DIALYSIS CULTURE IN ANIMAL CELL GROWTH AND PROTEIN PRODUCTION

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Abstract

Hybridoma cells were grown in dialysis perfusion culture using a stirred reactor within which a tubular membrane was suspended. Nutrient and product flows occurred by diffusion processes alone, and were both to and from the culture environment A mathematical model of the transfer and reaction allowed prediction of steady state cell and metabolite concentrations. Steady states in cell concentration were observed for a range of perfusion rates and membrane areas. However the model could not be applied to predict steady state cell concentrations between changes in the medium. The perfusate consisted of basal medium only. Serum addition to the reactor itself resulted in decreased steady state cell densities except when it relieved a glucose limitation.

Antibody was accumulated to high concentrations and yields on both basal medium and serum were many times those achieved in standard batch cultures. Cell viability fell to 30-50% but product quality did not appear to be adversely affected by the low viability.

Recombinant CHO-320 cells also grew successfully under dialysis conditions and produced γ-interferon. Cell concentrations and viabilities were higher than those seen with the hybridoma. The insect cell line SF9 did not grow during dialysis perfusion, but post infection with a recombinant Baculovirus permitted the yield of β-galactosidase to double in dialysis culture.

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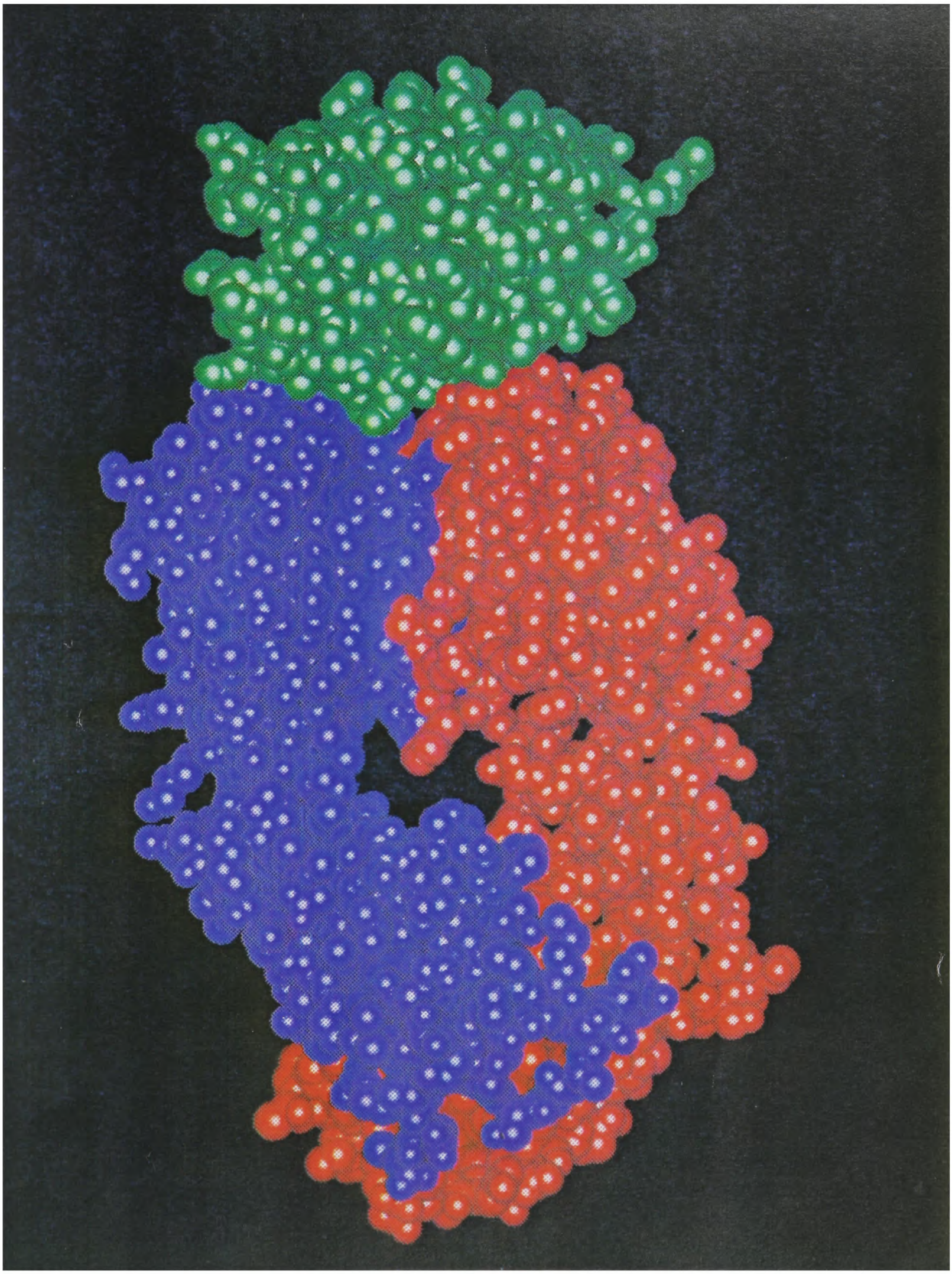
Dedication

To my parents for their love, support and encouragement.

Figure 1: A space filling model of an Fc fragment of IgG (red and blue) and its antigen (green



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Chapter 1

Introduction

1.1 Why grow animal cells?

Two discoveries during the 1970s prompted a renewed interest in growing animal cells which had for some time been used to produce viruses. The development of the hybridoma by Kohler and Milstein (1975) permitted the production of monoclonal antibodies, revolutionising the identification of complex biological molecules. The other discovery by Cohen *et al.* (1973) was a method of recombining DNA *in vitro*. This allowed for the first time the production of human proteins which previously were only available by purification from human tissue, often in vanishingly small concentrations. Genes could be expressed in organisms other than those in which the gene had evolved. Both discoveries were to have profound effects on the development of novel therapeutic drugs. To date there are about 20 proteins registered as drugs produced using these techniques (Drews, 1993), with about 150 recombinant proteins and more than 40 monoclonal antibodies at some stage of clinical development. In this project principally hybridoma but also recombinant systems were used.

1.1.1 Hybridomas

Hybridoma cells are developed from white blood cells. White blood cells are a crucial part of the immune system of all vertebrates and can be divided into two classes, myeloid cells and lymphoid cells. An average human adult has approximately 10^{12} lymphocytes, around 2% of total body weight and produces 10^9 lymphocytes per day (Roitt *et al.*, 1990). B-lymphocytes make up 5–15% of the circulating lymphoid pool and these cells produce a type of protein known as immunoglobulin, or antibody, which binds specifically to foreign matter within the body. Each B-cell produces only one specific antibody which, through a combination of chemical and steric factors, binds to a particular chemical structure, the antigen. A series of random genetic rearrangements give in excess of 10^8 possible different antibodies (Burnet, 1959). Those clones that produce antibody which correspond to antigens that are normally part of the body are held quiescent, or are deleted. This is known as the clonal selection hypothesis, first proposed by Burnet (1959).

Immunoglobulin is a major constituent of the protein of human serum. There are five classes of antibody: IgA, IgD, IgE, IgG and IgM. IgG accounts for 70–75% of the total immunoglobulin pool, and is a monomeric protein with a molecular weight of 146,000 consisting of 4 polypeptide chains; 2 light chains with a molecular weight of 25,000 and 2 heavy chains with a molecular weight of 51,000 (see Figure 2), held together by disulphide bonds. All immunoglobulins are glycoproteins, the carbohydrate content of IgG forming 2–3% of their mass. There are four subclasses of IgG, each having a different heavy chain. The heavy chains differ in amino acid sequence and inter-chain disulphide bond linkages.

Both the light and heavy chains have two sections made up of constant and

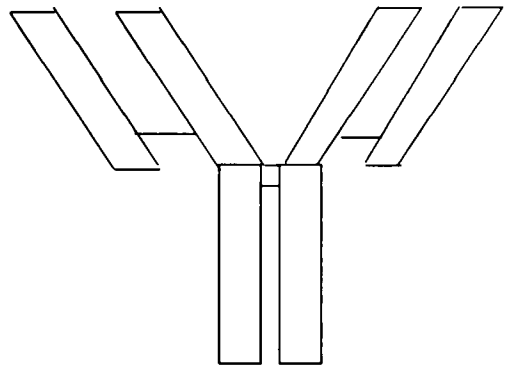


Figure 2: A schematic diagram of IgG

variable regions. The variable region of each light and heavy chain is unique to that particular B-cell clone. In the complete antibody molecule, this variable region forms an antigen binding site. IgG is a Y shaped molecule and possesses two such binding sites. Binding to the antigen is determined by steric factors (*it.* how well it fits into the binding site) and chemical factors (*it.* the presence of appropriate moieties to bind with). The *specificity* of the antibody/antigen complex can be very high. Antibodies are capable of distinguishing between small differences in the primary structure of the antigen, differences in charge and differences in steric conformation. For example, antibodies can be raised against α–helical tripeptide, which do not bind the same tripeptide in non-helical form. The constant region forms the remaining three domains of the four domain IgG and this part of the molecule includes sites specific for a variety of cells of the immune system and the activation of blood clotting, complement. The many binding functions of IgG have earned it the epithet **The Flexible Adapter** and its primary role in the immune system is to facilitate the recognition of foreign bodies by the cells of the immune system.

Hybridomas are produced by fusing Plasmocytoma cells with B-lymphocytes. Plasmocytomas are a type of B-lymphocyte cancer. It is possible to grow them

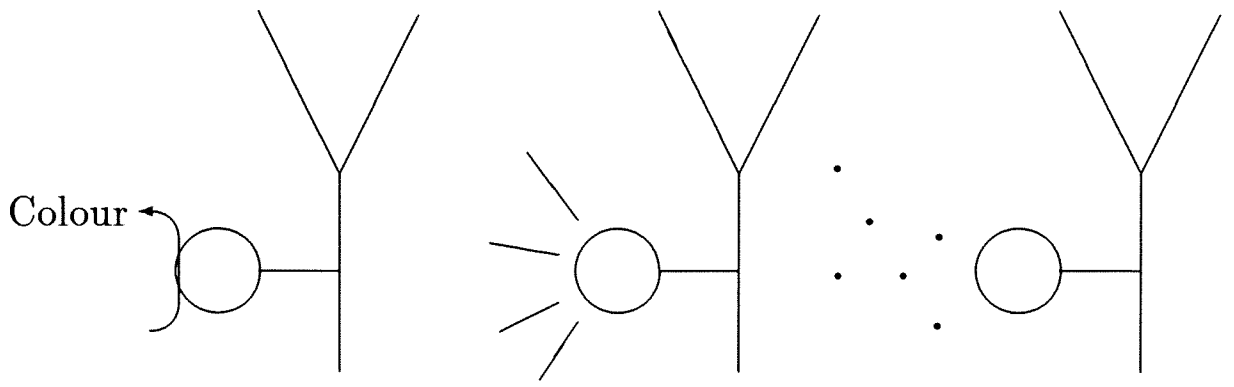
indefinitely *in vitro* while normal lymphocytes only survive for 1–2 weeks. A number of mouse derived plasmocytoma lines exist which do not produce antibody and also have lost the enzyme **Hypoxanthine guanosine ribosyl transferase** and consequently cannot synthesise deoxyribose nucleic acids via the *salvage* pathway and must rely on the *de-novo* pathway. Aminopterin inhibits **Dihydrofolate Reductase** thus blocking the regeneration of **tetrahydrofolate**, which is necessary for *de-novo* DNA synthesis. In the presence of aminopterin, these plasmocytoma cells die. It is possible to take the lymphocytes from a mouses spleen and fuse them with plasmocytoma cells. The majority of such fusions are genetically unstable and die. The cells are then grown in medium containing Aminopterin, along with Hypoxanthine and Thymidine to allow cell growth using the salvage pathway. Unfused myelomas die because of the aminopterin, unfused spleen cells die because they are not transformed. The resulting remaining cells are called **Hybridomas**. Diluting the cells, so that on average there will only be one cell per sample, allows separation of the different clones. Clones producing antibodies of interest are detected using ELISA (see below).

1.1.1.1 Why Grow Hybridomas?

Many biological molecules are difficult to identify with conventional chemical tests because of their complexity. The specificity of antibodies allows molecules of interest to be specifically labelled. The antibody can be tagged with a radioisotope, an enzyme, or a fluorescent chemical to allow identification (see Figure 3). If the antigen is attached to a solid support, then unbound antibody may be washed away and qualitative or quantitative assessment of either the antigen or antibody can be made. This technique is known as the Enzyme Linked Immunosorbant

Assay (ELISA). It is now used routinely in medical laboratories throughout the world for many diagnostic tests, two such uses being the determination of viral antigens *eg.* HIV and measurement of hormone concentrations *eg.* in pregnancy testing.

The antibodies for such tests can either be from a hybridoma and thus monoclonal, or from the serum of animals which have been immunised with the antigen of interest. Since one antigen may contain several potential sites for antigen binding, epitopes, and each epitope may be bound by several different antibodies then such serum is said to be polyclonal. Polyclonal sera, in most cases, are sufficiently specific for assay purposes, however, they are not sufficiently specific for other uses. Hybridomas may be grown in mice within a form of hollow tumor, known as an ascitic tumor and antibody may be recovered in 1-10 mg/ml concentrations in 1–10 ml volume. For production of small amounts of monoclonal antibody for diagnostic purposes, this has been the method of choice in many laboratories. However there are ethical concerns about its use, logistical problems if large quantities are required and, because of the animal host, difficulties in getting licenses for therapeutic purposes. Early *in-vitro* production methods used batch cultures, either in large agitated tanks, or in roller bottles, producing 10-100 μg/ml of monoclonal antibody, depending on the cell line, at scales of up to 10,000 litres. *In-vitro* production is more reproducible and consistent than ascitic culture and it is possible to produce the humanised antibodies that would be needed for therapeutic applications.



Identification Tag Enzyme Fluorescence Radioisotope

Figure 3: Conjugated tags used to identify the presence of antibody

1.1.1.2 Uses For Antibodies

Antibodies have many uses including:

- Diagnostics

  Many biological molecules are difficult to identify and quantify using conventional biochemical techniques, and antibodies are now routinely used for bacterial and viral identification, and quantification of proteins, hormones, *etc.*

- Tumor Imaging

  By attaching a radioactive label to an antibody directed against a cancer cell, tumors can be located then surgically removed.

- Drug Targeting

  Pharmaceuticals may be concentrated around a cell type of interest, *eg.* cancer cells, by attaching them to an antibody. This is known as Ehrlichs Magic Bullet.

- Direct Immunisation

  Antibodies raised against a disease-causing agent can be used to give short term protection.

- Purification

  A type of affinity chromatography, the antibodies recover the item of interest which is released by changing the pH or ionic strength of the buffer.

- Catalysis

  If the transitional state of a chemical reaction can be stablised, antibodies may be raised to it. In some cases, these antibodies then catalyse the reaction.

Table 1: Therapeutic products of recombinant DNA technology and animal cell culture

Growth Factors	Cancer, anemia, wound healing, viral and bacterial infections, bone marrow transplantation
Hormones	Diabetes, growth disorders, osteoporosis
Interferons	Cancer, viral infections
Interleukins	Cancer
Fibrinolytics	Cardiovascular diseases
Vaccines	Hepatitis-B, AIDS, malaria, pertussis, typhus, influenza
Recombinant Antibodies	Cancer, infections, inflammation
Soluble Receptors	Inflammation, HIV infection
Enzymes	Enzyme deficiencies

1.1.2 Recombinant DNA technology

The second development mentioned earlier, recombinant DNA technology has permitted tremendous developments in the understanding of biological systems over the last 20 years. The ability to transfer genes from one organism to another and to modulate the level to which they are expressed, has offered the possibility of new and improved therapies for a range of diseases. These are just starting to reach the market and products of recombinant DNA technology are currently undergoing clinical trial (Table 1). (Drews, 1993).

The tools for performing DNA recombination come from bacteria, from which can be isolated both a variety of enzymes that split DNA at very specific sequences and others that rejoin the DNA. Careful use of these tools allows the production of a new length of DNA encoding a novel combination of functions enabling the production of the protein of interest. For successful protein production a number of different functions must be performed, many of which are controlled by

the DNA sequence. Firstly, the DNA must be able to enter the host organism, secondly, in many cases, it needs to be transferred with the host DNA when the cell divides, thirdly, it must be translated and transcribed in the host organism; and fourthly it may need to be modified and secreted. A **Vector** is a length of DNA which combines those functions which are controlled by DNA for expression of the protein. Vectors are often based on naturally occurring viruses. Other functions are controlled by the host organism, or **expression system**, which in turn influences the vector chosen.

The first function, insertion of the DNA, can be achieved in a number of ways. The DNA molecule may be injected in to the cell, the cell may made permeable using a variety of chemical or electrical treatments, or, if the vector is a suitable virus, the molecule may be packaged in the coat proteins of the vector and the normal viral uptake mechanism may be used.

The second function, transfer of DNA to progeny, can be ignored if the vector is a virus that efficiently infects, then lyses its host, such as the baculovirus system. This gives transient protein production. Normally continuous production is desired, to ensure transmission of the recombinant molecule to the progeny. This may be achieved by a number of different strategies, for example the vector may recombine itself with the host genome or be present in high copy number, it may encode resistance to a cytotoxic drug, or conferring an essential metabolic function.

The third function is protein production. The controlling stage here is initiation of transcription, which is regulated by a DNA sequence known as the promoter. Promoters vary in the strength of their action, by about 100 fold and may be either constitutive, always on, or inducible, may be switched on and off.

Most vectors contain strong constitutive promoters, often from viruses such as simian virus 40 (SV40).

The fourth function, correct modification and secretion of the resulting protein is strongly affected by the type of host organism. Recombinant proteins may be expressed in many different organisms, as John Hodgson says in *Expression Systems: a user's guide* (Hodgson, 1993):

> *...producer organisms...span six orders of magnitude from* E. coli *weighing in at around one micrometer in length to the heavyweight lactaters and bleeders,* Bos taurus, Ovis aries *and* Sus domesticus *at over one meter.*

The choice of which organism to express your protein in is a complex one. Many human proteins are substantially altered subsequent to translation; peptides may be cleaved from them, certain amino acids are chemically altered, and carbohydrate groups may be added. In addition human cells have a number of proteins known as chaperonins which assist the nascent peptide chains to fold into the correct tertiary structures. These modifications may be necessary for the functioning of the protein. Also, incorrectly modified protein may provoke an immune response if used *in vivo*. Bacteria lack the ability to perform many post-translational modifications, and many proteins expressed intracellularly form insoluble denatured pellets known as inclusion bodies which must be refolded to become active. However bacteria are substantially cheaper to grow and yield higher concentrations of protein in shorter periods of time. A recent study (Datar *et al.*, 1993) compared the production of **tissue Plasminogen Activator** (tPA), which is given to myocardial infarction patients to stimulate blood clot dissolution, in *Escherichia coli* and Chinese Hamster Ovary (CHO) cells. *E.coli* produced 460 mg l^{-1} in

1–2 days, CHO produced 33.5 mg l^{-1} in 5–7 days. Bacterially produced tPA was present as a denatured mass and the costs involved in refolding the tPA made it more expensive per gram than the CHO tPA.

1.2 What Controls Reactor Productivity?

Both hybridoma and recombinant DNA technology involve using animal cells to make the product protein. However animal cells are expensive to grow because of the high medium cost, low cell densities and low specific productivities. It is therefore important to understand what factors affect the productivity of any production system. This may be considered at two, interlinked, levels; firstly factors that operate at the level of the reactor; secondly factors that operate at the level of the cell.

1.2.1 Reactor Design

The amount of antibody produced by a reactor is a complex function of a number of different parameters (see Figure 4). The two principle effects of reactor operation are **cell density** and **specific productivity** which is dealt with in the next section.

Cell concentration is a variable with a considerable scope for adjustment in animal cell culture. Batch *in-vitro* cultures of hybridoma cells generally reach a maximum cell number at around $1\text{–}4\times10^6$ cells ml^{-1} and there is often little or no stationary phase. Mammalian tissue has a cell concentration of around 1×10^9 cells ml^{-1}, five hundred times higher than that of the batch culture and this cell density is maintained, not transient as in the case of a culture. So, a 1 litre

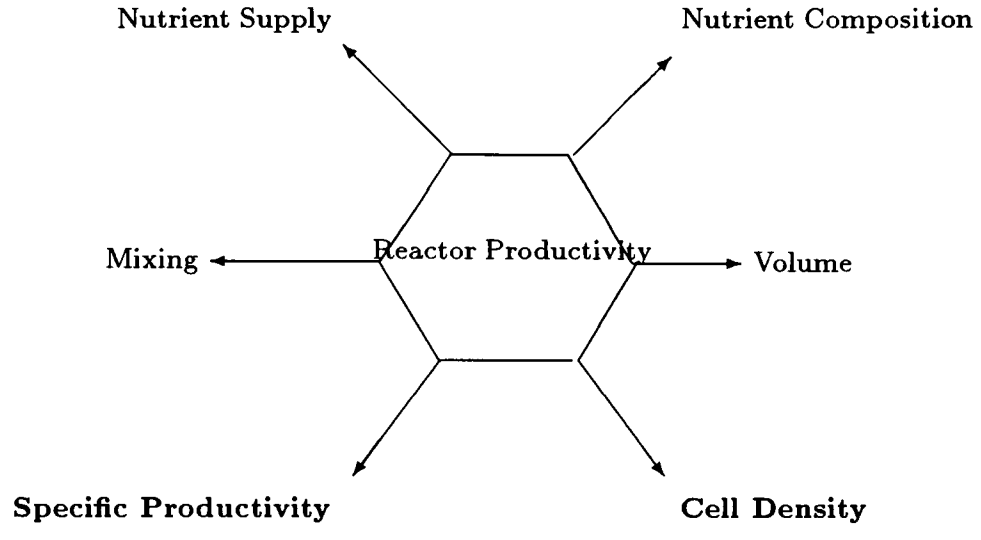


Figure 4: Factors affecting reactor productivity

bioreactor of the size and cell density of a human liver, could replace Celltech's 2000 litre airlift bioreactor (Birch *et al.*, 1985) if *in-vivo* cell densities could be reproduced.

Nutrient supply modes affect both cell density and specific productivity. Supply may be either batch or continuous. Batch operation modes are inherently limited by nutrient supply or toxin removal and therefore cell densities are low. Continuous operation modes produce a homeostatic control of chemical conditions within the reactor, giving intensified cell concentrations and extending operation. Cells at high cell densities require less (expensive) serum (Petricciani, 1987; Lee *et al.*, 1991; Ray *et al.*, 1989) and they use less nutrients (Wohlpart *et al.*, 1990). This may be due to production of high molecular weight effectors produced, called autocrine factors (Lee *et al.*, 1991). Some such intensive systems, for example hollow fibre systems, can reach cell densities in the $\times 10^8$ ml^{-1} range and maintain them for periods of months (Wang *et al.*, 1989). For most systems, the resulting

antibody concentration is much higher than that observed in batch culture, as is its ratio to the total culture protein concentration and this eases the purification of the antibody. However the practical problems of avoiding concentration gradients particularly of oxygen in such devices limits the overall volume.

Continuous nutrient supply accrues advantages in reactor operation; for example the frequency with which the reactor needs to be turned round is decreased. Also the use of a smaller inoculum permits a reduction in the number of stages required in inoculum preparation. However the systems are more complex than batch systems and with complexity comes increased probability of error. Also, more complicated systems are more costly to validate.

A larger volume obviously gives more cells and thus more product. But, because oxygen is poorly soluble in water, it is difficult to supply it sufficiently fast at the highest cell densities ($\times 10^8$ cells ml^{-1}) unless the diffusion path the oxygen has to take is minimised by putting its supply very close to the cells, for example in hollow fibre devices.

Medium composition also affects the cell density achieved. Each cell line's requirements are, however, individual. Optimal medium composition also depends on how the reactor is operated. Batch operation may require higher concentrations of some nutrients than do continuous operation modes, to achieve the maximum possible cell density.

1.2.2 Specific Productivity

- **mRNA concentration** The level of mRNA transcripts of a particular gene is the result of a balance between transcription and transcript degradation. Since protein synthesis is normally limited by the rate of mRNA

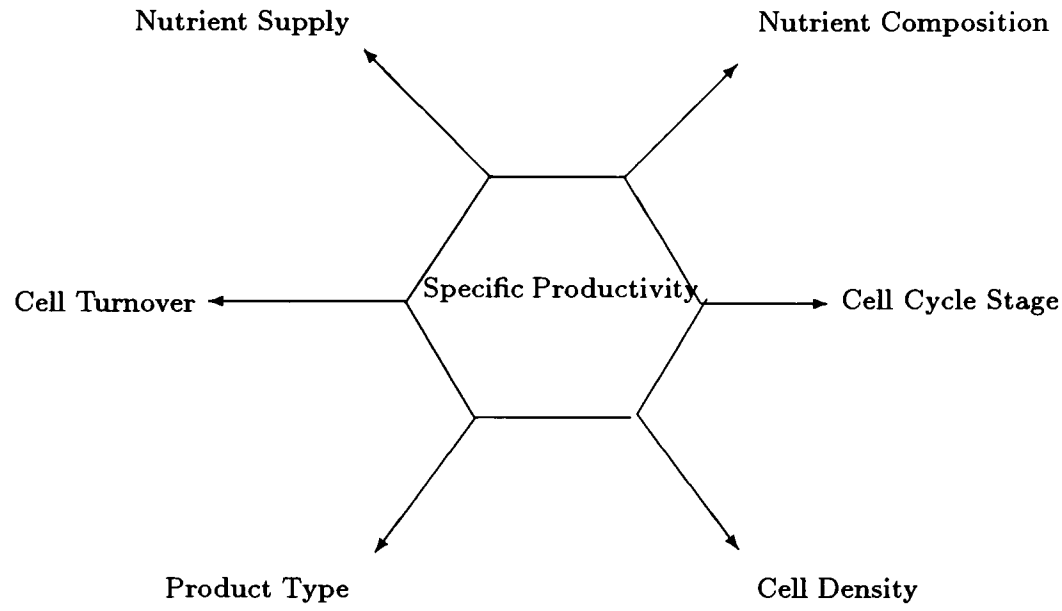


Figure 5: Factors affecting specific productivity

to Ribosome binding, altering the mRNA concentration, for example by increasing the gene copy number, will increase the amount of transcribed protein. There is little evidence however that changes in reactor conditions alter gene expression levels *per se*. For example, changes in growth rate produce little variation in light and heavy chain mRNA levels in hybridomas (Merton *et al.*, 1991; Bibila and Flickinger, 1991).

- **Cell Cycle** Several authors have shown that specific productivity increases under conditions of stress, for example; during the death phase of batch cultures, at low growth rates in chemostats and under conditions of mechanical stress (Ray *et al.*, 1989; Feder, 1987; Lee *et al.*, 1992). It has been shown that at low growth rates, hybridoma cells spend longer periods of time in the G1 portion of the cell cycle and in this phase they synthesise more antibody (Al-Rubeai *et al.*, 1990; Ramírez and Mutharasan, 1990).

- **Cell Turnover** Hybridoma cells store a portion of the antibody they produce and this is released when the cells die (Al-Rubeai and Emery, 1990) so conditions of stress, where death rates increase passive release of antibody, contribute to improved specific productivity.

- **Reactor Operation Mode** Steady state conditions, arising in some continuous operation modes, allow the cells to expend less energy on continuous physiological adaption to a continuously changing environment (Miller *et al.*, 1989a).

- **Medium Composition** Both product production and quality can be affected by the composition of the medium in which they are grown. Dalila and Ollis (1989) showed for a hybridoma cell line that specific productivity increased linearly with serum concentration. Al Rubeai *et al.* (1992) showed that addition of Pluronic F68 increased the specific productivity of a hybridoma cell line in continuous culture by increasing the proportion of time that the cell spends in G1. They also showed that addition of unphysiologically high concentrations of thymidine, arrests cells in G1 and so increases productivity. As for product quality, Hayter *et al.* (1992a) showed for recombinant CHO producing human interferon-γ, that the proportion of the product that was glycosylated decreased when the cell was grown in a glucose limited chemostat.

1.2.3 Problems in Comparing Systems

Comparison between different reports in the literature of systems used for cell culture is difficult because groups have used different cell lines, and have not reported

their results in a consistent manner. Hybridoma cell lines can be physiologically very different from each other.

- Cell specific antibody productivity can vary from zero to 70×10^{-9}g cell^{-1} day^{-1}.
- Many hybridoma cell lines contain a non-producing subpopulation, which can vary in proportion according to the growth conditions (Chuck and Palsson, 1992).
- Metabolism can vary considerably between clones, affecting the efficiency of the use of the medium. For example, ammonium toxicity varies between cell lines (Ozturk *et al.*, 1992; McQueen and Bailey, 1991; Hassell *et al.*, 1991).

For reactor systems where cells are attached to a surface, reactor volume is not a good indicator of the total cell capacity of the system. Surface area, or an indication of surface area, is more apposite. Some system configurations retain antibody which is harvested periodically and is therefore present at concentrations of mg ml^{-1}. Others do not and antibody is then present at 100's of μg ml^{-1}. Different system configurations are thus difficult to compare. Many different indicators of reactor "efficiency" are used. For example, total mass of antibody produced, reactor productivity in grams per hour, or grams per litre per hour, yield on medium, cell specific productivity and process improvement factors over batch culture. Many authors do not report their reactor productivity adequately to allow comparison between systems, though a few comparative studies have been attempted (Heath and Belfort, 1987; Schlaegar and Schumpp, 1992). The results of a batch culture form a good basis for comparison as this is widely regarded as being the base case. All reports on reactor systems should detail the performance

of their cell lines in batch culture, using the same, or as similar as possible, media to aid comparison, quoting maximum cell density, viability index (total number of viable cell hours), maximum antibody concentration and cell specific antibody production rates; quoting as many as possible of these for the intensified reactor system also.

1.3 How to intensify

Cell cultures are generally limited either because one or more nutrients are entirely consumed, or because of the accumulation of toxic metabolites. Intensified production is achieved by exchanging culture fluid for fresh medium, whilst retaining the cells within the reactor. This is known as perfusion. In hybridoma culture, the main energy sources are glucose and glutamine, whilst their metabolism produces toxic compounds, including ammonia from glutamine and lactate from glucose and glutamine. Hybridoma batch cultures are reported to be limited by one of these four compounds (Reuveny *et al.*, 1987). The concentrations of glucose and glutamine in the medium are constrained by the tolerance of the cells for their by-products. Local concentration gradients within a system could lead to cell death while the overall concentrations were not limiting. Therefore most systems are designed to minimise these gradients by one of two methods; either the cells are kept in a stirred homogeneous environment, in which case cell densities in the range of 1×10^6–5×10^7 are achieved and large volumes may be used ($\leq$ 1000 litre); alternatively, the cells are confined to a support and the maximum distance between the medium supply and the cells is kept short. These are the so called heterogeneous cultures, in which cell densities of 1×10^7–5×10^8 can occur,

volumes are however much smaller ($\leq$ litre) and it is often difficult to access the biomass.

1.3.1 Heterogeneous Culture Systems

By attaching cells to a solid surface, or confining them behind a membrane, cells may be retained within the reactor. Such intensified systems tend to reach very high cell concentrations, up to 5×10^8 cells ml^{-1}. As some or all of the the cells are attached to a surface, they are locally at tissue-like cell densities. Extracellular matrix and desmosomes (cell junctions) may be produced, which can affect cell growth and protein production (Vournakis and Rundstadler, 1989). The previously mentioned autocrine effects will be at their most effective. Also mechanical stresses caused by bulk fluid flow, or gassed aeration, which can cause cell damage, are avoided.

Most heterogeneous systems do not, however, successfully avoid heterogeneity in their cell populations caused by local concentration gradients (Hu and Peshwa, 1991). The range of conditions means that exact control over cell physiology is, therefore, not possible.

1.3.1.1 Entrapment

Cells may be entrapped within polymeric meshes. For example, alginate, carrageenan, agarose and polyacrylamide have all been used to produce beads entrapping cells at densities of approximately $\times10^7$ cells ml^{-1}. The entrapping polymer must exhibit a matrix with pore sizes as large as possible, to allow nutrient transport through the gel whilst being fine enough to entrap the cells and the method of entrapment must be gentle, so as to not damage the cells. For

example, polymeristion of alginate is achieved by dropping the cell-alginate suspension into a $CaCl_2$ solution. This can be done at physiological temperature and osmolarity. However bead size should be kept small. Al-Rubeai *et al.* (1989) found for alginate entrapped hybridoma, that cells were metabolically active only in the outer 0.24 mm. Thus bead diameter should be kept to less than 0.5 mm, as diffusion limitations can result in heterogeneous cell populations. Cell numbers of 5×10^7 cells ml^{-1} have been recorded within the beads themselves (Smidsrod and Skjok Broak, 1990), though the beads occupy 20–50% of the available space in the reactor, so the effective cell density is lower.

1.3.1.2 Membrane Systems

Cells my be entrapped behind membranes of a number of different configurations, the most popular being the hollow fibre. Hollow fibre cartridges have been likened to artificial capillaries. Cells are normally grown in the extracapillary space, where they may be in suspension, or attached to the wall of the hollow fibre, which in some types of fibre has a support material on the outer surface to which cells may attach. Medium is passed through the lumen of the fibre wherein diffusive exchange may occur.

Mixing inside the extracapillary space is caused by the flow of liquid in the lumen, the Starling effect. However nutrient and oxygen gradients occur both axially and radially. The hollow fibre may be operated in a convective filtration mode, with medium being forced through the hollow fibre from the cartridge side and out through the lumen. Nutrient gradients are reduced, but membrane clogging may occur. Hollow fibres are available in a range of molecular weight cut-offs and generally, for hybridoma culture, a value is chosen that will retain

the antibody within the extracapillary space, the antibody may be harvested periodically. Cell concentrations reach 5×10^8 cells ml^{-1} and systems have been run for months (Kidwell, 1989; van Erp *et al.*, 1991b; Wang *et al.*, 1989). In retained antibody systems, antibody concentrations can reach mg ml^{-1} levels. In practice, hollow fibre cartridges are limited in transfer area to a maximum of 2–4 m^2 each and further expansion must be by using multiple cartridges.

There are also several types of flat sheet membrane reactors (Kargi *et al.*, 1989). These systems allow improved access to the biomass and the distance between the cells and the medium supply can be closely controlled. However these have lower surface area to volume ratios than hollow fibre systems and are more difficult to construct as well as to operate.

Another form of membrane reactor is the microcapsule, first commercialised by Damon Biotechnology Inc (Duff, 1985). Cells are entrapped in alginate beads, which are then coated in polylysine (Duff, 1985) or other materials (Grootwassink *et al.*, 1992; Tsai and Chu, 1989; Gharapetian *et al.*, 1986), and the alginate gel may then be reliquified. The polylysine membrane pore size can be controlled and, for monoclonal antibody production, is adjusted so that antibody is retained. Cell concentrations in the range of $\times10^8$ ml^{-1} are observed within the microcapsules and antibody is recovered at mg ml^{-1} concentrations when the microcapsules are filtered off and opened up.

1.3.1.3 Adsorption

Microcarriers were developed by (van Wezel, 1967) for growth of attached cell lines. Many different materials have been used, including glass, polystyrene, cellulose, gelatin and dextran. However hybridomas attach only poorly to surfaces.

Glass microcarriers have been used in packed beds (Ramírez and Mutharasan, 1989), where the cell retainment is partly by entrapment. More successful has been the use of porous support materials, for example macroporous microcarriers. The use of macroporous microcarriers increases the area available for attached cell growth through the use of large pores which may make up 90% of the volume of the carrier. Cells within the pores are also protected from mechanical stresses. Verax Corporation has heavily promoted the use of collagen macroporous microcarriers (Vournakis and Rundstadler, 1989), which are used in a fluidised bed and it is claimed that pressure differences across the height of the bed cause flow of culture fluid through the bead and, when this is combined with tumbling of the bead good fluid exchange occurs within the bead. Racher *et al.* (1990) have grown hybridoma on glass macroporous microcarriers in a packed bed, giving 3×10^7 cells ml^{-1}. They concluded that monoclonal antibody production was limited by the rate of oxygen diffusion into the microcarrier. Many other porous support materials have been tried in fluidised or packed beds, including for example polyester, polyurethane or polystyrene (Jan *et al.*, 1992; Yamaji and Fukuda, 1992) or reticulated carbon (Kent and Mutharasan, 1992).

1.3.2 Homogeneous Culture

1.3.2.1 Filtration

Hollow fibres and wire mesh have been used successfully for separating cells from the medium. Tangential flow filtration involves placing a recirculation loop externally to a reactor which includes a hollow fibre cartridge, generally with a 0.2 μm pore size (Feder, 1987; Velez *et al.*, 1990; Pinton *et al.*, 1991). Concentrations of cells an order of magnitude higher than in batch culture are obtainable and

hundreds of μg ml^{-1}of antibody can be produced in the product stream. The cells, however, must be pumped around the loop causing both mechanical stress and changes in the chemical environment around the cell. Membrane fouling may occur but, with an external filtration unit, replacement is possible.

The spin filter consists of a rotating wire cage, often mounted on the agitation shaft. Mesh sizes from 25–60μm can be used and, although this is larger than the diameter of the cell (10–18 μm) cell retention of 95% can be achieved (Jan, 1992). Process improvements similar to those in tangential flow filtration systems can be achieved and filter fouling may be avoided by the use of higher rotational speeds and by selection of appropriate materials for filter construction. It is however, more difficult to deal with when it does occur.

1.3.2.2 Sedimentation

Cells may be removed from the product stream by gravitational settling. The out-flow goes through a region where the flow is slowed by expanding the area available for flow. Cells settle in this region and return to the fermenter (Tokashiki and Arai, 1989). Cells may also be separated by centrifugation (Tokashiki *et al.*, 1990; Vanwie *et al.*, 1991), but these methods produce only moderate increases in cell concentrations, are tricky to set up and, particularly in the case of the centrifuges, are mechanically complex.

1.3.3 Dialysis Culture

Dialysis culture is a form of culture whereby molecules up to a certain size are allowed to diffuse freely across a membrane in both directions. Larger molecules will not pass through the pores. Dialysis will not involve any net transport of

mass unless there is a difference in mechanical or osmotic pressure across the membrane. The retention of high molecular weight components means that only low molecular weight medium need be fed in the permeating stream, which would give a considerable cost saving. It also means that high molecular weight products are retained within the reactor and may accumulate to high concentrations.

Dialysis culture was first used in 1896 by Metchnikoff, Roux and Salembini 1896, who used colloidal sacs filled with cultures of *Vibrio cholera* placed in the peritoneum of animals to prove the existence of a soluble toxin and the effect of anti–toxin. The first *in vitro* use was by Carnot and Fournier (1900) who used it to obtain toxin from *Pneumococci.* Since then dialysis has been used extensively *in vivo*, for the study of soluble toxins in disease, or the role of serum factors in cell growth. *In vitro*, it has been used to enhance production of toxins, enzymes, polysaccharides, *etc.*; or to study interactions between organisms.

The first *in vitro* growth of animal cells in a dialysis mode was in 1960 by Eagle (1960) who placed cells in basal medium inside a dialysis membrane, with serum containing medium outside, to investigate the requirement of cells for the protein component of serum. Adamson *et al.* (1983) in 1983 were the first to grow hybridomas in dialysis culture. They placed 1ml aliquots of cells inside dialysis tubing bags, which were freely suspended in roller bottles containing medium plus 10% serum. They achieved cell concentrations in the range of 1×10^7 cells ml^{-1}, i.e.ten-fold higher than that observed in batch culture. Antibody titres were also ten fold-higher. Since then many groups have reported similar results (Pannel and Milstein, 1992; Falkenberg *et al.*, 1993; Sjorgren-Jansson and Jeansson, 1985; Mathiot *et al.*, 1993) using the same or similar techniques. Pannel and Milstein (1992) have grown a panel of 7 different hybridomas in a system whereby 30ml

of cell suspension is placed in dialysis tubing attached diagonally across a roller bottle. An air space is left at the top so that, as the bottle rolls, the air bubble travels the length of the tubing, keeping the cells suspended. Three such tubes are placed in a 2 litre volume roller-bottle, filled with 1.5 litres of medium, without serum. The medium is replaced every other day. Their hybridomas produced between 1.1–2.3 mg ml^{-1} monoclonal antibody in 20 days. Comparing this with ascitic culture they found that ascitic culture, produced 7.3–10.5 fold higher IgG protein but, when they examined antibody activity the difference was only 1.5–4.7. In other words, the antibody was of a higher specific activity in the dialysis culture, less degraded and containing less contaminating antibody. They say:-

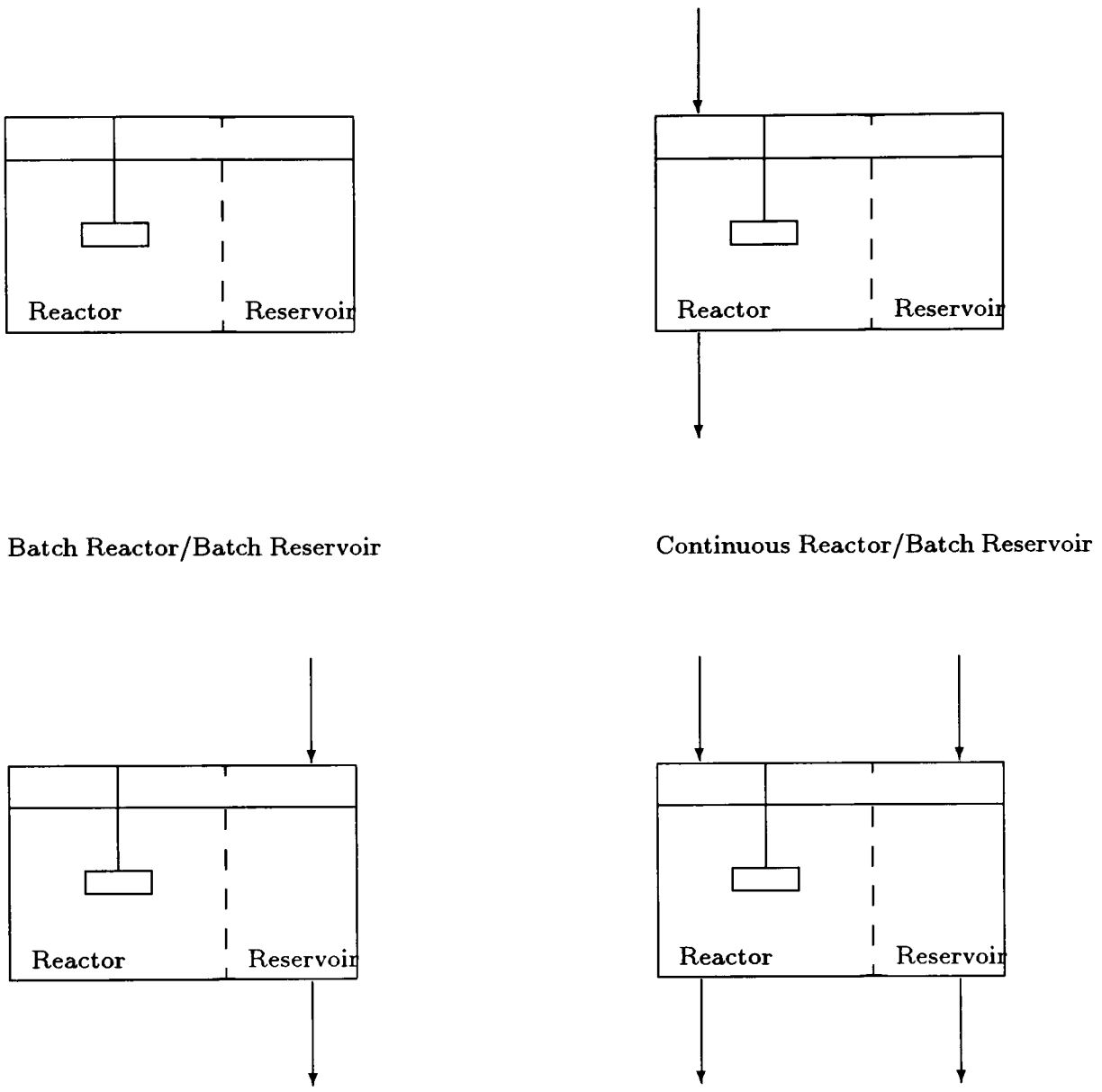
> "More antibody is produced within one bubble chamber than in one mouse, with little effort and without the need for animals"

Kasehagen *et al.* (1991) used more sophisticated control, by placing the dialysis tubing, with 1 ml aliquots of cells, within a pH and dissolved oxygen tension controlled bioreactor. Inoculating at cell concentrations of above 5×10^6 cells ml^{-1} no net cell growth was observed. Cell concentrations as high as 1×10^8 cells ml^{-1} could, however, be *maintained* over a period of 5 days. This mode of dialysis culture then is successful for producing small quantities of antibody at high concentrations and would be a satisfactory replacement for ascitic culture for a laboratory wishing to produce monoclonal antibodies for its own use. The volume of culture can be increased by ensuring that the cell compartment is mixed and by choosing appropriate medium and feeding strategies. However the final volume is limited unless good mixing is provided in the cell compartment. Moreover no online control of reaction conditions including for example pH and DOT *in the reaction space* is possibly with the methods described up to now.

The first serious attempt to solve this problem was by Gallup and Gerhardt 1963. They identified two basic issues, membrane placement and medium replacement mode. The obvious means are to turn the system inside out, i.e. to suspend a tubular membrane inside a homogeneous culture volume and pass medium through the inside of the tube. Alternatively the culture volume may be recirculated through an external exchange device. They decided on the latter because it allowed independent control of fermentation, agitation and dialysis functions. They grew the bacterium *Serratia marcescens* in it, achieving viable cells densities in excess of $\times 10^{12}$ cells ml^{-1}. Schultz and Gerhardt 1969, the best review by far of dialysis culture, describe four modes of medium supply (Figure 6). Mathematical models are derived for modes 1,2 and 4 describing cell concentration and nutrient concentration.

A comparison is shown in Table 2 of a number of systems reported, using the above medium. All of the reports have been published since this project was begun except the first two. All four modes have been used for growing animal cells. Different cell growth patterns were seen with the different modes. Modes with continuous dialysate replacement reached steady state cell concentrations. In all cases the authors felt it necessary to site the membrane inside the reactor to avoid cell damage in a circulation loop.

Most have used membranes with a molecular weight cut-off at around 10,000 Daltons, probably principally because these are the cheapest and most readily available, being the standard used for dialysing solutes away from protein purifications and in haemodialysis. The membrane area to reactor volume ratios are in the range 0.1–1 cm^2/cm^3. Schultz and Gerhardt 1969 suggested that ratios of around 1 $cm^2\ cm^{-3}$ were a reasonable target for the bacterial cultures with which



Batch Reactor/Batch Reservoir

Continuous Reactor/Batch Reservoir

Batch Reactor/Continuous Reservoir

Continuous Reactor/Continuous Reservoir

Figure 6: Modes of operation of dialysis culture devices.

Reference	Mode(s)	Memb. Type	Memb. Cut-off	Reactor Volume	Memb. Area to Volume
Gori (1965)	B/C (B/B)	D	10,000	2l	0.21
Rookes *et al.* (1989)	RB/B	HF	7,000	1l	(1.0)
Hagerdorn and Kargi (1990)	B/B	D	12,000	0.28l	0.26
Comer *et al.* (1990)	RB/B	HF	10–100,000	1–1000	0.1–0.3l
Comer *et al.* (1990)	C/C	D	1,000	1.3l	0.9
Bohmann *et al.* (1992)	C/RB	D	10,000	(0.9)	

Table 2: A comparison of some dialysis culture systems used for the growth of animal cells. Mode types reservoir first, then culture:B = batch, RB = repeated batch, C = continuous. D = dialysis tubing, HF = hollow fibre. Figures in brackets are estimates

they were concerned.

Gori 1965 was the first to grow a number of different cell types in a system consisting of a dialysis tube medium supply inside a 1.8 litre reactor. In batch reactor /batch reservoir mode (see Figure 6), they observed increases in maximum cell concentration which depended on the the ratio of the volume of the reservoir to the volume of the culture. They developed equations to predict the time till exhaustion of the medium in the reservoir and used this in a continuous reactor batch reservoir system, to predict when to change the reservoir.

Batch reactor/repeated batch reservoir systems were used by Boehringer Mannheim GMBH (Comer *et al.*, 1990), at up to the 1000 litre scale. Using hollow fibres of 10,000–100,000 dalton molecular weight cut off, an area for exchange was achieved of between 0.01 $m^2\ l^{-1}$–$0.3 m^2\ l^{-1}$. Reservoir sizes depended on the cultivation time, the medium and the product, with medium in the cell compartment either containing serum, or serum-free supplements. They used human and murine hybridoma and recombinant Chinese Hamster Ovary cells. They do not give full details for particular cell lines, or system configurations. Cell densities in excess of 1×10^7 cells ml^{-1} were achieved with viabilities greater than 90% and

increases in antibody concentrations over batch culture of between 2–15 fold for mouse-mouse hybridomas and 15–30 fold for human-human hybridomas.

Bohmann *et al.* (1992) have combined the dialysis bioreactor design of Markl *et al.* (1993) with a fixed bed of glass macroporous microcarriers and a special agitation system to induce radial flow through the bed.

Previous work in this laboratory used hollow fibre bundles with a 7 kilodalton cut-off, suspended within the reactor and a batch reactor/batch reservoir systems, with daily replacement of the perfusate volume and periodic serum addition (Rookes *et al.*, 1989). Cell numbers peaked at 8×10^6 cells ml^{-1}, antibody reached 600 μg ml^{-1}, 20$\times$ that seen in batch culture. The maximum cell number was transient and steady states did not occur. Subsequently to this, problems were encountered with the reliability of the hollow fibre after repeated steam sterilisation. Hollow fibre bundles are also rather expensive and both process reliability and economy were sought by using a cheaper membrane in the form of dialysis tubing, this forms the basis of the work reported here. Some of this work has been presented to the European Society for Animal Cell Technology (ESACT) as a poster paper (Amos *et al.*, 1992) and published in Enzyme and Microbial Technology (Amos *et al.*, 1994)

1.3.3.1 Advantages of Dialysis Culture in Homogeneous Reactors

Dialysis culture offers the following advantages over batch and other forms of perfusion culture.

1. Dialysis culture supports increased cell numbers over simple batch culture, for extended periods of time.

2. Nutrient and cell concentrations can be modeled.

3. Unlike heterogeneous systems, the system can be easily scaled up. Scaleability to 1000 litres has been demonstrated.

4. Unlike most homogeneous systems and some of the more easily scalable heterogeneous systems, for example macroporous microcarriers and gel bead entrapment), antibody is retained and is therefore recovered at very much higher concentrations, easing purification.

5. As only low molecular weight components need to be recirculated medium costs are reduced in comparison with systems that do not retain high molecular weight species

6. Dialysis culture can be conducted in ordinary reactors and no special equipment is needed. Nor is expensive consumables, such as macroporous microcarriers, or technically difficult processes such as gel bead formation, or encapsulation involved.

7. Culture homogeneity allows simple global control of the reactor and representative sampling as well as assessment of the physiological state of the cells.

8. Easy access to the biomass and the relatively simple technology will ease process validation which is a substantial cost burden in the production of pharmaceuticals.

1.4 Aims

The importance of animal cell culture in producing a wide variety of protein products has been explained. Dialysis bioreactors have been shown to be excellent bioreactors for the growth of animal cells. The theoretical advantages of membrane retained devices lie in their ability to accumulate product to high concentration and in savings in serum usage. However little is known about the physiology of cells in such devices, the fate of serum components and the effects on the product of spending an extended period in the culture medium at 37°C.

The aims of this project are to develop a dialysis culture device and use it to understand how cells were affected by long periods at steady state in terms of:

1. What controls cell concentration in such device?

2. What controls the level of cell viability, rate of cell death, in such a system.

3. What is the fate of high molecular weight components of the medium.

4. How is the product effected by long periods of time in, in some cases, low viability cultures.

Chapter 2

Materials And Methods

2.1 Cell Line and Culture Conditions

TBC3 is a mouse-mouse, NS1-derived hybridoma cell, a subclone of WC2 which produces antibody to the C2 region of human IgG (Jefferies *et al.*, 1982). PQXB1 is a mouse-mouse hybridoma derived from the X63 653 myeloma, producing Mab to paraquat and was donated by Mr. Andrew Dinsmore (ICI plc.-Pharmaceuticals Division.) CHO–K320 cell line is a dihydrofolate reductase (DHFR) deficient mutant of CHO–K1, and was kindly provided by Wellcome Biotechnology (Beckenham, Kent). Human interferon–γ is expressed. The gene was inserted using a plasmid vector derived from pSV2-dhfr, using the SV40 early promoter, coamplified with DHFR by methotrexate selection. SF9 is an insect cell line from *Spodoptera frugiperda*, the fall army worm. It was provided by the Department of Cancer Studies of the University of Birmingham. A recombinant *Autographa californica* nuclear polyhedrosis virus (AcNPV) expressing the *Escherichia coli* lacZ gene for the production of β-galactosidase was used to infect the cells. It

was constructed by homologous recombination of the E2 clone of AcNPV and the plasmid pAc360 and was provided by Luckow and Summers.

2.1.1 Routine Cell Maintenance

1. Cells from 2 or 3 day old 10ml or 50ml flasks were counted.

2. Details of cell line, passage no., cell no., viability, culture volume, pH and additional comments if necessary were logged on a data sheet.

3. About 1×10^5 cells ml^{-1} were inoculated into fresh 10ml or 50ml flasks e.g. from a flask containing 5×10^5 cells ml^{-1}

4. In a sterile flow cabinet 2ml of spent medium was withdrawn with a sterile pipette, ensuring that cells were washed from the surface of the flask by pulsing or scraping with a pipette.

5. 8ml of prewarmed, prepared medium was added to dilute cells back to approximately 1×10^5 cells ml^{-1}. The flask was labelled with: name, cell type, passage no., date and media supplements (if used).

6. Cells were counted again on days 2 and 3. On day 3 cells were diluted back to 1×10^5 cells ml^{-1}. Details were entered on a log sheet each time the cell lines were passaged or expanded to larger volumes.

2.1.2 Cell Counting

1. 0.1ml of sample was taken, added to a microwell and diluted 2 fold with 0.1ml of trypan blue stain. The sample was mixed well by pulsing with a pipette about 10 times.

2. Diluted samples were added to both sides of the haemocytometer slide.

3. Both sides were counted, as 2 samples, ensuring that there were no air bubbles present or that the coverslip was drying, before and after counting. All cells that overlap the 3 tramlines on the top and right sides were counted, those on the bottom and left not. Each grid has a volume of 0.001 ml.

4. Depending on the number of grids counted, the sample was multiplied by the appropriate dilution factor. 3 or 5 grids were usually counted. The more grids counted, the greater the accuracy.

5. Calculation of cell concentrations: The procedure was repeated 4–8 times to obtain an average cell count in cells ml^{-1}.

$$\text{Cell Count}=(\text{No. of Cells}/5)\times 2 \times 1000$$

N.B. Stained cells were noted as non viable cells. Only structures larger than a cells nucleus was counted. "Minicells" or stained blue fragments smaller than this were not counted as cells.

2.1.3 Cell Growth in Stirred Bottles

1. Only if the viability of the cells was high *i.e.* above 90-95%, were cells used for experiments. 50ml of cells at 5×10^5 cells ml^{-1} was taken and added to a sterile 250 or 500ml Duran bottle (Schott, England) containing a magnetic follower.

2. Cells were diluted up to the 200ml mark with fresh prewarmed medium ensuring that the pH was about 7.0 and then placed in a 37°C incubator.

3. To expand to 800ml, 200ml of cells was placed into a 1 litre heat-sterilised Duran bottle and prewarmed medium added to the 800ml mark. The bottle was placed in a 37°C incubator with the magneticfollowers stirring at 100rpm.

2.1.4 Freezing Cells

1. When cells had grown to 5×10^5 cells ml^{-1} in 50ml flasks, they were put in 2×25ml vials and centrifuged at 1000rpm for 5min.

2. The cell pellet was resuspended in freezing mixture to give a cell concentration of 1×10^7 cells ml^{-1}. 0.5ml of freezing mixture was placed into each cryotube (Nunc-Gibco), then labelled with code, cell type, viability, concentration and date.

3. Cryotubes were placed on to stage 1 of a cryogenic unit containing liquid N_2 for 15 min.

4. Cryotubes were then moved to stage 2 for 45 min to 1 hour. When frozen they were transferred to a nitrogen Dewar Flask containing liquid N_2, 10 XT (Jencons Scientific Ltd.).

5. Details of frozen cell sample were entered into the log book, *i.e.* date, cell line, concentration, viability, passage no. and name.

2.1.5 Thawing Cells

1. Medium with 10% FCS in a 20ml Sterilin vial was warmed to 37°C.

2. A cryotube of frozen cells was removed and warmed up in a 37°C water bath as fast as possible. The withdrawal was noted in the log book.

3. The contents of the cryotube were decanted into the universal of prewarmed medium.

4. The tube was mixed, a sample removed to count cells, and the rest centrifuged at 1000rpm for 5 min.

5. The medium was decanted and the pelleted cells resuspended into fresh medium at 1×10^5 cells ml^{-1} in a 10ml culture flask.

6. A sample was checked daily and resuspended in fresh medium if not healthy.

2.2 Routine Medium Preparation

2.2.1 Basal Medium

2.2.1.1 RPMI

50g of x10 strength powder RPMI 1640 with L-glutamine (Gibco, Paisley) was dissolved in 5 l of freshly distilled deionised water. $NaHCO_3$ at 2 gl^{-1} was added and the pH adjusted to 7.1–7.2 with 1M HCl after the medium was completely dissolved. Then the medium was filter sterilised through a 2 μm, 20 cm membrane filter in a 1 l Sartorius pressure barrel into sterile bottles and stored at 4 °C.

2.2.1.2 TC100

This was used for the growth of the insect cell line, and was purchased from Gibco. The powder supplied by Gibco was suspended in 4.5l of distilled deionised water.

pH was then adjusted to 5.0 with 3.5M HCl. When it was o completely dissolved 1.75g of Na HCO_3 was added. Next the pH was readjusted to 6.2 with 1M NaOH and the volume was made up to 5l with distilled deionised water

2.2.2 Media Supplements

2.2.2.1 Serum

500ml of sterile Foetal Calf Serum (FCS; GIBCO, Paisley), or Newborn Calf Serum (NBS; Advanced Protein Products, West Midlands) was dispensed into 25ml aliquots and frozen at $-20°C$. At least 2 days before use the complete medium of RPMI 1640 supplemented with 5% v/v of serum was made up. 25ml of frozen FCS or NBS was thawed and added to 500ml basal RPMI 1640. A 1ml sample was placed on an agar plate and 10ml removed into a Sterilin tube to incubate at 37°C to test for contaminants.

2.2.2.2 Pluronic F-68 - 0.125% (v/v)

1.25 grams of Pluronic F-68 (BASF, Germany) was dissolved in one litre of RPMI 1640 medium, and sterile filtered.

2.2.2.3 Freezing Mixture

FCS and 8.8% Dimethyl sulphoxide (DMSO) *i.e.* 20.7ml FCS and 2ml DMSO. The mixture was divided into 4ml aliquots.

2.2.2.4 Trypan Blue

5g trypan blue (Sigma, Poole) was dissolved in 100 ml distilled water.

2.2.2.5 Peptone and Biopro

Was added at the required concentration to RPMI and then sterile filtered.

2.3 Culture Vessel

2.3.1 Reactor Description

A 2l capacity, unbaffled, hemispherical bottom, top driven, glass bioreactor of vessel diameter 0.12m, kindly loaned by Setric Genie Industriel (Toulouse, France) was used. One 4-bladed, 45° pitch impeller (D=0.06m) of width 70mm was mounted 0.115m from the headplate for agitation. Headspace aeration at 100ml min^{-1} with agitation at 100rpm (the maximum rate) was sufficient for O_2 supply to a batch culture without the need for sparging. For high density cultures pure oxygen was used either sparged or via 1 metre of thin walled silicone tubing suspended within the liquid. The air/CO_2 mixture was controlled by automatic solenoid valves. Flow rates of the gases were controlled by needle valves, the supply of CO_2 being turned on if the pH rose above the set point, the supply of O_2 turned on if the dissolved oxygen tension fell.

2.3.2 Preparation

The following calibrations were necessary before use:-

1. The Ingold pH probe was calibrated with buffer solutions following the manufacturer's procedure.

2. The temperature probe was calibrated at 37°C against a mercury thermometer and the temperature was set to control at 37°C by an air jacket.

3. The indicated speed on the meter of the motor drive of the bioreactor was calibrated against the running speed with a TM 2011 electronic tachometer (Anders Ltd., London).

4. The DOT probe (Ingold) was calibrated by sparging air then nitrogen through distilled water at 37°C, placing the probe in it and setting zero and span.

Before use, the vessel was soaked overnight in Triton 7X solution (Flow Labs.), following which the vessel, probes and pipes were thoroughly washed with distilled water. The ports were covered with metal foil and autoclave tape and the vessel autoclaved at 121°C for 20-25min. After sterilisation the vessel was mounted on the drive unit and the instruments connected to the controllers.

The inoculum and medium, prewarmed to 37°C, 1 litre in volume, were mixed in a laminar air flow cabinet, put in an inoculation bottle and brought top the reactor. Therperature pH and DOT usually reached set point values within an hour. Each sample was taken in a 25ml Mc Cartney bottle, which was then replaced by a fresh sterile bottle after flaming its top.

2.3.3 Dialysis Mode Description

Figure 7 is a schematic diagram of the reactor used for this study. TBC3, was grown in homogeneous suspension culture in a standard SETRIC 2 litre Animal Cell Fermenter (Setric Genie Industriel (France)). pH was controlled at 7.0, automatically, by CO_2 addition, and the dissolved oxygen concentration was maintained at 50% air saturation by sparging with pure oxygen. 370 cm^2 of visking tubing (10 cm diameter) was suspended in the fermenter, in a coiled configuration,

supported by thick copper wire, acting as a shapeable former. The wire was sealed in silicone tubing, and this was threaded through the dialysis tubing. Medium, without serum, was circulated from a reservoir through the dialysis tubing. The reservoir was replenished constantly with fresh medium, the excess being forced by air pressure to a waste reservoir.

2.3.4 Continuous Culture

A 500 ml duran bottle was modified with ports for continuous culture. The culture volume was maintained at 300 ml by positive pressure from a 5% CO_2 in air supply. The reactor was placed in a 37 °C waterbath.

2.4 Assays

2.4.1 Glucose Assay

Glucose levels were measured using a glucose strip test kit (Boehringer-Mannheim). A drop of supernatant was placed on a test strip, left for 60 sec. and dried with a tissue. The strip was placed into the reader and after another 60 sec. the glucose level was displayed. The glucose readings were calibrated with standard solutions ranging from 0.2–2.5 g l^{-1}.

2.4.2 ELISA

The principle of the sandwich type ELISA is the formation of several layers of molecules, one attached to another in a single row, the last molecule being a reactive substrate which yields a yellow colour on reaction. Initially, antigen

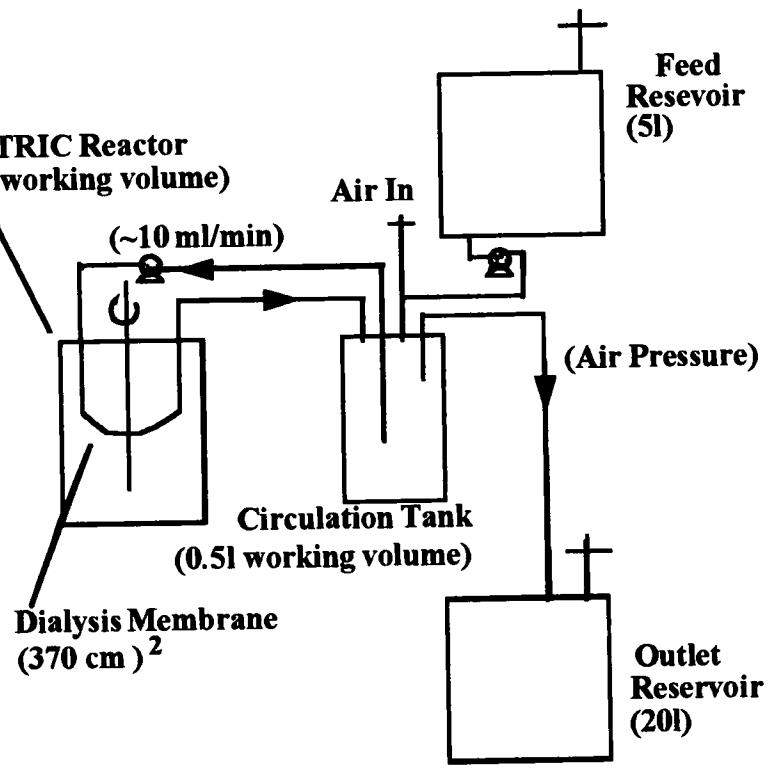


Figure 7: Schematic Diagram of Dialysis Reactor

(Human IgG) is added to bind to the surface of a well and when the supernatant containing mouse anti-human antibodies is added they bind to the Human IgG. Next casein solution is added to block all remaining binding sites. Then sheep anti-mouse IgG, with a peroxidase molecule, is added as the third layer of the sandwich to bind to the mouse antibody. Finally the citric acid substrate is added, forming the fourth layer, which reacts with the peroxidase molecules resulting in a yellow colouration. The intensity of the yellow colour is an indication of the concentration of mouse IgG attached to the wells.

Solutions (all chemicals were purchased from Sigma, Poole).

1. Coating Buffer - 0.05M Carbonate buffer pH 9.6. 0.795g Na_2CO_3 and 1.465g $NaHCO_3$ were dissolved in 500ml of distilled water. This was sufficient for 40 plates.

2. Washing Buffer A - Phosphate Buffered Saline (PBS) 10x stock solution pH 7.4. 400g NaCl, 10g K H_2PO4, 144.6g $Na_2HPO_4.12H_2O$, and 10g KCl were dissolved in 5 l of distilled water, and the pH adjusted with HCl or NaOH to 7.4. Working strength Buffer A 1 l of 10× solution was added to 9 l of distilled water.

3. Washing Buffer B - Working strength Buffer A with 0.05% Tween 20. 2.5ml of Tween 20 was added to 5 l of PBS.

4. Blocking Buffer - 2% Casein. 2g casein was added to 1 l PBS, stirred at 37°C (for 4 hours to dissolve) and stored frozen in 25ml aliquots in universals. One universal was used per plate.

5. Diluting Buffer - 0.525ml of 2% casein was diluted in 75ml of PBS.

6. Substrate solution citrate and phosphate buffer. Two solutions were made up as follows:- A 0.1M Citric acid - 10.5g in 500ml distilled water. B 0.2M $Na_2HPO_4.12H_2O$ 17.8g in 500ml distilled water. Fresh substrate solution sufficient for 2 plates was prepared:- 8mg of O-phenylene diamine (OPD), 8 μl of H_2O_2, and 12ml of A were added to 8ml of B. The pH was adjusted to 4.3-4.4 with NaOH and the solution stored in the dark.

7. Stopping solution 12.5% H_2SO_4. 12.5ml of concentrated H_2SO_4 was added to 100ml of distilled water.

Method

Samples for analysis, standards, antigen, conjugate and 2% casein, which were stored frozen at -20 C, were thawed out before starting work. Solutions for ELISA were prepared in advance.

1. Human IgG, 2mg ml^{-1} in PBS (Sigma) was diluted to 2.5 μl ml^{-1} in coating buffer. A 50 μl aliquot of Human IgG was added to 20ml of coating buffer (enough for 2 plates) and then 100 μl was applied to each microtitre well. The plate was covered with cling film and the antigen Human IgG was left to bind at 4 C overnight.

2. The plate was bang dried, then 100 μl of 2% casein in blocking buffer was added to each well and incubated at 37°C for 1 hour to eliminate the remaining binding sites on the plastic plate.

3. The plate was washed 4 times with buffer B and bang dried.

4. The standard primary antibody and test samples were added next.

   a) Sample preparation:- 100 μl of sample was added to 900 μl of PBS and

0.05% casein and mixed well, to give a 1 in 10 dilution. This step was repeated to give a 1 in 100 dilution. At least duplicate samples were taken, preferably quadruplicate samples for accurate readings.

b) Monoclonal antibody standard preparation:- 3 vials of 10 μl frozen standards were taken and 1ml of PBS and 0.05% casein was added to each vial to make a concentration of 100ng ml^{-1}. Standards of 70, 60, 50, 40, 30, 20, 10 and 5ng ml^{-1} were made by diluting as follows:-

Stds.(ng/ml)	Mab(ul)	PBS+0.05%casein
70	70	30
60	60	40
50	50	50
40	40	60
30	30	70
20	20	80
10	10	90
5	5	95

5. 1 set of 8 standards (100 μl per well) was applied on row 2 and another on row 11 of the plate and 100 μl of PBS and 0.05% casein were added to the remaining wells. Rows 1 and 12 were kept as blanks.

6. One set of 8 samples was placed on row 3. This gave a 1 in 200 dilution of the initial concentrations. The culture was mixed well and 100 μl of samples transferred to row 4 to give a 1 in 400 dilution; then 100 μl was transferred from row 4 to row 5 for a 1 in 800 dilution and finally 100 μl transferred from row 5 to row 6 for a 1 in 1600 dilution.

7. This procedure was repeated moving from row 7 to row 10 to give a duplicate set of readings.

8. The plate was covered with metal foil and incubated at 37°C for 45 minutes. After this the plate was banged dry and washed 3 times with buffer B to remove the excess unbound antibodies.

9. The secondary antibody - conjugate was applied next. Conjugate: Sheep anti-mouse conjugated to horseradish peroxidase samples were frozen in 10 μl aliquots. 2 vials of 10 μl of conjugate were taken and 20ml of PBS with 0.05% casein was added to them. This was sufficient for 2 plates. 100 μl of conjugate was added per well, the plate covered with foil and then incubated at room temperature for 45 min.

10. The plate was washed 6 times with buffer B and bang dried.

11. 100 μl of fresh citrate-phosphate substrate solution was added to each well and the plate covered with foil. This was incubated in the dark at room temperature until the 5ng standard started to show an increased orange colour over a blank well. Alternatively the plate was checked after 15min of incubation and the reaction stopped.

12. The reaction was stopped with 50 μl of 12.5% H_2SO_4 and the absorbance read on the ELISA plate reader at 492nm after blanking on an empty well. Plates were frozen if there was no time to read them.

2.4.2.1 ELISA for PQXB1

The same general technique was used, except that the plate was coated with Sheep anti mouse IgG. The assay therefore measures antibody protein concentration, not binding activity.

2.4.2.2 Interferon-γ ELISA

The same procedure was followed as in the IgG ELISA. Polyclonal anti human γ interferon serum was bound to the plate, then used to capture the test samples and standards (Sigma). Monoclonal anti-interferon-γ serum formed the next layer of the sandwich, and finally goat anti mouse serum (Sigma) conjugated to horseradish peroxidase. The anti-interferon monoclonal and polyclonal antibodies were a gift from Dr N. Jenkins of the University of Kent at Canterbury, U.K.

2.4.3 β-galactosidase Assay

Reagents Z-buffer: 0.01M K Cl, 0.06M Na_2 HPO_4, 0.04M NaH_2PO_4, 0.01M Mg SO_4. 0.05M 2–mercaptoethanol. Initiator: 4 mg ml^{-1} 2–nitrophenol-β-D-galactopyranoside (ONPG) in Z-buffer. Stop Solution: 1M Na_2 CO_3.

Method

1. The samples were diluted ten fold in 10 Z-buffer and 200 μ was applied to each well.

2. The samples were diluted, using doubling dilution down the plate, leaving 100 μl in each well.

3. 20 μl of Initiator was added and the sample incubated at 28°C for 30 minutes.

4. 100 μl of Stop solution was added.

5. The plate was read in a spectrophotometer at 415 nm

6. β-galactosidase activity= $\frac{OD_{450} \times dilution \times 0.22(lightpath)}{0.0045}$ in International Units ml^{-1}.

2.4.4 Lactic Acid Assay

Reagents 10mg of nicotinamide adenine dinucleotide (NAD); 2ml of glycine buffer; 4ml of distilled water and 0.2ml of lactate dehydrogenase were combined in a 25ml universal container.

Method

1. 0.98ml of reaction mixture was put in a 2ml cuvette and to this was added 16 μl of cell-free supernatant.

2. All samples were covered with cling film and incubated at 37°C for 30 min.

3. Lactic acid standards of 3, 6, 12, 20 and 25mM were made up and incubated at 37°C for 30 min.

4. The spectrophotometer was set to 340nm, and allowed to warm up for 30min.

5. After 30 min. cuvettes were removed from the incubator and the absorbance read after blanking on a cuvette with no supernatant.

6. Standards were plotted on a graph against the absorbance readings and sample concentrations determined from this calibration curve.

2.4.5 Ammonia Determination

From Fawcett and Scott (1960)

Reagents

A) 250 g Phenol, 1.35g Sodium Nitroprusside. Make up to 4.5 l with distilled water.

B) 132 ml 3.3M Sodium Hypochlorite, make up to 4 l with distilled water. To 80g Na OH add solution to make 4 l. The reagents are light sensitive, but store indefinitely in dark. *Method*

To 40 μl of sample (0-2mM) one ml of reagent A was added then one ml of reagent B, then mixed well. After incubation at 37°C for 15 minutes in the dark the extinction was read at 570 nm against air.

2.4.6 ^{3}H Thymidine Incorporation

Reagents

Thymidine cocktail (1mCi ml^{-1}) 25 μl of non-radioactive Thymidine at (10 μg ml^{-1}) with 100 μl of ^{3}H Thymidine (1mCi) and 875 μl of PBS. 10% Trichloroacetic Acid (TCA), 5% TCA, 95% ethanol and scintillation fluid, Optiphase X (LKB, Loughborough). *Method*

1. Cells were counted, then the sample concentrated or diluted to 5×10^5 cells ml^{-1}. 5ml of this cell concentration was placed into a universal tube. This procedure was repeated to give duplicate or triplicate samples.

2. 5 μl of Thymidine cocktail was added to each tube to a final ^{3}H thymidine concentration of 1mCi ml^{-1} and incubated at 37°C in a CO_2 incubator for 30 min.

3. Glass fibre filters (Whatmann GF/C) or 0.45 μm filters (Millipore) were placed on a Millipore vacuum filter.

4. Samples were filtered and washed twice with PBS.

5. Samples were washed with 10% TCA to precipitate the protein, then washed with 5% TCA and finally with 95% ethanol.

6. The filter was removed with a pair of pincers to dry in a scintillation tube. This was labelled and stored.

7. 10ml of Optiphase X was added to each sample tube and 10ml to a blank. Samples were taken to a Mk III scintillation counter for reading.

2.4.7 Flow Cytometry

Cells were analysed using a Coulter Elite flow cytometer (Coulter Instruments, Luton).

All samples were counted, then either resuspended in cold 70% ethanol at 1×10^6 cells ml^{-1} and stored at 4°C for at least 30 minutes prior to staining, or treated with DNase.

2.4.7.1 DNase Treatment

The DNA content of dead cells does not necessarily reflect the current status of live cells. DNase is able to penetrate only dead cells. It can therefore be used to remove DNA from these cells, removing them from the analysis.

1. DNase II (Sigma) suspended in a Sucrose Magnesium and Tris buffer (800 ml H_2O, 2.43g Tris adjust pH to 6.5 with 1 M HCl, add 85.6g Sucrose and

1.016g Magnesium, make up to 1 litre). Add 100 mg of DNase II (Sigma) to 200 ml SMT solution, store in 1ml aliquots at -20°C.

2. 5×10^5 cells ml^{-1} of sample was placed in a test tube and spun down at 1000 rpm for five minutes, after which the supernatent was discarded.

3. The cells were resuspended in 1 ml of DNase solution and incubated at 37°C for 30 minutes then spun down.

4. The cells were resuspended in cold 70% ethanol and stored at 4°C for at least 30 minutes prior to staining.

2.4.7.2 Propidium Iodide Staining

Propidium Iodide (PI) intercalates with two stranded nucleic acid. Upon excitation, which occurs at a maximum at 536nm, it emits fluorescence at 617 nm. Excitation of fluorescence can be achieved with an argon laser which emits light at 488nm (blue). It can be used to analyse the cell cycle status of a population of cells. Accuracy may be improved by prior removal of RNA, some of which is two stranded, using RNase.

1. 1ml of cells at 1×10^6 cells ml^{-1} were washed in PBS. Then resuspended in a solution of RNase in PBS, and incubated for 30 minutes at 3°C.

2. Resuspend in 1 ml PBS.

3. Add 76μl of 650μg ml^{-1} Propidium Iodide in PBS. Incubate for 10 minutes at room temperature.

4. Resuspend in PBS.

2.4.8 SDS Polyacrylamide Gel Electrophoresis

Proteins when placed in a high pH buffer have a net positive charge. This fact may utilised in gel electrophoresis to separate proteins on the basis of their molecular mass. In gradient gel electrophoresis proteins in a buffer solution migrate through a gel of an increasing degree of crosslinking, driven by an electric charge, until the pore size of the gel is smaller than the size of the protein. Sodium dodecyl sulphate denatures the proteins, removing effects caused by differences in shape.

Reagents.

1. Sample buffer, 10mM Tris-HCl pH 6.0: 1.0g Tris + 40 ml H_2O + HCl to pH 6.0 add H_2O to 100 ml. Add 2.5g SDS and 10 mg of Bromophenol Blue.

2. Stacking buffer stock: 0.5 M Tris-HCl, pH 6.0: 6.0g Tris + 40 ml H_2O + HCl to pH 6.0, approx 48 ml 1.0 M , add H_2O to 100 ml.

3. Resolving buffer stock: 3.0 M Tris-HCl, pH 8.8: 36.3g Tris + 48 ml 1.0 M HCl add H_2O to 100 ml.

4. Reservoir Buffer stock: 0.25 M Tris-HCl, pH 8.3 (10x stock): 30.0g Tris, 144.0g glyine in 1 litre. Adjust to pH 8.3 with 1.0 M HCl.

5. Acrylamide-bis: 30% acrylamide, 0.8% methylene-bis acrylamide: 30.0g acrylamide, 0.8g methylene-bis acrylamide, add H_2O to 100 ml.

6. SDS: 10% sodium dodecyl sulphate: 10g SDS, add H_2O to 100 ml.

The acrylamide solution was made up fresh each time a gel was prepared, the other solutions were stored. Table 3 shows the composition of the gels used.

Table 3: The composition of the reagents for the 6%–18% acrylamide gel

	Sealing Gel	Resolving Gel		Stacking Gel
		6%	18%	
Acrylamide-bis (ml)	4.0	2.4	7.2	1.2
Resolving buffer (ml)	0.75	1.5	1.5	2.5
10water (ml)	1.05	7.7	1.9	5.65
Sucrose (g)		1.8		
TEMED (μl)	5	5	5	7.5

2.4.8.1 Gel Preparation

Two 1.5 ml thick perspex spacers were lightly coated with vaseline, and placed parallel at either end of a sheet of clean glass. Another sheet was placed on top and the sandwich was clamped together. The gel plates were placed vertically in a small reservoir and 10 ml of blocking gel solution was poured in the reservoir. When the blocking gel had set, the resolving gel was poured in from the top and finally the stacking gel added.

2.4.8.2 Sample Preparation

To 20 μl of culture supernatent 10μl of 10% SDS solution was added after which the samples were placed in an oil bath at 4°C for 10 minutes. The same treatment was applied to the standards (Sigma).

2.4.8.3 Running the Gel

The gel and plates were placed in a vertical electrophoresis tank and reservoir buffer was placed in both top and bottom reservoirs. 50 mA was applied across the gel overnight or until the dye front had reached the end of the gel.

2.4.9 Western Blotting

Gel Electrophoresis is a powerful technique for protein separation, but in many cases the proper identification of a protein separated by this technique is also best achieved by stains with a high molecular weight, such as antibody and therefore limited mobility in the gel. Western blotting gets around this problem by transferring protein from the gel to the surface of a paper matrix, which is then easily accessed by high molecular weight stains. Transfer is encouraged by applying an electric field width-ways across the gel and blotting paper through a high pH solvent. In this case the protein was detected using the same anti-mouse polyclonal serum conjugated to horse radish peroxidase as is used in the TBC3 ELISA, together with a similar colourimetric reaction.

Reagents:

Buffer:

H_2O_2 (30%)	10μl
H_2O	15 ml

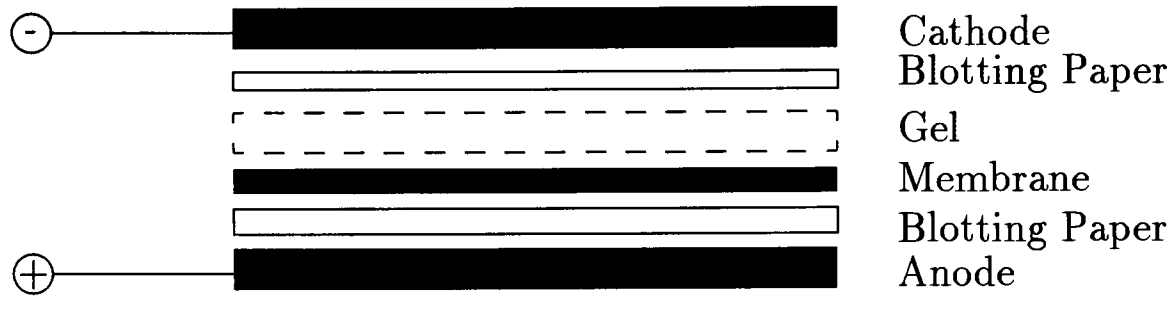
Stain:

Tris HCl (0.1M) pH 7.2	12 ml
4,3 diaminobenzidine 4 HCl (0.5% in Tris HCl	3ml
Ni Cl $6H_2O$ (8% w/v)	150 μl

Method

A piece of the membrane material, Poly vinylidene difluoride (PVDF) (Millipore, Watford, UK), was cut to the same size as the gel, along with two pieces of ordinary ink blotting paper. These were soaked in blotting buffer. The gel, membrane and blotting paper were arranged on the flat graphite electrode surfaces of an electroblotter (Biorad, Hemel Hempsted, UK.) carefully to avoid trapping any air between the layers (see Figure 8). An electric current of 50 mA was applied across

Figure 8: Schematic diagram of the arrangement of layers in the Western Blot.



the sandwich for 2 hours.

The membrane was incubated in a solution of 3% ovalbumin in PBS, to remove remaining protein binding sites. It was washed in PBS, then incubated with polyclonal anti-mouse IgG-HRP conjugate diluted in a PBS. It was washed with PBS 3 times for 5 minutes each, then dipped in the substrate solution until sufficient colour had developed, where-upon the reaction was stopped by washing in PBS.

Chapter 3

Experimental Programme

Cell lines differ from each other in their requirements for serum, rate of use of basal medium components, sensitivity to toxic metabolites, specific production rates, etc. This is particularly true of hybridomas which can have order of magnitude differences in specific antibody production rate for example. This laboratory has had considerable experience with hybridoma cell lines. Antibodies are stable products, which the laboratory had assays set up for. Therefore two hybridoma cell lines were chosen to asses their suitability for use in dialysis culture. Batch cultures were performed on these cell lines. The results of these batch cultures also formed a basis for comparison with dialysis cultures (see Problems in comparing systems, Page 15).

Ammonia and lactate are both toxic by-products of metabolism, which have been shown to limit the growth of cells in culture (Hassell *et al.*, 1991). The sensitivity of cells to them varies according to the cell line used (Hassell *et al.*, 1987). The sensitivity of TBC3 to both was identified using exogenously added lactate or ammonia in batch culture.

Two complex, undefined, nutrient sources were compared for their ability to increase cell growth, as both hybridoma lines proved rather low yielding.

To test their suitability under dialysis conditions both cell lines were grown in dialysis culture in small bottles. These devices were simple to construct and operate and were cheap to run. Few differences were observed between the two cell lines, so TBC3 was chosen for further examination as it was the one with which we had the greatest previous experience.

After successful growth in small bottles, more constant environmental conditions were sought by using a pH and DOT controlled bioreactor. Changing from a batch reservoir to a continuous reservoir system also helped to keep the environment constant. Steady state cell concentrations were observed to occur at a range of different membrane areas. The systems seemed to be limited by membrane area.

As the reactor was limited by membrane area and, since the mathematical models of Hagerdorn and Kargi, and Schultz and Gerhardt were available, though neither were fully applicable it was decided to develop our own model. This would allow the characterisation of performance with a view to identifying key process variables and provide a means of experimentally characterising the membrane and predicting the cell mass, nutrient and toxin concentrations.

The next stage was to verify the model experimentally. This was done by noting the cell, nutrient and toxin concentrations at a variety of fresh feed flow rates. This experiment was repeated for a second membrane area.

Serum must, normally, be added to the medium to allow cell growth. Due to the presence of a membrane it can be divided into two parts, that which can pass through the membrane and that which cannot. The importance of both parts

needed to be identified, as the lack of a continuous addition of fresh serum is a major difference between dialysis culture and other forms of perfusion culture. To see the effect of removing the low molecular weight components of the serum under batch conditions, cells were grown in dialysed serum. High molecular weight components of serum are retained within the reactor and may be deactivated by the conditions experienced. Periodic shots of serum were added to a reactor at steady state. The increases in cell number observed were accompanied by changes in glucose concentration. This suggested that glucose was the limiting nutrient. The experiment was then repeated with a higher glucose concentration, with continuous addition of serum.

Cell specific productivity has been shown to alter according to the physiologic state of the cell. Most types of membrane-based, total cell retention perfusion devices produce heterogeneous conditions, some making representative sampling of the biomass difficult. This device does neither. Cell size and cell turnover rates were determined for this system, as both affect specific productivity.

Chinese Hamster Ovary (CHO) cells are extensively used for the production of recombinant proteins. They have a higher cell yield on medium than the hybridoma cell we were using, and they are not susceptible to apoptosis (Singh *et al.*, 1994a). Interferon-γ is a commercially important product. Since, in other reports of dialysis culture eg (Schlaegar and Schumpp, 1992; Szperalski *et al.*, 1994) steady state cell viabilities are higher than with TBC3 it was decided to grow CHO in dialysis culture.

The virus *Autographa californica nuclear polyhedrosis* is a member of the baculovirus group. It has a gene expressed late in its lytic cycle, which is not essential to viral replication, which is expressed at very high levels producing up to 50% of

the total cell protein as its product, the polyhedrin protein (Cameron *et al.*, 1988). This virus has been used as the basis for a number of very effective expression vectors. The virus is normally added to cultures of insect cells midway through the exponential growth phase of a batch culture, as this produces the highest total yield. However a number of recent articles suggest that this is due to lack of nutrients for further production. Replacing the medium with fresh when the cells have reached the maximum cell density of a batch culture gives much higher specific productivity. Replacement of medium by a variety of perfusion cultures has been attempted, but, as Insect cells require expensive foetal calf serum, or equally expensive serum free formulations, this is an expensive option. Replacement of nutrients using dialysis allows cheaper basal medium to be used. A comparative experiment was attempted by growing a culture to maximum cell density, infecting it with virus, and then splitting it between two reactors, one dialysed, the other not.

Chapter 4

Experiments

4.1 Choosing a Hybridoma

For the work described in this section batch and small scale dialysis cultures were conducted. The purpose of the batch cultures was to develop familiarity with the cell line and equipment and to act as a basis for comparison with dialysis culture. Two cell lines were compared using both modes. As discussed in the introduction (Section 1.2.3) hybridoma clones can be very different from each other physiologically. Here we tested for gross differences in the cell line under dialysis conditions.

4.1.1 Batch Cultures

Figure 9 shows a typical batch culture of TBC3 in a pH and DOT controlled reactor (pH 7.0, DOT=50%). It was inoculated at 0.2×10^6 cells ml^{-1}, as a 10% volume inoculum, into RPMI 1640 with 5% NBS. A maximum cell density of 0.78×10^6 cells ml^{-1} was achieved after 51 hours, and there was no stationary

Table 4: Comparison of the growth of TBC3 and PQX/B1 in batch culture

	TBC3	PQX/B1
Max Cell Conc. $\times 10^6$ cells ml^{-1}	0.78	0.72
Viability Index $\times 10^6$ cells $ml^{-1}hr^{-1}$	42.3	34.5
Max Antibody Conc. μg ml^{-1}	45	35

phase, the cell concentration decreasing from 0.78×10^6 cells ml^{-1} to 0.22×10^6 cells ml^{-1} in 72 hours. Viability Index, which is the total number of cell hours, was 42.3 $\times10^6$ cells $ml^{-1}hr^{-1}$. It was calculated by taking the average of successive cell counts, multiplying this by the time interval between the counts, then taking the sum of all such measurements over the whole fermentation.

$$\text{Viability Index} = \sum_{t=0}^{t=n} \left(\frac{x_{t1} + x_{t2}}{2(t2 - t1)} \right)$$

Glucose was totally consumed reaching zero during the decline phase. Lactate and ammonia were produced (figure 10). Antibody was produced in all phases of the batch growth, the final concentration being 45 μg ml^{-1}.

Figure 11 shows a typical batch fermentation of PQX/B1 in a pH and DOT controlled reactor(pH 7.0, DOT=50%). It was inoculated at 0.1×10^6 cells ml^{-1} as 10% inoculum in RPMI 1640 with 5% newborn calf serum (NBS). A maximum cell density of 0.72×10^6 cells ml^{-1} was achieved after 58 hours, and again there was no stationary phase, the cell concentration decreasing from 0.72×10^6 cells ml^{-1} to 0.22×10^6 cells ml^{-1} by 96 hours. Viability Index was 34.5 $\times10^6$ cells $ml^{-1}hr^{-1}$. Glucose was also again totally consumed reaching zero during the decline phase. Antibody was produced in all phases of the batch growth, the final concentration being 35 μg ml^{-1}. Table 4 compares the two fermentations

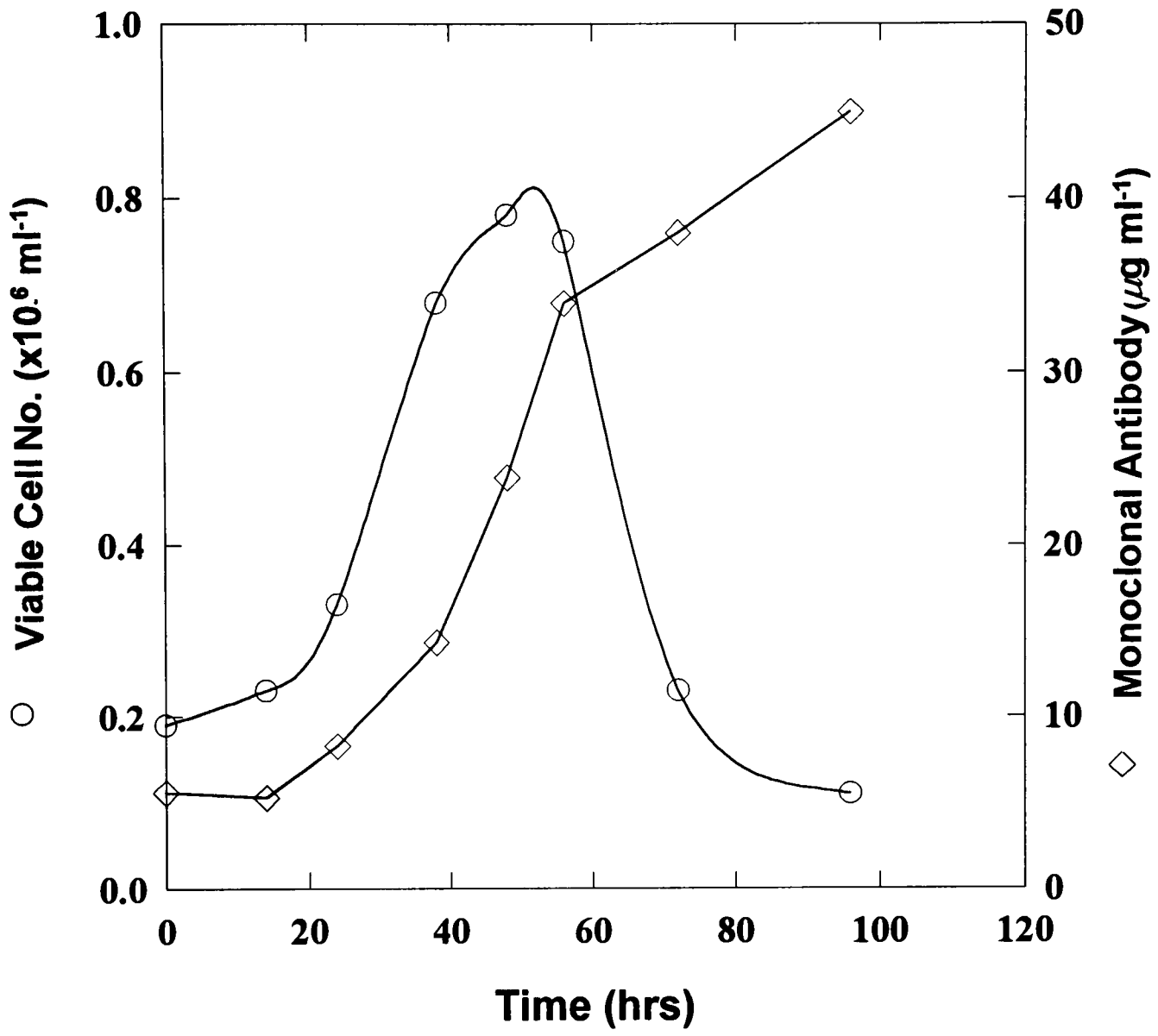


Figure 9: Batch growth of TBC3 (pH 7.0, DOT 50%, 100rpm, 10% inoculum volume, RPM1 1640 and 5% NBS). Cell Concentration (◯), Antibody Concentration (◇).

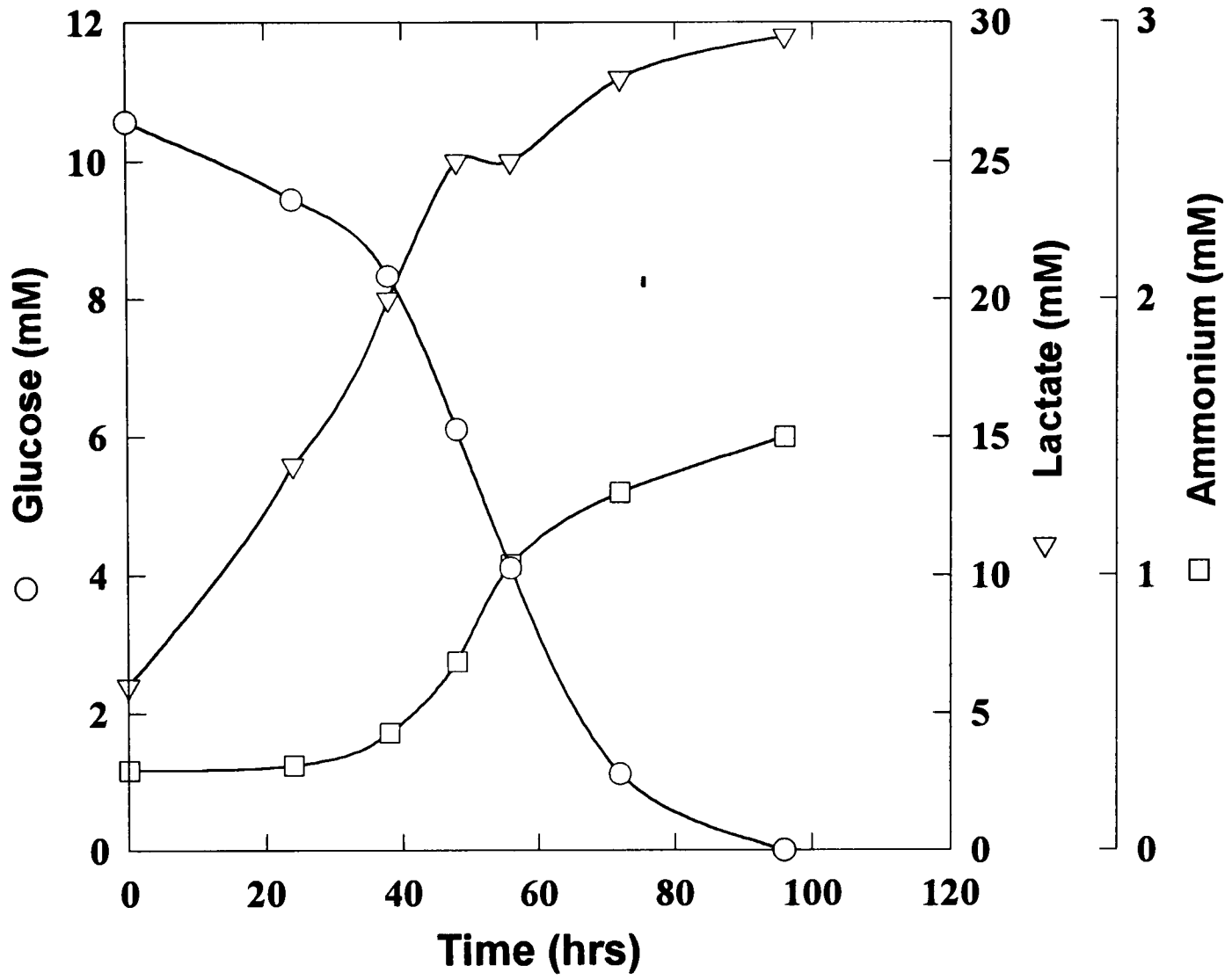


Figure 10: Metabolic profile of TBC3 batch fermentation. Glucose concentration (◯), Ammonia concentration (□), Lactate concentration (▽).

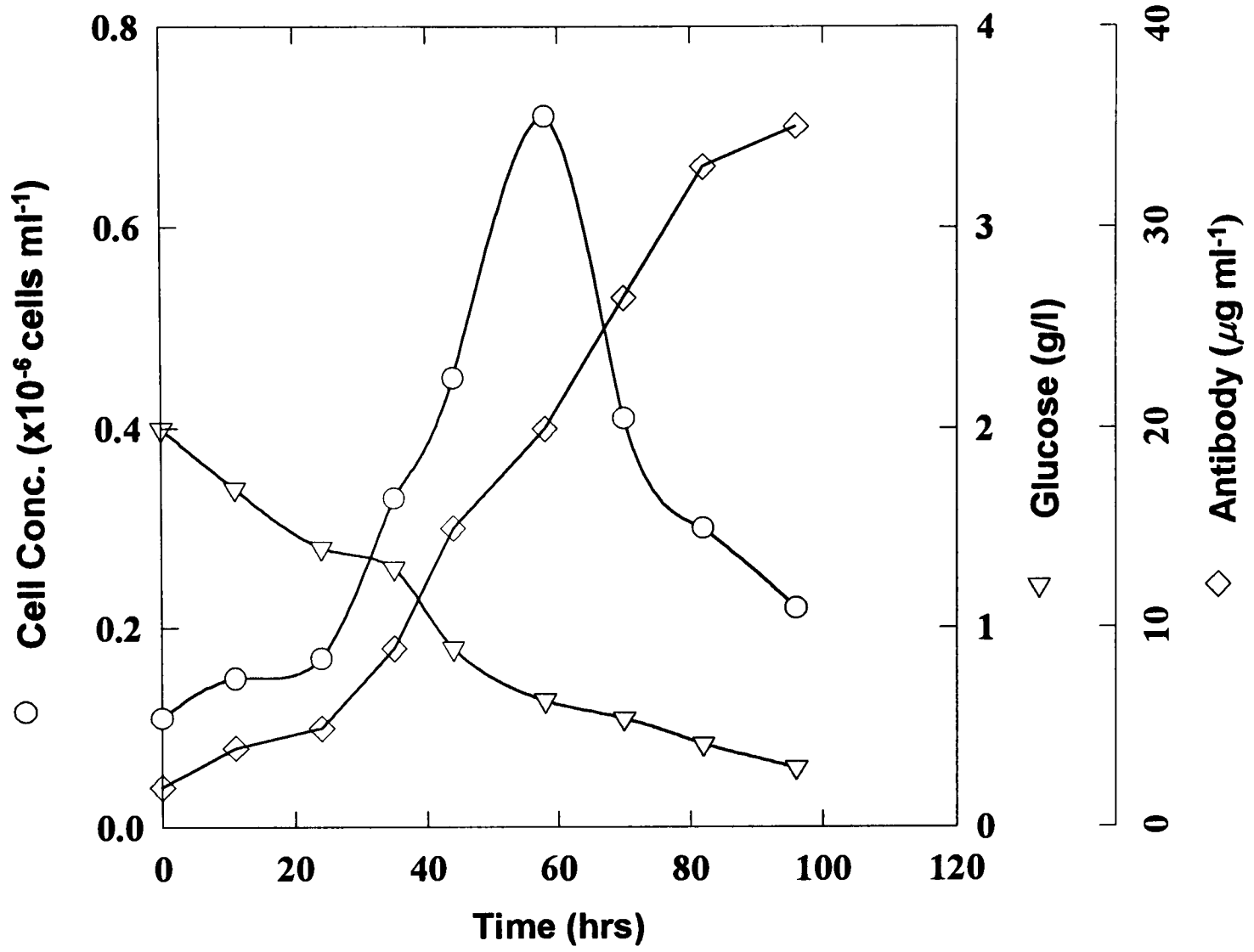


Figure 11: Batch growth of PQX/B1 in (pH 7.0, DOT 50%, 100rpm, 10% inoculum volume, RPM1 1640 and 5% NBS). Cell concentration (◯), Antibody concentration (◇), Glucose concentration (▽).

4.1.2 Small Scale Dialysis Experiments

It was decided that some initial experiments should be performed on a small scale as an inexpensive means of indicating the potential of the system. The system of Adamson *et al.* (1983) was adapted by placing a 2cm section of dialysis tubing, filled with 5 ml of 1.5×10^6 cells ml^{-1}, in a 250ml duran bottle, with 100 ml of basal medium, stirred with a magnetic flea. After 5 days the tube was opened, and the internal cell concentration found to be 9.6×10^6 cells ml^{-1}, at 86% viability. Dialysis was therefore capable of supporting high cell densities. Rookes *et al.* (1989) in this laboratory had previously grown TBC3 in a homogeneous dialysis system using suspended hollow fibre units. These units were expensive, would not stand repeated re-autoclaving and did not prove sufficiently reliable. Dialysis tubing was chosen as a cheap and simple alternative. Initial experiments were performed in small, magnetically stirred bottles. A 150 ml duran bottle was used at a volume of 50 ml. The lid was modified (see figure 12) to allow 4 metal ports to be inserted into the reactor; two were for surface aeration, the others were connected to 30 cm^2 of dialysis tubing suspended in the culture. 500 ml of medium was recycled from the medium reservoir through the dialysis tubing. The entire contents of the medium reservoir were replaced periodically. The reactor was placed in a water bath at 37 °C and 5% CO_2 in air was supplied at 10 ml per minute. The entire system was taken to a laminar flow cabinet for sampling. TBC3 and PQX/B1 have performed similarly in batch growth. They were therefore compared in dialysis mode.

Figure 13 shows the growth of TBC3 in dialysis mode. At 49 hours the perfusate was replaced with fresh basal medium. The cell concentration reaches a maximum of 2.5×10^6 cells ml^{-1} at 96 hours, dropping to 1.3×10^6 cells ml^{-1} by

150 hours. Glucose concentration varied around the 0.6–0.3 g l^{-1} mark. The maximum cell concentration was three times that observed in batch culture and the system was operating for 30% more time than the batch culture before becoming contaminated. Glucose was not limiting and actually increased towards the end of the culture, due to to the supply from the nutrient reservoir. This suggests that the system is limited by nutrient transfer, so when glucose demand decreases the glucose concentration increases to balance reactor and reservoir concentrations.

In Figure 14 a PQX/B1 fermentation is shown. The medium reservoir is replaced at 87 hours. The cell concentration peaks at 2.4×10^6 cells ml^{-1}, again approximately three times that observed for batch culture. The culture lasts for 187 hours, nearly twice as long as an ordinary batch culture. Again glucose concentrations are not limiting and are variable between 0.9–0.4 gl^{-1}

4.1.3 Discussion

In batch culture TBC3 out-performed PQX1 in all parameters: maximum cell concentration, viability index and final antibody concentration. PQX1 had a higher rate of glucose use than TBC3. Difference in growth yield on medium may explain the difference between the cell lines. The cell concentrations and growth rates seen are typical of hybridomas in batch culture.

The differences seen in the antibody concentrations may not be a fair comparison. The assay for the anti-human IgG antibody of TBC3 measures only that antibody which binds the antigen. PQX1 produces an antibody which binds to the weed-killer paraquat; the assay used measures all mouse antibody. If the antibodies are all intact and functioning these measurements should be identical. However if damage to the antibody occurs, then the total antibody measurements

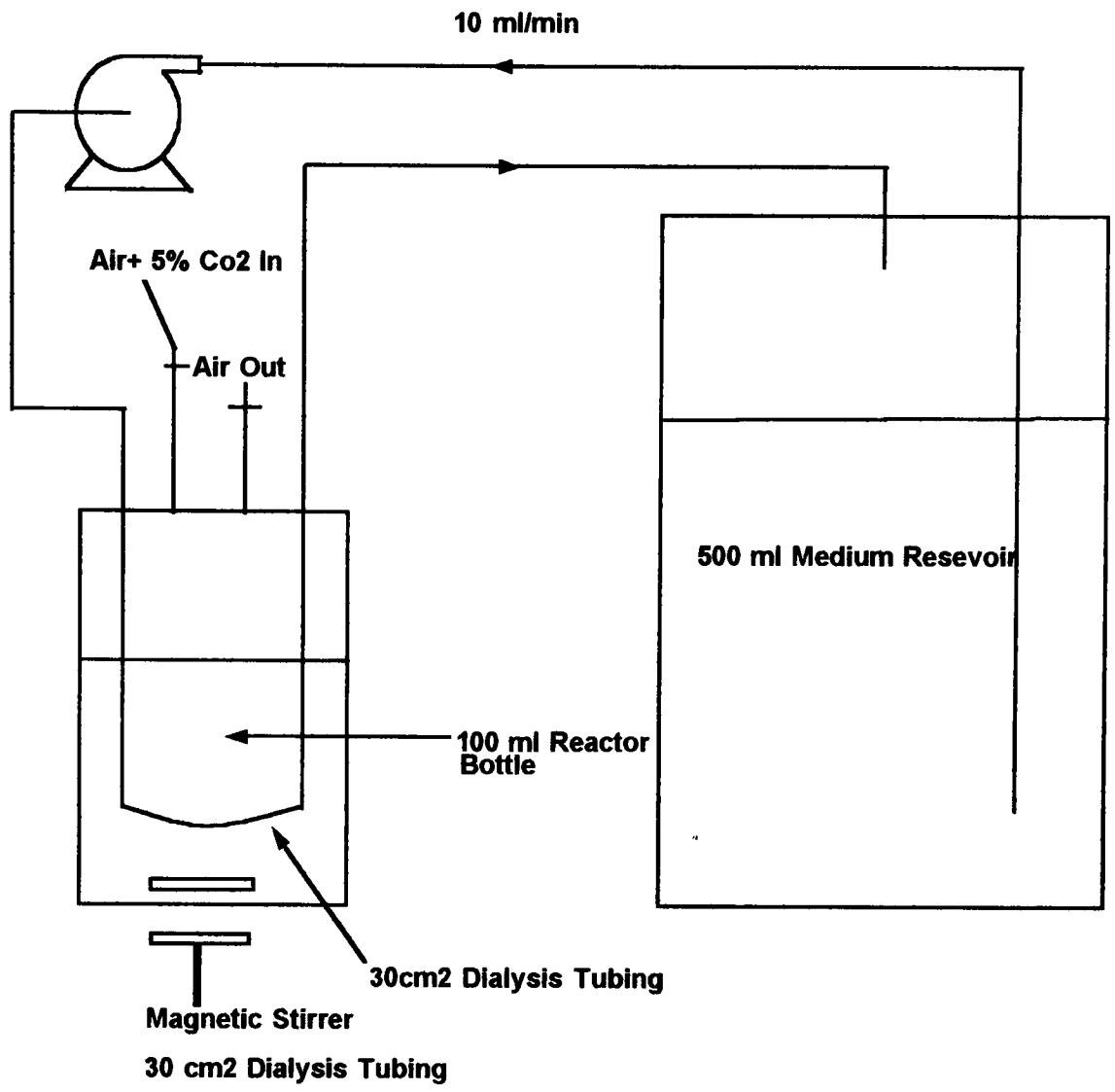


Figure 12: Schematic diagram of the dialysis bottle device.

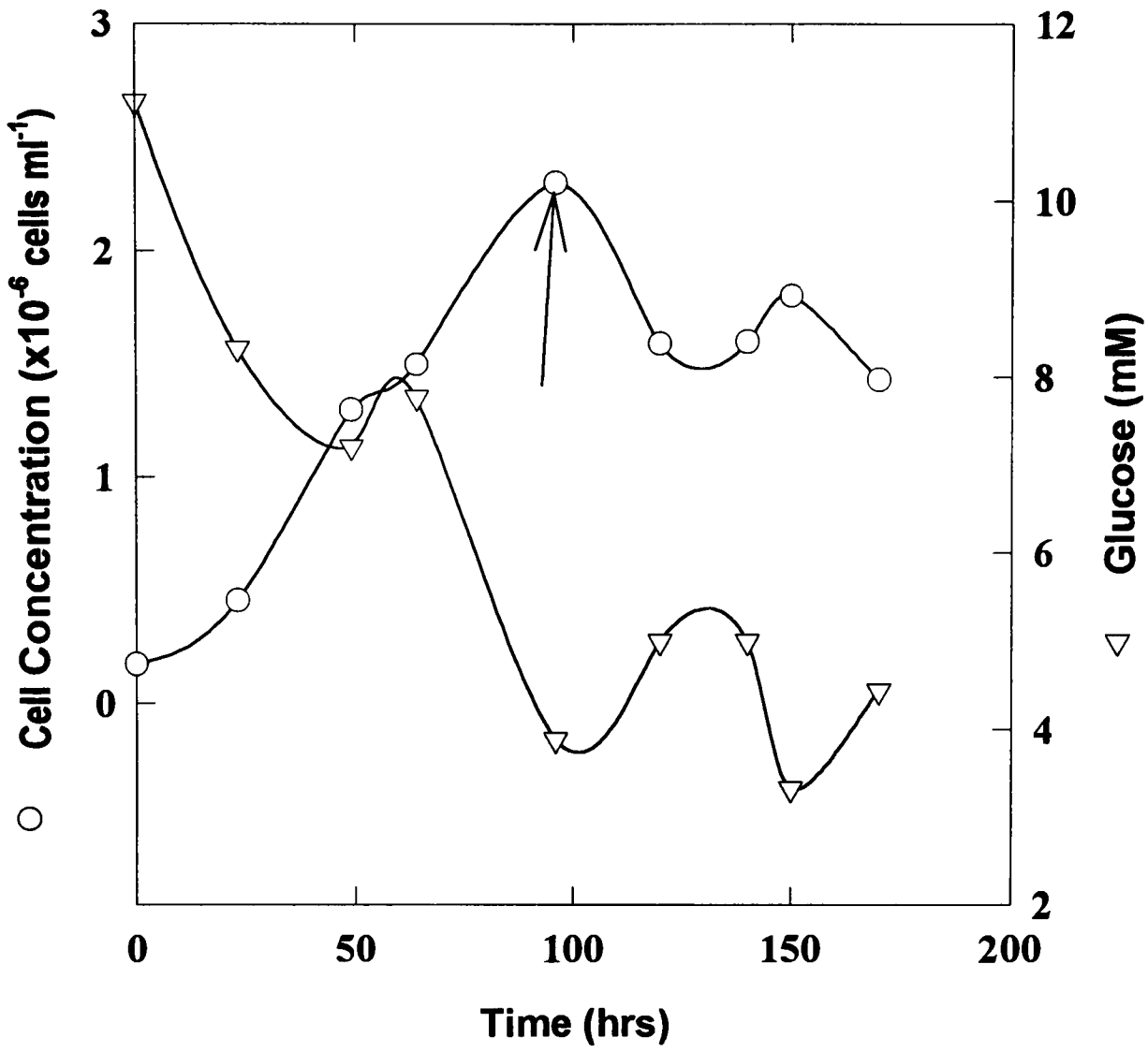


Figure 13: Dialysis Culture of TBC3 in dialysis bottle device. Cell concentration (◯), Glucose (▽), Medium change (↑).

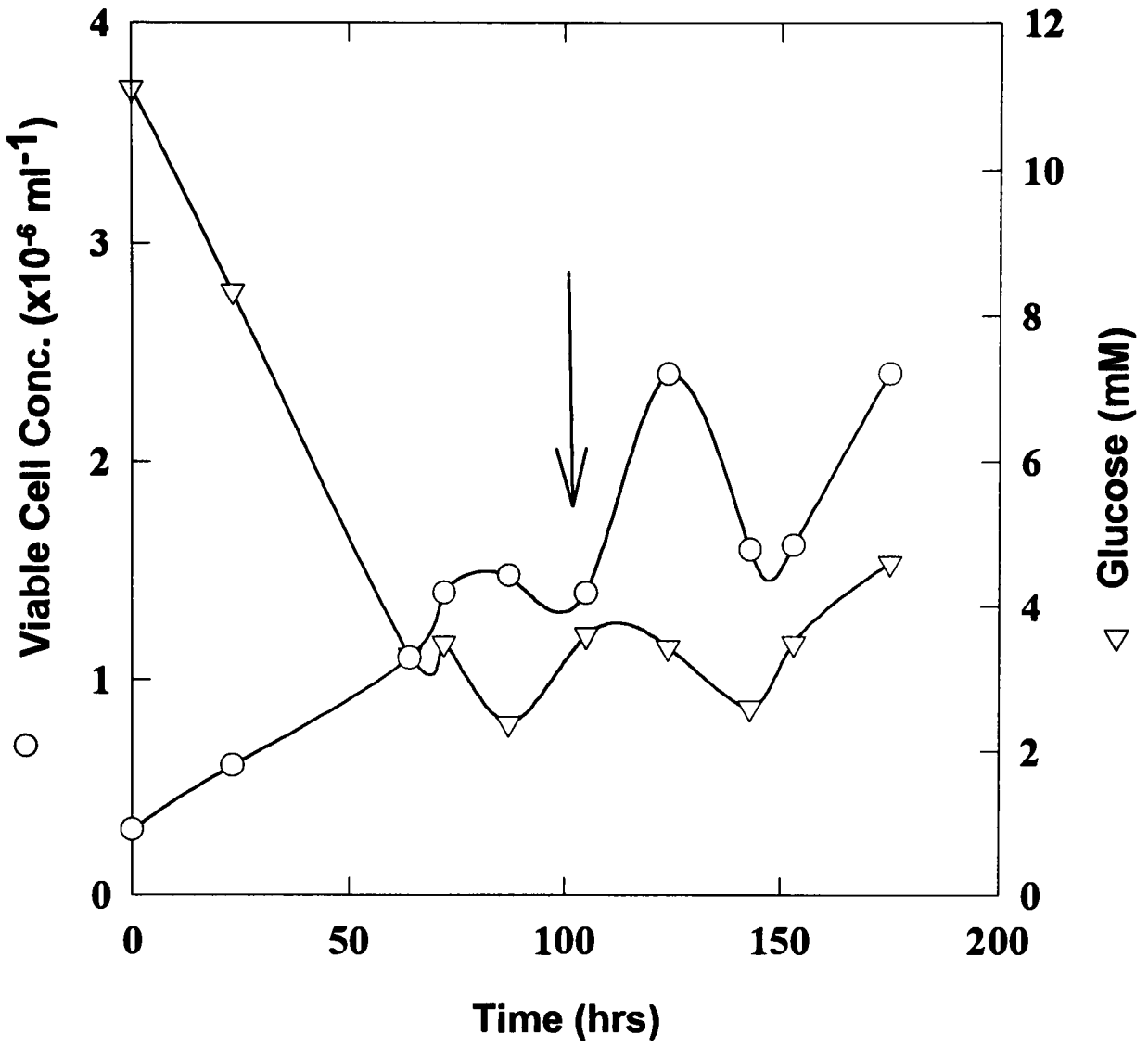


Figure 14: Dialysis Culture of PQX/B1 in dialysis bottle device. Cell Concentration (◯), Glucose (▽), Medium change (↑).

will over-read the amount of "active" antibody.

In the dialysis bottle cultures considerable benefits over batch culture were seen, with a tripling of the maximum cell concentration and batches lasting twice as long. No significant differences were observed between TBC3 and PQX1. Metabolite concentrations were variable however and thus temporal changes in cell physiology would occur, making the system performance difficult to predict.

TBC3 was chosen to concentrate on in further work because it exhibited a higher yield on medium, both of cells and of antibody and also because, as discussed above, the assay for antibody more accurately reflected the quantity of "useful" antibody. Finally there was more experience within this laboratory in handling TBC3.

4.2 Biopro as a medium additive

The reduction or elimination of the use of serum in animal cell culture is highly desirable because of its high cost and variability and the possible contamination with adventitious agents that it introduces. Most approaches involve the use of proteins fractioned from serum, for example albumin and transferrin and these are expensive. An alternative is the use of undefined protein or peptide sources. Peptone is an enzymatic digest of animal tissue which has been shown by several groups to be effective as a medium supplement increasing maximum cell numbers and monoclonal antibody titres. Jan (1992) has reported a doubling of TBC3 steady state cell numbers in a spin filter perfusion system with a 0.25% w/v addition of peptone and a corresponding increase in antibody concentration. Al-Rubeai and Emery (1989) were able to grow a hybridoma culture using peptone

and transferrin in place of serum. Peptone has been used extensively in this work.

In an attempt to explore other possibilities an investigation was made of the use of milk protein. Ramírez *et al.* (1990) grew hybridomas in bovine colostrum as a substitute for serum. Yamada *et al.* (1991) used casein as a serum additive and assigned its action to the slow release of amino acids from the casein by proteases secreted from the cell. Biopro is a preparation of milk proteins, produced by ion exchange chromatography, produced by Bio-Isolates Plc, Deeside, Wales. It is 95% protein, mainly β-lactoglobulin and α-lactalbumin, is not denatured and is soluble. It is used in a variety of food products because of its gelling, emulsifying and aeration properties.

Batch cultures were set up using 150 ml duran bottles and duplicate bottles were filled with 50 ml of RPMI containing either 0, 0.1, 0.25, 0.5, 0.75, or 1.0% w/v Biopro. The Biopro was dissolved in RPMI at the experimental concentrations and filtered into the bottles. The bottles were inoculated at 10% v/v at 0.1×10^6 cells ml^{-1} with TBC3 taken from one source.

Table 5 shows the effect of Biopro addition on cell number. The maximum cell number of 0.85×10^6 cells ml^{-1} was achieved at 0.25% w/v, compared with 0.7×10^6 cells ml^{-1} for the control. The maximum viability index was achieved at 0.5% w/v, 1.3 times higher than for no addition. The maximum final antibody titre was 50 μg ml^{-1}, achieved at 0.75 % w/v,representing an increase of 1.3×. Biopro does not seem to have any effect on cell specific antibody production, the increases in antibody concentration being proportional to the increased cell concentrations. For further experiments a concentration of 0.75% w/v was chosen.

If Biopro's nutritional effect is due to release of amino acids it may produce an improved growth even in cultures supplemented with foetal calf serum, which is

Table 5: Effect of Biopro on batch TBC3 growth in batch culture

Biopro %w/v	Max. Cell Density $\times 10^6$ cells ml^{-1}	Viability Index $\times 10^6$ cell$\times$hours ml^{-1}	Antibody Yield μg ml^{-1}
0	0.70	40.2	41
0.1	0.72	41.5	43
0.25	0.85	46.0	44
0.5	0.80	53.0	50
0.75	0.75	50.5	51
1.0	0.65	45.4	43

known to contain more nutrients than newborn calf serum. To test if there is any additional advantage of Biopro over foetal calf serum and peptone, the effect of which may also be due to amino acid release, a comparative experiment was set up. Table 6 shows the results of duplicate batch cultures prepared as described for the previous experiment. Foetal Calf Serum (FCS) has the largest effect on cell growth, and Biopro appears not to effect any extra advantage. Biopro was less effective at increasing maximum cell numbers and viability index than peptone.

A continuous culture was run at a dilution rate of 0.005 hr^{-1}, as described in Materials and Methods. A steady state cell concentration of 0.43×10^6 cells ml^{-1} was achieved for RPMI and 5% NBS and addition of Biopro increased this to 0.53×10^6 cells ml^{-1}.

In summary the use of Biopro increases both maximum cell concentrations and antibody productivity concomitantly, no particular effect on cell specific antibody production was noticed. The increases were smaller than those produced by peptone. Biopro appears to have no particular advantage over peptone. In all further experiments using TBC3 0.25% w/v of meat peptone was added to the medium.

Table 6: Comparison of the growth of TBC3 in medium supplemented with either 5% NBS, plus Biopro or peptone, or 5% FCS, with Biopro.

Serum	Supplement	Max. Cell Density $\times 10^6$ cells ml^{-1}	Viability Index $\times 10^6$cell$\times$hours ml^{-1}
NBS		0.97	34.8
NBS	Biopro	1.03	39.0
NBS	Peptone	1.19	40.0
FCS		1.25	47.6
FCS	Biopro	1.22	48.3

4.3 Lactate and Ammonium Toxicity

Lactate and ammonia are toxic by-products of metabolism which have been reported to limit batch cultures of hybridoma cells (Butler, 1987; Doyle and Butler, 1990; Murdin *et al.*, 1989; Reuveny *et al.*, 1987). The toxicity of lactate is presumed to be due to the increase in osmolarity caused by producing two lactate molecules for each glucose molecule consumed, as shown by controlled osmolarity chemostat studies (Omasa *et al.*, 1992). Ammonium toxicity is more complex but is thought to be due to intracellular acidifiction caused by active transport of NH_4^+ into the cell, disassociation into NH_3 and H^+ and diffusion of the non-polar NH_3 out (McQueen and Bailey, 1990). Hybridoma cells vary in their sensitivity to both (Hassell *et al.*, 1987).

In order to test the sensitivity of TBC3 to lactate triplicate 50ml batch cultures were setup with 0,20,40 and 60 $\times 10^{-3} Ml^{-1}$of lactic acid and the pH was readjusted to 7.0 with NaOH before inoculation. Figure 15 shows the growth of the cells. Maximum cell density decreased with increasing lactate concentration in a linear manner, so that at a concentration of 57 $\times 10^{-3} Ml^{-1}$no cell growth would be expected. In batch cultures of TBC3 the final concentration is 25–30

$\times 10^{-3} Ml^{-1}$(see Figure 10).

A similar experiment was set-up to measure ammonium toxicity, with ammonium added as ammonium chloride to make concentrations of 0,1,2 and 3 mM. pH was readjusted with HCl. A linear decrease in cell concentration was seen. By least squares linear regression of maximum cell density, cell growth would be expected to fall to zero at 5 $\times 10^{-3} Ml^{-1}$. Batch cultures of TBC3 produce approximately 1.5 $\times 10^{-3} Ml^{-1}$(see Figure 10).

The final concentration of lactate and ammonia in batch culture are less than 30 $\times 10^{-3} Ml^{-1}$and 1.5 $\times 10^{-3} Ml^{-1}$respectively (figure 36). These concentrations were less than athose observed to affect initial growth rate in the previous batch cultures and although the batch cultures both showed some decrease in maximum cell density by this point they would have accumulated at least double the accumulated amount of either metabolite. Thus the observed levels of lactate and ammonia in dialysis culture are in the range where they may be expected to affect the cells but they are both at the bottom end of this range.

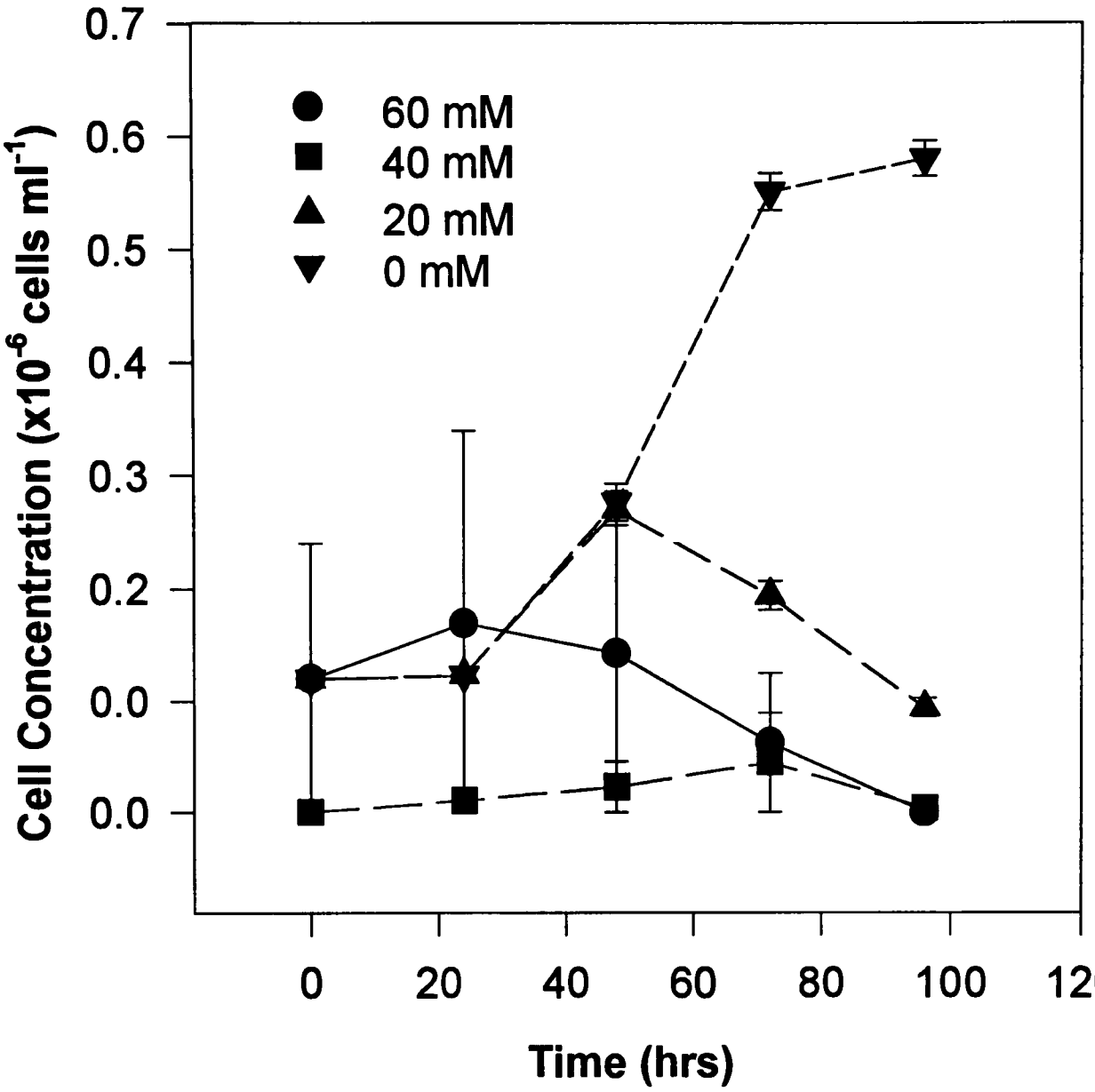


Figure 15: Effect of exogenous lactate addition on the growth of TBC3 in batch culture.

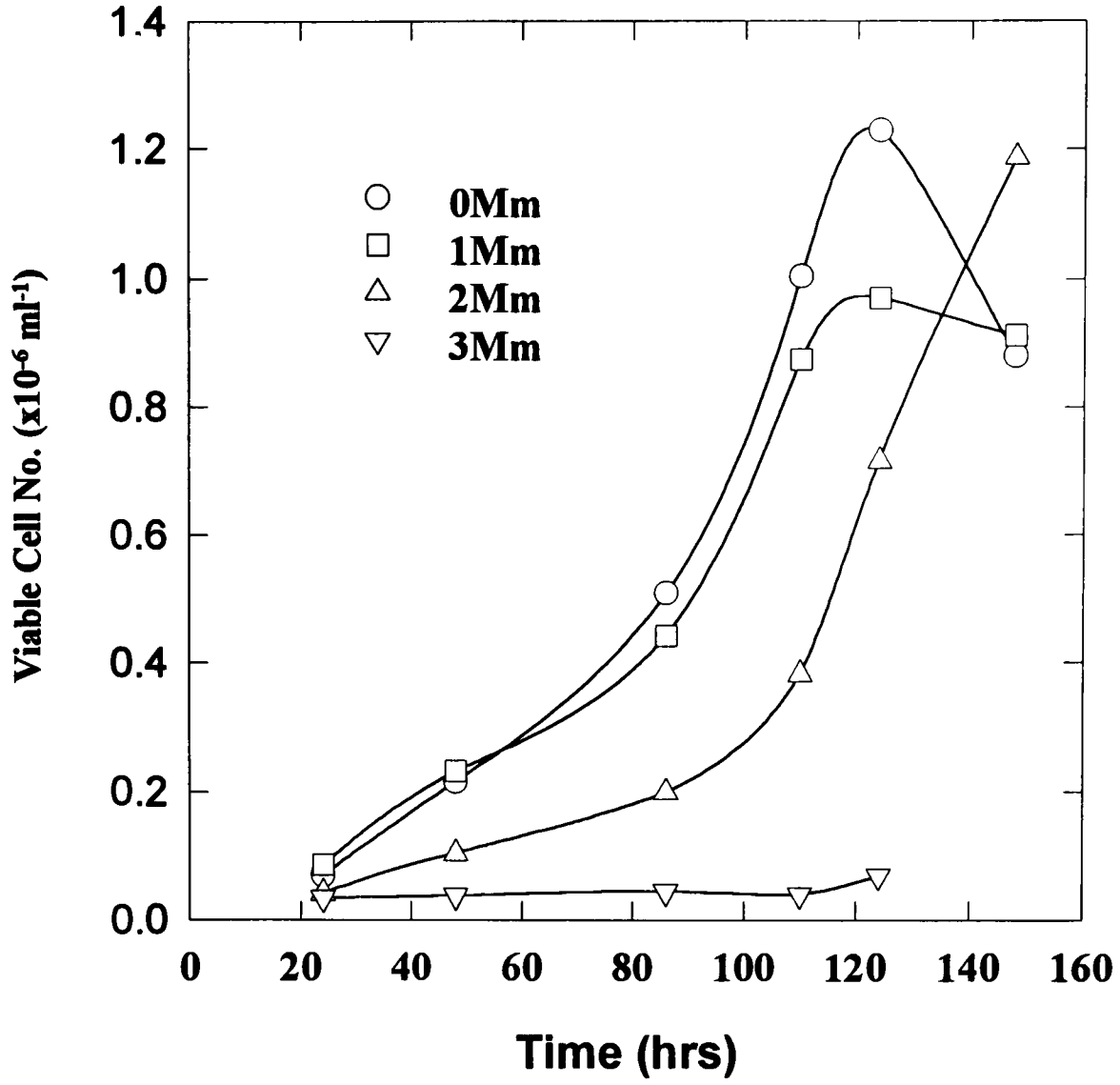


Figure 16: Effect of exogenous ammonia addition on the growth of TBC3 in batch culture.

Chapter 5

Large Scale Dialysis Culture

5.1 Results

5.1.1 Supply Mode

In the work described in the previous chapter TBC3 was grown in a dialysis bottle device. This showed that cell densities were enhanced and the reaction time was extended. However culture conditions were not precisely controllable, and the resulting cell and nutrient concentrations were variable. To improve the precision of parameter control and agitation, a one litre working volume stirred tank bioreactor with pH and dissolved oxygen control was used.

The bioreactor had a top driven agitator suspended at 1/3 of the distance from the bottom of the reactor to the waterline. To prevent the membrane from touching the impeller and to increase the area of membrane within the reactor, an idea was borrowed from Hagerdorn and Kargi (1990). A length of copper wire sealed into silicone tubing was threaded inside the dialysis tubing. The copper wire acts as a shapeable former. The former allows the tubing to be supported in

a helical shape away from the impeller and itself.

Four modes of nutrient supply to a dialysis device have been defined in the introduction (Figure 6). Since the principal advantages of the dialysis device stem from increasing cell densities and product concentrations and decreasing the use of high molecular weight medium components, the batch reactor configurations were chosen. Batch and continuous reservoir configurations were compared. The device described in the previous chapter was a batch reservoir and batch reactor device and this was used first.

The reactor was set-up in the same way as the dialysis bottle device (Figure 12). From a five litre reservoir basal medium plus peptone (0.25% w/v) and Pluronic F.68 (0.05% w/v) was recirculated at 10 ml per minute through 370 cm^2 of dialysis tubing (1 m length). The tubing was suspended in 900 ml of fresh medium (5% NBS, 0.25% peptone, 0.05% Pluronic), within a pH (7.0) and DOT (50%) controlled bioreactor agitated at 100 rpm. This reactor was inoculated with 100 ml of cell suspension at 1.3×10^6 cells ml^{-1}. 4.1 litres of basal medium was placed in the reservoir so that the total volume of fresh medium in the reactor/reservoir system was 5.0 litres out of the 5.1 litre total volume.

Figure 17 shows the results of such a run. Exponential growth rapidly increased the cell concentration to 3.8×10^6 cells ml^{-1}, which is five times the value achieved in standard batch culture. The cell number then fell to 3.3×10^6 cells ml^{-1} within eight hours after which it was steady for 24 hours. This type of steady-state is not normally seen in batch culture and may be the result of a limitation of transfer through the membrane. This hypothesis is supported by the observation that the death phase (decline in cell densities and viability) coincided with glucose running out. The time taken by the reaction was 165 hours, about 1.5 times that seen

Table 7: The viability index per basal medium ratio, for batch cultures and two modes of dialysis culture

Mode		Viability index per litre
Reactor	Reservoir	$\times 10^6$ cell·hrs l^{-1}
Batch	–	42.3
Batch	Batch	53.6
Batch	Continuous	76.0

in batch culture. The reaction time was not extended much because of the rapid growth to higher cell densities. The viability index was increased to 269×10^6 cell·hrs ml^{-1}, 6 times that seen in batch culture. The ratio of viability index to basal medium was slightly increased over a standard batch culture.

Figure 18 shows the results of a batch reaction with a continuous reservoir set up as shown in Figure 7. The conditions used were the same as in the previous example except that the total volume of the recirculation loop was 500 ml, and the contents of that loop were continuously replenished at 0.5 litres day^{-1}.

Cell concentration reached a maximum of 2.1×10^6 cells ml^{-1} then settled to a steady-state value at 1.9×10^6 cells ml^{-1}. This value is similar to that seen in the dialysis bottle device and 2.5 times that seen in batch culture. Cell concentrations between 1.9–2.1×10^6 cells ml^{-1} were maintained for a period of 200 hours. Viability fell at the beginning of the steady-state to 40% and remained there. Glucose reached a steady state concentration at 0.2g l^{-1}. The viability index was 500 $\times10^6$ cell·hrs ml^{-1}, 12 times that of a batch culture. Expressed as viability index per litre (Table 7) the dialysis culture achieves nearly double the ratio of viability index to basal medium that the batch culture does and 1.4 times that of the batch/batch dialysis reactor.

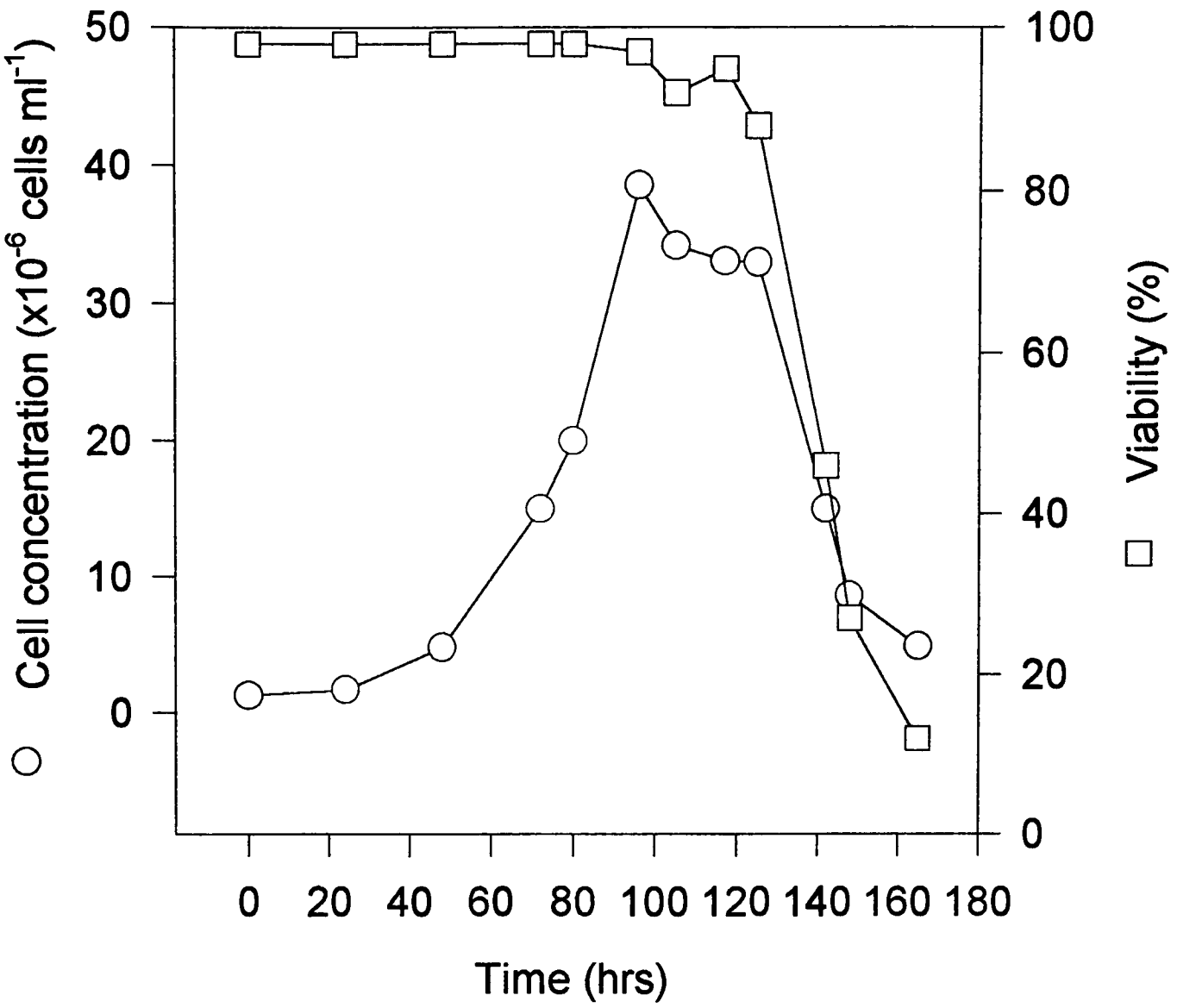


Figure 17: Growth of TBC3 in a controlled dialysis bioreactor with a batch cell space and batch reservoir. Cell concentration (◯), Viability (□).

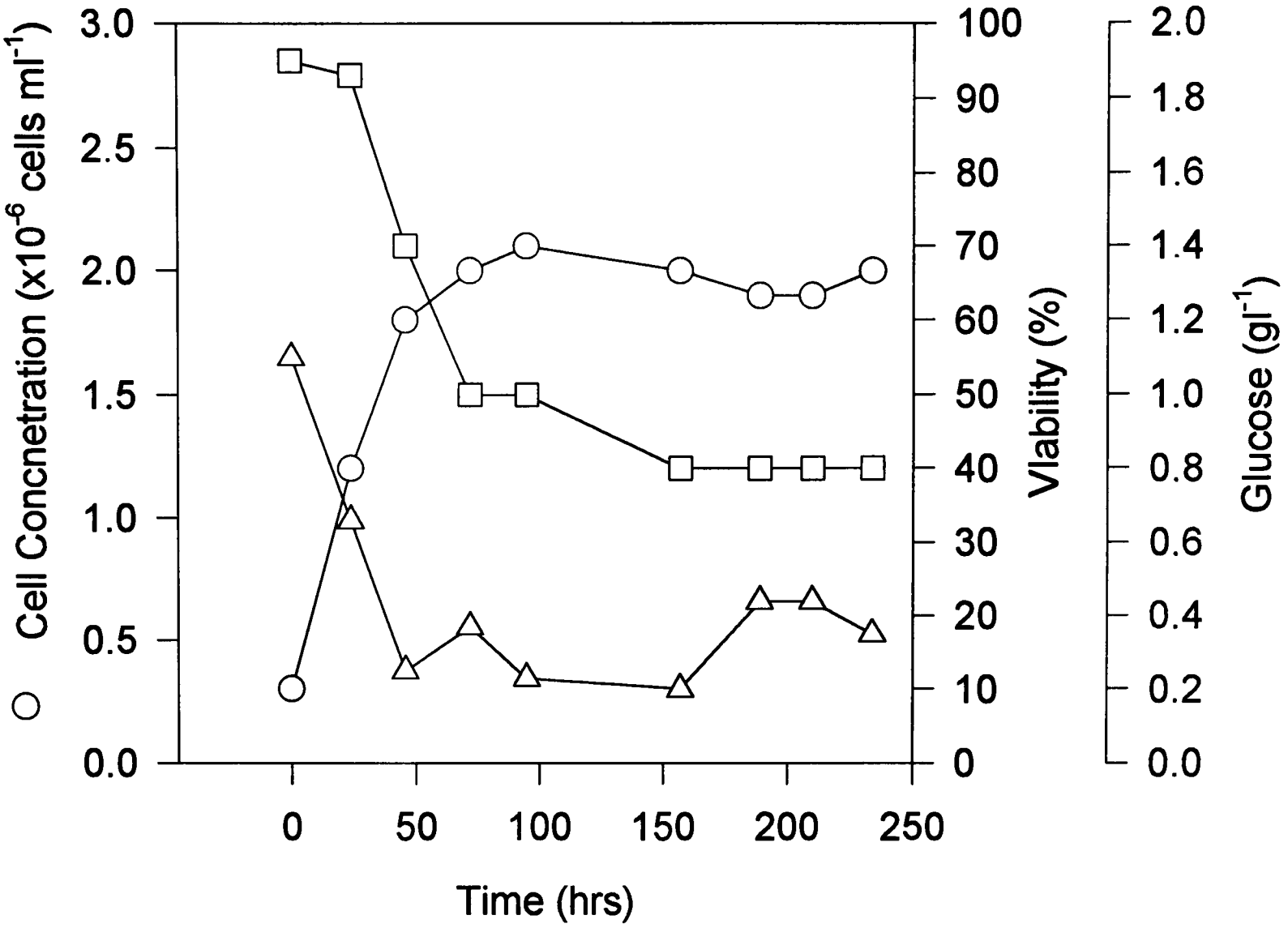


Figure 18: Growth of TBC3 in a controlled dialysis bioreactor with a batch cell space and continuous reservoir. Cell concentration (◯), Viability (□), Glucose (△)

Two modes of feeding have been compared. The batch system with a large reservoir leads to higher peak cell concentrations and a longer exponential phase. Continuous feeding gives steady-states in cell concentration, viability and nutrient concentration. The reaction run can be extended significantly over batch/batch systems. The production of cell hours per unit of medium is higher. Steady-state conditions are amenable to simple modeling which will help in understanding the constraints on the system. In the next section some physical factors affecting nutrient transfer and cell concentrations are explored.

A ^{3}H Thymidine incorporation experiment showed that DNA synthesis was occurring during the steady states. The average value for one steady state (Figure 29) was 689 $\pm$ 108 counts per minute (cpm), after correction for the blank (98.4 cpm); a control sample where the Thymidine was added immediately before quenching. This shows that cell division and, as the system is at steady-state, cell death is occurring.

To estimate the rate of cell division from the Thymidine incorporation rate it must be assumed that the relationship between the two is linear (Borth *et al.*, 1992). In a culture in the early exponential stages of batch growth the rate of cell death is likely to be small. In such a population the rate of cell division can be estimated by comparing the viable cell count between successive samples (Equation 1).

$$\mu = \frac{\ln x_1/x_0}{(t_1 - t_0)} \tag{1}$$

Then a constant of proportionality (c) can be calculated (Equation 2).

$$c = \frac{cpm}{\mu} \tag{2}$$

For sample **E** (Figure 29 a corrected cpm of 2064 was recorded. The growth rate as calculated by Equation 1 was 0.53 d^{-1}. The calculated value of μ for the steady

state is 0.16 $d^{-1} \pm 0.025$. This equates to an average cell lifespan of 4.3 days. The rate of turnover is significantly more rapid than may be expected. The fact that the steady state is a dynamic equilibrium has consequences for modeling specific protein production (Section 1.2.2) and therefore reactor design.

5.1.2 Investigating Design Factors

5.1.2.1 Effect of Membrane Area

The effect of membrane area on steady state cell number was studied by setting up fermenters with different membrane areas. The standard length of dialysis tubing used was one metre, giving a membrane area of 372 cm^2. For this experiment 0.75, 0.5 and 0.25 metres of tubing were used in consecutive runs. DOT was maintained at 50% by intermittent sparging with O_2, at maximum cell concentrations sparging occurred for no more than 10% of the time. pH was controlled at 7.0 by automatic CO_2 or Na OH additions. Fresh basal medium, plus peptone and Pluronic, was pumped at a constant rate from a 5 litre container to the perfusate reservoir.

Steady-state cell concentrations were obtained for a 0.5 v/v/d perfusion rate (see Figure 19) using each length of tubing. Cell concentrations increased linearly with tubing length (Figure 20) and was therefore apparently limited by transfer across the membrane under these conditions. The relationship between membrane area and steady-state cell number is not expected to be linear over the whole of its range. At zero membrane area the steady-state cell number should be zero, no nutrient transfer, however the regression line does not return to the origin. At large membrane areas cell concentration should be limited by factors in the culture fluid.

During these steady-states the cell viability was approximately 30%. The

precise value becomes very difficult to assess in low viability cultures because of the large quantity of debris.

5.1.2.2 Effect of Perfusion Rate

The cell number in an intensified system is chiefly controlled by the rate of exchange of low molecular weight nutrients. In a membrane–limited system this can be expected to be a complex function of medium perfusion rate. In order to study this a range of steady-states was achieved by perfusing medium at different rates (see Figure 21 and table 8). The fermenter was operated as described above, using medium with peptone and Pluronic. At 0.5 l d^{-1}, the steady-state cell concentration was 2.0×10^6 cells ml^{-1}. For a continuous culture without membrane retention at the same dilution rate, using the same medium and supplements, the steady-state cell concentration was 1.2×10^6 cells ml^{-1} (Al Rubeai *et al.*, 1992) and cell retention therefore seems to be responsible for increasing the yield of cells on medium by more than 50%. This is a reflection of the energetic costs entailed in synthesising biomass. Cell concentration increased with perfusion rate, the degree of increase tailing off at higher perfusion rates.

5.1.3 Calculation of Membrane Transfer Coefficients

To gain a better insight into the movement of nutrients through the system and the physical factors affecting it, a mathematical description of the system was constructed. Hagerdorn and Kargi (1990) describe a series of equations for the transport of nutrients into the fermenter from their perfusate reservoir which was not replenished *ie.* a batch reactor/batch reservoir system and this was adapted to our batch reactor/continuous reservoir system. Their equations are based on

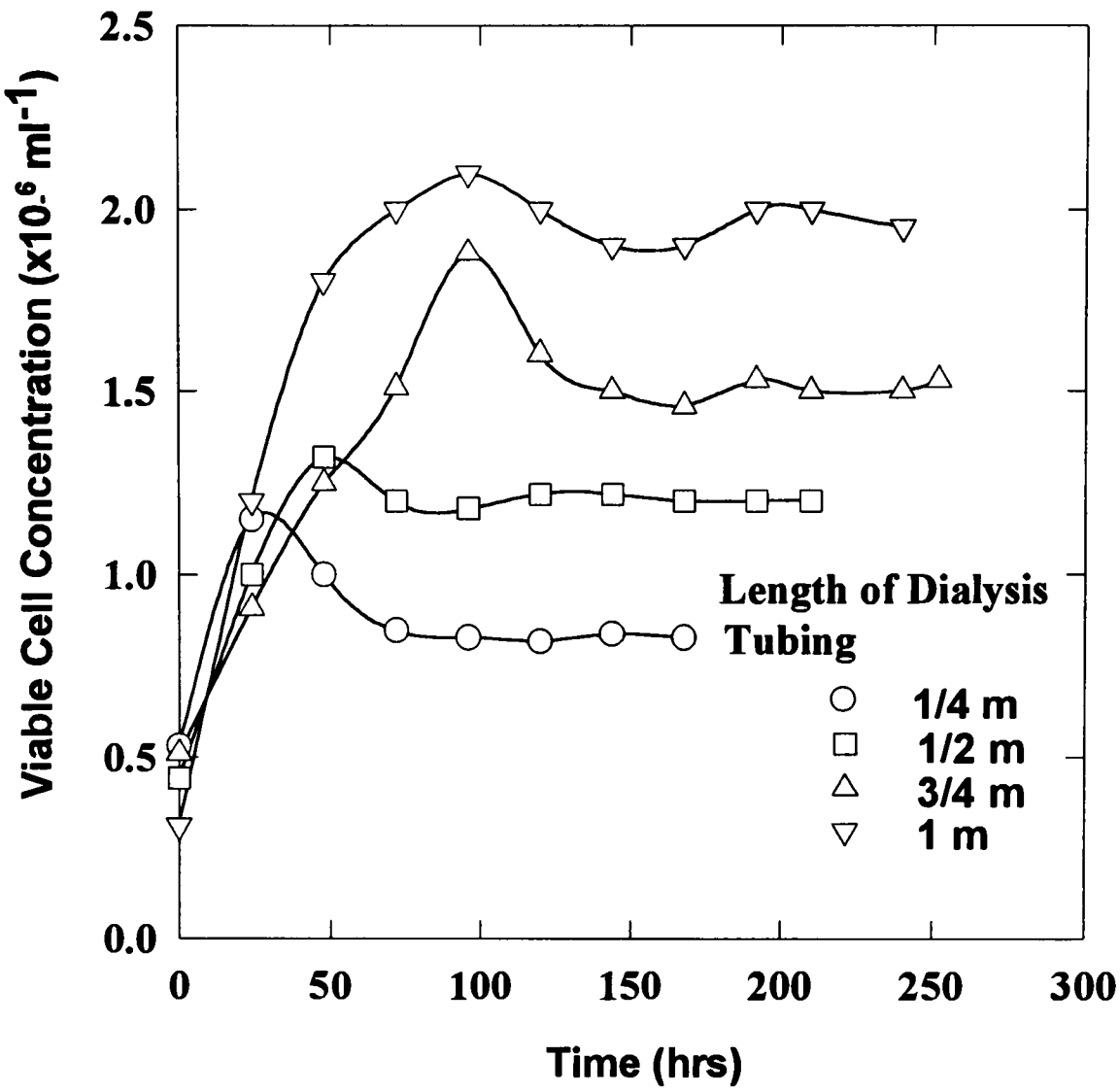


Figure 19: Cell growth of TBC3 in dialysis culture at 0.5 v/v/d perfusion rate with various lengths of dialysis tubing

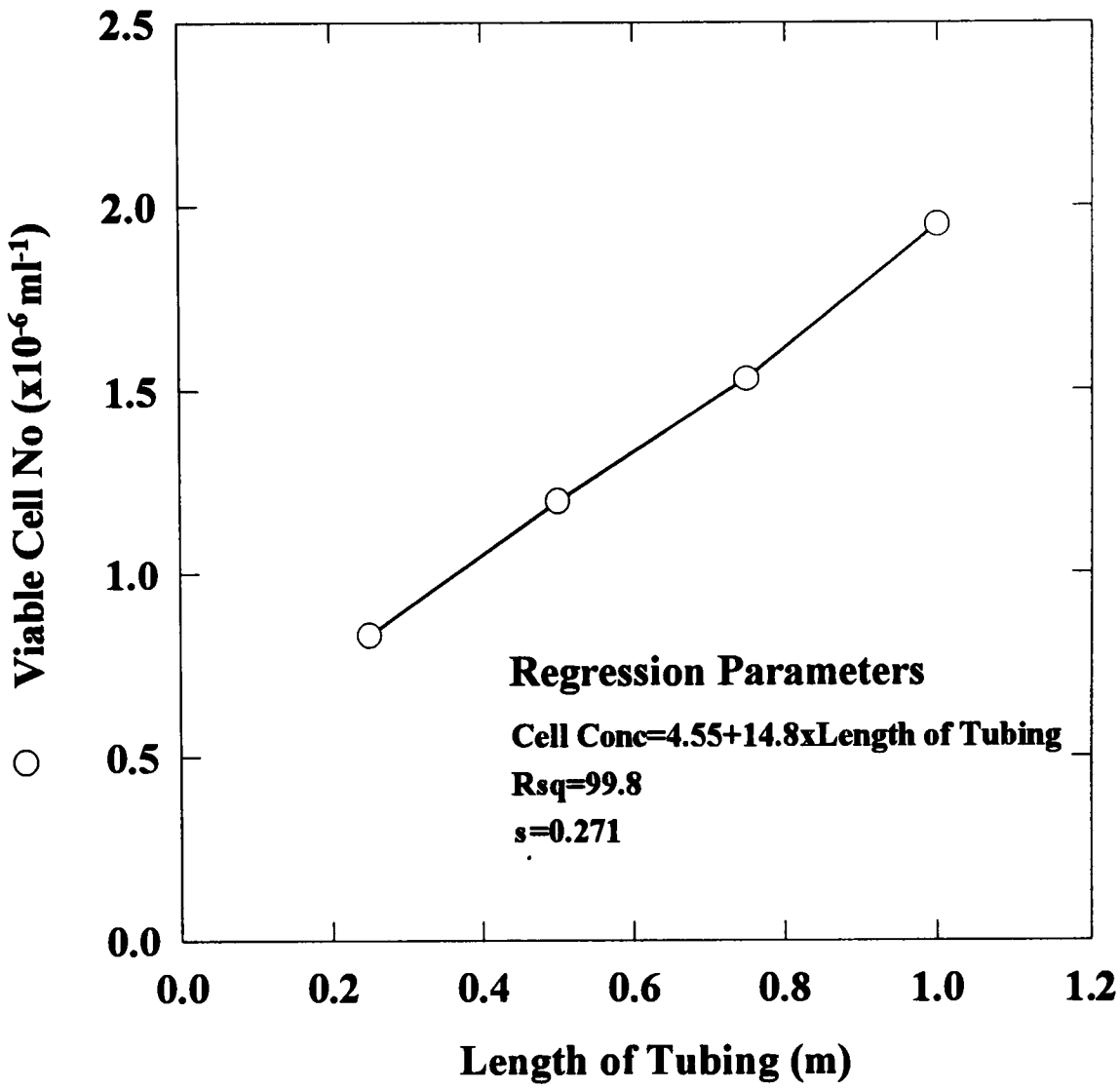


Figure 20: Effect of length of dialysis tubing on steady-state cell concentration. $\overline{ss}$ cell concentration (◯)

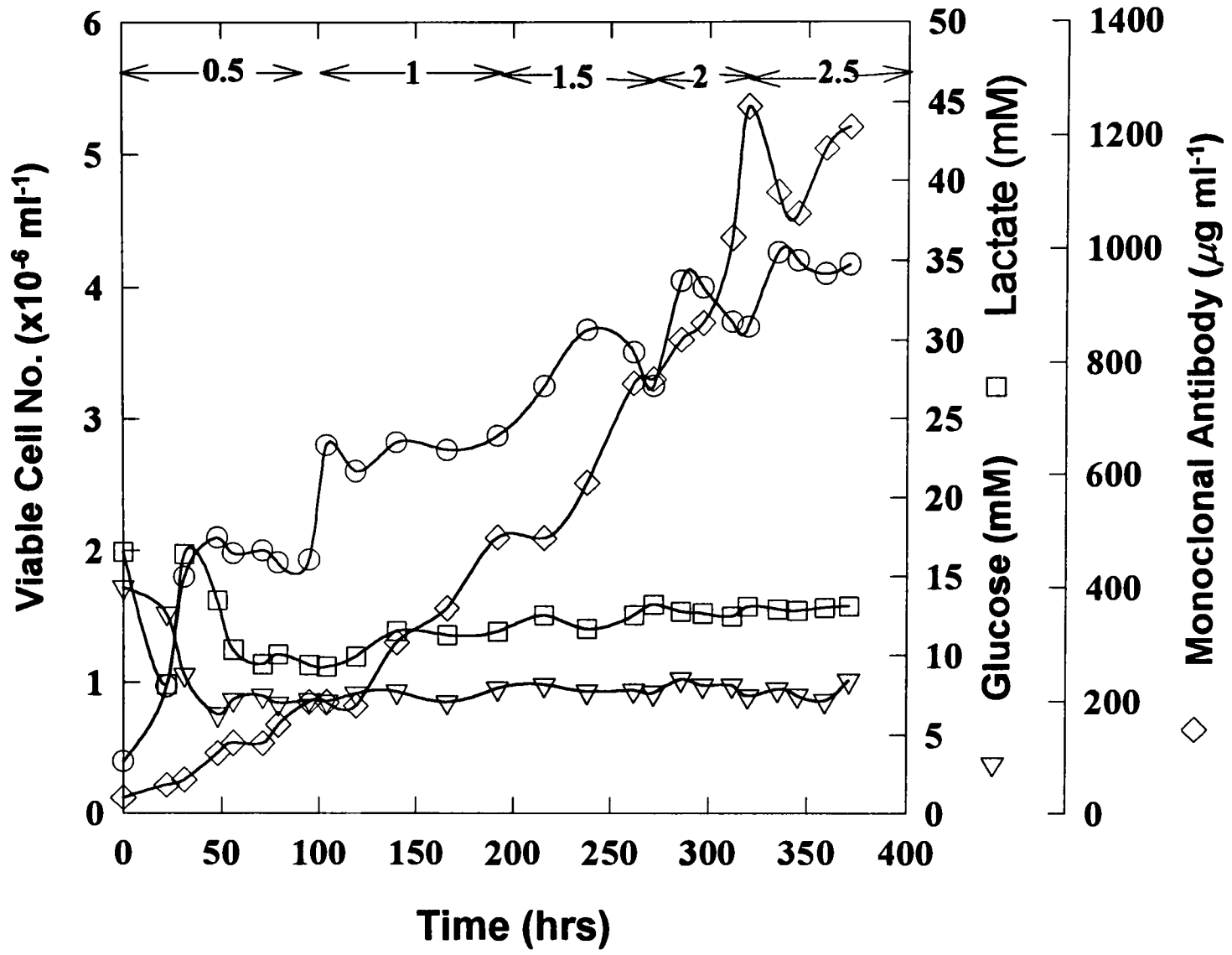


Figure 21: Effect of perfusion rate on steady-state cell concentration. Cell concentration (◯), glucose (▽), lactate (□), monoclonal antibody (◇).

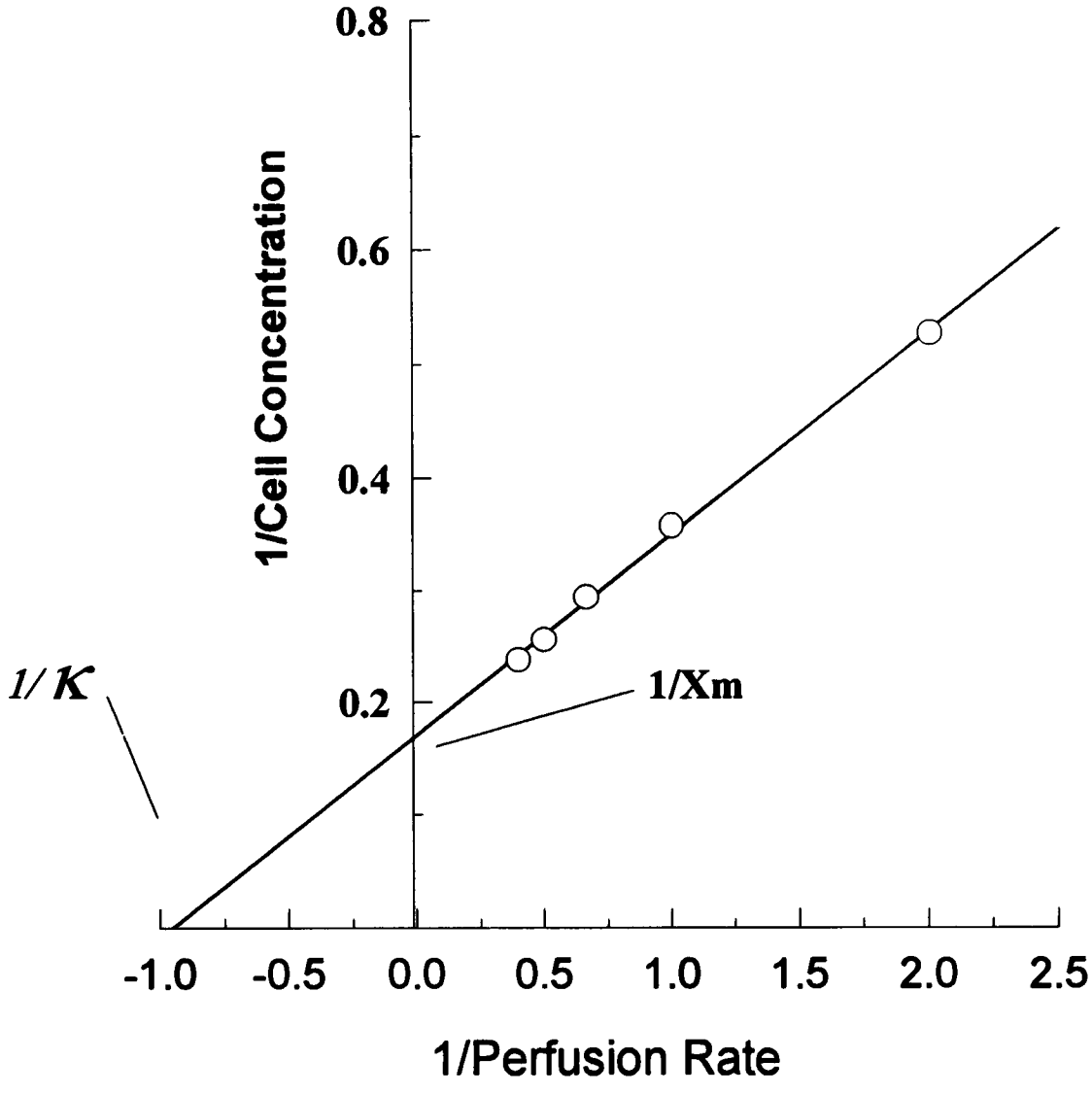


Figure 22: Plot of the reciprocal of perfusion rate against the reciprocal of the steady-state cell concentration.

Table 8: Steady-state cell concentrations $\times 10^5$ ml^{-1} at a range of Perfusion rates.

Perfusion Rate ld^{-1}	Cell Concentration $\times 10^6$ cells ml^{-1}
0	0
0.5	1.9
1.0	2.8
1.5	3.4
2.0	3.9
2.5	4.2

rate constants for the transfer of nutrients from the perfusate reservoir to the reactor across the membrane, multiplied by the ratio of membrane area to volume of culture. Figure 23 shows a schematic diagram of the membrane. Molecules pass through the membrane purely by a process of diffusion and the rate at which they do so will depend on the structure of the membrane, for example its thickness, pore density, pore size and structure and any charge on it; and the molecular type diffusing through in terms of charge, size etc. These factors should be constant for a particular membrane type and molecular species. Either side of the membrane there will be a region of stagnant liquid, through which the molecules must pass via a process of diffusion. The depths of these regions will depend on the flow of liquid in the tube and the bulk phase. In our system and in Hagerdorn and Kargi's, the flow through the dialysis tube was kept constant. The external agitation rate was also constant. Although the overall transfer coefficient should be related to the film transfer coefficients K_c and K_r and the membrane transfer coefficient K_m by $\frac{1}{K} = \frac{1}{K_c} + \frac{1}{K_m} + \frac{1}{K_r}$, in our experiments the agitation and flow conditions on either side of the membrane are constant and hence $\frac{1}{K}$ is assumed as an approximation to vary only with $\frac{1}{K_m}$.

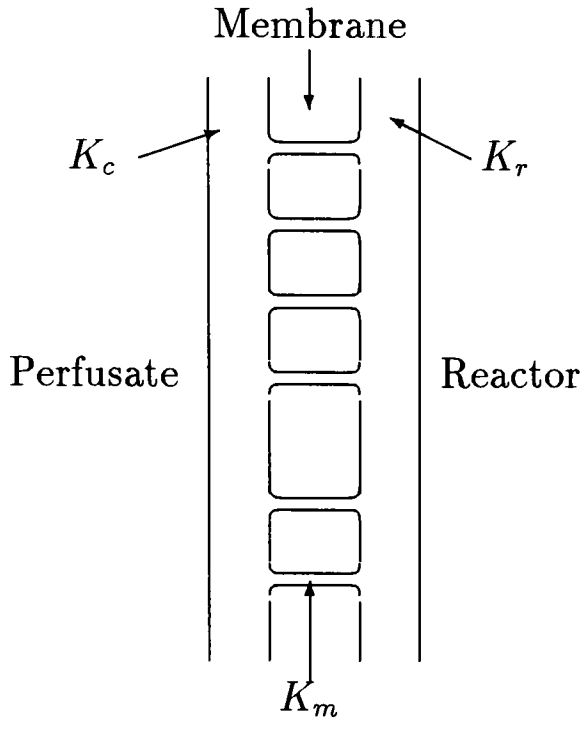


Figure 23: A schematic diagram of the dialysis membrane. K_m is the membrane transfer coefficient. K_r and K_c are the transfer coefficients for the stagnant liquid films on the reactor and perfusate faces of the membrane respectively.

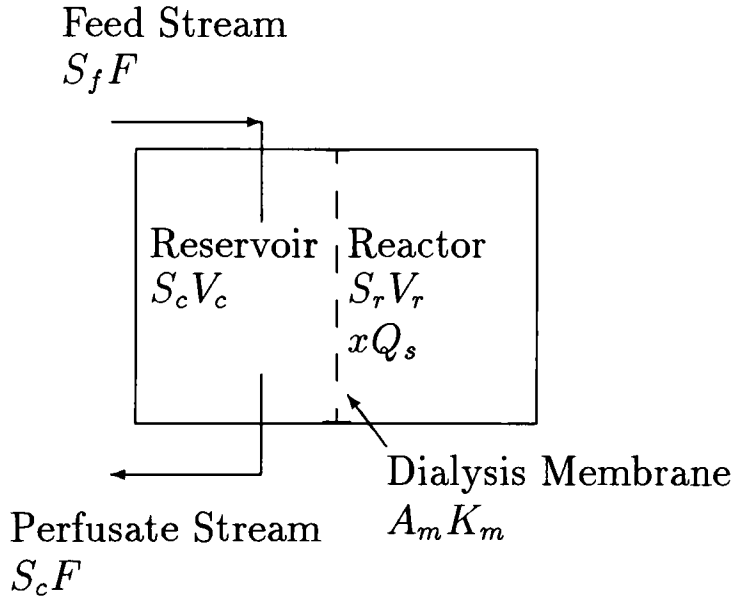


Figure 24: A simplified schematic diagram of our dialysis fermenter system. $F=$ flow rate, $V_c =$ volume of reservoir, $V_r =$ volume of reactor, $S_f =$ feed substrate concentration, $S_c =$ circulation volume substrate concentration, $S_r =$ reactor substrate concentration, $x =$ viable cell concentration, $Q_s =$ Specific rate of substrate use.

Figure 24 shows the distinction between the recirculated constant volume into which fresh substrate is fed and from which a product stream is bled and the reaction volume separated from it by the semi-permeable membrane. Substrate diffuses through the membrane in order to be consumed rapidly and irreversibly by the biomass in the reaction volume V_r. On the recirculating side the flow rate around the loop can be assumed to be high enough to produce an effectively constant concentration, S_c, of substrate in the mixing vessel and loop. The vessel is sufficiently well mixed that the fresh feed substrate (concentration S_f) can be considered to be instantly diluted and, since the product stream is taken from this vessel, this is also the concentration of substrate in that stream.

The substrate passes through the membrane due to the concentration gradient between the recirculated volume (S_c) and the reaction side (S_r). The membrane is characterised by an area A_m and a transfer coefficient K_m.

In the reaction volume V_r the substrate is consumed by the biomass at a specific rate Q_s. In the steady-state the biomass concentration is maintained at a fixed value x. This is not to imply that the net rate of cell growth–cell death is zero. Q_s therefore represents consumption both for growth and for maintenance.

Hence at steady-state, with no accumulation of substrate on either the recirculated side or the reaction side of the membrane, the consumption of substrate in the reaction side equals both the transfer rate through the membrane and the difference in substrate flux between the fresh feed and product streams, both at a flow rate F:

$$V_r x Q_s = F S_f - F S_c = A_m K_m (S_c - S_r) \tag{3}$$

and so:

$$K_m = \frac{F(S_f - S_c)}{A_m (S_c - S_r)} \tag{4}$$

The transfer coefficient for any substrate can therefore be calculated using only metabolic parameters. Similar balances can of course be made for products, including possible toxic metabolites such as ammonia and lactate. Replacing the S terms by P terms and assuming such components are not found in the fresh feed stream, then one can readily show that the product transfer coefficients are given by:

$$K_m = \frac{F P_f}{A_m (P_c - P_r)} \tag{5}$$

The central assumption of such an analysis is that Q_s and Q_p terms are both constant and independent of the S_r and P_r values. The same assumption has been used elsewhere (Wang *et al.*, 1989) to estimate cell numbers in heterogeneous reactors where direct measurements of cell numbers are difficult. (Miller *et al.*, 1989a, 1989b) showed however that Q_s for glucose and glutamine varies independently with concentration and with specific growth rate and pH for a hybridoma cell line in continuous culture. The series of experiments described here was carried out at constant pH and DOT and with significant glucose concentrations in the product stream at all states, the fresh feed medium being supplemented with 2 gl^{-1} glucose. The validity of the assumption is further supported by the fact that the observed nutrient and cell concentrations measured over the full range of flow rates used match very well (see Table 9) those calculated using a specific uptake rate calculated for the lowest flow rate.

A similar analysis by (Szperalski *et al.*, 1994) for non-steady-state conditions includes a term for fouling of the membrane. During our runs no such fouling was observed as evidenced by the K_m staying the same, keeping the S_c simulated and actual concentrations the same (see Table 9). This difference may be due to the different membrane configurations used, Szperalski *et al* used hollow fibre

membranes in bundles. The close spacing of the fibres may be responsible for trapping particles leading to the fouling. Some types of hollow fibre membrane are supported by an external spongy layer and these would be more susceptible to clogging. The smooth surface of the dialysis membrane used here should be less able to pick up material. At the end of a long dialysis run accretion is observed on the surfaces of the reactor, but none is seen on the membrane except on the upper side in the few places where the membrane touches one of the sampling ports or probes.

The K_m values for glucose and ammonia are identical to those produced by Hagerdorn and Kargi (1990) (see Table 10) and the value for lactate is similar. The transfer coefficient values differ between chemicals by more than two fold. It is possible then that the system might become limited by a factor which is poorly transferred across the membrane which normally would not be limiting in batch culture.

Membrane transfer coefficients supply necessary information for dialysis reactor design. A single steady-state experiment on a small scale, with sufficient analysis of metabolites, should permit calculation of transfer coefficients for all the major metabolites. With knowledge of metabolic parameters for a cell line, such as average nutrient uptake rates, lactate and ammonia production rates and toxicities and antibody production rates, it will be possible to optimise the medium.

Through a further simplified analysis it is possible to estimate the maximum cell number that a given reactor configuration can support. If it is assumed that the cell number in the reactor is limited by a substrate S which can pass freely through the membrane, then its concentration in the reaction volume S_r can be

Table 9: Comparison between experimental and simulated values for steady-state cell and nutrient concentrations. Simulated values were based on K_m values and Q_s values calculated from the 0.5 l/d perfusion rate. x=cell concentration, S=substratate concentration, (s)=simulated values.

D_r	x	x(s)	S_c	S_c(s)	S_r	S_r(s)
l d^{-1}	cells ml^{-1} $\times 10^6$		mM l^{-1}			
0.5	2.0	1.9	7.0	7.3	10	9.8
1.0	2.8	2.8	7.5	7.8	11.2	11.6
1.5	3.4	3.4	8	8	12.8	13.0
1.5	3.8	3.9	8.0	7.9	12.8	13.1
2.0	4.2	4.2	7.7	8.0	13.4	13.8

Table 10: Comparison of membrane transfer coefficients: experimental and from Hagerdorn and Kargi (1990).Units are m hr^{-1}.

	Exp	H+K
K_{mg}	1.6×10^{-3}	1.5×10^{-3}
K_{ma}	3.7×10^{-3}	3.7×10^{-3}
K_{ml}	2.2×10^{-3}	1.7×10^{-3}

Calculations of transfer coefficients

$$K_{MG} = \frac{21 \times 10^{-6} \times (16.6 - 9.7)}{0.037 \times (9.7 - 7.2)} = 1.6 \times 10^{-3}$$

$$K_{MA} = \frac{21 \times 10^{-6} \times 1.29}{0.037 \times (1.52 - 1.29)} = 3.7 \times 10^{-3}$$

$$K_{MA} = \frac{21 \times 10^{-6} \times 7.14}{0.037 \times (8.93 - 7.14)} = 2.2 \times 10^{-3}$$

assumed to be rather low, certainly much less than S_c, so that:

$$S_c = \frac{FS_f}{A_m K_m + F} \tag{6}$$

and also:

$$V_r x Q_s = A_m K_m \frac{FS_f}{A_m K_m + F} \tag{7}$$

At very high fresh feed flow rates $F \gg A_m K_m$ and the biomass that could be maintained approaches a theoretical maximum value x_m which is given by:

$$x_m = \frac{A_m K_m S_f}{V_r Q_s} \tag{8}$$

This equation contains only fixed variables and the maximum cell number achievable in a given reactor configuration is then fixed solely by the fresh feed substrate concentration. It will be noted that the performance is independent of the recirculating volume. In practice this need only be set by the practicality of achieving the conditions required for good mixing and high recirculation rates for a given membrane area.

Equation 7 can therefore be expressed in the saturation form:

$$x = \frac{x_m F}{K + F} \tag{9}$$

where $K = A_m K_m$

Equation 9 can be expressed in the double reciprocal form:

$$\frac{1}{x} = \left(\frac{K}{x_m} \times \frac{1}{F}\right) + \frac{1}{x_m} \tag{10}$$

A plot of $\frac{1}{x}$ against $\frac{1}{F}$ gives a straight line of slope K/x_m with intercepts on the x and y axes at $-\frac{1}{K}$ and $\frac{1}{x_m}$ respectively. Applying this to the data in Table 8 a least squares regression was used to calculate the equation of the line from which values for x_m and K were calculated (figure 22).

$$X_m \quad 5.88$$

$$K \quad 1.05$$

These equations have permitted prediction of steady-state cell concentrations without knowing the identity of the limiting nutrient. Increasing the flow rate bring diminishing returns at values above that of K, so a maximum useful perfusion rate will be reached based on economic factors. Using the K value from the regression to calculate the K_m for the limiting nutrient a figure of 0.12 cm^2 hr^{-1} was obtained, a smaller value than the others previously calculated. This suggests that the limiting nutrient/product is unlikely to be lactate or ammonia, which have larger transfer coefficients. Though it may be glucose, the more likely possibility is glutamine. The maximum achievable cell concentration for this system is, then, approximately 5.9×10^6 cells ml^{-1}, compared to the maximum we have achieved which is 4.0×10^6 cells ml^{-1}. To increase the cell concentration substantially from the present value would require large increases in perfusion rate. Increasing membrane area or using a different membrane type with a higher porosity, or molecular weight cut-off will increase the rate of transfer of the limiting nutrient and so will also increase the steady-state cell concentrations.

5.1.4 Testing

5.1.4.1 Half Membrane Area

To test the general applicability of the model derived above it was decided to use the parameters gained from the previous experiment to predict the cell concentrations under a different set of conditions. Equation 7 predicts and Figure 19 shows a dependence of cell concentration on membrane area, so it was decided to vary the latter. The area was reduced by half to 0.0185 m^2 and therefore the value of

Table 11: Cell concentrations predicted for a membrane area of $0.0185m^2$, using parameters from previous experiment

Flow rate ld^{-1}	Predicted Cell Concentration $\times 10^6$ cells ml^{-1}
0.5	1.47
1.0	1.96
1.5	2.20
2.0	2.35

K will be halved to 0.52; x_m is also halved (Equation 8) to 2.94. Using equation 9 the steady state cell concentrations can be predicted for a range of fresh feed flow rates. Table 11 shows the result of this calculation.

Figure 25 shows the result of a dialysis fermentation performed under identical conditions to those used for Figure 21, except that the membrane area was halved to 0.0185 m^2. Steady-states were obtained for fresh feed rates of 0.5, 1.0, 1.5 and 2 ld^{-1}. Figure 26 shows a comparison of the results obtained with those predicted on both straight and double reciprocal plots. The double reciprocal plot shows a straight line relationship, suggesting that the saturation kinetic argument holds in this case. However the value of K is 10 times higher than that predicted and the value of x_m is 4 times that predicted (see Table 12

The difference in the value of K must be due to variations in the value of K_m. These may be caused by differences in the permeability of the membrane or due to variations in the thickness of the surrounding liquid films.

The term for x_m contains K_m (Equation 8). However K_m changes by half the amount that x_m does. The other factor in x_m is $\frac{S_f}{Q_s}$ and since S_f has not changed then Q_s must have increased by a factor of 2.5.

The change in S_f/Q_s suggests either a genetic or physiological change in the

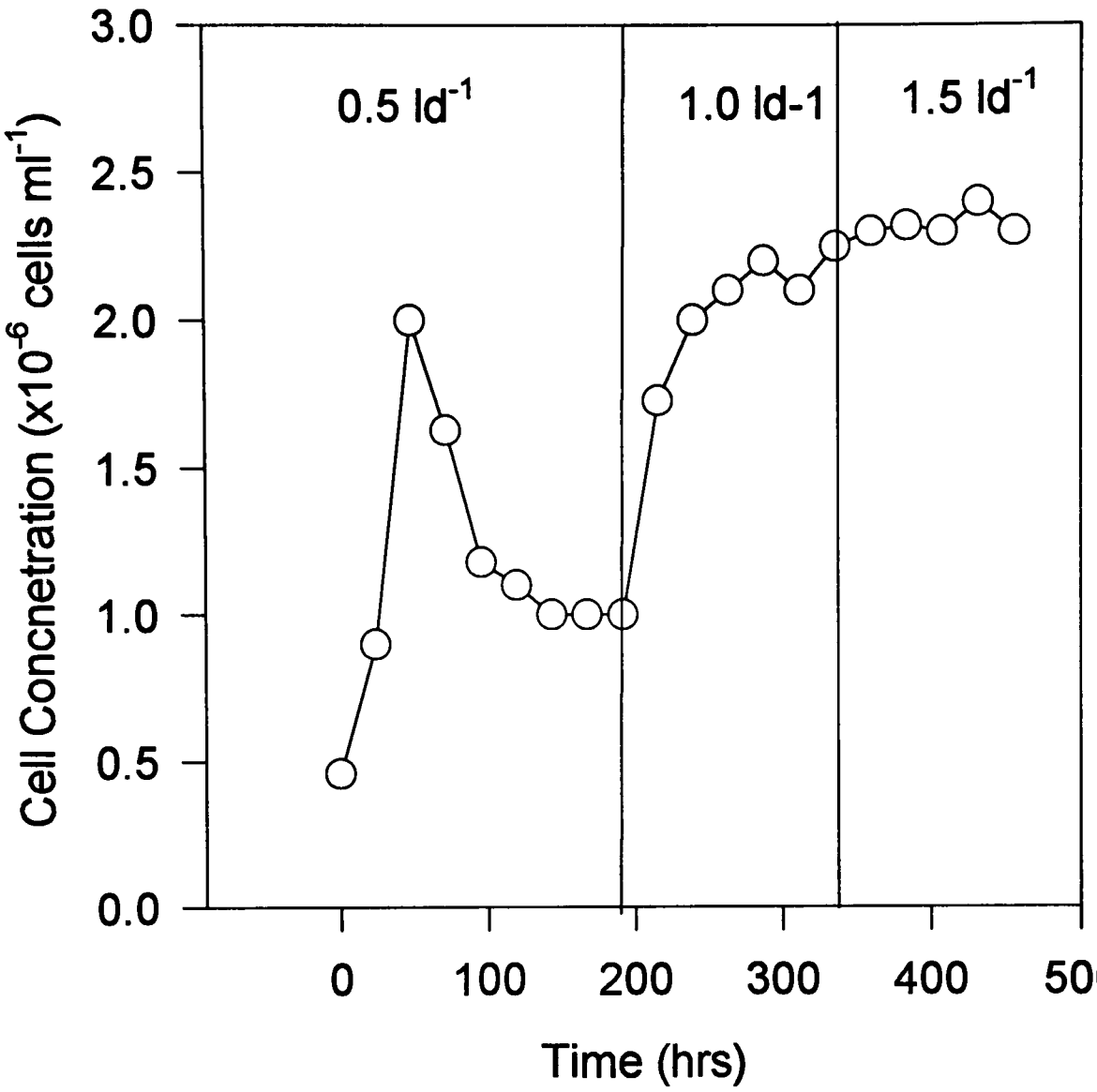


Figure 25: Dialysis culture of TBC3 using 0.0185m^2 of membrane. Cell concentration (◯).

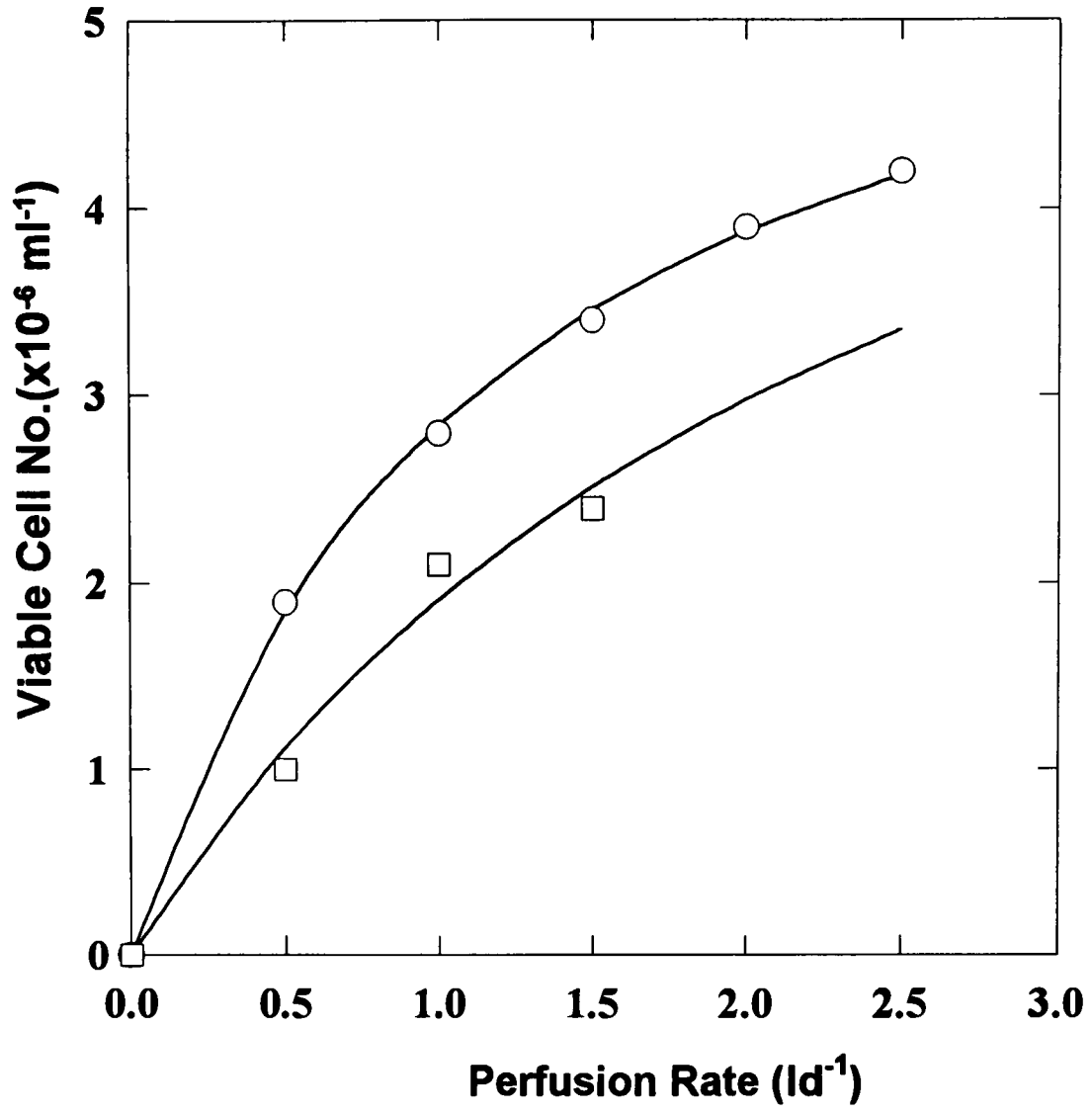


Figure 26: Simulated (line) and measured (◯) cell concentration for a range of feed rates.

Table 12: Comparison of K and x_m values for the perfusion rate experiments at full and half membrane areas

	Tubing Length			
	1.0m	0.5m		
		Measured (min,max)	Expected	Difference
K	1.05	5.04 (2,10)	0.5	10 (4,20)
x_m	5.88	11.7 (5.6,33)	2.94	4 (2,10)

cell line. This in turn may lead to a change in the identity of the limiting substrate. A different limiting substrate will have a different K_m value leading in part to the change seen here.

5.1.4.2 Double Strength Medium

Equation 7 suggests that the variables that control the steady state cell concentration are area/volume ratio, flow rate and limiting substrate concentration. The gains in cell concentrations with increasing flow rate diminish at it increases. Area to volume ratio follows a similar kinetic. Limiting substrate concentration, however, has a predicted linear relation to steady state cell concentration and is simple to alter. Since the identity of the limiting component is not known all the medium components must be changed. Jo *et al.* (1990) have grown a hybridoma cell line in medium based on RPMI 1640 but containing 5 times the concentration of amino acids and vitamins and 2.5 times the concentration of glucose. By lowering the concentration of Na Cl the osmolarity of the medium was kept at its normal value of 270 $mOskg^{-1}$ water. Final antibody concentrations increased, under batch cultivation, by up to 8 fold over standard RPMI 1640. The experi-

ments were repeated in this laboratory using commercial vitamin and amino acid concentrates (Sigma). Stoichiometric increases in viability index were achieved with double strength and quintuple strength medium (results not published) and normal batch culture behavior was seen. It was, therefore, decided to use double strength medium in the dialysis device.

Figure 27 shows the progress of a dialysis fermentation in the standard mode in the Setric fermenter, using 1 metre of tubing and 0.5 ld^{-1} of fresh feed medium. Two times RPMI 1640 was made by adding 50 ml of 100× RPMI 1640 vitamins (Sigma) and 100ml of 50× RPMI 1640 (does not contain glutamine), 10 g Glucose and 0.5g of glutamine with the standard Peptone and Pluronic supplements, whilst making up the medium as described in Materials and Methods. The medium does not contain double the quantity of glucose or peptone.

The reactor was started in batch mode with single strength medium and after 24 hours during which the cells doubled in concentration, the circulation tank was filled with medium and recirculation started. After a further 20 hours the cell density had again doubled. Perfusion was started at 0.5 ld^{-1}, but after 8 hours the cell number had not increased further, viability had fallen and the viable cells had become small and irregular in outline. The perfusion rate was increased to 1 ld^{-1} but cell density did not increase further. With the standard medium composition at 1 ld^{-1}, 3×10^6 cells ml^{-1} are to be expected, but this cell density is 40% of that value.

The most likely reason for these effects is toxicity. In batch culture this medium could be tolerated. The reason for this is not clear. It may be due to physiological adaption or differences in the physical conditions in the two systems. pH dropping in the batch culture may slow down metabolism allowing adaption to occur.

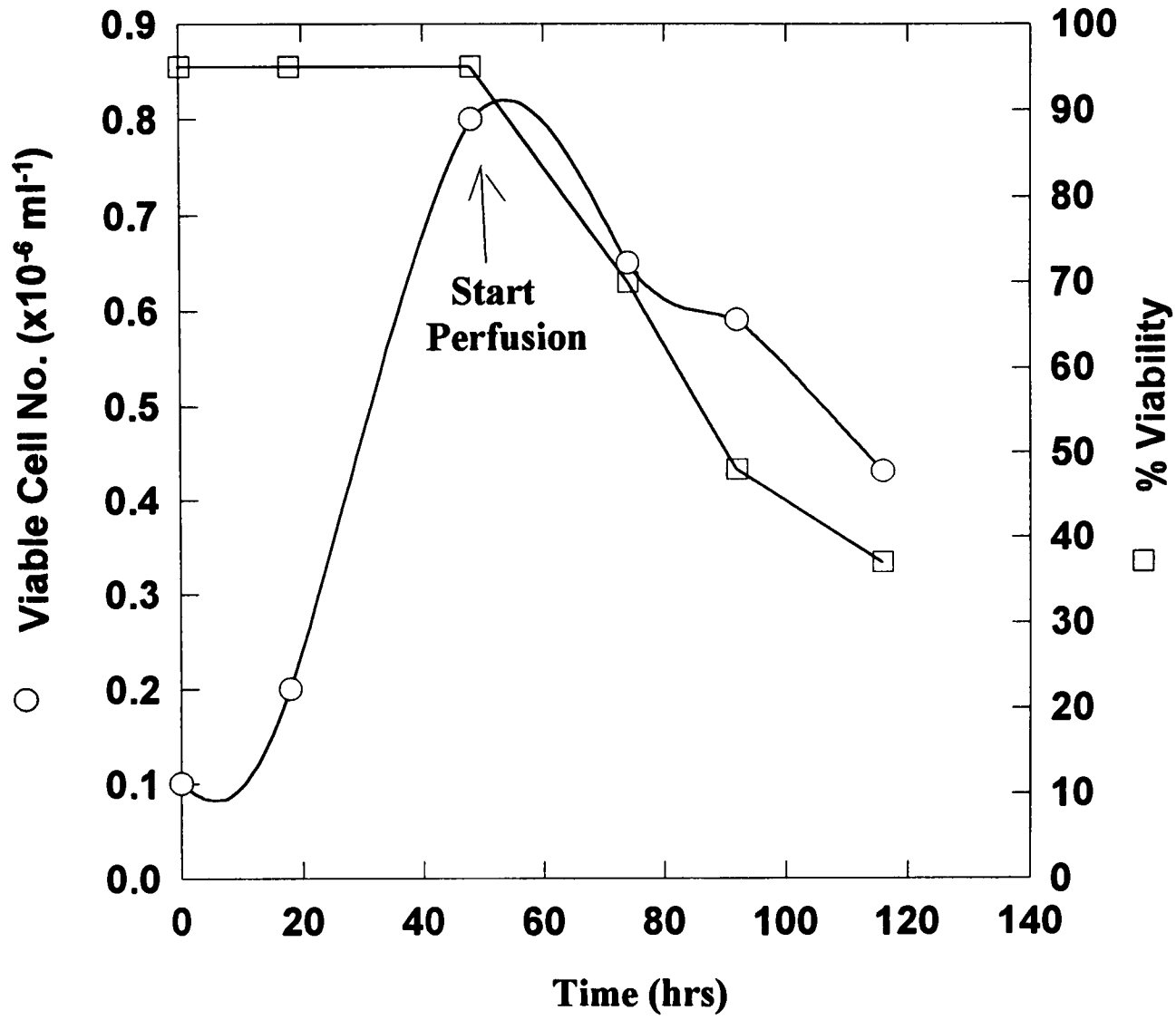


Figure 27: Dialysis culture of TBC3 using double strength medium. Cell concentration (◯), % viability (▽).

Alternatively the toxicity may lead to a weakening of the cells physically leading to damage in the stirred reactor.

5.1.5 Design and process improvements in dialysis culture

For a commercially useful device a method of arranging the dialysis membrane should be developed which allows it to be externally fabricated and then simply positioned within the reactor. It should be securely positioned so that it will not contact with the impeller and must be reasonably robust. It was decided that we should try the pleating solution as it was felt that this could be simply effected by placing the dialysis tubing over a rigid core. Medium is passed through the centre of the core and then flows upwards past the dialysis tubing, returning to the nutrient reservoir (see Figure 28). This design could easily be fabricated externally and then "plugged" into the fermenter.

For this run a further modification was also tested involving the implementation of a draw and fill operating regime for the cell-side bioreactor contents. Up to now this form of dialysis culture is essentially a batch process, however the production of the biomass inoculum for large fermenters is itself a costly process. One potential way to improve the process efficiency is to attempt a periodic selective harvest of clarified medium from the cell-side of the bioreactor whilst retaining the cells. In this way the antibody, and also hopefully any proteases, can be removed. A simple way in which this could be achieved is by letting the cells settle and then drawing off the culture fluid above it.

Figure 29 shows the growth of TBC3 in a dialysis reactor using a $1m^2$ pleated membrane. As in previous experiments, pH and dissolved oxygen tension were controlled at 7.0 and 50% of air saturation respectively. The perfusate consisted

of RPMI 1640 with 0.25% w/v of meat peptone and 0.12% Pluronic F68 and an additional 5.5 mmol litre of glucose was added. The cells were inoculated at 0.7×10^6 cells ml^{-1} and perfusion was started immediately. Steady-state cell concentration was achieved at a perfusion rate of 0.5 v/v/d giving 1.5×10^6 cells ml^{-1}. This compares with 2.0×10^6 cells ml^{-1} observed using 1 m^2 of membrane in the coiled configuration (see Table 8). This suggests that the membrane was acting at the same efficiency as $0.75m^2$ of membrane in a coiled configuration. Observation of the membrane within the reactor shows that upon medium recirculation some areas between the pleats became tightly folded denying access to the culture fluid and therefore not all the membrane area was available for dialysis.

At 270 hours one litre of RPMI 1640 with 5% new born calf serum was added to the reaction volume, the cells were allowed to settle for a short while and then one litre of culture fluid was removed from the reactor. The process of removing the culture fluid disturbed the cells and following removal of the fluid the cell count had gone down from 1.6×10^6 cells ml^{-1} to 0.87×10^6 cells ml^{-1}. Perfusion was continued at 0.5 litres per day and the cell concentration returned to its normal value. The two antibody harvests, both of one litre, contained 420 μg $ml^{-1}$and 730 μg ml^{-1}respectively. To make this procedure more effective a method of withdrawing medium without withdrawing cells should be investigated.

5.2 Discussion: What controls cell density?

The value of the steady-state cell concentration achieved by the dialysis culture device depends on 2 sets of factors; physical factors affecting the rate of nutrient transfer to and from the system and physiological factors affecting the specific

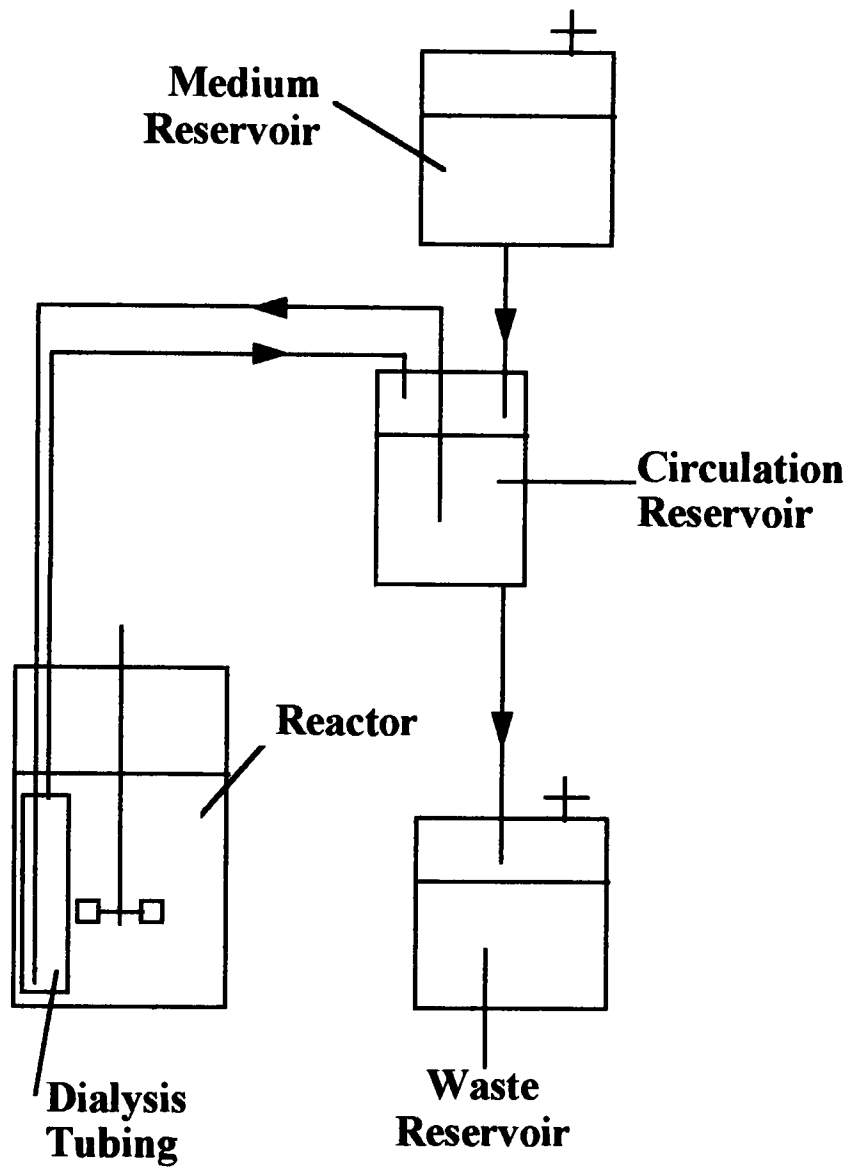


Figure 28: A method of placing dialysis tubing within a bioreactor. The tubing is threaded onto a stainless steel tube that reached the bottom of the reactor. One metre of tubing was bunched up on 0.15 metre's of tube. At either ends the tubing was sealed over a rubber bung which contained appropriate holes to allow medium to be recirculated. Medium passed down the inside of the stainless steel pipe, up through the dialysis tubing then back to the reservoir.

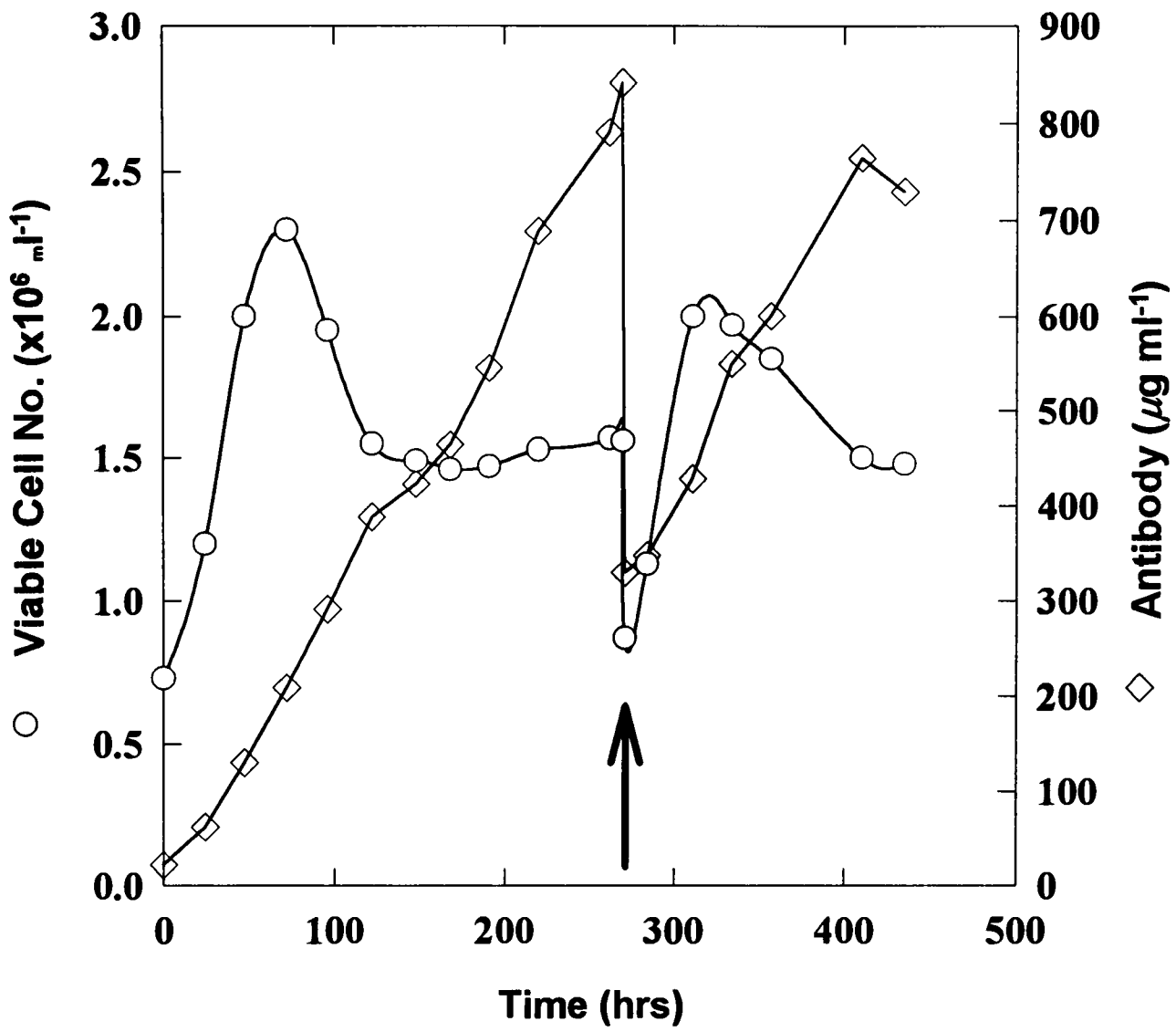


Figure 29: TBC3 in dialysis culture using the method of membrane placement shown in Figure28. At the point shown by the arrow half the culture was removed and replaced with fresh medium

requirement of the cells for nutrients and inhibition caused by the presence of toxic by-products of metabolism.

5.2.1 Physical Factors

The parameters affecting the transfer of nutrients have been simply modeled in section 5.1.3. The model presented there shows the importance of the values of the surface area to volume ratios, the transfer efficiency of the membrane, flow rate of the fresh feed and the feed substrate concentration.

5.2.1.1 Surface area to volume ratio

Experiment 21 showed that the dialysis system as it stands is limited by the absolute rate of transfer of components across the membrane. One possible way of increasing the steady-state cell concentration is to increase the membrane area in the reactor. Schultz and Gerhardt (1969) suggest that a surface area of up to 10 $cm^2\ ml^{-1}$ could be achieved with an external dialyser of 10% of the reactor volume. Our system achieves 0.4 $cm^2\ ml^{-1}$. This value can be increased but at the expense of the homogeneity of the biomass and accretion of debris caused by stagnant regions. Smaller tubing diameter will give a higher surface area to volume ratio. Boehringer Mannheim use and have patented the use of hollow fibre bundles fixed internally, which is the logical conclusion of using smaller tubing diameter. However they have observed and had to allow for in their model, significant clogging of the membrane (Szperalski *et al.*, 1994). This has not been apparent with our membrane configuration as the K_m values calculated do not decrease during the course of the reaction run. The clogging observed in the hollow fibre system may be due to the membrane type, or it may have occurred because close placement

is over 10 times the figure achieved in the reactor we operated. Reducing the radius of the tubing allows greater surface area per unit volume, at the expense of increasing the length of tubing to be arranged in the reactor. For example, from equation 11, the 0.6 litre holdup calculated above equals 2.1 metres of tubing. Methods of placing this within the reactor would become increasingly important as the length increases and this raises two issues; supporting the membrane rigidly away from its neighbours and the impeller and avoiding kinks in the tubing as it is coiled. Using the standard coiled configuration the copper former is successful within each turn of the coil, however inter-turn support of the coil would be beneficial and this could be achieved with a hook and frame arrangement. Dialysis tubing produced with a natural coil would solve the second issue. Alternatively, lengths of tubing could be suspended vertically connected by a distributor plate at the top and bottom (Figure 30). If the requirement for a standard stirred tank reactor is dropped, reactors with external loops which allow forced circulation past the membrane could be used, giving substantially higher membrane to liquid ratios

5.2.1.2 Transfer Coefficient

The amount of material transferred through a membrane depends on the difference in concentration across the membrane and the resistance to transport caused by the membrane. Two factors affect the size of the membrane transport coefficient:

- The membrane itself hinders the diffusion of the solute across it.
- Concentration gradients may exist in the liquid either side of the membrane

of the fibres allowed accretion between them.

What is the maximum surface area to culture volume ratio achievable in this system? The dialysis tubing has a volume (V_t), the radius (r) of which affects the working volume of the reactor. Surface area and volume are related as below:

$$A_m = \frac{2V_t}{r} \tag{11}$$

If a standard stirred reactor is used, then space must be left for the impeller and other projections into the culture volume. Tubing can then take up as large a fraction as is consistent with good mixing to minimise accretion and Nernst films (see section 5.2.1.2.2). This is not simple to assess as it will depend on the reactor geometry, impeller type, etc. Impeller radius is a fraction (w) of the radius of the reactor. Since the height of the liquid in the reactor depends also on the volume of the tubing the approach we take is to consider the volume taken by the dialysis tubing as an area and a fraction (g) of the remaining area after removal of the area taken by the impeller (w) which when multiplied by the culture volume gives the holdup.

$$V_t = \text{Culture Volume}\frac{\text{Tubing Area}}{\text{Total Area - Tubing Area}} \tag{12}$$

$$V_t = V_r\frac{\pi g(r^2 - (rw)^2)}{\pi r^2 - \pi g(r^2 - (rw)^2)} \tag{13}$$

Dividing by $\pi g r^2$ this simplifies to:-

$$V_t = \frac{V_r \times (1 - w^2)}{1/g - (1 - w^2)} \tag{14}$$

Given $V_r = 1$ litre, with an impeller of half the diameter of the reactor (w=0.5) and half the remaining area as tubing (g=0.5) the tubing volume V_t equals 0.6 l. Applying equation 11 the specific area produced by this is 4.0 cm^2 ml^{-1}. This

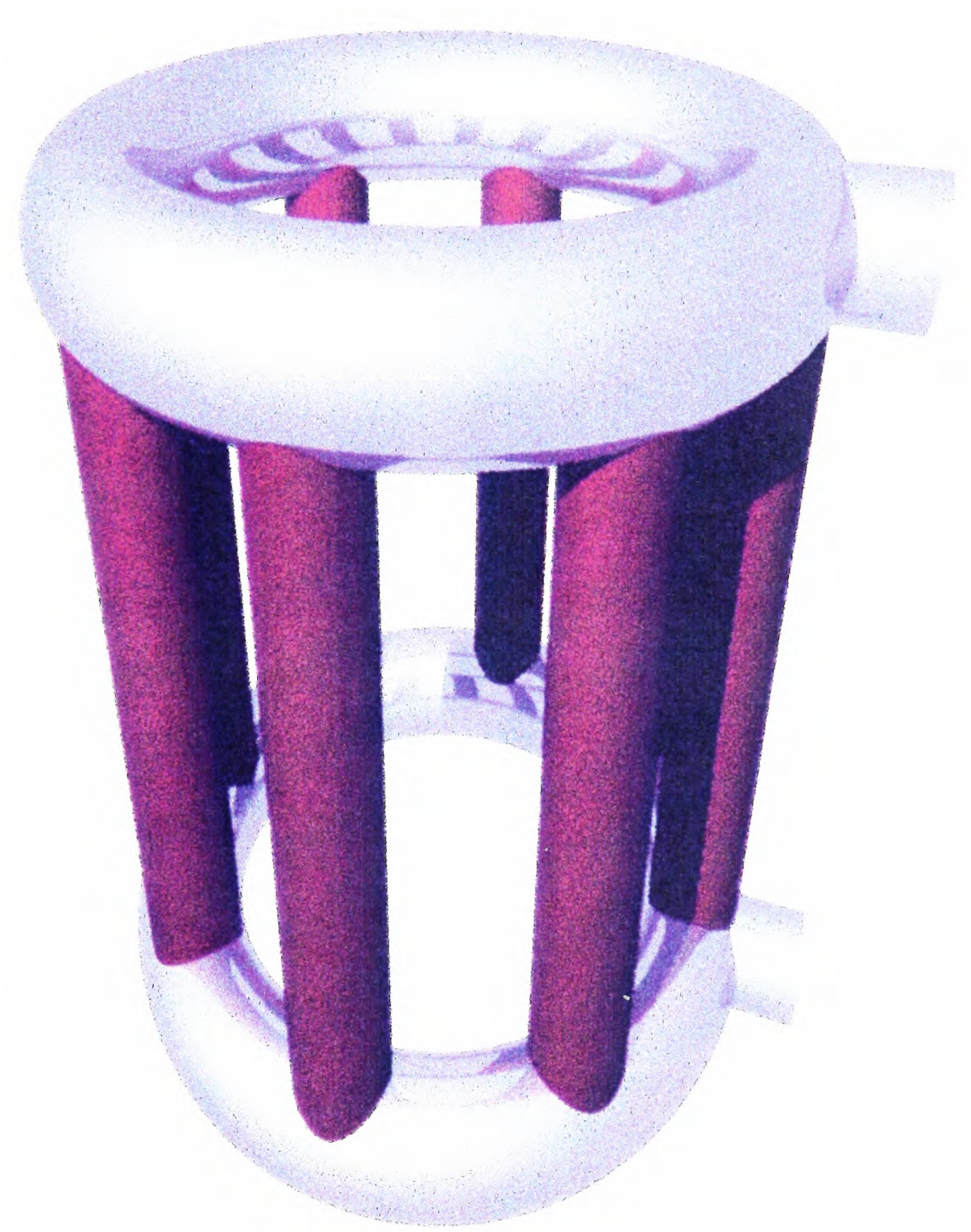


Figure 30: Another way of placing dialysis tubing within a reactor, consisting of circular tubes top and bottom with the dialysis tubing connecting them. Medium enters a toral distributor plate (silver) and flows up through lengths of dialysis tubing (red) to another distributor plate and is recycled.

5.2.1.2.1 Hindrance to transport in the membrane Semi-permeable membranes do not consist of an array of even sized, straight sided, holes that stretch the width of the membrane, rather they consist of an irregular three-dimensional net of different sized holes. A molecule passing the membrane would therefore have to negotiate a maze of interconnected passages. The rate of passage of molecules through the membrane will depend on the average length of the passage of the solute through the membrane and this is affected by the depth of the membrane and the way the passages are connected. These terms are summed as the tortuosity (τ). The percentage of the volume of the membrane that is accessible to the solute is known as the porosity (ϵ). An ideal semi-permeable membrane would offer the same hindrance to transport to all solutes of molecular weight less than its cut-off. In practice larger solutes may not be able to enter smaller pores and thus they see a smaller porosity. This effect is known as the sieving factor (H) and an empirical formula had been developed to account for it (Mason and Lonsdale, 1990)

$$H = (1 - m/M)^2 \tag{15}$$

where **m** is the radius of the molecule and **M** is the average radius of the passages. Similarly, larger molecules will experience a greater degree of drag due to edge effects (I) and an empirical expression for this factor is:

$$I = \frac{1 - (m/M)^{2.5}}{1 - 0.3956(m/M) + 1.0616(m/M)^2} \tag{16}$$

The size of the membrane portion of the transfer coefficient can therefore be estimated from those characteristics of the membrane which have the effect of modulating the diffusivity of the solute in free solution D_o.

$$K_m \approx \left(\frac{\epsilon}{\tau}\right) HID_o \tag{17}$$

Table 13: Estimation of the hindrance to solute movement caused by the membrane for a number of different solutes and a 12,000 dalton cut off regenerated cellulose membrane. Based on the method of Mason and Lonsdale (1990)

	Glucose	Lactate	Ammonia	Pore
Molecular weight ($g\ i^{-1}$)	180	90	18	12,000
$\sqrt[3]{M.Wt.}$	5.65	4.48	2.62	22.89
m/M	0.247	0.196	0.122	
Diffusivity ($cm^2\ hr^{-1}$)	19.2	25.0	47.2	
HI	0.290	0.390	0.591	
Calculated membrane transport coefficient ($cm\ hr^{-1}$)	0.92	1.61	4.58	
Measured membrane transport coefficient ($cm\ hr^{-1}$)	0.16	0.22	0.37	

Table 14: Normalised values for the diffusion of metabolites. Free: in dilute aqueous solution; Experimental: as measured using equation 21 and seen in table 10; Calculated: as shown in table 13

Solute	Diffusion coefficient		
	Free	Measured	Calculated
Glucose	1	1	1
Lactate	1.3	1.37	1.75
Ammonium	2.45	2.3	5.00

Values of ϵ (0.33) and τ (2) for similar membranes can be found in the literature (Sakai *et al.*, 1987). Molecular radius can be estimated from the the square root of the molecular weight and diffusivities of solutes in water are available in the literature (Green and Malony, 1984). Table 13 shows these calculations for the metabolites measured during the dialysis runs.

The values calculated are approximately ten times higher than those measured. This suggests that the boundary layer diffusion resistance could be significant. If this is so then the variations in the membrane transport coefficients should reflect the free diffusivity of the molecule more than the membrane hindered diffusion. Table 14 shows the values for the diffusion coefficients normalised to the value for glucose, from which it can be seen that this is indeed the case.

5.2.1.2.2 Hindrance to transport in liquid films Fluids flow in either a laminar or turbulent manner. Turbulent liquid flow will minimise the depth of the layers of stationary liquid either side of the membrane known as the Nernst films. Whether the liquid flow is laminar or turbulent can be predicted by the Reynolds Number of the system (Coulson *et al.*, 1990).

Because the pump speed is fixed, the circulation velocity of the liquid inside the membrane is constant. Treating the dialysis tube as a a cylinder the Reynolds Number can be estimated as :-

$$Re = \frac{rv\rho}{\phi} \tag{18}$$

where r is the diameter of the tubing, v is the velocity of the liquid, ρ is the density of the fluid and ϕ is the viscosity. Using the values for water as approximations the density equals 1×10^{-3} kg m^3 and the viscosity is 1×10^{-3} Ns m^{-1} (Coulson *et al.*, 1990). The dialysis tubing used was 0.012 m in diameter and the flow rate was 10 ml min^{-1} and hence the Reynolds number can be calculated to be 18 *i.e.* it is in the laminar region. To obtain turbulent flow a considerable increase in the flow rates would be required and this would only be possible by putting a significant pressure difference across the membrane, which could in turn lead to significant convective transfer of liquid across the membrane (see section 5.2.1.3).

Flow on the outside of the membrane is controlled by the impeller. The impeller speed used in this series of experiments was limited to 100 rpm. Impeller size was limited by the necessity of placing the membrane within the reactor. The impeller Reynolds number of a reactor with a Rushton turbine is given by the following equation:

$$Re = \frac{\rho D^2 N}{\phi} \tag{19}$$

where $\rho + \phi$ are as above N is the rotation rate of the agitator in hertz (0.167)

and D is the impeller diameter in meters (0.04). This gives a Reynolds number of 2667. This is just in the turbulent region for stirred tanks (Coulson *et al.*, 1990). Nevertheless more vigorous mixing may be desirable to minimise the size of the Nernst films and minimise any accumulation of debris on the upper surfaces of the membrane.

Studies performed in this laboratory (Oh *et al.*, 1992), with TBC3 show that the cell is capable of growing whilst being subjected to greater rates of agitation. Greater Reynolds numbers may be achieved with a larger impeller but this takes up more space.

Referring to the term K in Equation 8, it has been shown above that the values of A_m and K_m may both be increased, under ideal circumstances by a factor of 10. By assuming that overall an increase in K by a factor of 10 is possible and using this in Equation 8 cell concentration against perfusion rate was estimated (Figure 31). At 2.5 l d^{-1} the estimated cell concentration rose from 4×10^6 cells ml^{-1} to 11×10^6 cells ml^{-1}.

5.2.1.3 Pressure Differences

In theory concentration gradients of solutes, or pressure differences across the membrane, could cause significant transfer of volume via pressure differences. In practice this was not observed in the experiments conducted probably because the various effects canceled each other out. However, in some situations such as a pressure gradient caused by either poor gas exit from the reactor, or by pressure caused by pumping in the circulation loop, substantial transfer could occur (Schultz and Gerhardt, 1969).

The rate of dialysis through a semi-permeable membrane is defined by the

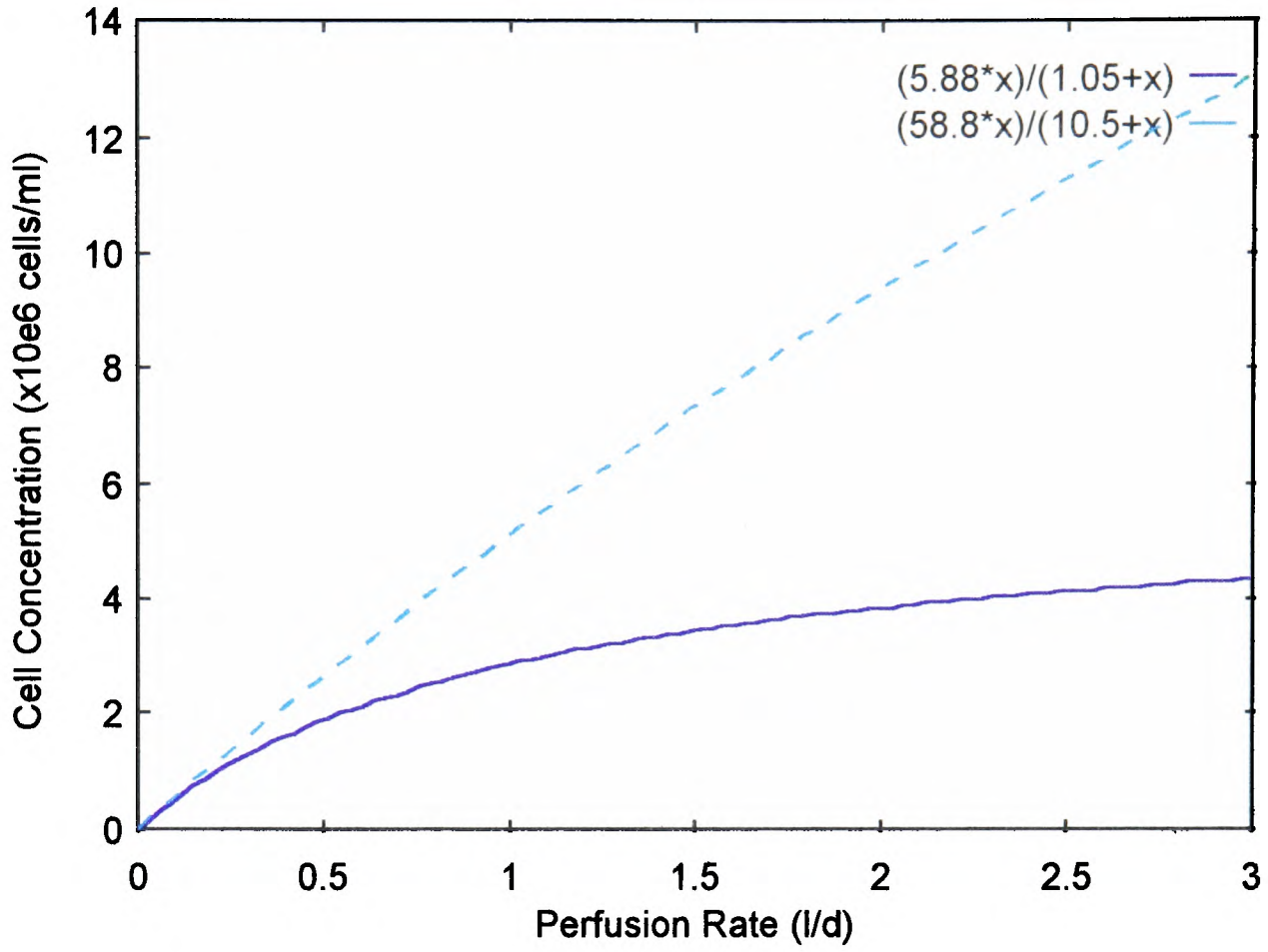


Figure 31: Cell concentration verses perfusion rate as modeled by Equation 8. From experimental values (—), calculated assuming that K may be increased by a factor of 10 (...)

following equation (Staverman, 1952).

$$Q = K_f(\Delta P - RT \sum_i \sigma_i \Delta S_i) \tag{20}$$

where Q is the filtration rate through the membrane ($cm^3\ hr^{-1}$), K_f is an empirically determined constant, ΔP the difference in pressure across the membrane and R is the gas constant, T is the temperature in degrees kelvin, ΔS_i is the concentration gradient of a solute, σ_i is the reflection constant for that solute which accounts for the non-ideal semi-permeable behavior of membranes (1 for non-permeable solutes to 0 for freely permeable solutes).

If ΔS is considered to be zero, then the equation simplifies to.

$$Q = K_f P \tag{21}$$

K_f has been estimated, for cellulose dialysis membranes, to be approximately 6×10^{-2} $cm^3 atm^{-1}\ hr^{-1}$ (Renkin, 1954). Given a pressure difference of 0.1 atm, Q=2.4 $cm^3\ hr^{-1}\ cm^{-2}$. This gives a total transfer rate for the dialysis reactor of 57.6 $cm^3\ day^{-1}$, *i.e.* one whole volume of the reactor (1 litre) in 418 hours. Pressure differences therefore must be considered when designing and operating dialysis reactors. Osmotic pressure differences could be used to advantage to replace liquid volume in the reactor or to concentrate the contents of the reactor (Schultz and Gerhardt, 1969).

5.2.1.4 Fresh Feed Flow

Fresh feed flow rate is the operational variable that controls the value of the steady-state cell concentration. Increases in flow rate give diminishing returns in increased cell concentration because the perfusing medium tends to its feed concentration. Given that specific productivity is not a variable of flow rate or

cell concentration then volumetric productivity is a linear function of flow rate. The value of the feed rate chosen will depend on the required rate of production.

5.2.1.5 Nutrient Concentration

Increasing the nutrient concentration, according to the model, gives linear increases in cell concentration. The model however does not account for the toxic effects of metabolic by-products. In practice, attempts to increase the medium concentration led to the cells dying in dialysis culture, but not in batch culture. The optimum balance of nutrients in medium for a dialysis culture may well be different to that for batch culture.

5.2.2 Physiological Factors

Given set physical conditions, the cell concentration achieved depends on a range of physiological factors. In Figures 35 and 48 it can be seen that given similar physical conditions and similar medium conditions significantly different steady-state cell densities were achieved by TBC3 and CHO (see 8.1). These differences must be caused by differences in cellular physiology.

Section 5.1.1 showed that significant rates of cell turnover were occurring during so-called steady-states. The rates of cell division and cell death are independent of each other but are influenced in opposite directions by similar factors, principal amongst them being the metabolite/product concentrations (Figure 32). Energy status *i.e.* the balance between energy generation and consumption, is also influenced by the concentrations of both substrates and products. Energy status may affect the rate of both the growth and death, and could be the mechanism for the effect of substrate and product concentrations. In the following sections

we discuss some of these factors

As discussed previously (Section 4.3) the levels of the principal toxic metabolites, lactate and ammonium, seen in our dialysis system, are less than have been implicated in significant inhibition of cell growth. In addition to this the data from section 5.1.3 for the K_m value of the limiting factor suggests a higher molecular weight than that of either lactate or ammonia, although this does not rule out the possibility of some unknown inhibitor.

5.2.2.1 Cell Turnover

Cell turnover rate influences the quantity of energy used in biomass construction, and thus the achieved cell concentration in energy limited systems, the viability, the cell-specific productivity (Al-Rubeai and Emery, 1990) and the release of intracellular proteases (see section 7.2.1).

The observed turnover of cells during the steady-state consists of three rates (see Figure 33). Firstly the rate of cell growth (μ), secondly the rate of cell death (k_d) and thirdly the rate of dead cell disintegration (k_r).

$$x_v\mu = x_v k_d = x_d k_r \tag{22}$$

Since we can measure the rate of cell division using DNA synthesis measurements, the other rates can be calculated given the cell concentration and the viability. At steady-state cell concetration and viability $\mu = k_d$. Measurements of DNA synthesis for TBC3 give estimates for the cell division rate, at steady-state and thus also the cell death rate as 0.11–0.19 d$^-$1. With viability at 40% $k_r = 0.165 - 0.285d^{-1}$.

Measured viability is a dynamic equilibrium between cell death and dead cell disintegration. Many authors assume that no cell turnover is occurring during

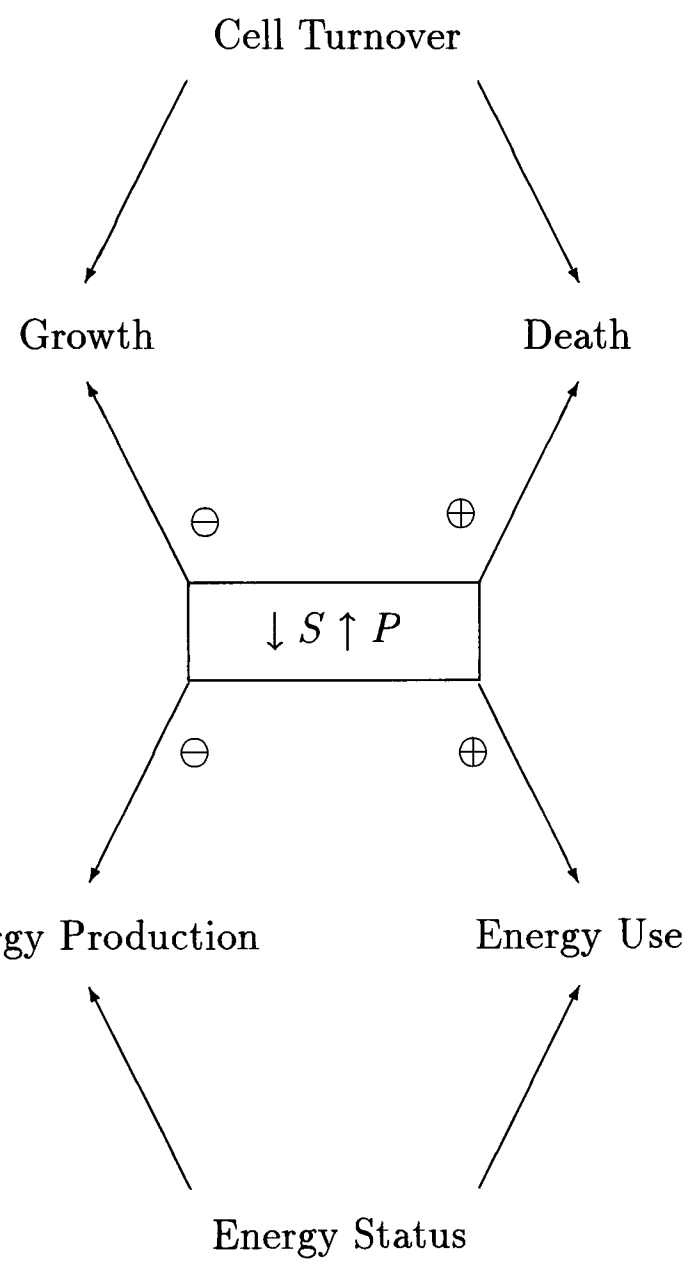


Figure 32: The effect of energy substrate/toxic by-product concentration on cell density and cell energy status. S=substrate concentration, P=product concentration, $\oplus, \ominus$ positive and negative influences respectively.

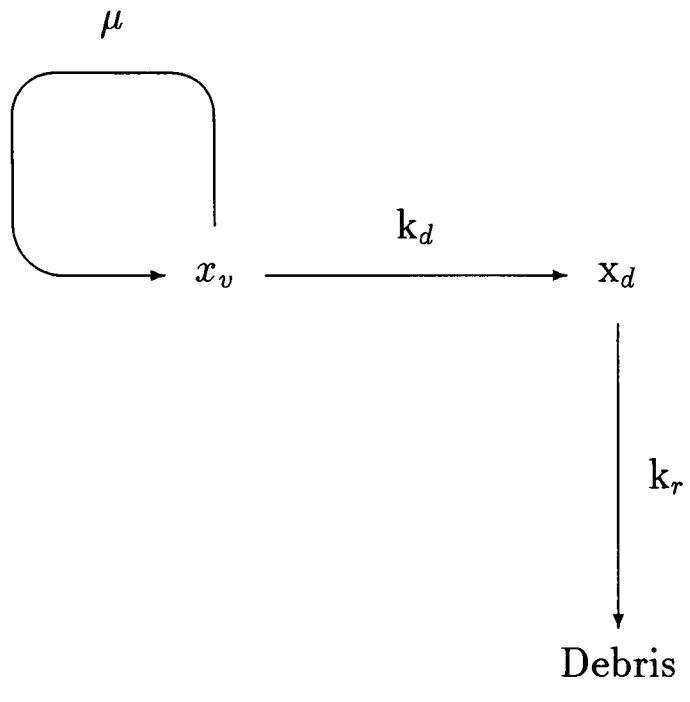


Figure 33: The three rates that control the value of steady-state cell concentration and viability as defined in Equation 22. Rates:μ=cell division, k_d=cell death, k_r=dead cell disintegration. Concentrations:x_v=viable cell concentration, x_d=dead cell concentration.

extended steady-state operation of their bioreactors (Ackermann *et al.*, 1994; Robinson *et al.*, 1994). Table 15 shows rates of cell turnover during apparent steady-states for CHO, BHK and several hybridoma cell lines and a variety of culture systems. Cell turnover rates are in the range of 0.05–0.4 d^{-1}. It seems likely that cell turnover is occurring during steady states in most perfusion systems.

Most of the references calculated turnover rate by assessing the rate of release of the intracellular enzyme lactate dehydrogenase (De Tremblay *et al.*, 1992). Another assessed the rate of DNA synthesis by blocking cell division with colchicine and measuring the accumulation of cells in the G_2 stage of the cell cycle using flow cytometery (Borth *et al.*, 1992). The final method used a measurement of the dead cell disintegration rate in a balance (as Equation 22) with the viable and dead cell concentration (Jan *et al.*, 1991).

When the limiting substrate concentration falls to that of the order of the K_m value, not the same as the membrane transfer coefficient, for the limiting enzyme in the pathway the growth rate will decrease significantly. At the same time as metabolic stress is put on the cells the death rate will increase until it balances the rate of cell growth and steady-state is achieved. The cell concentration is ultimately determined by the efficiency of use of medium ($Y_{\frac{x}{m}}$). The rate of cell turnover is determined by the rate of cell death.

Cell death may occur by either of two mechanisms (Scwartz and Osborne, 1993). Necrosis is a passive process in which osmotic balance is disturbed due to plasma membrane damage which allows passive entry of ions most notably Ca^{2+}, this leads to the cell swelling. The Ca^{2+} concentration increases inhibiting many enzymatic pathways including ATP production and stimulating other such as proteolysis.

Apoptosis is an active process, it requires protein synthesis in order to proceed and is a significant source of cell death in bioreactors for some cell types, especially lymphoid cell lines (Singh *et al.*, 1994b). The surface membrane boils and "blebs" then pinches off to produce "apoptotic bodies". Chromatin condenses along inner surface of the nuclear membrane. DNA is digested by an endogenous nuclease which cleaves between nucleosomes leading to a distinctive banding effect after DNA electrophoresis with a repeat of 180 basepairs, the intra-nucleosomal repeat distance. Apoptosis is triggered by many things including growth factor concentration, irradiation, heat-shock, a variety of xenobiotics, and nutrient limitation. Cells will die of apoptosis under these conditions unless the damage is severe enough to prevent protein synthesis in which case the cells die of necrosis. Necrosis will occur if the cell is subjected to mechanical damage or severe

metabolic stress.

The *bcl-2* proto-oncogene can inhibit apoptosis in a variety of cell types. BCL-2 has been expressed in Burkitt's lymphoma cells (Gregory *et al.*, 1990). Comparison of batch cultures of transfected and non-transfected examples of this cell line have shown that BCL-2 product increased the maximum cell density and viability index of stationary cultures (Singh *et al.*, 1994a). In another experiment cells were resuspended in glutamine-free medium. The apparent death rate of the transfected cells was considerably decreased in comparison to non-transfected cells. If these increases also occur in dialysis culture steady-state cell density could be improved by transfecting BCL-2 into hybridoma cells. Unless there is a change in efficiency of medium use this will not lead to increased steady-state cell densities. However, if the rate of cell turnover decreases the energy used to synthesise biomass will be saved. This will lead to an increased cell concentration.

The immediate death of TBC3 in double strength medium under dialysis but not batch conditions may be caused by triggering of apoptosis. One common factor amongst many of the triggers for apoptosis is that they increase the level of oxidative stress in cells (Buttke and Sandstrom, 1994). Serum contains a variety of low and high molecular weight anti-oxidants. The low molecular weight anti-oxidants would be removed by dialysis. The double strength medium may provide a variety of metabolic stresses on the cell such as an increased ammonia concentration. This hypothesis is supported by the observation that the stressed cells had shrunk.

Table 15: Cell turnover rate in immobilised cell systems. Culture systems= Alginate entrapment (AE), microcarrier (MC), macroporous microcarrier (MMC), fibre pad (FP) cross-flow filtration (XFF) Hollow fibre, cells in extracappiliary space (HF) and dialysis (DLS). The turnover rates were calculated by measuring DNA synthesis rate, cell death rate by release of intracellular lactate dehydrogenase (LDH), or calculated from measurements of dead cell destruction rate and total and viable cell concentrations.

Turnover Rate (d^{-1})	Cell Line	Culture System	Method	Reference
0.05–0.125	CHO	MC	DNA synthesis	(Borth *et al.*, 1992)
0.10	BHK	MMC	LDH	(Kratje *et al.*, 1994)
0.10	Hyb	MC	LDH	(De Tremblay *et al.*, 1992)
0.30–0.40	Hyb	FP	LDH	(Yamaji and Fukuda, 1992)
0.17	Hyb	HF	Model	(De la Broise *et al.*, 1992)
0.30–0.33	Hyb	AE + XFF	Model	(De la Broise *et al.*, 1992)
0.24	Hyb	XFF	LDH	(Pinton *et al.*, 1991)
0.30	Hyb	DLS	Model	(Hagerdorn and Kargi, 1990
0.12	CHO	DLS	DNA synthesis	Own
0.15	Hyb	DLS	DNA synthesis	Own

5.2.2.2 Energy Production and Maintenance Energy

Cells have two basic uses for energy; for production of new biomass; and for a variety of functions needed to keep the cell viable, such as protein turnover, maintenance of ionic gradients etc., collectively known as maintenance. About 60% of the ATP demand in a hybridoma cell line growing at 0.66 hr^{-1} was used for maintenance according to Miller *et al.* (1988). The proportion of energy used for maintenance could be expected to rise as the cell division rate decreases. The specific demand for maintenance energy and the actual growth rate during apparent steady-states, determine the cell concentration achieved in those steady-states.

Adenosine tri-phosphate is the universal currency with which energy is exchanged within the cell (Alberts *et al.*, 1986). The rate at which it is produced can be simply estimated from oxygen uptake and lactate production data (Glacken, 1988). This can vary according to the conditions experienced including growth rate (Hiller *et al.*, 1993) and nutrient supply. In *Homo sapiens* the rate of ATP production has a range from 0.4–9.0 g ATP/Kg body weight/hour (Erecinska and Wilson, 1981).

ATP production in hybridoma cells is apparently rather inefficient (Glacken, 1988; Mancuso *et al.*, 1994). Most of the glucose is only partly oxidised to lactate instead of the much more efficient oxidation to CO_2 and H_2O. Glutamine is also partly oxidised with production of alanine and aspartate. It has been suggested that the high glucose and glutamine fluxes are necessary to achieve sensitive control of nucleic acid and ribose phosphate synthesis (Szondy and Newsholme, 1990). Increases in efficiency of ATP production are therefore theoretically possible. Indeed the natural dynamic range of ATP use in man is 20 fold (Erecinska and

Wilson, 1981).

Maintenance energy may also be variable. Ammonia's toxic effects may be due to an increase in the need for ATP consuming, ion pumping, work which increases the level of the maintenance energy demand (Martinelle and Häggstrōm, 1993; Newland *et al.*, 1994). Lactate toxicity is probably due to increasing the osmolarity of the medium (Omasa *et al.*, 1992), thus increasing the osmotic work required to maintain intracellular ion concentration and thus also the maintenance energy demand.

In summary both the requirements for energy use by cells and their ability to produce it are variable depending on the cell type and the conditions experienced. The balance of these values will effect steady-state cell concentrations.

Chapter 6

Serum addition in dialysis mode

6.1 Introduction

Serum contains a variety of high and low molecular weight components which are often important for cell growth (Anderson and Lunden, 1976). Human plasma contains 70–80 gl^{-1} of protein, including more than 150 enzymes. These proteins perform many functions, principle amongst them being transport of a variety of substances to and from the cells. Serum Albumin transports fatty acids, steroids and metal ions, Lipoproteins transport lipids and cholesterol, Transferrin transports iron. Other functions include; Protease inhibition, provision of growth factors, hormones, attachment factors, and immunoglobulins, and the actions of a variety of tissue derived enzymes (Fisheman and Doellgast, 1975).

Low molecular weight components present in serum include all the amino acids, vitamins and ions present in basal media such as RPMI 1640. Additionally it contains trace elements, lipids, cholesterol, steroid hormones and components that influence the redox potential of the medium.

Many groups have been able to grow hybridomas in serum-free medium formulations (Al-Rubeai and Emery, 1989; Jager, 1991; Nielsen and Bertheussen, 1991; Wyatt, 1994). These formulations come in two types, those in which protein functions are replaced by a number of individual proteins either purified from tissue sources or produced by recombinant organisms in culture; and those other types of formulation, essentially protein free media, in which these functions are replaced by low molecular weight components. These media avoid the inherent variability of serum and, since they have a lower protein content, aid downstream processing, as well as presenting a smaller chance of introducing adventitious agents.

These media show that the functions of both high and low molecular weight components of serum are either not essential, or are replaceable *in vitro*. Why then use dialysis to retain high molecular weight medium components if they are not necessary?

Serum-free media have many disadvantages, which include: lower maximum cell densities and growth rates; loss of the protection from mechanical and oxidative stresses normally provided by serum; likewise less protection from proteolysis. However the serum-free formulations are more specific to individual cell lines than serum-containing formulations, and thus must often be reformulated for each new cell line. The protein-containing formulas are invariably more expensive than serum-containing formulations.

The basal medium that we used contains peptone which is a source of peptides and probably also lipids and trace elements. It also contains Pluronic F68 which has been included in many serum-free media formulations to protect against mechanical damage to the cell. Dialysis will remove the low molecular weight part of the serum. Previous experiments have shown that the cells are capable of grow-

ing without them in this medium for periods of hundreds of hours, so the basal medium is therefore competent for cell growth. But do low molecular weight serum components stimulate cell growth and increase steady state cell densities?

Dialysis will remove the molecular weight components below 12,000 Daltons, thus retaining the majority of the proteins,those with approximately 50 or more amino acid residues, within the reactor. These proteins will be within the reactor for extended periods of time and may be used up by the cell, or be degraded chemically or by proteases. Previous experiments do not show any fall off in steady state cell densities over time and this suggests that this effect is not significant over the time period studied. However higher concentrations of high molecular weight serum components may increase steady state cell densities.

In the experiments described in this section we investigate the role of both fractions of serum in the extended stationary states encountered in membrane bioreactor devices.

6.2 Results and Discussion

Duplicate batch cultures of 50 ml in stirred duran bottles were set up with a 10% inoculum of TBC3. A 10 ml aliquot of serum was placed in dialysis tubing and dialysed against 1 litre phosphate buffered saline overnight at 4°C. A control aliquot was also kept at 4°C, but not dialysed. Glucose was measured at 0.71 g/l in the dialysed serum and was not detectable (less than 0.05 g/l) in the non-dialysed serum. Batch cultures were set up with RPMI plus peptone and Pluronic, with whole serum or with dialysed serum.

Cell growth in dialysed serum was higher than in non-dialysed serum (Fig-

ure 34). The viability index increased from 35.6×10^6 cell hours to 41.5×10^6, and maximum cell density increased from 0.67 to 0.87 $\times10^6$ cells ml^{-1}. Low molecular weight components of the serum are, it seems, not required at the concentrations high molecular weight components are, if at all. Multi-generational studies and more thorough dialysis would be needed to confirm a total lack of requirement.

Figure 35 shows that cells have been maintained in dialysis mode for 280 hours without addition of serum. pH and dissolved oxygen tension were maintained at 7.0 and 50% air saturation respectively. The medium used was RPMI with 0.25% w/v meat peptone and 0.12% Pluronic F68. The reactor was inoculated with 0.4×10^6 cells ml^{-1}, and perfusion was started immediately at 0.5 litres per day. A steady state cell concentration was achieved at 1.5×10^6 cells ml^{-1}. Cell viability dropped to below 50% where it stayed for the remainder of the run. At 170 hours the perfusion rate was increased to 1.0 litres per day, and the viable cell concentration increased to 2.0×10^6 cells ml^{-1}. Subsequent periodic additions of 25 ml aliquots of NBS led to temporary increases in steady state cell concentration of 30–50%. After approximately 2 days the viable cell concentration then returned to the original level. These increases would therefore appear to be due to factors that were either of low molecular weight and so rapidly diluted, or were short lived, or were rapidly sequestered. The fermenter glucose concentration was however zero during this period (figure 36).

In order to examine the possibility that the reaction shown in figure 35 was glucose limited, another dialysis fermentation was then set up (figure 37). The same medium was used as in the previous experiment, but with an additional 5.5 mM per litre of glucose, bringing the total for the perfusate to 16.6 mM per litre perfusate. A steady state cell concentration at a perfusion rate of 1.0 ld^{-1} of

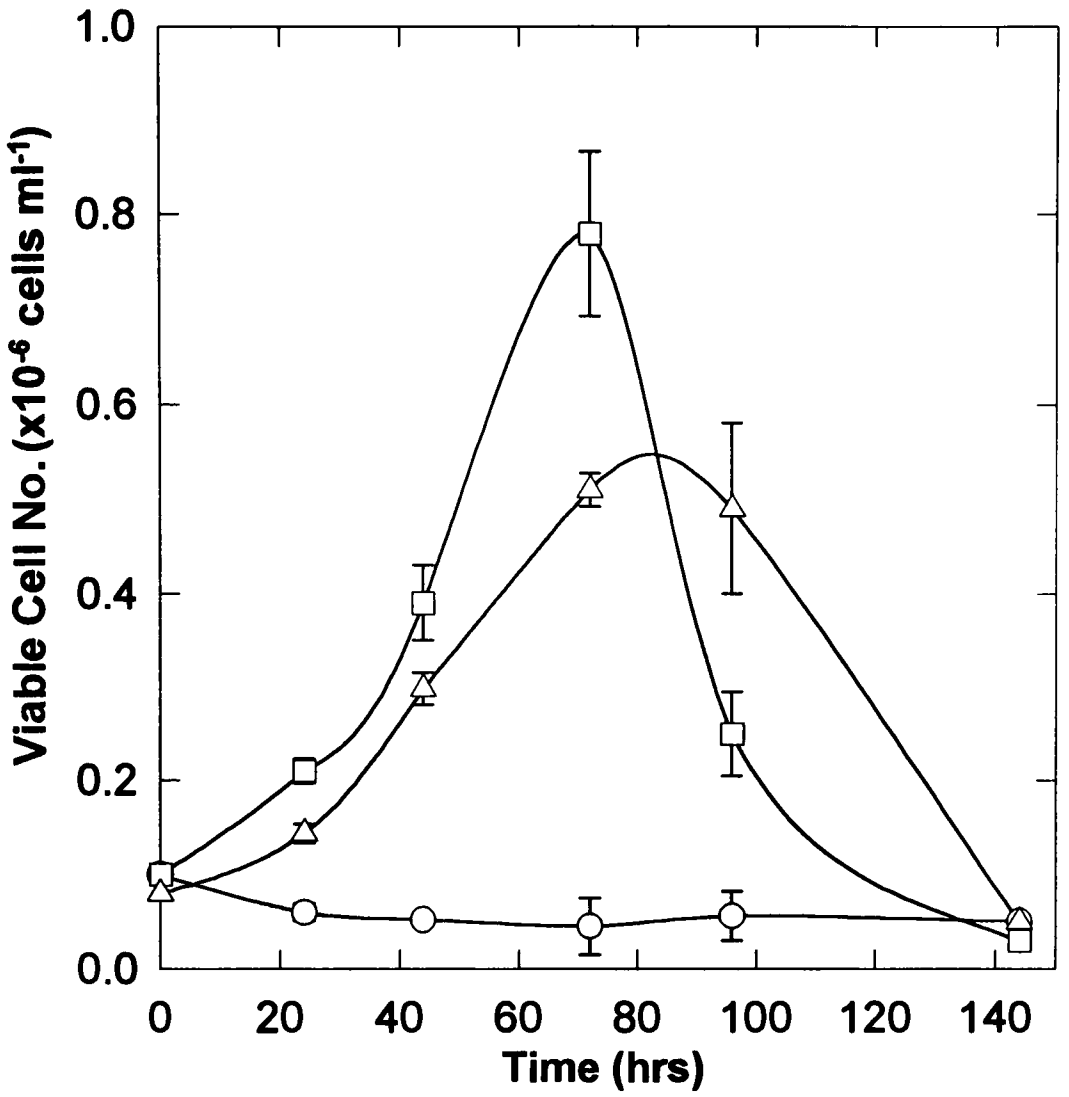


Figure 34: Comparison of the batch growth of TBC3 in dialysed and non-dialysed serum. Dialysed (□), Nondialysed (△), No serum (◯)

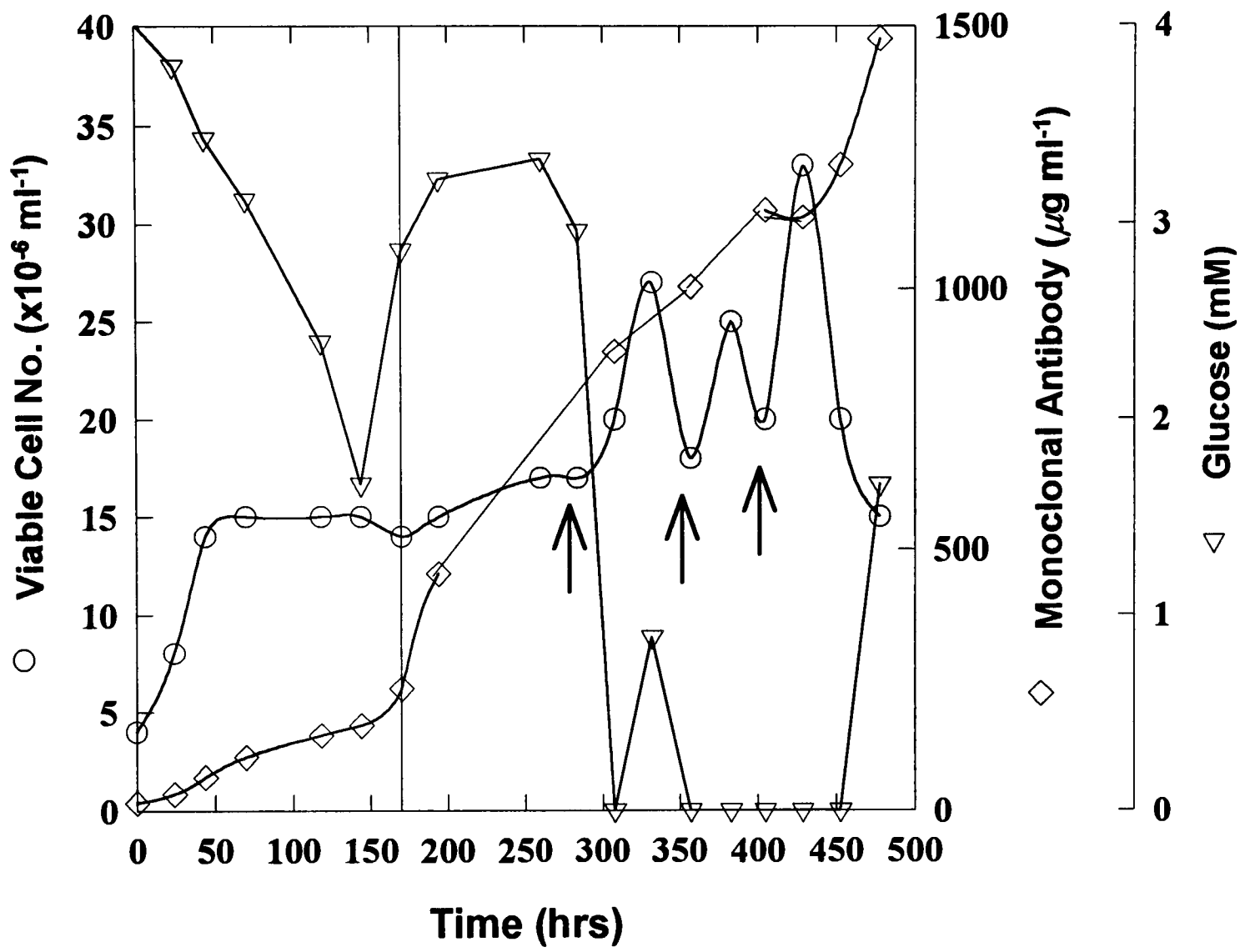


Figure 35: TBC3 grown in dialysis culture with periodic additions of serum. (○) cell concentration, (◇) Antibody Concentration, (▽) glucose concentration, (↑) serum additions.

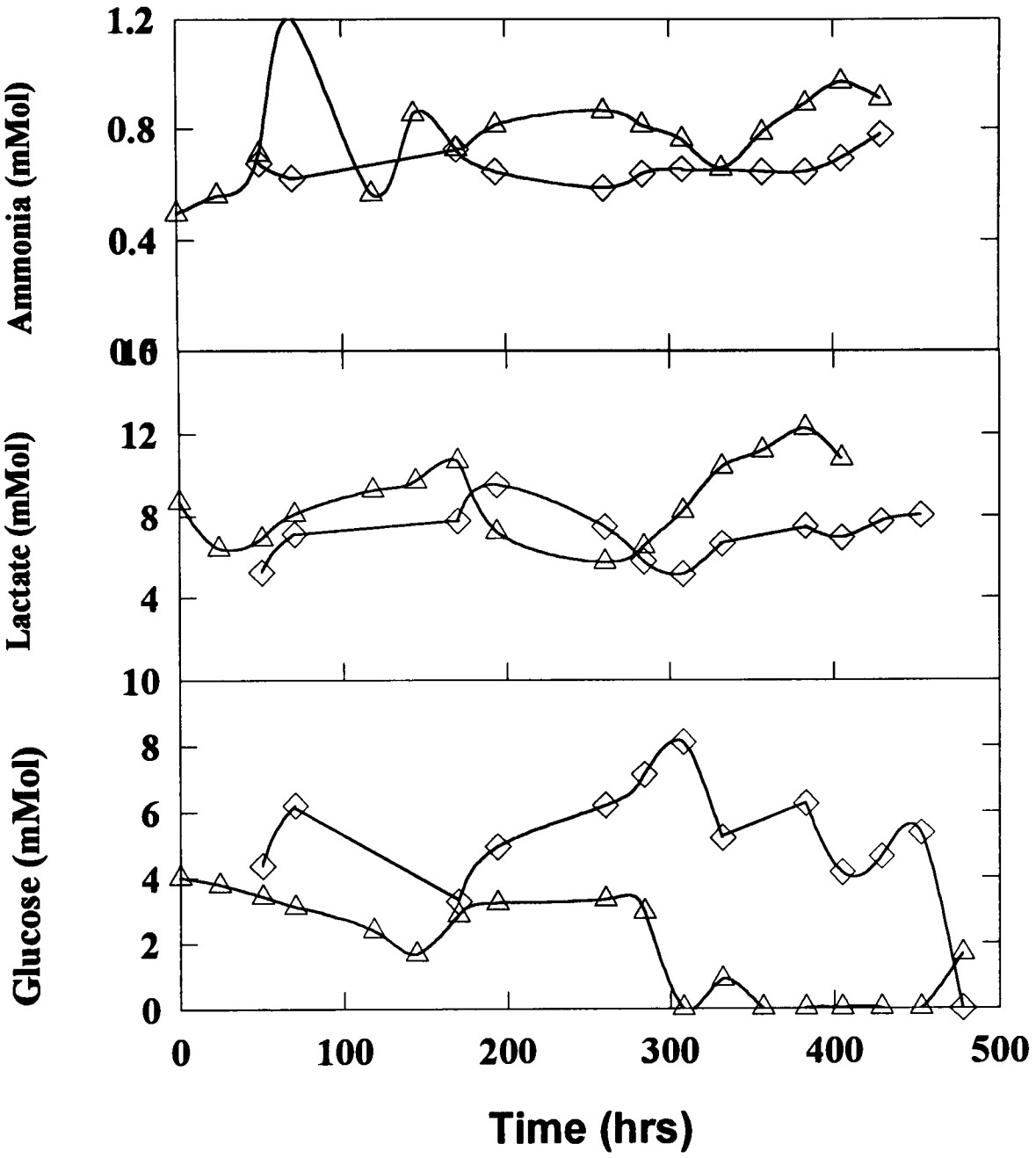


Figure 36: Metabolite concentrations for the previous fermentation. (△)Reactor metabolite concentrations, (◇) perfusate metabolite concentrations).

3.1×10^6 cells ml^{-1} was recorded, compared with 2.0×10^6 cells ml^{-1} in the previous run (figure 35). The residual glucose concentration was now 3×10^{-3}M $litre^{-1}$. At 192 hours a 10% v/v solution of NBS in RPMI 1640 was added continuously at such a rate that 1% v/v of serum was added to the reaction volume daily. Volume was maintained at one litre by removing the same volume daily when samples were taken. Under these conditions cell concentrations appeared to decrease from their previous values. The effect then of adding additional serum appears to be less than the effect of removing cells. Serum additions into the reactor appear to have, if anything, a negative effect on steady state cell concentrations. The batch experiment showed the presence of inhibitory low molecular weight component(s). The continuous addition of serum showed that they were at least not stimulatory. It appears that the initial shot of serum is sufficient for periods of at least 450 hours.

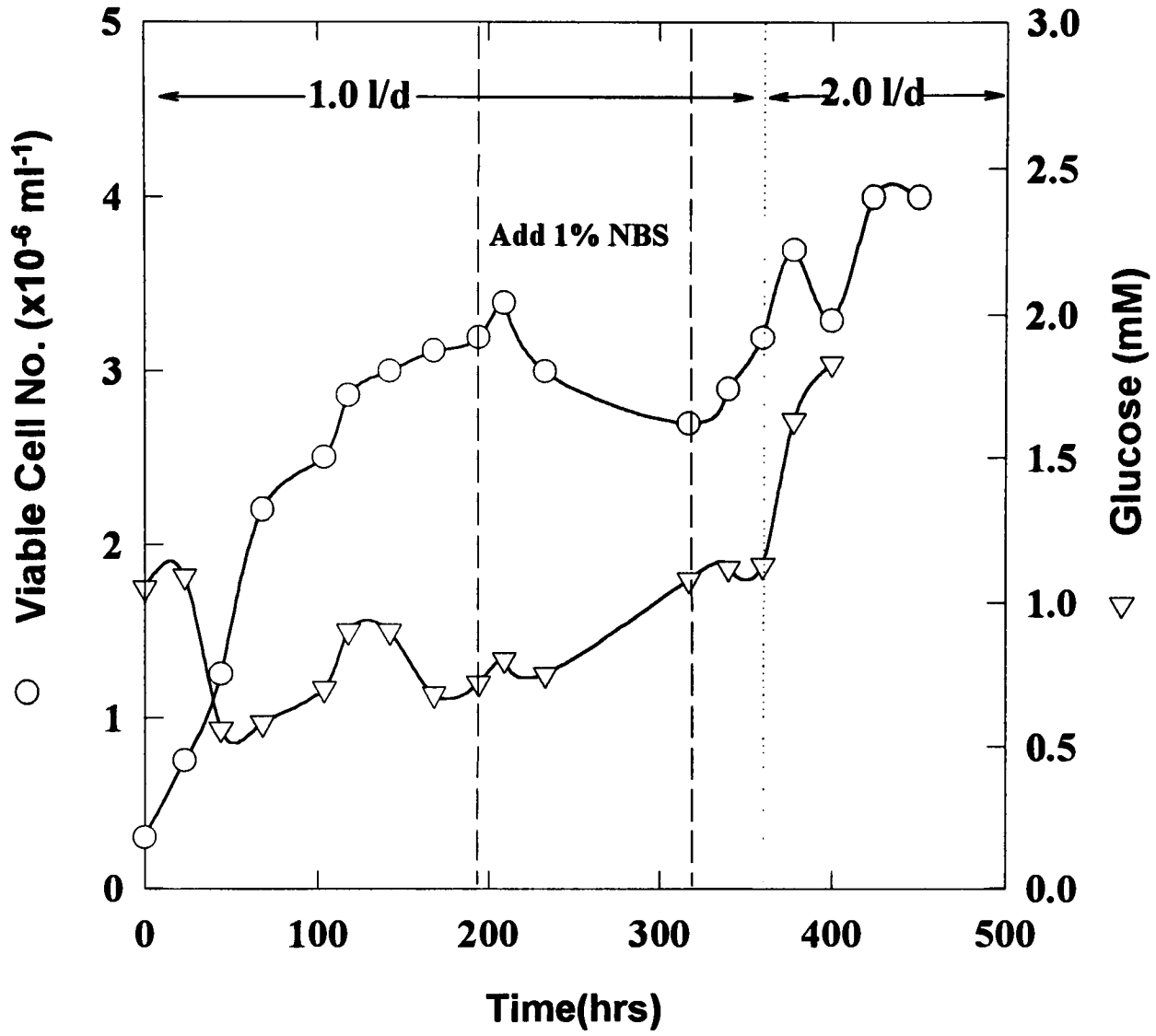


Figure 37: TBC3 grown in dialysis culture with continuous serum addition. (◯) cell concentration (▽) glucose concentration.

Chapter 7

Antibody Production

7.1 Product Accumulation

In dialysis culture antibody is retained within the system and, because the antibody molecule is relatively stable, it is accumulated to high concentrations. In Figure 35 1440 μg ml^{-1}of antibody seems to be the final concentration, while in Figure 21 the final antibody concentration is 1200 μg ml^{-1}. These values compare with those found in ascitic culture (1–10 mg ml^{-1}) (Pannel and Milstein, 1992), and are 20–30 times that seen in simple batch culture. The antibody is produced throughout the culture. However Figure 38 shows a different pattern of antibody production. Antibody concentration peaks at about 600 μg ml^{-1}and thereafter drops on two separate occasions. These drops in concentration may be due to release of proteases by the dead cells: van Erp *et al.* (1991a, 1991b), using hollow fibre bioreactors, noticed proteolytic activity leading to antibody degradation, and identified a cathepsin D type protease. This protease is normally found intracellularly, but may be released by dying cells. As our dialysis cultures at steady

state had viabilities in the region of 30–50%, it is reasonable to expect that there will be considerable protease activity present. Serum contains protease inhibitors so, in for example Figure 35, the lack of obvious protease activity may be due to the periodic additions of serum.

Specific cell productivity overall[1] in Figure 35 is 4.10 mg 10^6cells^{-1} hour^{-1} compared to 2.40mg 10^6cells^{-1} hour^{-1} for a typical batch culture (table 4). Comparing the data with other data for this cell line in a variety of different culture devices and conditions (Al Rubeai *et al.*, 1992) Figure 39 shows that the value is similar to that seen in a spin-filter device operated at high cell density and higher than seen in batch, continuous and the spin-filter system operated at low cell density. The reasons Al Rubeai *et al.* (1992) ascribe to this high productivity in spin filter systems would equally apply to this system:–

- Prolongation of the more productive G_1 and S phases of growth.
- Release of antibody from retained dead cells.

It has been shown that this cell line stores part of the antibody it synthesises (Al-Rubeai and Emery, 1990) so the low viability seen in this culture will allow a lot of this extra antibody to be released. Cell turnover will allow this release from dead cells to continue throughout the duration of the reaction.

No large variations in the specific production rate were ascribable to changes in perfusion rate or serum addition. Smaller variations may well be missed, however, because of the inherent difficulties in measuring this derived variable.

- The ELISA assay is precise only to about 10% coefficient of variation (on a good day!) and the cell count only to 5%.

[1]Calculated by dividing final antibody concentration by the total number of viable cell hours

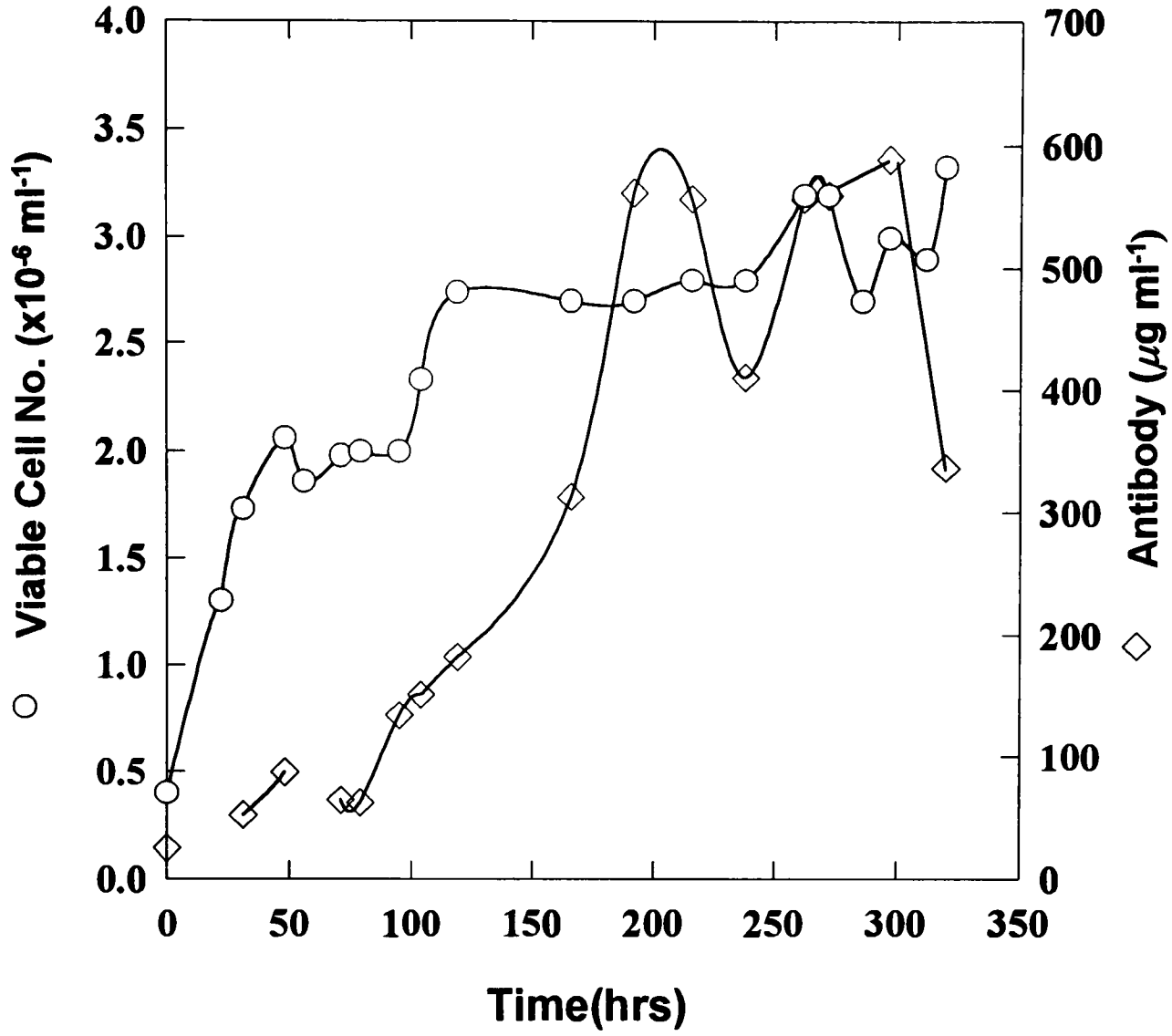


Figure 38: Dialysis fermentation with no addition of serum. (◯) Cell concentration, (◇) antibody concentration.

- Protease action will vary according to the quantity of protease inhibitors and the rate of protease release from dying cells.
- Release of antibody by dead cells will vary with the death rate of cells.

7.2 Cell size

Specific productivity has been measured on a per cell basis. However it has been suggested that a more reasonable basis for calculation is per weight of biomass (Spier, 1989). This is supported by evidence that the production of antibody is controlled at the level of translation (Bibila and Flickinger, 1991; Leno *et al.*, 1991). Measurements of this parameter were not done, because direct measurement of cell dry weight is difficult at this scale, due to the large quantity of biomass needed. Using forward scatter measurements in flow cytometry it is simple to measure relative cell size (Al-Rubeai and Emery, 1993). In Figure 40 forward scatter measurements are recorded for a dialysis culture (figure 41). It can be seen that the median cell size increases from that in the earlier samples. The cells during steady state are bigger and therefore the increases in specific productivity seen in dialysis culture over batch culture are partly due to an increase in the quantity of biomass.

7.2.1 Product Quality

Protein products in cell culture environments are exposed to a variety of chemical and enzymatic agents that can alter them. Animal cells contain lysosomes, membrane bound sacs that contain enzymes for the destruction of amino acid, nucleic acid and carbohydrate polymers. They have earned the nickname of "Suicide

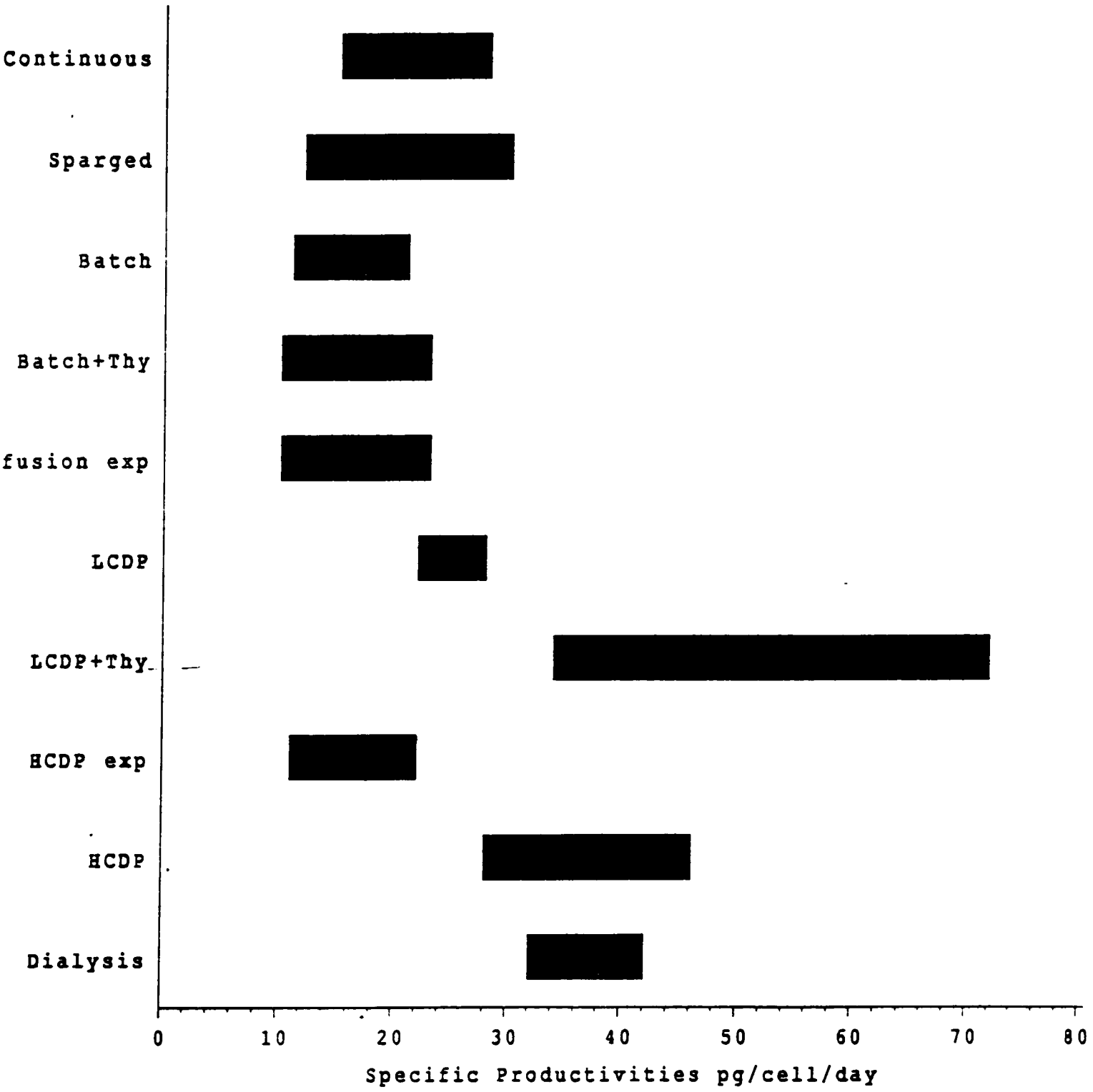


Figure 39: Comparison of the specific productivities of TBC3 in a number of different cell culture systems. Abbreviation: exp=exponential, Thy=Thymidine, LCDP= low cell density perfusion, HCDP= high cell density perfusion. Perfusion experiments used spin filter devices for cell retention.

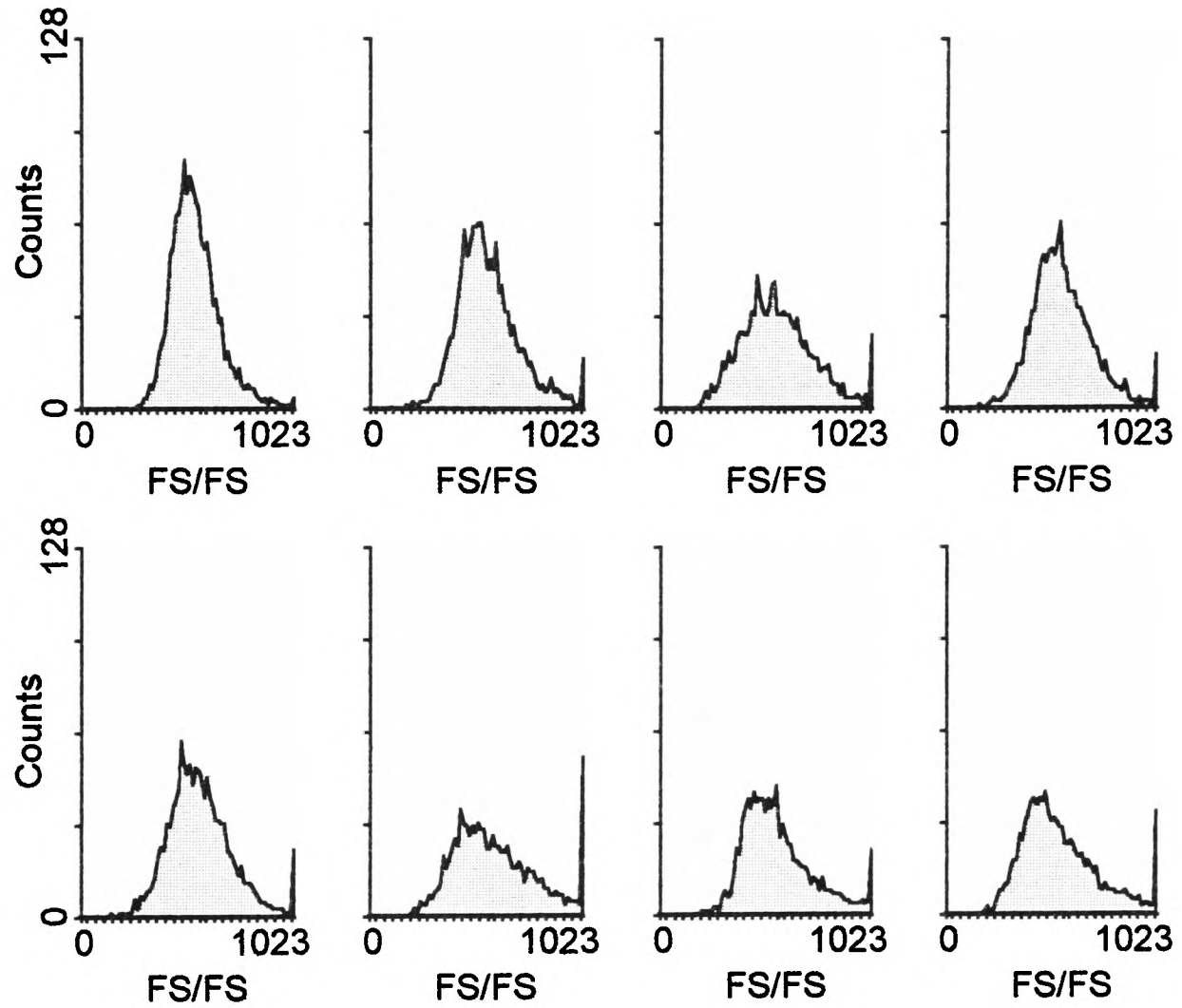


Figure 40: Forward Scatter flow-cytometry measurements for TB/C3 in dialysis culture (see figure 41).

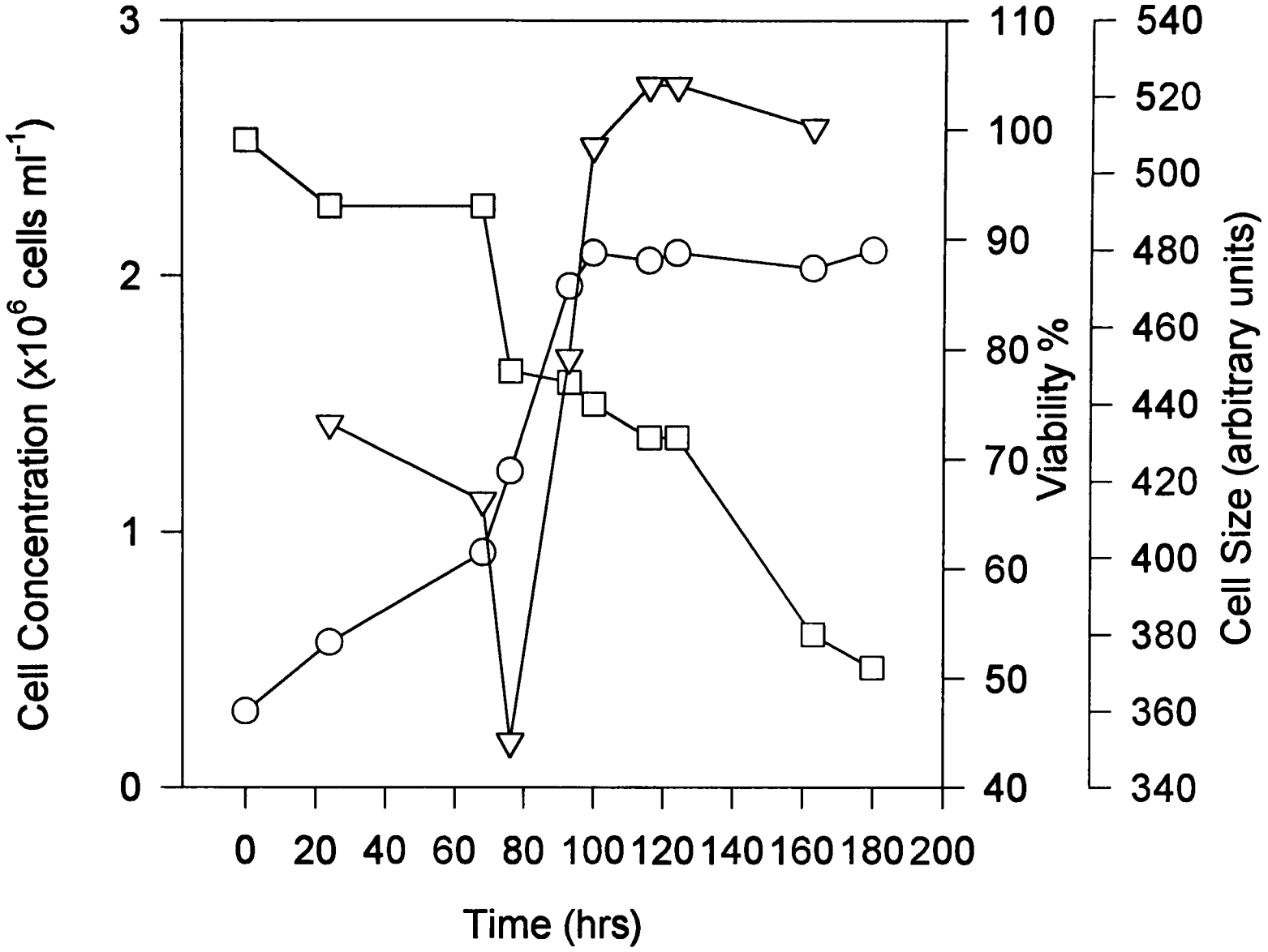


Figure 41: Dialysis culture of TB/C3, standard conditions, a perfusion rate of 0.5 ld^{-1}. Cell concentration (◯), viability (□), Cell size (▽).

Sacs". In the healthy cell these enzymes are used for orderly destruction of cellular components (Stryer, 1981). Their contents may be released from dead cells into the culture. Several groups have reported the presence of proteases in hybridoma cell supernatents (van Erp *et al.*, 1991a; Karl *et al.*, 1993; Kratje *et al.*, 1994; Mohan *et al.*, 1993), and these have been implicated in the degradation of antibody. Similar reports have been made for insect cell supernatents (Jager *et al.*, 1992), and CHO supernatents (Kratje *et al.*, 1994). The situation with carbohydrate residues is more complex as the addition of carbohydrate residues to proteins depends on the physiological condition of the cell and the nutrient environment that it is in for both Hybridoma (Patel *et al.*, 1992), CHO (Hayter *et al.*, 1992a) and insect cells (Fordham *et al.*, 1992). Several groups reporting on antibody quality in extended culture systems do not notice any significant degradation (Ackermann *et al.*, 1994; Robinson *et al.*, 1994)

Dialysis culture, as it has been presented here, apparently optimises conditions which promote degradation, *ie.* the product spends long periods of time in low viability culture. However, because of the retention of high molecular weight components, it becomes more economic to add protein protease inhibitors. A large fraction of the protein component of serum is protease inhibitor and it is indeed one of the concerns over the use of serum-free media that product is more liable to degradation as no inhibitors are present and the product forms a larger fraction of the total protein (no competition effect). Evidence of the protective effect of serum is contradictory. van Erp *et al.* (1991a) ascribe the protective effect of Foetal Calf Serum on antibody integrity mostly to competition effects, Pepstatin A was also found to protect the antibody. Jager *et al.* (1992) added Antipain, a serine protease inhibitor, to insect cell supernatents and observed

inhibition of proteolysis. Kratje *et al.* (1994) found that Aprotonin and Antipain were effective at inhibiting culture supernatant protease activity. There is evidence that different hybridoma cell lines release different proteases. For example Phenyl Methyl Sulphonyl Fluoride (PMSF) both inhibits (Kratje *et al.*, 1994) and does not inhibit (van Erp *et al.*, 1991a) protease activity in different cell lines.

Proteins are not homogeneous in their structure in cell culture supernatents. The question then is as to what degree of variation from the complete product is acceptable? This obviously depends on what it is to be used for. The most stringent requirements for use are likely to be those applied if the product is intended to be an *in vivo* therapeutic. The United States of America's Federal Drug Administration (FDA) guidelines for manufacturing protein drugs operate through the concept of licensing a product together with the facility used to produce it. This means that in the later stages of registration the precise details of the process become fixed and the product is defined by this. The precise state of the product that is acceptable is defined for every individual process, as it is for registration.

To obtain Figure 42: 20μg of supernatant from the experiment referred to in Figure 25 was added to each track of a non-reduced SDS polyacrylamide gel. The gel was run and then blotted. Four tracks were stained for protein with Coommassie brilliant blue, the rest were conjugated with sheep anti mouse IgG polyclonal antisera conjugated to peroxidase and developed with a di-amino-benzidine.

The presence of multiple banding in Figure 42 shows that some of the antibody protein is present as part molecules. Many of the bands are present as pairs. These pairs may be due to some of the molecules being un-glycosylated. Carbohydrate residues form 2% of the mass of a complete molecule. The intensity of

the bands increases with time as the antibody concentration increases. However there is no apparent change in the relative intensity of the bands. Since the early samples come from a culture at a high viability and thus, presumably a low concentration of, normally intracellular, protease, this suggests that any proteolytic processing that may be occurring is not critically dependant on released intracellular protease. Another potential source of incomplete antibody is the release of partially completed antibody from inside the cell. The above argument applies also to this source. Additionally, incompletely assembled antibody should show banding molecular weights which are multiples of that of the individual chains (25 kD and 50 kD). Thus banding at 25 kD, 50kD and 75 kD would be expected. On the gel no bands can be seen below 60 kD. This was also observed by Mohan *et al.* (1993). Finally the rate of release of intracellular antibody is rather low (see section 5.2.2.1).

There is evidence then for the presence of incomplete antibody product. However there is little evidence that this is related to cell viability. Another source of this proteolytic effect may be secretion of proteases. Lind *et al.* (1991) showed that levels of proteolytic activity followed the viable cell concentration and suggest that this is due to secretion of proteases. The multiplicity of different findings suggest that protease and proteolytic patterns are probably clone specific in hybridomas.

7.2.2 Economic Considerations

The cost of medium for hybridoma growth can vary tremendously depending on the costs of the supplements. Basal media, such as RPMI and DMEM, are relatively cheap. One litre of basal medium bought as a powder costs approximately

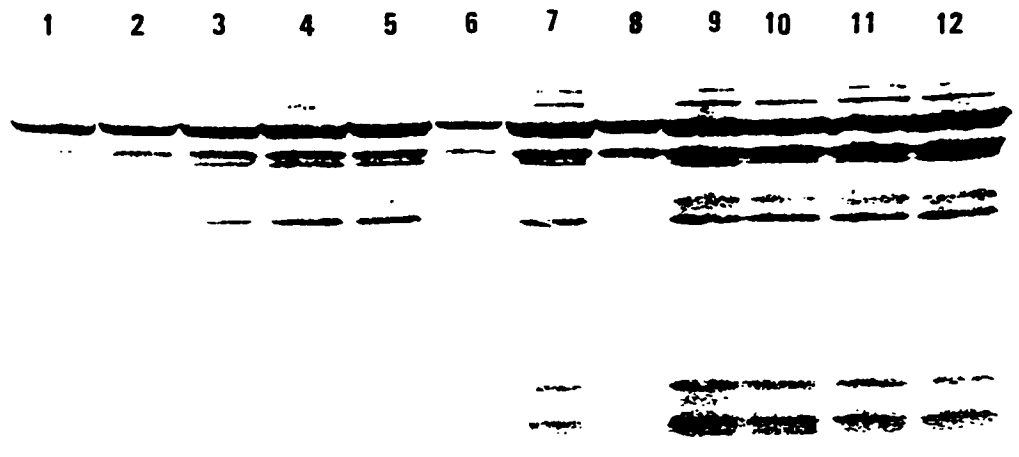


Figure 42: A western blot of samples from a dialysis culture (Figure 25). Each lanes consists of 20μl of culture supernatent was run on a 8-20% non-reduced SDS poly-acrylamide gel, then electro-blotted probed with polyclonal anti-mouse IgG peroxidase conjugate and developed with di-amino-benzidine. The numbers above the lanes denote the time in days when the sample was taken.

£1.05 (based on 1994 Gibco list prices) and considerable savings can be made by bulk purchase. A 5% supplement of New Born Bovine Serum costs £1.26, but many groups use 10% Foetal Bovine Serum and this costs £12 (1994). Serum-free medium supplements, such as Bovine Serum Albumin and Transferrin are costly. Antibiotics are also an expensive part of animal cell culture medium, but their use can be avoided by good aseptic technique. Dialysis culture's ability to avoid almost totally the use of serum, reduces the medium costs of production considerably (see Table 16). The yield of antibody on basal medium is double that of batch culture and the yield of monoclonal antibody on serum is 19 times that of a batch culture. The medium cost per gram of MAb is therefore decreased from £48.80 to £0.93. Furthermore that antibody represents 25% of the total of five grams of protein. By contrast, the culture protein mass' in batch mode was 1.1 gram, but the antibody formed just 2% of this total protein (Table 16).

7.3 Discussion: Specific Productivity

Specific productivity is a rate function and thus it doesn't have an instantaneous value. It is generally measured by comparing the antibody concentration in two separate samples. Comparing samples from the beginning and end of the dialysis run gives an overall rate, comparing adjacent samples gives a local rate. Figure 39 shows a comparison of the overall rate of specific productivity in the dialysis reactor from a number of runs to similar data for some other reactor configurations with the same cell line. The rate of specific productivity in the dialysis can be seen to be higher than that in batch cultures and similar to that seen in spin-filter perfusion culture. However local variations in specific productivity are

Table 16: Comparison of product yields and medium costs for a typical batch and dialysis culture. Estimates for the dialysis culture were taken from the last point prior to any serum additions.

	Batch	Dialysis
Figure Number	9	35
Total Medium Used (litres)	1.0	16.4
Total Serum Used (litres)	0.05	0.125
Duration (hours)	96	478
Maximum Cell Conc. ($\times 10^6$ cells ml^{-1})	0.85	3.3
Viability Index ($\times 10^6$ cells ml^{-1}hr^{-1}	45.0	835.3
Protein Concentration (grams litre^{-1})	1.1	5.0
Ab concentration (grams litre^{-1})	0.045	1.470
Yield Ab/Basal Medium (grams litre^{-1})	0.045	0.094
Yield Ab/Serum (grams litre^{-1})	0.090	1.700
Ab/Total Protein	2%	25%
£/ gram Ab	48.8	9.30

Yield Calculations—

$$\text{YieldAb/BasalMedium} = \frac{\text{Final antibody concentration}(gl^{-1})}{\text{Total basal medium used}(l)}$$

$$\text{Yield Ab/Serum} = \frac{\text{Total Antibody harvested}(g)}{\text{Total serum used}(l)}$$

$$\text{Ab/Total Protein} = \left(\frac{\text{Total Antibody harvested(g)}}{\text{Total Protein harvested(g)}}\right) \times 100$$

$$£ = \frac{20.8\times\text{Litres of serum used} + 1.18\times\text{ Litres of medium used}}{\text{Grams antibody harvested}}$$

seen in several of the dialysis runs, including an overall reduction in antibody concentration seen in Figure 38. The notorious inaccuracy of the ELISA will account for some of the variations seen.

Hybridoma cells are genetically unstable. As fusions of two cells, they retain more than the standard diploid complement of chromosomes and are susceptible to chromosome loss. The antibody gene family has been shown in myeloma cells to be particularly susceptible to mutation (Morrison and Scharff, 1981). This may be because recombination is the basis of antibody diversity. Also one of the two immunoglobulin alleles is excluded from expression, regardless of whether the other allele is functioning. Non-producing clones may have a higher growth rate, as antibody production is a metabolic burden on producing cells. Frame and Hu (1990) showed that in a continuous culture over 2000 hours, the antibody titre fell by 50% and that this could be related to the increase in a non-producing population. They later showed (Frame and Hu, 1991) that five out of a panel of six non-producing clones had a higher maximum specific growth rate than the producing clone, along with a higher yield of cell mass on glucose and glutamine.

Periodic checks for non-producing clones along with proper cell banking arrangements are required to avoid productivity loss. Sub-cloning of the cell line is an efficient method for removing non-producing clones and selecting clones that produce higher concentrations of antibody.

The factors affecting specific productivity have been discussed in the introduction (Section 1.2.2). Two of the factors that are affected by the mode in which a reactor is operated are cell cycle state and cell turnover rate.

7.3.1 Cell Cycle

The positive association between percentage of cells in the G1 stage of the cell cycle and specific productivity suggests that devices that encourage slow growth should also encourage high specific productivity, as Hybridoma cells extend the G1 portion of their cell cycle as their growth rate declines (Park and Ryu, 1994). Cells in the dialysis reactor spend long periods of time with no net growth, therefore apparently no cell turnover. In fact cell turnover is occurring (see next section).

The flow cytometry results for TBC3 cell cycle are confusing because there appears to be an oscillation in the proportion of cells in the different stages of the cell cycle. This suggests that the cells are dividing in a synchronous manner. Other reports of this cell line have shown a more constant cell cycle pattern with time in continuous culture steady states (Al-Rubeai and Emery, 1993).

7.3.2 Cell turnover

Cell turnover has been discussed generally in the introduction (Section 1.2.2) and in relation to cell concentration (Section 5.2.2.1). Its importance in specific productivity lies in the fact that TBC3, along with other cell lines, stores IgG (Al Rubeai *et al.*, 1992). This antibody may be released upon cell disruption, adding to the measured pool of antibody and increasing the productivity. TBC3 has been shown to store 15 $\times 10^{-9} g$ IgG per cell (Al-Rubeai and Emery, 1990). Given a turnover rate of 0.15 d^{-1} (see Section 5.2.2.1) the antibody release rate is 0.1 $\times 10^{-9} g$ $cell^{-1} hour^{-1}$. This is 2.3% of the overall antibody production rate.

Cell lines vary in the quantity of IgG that they store and therefore of the effect of this parameter. For example with the cell line I.13.17 no stored antibody was detected (Al Rubeai *et al.*, 1992).

Along with functional antibody there will be quantities of non- or partly-assembled chains as unglycosylated antibody present in the cell, which will also be released. Our ELISA is activity based and will pick up whole or part molecules. Section 7.2.1 showed that unassembled chains did not form a significant part of the final product.

Chapter 8

Other Cell Lines

8.1 Recombinant Interferon-γ production in Chinese Hamster Ovary cells

We have seen with a hybridoma cell line that dialysis culture allows higher cell densities to be maintained in quasi-steady states and also the accumulation of antibody product. However cell lines are known to differ from each other in many ways (see Section 1.2.3) and information gained from one cell line does not necessarily apply to another. The very low steady state cell viabilities seen with TBC3 in dialysis culture have not been observed in other reports of dialysis culture (Butler, 1987; Kasehagen *et al.*, 1991). Low viabilities are suggestive of a relatively high cell death rate and thus a high rate of release of intracellular proteases (see section 7.2.1). We next examined a different cell line/product system to identify under dialysis culture conditions whether different dynamics of cell and product turnover would be exhibited.

Chinese Hamster Ovary (CHO) cell lines have been extensively used for recom-

binant gene expression because of their genetic stability and high plating efficiency Many auxotrophic mutants are available allowing a choice of selection systems. CHO cells grow well in suspension or in monolayers.

Interferon-γ is a cell regulatory glycoprotein produced by T-lymphocytes and Natural Killer cells (another type of lymphocyte) in response to virus infection, endotoxin and a variety of mitogenic and antigenic stimuli (Dawson, 1991). It is a small protein consisting only of 176 amino acids. There are two glycosylation sites, both of which may or may not be filled. Interferon-γ is one of a group of proteins that induce a non-specific anti-viral state in neighbouring cells. Interferon-γ also powerfully inhibits cell growth of both normal and transformed cells. Possible clinical uses include antiviral treatment, particularly of AIDS, tumor therapy and as a replacement in the treatment of certain forms of autoimmune disease. In the human body it is present in tiny quantities, so the cost of purifying it from human tissue is prohibitive. Interferon purified from this source may also contain contaminating virus and other cytokines which can lead to side effects *in vivo*. The interferon-γ gene has however been cloned and been expressed in a number of different cell lines, including *Escherichia coli* and CHO cells (Hodgson, 1993). Currently 18 different companies are developing or have developed expression systems for Interferon-γ (Hodgson, 1993).

Figure 43 shows a dialysis culture of CHO cells. An inoculum of 0.4×10^6 cells ml^{-1} in RPMI 1640 with 1 mg ml^{-1} of methotrexate which maintains a selection pressure for the plasmid containing the interferon gene, was placed in an LSL 2 litre reactor, setup for dialysis as in Figure 28. pH was controlled at 7.0 by automatic NaOH and HCl addition, DOT was controlled at 20% by intermittently passing pure oxygen through 1 metre length of thin walled silicone tubing. Dialysis

was begun immediately after inoculation, with a fresh feed flow rate of 0.5 ld^{-1}, using RPMI 1640 with methotrexate.

The cell concentration increased for the first 284 hours. The increase was a linear function of time, suggesting therefore that cell growth was limited by the supply of some component of the medium, which was being incorporated into the biomass. TBC3 under similar conditions increased exponentially in concentration towards its steady state and therefore was limited by the maximum rate at which it could grow. The rate of growth was 0.3×10^6 cells ml^{-1} day^{-1}. Growth rate appears to be controlled by the fresh feed flow rate. Such growth would continue until the cells became limited by the demand for energy for maintenance, which is high in animal cells in comparison to bacteria (Miller *et al.*, 1989b).

The maximum cell density achieved was 4.2×10^6 cells ml^{-1}. Under similar conditions TBC3 would achieve 1.5×10^6 cells ml^{-1}. CHO has a much higher growth yield on the basal medium than TBC3, in terms of cell numbers, though of course not necessarily in terms of weight of biomass which is considered the more important indicator. Greater efficiency of use of medium means that very high cell concentrations could be achieved with this cell line. Using the same membrane area acting at its most efficient and a fresh feed flow rate of 1.0 l d^{-1}, 7.5×10^6 cells ml^{-1} (from equation 9) should be achievable Also medium optimisation should be possible to further increase the maximum cell density. From 284 hours to 380 hours the cell concentration decreased in a linear manner, at a rate of 0.16×10^6 cells ml^{-1} day^{-1}. Population viability decreased slowly over the course of the culture eventually reaching 75%. The dramatic decreases in viability observed with TBC3 as the culture reaches its steady state cell density did not occur. The Interferon-γ concentration was determined at three points in the culture.

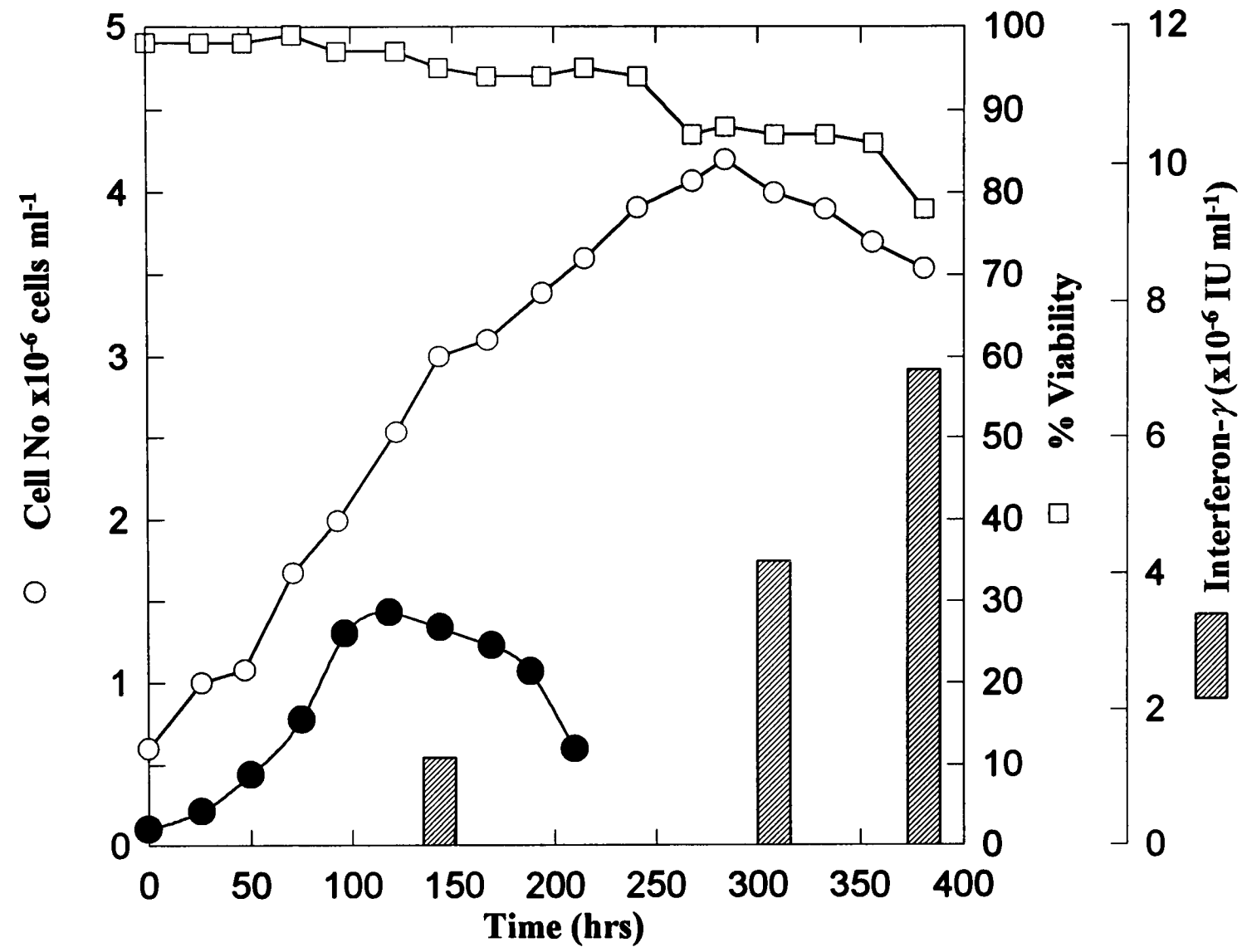


Figure 43: Growth and interferon-γ production of CHO under dialysis culture. Cell concentration (◯ dialysis culture, • batch culture) ,% viability (□) interferon-γ concentration (▨)

Table 17: Production of Interferon-γ: a yields in dialysis and continuous culture.

	Dialysis	Continuous
Basal Medium Used (litres)	9	3.81
Serum Used (litres)	0.05	0.19
Duration (hours)	381	381
Maximum cell concentration ($\times 10^6$ cells ml^{-1})	4.2	1.1
Viability Index ($\times 10^6$ cells ml^{-1}hr^{-1})	1250	418
Interferon-γ concentration ($\times 10^6$ International Units ml^{-1})	7	0.5
Specific Production ($\times 10^3$ IU 10^6cells ml^{-1})	5600	5000
Interferon-γ/Basal medium ($\times 10^6$ IU l^{-1})	0.78	0.5
Interferon-γ/Serum ($\times 10^9$ IU l^{-1})	140	10
Medium Cost (£/10^9IU)	£2.27	£14.20

The final concentration achieved was 7×10^6 International Units ml^{-1}. This is consistant with a specific production rate of 5.6×10^3 IU 10^6cells ml^{-1} (Table 17). A continuous culture of the same cell line and medium, at a dilution rate of 0.01 hr^{-1} gave a steady state interferon-γ concentration of 5×10^6 International Units ml^{-1} giving a specific productivity of 4.5×10^3 IU 10^6cells ml^{-1}. The specific productivity is roughly constant throughout the run. There is however a serious discrepancy between these results and those of Hayter *et al.* (1992b, 1992a) for the same cell line. These specific productivities are a factor of 1,000 higher. Hayters group use a serum free medium formulation but this is unlikely to explain such a large difference. Alternatively, genetic variation may account for the differences. A more likely explanation of the difference is an error in the concentration of the standards used to calibrate the ELISA. Smiley *et al.* (1989) with another CHO cell line producing interferon record specific productivities of 7.5×10^3 IU 10^6cells ml^{-1}.

The yield of inteferon on basal medium is slightly increased over that observed in the continuous culture, this is probably a reflection of the higher specific productivity. The use of a membrane to retain the high molecular weight fraction of the serum has once more led to a substantialy higher yield of Interferon on serum (14 fold). The difference in the yield on serum is the principal reason for the savings in medium cost (by a factor of 6). The Foetal Calf Serum is substantially more expensive than the New Born Calf Serum and thus the reduction in cost of medium is much more dramatic than that seen with TB/C3 (see table 16.

Figure 44 shows another dialysis cultivation of CHO cells, identical to the previous one except that flow rates of 0.1 ld^{-1} and 0.25ld^{-1} were used. Steady state was quickly achieved of 0.75×10^6 cells ml^{-1} at 0.1 ld^{-1}. The dilution rate was then changed to 0.25 ld^{-1} and a steady state cell density of 2.0×10^6 cells ml^{-1} and a viability of 83% was achieved. The steady state cell densities of Figures 43 and 44 when plotted against fresh feed flow rate fall on a straight line with an intercept at zero.

Flow cytometry was used to determine cell size and the percentage of cells in each stage of the cell cycle. Forward scatter measurements showed that the cells were bigger at the start of the quasi-steady state but subsequently decreased showing that the cells were not truely at a physiological steady state (Figure 45 and Figure 47). The cells were stained with Propidium Iodide which intercalates with double stranded nucleic acids. The DNA content of each cell can thus be measured and the proportion of cells in each stage of the cell cycle estimated (Figure 46). An initial periodicity of the percentage of cells in each cell cycle stage settled down later during the steady state (Figure 47). Because of the periodicity in the early readings no inference about differences between exponential and stationary phase

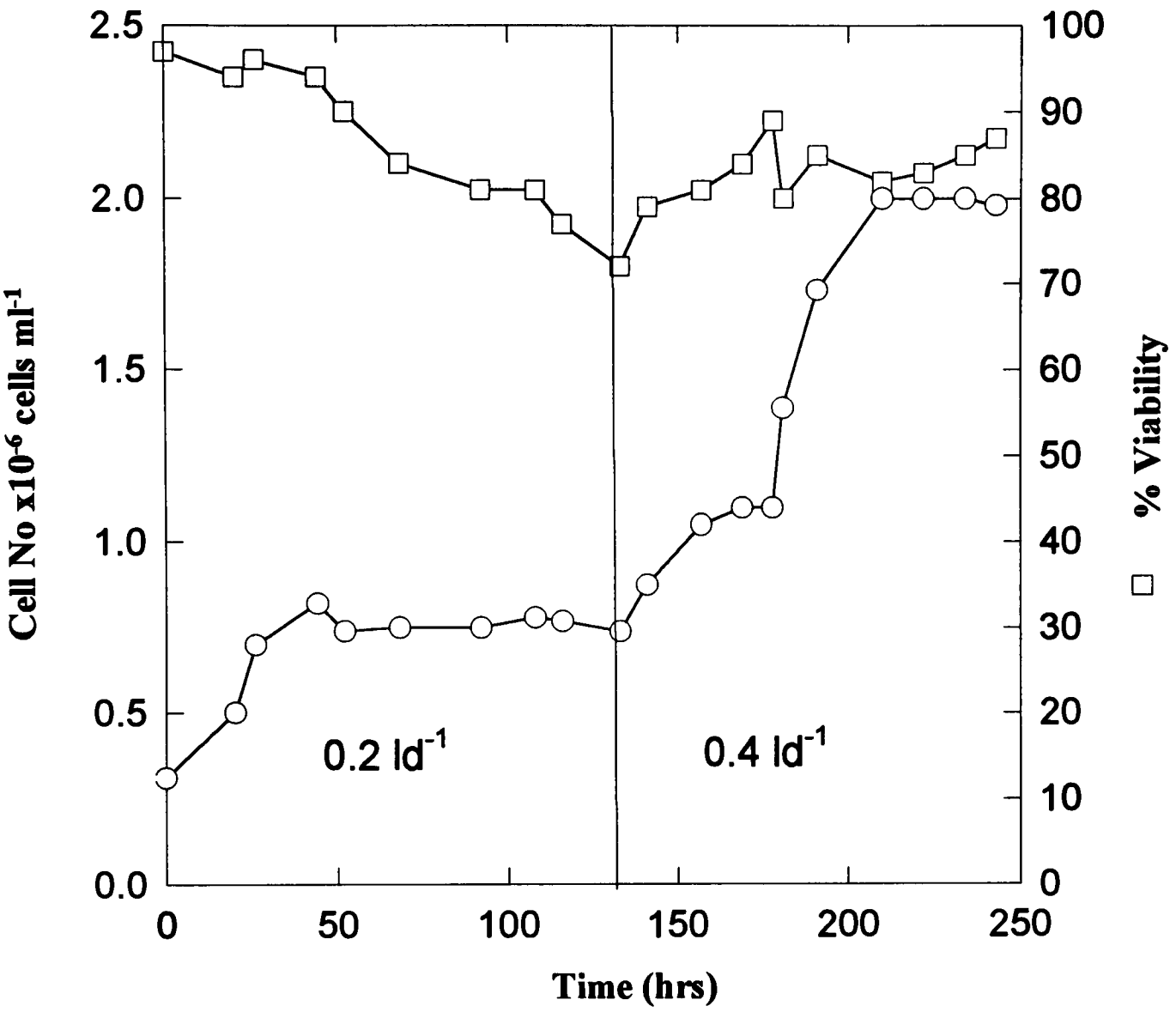


Figure 44: Growth and interferon-γ production of CHO under dialysis culture at low perfusion rates. Cell concentration (◯),% viability (□)

cells could be drawn which might relate cell cycle state to specific productivity.

Referring to Figure 33 cell viability is a balance between cell growth, cell death and dead cell disintegration. To measure the cell growth rate we compared the rate of incorporation of tritiated thymidine into DNA from cells from a batch culture growing at μ_{max} and that of the dialysis culture. The average counts per minute (cpm) of triplicate samples of CHO cells was 2921, the cell count was 1.7×10^6 cells ml^{-1}. 24 hours later the cell count was 3.7×10^6 cells ml^{-1}, which gives a growth rate of 0.0377 $hours^{-1}$. The average cpm of the cell count was 645, or 22% of the cell count for the batch culture. This gives a cell growth rate of 0.008 $hours^{-1}$. From equation 22 therefore $k_d = 0.008$ and $k_r = 0.034$ $hours^{-1}$. Hayter *et al.* (1992b) operating a continuous culture at a dilution rate of 0.01 observed a steady state cell viability of 80%. The similar viability suggests that the mechanisms of cell death and dead cell disintegration were similar in this system.

Specific productivity has been shown to be a linear function of growth rate for CHO by a number of workers (Hayter *et al.*, 1992b; Leelavatcharamas *et al.*, 1994). Leelavatcharamas *et al.* (1994) in this laboratory also found a direct relationship between the proportion of the cells in the S phase of the cell cycle and growth rate. In Table 18 specific productivity, DNA synthesis rate and flow cytometry data are presented. Growth rate during apparent steady states is approximately 0.008 hr^{-1}. Specific productivity was measured at 5.60 and estimated at 5.00 . The specific productivity of a chemostat at a dilution rate of 0.01 was 5.6×10^3 IU 10^6cells ml^{-1}. In a continuous culture at 100% viability specific growth rate is equal to the dilution rate. Below 100% cell viability the growth rate is increased

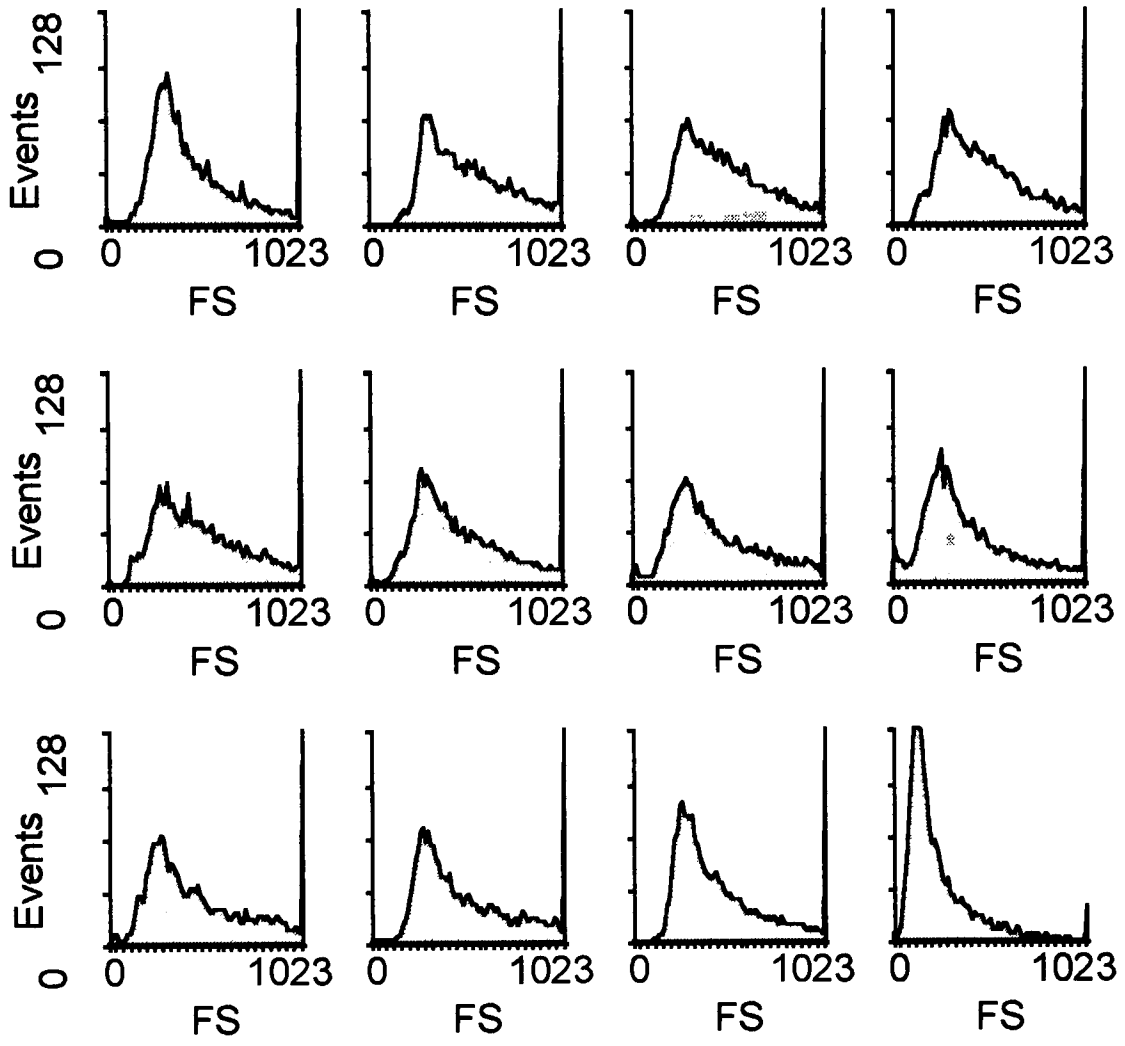


Figure 45: Forward scatter measurements of the CHO cells in Figure 44

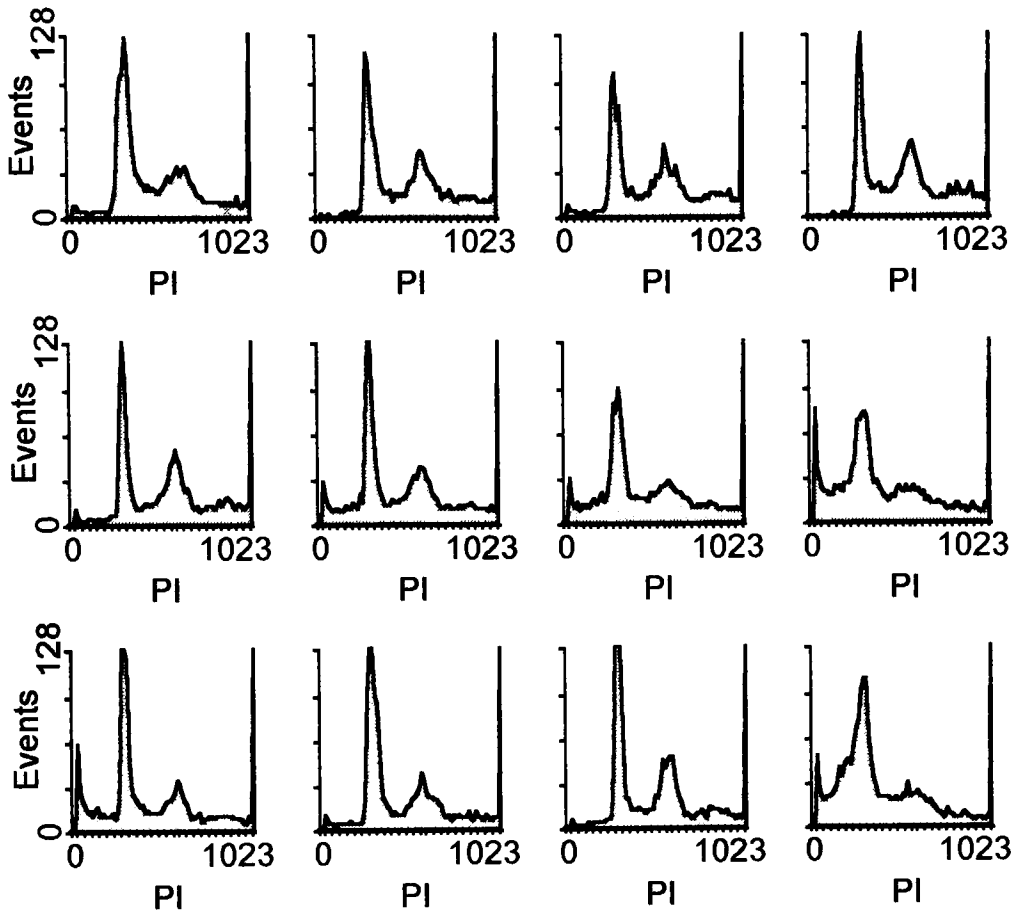


Figure 46: Cell cycle measurements of the CHO cells in Figure 44

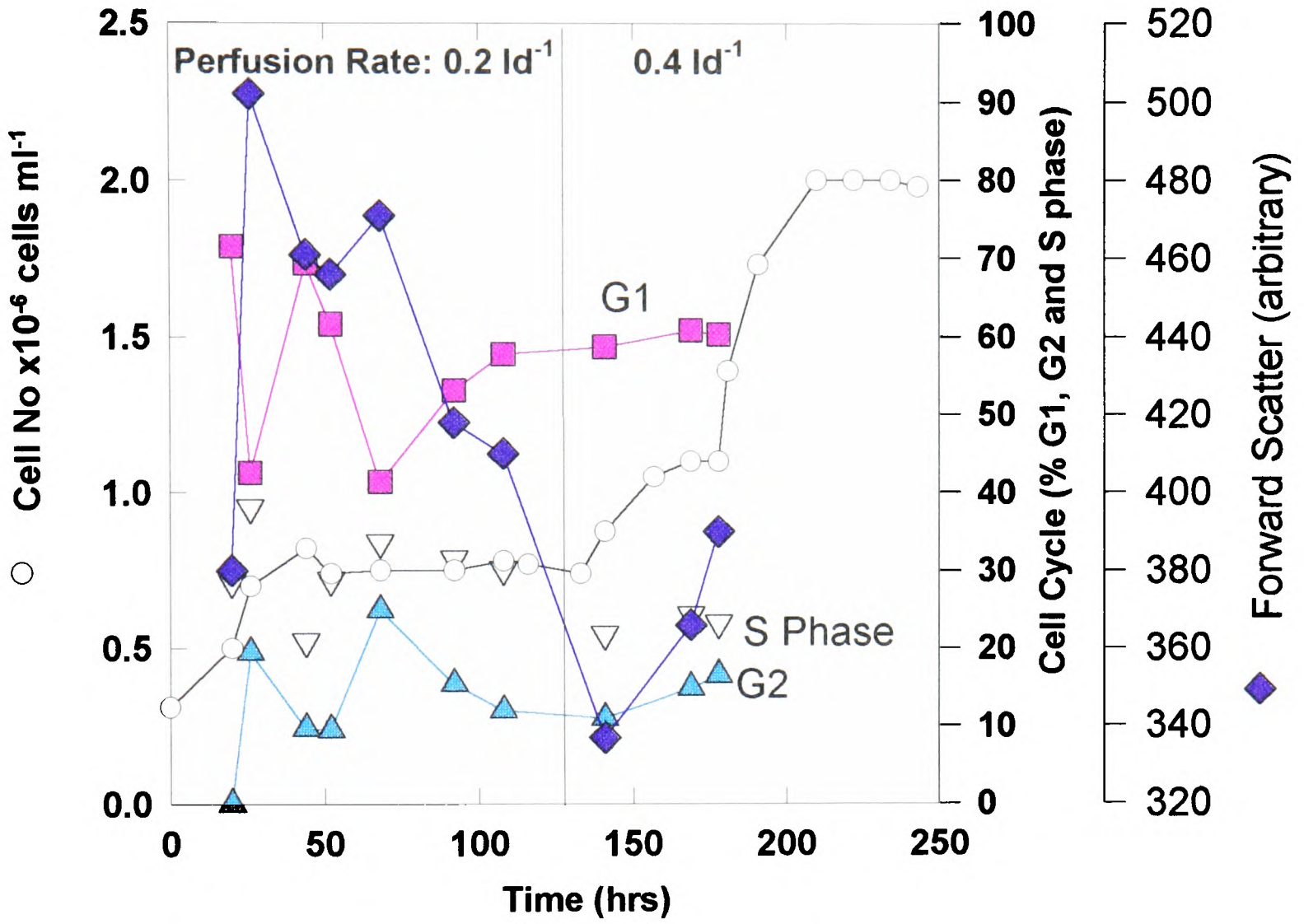


Figure 47: Percentage of cells in each stage of the cell cycle and median forward scatter measurements plotted against time with the viable cell number data from Figure 44

Table 18: Comparison of predicted levels of % S phase cells, growth rate and specific productivity from their measured values.

	S Phase Cells %	Growth rate hr^{-1}	Specific Productivity $\times 10^3$ IU 10^6cells ml^{-1}
Specific Productivity	32	0.009	5.6
DNA Synthesis	30	0.008	5.0
%S Phase	35–25	0.012–0.002	0.6–6.5
	30	0.08	5.0

from the dilution rate by the death rate in the culture (Kwong *et al.*, 1989).

$$\mu = D + \mu_d \tag{23}$$

where the death rate μ_d is the dilution rate multiplied by the proportion of viable cells.

$$\mu_d = D\left(\frac{x_d}{x_v}\right) \tag{24}$$

The cell viability of the continuous culture is 93% and therefore $\mu = 0.0108$. The specific productivities of the dialysis and continuous systems relate to similar growth rates (0.008 vs. 0.01).

8.2 Insect Cell Growth under Dialysis Culture

The *Baculoviradae* are a large group of viral pathogens of arthropods, which mainly infect only a single or a few related species (Vlak, 1992). They are large double stranded circular DNA viruses that produce large proteinaceous capsules called polyhedra. The polyhedra principally consist of two proteins: the **polyhedrin** protein and **p10** whose role is not understood. They are produced to very high concentrations in the host cell. Together these two proteins can constitute up to 50% of the total cellular protein, and *in vitro* both these proteins are dispensable. Promoters of both genes have been used in the construction of expression vectors. The most common combination has been the use of *Autographa califonica* nuclear polyhedrosis virus (AcNPV) with cell lines from *Spodoptera frugiperda e.g.* SF9. Insect cells grow well in static or suspension culture and perform many of the necessary post-translational modifications of mammalian cells. This system has therefore become popular for the production of quantities of protein for research use, because production of the recombinant vector is simple and yields of correctly folded and modified protein are high. However, it does not correctly glycosylate all proteins, there is a high degree of N-terminal degradation of secreted protein, also some proteins are not secreted efficiently (Grabenhorst *et al.*, 1992) and this system is therefore not suitable in all cases.

The initial step in the infection process is the uptake of the viral particle via receptor-mediated endocytosis. The nucleocapsid then enters the nucleus and is uncoated, host macromolecular synthesis is shut down and the cytoskeleton rearranged. Transcription of the "very late" genes including p10 and polyhedrin, begins at around 18 hours post infection. Production continues for 3–5 days after this as the cells are killed.

Many authors have found that maximum production of recombinant protein occurs if the cells are infected whilst in exponential phase (Summers and Smith, 1987). However recently several groups have found that by replacing the medium with fresh, immediately prior to infection maximum specific productivities can be obtained throughout the batch growth cycle, at least until cell viabilities fall (Radford *et al.*, 1992; Reuveny *et al.*, 1993; Wang *et al.*, 1993). Therefore it is possible to wait until the maximum cell density occurs before infecting the cells.

Medium exchange in the work referred to above was achieved by centrifuging the cells out of the medium. It would be much more practical on a larger scale to exchange medium by filtration or dialysis. Indeed there are reports of the use of spin-filter devices (Weiss *et al.*, 1992) and filtration devices (Caegn and Bernard, 1992; Guillaumme *et al.*, 1992; Jager *et al.*, 1992; Kloppinger *et al.*, 1990) in growth and infection, but not infection alone. Dialysis culture is another possible method of performing this medium change operation. Its advantage over filtration methods is that there is no necessity to add serum to the perfusing medium and that product is accumulated to high concentrations

Figure 48 shows the growth of SF9 cells in TC100 medium plus 5% foetal calf serum, in an LSL bioreactor fitted with a perfusion device (as in Figure 35). DOT was controlled via headspace gassing with pure oxygen, pH was not controlled, but only varied by +/- 0.2 unit. One metre of dialysis tubing was used. The dialysate medium, basal TC100, was fed at 0.5 ld^{-1}.

Dialysis was begun at 63 hours, at which point cell growth was already slowing down and the cell concentration then only increased to 1.3×10^6 cells ml^{-1}. This is less than half of what has been achieved in batch culture in this lab with the same cells and medium (Hild *et al.*, 1992). Addition of 5% FCS to the feed did

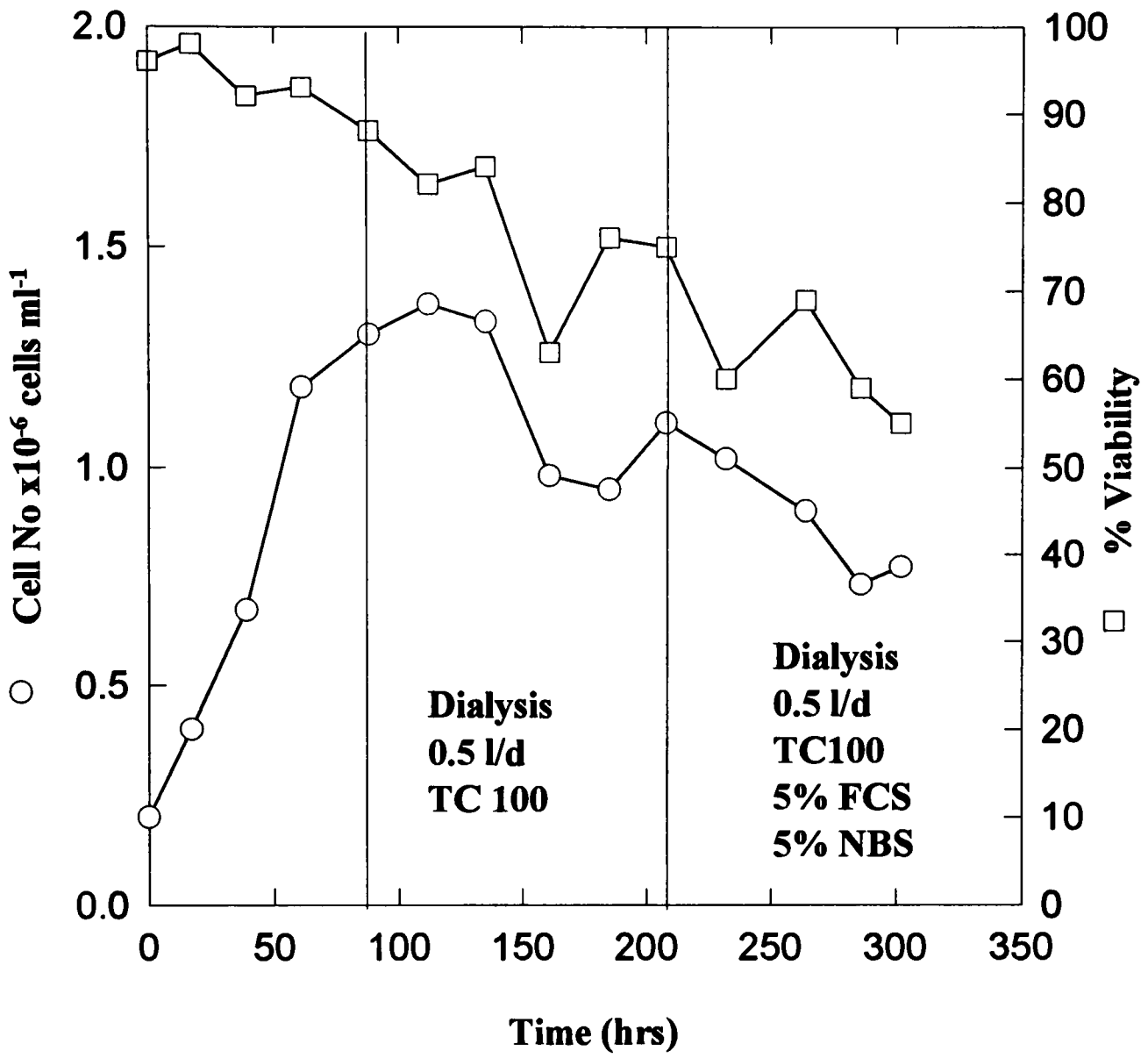


Figure 48: Growth of an SF9 culture in a dialysis reactor. Cell concentration (◯), % viability (□).

not enhance the cell density. Viability decreased over the entire course of the experiment. Despite the lack of growth glucose use by the cells increased during dialysis, the concentration reaching zero as the cell numbers started to decline. No contamination could be detected.

To try and assess whether the low cell density achieved was due to the physical conditions in the fermenter, or to the removal of some essential low molecular weight substance present in the serum a 30 ml sample was removed from the fermenter at 161 hours, 1×10^6 cells ml^{-1}, 65% viable. This sample was split into 7.5ml aliquots and various supplements were added, following which they were incubated in T-flasks at 28°C for 52 hours. The supplements were 5% FCS, 5% NBS, 1% lipid mixture (Gibco) 5% of 5% meat peptone in PBS and a control with no supplementation. No cell growth was observed in any of the flasks. Table 19, shows however that cells in the FCS flask maintained their concentration for longer than the others, lipids and NBS were equally ineffective and peptone had a negative effect, *i.e.* death was accelerated.

Significantly higher cell concentrations can be achieved in batch cultures by the use of optimised media (Weiss *et al.*, 1992). This suggests that the TC100 basal medium used is far from being optimal for cell growth. A more complete medium may allow cell growth under dialysis conditions. The additions made to samples from dialysis culture may have been ineffective because of a physiological change in the cell caused by earlier stresses.

Figure 49 shows the post inoculation behaviour of cells infected with a recombinant AcNPV for *Escherichia coli* β-galactosidase. SF9 was grown in four stirred Duran bottles at 500 ml volume until the maximum cell density was reached at 0.9×10^6 cells ml^{-1}. The contents of the bottles were mixed and divided between

Table 19: Growth of SF9 cells in batch, taken from a dialysis culture in stationary phase, with various supplements

Supplement	Cell Conc. $\times 10^6$ cells ml^{-1}	Viability
Control	0.63	0.56
FCS	0.93	0.62
NBS	0.75	0.62
Lipids	0.75	0.62
Peptone	0.53	0.51

two 1 litre reactors, operated as described in the previous experiment. The reactors were identical except that one was set up for dialysis. The fresh feed flow rate was 0.5 l d^{-1} of TC100.

Cell concentration and viability declined at the same rate in both reactors. Cell concentration started to fall 30 hours post infection, reaching 0.2×10^6 cells ml^{-1} at 120 hours. Viability declined linearly with time, from 95%, reaching 20% by 120 hours. β-galactosidase production started at 20 hours post infection in the dialysed reactor and 40 hour in the batch reactor. By 120 hours there was 52 $\times 10^6$ units ml^{-1} in the non-dialysed culture and 50 $\times 10^6$ units ml^{-1} in the dialysed culture, an increase of 1.7$\times$.

Medium change, via dialysis seems responsible for a significant change in the yield of protein, from 58 units $cell^{-1}$ to 100 units $cell^{-1}$ (Table 20). That this should occur is surprising since the cells would not grow under the same conditions. However the change in cell physiology upon infection and therefore probably the cells nutritional requirements is profound.

On a per litre of basal medium basis however, dialysis is half as efficient as batch, leaving an overall cost of medium per unit slightly higher under dialysis. Insect cell culture basal media are more expensive than mammalian cell culture

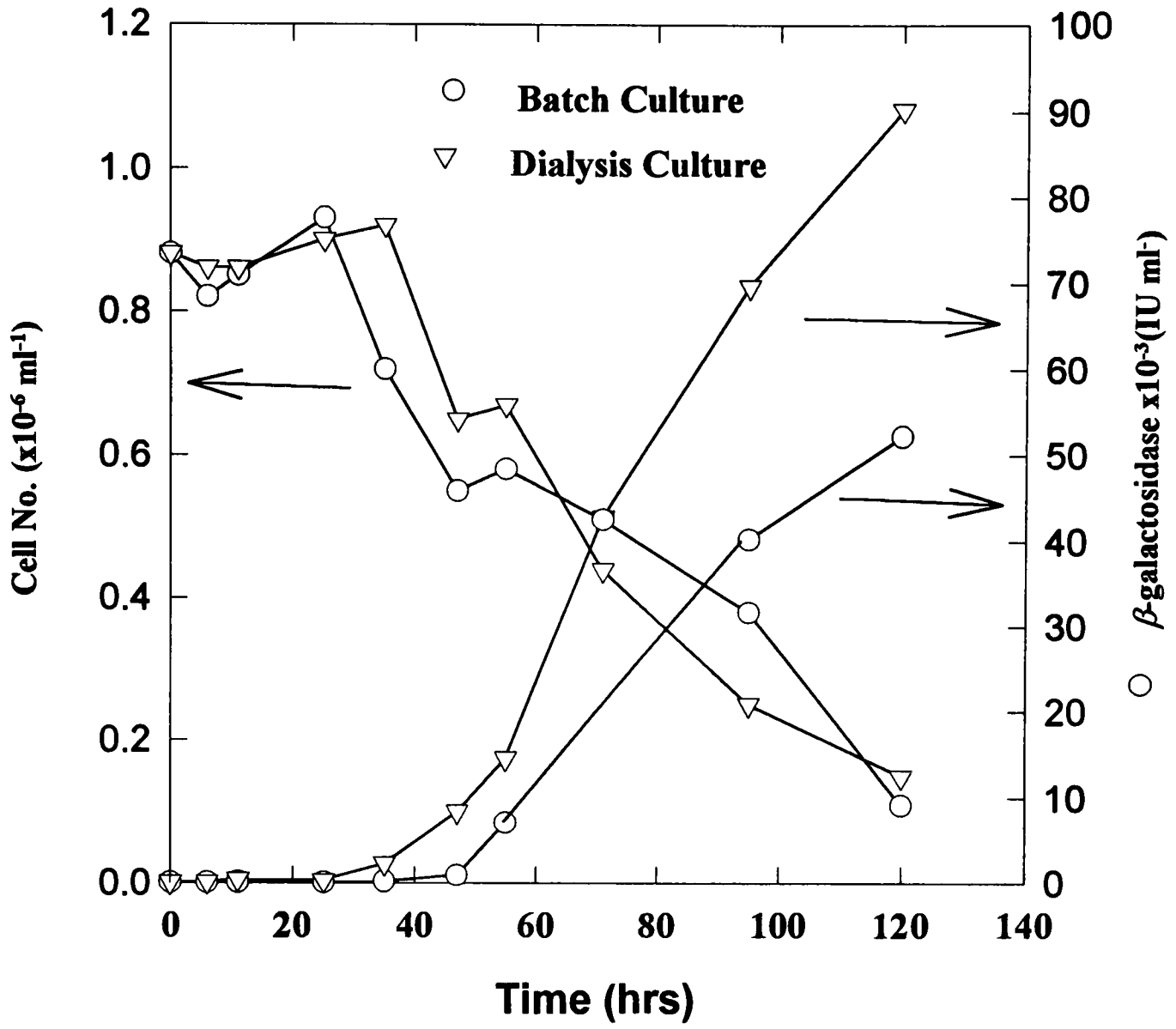


Figure 49: Comparison of the the behaviour of a SF9 culture infected with Ac-NPCV recombinant for β-galactosidase. The culture was split in two parts and put in two identical bioreactors, one of which was operated in batch mode (o), the other in dialysis mode ($\bigtriangledown$). Time is post infection.

Table 20: Comparison of the productivities of batch and dialysis culture of SF9 for the production of β-galactosidase

	Dialysis	Batch
Basal Medium (l)	3.5	1
Serum (l)	0.05	0.05
Duration (hrs)	120	120
Initial cell concentration ($\times 10^6$ cells ml^{-1})	0.9	0.9
β-galactosidase concentration $\times 10^6$ IU l^{-1}	0.90	0.52
β-galactosidase/cell (IU cell^{-1})	100	58
β-galactosidase/serum ($\times 10^9$ IU l^{-1})	18	10
β-galactosidase/basal medium ($\times 10^6$ IU l^{-1})	260	520
£/10^9 IU ml^{-1}	0.20	0.18

basal medium because of the large amounts of amino acids they contain. Reductions in the amount of medium used may be possible without compromising the yield of recombinant protein. This is because the rate of medium feeding was not optimised. There is an increase in metabolic activity immediately subsequent to infection which lasts for about a day (Kioukia *et al.*, 1994). After this time the rates of nutrient utilisation decrease and cells start to die. Perfusion should be concentrated in this time period and afterwards decreased. One possible method of deciding the perfusion rate is by controlling the glucose concentration.

Chapter 9

Conclusions

1. Hybridoma cells grew in dialysis culture.

   1a. A steady state viable cell concentration of 4×10^6 cells ml^{-1} was achieved, five times that seen in batch culture.

   1b. Steady state viability was 30-50%.

   1c. During the steady states TBC3 was dividing at $12\times10^{-3}hr^{-1}$. The steady state viability was therefore the result of a dynamic equilibrium between the rates of cell division, death and disintegration.

   1d. No serum was added to the perfusing medium, and apart from that initially batched, no further serum addition to the reaction volume was required.

   1e. The system produced considerable savings in medium cost because of savings in serum use.

   1f. Some evidence exists for proteolytic degradation of the monoclonal antibody product.

2. The value of these steady states can be simply predicted.

   2a. Under the conditions pertaining in the experiments cell density was limited by transport of metabolites across the membrane and depended on the cell type and medium used.

   2b. A model was constructed and verified for a range of perfusion rates and membrane areas.

   2c. Cell density was related to fresh feed flow rate following a saturation kinetic argument.

   2d. The model did not predict performance when the medium concentration or the cell physiology changed.

3. CHO-320 cells also grew in dialysis culture.

   3a. A viable cell density of 4×10^6 cells ml^{-1} at 0.5 ld^{-1} perfusion rate were achieved.

   3b. Steady state viabilities were 70-80%

   3c. CHO-320 cells grew in linear fashion during their approach to the steady state. This suggests that this growth was limited by the supply of some component of the biomass which was being supplied in a linear manner.

   3d. Interferon-γ was expressed and accumulated to high concentration (7×10^7 IU ml^{-1}).

4. Expressed protein product yield was strongly influenced by dialysis when using recombinant virus infection of the insect cell line SF9.

4a. Exchange of nutrients via dialysis permitted recombinant virus infected SF9 cells to produce twice as much β-galactosidase.

4b. SF9 did not grow in dialysis culture with basal medium alone, nor could growth be restarted by adding serum to the perfusing medium or by removing samples and growing them in batch culture.

Chapter 10

Recommendations for Future Work

10.1 Reactor Design

Cell concentrations within the current design are limited by transfer through the membrane. Since the majority of the hindrance to transport is in the Nernst films on either side of the membrane, methods of increasing the Reynolds Numbers should be investigated.

On the outer (reactor) side of the membrane efficient impeller design and operation strategies should allow the Reynolds Number to be maximised whilst minimising cell damage.

On the inner side of the membrane the Reynolds numbers are extremely low. Strategies immediately presented include decreasing the tube diameter and increasing the liquid flow rate. Both these solutions would result in increased pressure drop across the membrane, leading to liquid transfer into the reactor. This

transfer could be allowed for in reactor operation.

Methods of increasing flow rate whilst decreasing the pressure drop across the membrane should be explored. These might include; minimising the lengths of each piece of tube, arranging the tubing so it is straight not coiled; balancing the pressures on either entry or exit to the tubing, placing devices in the tubing to encourage turbulance; introducing the perfusate to the membrane with a rotational motion.

Scale up of reactor volume is also mainly a problem of arranging increasing lengths of tubing in the reactor. If the diameter of the tubing and the volume it takes up in the reactor is kept constant then the length increases in proportion to the volume. Methods of arranging and supporting this length of tubing will become increasingly mechanically critical.

The dialysis membrane could also be used for bubble-free oxygen transfer into the culture. A one litre reactor with a cell density of 2×10^6 cells ml^{-1} would have an oxygen demand of 0.05-1.0 mM O_2 hr^{-1}. Under present conditions using oxygen saturated medium (1mM l^{-1}) 0.6 mM O_2 hr^{-1} passes the membrane. Assuming a membrane transfer coefficient for oxygen of 0.16 (similar to that of NH^4) and the standard configuration then the oxygen transfer rate will be 0.06 mM hr^{-1}. The transfer rates of oxygen should be experimentally determined. Given the possibilities for increases in membrane area, and increasing membrane transfer coefficients this value could be substantially increased.

10.2 Cell Physiology

This is an interesting system in which to study mechanisms of cell death in a bioreactor. Unlike batch culture the chemical environment is kept stable over extended periods of time, and unlike continuous culture the growth rate is determined solely by the environment. Additionally the effects of low and high molecular weight factors medium, and mechanical damage can be separated. For example a cell line transfected with Bcl-2 should have a lower turnover rate in dialysis culture than the same clone not transfected with Bcl-2.

The separation of high and low molecular weight effects and steady state metabolite concentrations could be used to design efficient basal media for high cell density culture.

The reason for the apparent degradation of the antibody should be investigated. This could be done by assaying protease levels and/or by assessing the rate of decrease in antibody concentration using ELISA. Further assessment of the quantity of antibody fragments, using gel electrophoresis and protein blotting, to show the extent and type of degradation that occurs is necessary. Glycosylation status of the product should also be followed. Comparisons between a range of cell lines should be attempted to see if this effect is cell line specific.

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Appendix A

Appendices

A.1 Data

A.1.1 Batch Experiments

Batch growth of TBC3, pH 7.0 DOT=50%, agitation speed=100 rpm, medium= RPM1 1640, 5% NBS. 10%volume inoculum. Figures 9 and Figure 10

Time hrs	Cell $\times 10^5$ cells ml^{-1}	Viability %	Glucose mMol l^{-1}
0	6.2	98	8.9
16.5	13	98	4.3
24	13.8	98	3.8
46	20.3	98	4.1
48	20.6	96	4.5
63	18.3	82	2.1
88	12	45	1.3

Batch growth of PQX1, pH 7.0 DOT=50%, agitation speed=100 rpm, medium= RPM1 1640, 5% NBS , 10%volume inoculum. Figure 11.

Time hrs	Cells $\times 10^5$ cells ml^{-1}	Glucose mMol l^{-1}	Antibody μg ml^{-1}
0	1.1	2	2
11	1.5	1.7	4
24	1.7	1.4	5
35	3.3	1.3	9
44	4.5	0.9	15
58	7.1	0.64	20
70	4.1	0.55	26.5
82	3	0.42	33
96	2.2	0.3	35

A.1.2 Biopro Experiments

Effect of Biopro Concentration on the batch growth of TBC3. 50 ml volume in 150ml stirred bottles. 10% volume inoculum. Medium= RPM1 1640 and 5% NBS.

Time	Biopro Concentration %w/v											
hr	0		0.1		0.25		0.50		0.75		1.00	
0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
24	2.0	2.1	1.8	1.6	2.2	2.4	2.4	2.4	2.4	2.2	2.6	2.3
48	2.9	2.7	2.4	2.4	4.5	4.4	6.0	5.5	5.4	5.5	6.5	6.4
64	4.0	4.2	3.5	4.0	4.6	4.6	6.5	63	5.0	5.0	4.7	4.2
74	6.9	7.0	7.5	7.0	8.5	8.5	7.8	8.1	7.5	7.5	4.2	4.2
88	4.1	4.0	5.8	6.3	5.3	5.2	5.6	6.1	5.7	5.7	5.3	5.6
115	1.2	1.1	4.8	3.9	3.1	2.8	3.6	3.6	4.2	4.8	2.2	2.2

Comparison of Biopro with FCS and Peptone, in the batch growth of TBC3. 50 ml volume in 150ml stirred bottles. 10% volume inoculum. Medium= RPM1 1640 and 5% NBS.

Time	NBS		NBS		NBS		FCS		FCS.	
hr			Bp		Pep				Bp	
1	1	1	1	1	1	1	1	1	1	1
24	2.5	2.7	2.5	2.9	3.0	2.8	2.8	3.1	3.0	3.3
31	4.7	4.7	4.9	5.1	7.0	6.9	6.9	7.2	.7.0	6.6
44	4.2	4.6	10.2	9.9	12.0	11.8	12.2	12.8	12.8	11.5
55	9.6	9.8	10.1	10.4	9.0	8.5	12.0	10.4	12.1	12.0
68	5.0	5.8	8.1	8.5	4.3	4.0	10.0	9.0	11.0	10.4
75	2.8	2.5	4.3	4.6	1.6	1.5	4.2	3.6	4.0	3.5
95	0.25	0.4	1.25	1.0	0.15	0.1	0.5	0.3	1.2	1.3

A.1.3 Added Lactate and Ammonia Batch Cultures

Batch cultures of TB/C3. Ammonium chloride was added then pH was adjusted back to 7.0 before the medium was inoculated by spinning down the cell and resuspended in the medium. Cell Number (CN) in $\times 10^6$ cells ml^{-1}, Viability (VI) in % 16

-	Added Ammonia concentration							
-	0 mM		1 mM		2 mM		3 mM	
Time	CN	VI	CN	VI	CN	VI		
0	0.2	98	0.2	98	0.2	98	0.2	98
24	0.070	94	0.086	95	0.042	93	0.035	86
48	0.22	85	0.23	87	0.10	79	0.038	80
86	0.51	95	0.44	97	0.20	92	0.045	75
110	1.0	96	0.87	97	0.38	91	0.040	50
124	1.2	90	0.97	93	0.72	88	0.069	39
148	0.88	70	0.91	71	1.19	92	0.00	-

Batch cultures of TB/C3 with added Lactate. Figure 15

Time (hrs)	Cell No ($\times 10^6$ cells ml^{-1} Added Lactate conc.								Viability (%) Added Lactate conc.							
-	60mM		40mM		20mM		0mM		60mM		40mM		20mM		0mM	
0	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	96	96	96	96	96	96	96	96
24	0.17	0.17	0.14	0.13	0.12	0.13	0.13	0.12	99	97	98	97	97	100	99	50
48	0.14	0.15	0.18	0.21	0.26	0.28	0.27	0.29	100	98	98	97	98	35	99	95
72	0.07	0.05	0.10	0.13	0.19	0.2	0.54	0.56	88	33	62	53	61	80	98	96
96	0	0	0.05	0.06	0.1	0.09	0.57	0.59		32	31	56	34	94	95	

A.1.4 Small Scale Dialysis Experiments

Dialysis culture of TBC3, in bottle device, media= RPM1 1640, serum=5% NBS. Perfusate= RPMI 1640 only. Figure 13.

Time hr	Cell $\times 10^5$ cells ml^{-1}	Glucose mMol l^{-1}	Ammonia mMol l^{-1}
0	1.7	11.1	2
23	4.5	8.3	1.5
49	13	7.2	1.3
64	15	7.7	1.4
96	23	3.8	0.7
120	15.9	5	0.9
140	16	5	0.9
150	18	3.3	0.6
170	14.3	4.4	0.8

Dialysis culture of PQX1, in bottle device, media= RPM1 1640, serum=5% NBS. Perfusate RPMI 1640 only. Figure 14

Time hrs	Cell $\times 10^5$ cells ml^{-1}	Viability %	Glucose mMol l^{-1}	Ab μg ml^{-1}
0	2.66	97	11.1	1
11.5	3.7	98	8.3	2
23	6	99	7.2	10
32	9.7	99	7.8	17
48	13	97	3.9	32
62	17	96	5.0	54
82	23	95	5.0	62
94	24	94	4.5	87

A.1.5 Large Scale Dialysis Experiments

Effect of Membrane Area on steady state cell concentrations. All at 0.5 v/v/d perfusion rate. DOT=50%, pH=7.0, agitation rate=100 rpm. Medium= RPMI 1640 and 5%NBS. Perfusate= RPMI 1640 plud an additional 5.5 mMol l^{-1}Glucose; and 0.25 % w/v Peptone, 0.05% Pluronic F68. Figure 19.

Time	Cell Concentration				^{3}H Incorporation	
	Length of Membrane				0.5	Control
hrs	0.25	0.5	0.75	1	cpm	cpm
0	5.3	4.4	5.1	3.1	760	112
24	11.5	10	9.1	12	770	75
48	10	13.2	12.5	18	657	111
72	8.5	12	15.1	20	704	90
96	8.3	11.8	18.8	21	607	76
120	8.2	12.2	16	20	553	86
144	8.4	12.2	15	19	691	64
168	8.3	12	14.6	19	547	107
192		12	15.3	20	700	84
210		12	15	20	814	74
240			15	19.5		
252			15.3			

Dialysis culture of TBC3. pH 7.0, DOT 50%, agitation rate= 100 rpm. Medium= RPMI 1640 with 5% NBS. Perfusate: RPMI 1640 with an additional 5.5 mmol/l glucose, 0.25 % peptone, 0.05% Pluronic F68. Reac= reactor, Perf= Perfusate Figure 21.

time	PR	Cell	Ab	Concentration Glucose		Lactate		Ammonia	
				Reac	Perf	Reac	Perf	Reac	Perf
hr	l/d	$\times 10^5$ cells ml^{-1}	μg ml^{-1}	mmol/l					
0	0.5	3.8	28.3	14.4	16.5	0.3	0.2	2.1	1.4
22	0.5	9.7	50.8	12.7	13.5	0.8	0.7	4.9	3.4
31	0.5	17.6	60.5	8.8	10.4	1.2	1.1	8.0	5.7
48	0.5	21.0	108	6.3	9.5	1.6	1.3	10.2	8.0
56	0.5	19.8	130	7.3	10.1	1.5	1.3	9.8	6.9
71	0.5	20.1	130	7.5	9.4	1.6	1.3	8.1	7.2
79	0.5	19.1	160	7.0	9.3	1.5	1.3	8.3	7.3
95	0.5	19.4	200	7.2	10.0	1.5	1.3	9.5	7.2
104	1	28.1	205	7.1	11.6	1.3	0.9	7.6	5.3
119	1	26.0	250	7.6	11.3	1.3	1.0	8.3	4.7
140	1	28.3	300	7.8	11.6	1.2	1.0	7.8	4.6
166	1	27.6	370	7.1	12.6	1.2	1.0	9.0	5.1
192	1	28.7	490	8.0	11.7	1.2	0.9	8.0	5.1
216	1.5	32.5	500	8.2	12.6	1.1	0.8	7.7	4.0
238	1.5	36.8	586	7.8	13.3	1.1	0.7	7.2	4.0
262	1.5	35.2	760	7.8	12.8	1.1	0.7	8.3	4.5
272	1.5	32.4	770	7.7	12.7	1.1	0.8	7.0	4.0
286	2	40.6	840	8.5	12.5	1.1	0.7	7.1	3.4
297	2	40.0	870	8.1	13.1	1.0	0.7	8.1	3.5
312	2	37.4	1020	8.1	13.0	1.1	0.7	7.3	3.4
320	2	37.0	12 1	7.5	12.8	1.1	0.7	7.6	3.6
335	2.5	42.6	1100	7.9	13.0	0.9	0.6	7.5	3.3
345	2.5	41.8	1060	7.5	13.1	1.0	0.5	7.1	3.0
359	2.5	40.9	1170	7.2	14.2	1.1	0.6	7.2	3.1
371	2.5	41.8	1220	8.4	13.1	1.0	0.6	7.1	3.4

Dialysis culture of TBC3 at half normal membrane area, effect of perfusion rate. Figure25

Time	Cell No.	Viability	Perfusion Rate
0	4.6	95	0.5
24	9.0	97	
48	20.1	98	
72	16.3	72	
96	11.8	51	
122	10.6	46	
145	10.3	42	
169	10.1	39	
192	10.0	33	
216	10.3	35	1.0
240	17.3	48	
264	20.0	31	
288	21.0	32	
312	22.8	28	
336	21.3	32	
360	22.5	34	1.5
384	23.0	31	
408	23.2	27	
432	23.0	26	
456	24.0	28	2.0
480	33.0	30	
504	38.0	35	
528	37.0	40	

Dialysis culture of TBC3. pH 7.0, DOT 50%, agitation rate= 100 rpm. Medium= RPMI 1640 with 5% NBS. Perfusate= RPMI 1640 plus 0.25 % peptone, 0.05% Pluronic F68. Figure 35.

TIME HRS	Cell $\times 10^5$ cells ml^{-1}	Viab %	PR V/V/D	PV L	SERUM ml	Protein mg/ml	Antibody $\mu g\ ml^{-1}$
0	4	95	0.5	0.5		1.35	15
24	8	95					32
50	14	80					64
70	15	50		1			102
118	15	30					143
144	15						162
170	14		1			1.2	232
194	15						454
260	17						
284	17				25		
308	20						881
332	27					3	
357	18				25		1005
383	25						
405	20						1152
429	33				25		1138
453	20					5	1239
478	15						1477

Metabolite concentration for the previous table. Figure 36

FER	PER	FER	PER	FER	PER
Glucose		Lactate		Ammonia	
mMol l^{-1}	mMol l^{-1}	mMol l^{-1}	mMol l^{-1}	mMol l^{-1}	mMol l^{-1}
4.01		8.711		0.4942	
3.80		6.41		0.56	
3.44	4.38	6.90	5.24	0.71	0.67
3.13	6.20	8.09	7.11	1.20	0.62
2.40		9.27		0.56	
1.67		9.72		0.85	
2.87	3.28	10.68	7.77	0.73	0.73
3.23	4.95	7.22	9.53	0.81	0.64
3.33	6.20	5.72	7.48	0.87	0.59
2.97	7.14	6.51	5.78	0.81	0.64
0.00	8.13	8.23	5.15	0.76	0.65
0.89	5.21	10.40	6.64	0.66	
0.00		11.20		0.79	0.64
0.00	6.25	12.27	7.50	0.89	0.64
0.00	4.17	10.79	6.93	0.97	0.69
0.00	4.64		7.74	0.91	0.78
0.00	5.37		8.03		
1.67	0.00				

Dialysis culture of TBC3. pH 7.0, DOT 50%, agitation rate= 100 rpm. Medium= RPMI 1640 with 5% NBS. Perfusate= RPMI 1640 with an additional 5.5 mmol/l glucose, 0.25 % peptone, 0.05% Pluronic F68. Figure 29.

Time hrs	Cell Concentration $\times 10^5$ cells ml^{-1}	Viability %	Antibody Concentration $\mu g\ ml^{-1}$
0	7.3	97	24
24	12	98	67
47	20	92	130
72	23	89	221
96	19.5	70	308
122	15.5	43	385
148	14.9	41	452
168	14.6	44	502
191	14.7	38	560
220	15.3	39	634
262	15.7	43	744
270	15.6	40	766
271	8.7	38	330
284	11.3	38	362
311	20	42	434
334	19.7	45	511
357	18.5	33	586
410	15	36	737
435	14.8	35	800

Dialysis culture of TBC3. pH 7.0, DOT 50%, agitation rate= 100 rpm. Medium= RPMI 1640 with 5% NBS. Perfusate= RPMI 1640 with an additional 5.5 mmol/l glucose, 0.25 % peptone, 0.05% Pluronic F68. Figure 37.

Time hrs	Cell $\times 10^5$ cells ml^{-1}	Glucose mMol l^{-1}	PR l/d	Serum Addition
0	3	5.8	0.5	
20	7.5	6.1	0.5	
44	12.5	3.2	1.0	
68	27	3.2	1.0	
104	35	5.0	1.0	
118	29	5.0	1.0	
142	30	5.0	1.0	
168	31	4.9	1.0	
194	32	4.0	1.0	start
209	34	4.4	1.0	
233	30	4.2	1.0	
317	27	4.1	1.0	stop
340	29	6.0	1.0	
360	32	6.2	1.0	
378	37	6.3	1.0	
400	33	9.1	2.0	
424	40	9.2	2.0	
450	40	10.2	2.0	

Batch Growth of TBC3 in 50ml stirred bottles, with dialysed and whole serum. Medium= RPMI 1640, 0.25% peptone, 0.05%pluronic F68, 5% NBS.

time	Dialysed		Nondialysed	
hr	$\times 10^5$ cells ml^{-1}		$\times 10^5$ cells ml^{-1}	
0	1.0	1.0	1.0	1.0
21	1.5	1.8	1.9	1.9
31	2.4	2.6	2.5	2.5
45	5.2	5.2	4.7	4.4
56	5.4	5.6	5.5	5.5
70	6.4	7.1	8.6	8.8
80	5.1	5.6	8.6	8.6
91	4.0	3.1.	5.4	5.3

Dialysis culture of TBC3. pH 7.0, DOT 50%, agitation rate= 100 rpm. Medium: RPMI 1640 with 5% NBS. Perfusate: RPMI 1640 plus 0.25 % peptone, 0.05% Pluronic F68. Figure 38.

time hrs	Cell $\times 10^5$ cells ml^{-1}	glucose mMol l^{-1}	antibody μg ml^{-1}
0	4		25
22	13	4.3	
31	17.3	2.3	52
48	20.6	2.9	87
56	18.6	2.9	
71	19.8	1.8	64
79	20	2.3	62
95	20	2.6	134
104	23.3	2.3	151
119	27.4	1.7	182
166	27	1.8	313
192	27	1.0	562
216	28	0.0	557
238	28	0.0	410
262	32	1.0	557
272	32	1.6	560
286	27	2.3	
297	30	2.8	589
312	29	2.9	
320	33.3	2.9	337

A standard dialysis culture of TB/C3 perfusion rate of $0.5ld^{-1}$ 41

Time hrs	CN $\times 10^6$ cells ml^{-1}	Viability % %	Median Forward Scatter arbitrary
0	0.3	99	
24	0.57	93	435
68	0.92	93	414
76	1.24	78	352
93	1.96	77	452
100	2.09	75	507
116	2.06	72	523
124	2.09	72	523
163	2.03	54	512
180	2.1	51	

A.1.6 Other Cell Lines

CHO grown in Dialysis culture. pH=7.0, DOT=30rate=100 rpm. Medium=RPMI1640 with 5% FCS, Perfusate=RPMI1640. Batch data provided by Mr Leelavatcharamas for the same reactor and medium. Figure=43

-	Dialysis			Batch	
Time hr	CN $\times 10^5$ cells ml^{-1}	Viability %	Interferon-γ $\times 10^7$ IU ml^{-1}	CN $\times 10^5$ cells ml^{-1}	VI %
0	0.6	98		0	1
26	1	98		26	0.21
47	1.08	98		50	0.439
71	1.67	99		75	0.78
93	1.99	97		96.5	1.3
122	2.53	97		118.5	1.43
143	3	95	1.3	143.5	1.34
167	3.1	94		169	1.23
194	3.39	94		188	1.07
215	3.6	95		210	6
241	3.91	94			
268	4.07	87			
284	4.2	88			
308	4	87	4.19		
333	3.9	87			
356	3.7	86			
381	3.54	78	7		

Dialysis culture of CHO under Figure 44

Time (hrs)	Cell No $\times 10^6$ cells ml^{-1}	Viability %	G1 %	G2 %	S %	Forward Scatter Arbitrary units
0	0.31	97				
20	0.5	94	71.5	0	28.5	425.19
26	0.7	96	42.5	17.5	38	503.74
44	0.82	94	69.4	9.7	20.9	479.83
52	0.74	90	61.6	9.5	28.8	467.99
68	0.75	84	41.3	24.9	33.6	475.39
92	0.75	81	53.1	15.4	31.5	447.55
108	0.78	81	57.8	12	30.1	435.18
116	0.77	77				
133	0.74	72				
141	0.875	79	58.7	11	21.8	378.56
157	1.05	81				
169	1.1	84	60.8	14.9	24.3	428.66
178	1.1	89	60.3	16.5	23.2	457.83
181	1.39	80				425.73
191	1.73	85				218.2
210	2	82				
222	2	83				
234	2	85				
243	1.98	87				

Insect Cell Line SF9 in dialysis culture. pH=5.0 DOT=30%, agitation rate=100rpm. Medium=TC100 with 5% FCS, Perfusate= TC100. LSL reactor configuration. Figure 48

Time	CN	% Viability	Dialysis Conditions
0	0.2	96	Batch
17	0.4	98	
39	0.67	92	
61	1.18	93	
88	1.3	88	Dialysis TC100
112	1.37	82	
135	1.33	84	
161	0.98	63	
185	0.949	76	
208	1.1	75	Dialysis TC100 +5% FCS + 5% NBS
232	1.02	60	
264	0.9	69	
286	0.73	59	
302	0.77	55	

Insect cell:SF9. Comparison between dialysis and convential batch (no) feeding regimes post infection. Identical LSL reactors. pH=5.0, DOT=30%, Medium=TC100 with 5% FCS, Perfusate=TC100. Figure 49

Time	Batch			Dialysis		
	Cell	Viab	B-gal	Cell	Viab	B-gal
hr	$\times 10^6$ cells ml^{-1}	%	$\times 10^6$ cells ml^{-1}	%		
0	0.88		94	0.88	93	
6	0.82		91	0.86	93	
11	0.85		92	0.86	93	
25	0.93		84	0.9	87	
35	0.72		72	0.92	82	
47	0.55		52	0.65	62	
55	0.58		53	0.67	63	
71	0.51		49	0.44	56	
95	0.38		47	0.25	36	
120	0.11		20	0.15	25	