer: The heat transfer rate from a thin wire or film heated by electric current is affected by the rate of liquid flow. This is used for the measurement of flow and fluctuation velocity.

Electrochemical method: It is based on the principle that the boundary current intensity is proportional to the diffusing amount of ions in an electrochemical reaction. A relation between mass transfer and flow rate is obtained and can be used indirectly for measuring liquid flow rate.

Laser Doppler anemometer (LDA): One of the techniques for measuring fluid flow is to **tag** the fluid and then measure the velocity of the **tagging** material. In laser Doppler anemometry, particles moving with the fluid (either naturally occurring or added) are illuminated with a focussed laser beam and act as sources of scattered light. The velocity of these particles is then determined from the change in frequency (Doppler shift) of scattered light due to movement of the particles as observed by a stationary detector. From the particle velocity the fluid velocity can be determined. A laser is used as the light source because it is coherent and can be easily focussed.

The principle that is used in LDA is that the phase of light wave scattered from a moving scattering center depends on particle velocity and direction of incoming and scattered light waves. The velocity of the particle is described by the vector U(X,t) depending upon position (X) and time (t). The light waves decompose into plane waves and are given by the wave vectors $K_i (i = 1,2)$ and K_s

where K_i and K_s describe the incident and scattered beam respectively, with frequency ωI and the original laser frequency ωD. The Doppler shift is given by the relation

$$\omega D = U.(K_s - K_i) \qquad (4)$$

In the present work LDA has been used in the dual beam mode. The beam which combines two scattered beams derived from two different incident beams. The detector measures the difference between the two Doppler shifted scattered beams (Fig. 3.1)

$$\omega D_1 = U.(K_s - K_1) \tag{5}$$

$$\omega D_2 = U.(K_s - K_2) \tag{6}$$

$$\omega D = \omega D_2 - \omega D_1 = U.(K_1 - K_2) \tag{7}$$

this can be written as

$$f_D = \frac{2Ux}{\lambda} \sin \frac{\theta_B}{2} \tag{8}$$

This equation is used for velocity measurements in LDA (Ranade, 1988).

In the present work studies have been carried out to examine the effect of turbulent shear on a human lymphoblastoid cell line KG-1 and the effect of laminar shear on a human lung fibroblast cell line WI 38.

The cells were subjected to shear by agitating them in a spinner vessel on a magnetic stirrer at different agitation speeds. The shear effect was studied by performing total and viable cell counts of the treated population. The glucose utilization and proliferation of these cells was also studied. Changes in the actin network on treatment to shear were observed by immunofluorescent

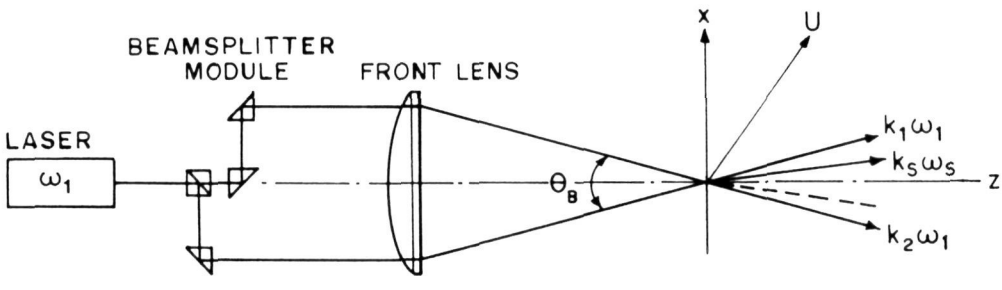


FIG. 3-1: VECTOR DIAGRAM-DUAL BEAM MODE

staining. The effect of shear stress on various parameters such as morphology of the cells, proliferation rate and changes in the cytoskeletal structure have been investigated. The protective effect of foetal bovine serum in minimizing damage under turbulent conditions has also been examined. The shear forces existing in the stirred vessel have been characterized by using laser Doppler anemometer.

The effect of laminar shear stress on adherent monolayer cultures of human lung fibroblast cells, WI 38 was studied by subjecting them to shear in a parallel plate flow chamber. The changes in the morphology of cells as a result of exposure to shear have been studied by carrying out staining of the cells. The viability of the cell and their proliferation after shear treatment has also been examined. The effect of shear on the cytoskeletal structure has been studied by immunofluorescent staining of actin and vimentin in treated and control cells.

3.1 MATERIALS AND METHODS

Cells and Medium:

KG-1 lymphoblastoid cell line grows as suspension culture and the cells have characteristics similar to hybridoma. These cells were used to facilitate the immunofluorescent staining, as in the case of hybridomas the antibody secreted by the cells themselves interfere in the immunofluorescent staining procedure using monoclonal antibodies of murine origin. In suspension culture the cells do not form aggregates or clumps but grow as single cells. The cells were routinely grown in RPMI-1640 medium (Flow Laboratories, USA.) supplemented with 10% (v/v) foetal bovine serum (FBS), supplied by Biologicals, Israel. Cultures were maintained in a medium free from antibiotics. They were normally grown in 75 cm^2 and 125 cm^2 tissue culture flasks (T-flasks), placed in an incubator at 37°C in a 5% CO_2 atmosphere and 95 % relative humidity.

Normal human diploid fibroblast cell line WI 38 was routinely maintained as a monolayer culture in Basal medium Eagle's, with Earle's salt, (BME(E), Gibco BRL., USA), supplemented with 10% foetal bovine serum (Biologicals, Israel), without addition of antibiotics. Cultures were split one to two when confluent by the standard method of trypsinization. Trypsinization for a brief period (1-2 min) after two rinses with medium were sufficient to detach cells from the surface of the bottle. Cells were flushed out in complete medium and then seeded into fresh culture bottles. Cultures were incubated in 5% CO_2 atmosphere at 37°C. Cultures were seeded on to glass coverslips (10 x 22 mm) 48 h prior to

their use for most of the experiments for laminar shear treatment.

Shear Treatment of KG-1 Cells in Spinner Flask:

The cells grown in T-flasks under static conditions were transferred to a spinner bottle (Belco, 125 ml, with a 42 mm stirrer bar and no baffles) and were agitated on a magnetic stirrer at different speeds. The spinner flask is a commonly used device for cultivation of suspension cells on a laboratory scale as well as for inoculum build up for large scale cultivation. As the spinner flask is unbaffled, agitation results in vortical flow and this effect is pronounced at higher agitation speeds. Samples were withdrawn at fixed time intervals and both total and viable cells were counted. The cells subjected to shear under these conditions were seeded into T- flasks with fresh nutrient medium and their growth pattern observed for a period of 6 days. These experiments were performed at least twice in triplicates and the results presented below are from a representative set of experiments. The effect of shear on cytoskeletal structure was studied by carrying out staining of actin network in cells by indirect immunofluorescence method. Smears of the treated cells were prepared by 'Cytospinning' a suspension of the cells on to glass slides. This was then fixed and stained with specific monoclonal antibody. Actin was stained using mouse monoclonal antibodies to α-actin (Boerhringer Mannheim, Germany). This was visualized by using antimouse antibodies conjugated with FITC, under fluorescent microscope.

Shear Treatment of Fibroblast Cells WI 38 in A Laminar Flow Chamber

Cells grown on coverslips were placed in a laminar flow chamber constructed according to Levesque and Nerem (1985), with dimensions (7.6 x 4.8 x 0.25 cm). Laminar shear stress was generated by recirculating complete medium BME(E) + 10% FBS supplemented with benzyl penicillin 50 IU/ml and streptomycin sulphate 250 ug/ml through the flow chamber at a controlled flow rate using a peristaltic pump (Watson and Marlow). The chamber was placed in an incubator with controlled 5% CO_2 environment at 37°C during the period of treatment. After the time of treatment, coverslips were removed, cells were fixed and processed for immunofluorescence staining of cytoskeletal structures. The viability of the cells and changes in the morphology were also observed by using suitable staining methods.

Biological Assays:

The total and viable cell counts and the estimation of glucose were performed according to the procedures described in Chapter 2.

Viability Staining of Monolayer Cultures of WI 38 :

The coverslips were removed from the flow chamber and placed in a petridish containing 0.4% Erythrosin B in phosphate buffered saline (PBS), pH 7.2. These were then mounted in the same solution and observed under the microscope. Viable cells were estimated by counting the number of stained and

unstained cells in a field. Counts were taken randomly to cover the entire coverslip. A minimum of 200 cells was counted, to obtain a statistical estimate of the viable cell count.

Staining for Observation of the Morphology of the Cells:

Fixing and staining to observe the morphology of the cells was done according to the procedure of Fox (1977), with some modifications. The cells were fixed in glutaraldehyde (3%)-formaldehyde (1%) for 60 min. at 4°C. Coverslips were rinsed in 70% ethanol and stained with Giemsa's stain (BDH Chemicals) diluted 1: 50 with Sorenson's buffer (pH 6.8). The coverslips were air dried and mounted in DPX mountant. The stained preparations were observed under light microscope (Zeiss). The size of the cells was estimated from Camera Lucida drawings made under the microscope (75 x).

Immunofluorescence Staining:

Fixation of cells and immunofluorescence staining to observe the cytoskeletal structure was carried out by following the procedure described by Guha Roy and Bhisey (1991). The coverslips, after shear treatment were removed from the chamber and rinsed quickly in PBS pH 7.2, (twice). Fixation was done in chilled methanol at 0-4°C, for 15-20 min. The coverslips were then rinsed in PBS and overlaid with 1% BSA for 30 min at room temperature. Monoclonal antibody against α-actin and vimentin (Boerhringer Mannheim) were used at dilutions of 1:10 and 1:3 respectively for 60 min at room temperature. After five washes of 10 min each with PBS the fluorescent second antibody i.e.,

sheep anti-mouse IgG labeled with FITC (Boerhringer Mannheim) was applied to the coverslips for 60 min at room temperature. The coverslips were then washed thoroughly in PBS and mounted in 0.1% phenylene diamine in 1:1 glycerol-PBS (pH 9.5) and the cells were observed and photographed under a Zeiss Axiophot microscope equipped for transmitted light and epifluorescence. Fujicolor 1600 film was used for photomicrographs.

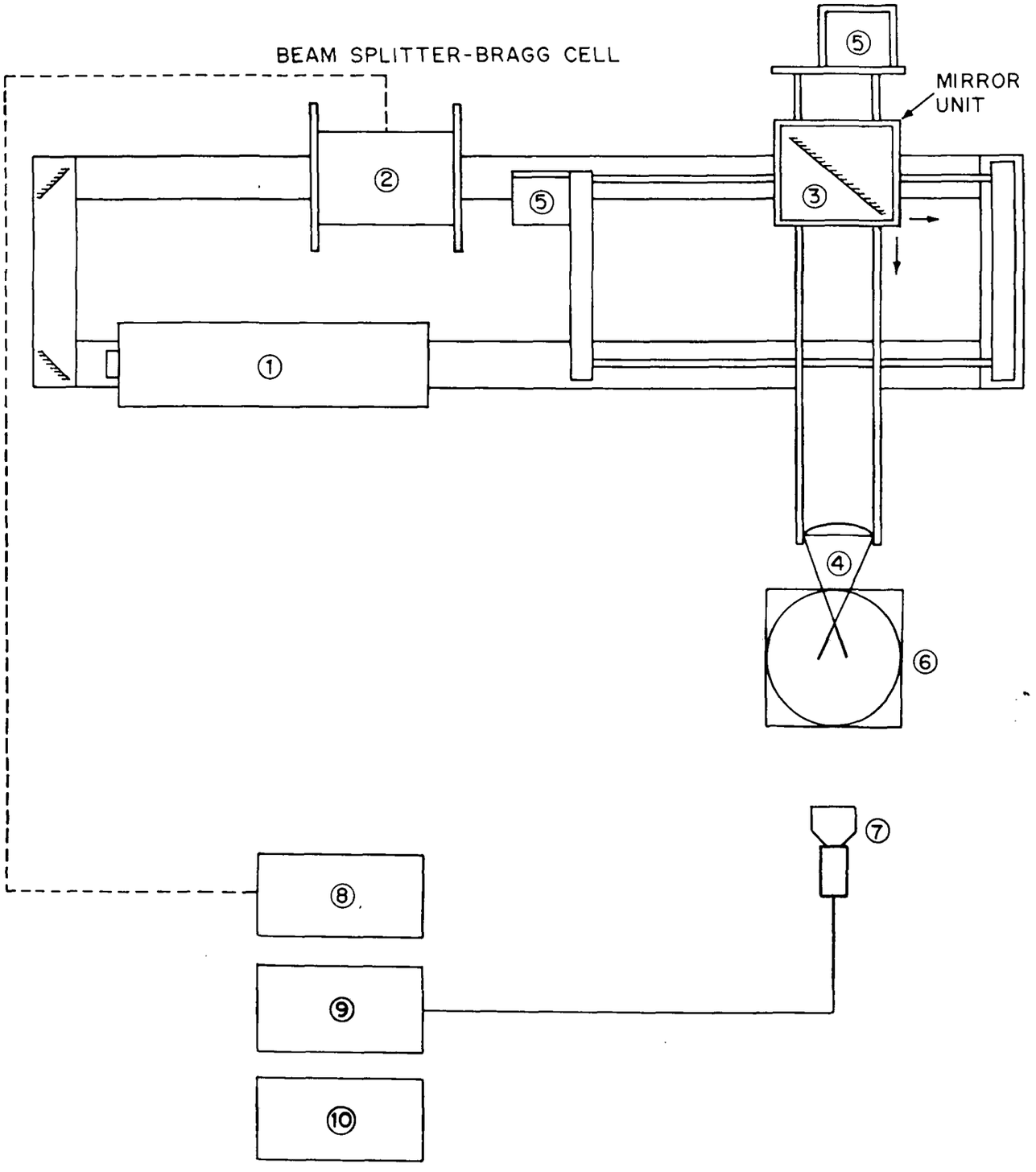
The cytoskeletal structure in the case of KG-1 was studied by staining the actin network of the cells. Smears of the control and treated cells were prepared by 'Cytospinning' a suspension of cells onto glass slides. These were then stained according to the procedure described above for monolayer cultures.

Measurement of Shear Stress in the Stirred Vessel:

In order to bring about a complete suspension of cells in the medium it is necessary to operate the spinner flasks in the turbulent region. The flow in a stirred tank under turbulent conditions is characterized by time and position dependent velocity fluctuations. These velocity fluctuations were measured by using a laser Doppler anemometer (Ranade and Joshi, 1989; 1990). The root mean square (rms) fluctuations can be determined from the mean velocities by squaring the fluctuations, averaging and taking the square root (Nagata, 1975; Oldshue, 1983). The rms values of fluctuation velocity in the axial and radial directions are denoted as u and w respectively. The rms velocities u and w are both proportional to the impeller speed and contain all the velocity components.

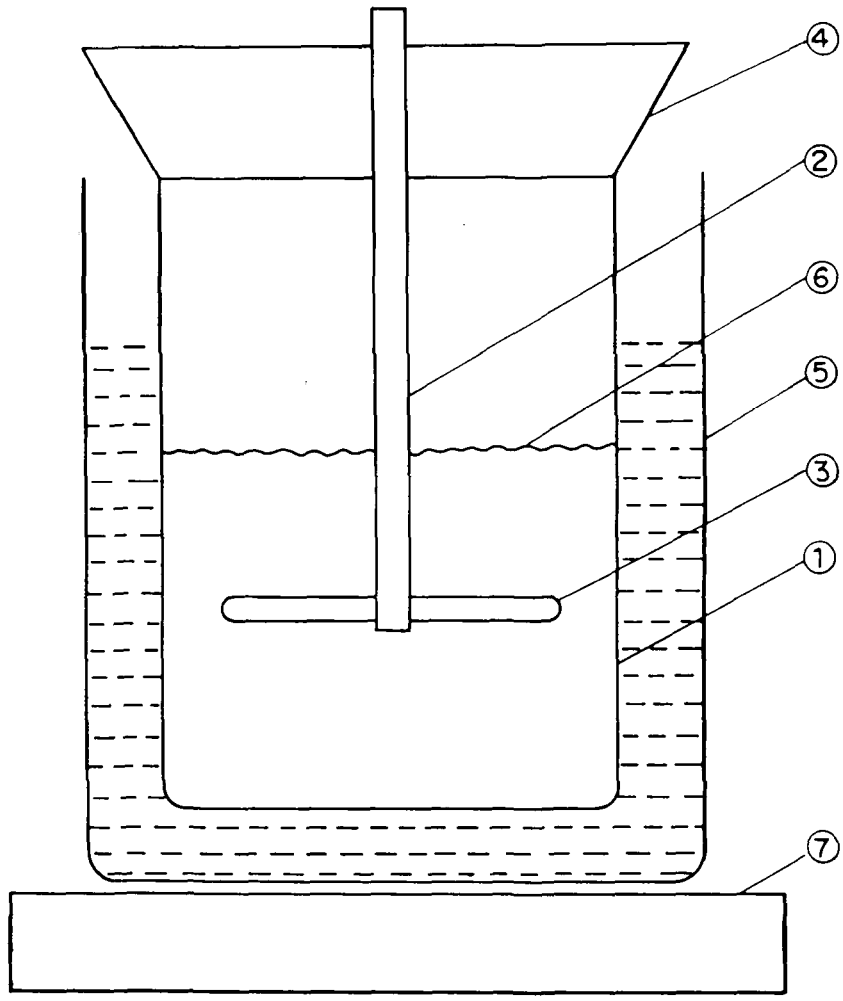
The measurement of turbulent rms velocity and turbulent stresses was made by using a laser Doppler anemometer (Dantec Inc). A schematic diagram of the experimental set up used for the LDA measurement is shown in Fig. 3.2. The spinner bottle was kept in a rectangular vessel for eliminating the lens effect of the cylindrical shape of the bottle (Fig. 3.3). Light beam of wavelength 632.8 nm from a 15 mW He-Ne laser was used. The beam was split by means of a beam splitter and the distance between the split beams was 60 mm. One of the beams was frequency shifted by an ultrasonic transducer. The beams were crossed with the help of a 310 mm converging lens. The point of crossing of these beams became the point of measurement, where interference fringes are formed. When any particle passes through the fringe pattern, the light gets scattered according to the Doppler principle. The scattered light was collected by a photomultiplier, and the frequency was measured by using Burst Spectrum Analyser (Dantec 57 N10).

The components of instantaneous velocity were measured by properly selecting the orientation of beams. At the point of measurement, that component gets measured, which is in the plane of two beams and perpendicular to the axis of two beams. Thus, the radial and axial components were measured at points such as N. For the radial component, the beams were oriented in the horizontal plane (Figs. 3.4A and 3.4B). For the tangential component, the measurements need to be made at points such as M. However, such points could not be seen by the photomultiplier tube because of the presence of the shaft. Therefore, tangential velocities were not measured. All the measurements were made in the absence of cells.



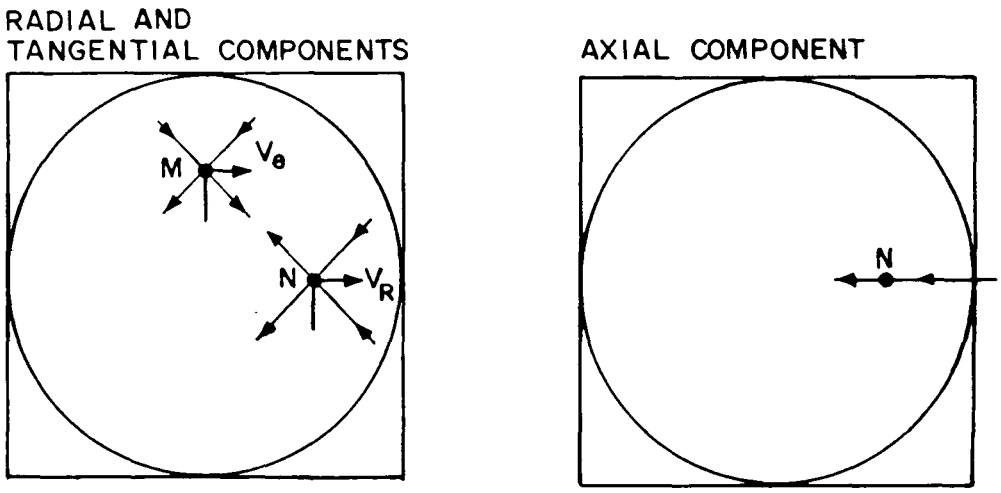
① He-Ne Laser, ② Beam splitter + Bragg cell, ③ Mirror casing, ④ Converging lens f=310mm, ⑤ Motorised traversing units, ⑥ Cross section of spinner bottle at the centre of beam crossing, ⑦ Photomultiplier, ⑧ Frequency shifter, ⑨ Counter processor + H.V. supply, ⑩ PC/AT/386

FIG. 3·2: SCHEMATIC REPRESENTATION OF THE EXPERIMENTAL SET-UP FOR LDA MEASUREMENTS



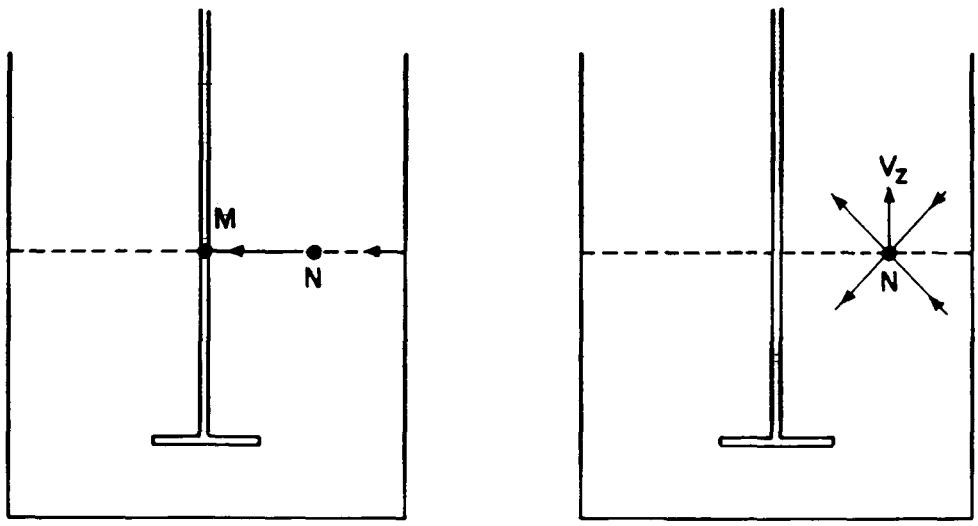
① SPINNER BOTTLE (59mm i.d.), ② GLASS ROD,
③ TEFLON COATED STIRRER BAR (42mm length), ④ STOPPER,
⑤ RECTANGULAR VESSEL, ⑥ LIQUID LEVEL IN THE SPINNER BOTTLE,
⑦ MAGNETIC STIRRER

FIG.3·3: SCHEMATIC DIAGRAM OF THE SPINNER BOTTLE USED FOR SHEAR TREATMENT AND LDA MEASUREMENTS.



TOPVIEW OF RADIAL (V_R), TANGENTIAL (V_θ) AND AXIAL (V_Z) VELOCITY COMPONENTS

FIG. 3·4A



FRONT VIEW OF RADIAL, TANGENTIAL AND AXIAL VELOCITY COMPONENTS

M) POINT OF MEASUREMENT FOR TANGENTIAL VELOCITY
N) POINT OF MEASUREMENT FOR RADIAL AND AXIAL VELOCITY

FIG. 3·4B

3.2 RESULTS

Effect of Shear on KG-1:

Human lymphoblastoid cells (KG-1) were grown in RPMI-1640 supplemented with 10% FBS. These were subjected to shear by agitating in a spinner flask, at different agitation speeds for 120 min. The measurement of cell damage after exposure to shear by dye exclusion method using Erythrosin B (Table 3.2) shows only a marginal reduction in viable cells, even at higher agitation speeds of 5 and 7.5 r/s. The agitation at 5 and 7.5 r/s does not result in lysis of cells as can be seen from the fact that the total cell count did not differ from that observed in the static control. Exposure to these higher speeds reduced the viability by 8% and 12 %, respectively. The cells were subjected to agitation at the above speeds and it was seen that they did not show alterations in the morphology when examined microscopically with respect to their shape. The cells exhibited their usual rounded and smooth shape.

Earlier studies on suspended animal cells and shear sensitivity have shown that cells suffer damage if exposed to agitation speeds above 3.3 r/s. The present experimental observations differ from those reported earlier (Backer *et al.,* 1988; Abu-Reesh and Kargi, 1991) in that the damage as assessed by dye exclusion was negligible even at very high agitation rates. The cell damage as evaluated by staining methods has a serious disadvantage due to the fact that if the membrane is not immediately affected after the treatment, the damage caused to the cell would be underestimated (Cook and Mitchell, 1989). Therefore

the reproductive potential of the treated cells is required to be studied in order to ascertain the damage to the cells caused under conditions of shear. This was studied by diluting the cells exposed to shear suitably in fresh medium containing serum. The cells were allowed to proliferate in T-flasks under static conditions. The proliferation was assessed by determining the growth rate in logarithmic phase. The results presented (Fig. 3.5A), show that although there is only a marginal decrease in the viability as measured by vital staining, the cells which have been exposed to shear are unable to proliferate. This is evident in the reduction of growth rate (μ) in the exponential phase, from 0.23 d^{-1} for control to 0.11 d^{-1} and 0.16 d^{-1} at 5 and 7.5 r/s, respectively. The viability of the population also indicates that only few cells from the entire population are able to divide and proliferate (Fig. 3.5B), as can be seen from the decreasing viabilities of the treated populations during the growth period. The viability of the control remained at approximately 80 % during the same period. Glucose utilization profiles of the treated and control population shows that the consumption of substrate on the first two days of culture is not significantly different (Fig. 3.5C). This may be due to an initial lag period resulting upon transfer to a fresh medium. Thereafter, the control population is able to utilize the glucose and proliferate, whereas very little glucose is consumed by the treated population even on day six of culture. These observations support a conjecture that the cells exposed to shear are metabolically inactive as evidenced by their inability to utilize the glucose in the medium.

Fig. 3.5 **Effect of agitation on cell proliferation and metabolism of KG-1 cells:** KG-1 cells were allowed to proliferate in RPMI-1640 with 10% FBS under static conditions after exposure to shear by agitation (-o- 0 r/s.; -●- 5 r/s; -Δ- 7.5 r/s) for a period of 2h. The growth was monitored as

A) Total cell count
B) Percentage viability and
C) Residual glucose in the medium.

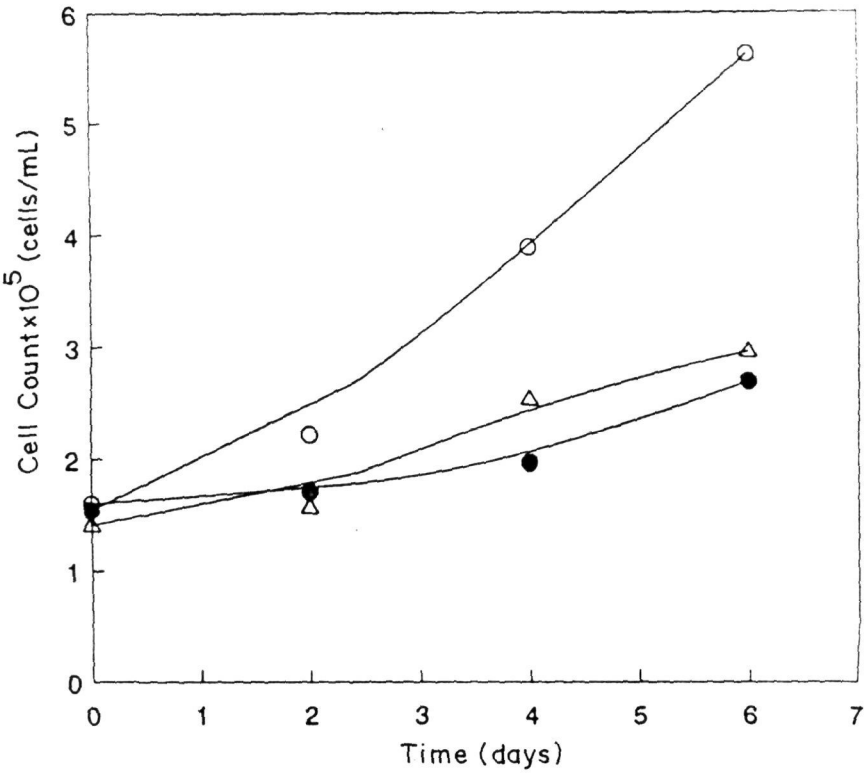


Fig. 3·5A

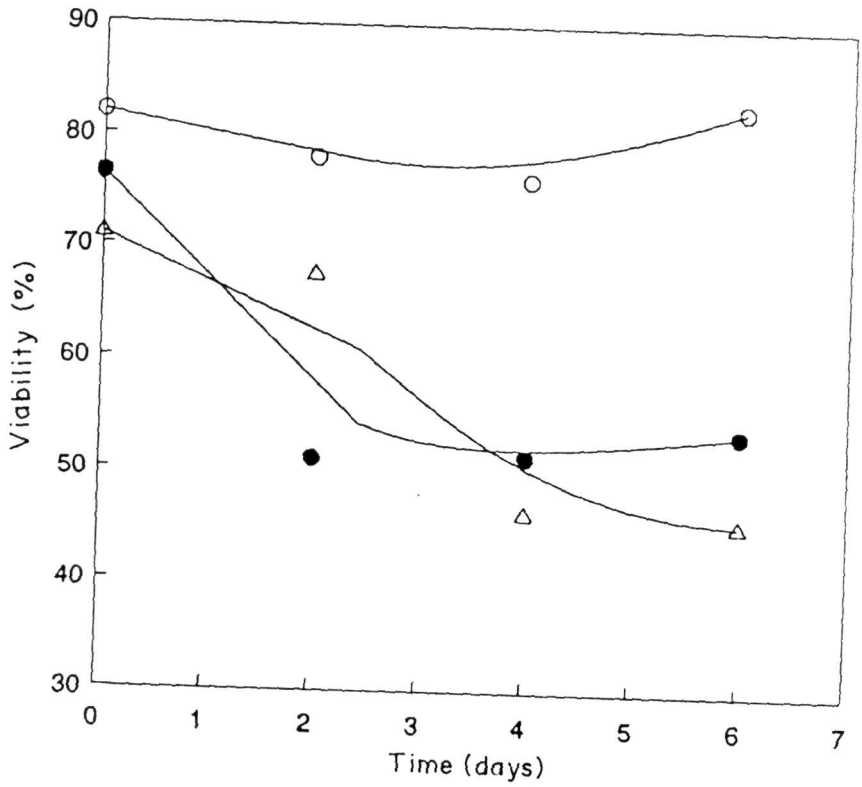


Fig. 3·5B

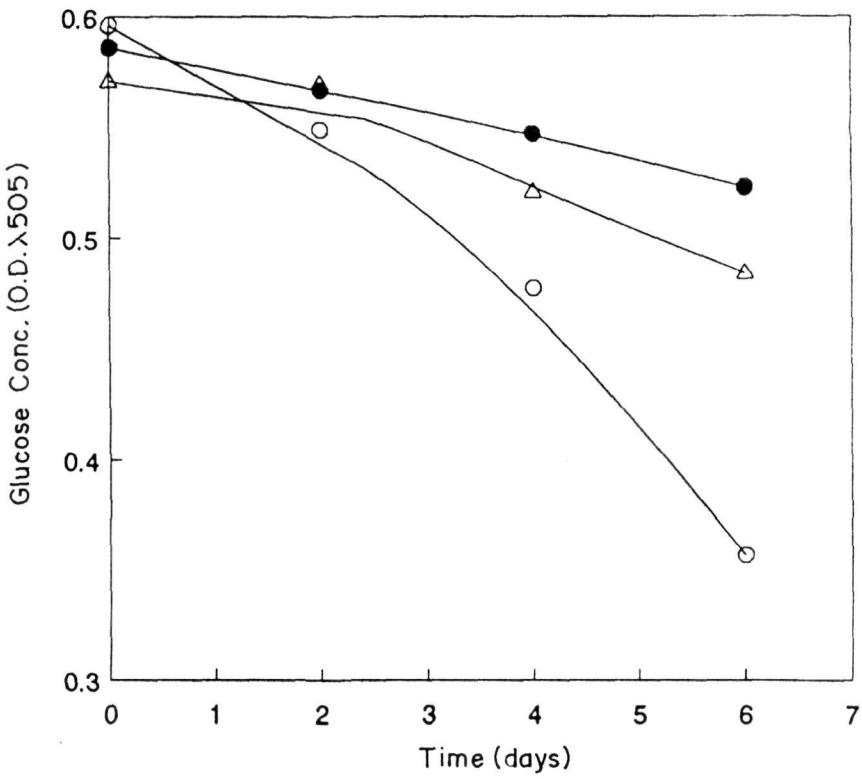


Fig. 3·5C

Agitation of KG-1 cells at 5 r/s for different time intervals showed that shear damage to cells is dependent on the time of exposure (Fig. 3.6). The cells exposed to shear for a shorter period (50 min) did not suffer from any significant damaging effect as can be seen from the growth rate which is similar to the control population. However, the cells exposed to shear by agitation for longer periods of 100 min and 150 min showed decreased growth rates and decreased viability of the population.

Protective Effect of FBS on Shear Sensitivity of KG-1 Cells:

Many workers have reported the use of additives such as Pluronic-F68 and foetal bovine serum as shear protectants for the cultivation of animal cells in stirred vessels. The effect of FBS at a concentration of 10% in the medium was studied. The cells were grown in a medium supplemented with 10% FBS and these were centrifuged, collected, and resuspended in medium i.e. RPMI-1640, with and without FBS. These cells were then subjected to shear by agitation at 2.5 r/s for 120 min. The cells exposed to shear at this agitation speed in the presence of 10 % serum did not show any damage in terms of total cell count, viable count and rate of proliferation, as was observed in earlier experiments. The cells exposed to shear in the absence of serum did not show any variation in total or viable count (Table 3.3). To ascertain the viability of these cells, they were transferred to fresh medium containing FBS at 10% concentration in T-flasks and allowed to proliferate under static conditions. The results clearly indicate a protective effect of serum as can be seen from the growth of cells exposed to shear in the presence of serum as compared to the cells exposed in the absence of serum (Fig. 3.7A).

Fig. 3.6 **Effect of time of shear exposure on proliferation of KG-1 cells:** KG-1 cells were grown in RPMI-1640 with 10% FBS in T-flasks after exposure to shear by agitation at 5 r/s (-o- 0 min, -●- 50 min, -Δ- 100 min, -▲- 150 min). The growth was monitored as the total cell count.

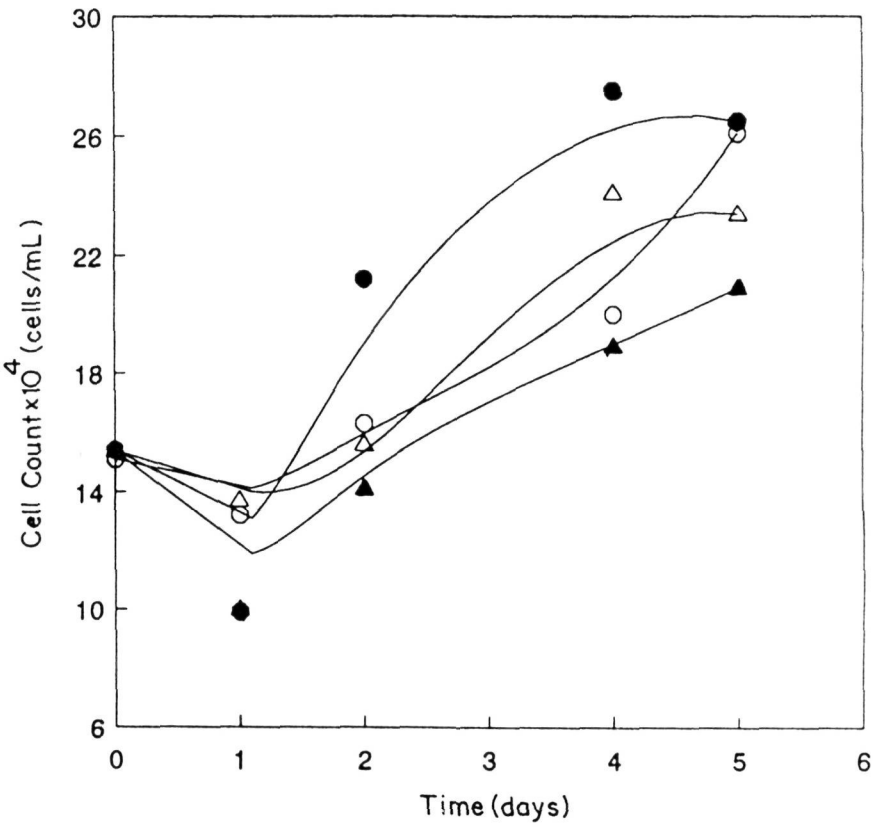


Fig. 3-6

TABLE - 3.2

EFFECT OF AGITATION ON THE TOTAL AND VIABLE CELL COUNTS OF KG-1 CELLS

Agitation speed rpm	Total count $x10^5$ cells/ml	Viable count $x10^5$ cells/ml	Viability %
Control	1.54 (100%)	1.26 (100%)	82
150	1.41 (93%)	1.25 (99%)	88
300	1.60 (103%)	1.22 (92%)	76
450	1.41 (93%)	1.00 (80%)	71

TABLE - 3.3

TOTAL AND VIABLE CELL COUNTS OF KG-1 CELLS SUBJECTED TO SHEAR IN THE PRESENCE OF SERUM.

FBS %	Total count $x10^5$ cells/ml	Viable count $x10^5$ cells/ml	Viability %
10 (Control)	2.8 (100%)	2.36 (100%)	83
0	2.9 (103%)	2.26 (95%)	78

The growth rate of cells exposed to shear in the presence of serum was 0.26d^{-1} and the viability of population remained at approximately 80% throughout the growth period. As compared to this, the cells exposed to agitation in the absence of serum had a growth rate of0.11d^{-1}, and their viability decreased to 54 % after two days and remained at the same value up to six days of culture (Fig. 3.7B). When the cells were agitated without serum at 5 r/s, the viability decreased to 20 % after six days in culture (results not shown). These results are further supported by the glucose utilization pattern of cells exposed to shear in presence and absence of serum (Fig. 3.7C). The difference is exemplified in the glucose utilization patterns after an initial period of two days.

Effect of Shear on Actin Stress Fibers in KG-1:

The cytoskeletal structure of cells of lymphoblastoid origin has been characterized by using fluorescently labeled antibodies against actin and tubulin. The stained preparations showed diffuse and weak labeling of cytoskeleton along the membrane and is difficult to detect on the nuclear area. (Gabbiani *et al.*, 1977). To find out the nature of the actin polymers after shear treatment, KG-1 cells were stained before and after shear treatment with anti α-actin antibodies. The immunofluorescently labeled preparations showed considerable difference in the thickness of the actin bundle, below the cell membrane. The structure of the actin bundle was diffuse but considerably thick in the untreated cells, whereas upon subjecting them to shear, depolymerization of actin fibers was

Fig. 3.7 **Effect of FBS on the shear sensitivity of KG-1 cells:** KG-1 cells were grown in complete medium. The cells were collected by centrifugation and resuspended in RPMI-1640 containing 10% FBS (-o-) and RPMI-1640 without FBS(-●-). The cells were then agitated at 2.5 r/s for a period of 120 min and then allowed to proliferate in RPMI-1640 supplemented with 10% FBS under static conditions. The growth was monitored as

A) Total cell count
B) Percentage viability
C) Residual glucose in the medium.

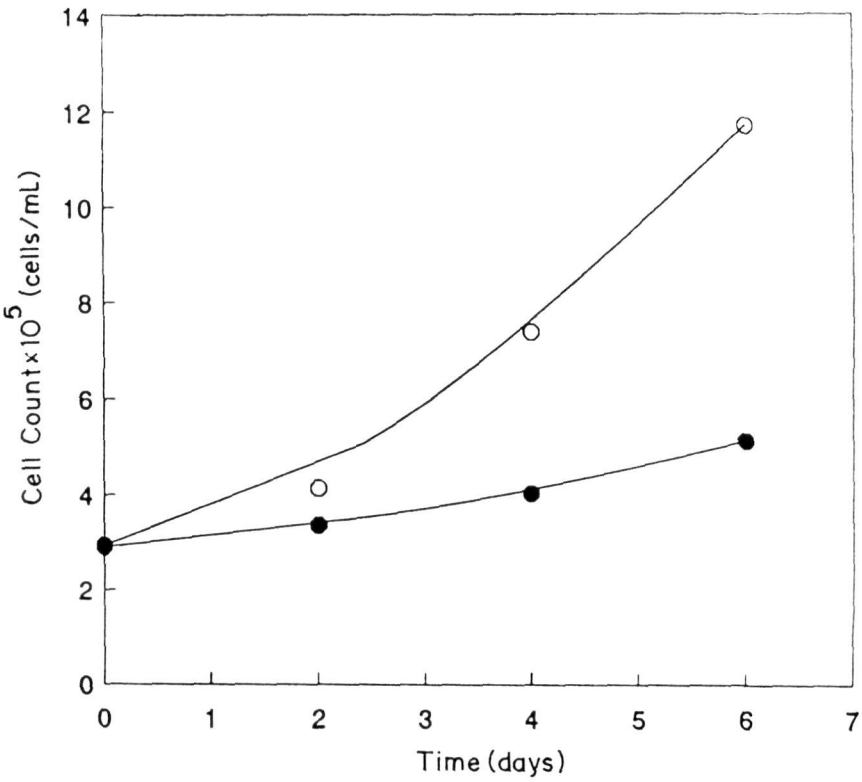


Fig. 3·7A

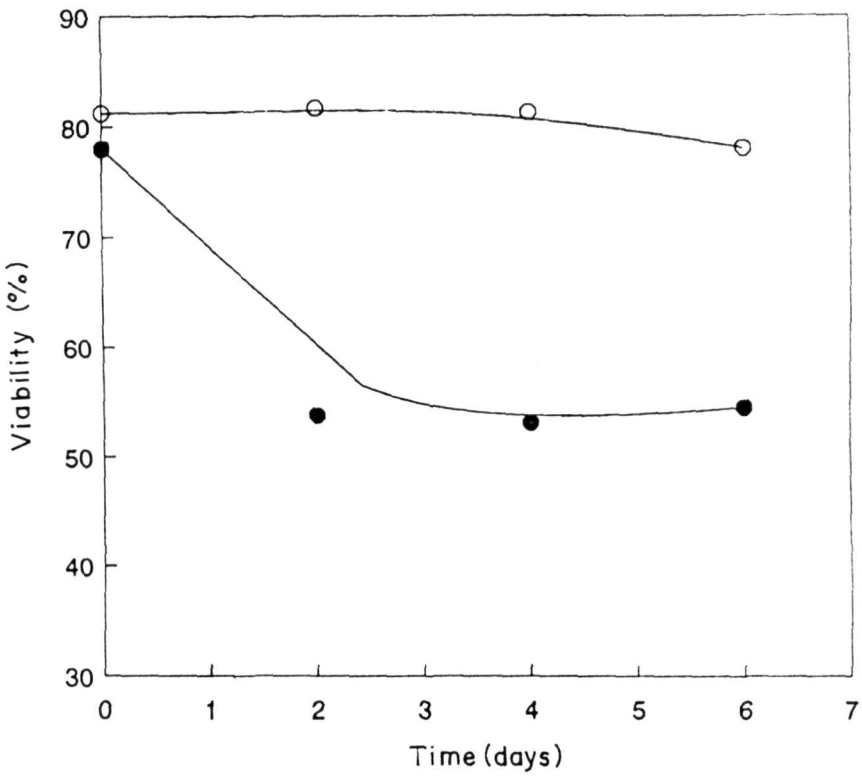


Fig.3-7B

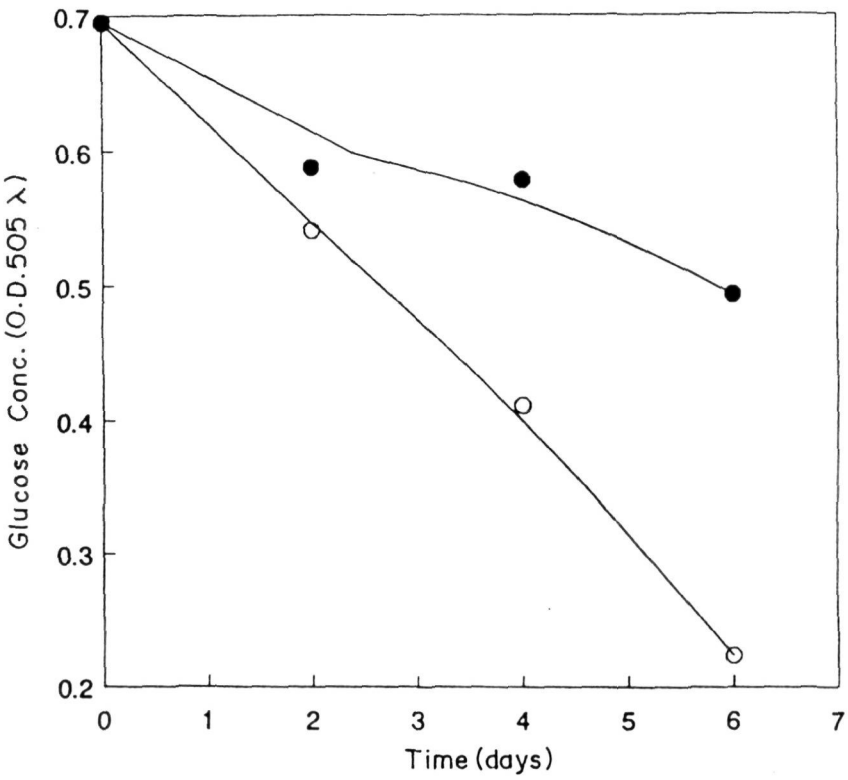


Fig.3·7C

found to occur, resulting in a reduced thickness of the actin network below the membrane (Figs. 3.8A and 3.8B). This indicates that shear has an effect on the cytoskeletal structure of KG-1 cells.

Effect of Shear on Viability and Morphology of WI 38 Cells:

WI 38 cells were cultivated on glass coverslips and were exposed to shear for different time periods. There was no loss of cells from the coverslips up to an exposure time of 6 and 12 h. The viability staining of these coverslips showed that there was no significant reduction in the viability in the cultures exposed for 6 h. However, longer exposure times of 12 and 24 h led to decrease in the viability of cells which were still attached to the coverslip. The viability decreased to 82% and 75% when cultures were exposed for 12 and 24 h respectively. The viability of cultures exposed for 6 h was further ascertained by studying their proliferation ability. This was done by carrying out [^{3}H] thymidine incorporation assay. For this experiment subconfluent cultures, incubated for 8 h after seeding on coverslips were used. They were exposed to shear for 6 h, and were then allowed to proliferate further for 48 h in medium containing [^{3}H] thymidine. The incorporation of [^{3}H] thymidine by these cultures showed that the treated cultures could incorporate [^{3}H] thymidine (1.90×10^4 cpm/ coverslip) to the same extent as the controls (2.05×10^4 cpm/ coverslip).

The stained preparation of the control cells showed varied morphology from typically spindle shaped cells with bipolar tapering processes at opposite ends to irregularly shaped large cells with a number of these processes. The cells

Fig. 3.8 Immunofluorescent staining of α-actin in KG-1 cells: Immunofluorescent staining of α-actin in KG-1 cells

A) Control cells showing thick actin bundle below the membrane (x 820)

B) Cells treated to shear by agitation at 5 r/s for 2h showing reduced thickness of the actin bundle and diffuse staining throughout the cell (x 820).

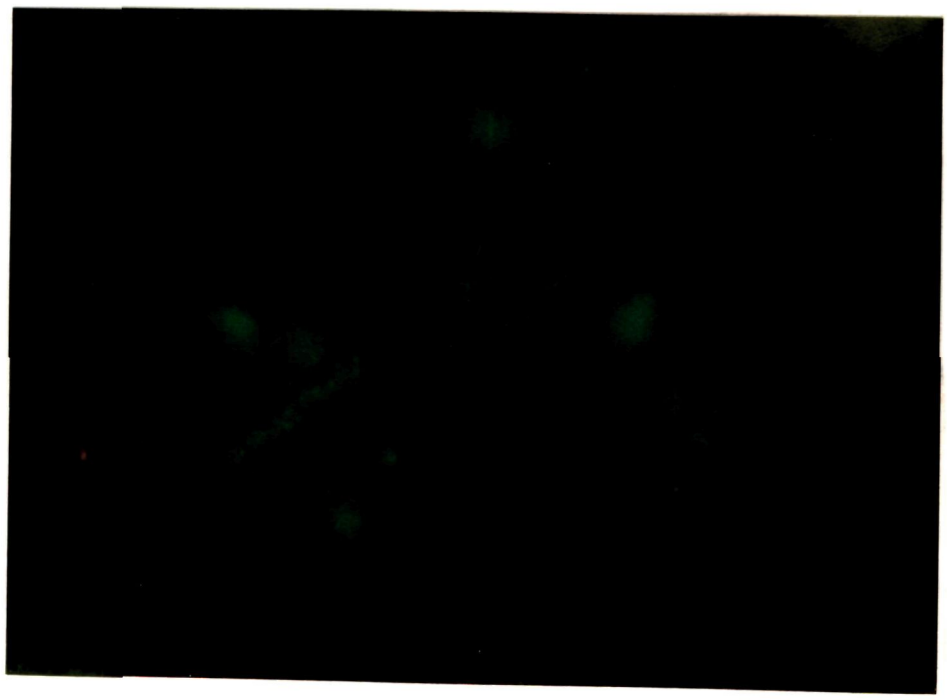


Fig. 3·8 A

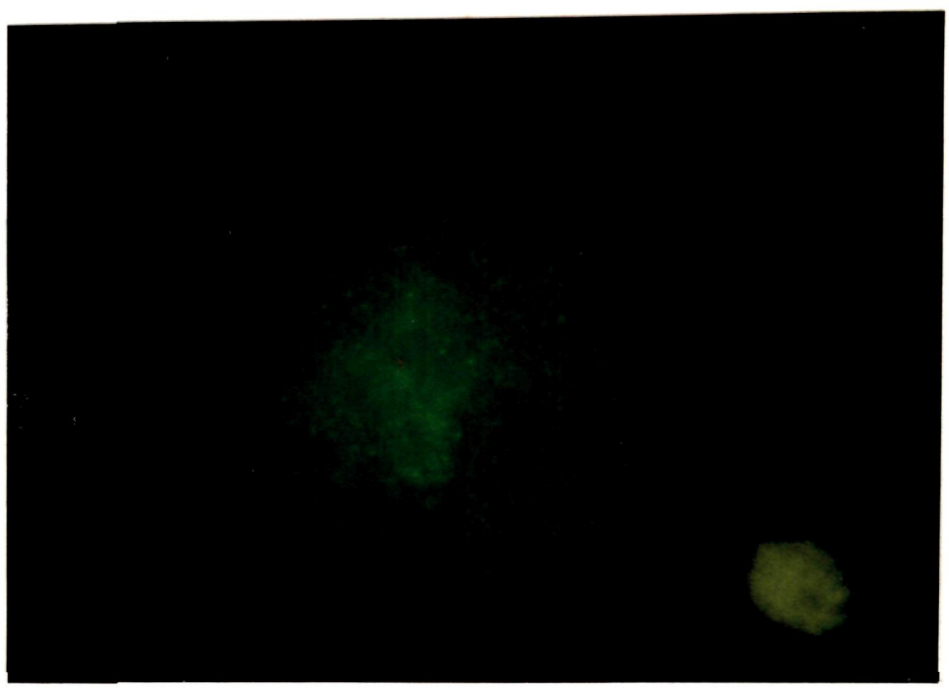


Fig. 3·8 B

on exposure to shear (for 6 h) were found to have an increased surface area, indicating that the cells were flattened (Fig.3.9). In the case of the control cell population nearly 40% of the cells had a surface area of 50 - 100 μm^2 . After shear exposure the surface area of the cells increased and 36% of the population was found to have an area of 100 - 150 μm^2. Rounding of cells as observed in BHK21 cells (Ludwig *et al.*, 1992), or reorientation of cells as in the case of endothelial cells (Levesque *et al.*, 1989) was not observed in this case. Longer exposure times did result in some alterations in the morphology, which however, were not of a drastic nature.

Effect of Shear on Cytoskeletal Structure of WI 38 Cells:

The control cells when stained with anti α-actin antibodies revealed the morphology of the F-actin polymers. These appeared straight, long and run parallel to each other along the axis of the cells. Some of these fibers were found to overlap each other yielding a stable network of actin (Figs. 3.10A). In shear treated cells (6 h exposure) only very few F-actin polymers were observed. The number of fibers and their length and thickness varied from cell to cell as evidenced by the reduced intensity in fluorescence. Diffuse fluorescence was observed throughout the cells indicating depolymerization of actin filaments (Figs 3.10B). Similar pattern was observed in the case of vimentin, where fibers had a wavy appearance and were highly interwoven and prominent. Vimentin fibers in the control cells were found to extend fully up to the cell membrane (Fig 3.11A). In the treated cells (6 h exposure) there were few changes in the vimentin fibres. These became reduced in length and did not extend up to the cell membrane (Fig. 3.11B).

Fig. 3.9 **Effect of laminar shear stress on the surface area of attachment of WI 38 cells:** WI 38 cells grown as monolayers on glass coverslips were subjected to laminar shear stress in a parallel plate flow chamber for 6h. The coverslips were then fixed and stained with Giemsa. Camera Lucida drawings (75x) were made and analyzed for surface area of cells (-■-) control, (-▨-) treated

A: 1-25μm^2; B: 25-50μm^2; C: 50-75μm^2; D: 100-125μm^2; E: 125-150μm^2; F: 150-175μm^2; G: 175-200μm^2.

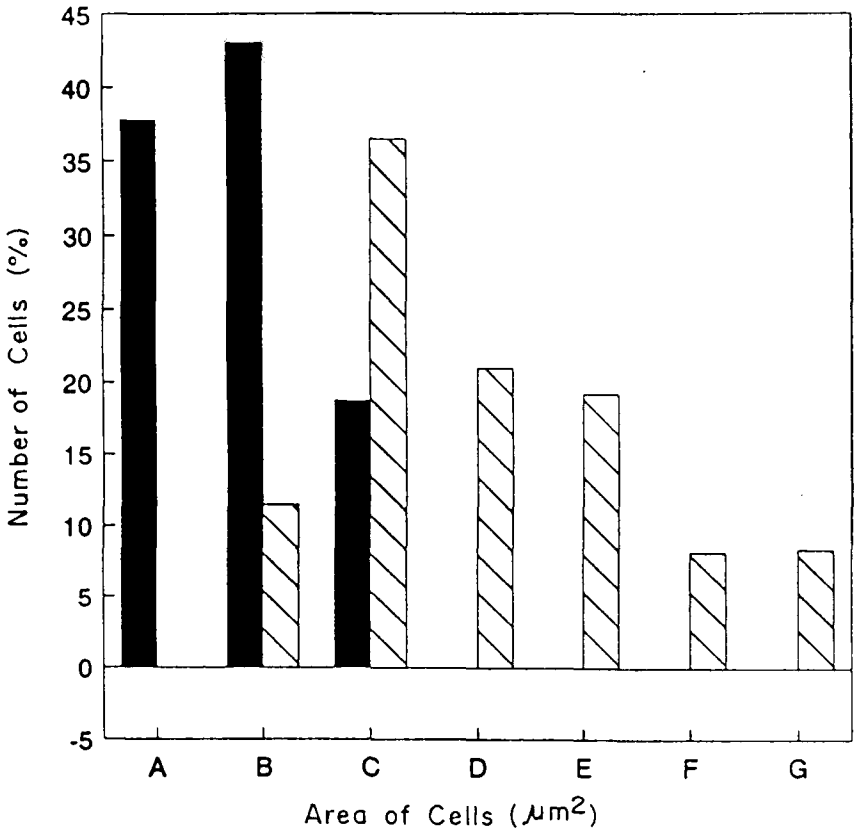

45
40
35
30
25
20
15
10
5
0
-5
Number of Cells (%)
A
B
C
D
E
F
G
Area of Cells (μm²)

Fig. 3·9

Fig. 3.10 Immunofluorescent staining of α-actin in WI 38 cells: Immunofluorescent staining of α-actin in WI 38 cells

A) Control cells showing actin fibers running parallel to the axis of the cells (x 820)

B) Treated cells showing few actin fibers and diffuse fluorescence throughout the cells (x 820).

Fig. 3·10A

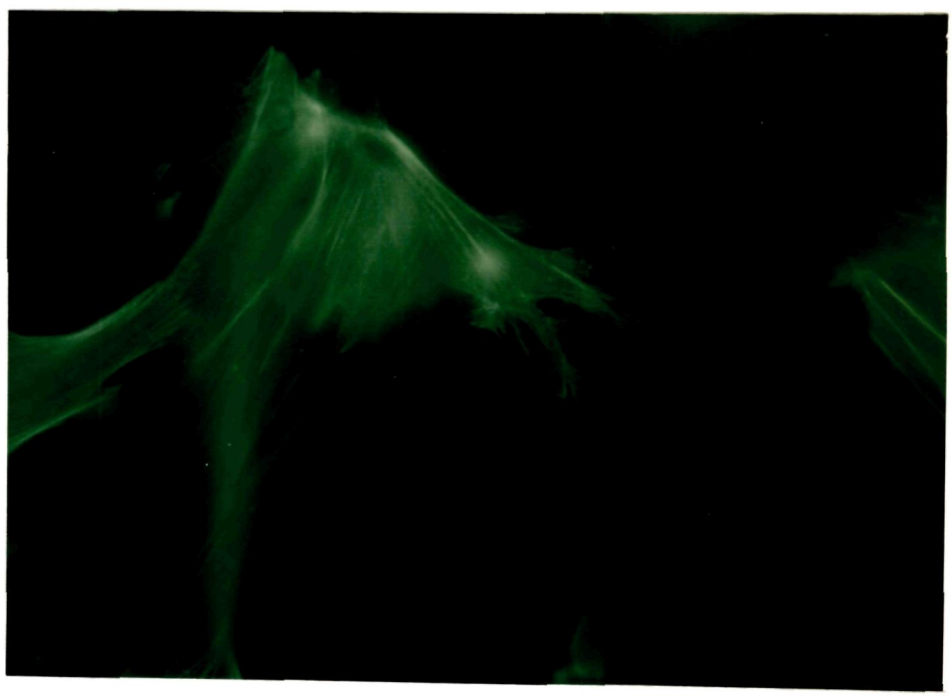


Fig. 3·10B

Fig. 3.11 Immunofluorescent staining of vimentin in WI 38 cells: Immunofluorescent staining of vimentin in WI 38 cells

A) Control cells showing interwoven vimentin fibers extending upto the cell membrane (x 820)

B) Treated cells with shorter vimentin fibers not extended till the cell membrane (x 820).

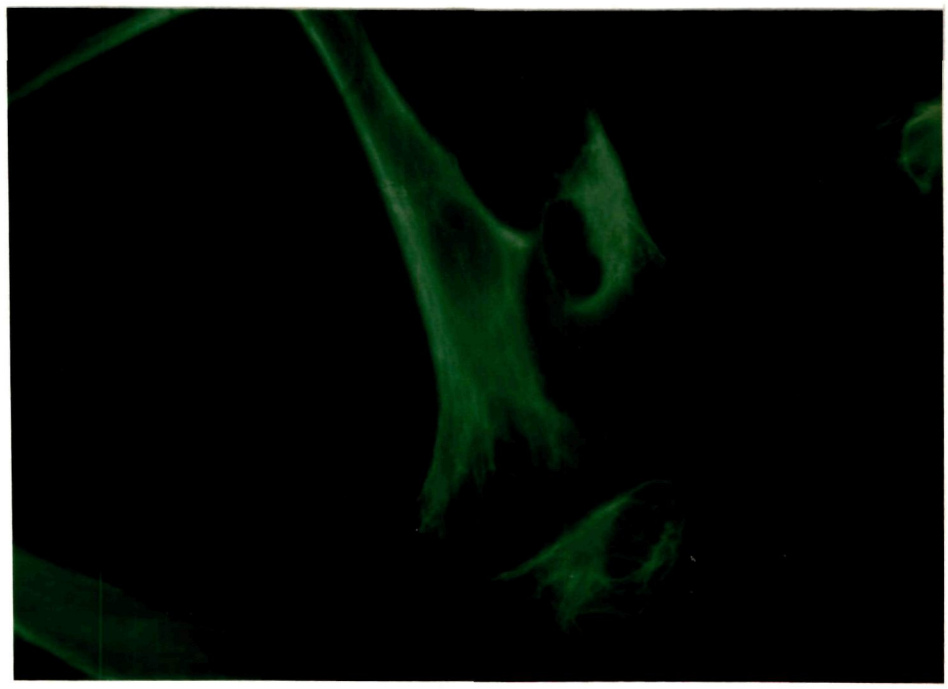


Fig. 3·11A

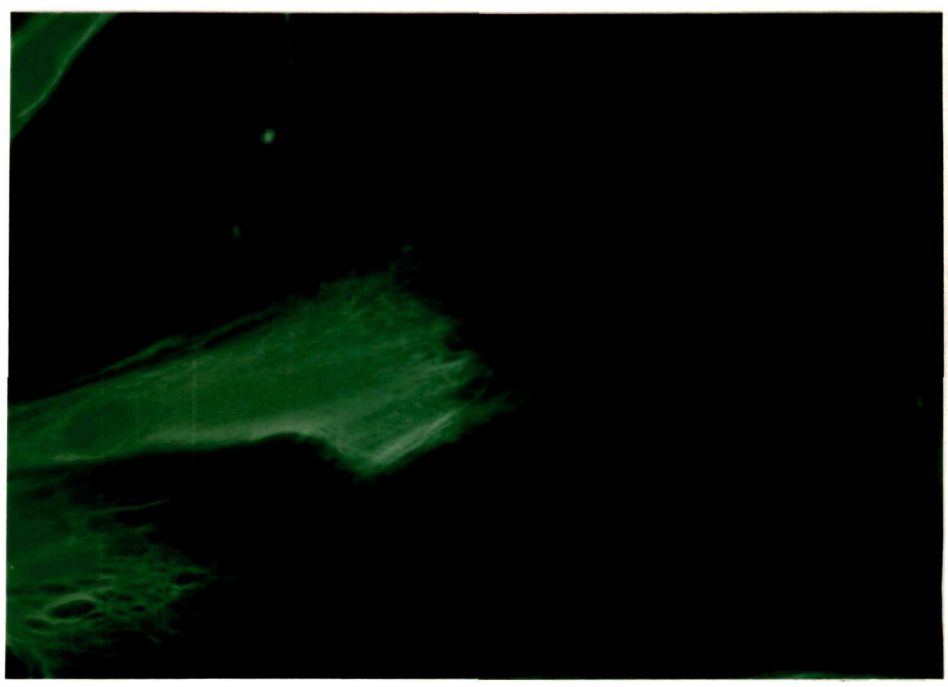


Fig. 3·11B

Measurement of Shear Stresses Using Laser Doppler Anemometer:

Measurements of rms velocity and Reynolds stresses using laser Doppler anemometer were made at six radial and two axial locations, in the same spinner bottle which was used for the earlier studies. One axial location was 6 mm above the impeller and the other was 12 mm above the impeller. The radial mean velocity profiles in the axial and radial directions at agitation speed of 2.5 r/s and 5 r/s are shown in (Figs. 3.12 and 3.13) respectively. The radial variation of axial and radial rms velocity at the two axial locations for the two speeds (2.5 and 5 r/s) are shown in (Figs. 3.14 and 3.15 respectively). It can be seen that the intensity of turbulence was higher at the lower position. From the profiles obtained it is clear that the mean velocity and the rms velocities increased with increase in the agitator speed. Consequently the values of Reynolds stresses will also be higher at this location. It is obvious that the region of high Reynolds stresses determines the viability and growth rate of cells. In the previous sections we have described the effect of addition of 10% FBS. It was observed that FBS exhibited a protective effect against shear damage and the cells exposed to shear in the absence of FBS suffered greater damage in terms of decreased viability and lower growth rates. Therefore, LDA measurements were also made in the presence of 10% FBS. From the mean velocity and rms velocity profiles obtained (both axial and radial) at the two impeller locations at 2.5 and 5 r/s in the presence of 10% FBS (Figs. 3.16, 3.17, 3.18 and 3.19 respectively), it can be inferred that, the mean velocity as well as the rms velocities were reduced and exhibited a more uniform behaviour in the presence of serum. Thus it is evident

Fig. 3.12 **Axial and radial mean velocity at 2.5 (r/s) in medium without FBS:** Radial profiles of axial (-o- 6mm and -Δ- 12mm above impeller) and radial (-●- 6mm and -▲- 12mm above impeller) mean velocities in the spinner bottle at an agitation speed of 2.5 r/s in medium without FBS.

Fig. 3.13 **Axial and radial mean velocity at 5 (r/s) in medium without FBS:** Radial profiles of axial (-o- 6mm and -Δ- 12mm above impeller) and radial (-●- 6mm and -▲- 12mm above impeller) mean velocities in the spinner bottle at an agitation speed of 5.0 r/s in medium without FBS.

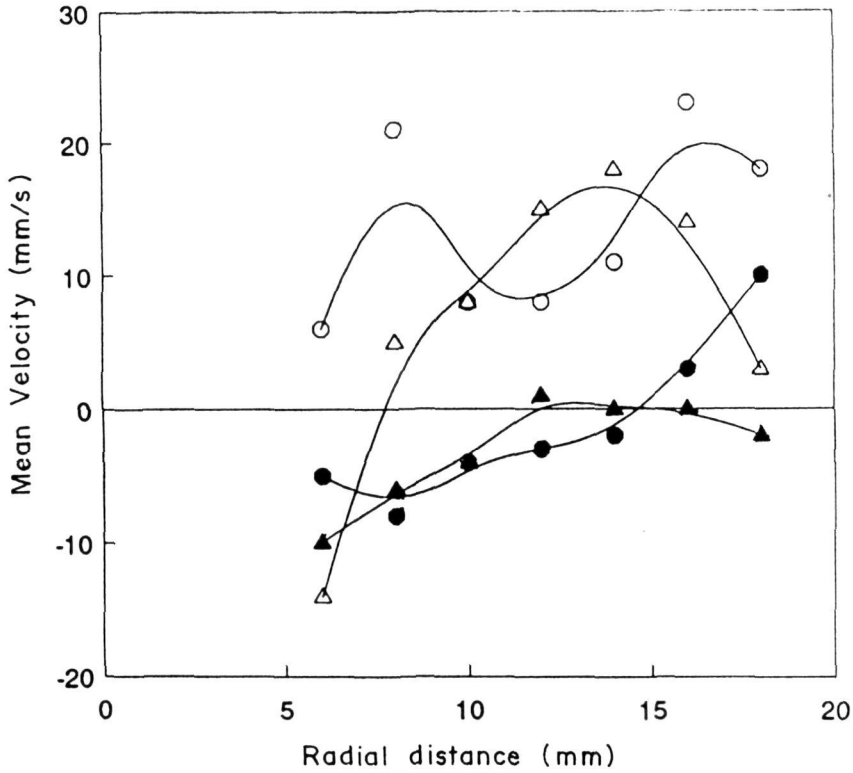


Fig. 3·12

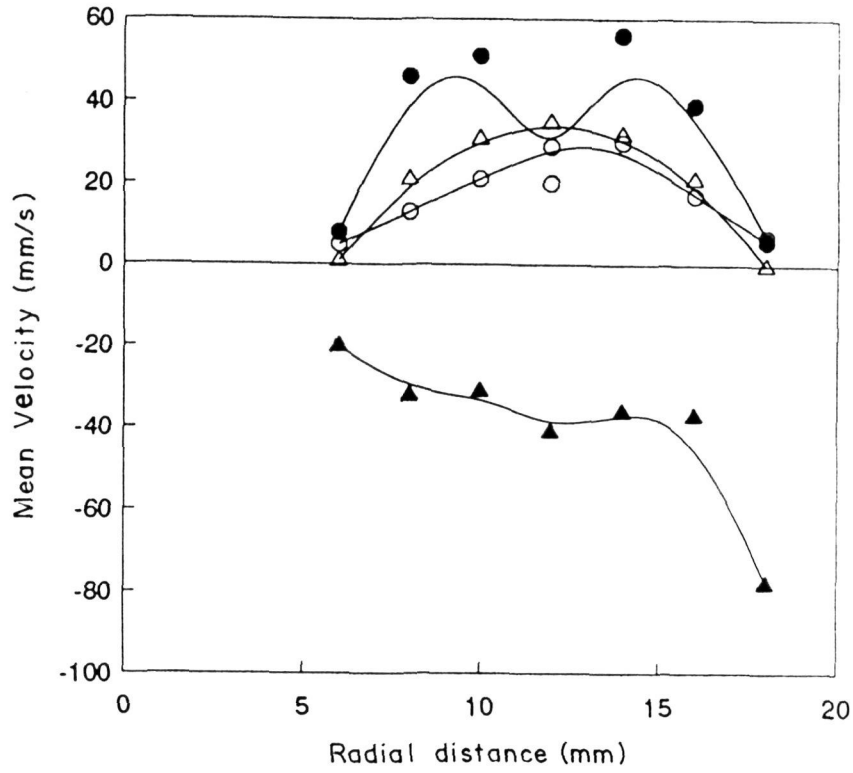


Fig. 3·13

Fig. 3.14 **Axial and radial rms velocity at 2.5 (r/s) in medium without FBS:** Radial profiles of axial (-o- 6mm and -Δ- 12mm above impeller) and radial (-●- 6mm and -▲- 12mm above impeller) rms velocities in the spinner bottle at an agitation speed of 2.5 r/s in medium without FBS.

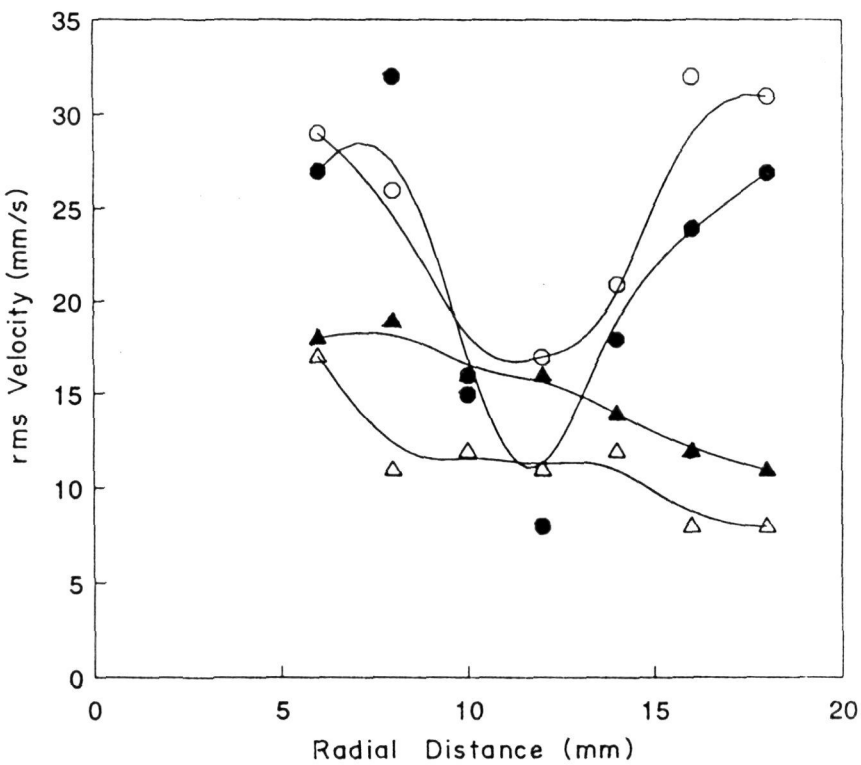

35
30
25
20
15
10
5
0
rms Velocity (mm/s)
0
5
10
15
20
Radial Distance (mm)

Fig. 3·14

Fig. 3.15 **Axial and radial rms velocity at 5 (r/s) in medium without FBS:** Radial profiles of axial (-o- 6mm and -Δ- 12mm above impeller) and radial (-●- 6mm and -▲- 12mm above impeller) mean velocities in the spinner bottle at an agitation speed of 5 r/s in medium without FBS.

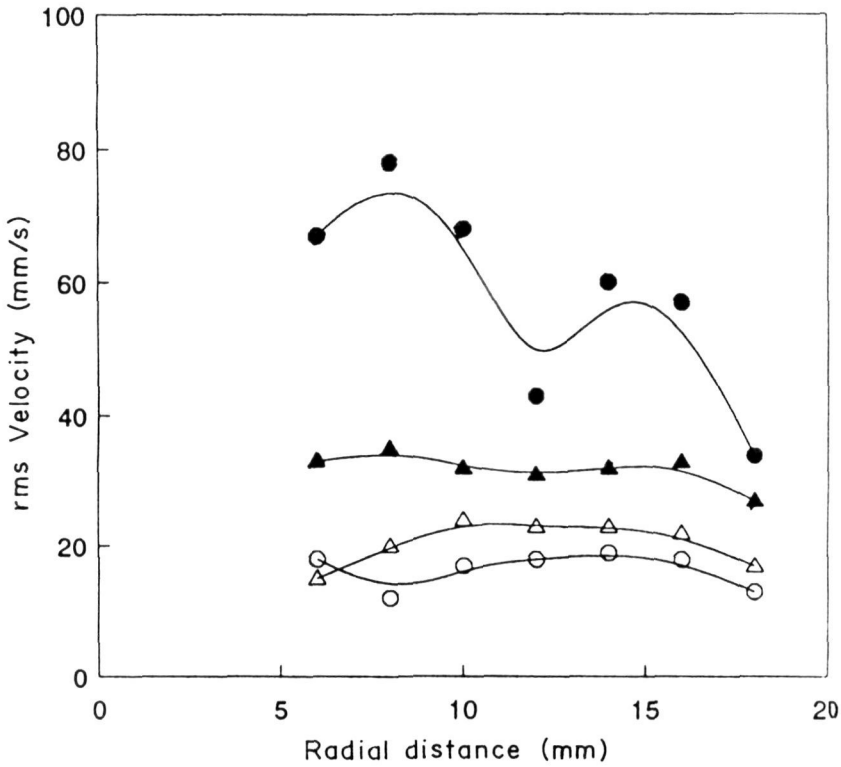

100
80
60
40
20
0
rms Velocity (mm/s)
0
5
10
15
20
Radial distance (mm)

Fig. 3·15

Fig. 3.16 Axial and radial mean velocity at 2.5 (r/s) in medium with FBS: Radial profiles of axial (-o- 6mm and -Δ- 12mm above impeller) and radial (-●- 6mm and -▲- 12mm above impeller) mean velocities in the spinner bottle at an agitation speed of 2.5 r/s in medium containing 10% FBS.

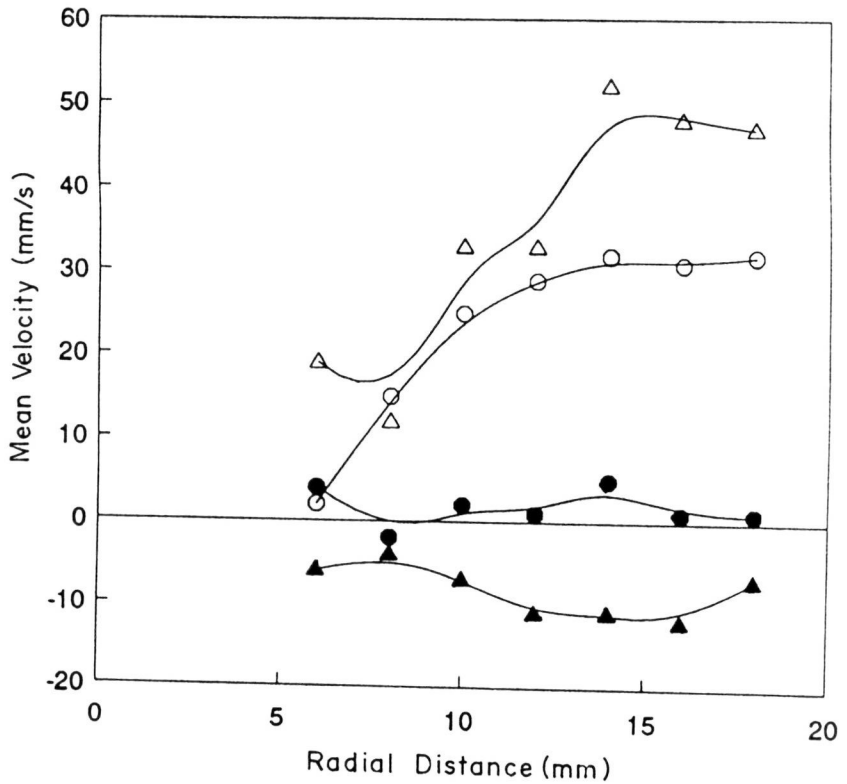

60
50
40
30
20
10
0
-10
-20
Mean Velocity (mm/s)
0
5
10
15
20
Radial Distance (mm)

Fig. 3·16

Fig. 3.17 Axial and radial rms velocity at 2.5 (r/s) in medium with FBS: Radial profiles of axial (-o- 6mm and -Δ- 12mm above impeller) and radial (-●- 6mm and -▲- 12mm above impeller) rms velocities in the spinner bottle at an agitation speed of 2.5 r/s in medium containing 10% FBS.

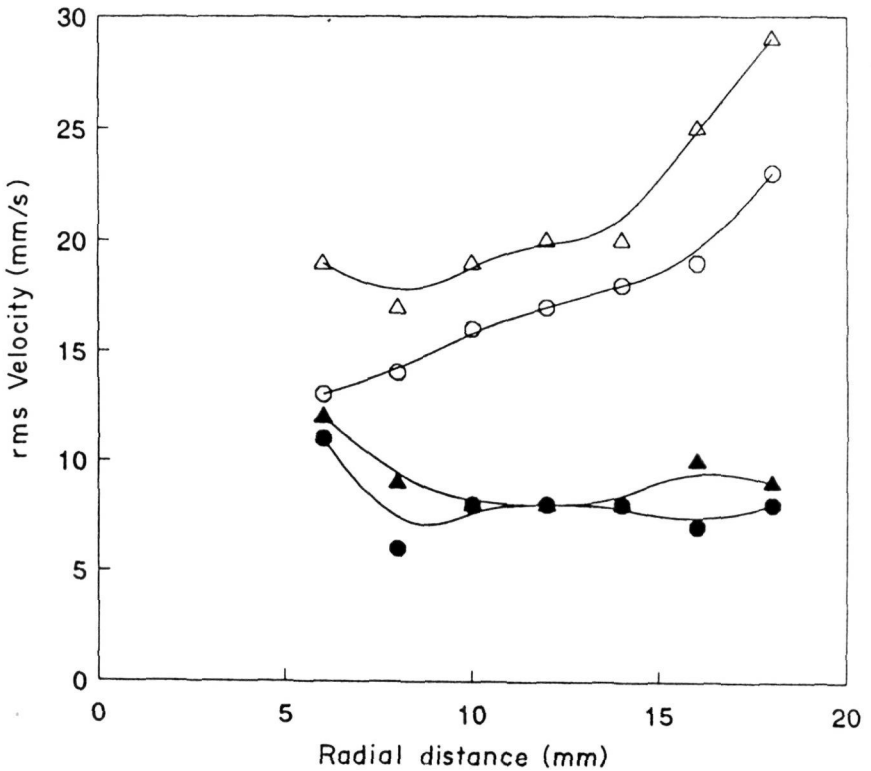

30
25
20
15
10
5
0
rms Velocity (mm/s)
0
5
10
15
20
Radial distance (mm)

Fig.3·17

Fig. 3.18 **Axial and radial mean velocity at 5 (r/s) in medium with FBS:** Radial profiles of axial (-o- 6mm and -Δ- 12mm above impeller) and radial (-●- 6mm and -▲- 12mm above impeller) mean velocities in the spinner bottle at an agitation speed of 5 r/s in medium containing 10% FBS.

Fig. 3.19 **Axial and radial mean velocity at 5 (r/s) in medium with FBS:** Radial profiles of axial (-o- 6mm and -Δ- 12mm above impeller) and radial (-●- 6mm and -▲- 12mm above impeller) mean velocities in the spinner bottle at an agitation speed of 5 r/s in medium containing 10% FBS.

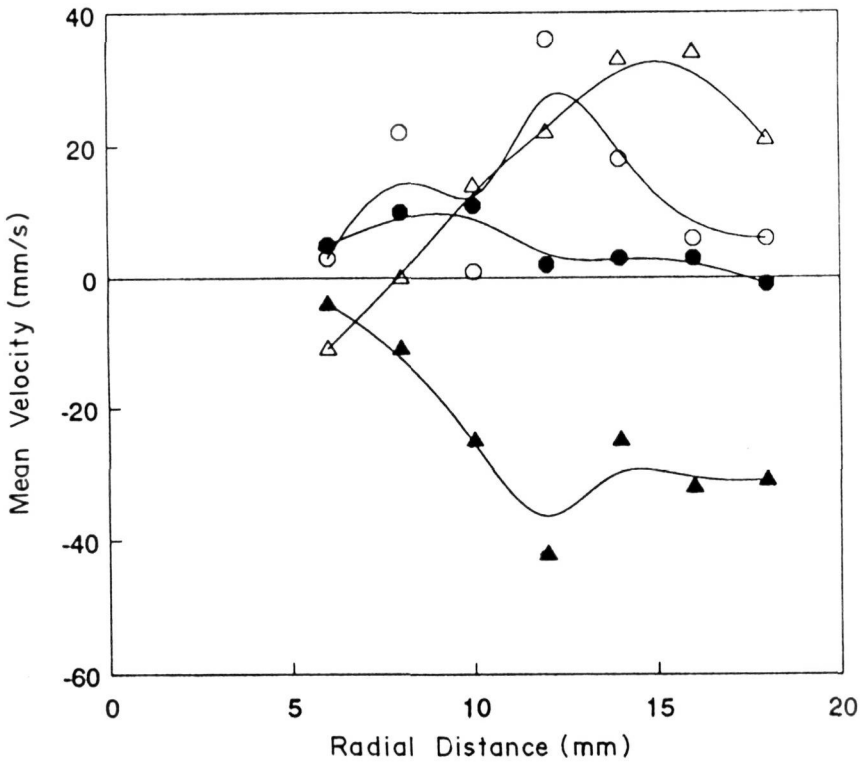


Fig.3·18

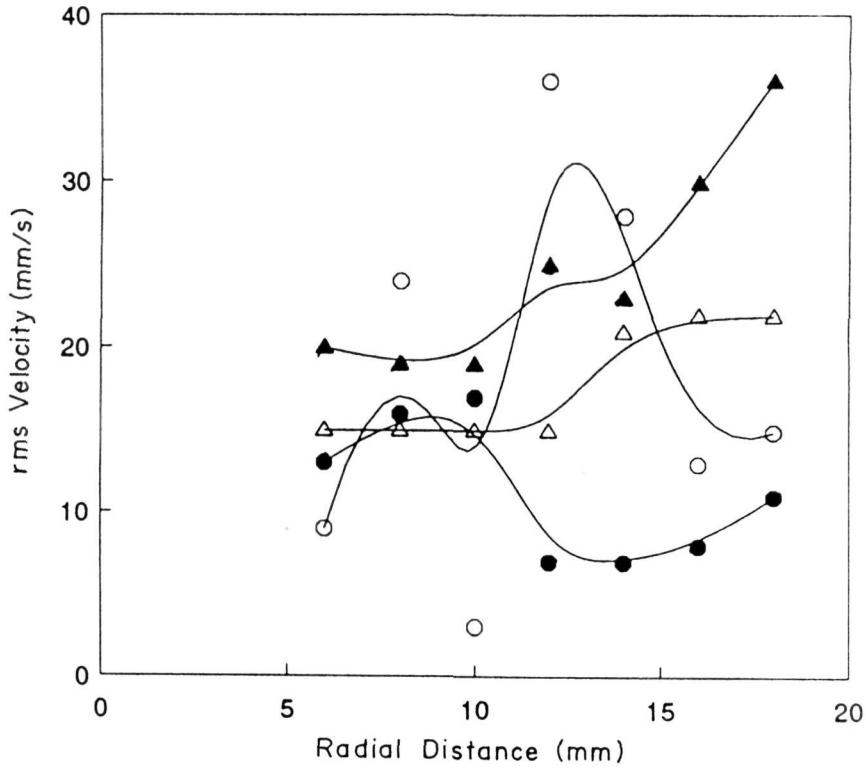


Fig.3·19

that the intensity of turbulence was largely reduced in the presence of 10% FBS. Since the values of radial and axial velocities were within $\pm$20%, it was thought to estimate the Reynolds stress on the basis of ρ, u and w, where u and w are radial and axial components of rms velocity. Reynolds stress is calculated as the product of ρ, u and w, where ρ is the density of the fluid. The results of the Reynolds stresses at the two speeds are shown in Figs. 3.20 and 3.21 respectively. The values of Reynolds stresses in the presence of 10% FBS are also shown in the same figures. It can be seen that the Reynolds stresses were 2-4 times higher in the absence of FBS. Further, the stresses were more uniform when FBS was added to the medium. There was reduction in Reynolds stress at 5 r/s with the addition of 10% FBS as in the case of 2.5 r/s, however the stresses were higher than those present at the lower speed. It had been observed in the earlier experiment that cells agitated at 5 r/s in the presence of 10% FBS suffered from shear damage. Though the effect of agitation was also studied at an impeller speed of 7.5 r/s the LDA measurements at this speed showed interference from the vortex generated and are hence not discussed here.

Fig. 3.20 Reynolds stresses at 2.5 (r/s): Radial profile of Reynolds stress at an agitation speed of 2.5 r/s in the spinner bottle for medium without FBS (-●-) and with 10% FBS (-o-).

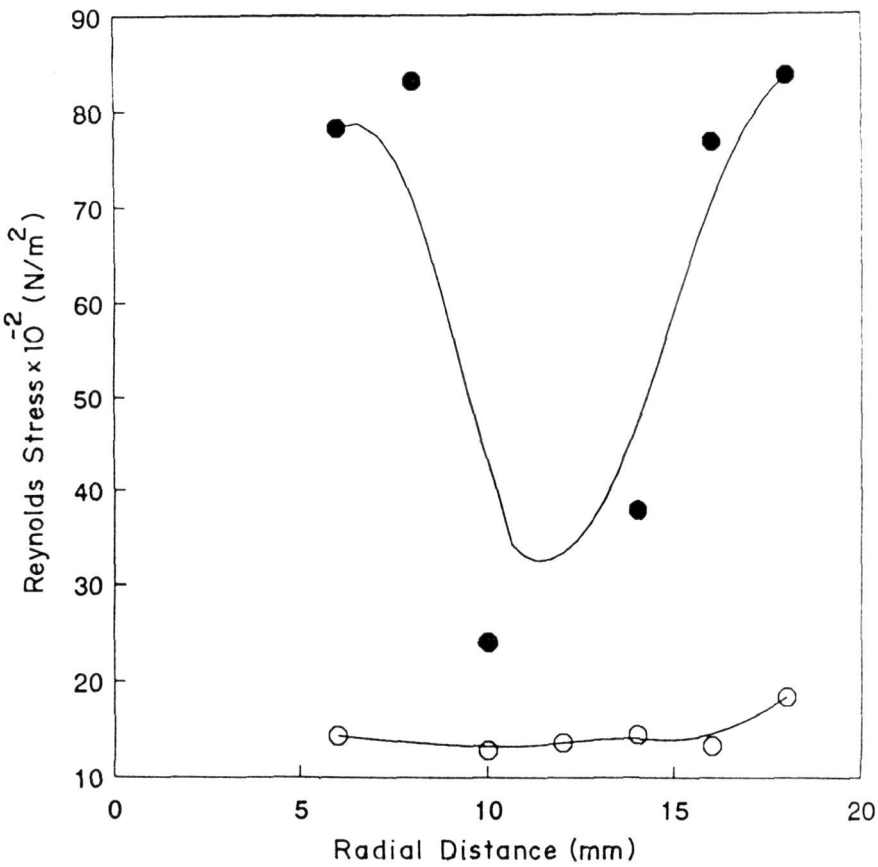

90
80
70
60
50
40
30
20
10
0
5
10
15
20
Reynolds Stress x 10^-2 (N/m^2)
Radial Distance (mm)

Fig.3·20

Fig. 3.21 **Reynolds stresses at 5 (r/s):** Radial profile of Reynolds stress at an agitation speed of 5 r/s in the spinner bottle for medium without FBS (-●-) and with 10% FBS (-o-).

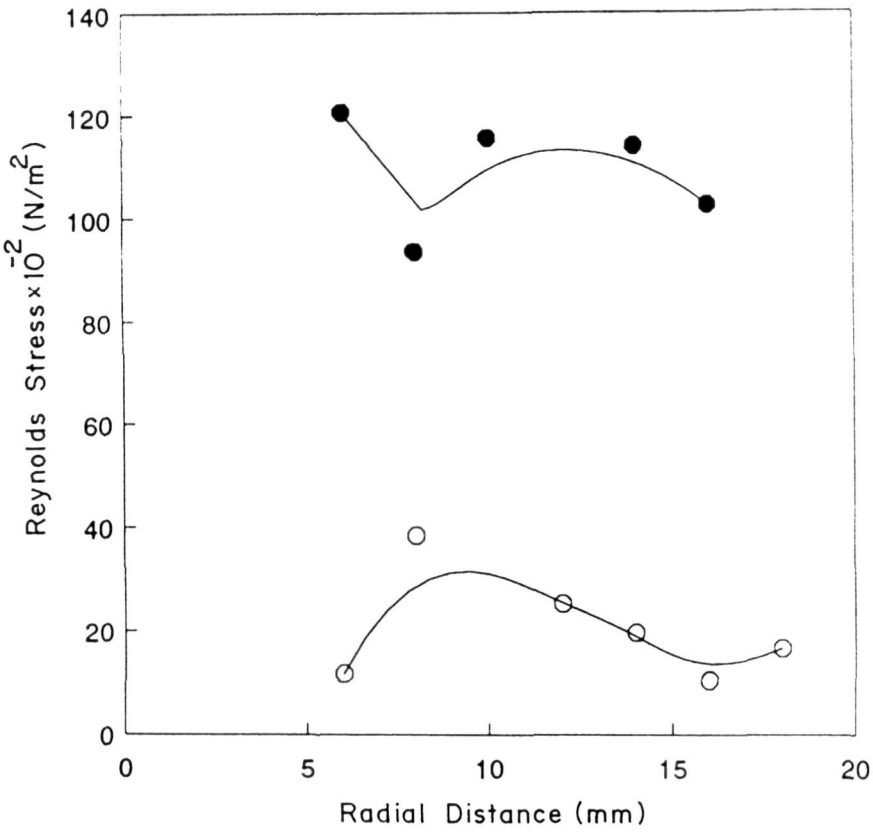

140
120
100
80
60
40
20
0
0
5
10
15
20
Reynolds Stress x 10^-2 (N/m^2)
Radial Distance (mm)

Fig.3·21

3.3 DISCUSSION

A significant extent of research has been directed towards studying the response of suspension, adherent and microcarrier cultures of animal cells to shear stress. Our results indicate that the exposure of suspension animal cells to shear does not show any damage as seen by the total count and viable count, and their metabolic activity as seen by glucose uptake. Viability of the cells by dye exclusion method has been questioned by many authors. As observed by Abu-Reesh and Kargi (1989), the metabolic activity assessed by MTT assay, in the case of shear damaged hybridoma cells was found to be affected before loss of cell integrity (by trypan blue staining), but their data indicate that there are no large differences between the quantity and the time of applied shear and the percentage reduction of these parameters. Viability of these cells can only be assessed by their capacity to proliferate. The shear exposed cells lose their capability to proliferate as seen by their decreased growth rates. This is in agreement with the results obtained by Chittur *et al.,* (1988). They studied cell proliferation by following [^{3}H] thymidine incorporation into DNA by B-lymphocyte cells exposed to shear forces. Assessment of shear damage in bioreactors may not be feasible by using proliferation assays as the time taken to complete the assay may vary from two to six days in culture. One of the immediate effects as observed in this work is the change in the morphology of the actin network. This actin network gives mechanical strength to the cells.

Reduction in the mean bursting tension and compressibility modulus of cells upon shear exposure as observed by Zhang *et al.*, (1992) may be due to depolymerization of actin network. The role of the cytoskeletal network in shear resistance of animal cells was also described by Papoutsakis *et al.*, (1991).

The shear protective effect of various substances (Michaels *et al.*, 1991) and especially of FBS has been documented (Table 3.1). From this table it is evident that the exact mechanism by which these substances protect the cells from shear damage is still not very well understood. Two mechanisms have been proposed, the protection may be merely physical, which means it is a non-specific mechanism, involving reduction of the shear forces acting on cells, or it may be biological in nature, which would involve a specific interaction of serum and cells. The results as presented here support the view that the protection offered to the cells is more of a nonspecific physical nature than being of a biological nature. This is indicated by the fact that the cells were cultivated throughout (48 h) in medium containing serum and were deprived of serum only during the short period of exposure to shear i.e., 120 min. After shear exposure the cells were supplied with serum again, and incubated further. However, the cells which were subjected to agitation in the absence of serum were unable to proliferate even though the agitation speed was only 2.5 r/s, which was seen to be non-damaging to the cells in earlier experiments. This indicates that serum protects the cells from shear damage by physical reduction of the shear forces. Similar observations have been made by van der Pol *et al.*, (1992) for hybridoma cells.

The role of actin network in shear sensitivity of animal cells has been described for endothelial cells, hybridoma cells and fibroblast cells. In the case of endothelial cells it is observed that shear treated cells align in the direction of the fluid flow and stress fibers are formed which run parallel to the flow direction. Preliminary studies on mouse embryo fibroblast cells NIH 3T3 grown on microcarriers have shown that agitation leads to alterations in the cytoskeletal structure, and F-actin polymers are not developed (du Laney *et al.,* 1992). The nature of actin network in cells grown on curved surfaces such as microcarriers is not well understood. In the present paper normal human lung fibroblasts were cultivated on flat surface and were subjected to laminar shear. Cytoskeletal structure revealed by indirect immunofluorescence staining confirmed the earlier observations of du Laney *et al.,* (1992) that shear leads to depolymerization of actin network. It is emphasized here that in the present study the controls and the treated cells were grown under identical conditions on flat surfaces unlike in the former case. The observations also indicate that the first response to shear is change in the cytoskeletal structure. Reduction in the viability and the proliferating ability and detachment from the surface occur later during prolonged exposure to shear conditions.

The measurement of Reynolds stresses indicates the momentum transfer in the fluid associated with the fluctuating velocity components. A complete description of turbulent flow is as yet not available however, it has been described to a great extent based on the Kolmogoroff theory of isotropic turbulence (Nelkin, 1992). The flow comprises large or primary eddies which are unstable and

decay transferring energy to a chain of smaller eddies, where viscous dissipation can occur. Primary eddies carry most of the turbulent kinetic energy of the flow. The interactions between the cells and the fluid in a turbulent flow depends on the relative sizes of the eddies and the particle. Eddies that are large, relative to the size of the cell will entrain the cell resulting in little relative motion. The smaller eddies will be unable to entrain the particle and hence act at its surface. The eddy-eddy and cell-eddy interactions lead to damage to the cells under turbulent conditions. Fluctuations of velocity in the fluid will produce fluctuations across the particle (cell) giving rise to hydrodynamic shear which causes damage (Thomas, 1964). Therefore, it can be inferred that damage to cells will be minimized, if the fluctuations in velocity are minimized thereby reducing the Reynolds stresses.

The effect of drag reducing additives such as polyethylene oxide and polyacrylamide on turbulent flow has been studied (Berman, 1978). The turbulence structure is known to be affected by macromolecules dissolved in the liquid (McComb, 1990; Wei and Wilmarth, 1992). The reduction of turbulence intensity upon addition of long chain molecules and similar additives has been attributed to the ability of these to reduce the small scale turbulence (Mashelkar, 1973). In a stirred vessel the shear rate in the vortex system was found to be ten times higher than the average shear in the vessel. The addition of polymers results in a resistance to vortexing thereby reducing the turbulent shear stress (Quraishi *et al.*, 1976). Similar drag reducing effect has been observed with biopolymers such as polysaccharides (Hoyt, 1985), proteins (Berman, 1978) and deoxyribonucleic acid (Hoyt, 1966; Parker and Hedley, 1974).

The measurement of rms velocity and Reynolds stresses by LDA showed that in the presence of FBS at a concentration of 10%, the intensity of turbulence is reduced. It was also observed that the rms velocities in presence of FBS are more uniform. This could explain the observation that there is less damage in the presence of serum, as it has been shown earlier that the rms velocity fluctuations cause intense shear in the neighbourhood of suspending particles and can be reduced by drag reducing additives. In the present work it was seen that the values of Reynolds stresses are also lower in the presence of serum. This effect could be also explained based on the recent work of Ranade and Mashelkar (1993) who showed that the presence of polymer molecules at a low concentration causes damping of turbulent stresses over eddy scales corresponding to the smaller eddies resulting in lowering of overall turbulent stresses. The addition of serum does not lead to a significant increase in the viscosity of the medium (viscosity of a 10% FBS containing medium was 0.809 cP as compared to 0.8 cP for medium only), but mainly serves to reduce the turbulent shear stress and hence protect the cells from shear damage. When the agitation speed was increased to 5 r/s, the cells suffered shear damage inspite of the presence of 10% FBS in the medium. From the observations it can be seen that as the impeller speed was increased from 2.5 r/s to 5 r/s, there was approximately a twofold increase in Reynolds stress. In the presence of FBS, there was a decrease in stress in both the cases. However, at 5 r/s the maximum shear stress is about two times the maximum Reynolds stress at 2.5 r/s. This would explain the earlier observation that the cells exposed to shear by agitation at a speed of 5 r/s and above show a decreased growth rate and viability. These results support the view

that the protective effect of serum is by physical reduction in the shear forces existing in the stirred vessel. The effect of serum on the turbulence structure may be due to the many dissolved proteins present in it. A similar effect may also be found in the case of Pluronic F68 and other polymeric surfactant molecules which also protect the cells from shear (Cherry, 1993). As the serum is a complex mixture of a variety of molecules, the exact mechanism of action in the case of serum requires further investigation to identify the components of serum which act as drag reducing agents or as shear protectants.

It would also be interesting to study the mechanisms involved in the shear induced destabilization of actin network and the mediators that take part in the molecular events following shear induction. The effect on turbulence structure of serum which contains many dissolved proteins may provide an understanding of the mechanism by which substances such as serum, Pluronic-F68 and other polymeric surfactant molecules protect cells from shear forces (Cherry 1993).

CHAPTER - 4

A STUDY OF THE RESIDENCE TIME DISTRIBUTION IN THE EXTRACAPILLARY SPACE OF THE HOLLOW FIBER BIOREACTOR

4.0 INTRODUCTION

The potential use of hollow fiber bioreactors (HFBRs) for animal cell culture was demonstrated by Knazek et al., (1972), who successfully cultivated mammalian cells on artificial capillaries. The HFBR has been compared to the *in vivo* vascular system which provides nutrients to cells and tissues through the extensive capillary network. In the *in vitro* system nutrients diffuse from the lumen across the membrane to the cells growing outside and the toxic metabolites diffuse back to the lumen. The stabilized perfusion cell culture environment supports the growth of cells to about 100 fold higher densities than those achieved in conventional cell culture devices (von Wedel, 1987). This system has been used since the early 1970s for the production of cancer (David *et al.,* 1978; and Hager et al., 1982) and viral antigens (Johnson *et al.,* 1978; McAleer *et al.,* 1983). The development of hybridomas (Kohler and Milstein, 1975) for monoclonal antibody production and the cultivation of these cells in HFBRs (Schoenherr *et al.,* 1986; Altshuler *et al.,* 1986) led to the use of these reactors on a commercial scale.

Traditionally monoclonal antibodies were produced in mice. The hybridoma cells were injected intraperitoneally into the ascites of mice and grew as tumors for a couple of weeks. The ascites fluid containing the monoclonal antibody was then collected after sacrificing the animal. Thus, these animals were used as culture chambers. This system is beset with several problems which include the handling of a large number of animals, batch to batch

variation in the product, and a requirement for histocompatibility between the hybridoma and the mouse strain used. The HFBR system provides an attractive alternative to the mouse system for production of monoclonal antibodies (Fig.4.1). The antibody concentration obtained in HFBRs is comparable to ascites fluid, however, the contaminating proteins are minimized by the use of defined media. This is advantageous in the further downstream processing of the product. There are several reports on the use of HFBRs for mammalian cell cultivation (Randerson, 1985; Piret and Cooney, 1990b; Hu and Piret, 1992). The HFBRs have also been recently applied in the emerging area of tissue engineering, for the development of artificial tissue systems such as pancreas (Nyberg *et al.,* 1993), bioartificial liver (Rozga *et al.,* 1993) and human bone marrow cells (Palsson *et al.,* 1993).

Physical Characteristics of the HFBR

The HFBR is constructed like a shell and tube heat exchanger (Winkler, 1990). A bundle of hollow fibers are arranged in parallel inside a cylinder with the ends of the fiber mounted in plastic blocks which seal the cylinder and provide mechanical support. Hollow fiber systems are also constructed with looped fibers that have both ends mounted at the same end of the cartridge. The medium in this case is fed at the opposite end of the cartridge and diffuses through the cell mass into the lumen (Lambe and Walker, 1987). Hollow fiber membranes in bioreactors are made of cellulose, modified cellulose, cellulose acetate, polysulfone, polypropylene and other polymers (Belfort, 1989). Hybrid bioreactors are also available wherein in addition to the

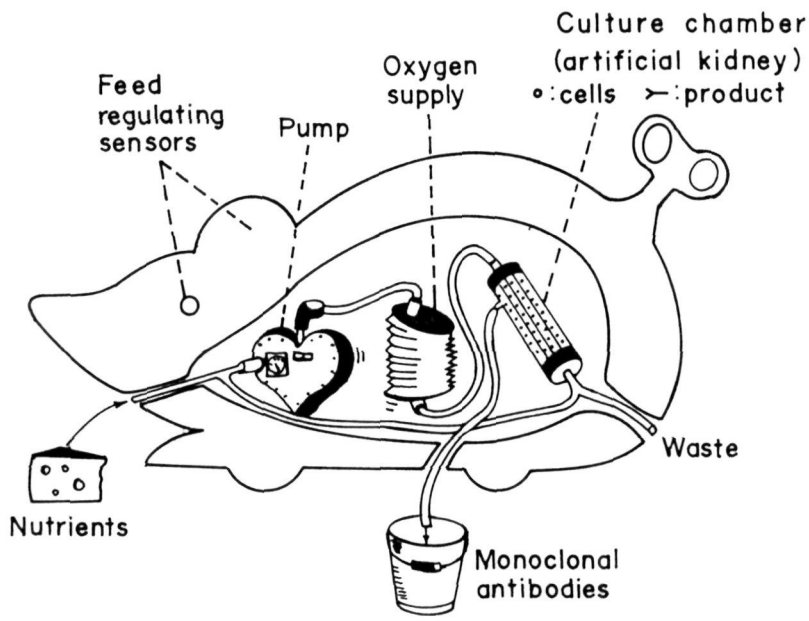


FIG.4·1: MONOCLONAL ANTIBODY PRODUCTION IN HOLLOW FIBER BIOREACTORS

(REPRODUCED FROM SCHONHERR AND VAN GELDER, 1988)

hydrophilic membranes provided for nutrient supply, silicone based hydrophobic membranes are also present, through which a gas mixture containing air and CO_2 is passed facilitating gas-liquid exchanges.

The system comprises capillary tubes, consisiting of a high porosity wall (0.07 mm thick) and a central tubular cavity (0.2 mm in diameter). The membrane protects the cells from fluid shear and allows nutrients to diffuse across. These reactors provide a higher surface area to volume ratio resulting in high cell density culture. Selection of membranes with appropriate molecular weight cut-off enables simultaneous concentration of the protein product in the cell space (Lambe and Walker, 1987).

Salient Features of the HFBR

The HFBR provides a low shear environment for the cultivation of anchorage dependent cells, resulting in cell culture systems which are smaller and yiel products at higher concentrations. A major advantage of these systems is that since the cell densities are very high, a 100 fold higher productivity can be achieved for the same reactor volume. Another advantage of this system is the reduction in serum requirement as cells growing at high densities are less dependent on serum and may even grow in its absence (Griffiths *et al.*, 1987b).

The tissue like densities of cells produced in the reactor give rise to several problems. It is of great importance to maintain a rate of medium replacement through the cell mass such that inhibitory metabolites are removed

and fresh nutrients and oxygen are continuously supplied. The dense cell mass poses difficulties in maintaining a uniform nutrient flow pattern throughout the reactor. The fibers may also rupture due to excessive cell growth and gas production (Belfort, 1989) and the membrane may also become clogged.

Mass Transfer in the HFBR

The transport in membrane systems is mainly through diffusion in cases where the medium flow rates are low, the porosity is small and the cell region is dense with low permeability. The early models were described for enzyme reactors (Rony, 1971; Horvath *et al.,* 1973; Waterland *et al.,* 1974; Kim and Cooney, 1976). These were followed later by models describing whole cell immobilized reactors (Webster and Shuler, 1978; 1981; Webster *et al.,* 1979; Davis and Watson, 1985; 1986; Heath and Belfort, 1987; and Chresand *et al.,* 1988). Kim and Cooney (1976), considered the general case, representing the system as a plug flow reactor and showed the existence of axial concentration gradients in the fiber lumen. The nutrient and metabolite gradients have also been studied by Piret and Cooney (1991) and Piret *et al.,* (1991). Webster and Shuler (1981), assumed the lumen side to be well mixed and described the substrate concentration profiles at the hollow fiber mid point at the outer surface of the cell annulus. The resistance to mass transfer offered by the tube side stagnant liquid film and hollow fiber wall was found to be negligible as compared to the cell suspension mass transfer. Heath and Belfort (1987), modeled the axial and radial substrate concentration profiles in a mammalian cell hollow fiber

system. They used the Thiele modulus (which is the ratio of observed reaction rate to the rate in the absence of diffusional limitations) as an indicator of the diffusional limitations in the system.

In most hollow fiber systems in addition to the diffusive mass transfer, convective transport also occurs due to the hydrostatic pressure difference across the membrane. This occurs in two ways, under conditions of an open shell operation fluid will flow through the membrane when pressure in the lumen is greater than that in the cell region. In a closed shell operation fluid flows through the membrane into the cell region near the reactor entrance and reenters the lumen at the exit. This is often referred to as Starling flow as it is analogous to fluid transport *in vivo* in the venous and arterial networks (Starling, 1896; Apelblat *et al.*, 1974). The convective flow in the cell region may cause heterogeneous distribution of cells affecting their growth and reactor productivity.

Fluid Flow in the HFBR

The fluid dynamics in the cell compartment can play a crucial role in the performance of the HFBR. There are very few reports in literature concerning the flow in the cell compartment. Reach *et al.*, (1984), measured the convective flow through the membranes, using bioartificial pancreas and demonstrated that convective flow plays a significant role in transport of glucose across the membranes. Park and Chang (1986), studied the flows in the tube side of the HFBR using a tracer dye. An analysis of the flows and pressure in hollow fiber system has

been carried out for an incompressible pure fluid with a constant pressure in the ECS and either laminar or turbulent flow in the lumen (Bruining, 1989). The pressures and flows in the reactor have been described by two dimensionless groups, a dimensionless flow rate and a dimensionless transport modulus relating the viscous flow resistance inside the fiber and the permeability of the fiber walls. Drury *et al.*, (1988), Pangrle *et al.*, (1989) and Hammer *et al.*, (1990), used NMR imaging to measure velocity profiles in the shell side space of HFBR modules. Heath *et al.*, (1990) and Donoghue *et al.*, (1992), employed magnetic resonance imaging (MRI) to measure the axial velocity of water in the shell space due to convective leakage.

A model which combined convective and diffusive mass transport was described by Schoenberg and Belfort (1987) and Salmon *et al.*, (1988). The convective transport was found to be negligible due to the low permeabilities in the densely packed cell region. Piret and Cooney (1990a) studied the distribution of cells and high molecular weight proteins in the ECS of ultrafiltration HFBRs. The effect of convective flow in the ECS on high molecular weight protein distributions was studied by visual observation of azoalbumin, a red dyed derivative of albumin. They also made transverse sections of the reactor and examined the cell and protein concentrations in each section. Downstream polarization of cells and high molecular weight proteins was observed. They reasoned that this distribution was the result of shell side convective fluxes and demonstrated that the distribution could be made more uniform by periodic reversal of recycle flow in the ICS.

The macromixing in HFBRs has not been considered in the previous studies on these reactors. In the present work tracer studies have been carried out to characterize the residence time distribution (RTD) in the ECS of the HFBR. The RTD has been used as a tool to investigate the flow non- idealities in the system.

Residence Time Distribution

Feed particles entering a reactor (which may be molecules, Brownian particles or fluid elements) remain in the reactor for different times. This results in a spread of the residence time of the fluid elements called as the **residence time distribution**, which is represented by an age distribution function (Danckwerts, 1953). The distribution of residence times is a direct result of the specific flow pattern existing in the reactor and governs the overall extent of reaction. The fluid element mentioned above may be considered to be made of molecules distributed within an enclosed boundary. The element may be as small as consisting of a single molecule (microfluid) or it may be large enough for a concentration within the element to be defined resulting in a macrofluid. The mixing on these two scales are called as micromixing and macromixing respectively. A complete characterization of the fluid flow in a reactor requires a knowledge of the pattern of fluid passage through the reactor. This can only be determined from the complete history of all the fluid elements passing through the reactor. However, the complexities involved in real reactors make this impractical and impossible. Valuable information regarding the flow behaviour in a real process

can be obtained from the age distribution of fluid elements in the exit stream of the reactor or the residence time distribution (Levenspiel, 1972; Wen and Fan, 1975).

The RTD profile can be predicted if the flow pattern is completely known. Since this is possible only for well defined laminar flows, RTD in the case of non-ideal flows is based upon some experimental procedures. These experimental techniques are called as stimulus response techniques. The stimulus is generally a tracer input into the fluid entering the vessel and the response is a time record of the tracer concentration at the exit of the vessel. A tracer can be any material that can be detected and does not disturb the flow pattern in the vessel. A small pulse of concentrated tracer is injected into the feed stream at a specified time (reference time) and the concentration of the tracer in the outlet stream is measured at various times, T. The time required for injection is very small. The amount of tracer (Δ N) leaving the reactor at time $t + \Delta t$ is given by the following expression

$$\Delta N = C(t) v \Delta t \qquad (1)$$

v is the volumetric flow rate, and C(t) is the concentration of tracer exiting between time t and $t + \Delta t$. If N_o is the total amount of tracer injected then

$$E(t) = \frac{vC(t)}{N_o} \qquad (2)$$

E(t) is called the residence time distribution function (Fogler, 1986). The mean residence time ($\bar{t}$) of the reactor is defined as

$$\bar{t} = \frac{v}{V} \tag{3}$$

where V is the volume of the reactor and v is the volumetric flow rate.

The input signal can be of various types such as random, periodic, step or pulse. In a step input at the reference time the concentration of the tracer is increased to a predefined value and maintained at that level for a fixed time interval. For a given system the same information can be obtained by using any one of the above signals. The step and the pulse input signals are commonly used as they are simpler to generate and analyze.

The RTD information depicts the large scale fluid dynamics or mixing at the macroscopic scale. In the case of first order reactions the RTD is sufficient to estimate the performance of the reactor e.g., mass transfer, chemical reaction or heat transfer. The RTD can also indicate any anomalies in the expected flow pattern such as channeling, by-passing, existence of stagnant dead zones, etc. Thus, it can also be used *in situ* for evaluating reactor performance.

In the present study the flow pattern in the ECS of the HFBR was studied using a high molecular weight tracer, bovine serum albumin (BSA). As it would have been difficult to obtain these profiles in the presence of cells (interaction of cells and tracer cannot be eliminated), all the experiments were carried out

in the absence of cells. This also ensured that the flow patterns observed under different conditions were not affected by the distribution of cells in the reactor.

4.1 MATERIALS AND METHODS

HFBR Cartridge

The Acusyst-R HFBR (Endotronics Inc., USA) used in this study essentially consisted of a bundle of hollow fibers potted at the ends with epoxy resin and enclosed in a transparent polycarbonate casing (23 cm x 2.0 cm dia). The reactor is divided into two compartments, the lumen or the intracapillary space (ICS), and the shell space or extracapillary space (ECS) with a void volume of approximately 80 ml.

The geometric construction of the reactor and the flow distributor at the inlet and outlet of the ECS were examined by making transverse sections of the cartridge. This is shown schematically in Fig. 4.2. The dimensions of the individual wetted hollow fibers were measured using a micrometer slide under an optical microscope (Zeiss, Axiophot).

Tracer Studies

The HFBR was equilibrated with 0.85% NaCl (filtered through 0.2 µm, filter, Milli-Q). A Coomassie Brilliant Blue (G-250, Sigma Chemical Co., USA) stained preparation of BSA was dialyzed extensively against 0.85% NaCl to remove unbound stain molecules and used as tracer. The stained protein facilitated the visual observation of the tracer. A concentrated stock solution (60 g/L) of the dye-protein complex was made in saline (0.85% NaCl). A small aliquot (about 200µl) of this was injected into the ECS feed stream of the HFBR using

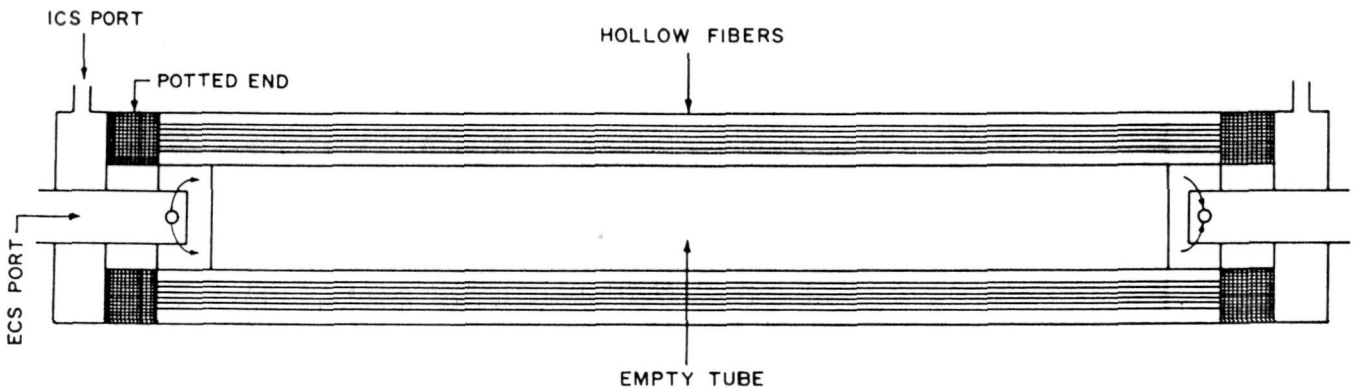


FIG.4·2 : SCHEMATIC DIAGRAM OF THE GEOMETRIC CONSTRUCTION OF THE HFBR

a hypodermic syringe. The exit concentrations of the tracer was determined by monitoring the absorbance (at 220 nm) of the effluent using a spectrophotometer equipped with a flow cell. Equal flow rates at the ECS inlet and outlet were ensured by the use of peristaltic pumps at both ends.

The fiber membrane permeability was measured according to the procedure described by Patkar *et al.,* (1993). The lumen inlet port was connected to a constant head water tank, and the oulet port of the lumen was closed. Both the ECS ports were open and the ECS permeate rate was measured. The lumen pressure was measured near the upstream (Pi) and downstream ends (Po) of the reactor using a manometer connected to the inlet and outlet ends. The transmembrane pressure (ΔP) was calculated as

$$\Delta P = \frac{P_i + P_o}{2 - P_e} \tag{4}$$

Here P_e is the ECS pressure, which was assumed to be the atmospheric pressure. The permeability (κ_p) was calculated as

$$\kappa_p = \frac{Q}{A\Delta P} \tag{5}$$

where Q is the permeate flow rate, and A is the fiber surface area based on the fiber outer diameter. The secondary Starling flow in the ECS was approximately calculated by assuming linear axial pressure drop in the ICS (Tharakan and Chau, 1986a; Heath *et al.,* 1990) and ECS (Appendix I). The expected tracer residence

time was calculated from the ECS volumetric flow rate and the volume. For calculating the average residence time and variance from RTD data, the tail of the curve was approximated as an exponential decay (Fogler, 1986).

4.2 RESULTS

The tracer studies were performed under different flow conditions in the ICS and ECS. The physical parameters of the reactor and the experimental conditions used are summarized in Tables 4.1 and 4.2. In the absence of ICS flow the tracer concentration profiles were essentially symmetrical Gaussian RTD curves with an extended tail portion (Figs. 4.3 and 4.4). The Gaussian portions of the curves indicate significant deviation from plug flow in the ECS. This dispersion may occur due to nonhomogeneous fiber packing and nonuniform ECS flow distribution. The extended tail could possibly be due to stagnant zones in the reactor and/or slow desorption of protein adsorbed on fibers. The ECS feed enters a small cylindrical region from where it is distributed to the ECS (Fig.4.2). The reverse process occurs at the ECS outlet. Stagnant regions could be present in both these distribution regions resulting in a long tail in the RTD. An increase in the ECS flow rate resulted in a decrease in the mean residence time. The mean residence time and the expected residence time calculated from the ECS flow rate and volume were in good agreement in experiments performed at zero ICS flow.

Highly asymmetric protein concentration profiles, with long tails were obtained in the presence of ICS flow, either cocurrent or countercurrent to the ECS flow (Figs.4.5 and 4.6). This asymmetry indicates a significant increase in dispersion (Levenspiel, 1972). With cocurrent ICS flows, the observed residence

TABLE - 4.1

PHYSICAL PROPERTIES OF THE HFBR SYSTEM USED

Inner radius of fiber	1.05×10^{-4} m
Outer radius of fiber	1.26×10^{-4} m
Krogh radius	2.07×10^{-4} m
Length of the fiber	0.23 m
Number of fibers	5070
Area of the ECS	0.92 m^2
Membrane permeability (κ_p)	4.94×10^{-10} m/Pa.s
Fluid viscosity (μ)	1×10^{-3} kg/m^3

TABLE - 4.2

ECS MEAN RESIDENCE TIME AND STARLING FLOWS UNDER THE EXPERIMENTAL CONDITIONS USED

Expt. Number	ECS Flow Rate (mL/min)	ICS Flow Rate (mL/min)	Flow Direction*	Calculated Residence Time (h)	Exptl. Residence time (h)	Starling Flow (mL/min)
1	0.77	0	NA	11	10.66	0
2	1.67	0	NA	1.1	1.31	0
3	1.67	100	+	1.1	0.747	5.32
4	1.67	200	+	1.1	0.51	10.73
5	1.67	100	-	1.1	0.796	5.50
6	1.67	200	-	1.1	0.856	10.92

* + - Co-current to ECS flow

\- -Counter current to ECS flow

Fig. 4.3 **The exit tracer concentration profile when the flow is only in the ECS:** The tracer protein concentration was monitored at the exit of the ECS at a flow rate of100mL/h and no flow in the ICS.

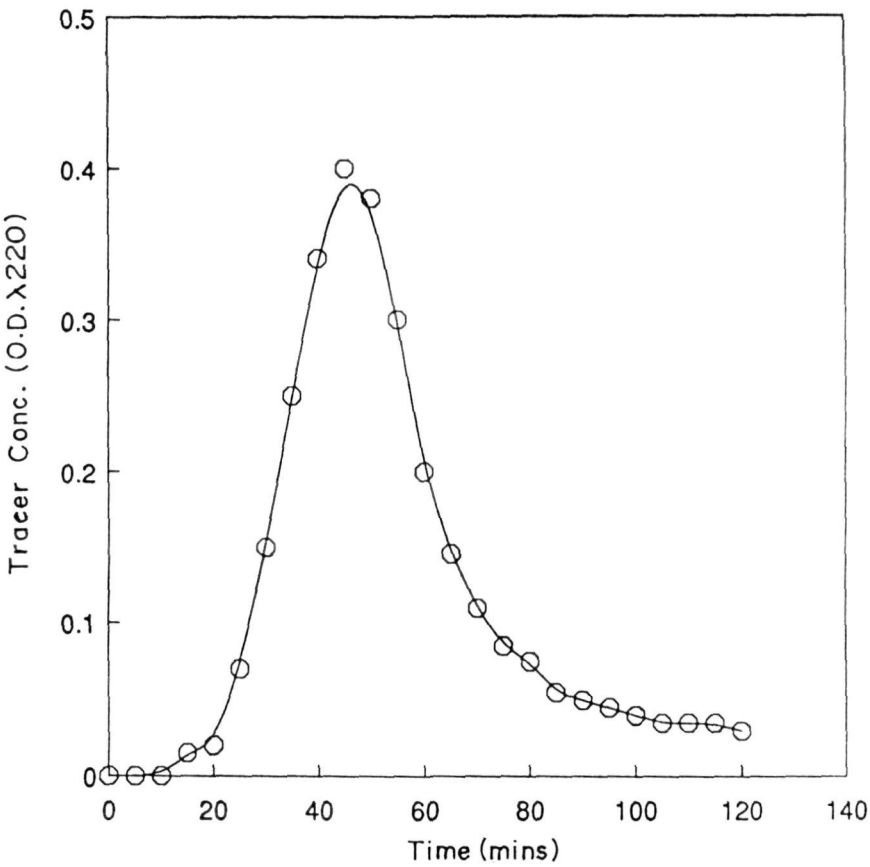

0.5
0.4
0.3
0.2
0.1
0
Tracer Conc. (O.D.λ220)
0
20
40
60
80
100
120
140
Time (mins)

Fig. 4·3

Fig. 4.4 **The exit tracer concentration profile when the flow is only in the ECS:** The tracer protein concentration was monitored at the exit of the ECS at a flow rate of 10 mL/h and no flow in the ICS.

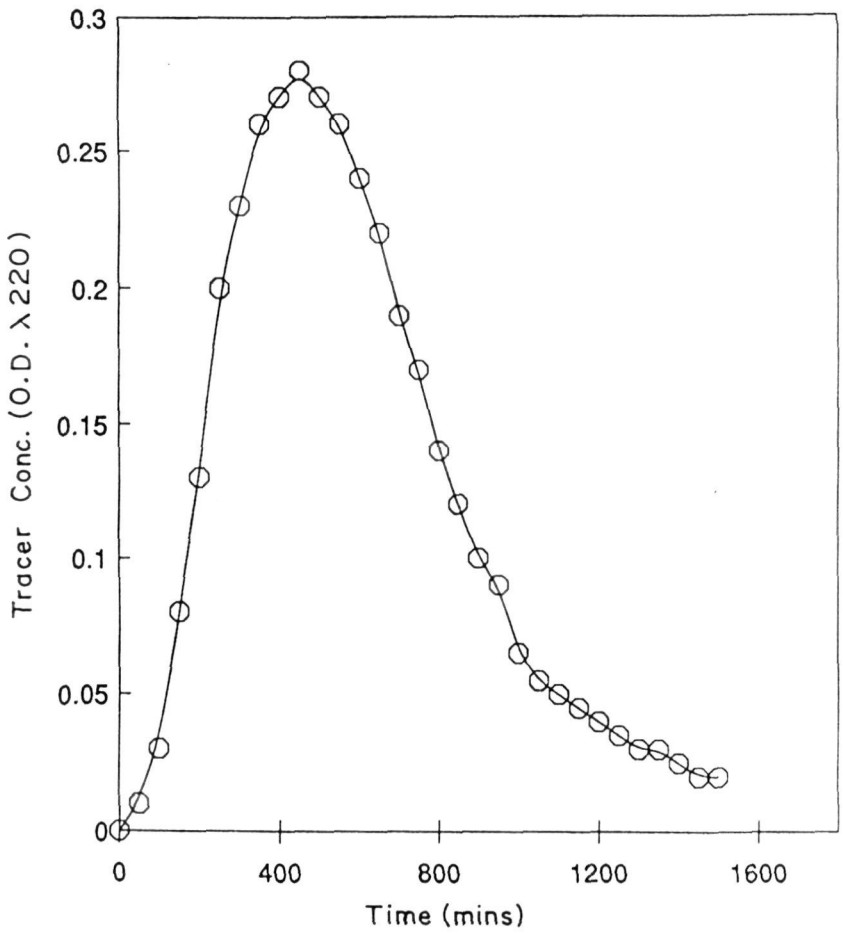

0.3
0.25
0.2
0.15
0.1
0.05
0
Tracer Conc. (O.D. λ 220)
0
400
800
1200
1600
Time (mins)

Fig. 4·4

Fig. 4.5 **The exit tracer concentration profile when ECS and ICS flows are cocurrent in direction:** The tracer protein concentration was monitored at the exit of the ECS at a rate of 100mL/h and the ICS flow rate was 100 mL/min (-o-) and 200 mL/min (-●-) and the direction of flow was cocurrent to the ECS flow.

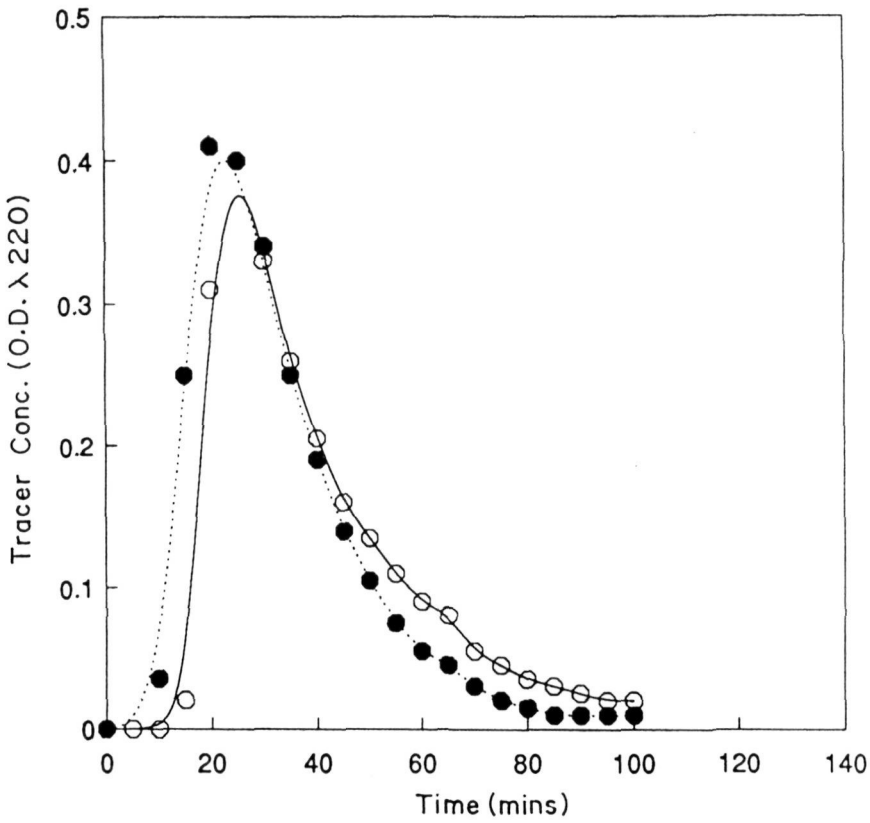

0.5
0.4
0.3
0.2
0.1
0
Tracer Conc. (O.D. λ 220)
0
20
40
60
80
100
120
140
Time (mins)

Fig. 4·5

Fig. 4.6 **The exit tracer concentration profile when ECS and ICS flows are countercurrent in direction:** The tracer protein concentration was monitored at the exit of the ECS at a flow rate of 100mL/h. The ICS flow rate was 100 mL/min (-o-) and 200 mL/min (-●-) and countercurrent to the ECS flow.

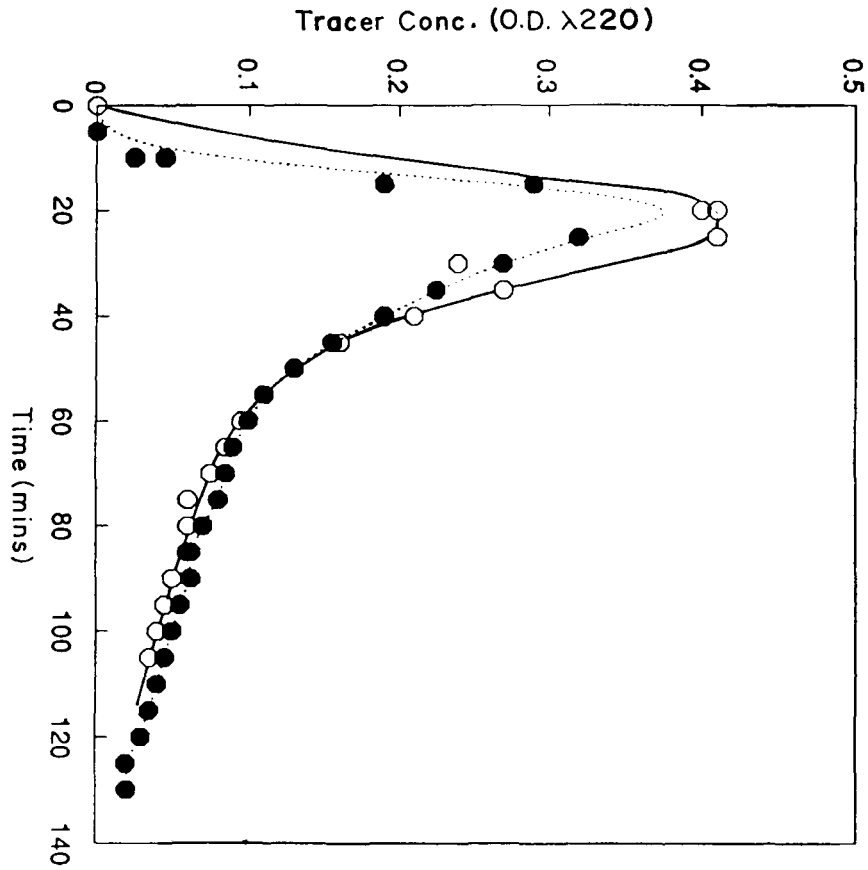

Tracer Conc. (O.D. λ220)
0
0.1
0.2
0.3
0.4
0.5
0
20
40
60
80
100
120
140
Time (mins)

Fig. 4·6

times were substantially less than the calculated residence times (Table 4.2). The mean residence times were lower even when the ICS flows were countercurrent, although the differences were less than that in the cocurrent case.

A possible qualitative explanation of these results is presented. The pressure gradients in the ICS and the ECS cause a secondary convective flow in the ECS (Tharakan and Chau, 1986a; Piret and Cooney 1990a). The ICS fluid enters the ECS through the fiber membrane near the reactor entrance and reenters the ICS near the reactor exit. A simple calculation based on ICS and ECS pressure gradients and membrane permeability was used to approximately estimate the magnitude of average Starling flow in the ECS (Table 4.2). The Starling flow was much greater (3-6 times higher at ICS flow rates of 100 mL/min and 200 mL/min respectively) than the superimposed ECS flow. The Starling flow increases with an increase in the ICS flow rate. The magnitude of the Starling flow is about 5% that of the bulk lumen flow. When the ICS flow is cocurrent the tracer is pushed towards the reactor exit at a faster rate due to the Starling flow. At the end of the reactor the fluid reenters the ICS and since the tracer cannot enter the ICS it leaves the reactor at the exit. This results in a channeling or bypassing, where the tracer protein is not distributed throughout the hollow fiber bundle and passes through only a small central portion of the entire bundle. This process is schematically described in Fig.4.7A. In the presence of counter-current flow a similar mechanism can be visualized where the tracer protein now travels towards the outer radius of the hollow fiber

bundle and bypasses the central region of the bundle (Fig.4.7B). These channeling and bypassing phenomena may cause a reduction in the observed residence times.

Fig. 4.7 **A schematic representation of the fluid flow pattern in the ECS of the HFBR:** The fluid flow pattern existing in the ECS of the HFBR is described schematically when

A) The ICS and ECS flows were cocurrent

B) The ICS and ECS flows were countercurrent

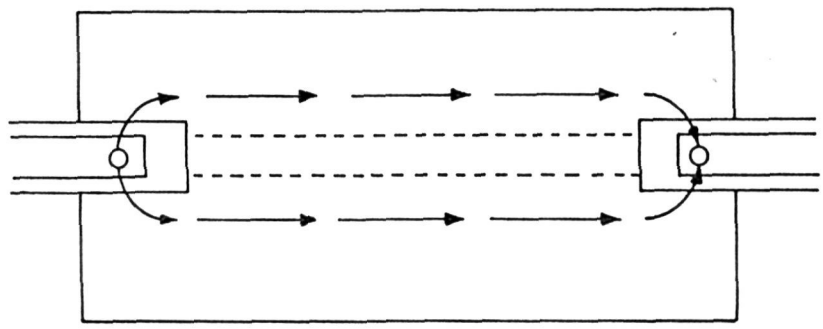


Fig. 4·7A

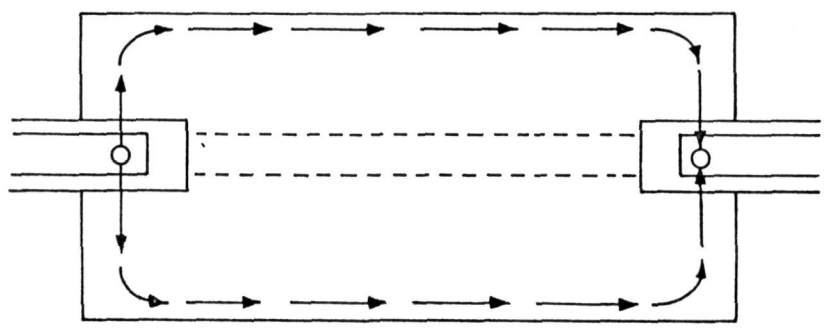


Fig. 4·7B

4.3 DISCUSSION

The results obtained indicate that the flow in the ECS deviates significantly from plug flow. The Starling flow was shown to have a significant effect on the RTD in the reactor. This has significant implications in the use of HFBRs for cell cultivation for the production of secretory products. During the operation of HFBRs the cells are supplied with high molecular weight nutrients like serum and growth factors through the ECS. Distribution of proteins in the ECS affects the distribution and amount of cell growth in HFBRs. Piret and Cooney (1990a), demonstrated that downstream polarization of growth factors (induced by Starling flow) resulted in downstream cell growth, under utilization of reactor volume and corresponding lower reactor productivity. Similar observations were made earlier in this work during the cultivation of AE9D6 hybridoma cells in the HFBR (Fig. 2.13). The cells were found to be packed at high density at the lower end of the reactor with very low cell concentrations towards the upper regions of the bioreactor. Ineffcient use of the reactor surface area means decreased reactor productivity. The Acusyst-R system used in the study was equipped with a mechanism for reversing the ICS flow direction. Nevertheless, this was not sufficient to bring about uniform distribution of cells. A study of the conditions leading to nonuniform cell distributions would be of great significance especially in the emerging area of tissue engineering, where HFBRs are being used as *in vitro* models of tissue systems like pancreas, liver and bone marrow (Koller and Palsson, 1993).

These phenomena may also play a major role in product recovery from HFBRs. This is normally done by passing medium (containing a small amount of

serum) through the ECS inlet and collecting the product in the ECS outlet stream. Assuming that the product is uniformly distributed throughout the ECS volume, a reduction in the mean residence time by 30-50% (when the ICS flow is co-current) implies that the effective ECS volume is correspondingly reduced and hence only 30-50% of the product is recovered. Considering that animal cells secrete products at very low concentrations, this loss is quite significant. Alternative designs and operational procedures of HFBRs may be used to alleviate some of the problems discussed above. The radial flow HFBR, which comprises hollow fibers surrounding a central flow distributor tube which ensures an axially uniform radial convective flow of nutrients across the fiberbed may be employed (Tharakan and Chau, 1986b). A design incorporating a sheath surrounding the hollow fiber bundle at the ECS inlet and outlet, through which the nutrients are added can also be visualized. An increase in the operating ECS flow rates could also help in minimizing the channeling and bypassing effects induced by the secondary convective flow. An in depth study and understanding of the fluid flow patterns in the ECS is desirable in order to develop better designs and operating procedures for HFBRs.

APPENDIX - I

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APPENDIX - I

Calculation of Starling flow in the HFBR

The Starling flows in the HFBR were approximately calculated as follows, using the measured permeability and assuming linear axial pressure drop in the ICS (Tharakan and Chau, 1986a; Heath *et al.,* 1990) and ECS.

$$Q_{\text{Starling}} = \int_{o}^{L} 2\pi\kappa_p N(P_I - P_{ECS})dz \qquad (1)$$

where κ_P is the fiber membrane permeability, r is the outer radius of the fiber, N is the total number of fibers and P_I and P_{ECS} are the pressures in the ICS and ECS respectively given as

$$P_{L_{(z)}} = \Delta P_I - \frac{\Delta P_L}{L} z \qquad (2)$$

where

$$\Delta P_L = \frac{8\mu L Q}{\pi R^4} \qquad (3)$$

where Q is the flow rate in the ICS, L is the length of the fiber, R is the inner radius of a single fiber and μ is the viscosity of the fluid

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$$P_{ECS} = \frac{-\Delta P_{ECS}}{L} z + \frac{(\Delta P_L + \Delta P_{ECS})}{Z} \quad (4)$$

$$\Delta P_{ECS} = \frac{8\mu L Q}{N\pi R^4 \left(1 - k^4 - \frac{(1-k^2)}{\ln k^2}\right)} \quad (5)$$

where R is the inner radius of the shell and

$$k = \frac{\text{outer radius of a single fiber}}{\text{Krogh radius}}$$

The expression for $Q_{Starling}$ can be derived by combining eqns. 1, 2 and 4 as follows

$$Q_{Starling} = \frac{\pi}{4} \kappa_p N L (\Delta P_L - \Delta P_{ECS})$$

CHAPTER - 5

GENERAL DISCUSSION AND CONCLUSIONS

The present investigation was initiated in order to understand the different aspects involved in the mass cultivation of animal cells. Animal cells can be cultivated on a large scale either in stirred systems or as high density cultures by immobilization or entrapment within gels, ceramic matrices or in membrane devices such as HFBRs.The major concern upstream of the bioreactor is the nutritional requirements of the cells and their growth and production kinetics. For a proper scale up of any cell culture process a complete understanding of the effect of the nutritional environment on the growth rates, cell yields and product formation is essential. A prior knowledge of the performance of the cells under different modes of operation such as batch, fed batch and continuous perfusion cultures is helpful in the selection of an optimal operational procedure for the cells under consideration.

The nutritional requirements of a murine hybridoma cell line (CC9C10) has been studied. The results obtained indicate that above a certain critical concentration of the major nutrients (glucose, glutamine and FBS), the growth and product formation rates were not dependent upon the initial concentrations present. Glutamine was found to be the limiting nutrient when added at concentrations below 2.0 mM. Glucose concentrations below 5.0 mM were also found to limit the growth of hybridoma cells. Accumulation of lactate above concentrations of 1.5 mg/L were found to be detrimental to the growth of cells. The results obtained are in agreement with the observations of earlier authors (Miller *et al.*, 1988). The growth rate of the cells was found to be independent of the FBS concentrations present initially, however, the production of antibody was reduced at the lower FBS

concentrations. The cells also exhibited inoculum cell density effects, and showed decreased growth rates at lower initial cell concentrations. The production of metabolite (lactate) and antibody was directly related to the concentration of cells present.

Batch culture studies were performed with another hybridoma cell line (AE9D6). The cell growth followed the typical batch culture pattern. In batch culture experiments glutamine was found to be the limiting nutrient as it depletes at a rate faster than glucose and is completely utilized by the end of the culture period. Lactate is produced as the major metabolic waste and accumulation of lactic acid resulted in a decrease in the number of viable cells. This is probably due to a decrease in the pH of the medium as addition of lactic acid to culture medium under controlled pH conditions was found to be nontoxic to cells (Eagle, 1973; Ozturk *et al.,* 1992). The cells were also cultivated in a fed batch mode, where complete medium was added to the culture flask as a single addition at fixed time intervals. The period of growth could be extended from 6 days upto 12 days before a significant decrease in growth occurred. Reuveny *et al.,* (1986b), also found that addition of glucose and glutamine to the culture extended the period of high viability of the population. However, such a method does not overcome the problem of accumulation of waste metabolites e.g. lactate which lead to decrease in the cell viabilities. At higher cell concentrations under static conditions with no external aeration, oxygen could also become a limiting factor resulting in a decline of the viable cell population.

The cultivation of hybridoma cells in HFBRs was studied and it was shown that the cells could be maintained in the reactor at high densities for extended periods with the continuous perfusion of nutrients and removal of toxic wastes. Non-uniform distribution of cells in the HFBR was observed. The RTD was used as a tool to investigate the fluid flow patterns existing in the ECS. The RTD profiles obtained showed that the flow in the ECS deviates from plug flow. A qualitative explanation of the RTD results is presented. The Starling flows induced by the ICS flows were estimated and were found to affect the RTD in the reactor. The pressure gradients in the ICS and ECS and the secondary Starling flow lead to channeling and bypassing in the ECS of the reactor. This leads to a nonuniform distribution of cells and proteins in the reactor. These phenomena are also important in the product recovery from HFBRs.

One of the major constraints in the large scale cultivation of animal cells in traditional stirred tank or airlift systems, is the shear sensitivity of the cells. The effect of turbulent shear stress on suspension lymphoblastoid (KG-1) cells was investigated. The effect of laminar shear stress on adherent monolayer cultures of human lung fibroblast cells (WI 38), was studied by subjecting them to shear in a parallel plate flow chamber. The KG-1 cells were sheared under turbulent conditions by spinning them at different speeds in a spinner flask. The effect of shear was studied by monitoring the total and viable cell counts of the population, their proliferating ability and the structure of the cytoskeletal network of the treated cells. The damage to the cells as assessed by performing total and viable cell counts was not significant. However, it was observed that

the cells exposed to agitation at speeds of 5 and 7.5 r/s were unable to proliferate, while the cells exposed to a lower agitation speed of 2.5 r/s continued to grow at the same rate as the cells grown under static conditions. These observations showed that viability as monitored by dye exclusion methods tends to underestimate the cell damage. This may be due to the fact that if the membrane damage does not occur immediately after the shear treatment the dye exclusion test would give false results (Cook and Mitchell, 1989). The cytoskeletal structure of the cells undergoes changes as seen from the immunofluorescent staining studies of the control and treated cells. Changes in the actin network of the treated cells were observed indicating that the actin plays a role in the shear sensitivity of animal cells. Earlier workers (Levesque *et al.*, 1989; Papoutsakis *et al.*, 1991) have also made similar observations. The shear protective effect of serum was studied by shearing the cells at a lower agitation speed of 2.5 r/s in the presence and absence of serum.

The previous studies in this area have not been able to provide a quantitative measure of the turbulent shear stress in a stirred bioreactor and relate it to the damage to the cells. In the present study an attempt was made to bridge that crucial gap and the turbulent shear stresses existing in the commonly used spinner flask were quantitatively estimated by using laser Doppler anemometer. The turbulent shear stresses were estimated in the same spinner flask as that used for subjecting the cells to shear. The shear protective mechanism of serum was examined by estimating the Reynolds stresses in the presence and absence of serum.

The exact mechanism by which serum and other shear protective additives protect the cells from shear is still not clearly understood. There are two mechanisms proposed, the protection may be merely physical i.e., the shear protective additives reduce the shear forces experienced by the cells or the effect may be biological involving certain specific interactions between the cells and the additive. The question of which of these two mechanisms is dominant or whether both the mechanisms are responsible for the shear protection is still unresolved. The results presented in this work support the view that the protection offered to the cells is more of a nonspecific physical nature than being of a biological nature. This is indicated by the fact that the cells (which were grown in the presence of serum) exposed to agitation in the absence of serum were damaged even at the lower agitation speed of 2.5 r/s. The measurement of rms velocity and Reynolds stresses in the presence and absence of serum showed that the intensity of turbulence was reduced at a FBS concentration of 10% v/v. The results also gave an indication of the critical Reynolds stress beyond which cells were damaged. This was ascertained by measuring the Reynolds stresses at 5 r/s in the presence of serum. The earlier experiments showed that cells agitated at this speed suffered from damage even in the presence of FBS. However, as serum is a complex mixture of several dissolved proteins (McLeod and Drummond, 1980) the exact protective mechanism of serum and identification of the components which act as shear protectants or drag reducers requires further investigation.

Most of the earlier studies on the effect of laminar shear dealt with endothelial cells (Levesque and Nerem, 1985; Nollert *et al.*, 1991). These cells

may be different from normal fibroblast cells with respect to shear sensitivity as they are constantly exposed to hydrodynamic flow *in vivo* in the blood vessels. Hence, the effect of laminar shear on cells such as fibroblasts which are not normally exposed to shear *in vivo* were considered in the present work. These cells did not show the elongation and orientation phenomena described earlier for endothelial cells. The WI 38 cells upon shear exposure were found to flatten as evidenced by an increase in the surface area of the cells. Changes in the actin and vimentin network of treated cells were observed by immunofluorescent staining. As in the case of KG-1 cells, the actin network was found to be affected by shear, implicating its role in providing mechanical support to the cells. The results of this work showed that the changes in the actin network are evident earlier than the damage to membrane as assessed by the dye exclusion methods. A detailed study of the mechanisms involved in the shear induced destabilization of the cytoskeletal structure and in the conversion of a fluid mechanical stimulus into a biochemical response would be of fundamental importance.

SUMMARY

SUMMARY

The work deals with different aspects of large scale animal cell cultivation, such as nutritional requirements, cultivation of cells under different operation modes and the shear sensitivity of animal cells.

The nutritional requirements of hybridoma cells in culture with respect to the major nutrients glucose, glutamine and FBS were studied for a murine hybridoma CC9C10. The inoculum cell density effect has also been examined. The cultivation of hybridoma cells (AE9D6) in different modes of operation such as batch and fed batch and under perfusion conditions in the HFBR has also been investigated.

The fluid flow patterns existing in the ECS of the HFBR has been studied using RTD as a tool. The hydrodynamics of the ECS plays an important role in the cell distribution and growth as well as in product recovery in the HFBRs.

The effect of laminar and turbulent hydrodynamic shear on animal cells *in vitro* was examined using adherent fibroblast cells (WI 38) and suspension lymphoblastoid cells (KG-1) respectively. The effect of shear on the morphology and function of cells (proliferating capacity, glucose utilization) was observed. The changes in the cytoskeletal network of the cells which has an important role in the maintenance of cell shape and function were also studied. The shear protective effect of serum was investigated. The turbulent shear stresses existing in a stirred vessel (spinner flask) were quantitatively estimated using laser Doppler anemometer and the results showed that the cells suffer from damage beyond a

critical Reynolds stress. The studies also helped in elucidating the protective mechanism of serum by demonstrating that the Reynolds stresses decrease in the presence of serum, thus supporting the view that the effect of serum is physical.

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PUBLICATIONS

The following papers based on the present work have been communicated for publication.

1. Shear Sensitivity of Human Lymphoblastoid Cells (KG-1) in Turbulent Suspensions: Turbulence Measurements and Elucidation of Protective Mechanisms
   Cynthia B. Elias, Rajiv B. Desai, Milind S. Patole, Jyestharaj B. Joshi and Raghunath A. Mashelkar.
   Biotechnol. Prog. (Communicated)

2. Residence time Distribution in the Extracapillary Space of Hollow Fiber Bioreactors
   Cynthia B. Elias, Milind S. Patole, Anant Y. Patkar and Raghunath A. Mashelkar
   Biotechnol. Bioeng. (Communicated)

ERRATA

1. *The second line in para 2, pg. 4 should read as*

   In the 1920s came the major development of glass bottles or culture vessels introduced by Carrel, who also used his surgical procedures for the development of aseptic techniques which have led to the success of long term *in vitro* culture.

2. *Equation (1) on pg. 18 should read as*

$$\frac{dx}{dt} = \mu X$$

3. *Line 5 para 1 on pg. 32 should read as*

   Fed batch cultivation of these cells was also carried out in a similar system using 125cm^2 T flasks with an initial volume of 30mL.

4. *The legend for Fig. 3.19 should read as*

   Axial and radial rms velocity at 5(r/s) in medium with FBS: Radial profiles of axial (-o- 6 mm and -Δ- 12 mm above impeller) and radial (-●- 6 mm and -▲- 12 mm above impeller) rms velocities in the spinner bottle at an agitation speed of 5r/s in medium containing 10% FBS.

5. *Equation (4) on pg. 93 should read as*

$$\Delta P = \frac{P_i + P_o}{2} - P_e$$