ENHANCEMENT OF PRODUCT RECOVERY IN MAMMALIAN CELL CULTURE.

A thesis submitted for the degree of
Doctor of Philosophy
by

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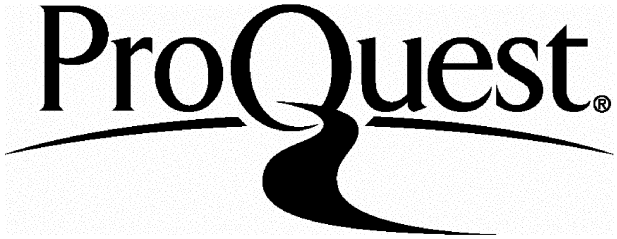

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For Philip.

"...shaking the tree..."

ABSTRACT.

The objective of this doctoral study was the enhancement of the early stages of protein purification from mammalian cell culture, specifically the removal of cells, cell debris and other contaminants from large-scale cultures producing antibodies by the aggregative action of long-chain polyelectrolytes.

To accomplish this, a detailed examination was made of the cell culture itself, and of the primary downstream processing stages. Assays were developed to monitor the levels of product and contaminants throughout the process, and a mass balance of the cell separation was produced.

Having established current separation practice and efficiency, flocculants were screened on a small scale to assess their ability to aggregate mammalian cells and debris. Assays were used to establish the ability of the flocculants to remove contaminants such as nucleic acids and extraneous proteins while leaving antibody levels and activity unchanged.

In order to assess the effect of adding flocculant to a large-scale cell culture, a scaled-down version of an industrial separation was performed. A theoretical efficiency index was used to compare two centrifuges, and assays were performed to evaluate the effectiveness of the flocculant when exposed to conditions similar to those found on an industrial scale. Conclusions were drawn on the desirability of using such chemicals to assist in the early purification of large-scale cultures of mammalian cells.

INDEX

INDEX OF FIGURES AND TABLES.

Plate 5	7.5	Control sample from Day 4 of scale-down study, with no PEI and no shear.
Plate 6	7.5	Sample from Day 4 of scale-down study, with 0.01% PEI and no shear.
Plate 7	7.5	Sample from Day 4 of scale-down study, with 0.01% PEI, after exposure to shear in 75 mm Instron capillary.
Fig 59	7.5	Comparison of IgM levels in samples exposed to different shear conditions, with no PEI added.
Fig 60	7.5	Comparison of IgM levels in samples exposed to different shear conditions, with 0.01% v/v PEI added.
Fig 61	7.5	Comparison of protein levels in samples exposed to different shear conditions, with no PEI added.
Fig 62	7.5	Comparison of protein levels in samples exposed to different shear conditions, with 0.01% v/v PEI added.
Fig 63	7.5	Comparison of DNA levels in samples exposed to different shear conditions, with no PEI added.
Fig 64	7.5	Comparison of DNA levels in samples exposed to different shear conditions, with 0.01% v/v PEI added.

INTRODUCTION.

1. MAMMALIAN CELL CULTURE.

This chapter is intended to give an overview of the development of mammalian cell culture processes, from initial conception to current methods, and to examine the relative advantages and drawbacks such a cell system offers the pharmaceutical industry in comparison with microbial methods.

1.1 The history of the development of mammalian cell culture.

Tissue culture developed from embryology techniques in use in the last century and are on record as far back as 1885, when Wilhelm Roux maintained the medullary plate of a chick embryo in warm saline solution for several days. The origins of cell culture (the cultivation of separate cells rather than tissue) date from 1903, when Jolly maintained salamander leucocytes in hanging drops for up to a month at a time, while making detailed observations on cell survival and division *in vitro*. In 1907, Ross Harrison's experiments on explants from the medullary tube region of frog embryos demonstrated unequivocally the continuation of normal cell functions *in vitro*. The explants, grown on clots of frog lymph, not only survived several weeks under aseptic conditions, but developed nerve fibres, growing out from the cells.

In 1910, Burrows replaced lymph clots with plasma clots and applied the method successfully to the tissues of warm-blooded animals. In using plasma from chick embryos, the discovery that foetal extract has strong growth-promoting effects on certain cells was made.

The limitations of the hanging drop culture method led in 1923 to the development of the Carrel flask, in which cultures could be grown on a larger scale. Carrel, a skilled surgeon, was himself responsible for the introduction of many of the basic tissue culture methods. During the 1920's two distinct areas of research developed; the investigation of cell growth, nutrition and multiplication, and that of cell differentiation and the organised development of embryonic tissue.

In 1938, Carrel and Lindbergh developed an early form of

perfusion system for the supply of oxygen and nutrients to the culture. This was inefficient however, and did not replace the use of roller bottles, which had been developed by Grey and his co-workers in 1933. Eventually the roller bottle system replaced the use of the Carrel flask.

Up until the beginning of the Second World War, tissue culture depended on the excision of material and its subsequent maintenance on plasma or serum. Such 'natural' media is even now difficult to define accurately and varies from batch to batch, so understanding of cell metabolism grew very slowly. To improve the situation, two things were required. The first was the development of standardised and uniform cell populations, similar to the pure strains in use by bacteriologists, and the second was the replacement of natural media with those of chemically designed composition. Great advances have been made in both areas. Cell lines now exist that can grow on totally chemically defined, serum free media, and the cell lines themselves are uniform in character, often having been cloned from single cells. Most of these lines, however, differ markedly from the original body cells from which they were derived.

The practical usefulness of such cell lines was demonstrated in 1948 when Enders and his colleagues showed that the poliomyelitis virus could be cultivated *in vitro* in the absence of nerve tissue (Phaff, 1981). By 1954, much work had been done on the cultivation of cells on a larger scale than before, using monolayers of cells. These monolayers were obtained by a method developed by the Mosconas in 1952, involving the use of trypsin to disperse viable cells from their parent tissues, then allowing the cells to settle on the flat surface of a bottle. For several years it was believed that cells could only survive and multiply if anchored in such a way.

The first suspension cultures, using rotating or shaking flasks, were not developed until 1954. Following that development, the use of tissue culture declined due to the difficulty of supplying such a system with oxygen and nutrients, and the tendency of the cells to differentiate

rather than divide (the two are mutually exclusive). Cell culture, however, is a technique that has grown steadily and rapidly since its inception. The discovery of the structure of DNA in 1953, and the elucidation of the DNA code in 1960 opened the door on the era of genetic engineering. The creation of hybridoma cells by Köhler and Milstein in 1975 made the large-scale production of monoclonal antibodies possible (Wyke, 1987).

There are two cell types used in mammalian cell culture.

'Normal' cells have finite lifespans of around fifty cell doublings, are non-oncogenic, nutritionally complex, and are anchorage-dependant (ie. they grow only when attached to a solid matrix). Examples of these cells include human embryo fibroblasts and primary monkey kidney cells.

Permanent cells occur spontaneously, or are induced by the action of a transforming virus, such as the Epstein Barr virus. They are characterised by an infinite lifespan (ie. no limit to the number of cell doublings), possible oncogenicity, less demanding nutritional requirements than 'normal' cells, and the ability to grow in suspension. Examples include the commonly-used Chinese hamster ovary cells (CHO), baby hamster kidney cells (BHK), monkey kidney cells (VERO) and NAMALVA, an Epstein Barr-transformed lymphocyte.

Another cell type is the hybridoma mentioned earlier. This is not a 'true' cell type, but is the fusion product of an antibody-forming lymphocyte taken from the spleen of a mouse immunised with a particular antigen, and a malignant, rapidly proliferating myeloma cell. These hybrid-myeloma cells, or hybridoma, express both the lymphocyte's specific antibody production and the myeloma's capacity for continuous proliferation. Such cells are used to produce monoclonal antibodies which, because of their specificity to one antigen only, can convey passive immunity against that antigen when administered in smaller doses than is the case with a conventional polyclonal antiserum, which typically contains more than 90% irrelevant immunoglobulins and will vary between preparations.

Mammalian cell cultures can be initiated from virtually any

cell source, though most work involves the cell types mentioned above because they are well-documented and carry less regulatory problems than previously unused cell lines. For all cell lines, including hybridoma, growth rates are slow, with doubling times of 10-24 hours (compare this with the bacterium *Escherichia coli*, which has a doubling time of only twenty minutes). Mammalian cells are nutritionally complex when compared with bacteria and yeasts, and achieve a low maximum cell density. They have a low oxygen demand but are nonetheless obligate aerobes. Most of their products are secreted freely into the surrounding media, except for certain surface antigens which adhere to the cell membrane.

1.2 The importance of mammalian cell culture to the pharmaceutical industry.

Because of the influence they exert on the human body, many naturally-occurring proteins are likely candidates for useful drugs, or as the basis of drugs. The main problem has always been one of supply; the body synthesises only minute quantities of many of the proteins, and their size and complexity make chemical synthesis impossible. For example, Tagamet, one of the world's best-selling chemically-made drug, has a molecular weight of only 250 Daltons, compared with 60,000 Daltons for tissue plasminogen activator (Wyke, 1987). Bacterial, yeast and mammalian cell systems have been used to produce proteins for the pharmaceutical industry. Each system has its own strengths and weaknesses.

1.2.1 Bacterial cell culture.

With the advent of genetic engineering it became possible to genetically modify microorganisms so that they produced many of these desirable proteins as part of their normal metabolism. Initially, bacteria were used because their expression systems were so effective and economical. *Escherichia coli* was particularly favoured, mainly because it was the most widely studied organism and thus the best

understood. However, in common with many other bacteria it does not usually secrete recombinant products, but often stores them as dense, insoluble, granular bodies within the cells, thus necessitating the use of lysis to release the protein into the supernatant. It also produces endotoxins and has a pyrogenic cell wall, both of which must be removed during processing. Even the use of alternative non-pyrogenic genera such as *Bacillus subtilis*, which also has the advantage of secreting many of its products, does not overcome all the problems associated with protein production in bacterial hosts. Of these, four are major drawbacks (Spier, 1983):-

--Glycosylation is the addition of highly variable carbohydrate oligomers to the protein molecule. It is an essential step which determines the antigenicity, longevity on injection into a recipient, and other properties of the fully- tailored molecule. Other less common post-translational modifications include phosphorylation, sulphonation, carboxylation and hydroxylation.

Bacterial cells do not glycosylate proteins. This lowers the activity of any protein that requires glycosylation, and risks unfavourable immunogenic responses when the protein is administered as a therapeutic. It may also affect protease resistance and the ability of the cell to secrete the protein (Atkinson *et al*, 1986). Most products are still utilisable, and examples of products made bacterially include α and β interferon and an HIV subunit protein. Most successful are those proteins which require no glycosylation to achieve full activation.

Another modification affecting the tertiary structure of the protein is the insertion of disulphide bridges. Bacteria, while capable of performing this modification, often do so incorrectly or insert only some of the required bridges. Saunders and Saunders (1987) noted that the reduced activity of human fibroblast interferon produced in *Escherichia coli* was due to the formation of incorrect disulphide bonds between cysteine residues in the protein.

--Aminoterminal residue removal is the second major problem associated with the production of mammalian proteins in

bacterial hosts. The initiator codon for eukaryotic proteins, a base triplet consisting of adenine, uracil and guanine (AUG) is also the prokaryotic codon for methionine, so this amino-acid is always added to the aminoterminal end of recombinant proteins produced in bacteria. Steps sometimes have to be taken to avoid this. One method is to synthesise the required protein as a hybrid with one normally produced by the host, and cleave it enzymatically at a later stage. For example, the hypothalamic hormone, somatostatin is made as a hybrid with β-galactosidase and is later cleaved by cyanogen bromide. A further advantage of this technique is that by expressing a protein as a fusion product, it may be secreted through *E. coli*'s cytoplasmic membrane into the periplasmic space, or even into the medium (Sherwood, 1991). Disulphide bond formation occurs with secretion, producing active products (Marston, 1987). However, this is not always the case, as was found when tissue plasminogen activator was expressed in *Escherichia coli*, fused to a normally efficient leader peptide. The protein still formed insoluble, inactive granules (Cartwright & Crespo, 1990). This method is also rendered useless by the presence of any further cleavage sites within the desired protein, thus severely limiting the usefulness of this technique. An alternative is to use a stepwise enzyme, which works along the amino-acid chain until inhibited by a certain residue. The enzyme dipeptylaminopeptidase I, for example, is stopped by a proline residue. As that happens to be the second amino-acid in human growth hormone, this method is ideally suited to the production and purification of that particular protein (Dalbøge *et al*, 1987). Reports have also been made of the development of a strain of *Escherichia coli* that simultaneously overproduces the required protein and the enzyme methionine aminopeptidase which removes the unrequired methionine residue (Ben-Bassat *et al*, 1987). While there are techniques available to circumvent this problem in specific cases, there is as yet no general method that can be applied successfully to all products that require modification.

--The basic differences between prokaryotic and eukaryotic

genes cause major problems in the manufacture of recombinant eukaryotic proteins in prokaryotic hosts. Firstly, the two cell types have different codon usages with respect to particular amino-acids. For example, the prokaryotic codon for the amino-acid leucine is cytosine, thymine, guanine (CTG) while in eukaryotes the cytosine residue is replaced with a second thymine residue (TTG). Secondly, eukaryotic genes often carry interspersed non-coding regions known as introns. These regions are enzymatically removed from the RNA during normal eukaryotic expression, but are retained in prokaryotic expression as bacteria do not carry the necessary enzymes for intron excision. A few proteins such as α and β interferon carry no introns in their DNA code, and as such are unaffected by this problem. It is possible to remove introns enzymatically from recombinant DNA fragments prior to insertion in the prokaryotic host although this would involve laboriously identifying, locating and excising the introns, and splicing the residual DNA.

--Bacteria that lose the inserted vector containing the code for the required protein will revert to their natural or 'wild' state and as such will almost always outgrow the cells that retain the vector, causing low or zero product yields. A safeguard is usually created by including resistance to a specific antibiotic in the vector, and then adding the antibiotic to the culture medium. Any cells that lose the vector thus lose their resistance and are killed. This is expensive however, and is frowned upon by regulatory authorities because of the risks of contamination of the product with the antibiotic. There are other methods for stabilizing vectors within bacterial cultures, including bioprocess strategies to nullify the growth advantage of the 'wild-type' cells or to separate them from the vector-retaining cells (Kumar *et al*, 1991).

Despite these various difficulties bacterial cell culture is widely used for the production of therapeutics. The lowered activity levels and the difficulty of purifying intracellular products is to a large extent mitigated by the rapid proliferation, simple growth requirements and ability to be

cultured on a large scale that characterises bacterial cell culture. Recombinant DNA techniques are facilitated by the high level of knowledge and understanding of bacterial chromosome layouts, and other methods of achieving the required post-translational modifications during downstream processing are available. The development of antibody fragments, which are much less complex structurally than the complete antibodies, has allowed manufacturers to return to bacteria as a means for producing them. Such fragments can be produced at much reduced manufacturing costs, and are expected to be used extensively within the diagnostics industry (Harris, 1991). However, in the area of highly pure, specific therapeutics, there is at present a limit to the usefulness of bacteria, due to the basic incompatibility of eukaryotic genes with the prokaryotic expression system.

1.2.2 Yeast cell culture.

The problems associated with expressing eukaryotic genes in a different host should in theory be reduced by the use of another eukaryote as that host. Yeasts are eukaryotic cells, and are typically unicellular, non-pathogenic, stable, easily immobilised, and facilitate product isolation by secreting many proteins. Carter *et al* (1987) note that, although eukaryotic, yeasts can be manipulated biochemically and genetically with the same ease as *Escherichia coli*. Recombinant DNA technology is assisted by the fact that some yeast strains carry plasmids. These are circular, extrachromosomal strands of DNA that replicate independently of the main chromosomes. In certain conditions, known as "relaxed control", 10-200 plasmids can form within a single cell. If the required vector is inserted into a plasmid, the independent replication of such a plasmid ensures that the vector is safely carried into new generations of cells.
However, yeasts also have drawbacks as far as the production of correctly structured proteins is concerned.
--Yeasts, in common with other eukaryotes, glycosylate many of their products, but not in the same manner as mammalian

cells. As a result, mammalian proteins expressed in yeasts may often be incorrectly glycosylated (Kikuchi & Ikehara, 1991; Teh-Yung Liu, 1992). This can result in a lower activity, though not as low as if the protein were totally unglycosylated, as from a bacterial source. The glycosylation apparatus of yeasts lies in the secretion pathway, with inner core carbohydrates assembled in the endoplasmic reticulum and the outer core added in the Golgi apparatus. The latter stage is different from that observed in mammalian cells (Carter *et al*, 1987).

--The same problem applies to intron excision as to glycosylation; the yeasts, being eukaryotic, have the means to perform this modification, but do not do so correctly on mammalian RNA. This can result in sections of RNA being removed from the wrong places, giving a lower product activity.

Yeasts may well be the area in which the future of engineered pharmaceuticals lies, as the knowledge of their genetic layout improves and the advantages of secretion, partial post-translational modification and intron excision are seen to outweigh those of working in the well-understood and well-established but sometimes impractical field of bacterial cell culture. As with bacterial systems, yeasts are now being used to produce antibody fragments (Hajela & Galzie, 1991). However, in areas where proteins of considerable complexity and glycosylation are required, yeasts are not currently able to compete with mammalian cell systems.

1.2.3 Mammalian cell culture.

The current role of mammalian cell culture is to create four basic groups of products:-

--Vaccines for both human use, such as the Rubella vaccine, and for veterinary use, such as that for rabies, are produced in mammalian cells. The first to be manufactured in such a way was the polio vaccine in 1954, using monkey kidney cells. Until recently effective vaccines were live, attenuated strains of the specific virus and thus inevitably carried some

risk of infection with the full-blown disease.
The advent of second-generation vaccines made of antigenic components, such as the hepatitis B surface antigen vaccine, has eliminated such risks. Many vaccines made in mammalian cell lines are much more immunogenic than those cultured in bacterial or yeast cells, as they are more accurately glycosylated.
--Natural products; these are compounds that are naturally made and secreted as a normal function of certain mammalian cells, and include the interferons and interleukins.
--Recombinant DNA products are those which, prior to the advent of genetic engineering, were not available commercially (Birch & Arathoon, 1990). They include hormones, enzymes, growth factors and other proteins. Examples of such products are tissue plasminogen activator, factor VIII and urokinase.
--The product of hybridoma cells, monoclonal antibodies are possibly the most important pharmaceuticals from mammalian cell culture to date. Their applications are varied and wide-ranging. In the area of immunopurification, the affinity of monoclonal antibodies (MAbs) to one antigenic site can be exploited in the creation of highly specific affinity purification systems, while in the field of diagnostics the ability to develop MAbs to recognise almost any organic molecule of more than 8,000 Daltons can be used to make diagnostic kits to measure the levels of different biological substances in the blood or other body fluids. The minimum in the size of the molecule to be detected can be circumvented by conjugating the smaller molecules to proteins. After the antibody has been developed, it will be able to recognise the molecule even when it is no longer attached to the protein. Limitations to this technology include the inability to produce MAbs to weak immunogens.
MAbs can also be used as *in vivo* diagnostic probes in tumour imaging by being radiolabelled. Finally, in the area of immunotherapy, MAbs can be conjugated to drugs and, because of their specificity to a single antigen, be able to carry the drug specifically to the site where it is required. This technique is known as targeted drug therapy. It could also be

used to suppress the rejection of transplanted organs and to remove undesirable substances from the bloodstream.
The main advantages of mammalian cell culture are that the product is virtually always secreted, facilitating downstream processing and making cell immobilisation feasible, the proteins are folded correctly, and post-translational modifications are carried out correctly, conferring full activity on the product (Kaufman, 1990; Bebbington & Hentschel, 1987). Disadvantages include the relatively low product yields from low density cultures (compared with yeasts or bacteria) growing on expensive, nutritionally complex media with a high protein content that makes subsequent purification processes more difficult. Another problem is that occasionally the alternative cell lines used do not give the exact post-translational modifications required by human proteins (Teh-Yung Liu, 1992). As human cells almost never undergo spontaneous immortalisation and are not easily transformed by somatic cell or chemical mutagenesis (Van Brunt, 1987; Hajela & Galzie, 1991), and with the future of mammalian cell culture expected to be in therapeutics rather than diagnostics, this is a potential limitation. The problem is not insurmountable; for example, the human anti-mouse (HAMA) response to rodent antibodies has been overcome by the development of 'humanised' antibodies that carry only a fraction of the original mouse amino-acid sequence. This small fraction, 2-3%, allows the retention of the binding specificity of the rodent antibody. Another approach is to replace the variable domains on both the heavy and light chains of a human antibody with those of a murine antibody which possesses the required antigen specificity, generating a 'chimeric' antibody (Cunningham & Harris, 1992). This can then be expressed in a mammalian cell line such as CHO or a myeloma cell line (Birch & Arathoon, 1990).

1.3 Current practices in mammalian cell culture.

1.3.1 Inoculation.

The inoculum is usually 10% of the total fermenter volume, as mammalian cells do not thrive if heavily diluted. For a large-scale fermentation several scale-up steps are therefore needed.

1.3.2 Fermentation.

The fermentation system used is decided basically by whether or not the cell line is anchorage dependant.

1.3.2.1 Anchorage dependant systems.

Roller bottles were developed early in the history of cell culture and were used in the original commercial cultivation of anchorage dependant cells. The cells adhere to the sides of the bottles, which are laid on their sides and slowly rotate about their horizontal longitudinal axis so that the cells are periodically immersed in the small amount of media in the bottle. The drawback of this system is that to harvest the product and replace exhausted media each bottle must be opened and the liquid replaced by hand. The process is labour-intensive, a problem which scale-up does not simplify, and carries a high risk of contamination (Rubinstein, 1985). Despite this, roller bottle cultures are surprisingly widely used today.

Microcarrier culture is the most common way of culturing anchorage dependant cells. The cells are immobilised on beads of about 100 μm diameter, made of gelatin, cross-linked dextran, modified DEAE-Sephadex or other such material, and are then grown in normal suspension fermenters.

Multiplate devices are another method of providing a large surface area for the cells to attach to, giving a large reaction area as the medium flows through the device.

Packed beds of glass beads, with cells immobilised on the

beads, also give a large reaction area. The medium flows through the packed bed that they form.
Both the polio vaccine and interferon are produced in anchorage dependant cell lines, using microcarrier systems.

1.3.2.2 Suspension culture systems.

These can be designed with or without cell retention (perfused). Of the two, the former is currently the most common.
Stirred reactors give an approximation of homogeneous conditions. They differ from bacterial stirred fermenters in that it is recommended they have a hemispherical base, no baffles or intrusions and a marine-type impeller. Lacking a rigid cell wall, mammalian cells are sensitive to shear and the above modifications protect them from this, to a certain extent. It should be noted, however, that mammalian cells are more resistant to shear than was originally thought (Spier, 1988) and in many cases can be grown in standard reactors designed for microbial systems, as long as the impeller speed is reduced. Oh *et al* (1988) report that marine impellers 0.06 m in diameter in 2 L bioreactors caused no damage to hybridoma cells when run at 450 rpm. The reactors were not sparged.
Another homogeneous system, and generally the method of choice, is the airlift reactor. The main body of the reactor has an internal cylinder, into the bottom of which air is sparged. The resulting drop in density causes the liquid in the inner cylinder to rise. At the liquid surface the air is lost and the liquid falls down the outside of the internal cylinder. At the base of the reactor, the air being continually sparged in causes the liquid to rise again, and so on. This method gives good mixing with low shear forces, and the fact that no impeller is needed means there are fewer glands through which contaminants might enter the vessel. The system is simple to construct and operate, easy to automate and control, and can be effectively scaled up.
Both suspension systems discussed so far can be modified for continuous or semi-continuous operation. However, for the cell

lines discussed in this thesis, immunoglobulin production continues well into the stationary and decline phases of the cell cultures, accumulating high product titres that enhance the efficiency of the subsequent product recovery steps. Other advantages of batch operation include the ease of operation and the clear definition of a batch for quality assurance purposes (Rhodes & Birch, 1988).

The advantage of perfusion systems, where the cells are retained within the bioreactor while the media passes over or through them, is that higher cell densities, often reaching 10^7 cells per ml, can be achieved. However, the perfusion rate of the media is also high, and the overall ratio of biomass to litres of media is the same as for homogeneous systems. The retention of such slow-growing cells has obvious advantages, especially as a lot of product formation occurs in the stationary or decline phases of many cell lines, and the cell-free product stream facilitates downstream processing. Of the various perfused systems available, the most commonly used is a method based on the *in vitro* simulation of capillaries, using hollow fibres. Cell densities of up to 10^9 per ml occur in living human tissues, interspersed with a network of capillaries, and this method endeavours to emulate this (Meldrum, 1987). The cells grow on the outside of the hollow capillaries, within an enclosing shell, and the medium is introduced through the capillaries. The product diffuses into the capillaries along with the cellular wastes. Reports have been made of the continuous operation of such a system for up to seven months. Other perfused systems include membrane encapsulation, where the cells are imbedded in beads of gel which are then coated with a permeable membrane, after which the internal gel is resolubilised. This has the advantage of protecting the cells from shear while allowing easy cell separation during downstream processing. There are, however, problems of oxygen and nutrient limitation within the capsules, and the manufacture of the capsules is a long, costly process that carries a high risk of contamination.

Most mammalian cells are cultured in media consisting of 5-10% serum, whose function is to provide trace elements, along with

the means to pass them through the plasma membrane (transferrin), hormone-like growth factors, non-specific 'protective' effects (albumin), materials for membrane biosynthesis, and attachment factors (Spier, 1988a). It also binds and neutralises toxins and is a carrier for labile or water-insoluble nutrients (Lambert & Birch, 1985). Despite the complexity and diversity of the functions of serum it has been possible to develop fully-defined serum-free media for some cell lines. Although expensive to develop, this provides a highly reproducible medium with assured long-term supply, free from variations from animal to animal and without the associated risks of medium-derived contaminants. Downstream processing is also simplified by the presence of fewer proteins. However, care must be taken that product stability is not affected. The cells are also more shear-sensitive and thus prone to bubble damage in the foam layer, as discussed in the following section. Even so, conversion to serum-free medium is of considerable benefit in the pharmaceutical industry, as serum can be the source of contamination in the form of bacteria, fungi, viruses, mycoplasmas and endotoxins.

1.4 Shear sensitivity of mammalian cells.

While there are conflicting opinions as to the degree of sensitivity to shear that mammalian cells show, there is no doubt that a certain amount of care must be taken to protect the cells from high shear forces.

The data that exists on shear sensitivity shows that robustness of the cell membrane varies not only between cell lines, (hybridoma are more sensitive than baby hamster kidney cells or myeloma cells, for example) but also between different phases of growth, generation number, or even with a change to fresh medium (Bliem & Katinger, 1988). Shear sensitivity is independent of metabolite or cell concentrations, though the addition of fresh media in routine subculturing dramatically increases shear sensitivity for a short while (Petersen *et al*, 1988). The sensitivity of the cells also depends on previous exposure to shear. Cells grown

under vigorous agitation (200 rpm) are more resistant than cells grown under mild agitation (60 rpm) or under stationary conditions (T-flasks). Petersen hypothesises that "...under sufficient agitation the culture can exhibit a phenotypic response (eg. change in membrane lipid composition, expression of shock proteins, overexpression of cytoskeletal proteins, etc.) that renders the cells less sensitive to shear."
Mouse myeloma cells are lysed at an average wall shear stress level of 1800 dyne cm^{-2} (McQueen *et al*, 1987). Hybridoma cells shear at levels of 800 dyne cm^{-2}. The shear was imposed by passage, under high pressure, through a constricted section of tubing about 50-80 times the diameter of the cells. Cell numbers decreased exponentially with the number of passes through the tube:-

$$\log. \left(\frac{x}{x_o} \right) = -kN$$

where:-
N = number of passes
$\left(\frac{x}{x_o} \right)$ = fraction of viable cells left
k = specific lysis rate (number of $passes^{-1}$) or probability of lysis per pass (Depends on average wall shear stress.)

The cell viability levels were unchanged; cells either broke open or remained viable, as measured by lactate dehydrogenase release into the media. An increase in residence time by increasing the tube length caused more lysis, showing that lysis is caused by turbulent flow effects as well as sudden constriction of the flow path. This work was extended to look at the shear-protective effects of serum on mammalian cells by studying cultures grown in different concentrations of serum (McQueen & Bailey, 1989). Those cultures grown in less than 10% serum suffered more from exposure to prolonged shear conditions than did those grown in 10% serum or more. Work done by Al-Rubeai *et al* (1990) on the shear sensitivity of serum-deprived cultures supports these findings. The protective action of serum is thought to be mostly physical,

but not related to any increase in viscosity of the medium (Kunas & Papoutsakis, 1989). Ozturk and Palsson hypothesise that growth stimulatory factors carried by the serum change the cytoskeletal structure, making the cells more sturdy. Ramirez and Mutharasan (1992) suggest that "the protective mechanism of serum against hydrodynamic damage relies, at least partially, on its ability to decrease the plasma membrane fluidity of hybridomas possibly through the transfer of cholesterol from the serum lipoproteins into the plasma membrane".

Petersen *et al* (1988) measured the shear sensitivity of mouse hybridoma producing IgM using a cup and bob viscometer equipped with a rotating outer cup for the generation of a stable, overall identical shear field.

$$t = \mu\gamma$$

where:-

t = shear stress ($N\ m^{-2}$)

μ = fluid viscosity ($N\ s\ m^{-2}$)

γ = shear rate (s^{-1}) (A function of the design equation of the viscometer, and the rotational speed.)

Petersen also measured cell lysis by the release of lactate dehydrogenase, and commented that the assay "...is somewhat more sensitive [than trypan blue] and can indicate low levels of cell damage not detectable by trypan blue exclusion." He hypothesised the existence of a subpopulation of cells that are extremely shear-sensitive.

Lee *et al* (1988) noted the importance of cell viability in relation to the levels of monoclonal antibody produced. Viable cells produce MAbs throughout their growth cycle, including the decline phase, if the correct precursors are present. Shear is therefore to be avoided, to keep the cells viable for as long as possible, particularly as cells in the decline phase of growth appear to be very shear-sensitive.

Another important advantage in minimising cell damage is the reduction of contaminant levels in the broth, making

subsequent purification easier. Brooks (1984) agrees that the damage to cell viability and the state of the product stream by cell shear is more serious than that caused by the release of intracellular enzymes.

Handa *et al* (1987) found two mechanisms of cell damage at the liquid:air disengagement site; from rapid oscillations caused by bursting bubbles, and from shearing in draining liquid films in foams. Local shear stress associated with the bubbles is in the region of 0.01 to 0.1 N m^{-2}, the same level as the impeller-generated shear stresses found in low shear operating conditions, while theoretical calculations show that the bubble shear rates could be much higher (Bliem & Katinger, 1988).

There is negligible damage caused by vessel wall:cell interactions given a smooth, unbaffled vessel.

The addition of compounds such as Pluronic (a commercial antifoam), PEG or foetal calf serum, stabilize the foam layer by generating a high surface viscosity. This reduces bubble breakup and prevents cells from being drawn into the foam layer (Papoutsakis, 1991). The use of larger bubbles and lower bubble frequencies, balanced against good oxygen transfer, also reduces shear damage. Serum-free media, lacking the 'protection factors' provided by serum, needs careful attention. The use of tall, slim vessels reduces the relative contact between the media and the surface.

2. DOWNSTREAM PROCESSING.

With the advent of genetic engineering the commercial manufacture of a wide range of hitherto unavailable therapeutic human proteins is now becoming possible. However, if these are to be widely available, such techniques must provide economically realistic answers to human needs. Reports show that the engineering 'bottlenecks' associated with bringing recombinant DNA products to the market are frequently due to the poorly understood problems of the purification processes, particularly in the primary stages (Datar, 1986). Downstream processing, the general term covering the series of post-fermentation operations necessary to refine the product to the required level of purity, is in some respects a poorly-researched area of biotechnology. The importance of expanding our knowledge in this field was stressed in the 1982 report to the Organisation for Economic Co-operation and Development (OECD) by Bull *et al*. In this, they state that "...molecule recovery and purification...are absolutely essential in ensuring that advanced techniques in genetic engineering or enzyme engineering do not simply remain forecasts, projects and newspaper articles, but become established processes capable of stimulating the industrial and economic development of our countries" (Bull *et al*, 1982). Often, no overview is taken of a production process as a whole, and downstream processing is seen as a discrete entity, separate from fermentation or cell culture. As a result the fermentation stage is not designed with purification in mind, although relatively simple changes to the fermentation process can reduce or even remove some downstream problems (Knight, 1989; Mather & Tsao, 1990). An ideal fermentation would present the broth for harvesting with the cells in a concentrated state in a chemically simple medium containing a minimum of contaminating proteins and no pyrogenic or infectious materials (Cartwright, 1987). The optimisation of the purification sequence itself is also vital. It is important to achieve this during the early stages of product development, since any licence granted to market the product

will cover the production and purification processes as well as the biochemistry of the product. Any change to the purification sequence at a later date is likely to be highly expensive, involving as it does reapplication for a product licence (Rosevear & Lambe, 1988; Asenjo & Patrick, 1990).

2.1 Definition of aims of downstream processing.

The main aims while designing an optimal purification sequence are the maximisation of yield and purity of the product while minimising cost. The level of purity required is defined by the intended use of the product. A therapeutic drug intended for repeated use must be highly pure so as to avoid any adverse immunogenic responses, caused by the presence of product variants, endotoxins, pyrogens, oncogenic DNA, or other contaminants. A protein intended for use in a diagnostic kit need only be pure enough to avoid secondary reactions affecting the accuracy of the results given by the kit; perhaps 80% pure, with no residual protease activity (Schmidt, 1989).
To minimise the cost involved it is wise to minimise the cost of each purification process, and also to minimise the number of processes used. The latter step will also help to keep the overall product yield as high as possible.

2.2 Protocols used to achieve aims.

Asenjo and Patrick (1990) suggest a strategy to be applied to protein purification. As stated above, the level of purity required should be established before the purification process is designed.
The starting material should be characterised by gathering as much physicochemical information as possible on the nature of the protein to be purified, and its contaminants. This should include details of size, molecular weight, charge, isoelectric point, biospecificity, surface properties, solubility, stability, and so on.
Possible separation processes should be defined in terms of

flowrate, binding ability of gel per unit volume, specificity, cost, reproducibility, ease of scale-up, and so on.

Using the knowledge gained from the previous steps, a sequence of unit operations should then be defined. The following criteria should be observed:-

-- Separation processes should be based on different physicochemical properties (ie. do not use two processes based on say, size).

-- In choosing possible processes, exploitation of the greatest difference between the product and the impurities should be ensured.

-- The first step should involve the removal of water, particularly if the product stream is dilute. This reduces the volume that requires further purification, an advantage if chromatographic steps requiring slow flowrates are to be used.

-- Substances likely to interfere with later steps should be removed as early as possible. For example, cell debris could foul ultrafiltration membranes and should therefore be removed before the ultrafiltration step. Similarly, lipids could foul affinity chromatography columns.

-- The minimum number of steps should be used. If affinity chromatography is to be used it should be included as early as possible, soon after the volume has been reduced to a manageable amount and any contaminants likely to foul the column have been removed. The earlier the technique is used, the greater its potential resolving power.

-- The mildest conditions feasible should be used, to avoid product denaturation.

Expert systems could greatly facilitate the design of an effective downstream processing sequence. Databanks of the physicochemical properties of different proteins could be built up, along with information on unit separation processes, and from this an optimum sequence could then be developed.

The purity of recombinant DNA products from transformed mammalian cell lines is particularly dependant on a safe and efficient purification sequence. Such products are derived from cells which are themselves potentially hazardous to health, and safety must therefore be assured by stringent

purification techniques followed by detailed physicochemical characterisation of the isolated product. Examples of such products include interferons, isolated from lymphoblastoid tumour cells, and monoclonal antibodies from hybridised tumour cells. The biological safety of such therapeutics is also addressed by rigorous biological control of the starting materials and stringent checks for in-process contamination, along with the use only of well-characterised and maintained cell stocks which have been examined at all stages of their lifespan and during the production process (Lubiniecki, 1988). Because of the difficulty of achieving product purity while maintaining the viability of viral vaccines, purification of such products is primarily concerned with the removal of unwanted immunogens, viruses and nucleic acid, coupled with careful control of the starting materials and stringent checks for in-process contamination; chemical purity of the product is not achieved (Cartwright, 1987).

2.3 Current methods.

This section gives a generalised layout of the typical steps used in the harvesting and purification of mammalian cells from batch culture on an industrial scale.

2.3.1 Harvesting of the cell culture.

The products of all the cell lines examined in this study are harvested at 10% cell viability: ie. when a trypan blue dye test shows that 90% of the cells have lost their ability to exclude the dye from their membranes. This harvesting point is selected because many products are not growth dependant, with a large proportion of the protein of interest being accumulated in the stationary and decline phases of the culture (Birch & Arathoon, 1990; Boraston *et al*, 1982). For such cell lines, the decision to harvest at 10% viability is sound. However, in the case of growth associated products the extra production during the decline phase is quite possibly not enough to be worth the problems caused by the release of

unwanted proteins into the broth by lysing cells. The point at which such a culture is harvested must balance the maximum product concentration with the minimum release of potentially damaging enzymes from the cells as they lyse.

2.3.2 Primary separation.

Having decided the optimum harvesting point of the culture, the next step is the primary separation of the cells and cell debris from the product stream. Because most mammalian cells secrete the products of interest (all cell lines in this study secrete their products) there is usually no need for a cell lysis step. A disc-bowl centrifuge with continuous liquid discharge and intermittent solids discharge is frequently the method of choice for solid-liquid separation. Although a tubular bowl centrifuge can give better dewatering through higher g-forces, the disc-stack has the advantage of allowing continuous operation. The processing of such large liquid volumes under sterile conditions is difficult, carrying a risk of aerosol formation from the entrainment of air through the centrifuge seals or from the compressed air used to actuate the solids discharge mechanism. There are also problems with heat generation, which must be combatted by the use of cooling systems and short residence times. (Baldwin, 1973; Birch *et al*, 1987; Asenjo & Patrick, 1990.) An alternative method for solids separation is the use of membrane filters. These are better suited to sterile operations with containment requirements, run at ambient temperatures, and would be particularly applicable to mammalian cell culture with its low solids content. The method is cheaper and less energy-demanding than centrifugation, but is subject to fouling by the build-up of solids on the membrane. This lowers the flux, and may increase processing times to unacceptable levels. This problem is mitigated to a certain extent by the use of cross-flow filtration, which reduces debris build-up on the active membrane face by shearing the surface at a rate ten times that of the transmembrane flux (Rosevear & Lambe, 1988). However, the membranes still need regular cleaning and backflushing as

some fouling does occur. At present a combination of centrifugation and filtration is often used to ensure total solids removal, with an initial centrifugation step followed by filtration as a polishing step (Whittington, 1990).
Because the separation of solid from liquid by means of centrifugation is central to this project, the method is discussed in more detail in Chapter 3. The centrifugation step is in many respects poorly characterised at present. Any additional information relating to this process would assist optimisation. Furthermore, the presence of a solids removal step opens up the possibility of flocculation being used, not only to improve the efficiency of the process and protect the cells from shearing by using lower centrifugal forces, but also to selectively remove contaminants. Practical problems that are likely to be encountered in this area include the inefficient flocculation of dilute solutions and the introduction into the system of contaminants which must later be removed.

2.3.3 Concentration.

The next purification step is the concentration of the product stream. This is important, as product levels from mammalian cell culture will typically be 20-50 $\mu g\ ml^{-1}$, though hybridomas can give yields of up to 500 μg MAbs ml^{-1}. The concentration step is usually performed immediately after solids separation, and uses tangential flow ultrafiltration which, using hydraulic pressure, separates the particles according to molecular weight by retention or passage at a membrane of defined pore size. The concentration factors achievable by this technique are high, though a factor of about ten is typically used. This results in a more manageable volume to be sent on for further purification. A variation, diafiltration, can be used to change the aqueous phase to one more suitable for subsequent purification steps. A problem with the use of ultrafiltration arises if the dissolved protein concentration reaches too high a level, resulting in membrane fouling problems similar to those mentioned earlier,

due in this case to the formation of a protein gel on the membrane. Hoare and Dunnill (1986) report a fall in trans-membrane flux with rising protein concentration. However, the protein levels in the tenfold concentrate from a mammalian cell culture reach approximately 25 g L^{-1}, a fraction of the levels discussed in Hoare and Dunnill's paper. It is unlikely that serious limitations would arise in the processing of mammalian cell culture, given the low concentration factors used.
Ultrafiltration may be followed by a pH drop from 7.1 to 5.5 as a virus-inactivating measure.

2.3.4 Sterile filtration.

The next step before therapeutic products are passed to the chromatographic steps is the removal of any remaining solids, possibly by sterile filtration through a series of dead-end filters to remove any particles of a diameter greater than about 0.2 μm. Diagnostic products often have azide added after filtration, as a preservative.

2.3.5 Chromatographic purification.

Subsequent purification steps are tailored to suit the requirements and characteristics of the particular product. Some of the more commonly-used techniques are discussed here, with particular reference to the purification of IgM and IgG, as these classes of monoclonal antibody were used in this study. IgM has a high molecular weight of around 900,000 Daltons and consists of five units, each with two light and two heavy chains. IgGs are approximately 150,000 Daltons in weight, with only one unit.
A widely-used chromatographic technique is affinity chromatography, which separates the product on the principle of adsorption of the required protein by biospecific selection (Schmidt, 1989). The ligand, a molecule with a specific affinity for the product, is bound to a matrix or backbone. The product is selectively bound to the ligand and is thus

removed from the feed stream as it flows over the matrix. It is later eluted by changing the column conditions. The specific interactions that can be exploited by this method include those between an enzyme and its inhibitor or substrate, an antibody and its antigen, and a hormone and its receptor (Scawen & Hammond, 1986). These examples are of true 'specific' ligands. Also available are 'pseudo-specific' dye ligands, which imitate the characteristics of other ligands, but which are cheaper to manufacture (Dean, 1986).

A ligand attracting much interest for mammalian cell products is Protein A, a soluble protein made by mutant strains of *Staphylococcus aureus* (Rosevear & Lambe, 1988). It binds with several IgG antibodies of interest and is one of the principle methods for their purification on a sub-kilogram scale. Other bacterial proteins, such as Protein G, have a wider specificity for immunoglobulins, including IgM.

Immunospecific methods are useful for the purification of monoclonal antibodies that have only a weak affinity for Proteins A and G, but depend on the availability of either the pure antigen to the antibody of interest, or a second antibody raised against it. Given the high affinity of such molecules, harsh conditions may be needed to elute the product (Hill *et al*, 1986). IgM has been purified from cell culture fluid using such immunospecific methods.

To maximise the benefits of an affinity technique, it should be used as early as possible in the purification process, otherwise its high resolving power is not fully utilised (Bonnerjea *et al*, 1986). Protein A has been used to purify IgG_1 directly from clarified concentrated culture supernatant, giving >95% purity and >90% yield (Birch *et al*, 1987a; Rhodes, 1989). This level of purity would be sufficient for many diagnostic applications.

The technique of affinity chromatography can be run either as a batch operation or using a chromatographic column; the latter is more usual. One drawback of the technique is the leaching of ligands from the matrix, a problem that reduces capacity and contaminates the product. It occurs through cleavage of the matrix-ligand bond, dissolution of the matrix

material or degradation of the ligand (Hill *et al*, 1986). This is thought to occur in most applications and is a particular problem when the affinity step is being used in the later stages of purification. Ligands such as Protein A or protease inhibitors are potentially dangerous contaminants and thus must be removed. This is most easily achieved by the use of an ion-exchange step (Rosevear & Lambe, 1988). The problem can be minimised by prewashing the column prior to use.

Because of the expense of an affinity chromatography step, a pretreatment column is often placed just prior to the affinity column to protect it from fouling by removing the last of the cell debris and lipids which would otherwise attach irreversibly to the ligands. Such a step need not give high purity, but should give a high yield. Options include ion exchange or hydrophobic interaction. Ion exchange chromatography works on the principle of electrostatic binding, in which the protein displaces the counterion and binds to the ion exchanger. Anion exchange has been used to remove contaminants, particularly albumin and transferrin, by binding them to the matrix. However, this results in loss of activity of the column matrix and more extensive regeneration requirements than for cation exchange (Schmidt, 1989). Cation exchange has been used to purify a wide range of antibodies, leaving the contaminants in the flow-through, and gives high purity and recovery levels. Hydrophobic interaction chromatography separates molecules according to differences in their hydrophobicity. All immunoglobulins are hydrophobic, and are often different enough from their contaminants to give a high degree of purification in one step. Problems include the fact that some proteases are co-purified with the immunoglobulin, and the conditions of high salt concentration that must be used.

To finish the chromatographic separation stage, a gel filtration step can be used to remove protein dimers and any affinity ligands that may have leaked from the column, and to desalt and buffer-exchange the product prior to storage. Gel filtration separates molecules according to size, and has the effect of diluting the product with buffer.

2.3.6 Proof of purity.

Because therapeutic products must be free from extraneous antigens and proteins, viral and microbial contaminants, pyrogens and nucleic acids, proof of purity is essential. A convenient initial test of purity is to elute the product in two systems using different separation principles. A pure product will give a single, symmetrical peak in each case. Alternatively, comparison of stained gels with a reference can be used, or partial sequence analysis of the product. Proof of the removal of specific contaminants is essential. Acceptable contamination levels are 100 ppm of extraneous protein and 10 pg nucleic acid per dose (these levels were at the limit of the detection range of the assays when set). No pyrogenic, microbial, viral or mycoplasmal contaminants should be present. The targets for purity are constantly under review as the sensitivity of assays improve. Other methods of proving the purity of a product include 'spiking' contaminants into the product stream and quantifying the ability of the purification processes to remove them. Stringent controls are also placed on the starting cell lines and media components. An example of the use of these techniques is given in the following paragraphs, which consider viral contamination.

Several approaches are combined to ensure that the products of mammalian cell culture are free from viral contamination (Inveresk Brochure, 1988). The diverse nature of viruses and the lack of a general feature that could facilitate the use of a single test to show the presence of viruses means that viral testing is a long and expensive process if it is to be done effectively. It is not feasible to test for viruses continually throughout a fermentation, so other methods must be employed to ensure the safety of the finished product.

Firstly, the cell lines are tested extensively by an establishment which has the facilities for wide-range virus testing, such as Porton Down. This process takes several months, and is performed before the cell line is used to produce anything on a commercial scale (Poiley, 1990). Secondly, all media components, particularly serum, are tested

to a much lesser degree, for example for the presence of the enzyme reverse transcriptase. Should this prove positive, the component is sent for further testing before it is used, if at all. Thirdly, the purification process itself is tested on a laboratory scale to see how efficiently it removes 'spiked' viruses. From this, the likelihood of viral contamination of the final product is calculated (Lees & Onions, 1990). Finally, limited testing of the product is used, again checking for the presence of reverse transcriptase. If this is found to be positive, more extensive tests are performed.

3. CENTRIFUGATION.

Centrifugal sedimentation is the application of high radial acceleration to a suspension of particles by rotational motion, pushing particles denser than their suspending medium outward to the perimeter of the centrifuge bowl. Centrifuges have played an important role in biochemical engineering and biotechnology processes since 1896, when they were first used for the separation of yeast cells from fermentation broths (Knight, 1988). They are ideal for soluble protein product recovery where poor protein transmission through a membrane or blinding of the filter and denaturation of the protein by the filter aids is a problem (Mannweiler *et al*, 1989). In their subsequent development, they have proved to be reliable and versatile tools in their four main areas of application:-

-- Separation. (Two immiscible liquids of significant density difference.)

-- Clarification. (Removal of solids present at 1-2% w/v in liquids.)

-- Desludging/decanting. (Removal of substantial amounts of solid from a liquid.)

-- Centrifugal filtration.

The role of the centrifuge in mammalian cell culture essentially falls in the area of clarification as the solids levels involved are very low. Wang *et al* (1968) noted that little was known of the effects of centrifugation on mammalian cells, other than that a high centrifugal force resulted in a low cell viability, as did high temperatures. More is known now about the optimisation of the centrifugation step to achieve maximum cell removal with minimum cell breakage; Shimazaki *et al* (1986) found that a relative centrifugal force of 100 g was optimal for cell recovery, while a force of more than 300 g damaged the cells (Merten, 1987).

Cell removal is frequently the first unit operation in the downstream processing sequence and as such affects all the later ones. The requirements of a centrifuge within the biotechnology industry are that it must be able to efficiently separate shear-sensitive cells and cell debris only marginally

more dense than water from fluids that can be slimy or sticky and highly variable with time, as rapidly as possible without heating the liquid excessively or rupturing the cells. The equipment should be sterilisable *in situ* using either detergents or steam, and should be designed so that no solids can lodge in crevices and remain unsterilised. Containment of potentially biohazardous materials is essential.
To improve the throughput of the centrifugal stage, continuous centrifugation can be used. In this mode of operation particle separation is controlled by two factors; the relative centrifugal force (RCF) applied and the feedstock flowrate. The former describes the number of times greater the applied centrifugal force is than gravity (for example, an RCF of 2 means the force applied is twice that of gravity). The latter controls the length of time any particle will be in the centrifuge.

$$\text{Residence time} = \frac{\text{centrifuge bowl volume}}{\text{feedstock flowrate}}$$

However, there are problems with high shear forces at the feed inlet where the process liquid is accelerated from rest to high velocity, and at the solids discharge port during continuous centrifugation. These problems limit the amount by which throughput can be improved by using continuous systems. Overall, the capital costs of installing a centrifuge are high, with low operating costs (despite their high power consumption) when compared with systems such as membrane filtration.

3.1 The theory of centrifugal separation.

If a solid/liquid system is left to stand for any period of time the suspended solids will sink to the bottom of the container under the influence of gravity, effecting the separation of the original mixture into two layers (this ignores any colloidal effects). In an ideal solution the rate at which an isolated particle sediments is described by Stokes' Law:-

$$v_t = \frac{D_p^2(\rho_s-\rho_l)g}{18\eta}$$

where:-

v_t = terminal velocity of settling particle (m s^{-1})

D_p = diameter of particle (m)

ρ_s = density of solid particle (kg m^{-3})

ρ_l = density of liquid (kg m^{-3})

g = acceleration due to gravity (m s^{-2})

η = viscosity of liquid (kg s^{-1} m^{-1})

In a centrifuge, the separation is performed by centrifugal rather than gravitational force:-

$$v_t = \frac{D_p^2(\rho_s-\rho_l)\omega^2R}{18\eta}$$

(Stanbury & Whitaker, 1984)

where:-

ω = angular velocity = $2\pi N$; N = revolutions per second(rps)

(thus $\omega = \frac{\pi(rpm)}{30}$)

R = distance between particle and axis of rotation (m)

The above equation assumes that the effect of gravity on the particle is negligible, and that the particle will therefore follow a horizontal path and achieve its terminal velocity. The terminal velocity is the factor that decides whether or not a particle will reach the outer wall of the centrifuge, and thus be separated out, before it is swept out of the bowl with the clarified liquid. Below a certain particle diameter for example, the velocity will not be great enough to remove the particle, and it will remain in the liquid stream. The cut-point diameter, D_{pc}, is the cutoff diameter between separation and non-separation, and is described as the diameter of those particles half of which stay in the liquid while the remaining half are separated (Ambler, 1959; Ambler, 1961). The density difference between the solid and the liquid also plays a part in determining the separation or retention

of a particle, as does the liquid viscosity.
From the original equation modified from Stokes' Law it is possible to derive the following relationship:-

$$Q = 2V_g\Sigma$$

where:-
Q = volumetric flowrate through the centrifuge ($m^3\ s^{-1}$)
V_g = characteristics of slurry = $\frac{g(\rho_s-\rho_l)D_{pc}^3}{18\eta}$ = settling velocity of slurry under gravity ($m\ s^{-1}$)
Σ = characteristics of centrifuge: that area of a gravitational settling tank with the same separation performance as the centrifuge (calculation depends on the type of centrifuge used) (m^2)

From this the maximum flowrate can be calculated for given slurry and centrifuge characteristics. This is useful for the scale-up of centrifuges of similar geometry.

$$\frac{Q_1}{\Sigma_1} = \frac{Q_2}{\Sigma_2}$$

As the Q/Σ value increases, centrifugal performance declines (Mannweiler *et al*, 1989).
All the above calculations are based on the behaviour of a single, spherical particle. In a true situation settling can be hindered by the proximity of one particle to another.

$$R'g_c = 3\pi\eta D_p v(1 + \beta_o \frac{D_p}{L})$$

where:-
R' = resistance exerted on spherical particles at velocity v, in hindered settling ($kg\ m\ s^{-2}$)
g_c = conversion factor
β_o = coefficient
L = distance between neighbouring particles (m)

In the case of mammalian cell culture, however, the cell count remains low enough that the ratio D_p/L approaches zero (Aiba *et al*, 1973), allowing R′ to be considered to equal R, where:-

$$Rg_c = 3\pi\eta D_p v$$

for single, spherical particles.

3.2 The disc-stack centrifuge.

This is frequently used for biotechnological applications, and is designed for the removal of low levels of solids from fluids. It is run continuously, with either continuous or semi-continuous solids discharge. The central inlet pipe is surrounded by a stack of stainless steel conical discs, each with spacers so that the stack can be built up. These discs ensure that the distance that the solids must travel before settling on a surface is minimised (Mannweiler *et al*, 1989a), thus allowing for lower rotor speeds and bowl sizes. The process fluid flows out radially from the central feedpipe, then up between the discs, where the solids are deposited by centrifugal force on the undersides of the discs and slide down to the edge of the bowl. If a solid bowl is used the rotor must be disassembled and the accumulated solids removed, which is labour-intensive and limits the volume of fluid that can be processed before the bowl fills with solids. For continuous discharge of solids, a series of nozzles are placed around the wall of a diamond-shaped rotor. Nozzle machines cannot be satisfactorily constructed below a certain scale and machines allowing intermittent discharge are used instead. If the solids are to be discharged intermittently they must be fluid enough to flow, thus making the process less efficient at dewatering than a solid bowl machine.
Most disc-stack centrifuges are of moderate speed only (5,000-15,000 rpm) and as such can be quite large without risking bowl distortion at high speeds. Typical sizes range from 20 to 100 cm in diameter, and contain 50-100 discs. The RCF obtainable is 700-22,000 g.

The characteristics of the centrifuge, Σ, for use in scale-up calculations is as follows:-

$$\Sigma = \frac{2\pi\omega^2(s-1)(R_x^3-R_y^3)}{2g \tan.\Omega}$$

(Ambler, 1952)

where:-
R_x = outer radius of discs (measured horizontally) (m)
R_y = inner radius of discs (m)
s = number of discs
Ω = half-angle of discs

The sigma (Σ) value also indicates the efficiency of the centrifuge, representing as it does the equivalent area of a gravitational settling tank required for the same degree of separation.
Commonly used in the mammalian cell industry, with its limited fermenter scale to date, is a disc-stack centrifuge with intermittent sludge discharge capability. The solids are collected in the periphery of the bowl and are intermittently discharged by the operation of a hydraulically driven piston. The centrifuge can be modified to allow steam sterilisation *in situ*.
Other types of centrifuge that have been used at a pilot scale in the biotechnology industry are multichamber and scroll-type centrifuges. All are slower, and are not as suitable for the removal of low percentage solids or contained operation (Whittington, 1990).

3.2.1 The efficiency of the disc-stack centrifuge.

In mammalian cell applications, most disc-stack centrifuges can give at least a 10-fold concentration of solids and a clear supernatant. At the levels of RCF attainable in industrial models it is possible that the rapidity of cell sedimentation in a disc-stack centrifuge is obtained at the expense of cell integrity. Cell lysis during centrifugation

can cause two problems; the generation of cell debris that is smaller and more difficult to remove by centrifugation, and the release of proteolytic enzymes which could subsequently degrade the product. To improve the performance of the centrifuge it would perhaps be better to lower the fluid flowrate or increase the particle diameter by flocculation, rather than increase the RCF. The former option results in a longer residence time, which could be a problem in the higher-speed industrial-scale centrifuges where a lot of heat is generated, risking the degradation of heat-labile proteins. The use of centrifuges with cooled double-walled rotors would assist in minimising this effect. It is also important that the feed zone of the centrifuge is designed to give the smoothest possible acceleration of the process fluid on entering the centrifuge, as this is also associated with cell lysis (Mannweiler *et al*, 1989).

3.3 Cell recycle.

Although it would be possible to retain the cells in a viable condition by using a low centrifugal force, the disc-stack centrifuge is not suitable for a cell recycling system. Density gradient centrifugation (based on density differences) or rate-zonal and differential centrifugation (based on size differences) would be more applicable. The density of mammalian cells range from 1.06 to 1.15 kg m^{-3} and their sizes range from 6 to 50 μm in diameter. "These techniques enable the separation not only of cells of different types but also of sub-populations of cells at different stages of the cells' life-cycle" (Griffiths, 1985). Mammalian cells in a viable state settle under gravity at a rate of 2.9 cm h^{-1}, while non-viable cells settle at the much slower rate of 1.1 cm h^{-1} (Batt *et al*, 1990). However, very little work has been done on continuous modes of density gradient centrifugation, and other methods of low-speed centrifugation are more likely to be used.

Although some work has been done on continuous culture with cell feedback at the 30 litre scale (Birch *et al*, 1987) and

at the 150 litre scale (Björling & Malmström, 1990), there are drawbacks to using either continuous mode or cell recycle at production scale. This is for safety reasons as well as economic ones; it is easier to trace and recall the products from one batch run than to try and calculate at what point a continuous run became contaminated. A more likely use of cell recycle would be to operate at high cell density for an extended 'batch' run.

3.4 Safety.

General hazards from centrifuges include the leakage of process fluid through the rotor shaft seals into the coolant fluid bathing the seals, and emissions in the exhaust air if turbine drives are used. Aerosols of liquid containing particles potentially dangerous if inhaled can form if the seals are breached, and are also a problem during solids discharge from older types of intermittent discharge disc-stack centrifuges. Although these problems are most dangerous when hazardous products such as viral particles are being purified, general precautions should always be taken as all transformed mammalian cell lines, and some normal ones, are likely to have viruses associated with them. These precautions include following the correct operating instructions supplied with the centrifuge, regular inspection and maintenance of the equipment, the avoidance of blockages or froth formation, careful sterilisation of the centrifuge, and filtration of all exhaust air and coolant.

4. AGGREGATION.

This section covers the definition and basis of both protein precipitation and flocculation of solids. The mechanisms of the two are comparable in many respects.

4.1 Definition and scientific basis of aggregation.

The terms 'flocculation', 'coagulation' and 'precipitation' are not used uniformly in science or industry, and confusion as to what each term means often arises. In this report, the following definitions will be adhered to:-
AGGREGATIVE MECHANISMS (Akers, 1975; Gregory, 1982).
-- Coagulation. The destabilisation of colloid dispersions by adding ions which cause a reduction in the mutually repulsive electrical double layer forces present at the solid/liquid interface (ie. charge neutralisation).
-- Flocculation. The aggregation of colloidal suspensions by the action of high molecular weight polymers soluble in the continuous phase by polymer bridging and, in the case of polyelectrolytes of opposite charge to the particles, charge neutralisation and 'electrostatic patch' effects. These effects can occur separately or simultaneously.
-- Precipitation. The formation of insoluble particles from a solution, utilising either of the above mechanisms, or solvent modification.
In their paper on the formation and centrifugal recovery of protein precipitates, Bell and co-workers (1983) defined protein precipitation as describing "an operation in which a reagent is added to a protein solution which causes the formation of insoluble particles of protein". They noted the similarity of such precipitation and recovery to the coagulation and flocculation of biological cells, and their separation. Like precipitation, flocculation is an aggregation of primary particles into larger structures, usually caused by the addition of some agent or flocculant. According to Bell *et al* (1982), the growth of such flocs or precipitates is due initially to molecular diffusion and subsequently to

perikinetic and/or orthokinetic mechanisms, depending on the particle and fluid properties. Perikinetic growth is controlled by diffusion and orthokinetic growth is controlled by fluid motion.

4.1.1 Mathematical model of protein precipitation.

The following assumptions were made by Glatz *et al* (1986):-
-- The protein comes out of solution very quickly, so only solids need be taken into account.
-- Aggregate growth is by collisions between the aggregate and particles, or between the aggregate and other small aggregates. There is no lasting binding between two large aggregates. Growth is thus the incremental addition of small units to the growing aggregates.
-- The effectiveness of the collisions of small particles with growing aggregates is independent of the size of the collecting species.
-- The aggregates will break up to form daughter fragments assumed to be of equal volume. The number of fragments obtained from any one parent aggregate is thus dependant on the size of that aggregate.
There are two stages of growth; perikinetic and orthokinetic. Perikinetic growth occurs in the absence of shear forces, and is controlled by the rate of diffusion of the molecules. When the particles so formed have reached a size at which fluid motion becomes important in promoting collisions, orthokinetic growth begins, controlled by the rate of fluid motion.

4.1.1.1 Perikinetic growth.

According to Smoluchowski's theory (Bell *et al*, 1983) for particles of the same size and charge:-

$$-\frac{\delta N}{\delta t} = K_A N^2 = \text{rate of decrease of particle number concentration.}$$

where:-
N = particle number concentration (m^{-3})
K_A = rate constant = $8\pi Dd$ ($m^3\ s^{-1}$)
D = diffusivity ($m^2\ s^{-1}$)

The limiting particle size for this type of growth ranges from 0.1 μm in a field of high shear to 10 μm in a field of low shear. The rate of association can be reduced by electrical barriers such as remaining surface charges.

4.1.1.2 Orthokinetic growth.

In sheared suspensions of particles where the particle diameter exceeds 1 μm, fluid motion will cause collision and aggregation. Assuming uniform shear field and particle diameter:-

$$-\frac{\delta N}{\delta t} = \frac{2}{3}\alpha G d^3 N^2 = \text{rate of decrease of particle number concentration.}$$

where:-
α = collision effectiveness factor (number of collisions which result in permanent aggregates)
G = shear rate (mean velocity gradient) (s^{-1})
d = particle diameter (m)

To experimentally observe orthokinetic agglomeration, shear should be applied to the suspension using a paddle stirrer or a Couette apparatus. Where no shear stress is applied (ie. no stirring or flow) perikinetic agglomeration only will occur (Ives, 1978).

4.1.2 Mathematical model of flocculation.

This is very similar to the orthokinetic aggregation theory described above, though it is complicated by shear breakup and polymer scission. The stability of a suspension depends on a

balance between attractive van der Waals forces and repulsive electrical forces between the particles; any disruption of the repulsive forces, denoted by a lowering of the electrical (or zeta) potential of the particles results in decreased stability and possible coagulation (Section 4.1.2.1). Such an effect is reversible. The mechanism of polymer bridging can be described in terms of a series of events: dispersion of the flocculant in the liquid phase of the suspension, diffusion of the flocculant to the solid-liquid interface, adsorption of the flocculant onto the solid surface, collision of the particle bearing the adsorbed polymer with another particle, adsorption of free polymer chain onto the second particle forming a bridge, and subsequent collision and adsorption reactions leading to the build-up of a floc.

The mechanisms involved in the adsorption of polymers onto particles are varied, as are the strength of the bonds formed by the different mechanisms (Gregory, 1987), though even the weaker single interactions can produce strong overall bonds due to the attachment of many polymer segments to one surface. Ionic (electrostatic) interactions occur between the polyelectrolyte and particles bearing oppositely charged ionic groups. This gives strong adsorption. Another mechanism is ion binding, which occurs between anionic polymers and negatively charged surfaces, though this often requires a certain concentration of divalent metal ions to promote adsorption. Other important interactions are hydrophobic bonding and hydrogen bonding.

As the particles to be aggregated are already solid there is no need to assume rapid dissolution as there was for precipitation. Instead, the rate of aggregation is dependant on the speed and effectiveness with which the agent is mixed, diffusion rate, attachment rate and so on. The rate of aggregation can be deemed to follow the Smoluchowski rate equations described earlier. In the case of polymer bridging, flocculation might be impeded if the agent needs to be rearranged at the particle surface. The floc size is increased if the polymer is bigger, up to a limiting value. Floc density is inversely proportional to floc size, in a linear log.-log.

relationship.
The breakup of flocs due to shear has various mechanisms:-
-- deformation due to the presence of pressure gradients across the floc.
-- fragmentation.
-- primary particle erosion due to hydrodynamic shear.
-- particle/particle or particle/surface collisions.

4.1.2.1 Zeta potential.

The zeta potential is defined by Ball (1985) as "the electric potential within a fluid where a liquid is in contact with an ionogenic solid". It is more fully described by Ives (1978); "The zeta-potential is strictly the potential at the surface of shear round the particle, being the interface between the fixed and mobile liquid phases when the particle moves in the electric field". The fixed and mobile phases referred to are the Stern and Gouy-Chapman layers respectively. The former is the layer of ions attracted to the overall charge (in the case of mammalian cells or protein in solution, this is normally negative) of the particle or molecule. The latter is the more diffuse layer that forms around the Stern layer. The characterisation of these layers can be related to the zeta potential. Bell *et al* (1983) note that for a stable, non-aggregating system, particle zeta potentials of the order of ±10 to ±40 mV have been reported. The Stern layer indirectly controls colloid stability by its size and magnitude, as it controls the ultimate approach of the aggregating species. The concentration and valency of ions in the solution will thus affect the stability of the colloid. Akers (1975) stated; "It is well known that increasing the ionic concentration of the suspending fluid will bring about a decrease in zeta potential and hence [in] stability ... (Schulze-Hardy Rule)." The stability of particles can thus be assessed to some degree by measuring their electrophoretic mobility and calculating their zeta potential. The repulsive force generated by the diffuse layers as two similarly charged particles approach one another is roughly proportional to the

square of the zeta potential of the particles, and decreases exponentially with increasing distance between the particles. The position and structure of the Stern layer and the diffuse layer, and the strength of the zeta potential, can be altered by the absorption of polymers. They act by reducing the charge intensity of the Stern layer and rearranging the counterions in the diffuse layer, thus moving the slipping plane. Such polymers generally adsorb to the particles or molecules in several places, generating a tightly-bound species which will not desorb on dilution. They extend beyond the diffuse layers and bind to other particles or molecules, provided they do not form a steric barrier to aggregation.

4.1.3 Methods of aggregation.

Although the primary aim of this project is the aggregation of cell debris, the intention is to examine the removal of other contaminants as well, such as lipids, proteins and nucleic acids. Various methods are used to achieve aggregation of such components, and some of these are discussed in this section.

4.1.3.1 Salting-out.

This involves the addition of high concentrations of highly soluble neutral salts to decrease protein solubility by denying the dissolved protein molecules water of solvation. It can be described as a balance between salting-in due to the electrostatic effects of the salt, and salting-out due to its hydrophobic effects. In the presence of high concentrations of salts the solubility of the protein falls as temperature increases, while the reverse is generally true at low salt concentrations (Van der Marel, 1985). Protein solubility is at its lowest at its isoelectric point, and the protein is thus more susceptible to precipitation at this point. Ammonium sulphate is one of the most commonly used salts. It is extremely soluble and may have some protective influence on the structural stability of proteins. It has been used in the

purification of interferons, interleukin, viruses and tissue plasminogen activator. The main drawback of such a method is the high concentration of salt required, making large-scale precipitation of dilute proteins, yielding particles of small size and density difference, very costly.

4.1.3.2 Isoelectric point precipitation.

The solubility of a protein is a function of the net charge of the weakly acidic and basic amino side-chains and thus decreases at the isoelectric point, where the molecule is neutral and electrostatic repulsion is at a minimum. The rate of precipitation is then determined by diffusion-controlled collision of the neutral molecules (Chan *et al*, 1986). The effect is enhanced for proteins of low hydration constant or high surface hydrophobicity, where interactions with the solvent are minimised anyway, with few charged groups to interact with salts (Scopes, 1982). The acids used to cause aggregation are cheap and often do not need to be removed. However, they can irreversibly damage proteins, whose sensitivity to low pH can be amplified by the presence of acid monovalent anions. An exception to this is interferon, which is acid-stable and has been successfully precipitated with trichloroacetic acid. An added advantage in this case is the inactivation of potential viruses (Cartwright, 1987). A drawback of this method is that the aggregates formed include many different proteins as well as particulate fragments and protein-nucleic acid complexes. If the initial composition of this mixture is changed, the desired protein may not exhibit the same pattern of solubility and precipitation as before (Scopes, 1982).

4.1.3.3 Organic solvent precipitation.

The addition of water-miscible organic solvents such as ethanol or acetone reduces the dielectric constant of the aqueous medium and thus increases the electrostatic interactions between the protein molecules, causing them to

aggregate (Scopes, 1982; Bell *et al*, 1983). There is a bulk displacement of water, coupled with the partial immobilisation of water molecules through hydration of the organic solvent. The method is effective at low temperatures (0-10°C) and is indeed better used in this range, as protein denaturation can be a problem at higher temperatures. Such a method has been effectively used for the purification of plasma proteins.

4.1.3.4 Non-ionic polymer addition.

This is thought to reduce the amount of water available for protein solvation by excluding the proteins sterically from part of the solution. An example of a regularly-used polymer is polyethylene glycol, or PEG, which has the following basic formula:-

$$HOCH_2-(CH_2-CH_2-O)_n-CH_2OH$$

Generally the molecular weight of the polymer used is 2000-20,000 Daltons, to avoid excessive viscosity. Protein denaturation is minimal even at room temperature as PEGs do not interact with proteinaceous materials. pH is critical in determining the amount of polymer necessary; at the isoelectric point, less PEG is required. The protein concentration is also critical, with low initial levels requiring more PEG to bring a given proportion out of solution than higher protein levels (Foster *et al*, 1973). A high salt concentration will reduce the amount of PEG needed.
This method is related to liquid-liquid two-phase partitioning. Viral precipitations are commonly performed using PEG, which has a stabilizing effect on proteins. Plasma proteins have also been precipitated by PEG, as have other low-solubility globulins. However other proteins, including albumins, may require such high concentrations of PEG to be precipitated that the viscosity and density of the solution would make centrifugation difficult, or phase separation could occur, resulting in a protein-rich heavy phase with a lighter phase above it (Scopes, 1982). This method is milder than

salting-out or the addition of organic solvents. Residual low molecular weight PEG can be recovered by gel filtration or during binding of the protein to an ion exchanger. Removal of high molecular weight molecules is more difficult, but low residual levels will not affect later processing steps, including chromatography.
Dextran with a molecular weight in excess of 40,000 Daltons has been used to induce aggregation of human erythrocytes (Knox *et al*, 1977) by polymer bridging. Dextran is a non-ionic polymer (Bell *et al*, 1983), but dextran derivatives can be charged. DEAE dextran is cationic, and dextran sulphate is anionic.

4.1.3.5 Addition of polyvalent metal ions.

These cause reversible or irreversible coagulation by direct interaction with protein molecules. The method is more effective than most in dilute protein solutions, and in the case of reversible association the metal ions can be easily removed by ion exchange chromatography or chelating agents. Zinc precipitation was once used to purify interferon and the rabies virus, but has now largely been replaced by metal chelate affinity chromatography (Van der Marel, 1985) in which immobilised metal ions bind proteins by the formation of coordination compounds with histidine and cysteine.

4.1.3.6 Affinity precipitation.

This method involves the covalent coupling of a specific ligand to a soluble carrier polymer. The carrier should be easily precipitated, for example by a pH change. After contact with the protein solution, the carrier is precipitated and the product separated by extraction/elution procedures. The method has been used to purify lactate dehydrogenase and to extract trypsin from bovine pancreas (Luong *et al*, 1987).

4.1.3.7 Precipitation by selective denaturation.

Severe changes in temperature or pH, or the addition of organic solvents, will denature proteins, often irreversibly. Such denaturation may well cause the precipitation of the protein. If the product is robust enough to survive such severe conditions without itself denaturing, it is possible to remove contaminants by this method. However, few proteins are purified in this way (Scopes, 1982). Work has been undertaken to examine the feasibility of using selective denaturation to remove contaminants from a broth, while leaving the required protein in solution. Takesawa *et al* (1990) studied the separation of thermostable β-galactosidase produced in *Thermus aquaticus* from mesophilic yeast debris, as a model to predict the efficiency of the method for recovery of thermophilic proteins cloned and expressed in mesophilic hosts. They found that 35 to 50-fold purification factors could be achieved.

4.1.3.8 Addition of charged polyelectrolytes.

Under appropriate pH conditions these can act as flocculating agents by effecting polymer bridging between particles, and can also cause protein precipitation by the same mechanism. Such interactions involve the adsorption of segments of the polymer onto different molecules or particles, thus creating a bridge between the two, and can be due to direct interactions between the functional groups of the flocculants and the proteins or particles, including hydrogen-bonding, chemical and co-ordination bonding, hydrophobic association through non-ionic hydrocarbon groups and long-range electrostatic attraction through ionisation of the functional groups. It can also be due to mutual interactions of the polymer and the protein or particle with bridging metal cations. Adsorption of polymers (Attia, 1987) is due to:-

-- Chemical forces : co-ordination bonding, hydrogen- bonding; these act over very short distances.

-- Physical forces : electrostatic (Coulombic) forces, dipole

bonds, hydrophobic bonding, London-Van-der-Waals forces; these act over longer distances.
-- A combination of the two.
Care must be taken that the particles do not become completely covered in the polymer, or they will be restabilised, and no flocculation will occur. Flocculation can also be achieved through the use of polyelectrolytes with an opposite charge of much higher density to that of the particles, through charge reversal of 'patches' on the particle surfaces. These patches then interact directly with the normally-charged surfaces of other particles, creating much shorter bonds than are seen in the previously described form of polymer bridging (Gregory, 1987; Gregory, 1989). Extensive studies have been undertaken on the cationic polyelectrolyte polyethyleneimine, and this method is thought to be the mechanism behind the effectiveness of the lower molecular weight PEIs, where the polymer chain length may be insufficient for bridging between particles (Akers, 1975), while other researchers believe it plays a dominant role in high molecular weight polyelectrolytes also (Treweek & Morgan, 1977a). This dominance is likely to be dependant on other factors; Lindquist and Stratton (1976) state that "... the relative importance between polymer bridging and electrostatic considerations is pH dependant. At pH 9 and greater, polymer bridging is the dominant mechanism of destabilization due to the low cationic charge of the PEI molecule. However, at pH less than 9, charge interaction between PEI and colloidal silica is the dominant consideration". More recent work (Bulmer, 1989) indicates that this explanation may be over-simplified; colloid titration shows that PEI retains substantial charge at pH 9.
Such methods of flocculation are much favoured because of the low polyelectrolyte concentrations at which flocculation is achieved. Cationic polyelectrolytes such as polyethyleneimine (PEI) are frequently used. The polymer is weakly cationic and is thus affected by the type and concentration of any counterions present, as well as pH. "The PEI's constitute a large family of polyamines of varying molecular weight and

degree of modification. They act as weak bases and exhibit a cationic character depending on the extent of protonation" (Horn, 1980). They have the following general formula:-

$$H_2N(C_2H_4NH)_nC_2H_4NH_2$$

PEIs form compounds with macromolecules containing anionic domains, including nucleic acids, some proteins, and cell debris (Jendrisak, 1987). Such complexing is thought to be due to the formation of 'patches' of charge neutralisation on the species to be flocculated. The effectiveness of this flocculation increases with higher PEI concentration (up to a maximum concentration, after which restabilisation of the colloid occurs) and molecular weight. However, charge density is thought to be the important factor rather than molecular weight, and that is pH-controlled (Eriksson & Hardin, 1987). Protonation of PEI rarely exceeds 75%, due to the proximity of some charged groups on the polymer to several others. Such mutual charge repulsion also leads to expansion of the molecules. The expansion, which is obviously pH-related, can be measured via viscosity or dynamic light scattering. The isoelectric point of PEI is 10.8. A reduction to pH 5.0 will give an expansion of up to 65%. The degree of polymer dispersion and the rate of polymer addition are also important factors in flocculation, as are the shear conditions. It is reported that if the flocs are disrupted by high shear conditions, they will reform as soon as the shear stops (Horn, 1980). To avoid viscosity problems with high molecular weight PEI, Klotz and Sloniewsky (1968) suggest using cross-linked or highly branched forms of the polymer, consisting of 25% primary amines, 50% secondary amines and 25% tertiary amines. All commercially available PEIs conform to these specifications. This could result in a lower degree of protonation, however, and would have to be balanced carefully to gain the optimum degree of flocculation while remaining at a manageable viscosity.

To function as a flocculant, a polymer must have an extended and flexible configuration, the former to achieve bridging

between particles and the latter to withstand shear (Attia, 1987). PEI is unusual in that the molecule is spherical in solution, and must thus have a large enough diameter to achieve polymer bridging. The ideal flocculant dosage is that which causes partial coverage of the particles with polymer, as polymer:polymer or 'patch':'patch' attachment is unlikely. Higher dosages will cause a reversal of the net surface charge of the particles, leading to particle repulsion and restabilisation of the suspension. Other factors affecting the efficiency of the polyelectrolyte at aggregating cells and debris include the release by the cells of proteins, nucleic acids and polysaccharides. PEI has been shown to remove these contaminants along with the cells, however (Milburn *et al*, 1990).

Polygalacturonic acid (PGA) is an anionic polymer made by deacylating pectin. Surprisingly, despite its overall negative charge, it can flocculate mammalian cells, which are also anionic overall. Furthermore, out of all the anionic polymers (including dextran sulphate, polyglutamic acid and polyvinylsulphate) tested by Celltech (European Patent Application, 25th April, 1985) it was the only one to exhibit this property. It is thought to be due to the matching of the charge distribution on the polymer with positive local regions of charge on the cell surfaces. Further work on PGA by Sirica and Woodman (Aunins, 1989) reported no aggregation of mouse leukaemia cells at neutral pH, nor did it aggregate CRL 1606 cells, which are murine-murine hybridomas.

Dextran sulphate, which is anionic, has been reported to aggregate erythrocytes in the presence of calcium by a postulated method of specific calcium adsorption to the cells, where it aids the coordination of a bridge between the polymer and cell membrane (Aunins, 1989). Further studies by Aunins showed that high molecular weight dextran sulphate failed to aggregate CRL 1606 cells, even in the presence of calcium.

Poly-L-histidine is a cationic polymer found to be effective at flocculating a range of cell lines (Aunins, 1989; Aunins & Wang, 1989). At pH 7, PLH is an insoluble precipitate, and is only slightly charged. As its pH drops, its charge and

solubility increase. By using PLH at neutral pH, Aunins suggested that selective flocculation of mammalian cells without membrane disruption would be possible.

Possible drawbacks with all the methods described here are that they all involve the addition of possibly toxic chemicals that may have detrimental effects on the product, or on later processing steps, and which must be proved to have been removed from the final product. This is not always the case; PEG with a molecular weight of 4,000 Daltons is used as a plasma precipitant. Furthermore, a flocculation process that for any reason was incomplete would bring no advantage to the purification system while still adding the problems mentioned above. Process-specific trials to optimise flocculation with minimum effect on product activity or subsequent processes would be vital before a decision could be made on the appropriateness of including a flocculation step in any purification sequence.

4.1.4 Choice of aggregating agent.

The flocs produced by polymers can be considerably stronger than those produced by direct interactions between particles. Flocs held together by polymers have numerous links, while those formed by the reduction or elimination of the electrical repulsion between cells have only weak van der Waals forces (Gregory, 1987). For this reason, most of the work in this study was undertaken using polymeric flocculants.
The biggest difference in surface chemical behaviour of the various particles should be exploited in order to precipitate only one, leaving the others in suspension. This can be difficult in the flocculation of cells and cell debris as the surfaces of biological particles are not well documented. Their metabolic and other activities cause continuous changes to the surfaces, which may also be coated with extracellular polymers. The nature of the flocculants and particles must be examined, as well as that of the suspending medium, which affects the electrochemical and physical properties of the

flocculant and particles. Usually, a polymer with an extended flexible configuration is chosen, with a high charge density. It is often linear, in the range of $>10^6$ Daltons, and water-soluble.
Various factors must be taken into account, including the following:-
-- The structural stability of the protein. Some precipitants are liable to denature proteins unless conditions are strictly controlled. This is unlikely to be a problem with the flocculants to be investigated in this project as the levels used are low and the protein product is intended to be left in solution.
-- Ease of use. Inflammable organic solvents should be avoided, as should ammonium sulphate, which produces ammonia in alkaline conditions. Polyelectrolytes are better as they can be applied as concentrated solutions in small amounts.
-- Removal of flocculant. This is often vital and must be easy to do, whether or not the flocculant is to be recycled.
-- Product end use. This decides the importance of the previous point, as it dictates the product purity required.
-- Selectivity. Ideally, the agent should flocculate either the product alone, or everything but the product. There should be a minimum overlap between these two options. In the case of cell flocculation, an ideal system would utilise some group on the cell surface that specifically binds with the agent. This has been achieved for the flocculation of yeasts, using borax to bind to cis-diol residues on the cell wall (Bonnerjea *et al*, 1988).

4.2 Characterisation of mammalian cell membranes and components.

To predict likely flocculants for mammalian cells and cell debris, some knowledge of the characteristics of the cell membrane and components is desirable. In their studies of the electrophoretic properties and aggregation of a range of mammalian cell lines Greig *et al* (1976) stated; "Whether a given cell is stable in a dispersion or aggregates must depend

to a large degree on the interactions between the plasma membranes of adjacent cells in close contact. The surface characteristics of the factors which affect the interactions between plasma membranes are thus of importance". Some of their results indicate that the aggregative tendencies of certain cell lines could be due to the presence of specific residues on the surface of the cells. Although there are few such papers concerning specific cell lines, some general points can be made. Such knowledge is also useful in designing assays, as it can give indications of sensitivities required.

4.2.1 Overall cell composition.

Griffiths and Riley (1985) provide a generalised table of cell composition with ranges of variation. It should be noted that the composition will vary not only between different cell lines but also between cells in different phases of growth.

	pg/cell	Range (pg)	% of total cell weight
Wet weight	3500	3000-6000	---
Dry weight	600	300-1200	---
Protein	250	200-300	10-20
Carbohydrate	150	40-200	1-5
Lipid	120	100-200	1-2
DNA	10	8-17	0.3
RNA	25	20-40	0.7
Water	---	---	80-85

(Cell volume: 4×10^{-9} cm^3)

This gives an approximate guide to animal cell composition. Its main use is in determining the sensitivities required of the assays.

4.2.2 Membrane structure and composition.

The basic structure of the membrane is a bimolecular layer, consisting primarily of proteins and lipids, with a thickness of 70-100 Angstroms (7-10 nm). There are pores present in the layer (Smith *et al*, 1983).
The phospholipid and protein components give a low interfacial tension to the membrane. Singer and Nicholson's fluid mosaic model (Griffiths & Riley, 1985) states that the membrane is plastic and that some components can diffuse laterally in the phospholipid bilayer, giving a changing surface composition to the membrane.
A typical percentage composition of a mammalian membrane is as follows:-

	%
Protein	48-58
Lipid	42-54
Carbohydrate	2-10

(Griffiths & Riley, 1985)

All vertebrate cells bear an overall negative charge, mainly due to sialic acid residues present on cell surface glycoproteins. The distribution and density of the cell surface anions influence cell-to-cell adhesion phenomena by governing the distance to which neighbouring cells may approach one another (Borysenko & Woods, 1979).
As previously stated, eukaryotic membranes contain 2-10% carbohydrates. These are in the form of glycolipids and glycoproteins. Neutral glycosphingolipids are important cell-surface components in animal tissues (Lehninger, 1975a), as are types of glycoproteins (Lehninger, 1975). In all mammalian cell lines studied, any sugar residues are located exclusively on the external surface of the plasma membrane (Stryer, 1981).
There are three main groups of lipid present in the membrane:-

-- Phospholipids.
These are the most abundant, and make up 40-90% of the total lipid content. They are derived from glycerol, to form phosphoglycerides, and sphingosine, to form sphingomyelin. The fatty acids associated with the phosphoglycerides usually consist of 16 to 18 carbon molecules in the cis form. There are very few free or unesterified fatty acids in cells and tissues. Phospholipids are not readily soluble in anhydrous acetone, and are best extracted in chloroform/methanol mixtures (Lehninger, 1975a). The main groups of phosphoglycerides are phosphatidyl choline, phosphatidyl serine and phosphatidyl ethanolamine.
-- Glycolipids.
These are derived from sphingosine, as are some of the phospholipids.
-- Neutral lipids.
The main neutral lipid present is cholesterol, which is found in most eukaryotic membranes but few prokaryotic membranes (Stryer, 1981).
The following table summarises the groups of lipids and their component units (Stryer, 1981):-

LIPID	HYDROPHOBIC UNIT	HYDROPHILIC UNIT
Phosphoglycerides	Fatty acid chains	Phosphorylated alcohol
Sphingomyelin	Fatty acid chain and hydrocarbon chain of sphingosine	Phosphoryl choline
Glycolipid	Fatty acid chain and hydrocarbon chain of sphingosine	One or more sugar residues
Cholesterol	Entire molecule except for -OH group	-OH groups at C3

From this it is difficult to establish any one feature of the

cell that is likely to bind selectively to a flocculant. The overall negative charge of mammalian cells makes it likely that cationic polyelectrolytes will be effective, but it is known that negatively-charged molecules can also bind to cells and debris (European Patent Application, 25th April, 1985). The use of neutral polymers to fuse cells in the production of hybridoma is mentioned by Neil and Urnovitz (1988), where they described the use of "a solution of polyethylene glycol to induce fusion by reversibly disrupting the membranes". Sodium dodecyl sulphate is also capable of disrupting most protein-protein and protein-lipid interactions in the membranes at a concentration of 1%.
Overall it appears that, unlike yeasts, there is not a constant residue or group on the surface of the cell, common to all mammalian cells, that can be selected as a target for cell-specific flocculation.

4.3 Review of published aggregation work to date.

Flocculation and protein precipitation are accepted stages in the purification processes of many products. This section covers some of the current or previous uses of the technique. Fractional protein precipitation and cell flocculation have been used in bacterial cell culture, for example in the production of β-galactosidase from *Escherichia coli*, to selectively remove either the product or the whole cells and debris. Another application of bacterial cell flocculation is in biological waste treatment, where it is used to remove microorganisms from the effluent of activated sludge. To flocculate cells, the three forces that keep bacterial cells discrete must be overcome (Warne & Bowden, 1987). Those forces are as follows:-
-- Coulombic: bacterial cells have an overall negative charge and therefore repel one another.
-- Hydration: the cells have an associated shell of bound water molecules because the cell surface is hydrophilic. The water molecules act as a thermodynamic barrier to aggregation, though Hannah *et al* (1967) state that this is negligible for

particles with diameters far greater than the thickness of the water shell.

-- Steric: the cell surface has protruding polymers which physically prevent contact between adjacent cells.

Factors likely to affect the degree of flocculation include the bacterial genus, the nature of the suspending medium, the temperature, the physiological age of the cells, the flocculant type and the presence or absence of surface shear (McGregor & Finn, 1969) and also the pH of the medium and the degree of release of extracellular polymers by the cells. Most flocculation of bacterial cells has been performed near neutral pH, and most of the variables are related to the release by the cell of proteins, polysaccharides and nucleic acids. Eriksson and Härdin (1987) note that in the flocculation of *Escherichia coli* by the cationic polyelectrolyte chitosan the degree of flocculation is related to the amount of extracellular polymer released by the organism. The flocculant, a derivative of chitin, needs a high charge density as well as being a long, flexible molecule, to overcome this barrier.

Various methods are available for the selective flocculation of yeast cells and debris. The mannitol and sorbitol residues found on the cell walls contain 1,2 cis-diols that readily bind to borate anions, giving good clarification (Bonnerjea *et al*, 1988). The higher the pH, the more effective the clarification, even at lower concentrations of borax. Polyethylene glycol of a molecular weight of 6,000 Daltons is commonly used for flocculation, though the selectivity of the technique is still effective with polymers as small as 400 Daltons (Hönig & Kula, 1976). 6% PEG is routinely used to precipitate foot-and-mouth disease virus (Wagner *et al*, 1970; Bachrach *et al*, 1975). Studies have also been performed on the ability of PEG to selectively precipitate proteins (Hönig & Kula, 1976), in which it was found that PEGs of lower molecular weight than are generally employed were able to selectively purify α-glucosidase from crude yeast extract. Polyethyleneimine has also been used in the purification of proteins from yeast and, if added after a borax flocculation

stage, effectively removes all lipid and nucleic acid as well as cell debris. The removal of non-protein contaminants prevents the fouling of high-resolution chromatographic supports, as does the removal of non-soluble proteins (Milburn *et al*, 1990).

Gasner and Wang (1970) note the importance of shear in the flocculation of cells with polyelectrolytes. In their work on *Candida intermedia*, they found that low shear did not produce enough cell contact, while high shear caused the flocs to break up. In the case of strong anionic polyelectrolytes such as polystyrene sulfonate, they postulated an irreversible ionic bridging mechanism in floc formation. High local shear conditions could break the polymer free from one particle and cause it to wrap itself round the other particle, bonding itself in several places and rendering that particle no longer susceptible to flocculation. The shear must be carefully controlled to find a balance between the two extremes.

Atkinson and Jack (1973) used PEI with a molecular weight of 20,000 Daltons to precipitate bacterial nucleic acids. They found that the best results were obtained using a final concentration of 0.294% PEI and 0.02M sodium chloride. This successfully removed 89% of the DNA present, and 96% of the RNA. Oku *et al* (1986) report the aggregation of liposomes composed of phosphatidyl serine and phosphatidyl choline by PEI, and postulate that the aggregation is due to multivalent binding of amine residues to negatively charged head groups of phospholipids.

Much less work has been done on the flocculation of mammalian cells than on microbial cells. Some results are available on the removal of contaminating proteins and fragments of casein from human leucocyte interferon (Rubinstein *et al*, 1979). Ammonium sulphate and organic solvents proved to be unsuitable for such work, as both coprecipitate interferon. The addition of detergent was found to reduce this, leading to the conclusion that the coprecipitation was caused by hydrophobic interactions.

As mentioned in Section 4.2.2, polyethylene glycol is used in the preparative production of hybridoma cells. It induces cell

fusion by the reversible disruption of the membranes.
Polygalacturonic acid has been found to be suitable for the flocculation of rat or mouse hybridoma cells as it has little effect on the immunoglobulins produced (European Patent Application, 25th April, 1985). Any concentration of the flocculant can be used, with a maximum value set by the concentration at which a gel forms, and a minimum set by the desired level of flocculation. Experimentally, the optimum for the mouse hybridoma cells used was found to be 0.03% w/v. The use of an acid pH to ensure protonation of amino groups and histidine residues on the cell surface effectively increases the cell area. pH in the range 5-6 was found to be preferable, though the technique is very flexible; PGA remains ionised at pH values below 3, and some flocculation occurs at least up to pH 9. Polyethyleneimine has also been used effectively on mammalian cells (Horn, 1980). It has been noted that quaternary PEI, in common with a few other classes of polycation, has a highly selective adsorptive activity. They have been found to inhibit the growth of allogenic and syngenic tumours in mice at non-toxic levels to the host, apparently by binding specifically to tumour cells while non-tumour cells remain unaffected. Other types of PEI bind tumour and non-tumour cells as well as complexing with lipids and lipoproteins.
Scopes (1987) notes that it is often possible to form aggregates in a tissue extract merely by lowering the pH to between 5.0 and 6.0, close to the isoelectric point. This not only aggregates soluble proteins but also particulate material such as ribosomes and membrane fragments.

EXPERIMENTAL WORK.

5. STATEMENT OF AIMS.

The overall aim of this project was to assess the feasibility of using an aggregative step to improve the separation of cells and debris from a mammalian cell culture. This section of the thesis examines the aims of each area of research.

As regards the large-scale cell culture study, the proteins of interest are produced by mammalian cell culture; their rate of production is related to the growth cycle of the cells. As a priority for this study, assays will be developed to quantify the major contaminants such as proteins, lipids, carbohydrates and nucleic acids, as well as tests specific for the product, and research will be performed to find the optimal harvesting point that maximises product yield while minimising cell lysis and contaminant release.
Mass balances will be constructed of the early downstream processing, so as to have a datum against which to compare any improvements through flocculation. Particular attention will be paid to the solids removal process.
At the levels of relative centrifugal force attainable in industrial-scale centrifuges, it is possible that rapidity of cell sedimentation is obtained at the expense of cell integrity. This area will be studied in more detail; if the cells are rupturing, many internal proteins will be released into the supernatant, and will have to be removed at a later stage. The release of proteases could have a detrimental effect on product level and activity.
Work will be done to ensure that the trends of cell growth and product formation observed on the large scale can be reproduced on the small scale.
Potential flocculants will be screened for their effects on samples (at small scale). Not only flocculating ability will be examined but also the effect, if any, on product and contaminant levels. A range of molecular weights and pH conditions will be examined.
Having determined the optimum concentration of flocculant, an estimation of the effects of adding flocculant to a large

scale culture will be made, by simulating the conditions experienced at large scale.

Work will be done to ensure that the assay results are genuine, and are not affected by the presence of the flocculant or other conditions of use. For example, cell viability readings obtained by trypan blue dye exclusion will be confirmed by other assays, such as the levels of extracellular lactate dehydrogenase or DNA, as not all cells that take up the dye are actually non-viable. The test is also the presence of soluble proteins such as those found in serum; trypan blue will bind to them rather than the 'non-viable' cells.

6. MATERIALS AND METHODS: ADDENDA.

The cell lines studied in the course of this thesis were as follows:-

#1: Rodent hybridoma producing IgM in serum-containing medium.
#2: Rodent hybridoma producing IgM. Large-scale cultures contain serum; small-scale cultures are serum-free.
#3: Human cell line, grown in serum-containing medium.
#4: Recombinant mouse hybridoma producing humanised IgG_1 in serum-free medium.
#5: Rodent hybridoma producing IgG_1 in serum-free medium.
#6: Rodent hybridoma producing IgG_{2a} in serum-free medium.

All cell lines were studied at large scale at Celltech Limited of Slough, Berkshire. Small-scale studies and flocculation work was carried out at University College, London, using line #2 cells and serum-free medium provided by Celltech.

The products assayed in this thesis were as follows:-

IgM: An immunoglobulin with a molecular weight of 900,000 Daltons, made up of five subunits. The heavy chains are μ class, and the light chains are κ or λ class. In humans it is found mainly in the blood plasma.
IgG: An immunoglobulin with a molecular weight of 150,000 Daltons, made up of one subunit. The heavy chains are γ class, and the light chains are κ or λ class. In humans it is found in the blood and interstitial fluids.

6. MATERIALS AND METHODS.

This chapter covers the assays and other methods used to gather information in the course of this project. An initial selection of the parameters felt to be of significance to the project was made, then a study of the available techniques for measuring them was undertaken. For most assays there are many methods to choose from, based on different properties or reactions, and each has relative merits and disadvantages. For each parameter a method or combination of methods was selected, and the reasons for the selection are covered in this chapter along with details of materials and methods used. All flocculation studies and assays were undertaken on samples at room temperature.

6.1 Cell quantification.

Three measurements can be made; dry weight, total cell number and viable cell number.

6.1.1 Dry weight of centrifuge sludge.

This was measured by placing a known volume (10 ml) of sludge in weighed crucibles, reweighing the crucibles, and drying them in an oven at 65°C (Bell & Dunnill, 1982a). The crucibles were reweighed after 24 hours and again after 48 hours to ensure complete dehydration, and the results were expressed as a percentage of both the initial weight and the volume. A series of blanks, performed by treating 10 ml samples of media in an identical manner, gave an average reading of 0.15 g per sample, indicating that the contribution of the salts present in the media to the final dry weight was low.

6.1.2 Packed volume of cells.

The percentage solids in culture broth and centrifuge feed and supernatant were measured using 10 ml glass solids determination tubes (Westfalia Separator Limited), as shown

in Figure 1. The tubes were filled and spun in a bench top centrifuge (MSE Centaur 2, Fisons, Crawley, Sussex) for 15 minutes at 3000 rpm (170 g), and packed volume readings were then taken from the graduated capillary at the base of the tubes. Depending on the origin of the sample, different capillary sizes were used, ranging from 0.2% to 3% of the total volume.

6.1.3 Total cell number.

Because of the large size of mammalian cells and the relatively low cell densities reached, it is possible to make a direct count of cell numbers using a haemocytometer, a suitable dye and a magnification of x200 on a microscope (Olympus BH-2). On some occasions, photographs were taken of the samples using an Olympus OM-2_N camera attached to the microscope.

100 μl of a well-mixed cell culture sample was placed in a Treff tube and an equal volume of a 0.4% (w/v) solution of trypan blue (Sigma, T6146) was added. A haemocytometer with Modified Fuchs-Rosenthal grid (Philip Harris Scientific, London) was prepared and cell/dye mixture introduced under the cover slip, allowing the chamber to fill by capillary action. A hand counter was used to record the number of cells in three sections of the haemocytometer grid, and a simple calculation was then used to find the number of cells per millilitre of broth.

It is also possible to convert between dry weight and cell count using the following equation:-

Absolute cell number x (4.9×10^{-10}) = dry weight in grams.

(Pers. comm: S. Froud, Celltech Ltd., Slough, Berkshire.)

6.1.4 Cell viability.

This can be measured in various ways.

6.1.4.1 Dye exclusion.

This method was used as an extension of the measurement of total cell number. It is based on the principle that live cells, having an intact, functioning membrane, will exclude certain dyes, while dead cells will become stained as their membranes become permeable. Dyes commonly used include trypan blue, eosin yellow and erythrosin B. The latter is particularly useful for culture media containing serum as, unlike trypan blue, it does not stain serum proteins (Griffiths, 1985; Phillips, 1973). However, no particular problem was encountered with the use of trypan blue provided that the cells were counted within five minutes of mixing with the dye, as viable cells had a tendency to take up the dye if left for longer. Results were recorded as a percentage of the total cell count. The main drawback with the dye exclusion method is that it is a measure of membrane function, while the strict definition of a viable cell is one that will replicate in culture (Petersen *et al*, 1988). Furthermore, this method does not take into account cells which have lysed (Marc *et al*, 1990). The permeability of a membrane can be affected by recent trypsinisation or by freezing and thawing, especially in the presence of dimethyl sulphoxide, so cells recently revived from frozen storage in a cell bank must be counted with care; many cells indicated as 'non-viable' by the dye exclusion method will recover and replicate normally. The pH and concentration of the surrounding media also affects the membrane characteristics (Griffiths, 1985). It is therefore usual to combine this method with another, and take account of the results of both tests.

6.1.4.2 Measurement of the extent of cell lysis.

This can be determined by monitoring the extent of release of the cytoplasmic enzyme lactate dehydrogenase into the surrounding medium. Problems with the assay method, and degradation of the enzyme standard meant that the method was not included in these studies. The method is described in the

section concerning future work (Section 8.1.3).

6.2 Tests for contaminating microorganisms.

The following tests are routinely used to check the sterility of mammalian growth media and can also be used to test for the presence of contaminants during a fermentation.
The earliest sign of contamination with aerobic bacteria is an increase in the oxygen demand of the cell culture. This is followed by a colour change of the phenol red indicator in the growth media from red to yellow as a result of the acidic conditions generated by the bacteria, usually *Bacillus*.
A further test is the inoculation and incubation at 20-25°C of tryptone soya broth (Oxoid CM129). The culture should be examined after 5-7 days, and again after 14 days. Cloudiness indicates the presence of aerobic bacteria.
To detect the presence of moulds and fungi, a sample of culture media should be used to inoculate a vial of sabouraud liquid media (Oxoid CM147), which is then incubated at 20-25°C and examined after 5-7 days, and after 14 days. Again, cloudiness indicates the presence of contaminants.
Fluid thioglycollate medium (Difco 0256-17) is used to test for anaerobic bacteria. The inoculated vial is incubated at 30-35°C and examined for cloudiness as previously described.
During the course of this study, such tests were used only to confirm the sterility of media, and when specific contamination problems necessitated the identification of the contaminating microorganism in order to trace its source.

6.3 Tests for viral contamination.

The cell lines used in the course of this study were all in commercial use, and had undergone the rigorous tests described in Section 2.3.6. For this reason no further tests for viral contamination were carried out.

6.4 IgM assay.

To decide upon a suitable harvesting point in the culture cycle, the level of the required product should be monitored. This can present problems if the product has not been analysed with regard to chemical composition (eg. antibodies or viruses). Indeed, some products can be evaluated only by applying them to animals or humans and measuring their effects as "a definable non-specific appearance or increase of a biological property (production of antibodies, resistance) specifically characteristic of the biological" (Fontaine *et al*, 1985). More usually, some specific characteristic of the product can be assayed and quantified. The cell line which was predominantly used in this project is a mouse hybridoma which produces immunoglobulin M (IgM). It can be assayed using an enzyme-linked immunosorbent assay (ELISA). In this case, a double antibody sandwich ELISA was used, in which the antibodies in the culture media are exposed to specific antigens which have been adsorbed onto the plastic ELISA plate. To detect the antibodies which have been bound to the antigens, a conjugate is added. This is another antigen, to which a particular enzyme has been linked. The test system is then washed clean of any uncombined conjugate and incubated with an appropriate substrate for the enzyme, and the product of the enzymic cleavage is assayed, for example by spectrophotometry.

Each sample was prepared to give a total of seven dilutions ranging from 1/10 to 1/1000, as was the IgM standard (Purified mouse IgM κ, TEPC 183, obtained from Organon Teknika (Cat.#50336) or Sigma (M2770)). The standard concentrations ranged from 10 to 1000 ng ml^{-1}. 100 μl volumes were added in duplicate to 96-well, flat-bottomed microtiter plates (EIA II microplates, ICN Flow, High Wycombe, Bucks.) that had been coated overnight in a buffer containing coating antisera (Goat antimouse IgM, Tago Immunologicals Inc. (Cat.#4142)). After a period of time the plate was washed to clear it of any unbound antibody, using a Titertek Handiwash 110 plate washer (ICN Flow, High Wycombe, Bucks.), then a conjugate was added.

This consisted of a further antibody conjugated to the enzyme horseradish peroxidase, type VI (Goat anti-mouse IgM HRPO, Tago Immunologicals Inc. (Cat.#6442) or Anti-mouse IgM (μ-chain specific) peroxidase conjugate, Sigma (A8786)). Again after a period of time, the plate was washed clean of any excess conjugate and a substrate was added. Reaction with the horseradish peroxidase caused the development of a blue colour, the absorbance of which was read at 570 nm using a Titertek Multiskan Plus Mk. II microplate reader (ICN Flow, High Wycombe, Bucks.). A standard curve was constructed for each plate and the concentration of IgM in each sample was calculated from this. Studies of cell lines producing IgG in the course of this thesis were assayed using protocols and reagents supplied by Celltech Ltd. (Slough, Berkshire).

Ideally, another method would have been included to verify the results as the variance of the ELISA is ±25%. However, the options were limited by the small, dilute samples available. The use of a Protein A column to bind and release IgG is common, but Protein A does not bind IgM. The possibility of using thin layer chromatography was also explored, but the antibody is denatured by the sample preparation procedure, and appears as two faint bands -- light and heavy chains -- which are difficult to see even with silver staining. Quantification of such bands using densitometry was considered too inaccurate to be used. As a result the findings of the ELISA were not verifiable except by duplication, which was used both per plate and between plates to reduce error as much as possible. In a limited number of cases it was possible to verify the ability of the IgM to agglutinate blood cells by sending the samples to the Protein Fractionation Centre at the Scottish National Blood Transfusion Centre in Edinburgh. The samples were assayed using a method known as 'titre/immediate spin' which assesses the activity of the sample over a series of doubling dilutions from neat to 1/512. Such samples were of necessity restricted in number, and could by no means be used as a routine method of verifying ELISA results.

6.5 Total protein.

There are various techniques available (Scopes, 1982).
The Lowry method is based on the reduction of alkaline Cu^{2+} to Cu^{+} by proteins in a concentration-dependant manner. Bicinchoninic acid forms a purple complex with Cu^{+}, and the absorbance of this complex at 562 nm is directly proportional to protein concentration (Lowry *et al*, 1951). A modification of this is the Biuret method, using alkaline tartrate solution (Biuret Reagent) and phenol indicator. It is of low sensitivity. Another variation is Peterson's method, which is commonly used in mammalian cell culture. Lipoproteins are included in the assay by being dissolved in sodium dodecyl sulphate, and membrane proteins are also assayed. Again, absorbance is monitored. A deoxycholate-TCA precipitation technique can be used to remove interfering substances such as TRIS, sucrose, EDTA, etc. though these were not felt to be a problem in this study.
The Bradford dye binding method is based upon the colour change of Coomassie Brilliant Blue G-250 from red to blue upon binding to protein. Such a protein:dye complex has a high extinction coefficient and is thus a sensitive assay. The absorbance at 595 nm is proportional to protein concentration. The range in which this assay is effective is 10-200 $\mu g\ ml^{-1}$. The presence of sucrose, glycerol, EDTA and other compounds can interfere with the reaction, but this can be compensated for if the correct buffer is used (Bradford, 1976). Of the methods available, this was considered to be appropriate because of the small sample size involved and the sensitivity and speed of the method. The Lowry method is the only comparable assay in these respects, and it gives a much slower colour change.
Bovine serum albumin (BSA) was used to make up a set of standard dilutions in distilled water, and the samples were diluted to approximately 100 $\mu g\ ml^{-1}$ protein, also in distilled water. 25 μl volumes of sample or standard were added to the wells of a microtiter plate, and 250 μl volumes of assay reagent (Pierce, Cat.#23200) were added. After two

minutes, during which time any surface bubbles were burst with a needle to avoid interference, the absorbance was read at 600 nm with a microtiter plate reader. The absorbance peaks at 595 nm but such a filter was unavailable for the plate reader. However, the manufacturers of the assay reagent state in their accompanying literature that the standard curve is linear and reproducible even at 570 nm. Since most of the experiments were comparative, or had blanks and standards included, it was felt that this deviation from the optimum wavelength was acceptable. A standard curve was constructed and sample values read from this, with appropriate compensation for initial sample dilution. Each sample was repeated in four wells, and the samples themselves had duplicates. The variance of this assay was found to be ±2%.

6.6 Nucleic acids.

6.6.1 Deoxyribonucleic acid.

The level of DNA allowable in preparations for therapeutic use is very low -- no more than 10 pg per dose. As such, the amount of DNA present in the culture medium during downstream processing is carefully monitored.
Conventional methods of DNA isolation and quantification use precipitation of DNA from lysed cells with ethanol, and measurement of the absorbance at 260 nm (Boyer, 1986) or extraction of all nucleic acids using an aqueous/organic two phase system (Burton, 1956). Separation of RNA from DNA is achieved by the adsorption of the RNA onto a bed of activated charcoal (Zamenhof & Chargaff, 1951). The possibility of using such techniques is precluded by the nature of the samples to be assayed; small, with low DNA levels.
Another method of estimating DNA content is to bind a dye such as ethidium bromide, Hoechst 33258 (bisbenzimide) or 4',6-diamidino-2-phenylindole (DAPI) to the DNA, and measure the increase in fluorescence using a fluorometer. Such dyes can be highly sensitive, easy to use, and specific for DNA in the presence of protein, RNA and cell debris (Sterzel *et al*,

1985).

For the purposes of this investigation, DAPI (Sigma, D1388) was used. At a concentration of 10 ng ml^{-1} its binding with DNA gives a linear response over DNA concentrations from 0 to 70 ng ml^{-1} when excited at 372 nm and emission is read at 454 nm (Kapuściński & Skoczylas, 1977).

Highly polymerised freeze-dried calf thymus DNA (Sigma, D1501) was used to construct the standard curve for this assay. It was dissolved in 0.01 molar sodium chloride, sonicated to break the DNA down into short lengths, and the final concentration was adjusted to 50 μg ml^{-1}, giving an absorbance at 260 nm of 1.0 (Downs & Wilfinger, 1983) using a PYE Unicam PU8600 UV/VIS spectrophotometer. From this a standard curve was prepared to give final concentrations in the range 0 to 70 ng ml^{-1} when mixed with the correct proportion of DAPI. The samples were buffer-exchanged prior to assaying to ensure the correct salt concentrations, as at low ionic strength fluorescence is decreased (Brunk *et al*, 1979). Disposable NAP-5 columns (Pharmacia LKB Biotechnology, Cat.#17-0853-02) were used to buffer-exchange the samples into 5 mM N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid) (HEPES) in 18 mM sodium sulphate. The samples were then diluted if necessary, to bring them into the range of the assay, mixed with DAPI (0.4 ml sample and 2.6 ml DAPI) and read at 454 nm using a Perkin-Elmer MPF-44A Fluorescence Spectrophotometer. The temperature of the sample cell was maintained at 25°C by connecting it to a water bath. The results were used to produce a standard curve from which the DNA concentration of the samples was calculated, taking into account the dilution effect of mixing with the DAPI and any prior dilutions. The samples were read in duplicate, or more if several dilutions of the same sample were assayed. The variance of this assay was found to be ±6%.

6.6.2 Ribonucleic acid.

Ribonucleic acid measurements were not undertaken as a sufficiently sensitive and rapid assay was not found. The

options available are discussed in the section concerning future work (Section 8.1.4).

6.7 Lipids.

The techniques available for the quantification of lipids are discussed in Section 8.1.5, in the section concerning future work, as such measurements were not undertaken in the course of these studies.

6.8 Small-scale cell culture conditions.

The cell line cultured on a small scale was a mouse hybridoma designated cell line #2, which produced monoclonal IgM against human blood group B antigens. It was grown in Dulbecco's Modified Eagle Medium (DMEM) with supplements, all of which were supplied by Celltech Limited (Slough, Berkshire). No serum was added to the media. All culture work was undertaken using protocols supplied by Celltech, and all aseptic work was performed in a Nuaire Labgard 425 laminar flow cabinet, class II, type A/B3 (Nuaire Inc., Plymouth, Devon). The incubator used was from the Gallenkamp Incubator Plus series (INC-208-020F) supplied by Fisons of Crawley, Sussex. The incubator was equipped with a flexible drive module (INC-220-045A), two roller shelves (INC-230-515F) and an orbital/linear shaker base (INC-230-510P) from the same supplier. The plastics used for all small-scale culture work were Corning tissue culture products supplied by Imperial Laboratories Ltd. (Andover, Hants). They included 25 cm^2 and 75 cm^2 flasks, 490 cm^2 and 850 cm^2 roller bottles (all graded according to their internal surface area), 15 ml and 50 ml centrifuge tubes and 1 ml, 5 ml, 10 ml and 25 ml pipettes. All were polystyrene and were sterilised by gamma irradiation. The pipettes were used in conjunction with a Pipetboy (Tecnomara AG, Zurich) for ease of sterile liquid transfer. Further small-scale culture work was undertaken using a Techne biological stirrer system consisting of a MCS-104S stirrer unit and four 250 ml vessels (Techne Ltd., Cambridge, Cambs.) stirred at 60 rpm by

magnetically-impelled glass stirrers. Two 2 L stirred vessels were also used to culture cells. These vessels were glass, with no baffles, and were equipped with LH 502D agitators (LH Fermentation, Maidenhead, Berkshire) which ran at 100 rpm with two Rushton turbines each, and Anglicon Microlab fermenter control panels (Anglicon Instruments Ltd., Newhaven, Sussex). The control panels were built to specifications laid down by Celltech and UCL, and were linked to a Bio-pc process monitoring system (BCS Ltd., London) for data logging. Media used at UCL was filter-sterilised using an Amicon pressure vessel and Millipak-40 media filters (Millipore, Watford, Herts.). A cell bank was laid down at Celltech and was then transported back to University College, where it was stored in a Taylor-Wharton 10XT liquid nitrogen dewar (Taylor-Wharton, Indianapolis, Indiana, USA). All other samples were stored at -70°C in a Camlab Fryka TT80 chest freezer (Camlab Ltd., Cambridge, Cambs.). pH control of the 2 L stirred vessels was achieved using 1 M sodium hydroxide solution and 1 M hydrochloric acid, while control of the smaller cultures such as flasks, roller bottles and spinner flasks was achieved by periodically gassing out the container with a sterile mixture of 5% carbon dioxide in air.

6.9 Measurement of the extent of flocculation.

For accurate comparison between results, the method of flocculation must be well-established and adhered to. Various methods have been documented; addition of cells to the polymer while stirring at 1000 rpm (Eriksson & Hardin, 1987); addition of the flocculant to the cells while stirring at 82 rpm (McGregor & Finn, 1969); mixing by inversion (Milburn *et al*, 1989; Bonnerjea *et al*, 1988; European Patent Application, 1985). The only method used on mammalian cells was that described in the above patent application; all the others were applied to microbial cells. It was felt, therefore, that this was the appropriate method to use. To 10 ml samples of cell suspension at room temperature was added the relevant amount of flocculant made up in a 1% solution, with a balance of

water being added to each tube to ensure the same degree of sample dilution (Table 1). Each sample was then mixed by inversion several times and 100 μl samples were taken for examination under a microscope. The packed volume of each sample was measured by the technique described in Section 6.1.2. Duplicate 1 ml samples were taken from each spun sample and retained for later analysis using the methods described in sections 6.4 to 6.6. Initial studies included reading the residual optical density of the samples at 600 nm (as described by McGregor and Finn, 1969; Bonnerjea *et al*, 1988; Milburn *et al*, 1990; Persson and Lindman, 1987) but it was found that all the samples were clarified so efficiently by centrifugation, whatever the level of flocculant addition, that no measurable difference existed between them after centrifugation.

The effect of pH on the process of flocculation was also examined. This was done by adjusting 50 ml samples of cell suspension to pH 5, 6, 7, 8 and 9 by addition of either 1 molar hydrochloric acid or 1 molar sodium hydroxide solution. Both are routinely used in the pH control of small-scale mammalian cell cultures, and can achieve the required pH changes with only small volume additions. Each 50 ml sample was then mixed by inversion to ensure resuspension of the cells, and split into five 10 ml samples. These were then treated as described earlier in this section.

6.10 Measurement of particle size.

It is important to be able to measure not only the size of aggregates, to determine the effectiveness of the flocculating agent, but also the size of individual cells and debris, to determine the degree of cell destruction inherent in different processes. Aggregate size is inversely proportional to zeta potential, with maximum size being attained close to the isoelectric point of the particles or proteins.

The ideal instrument to measure particle size should provide a complete analysis of particle distribution using minimum additional data about the species or the medium in which it

is suspended. It should be capable of analysing a wide range of sizes, and should give a fast result with minimum ambiguity with respect to instrument response (Barth, 1984). Various methods and instruments are available.

The Joyce Loebl method of particle sizing, for example, is a variation on the basic centrifugal sedimentation method where the mathematics is complicated by changing particle velocity with radial distance from the axis. Instead, a density gradient is set up and turbidity is measured. The photosedimentometer consists of a rotating hollow disc in which an annulus of spin fluid is held. A small volume of the suspension to be studied is layered onto the inner surface of the annulus, and the particle sedimentation is monitored by the attenuation of a white light source near the outer edge of the annulus. Using Stoke's Law and assuming that the Reynolds' Number of the sample is less than 0.2, the size distribution is analysed. The main drawback of this method is the practical difficulty of creating a density gradient and smoothly applying the sample. Other density methods include the measurement of buoyant density in caesium chloride or caesium chloride/sucrose solutions, the centrifugation of particles in water-immiscible solvents, the use of dye sorption and absorbance measurement, and the measurement of the settling velocity of isolated aggregates under centrifugal forces. The first three can involve large errors and the last is restricted to particles of a maximum diameter of 10 μm, a range which does not cover viable hybridoma cells (Treweek & Morgan, 1977). Larger sizes can be studied using gravitational settling, though that method is time-consuming.

The HIAC optical pulse sensor is based on optical blockage, and is an ideal method for irregular shapes and a wide range of fluids (Bunville, 1984). It measures the change in voltage of a photodiode when a particle passes in front of its sensor, and is based on the following relationship:-

$$E = \frac{aE_0}{A}$$

where:-
E = voltage output of photodiode (V)
a = particle cross-sectional area (m^2)
A = cross-sectional area of light sensor (m^2)
E_0 = voltage from photodiode in the absence of a particle(V)

Problems occur in ensuring the passage of single particles in front of the sensor at any one time.
Another method is based on the scattering of a cone of light from a laser when it hits a particle. It can be based on time averaged measurements, where either the scattered intensity or its spatial distribution is measured, or on time-fluctuation measurement, which includes analysis of the spectral distribution of the scattered light. An example of such a technique is photon correlation spectroscopy. It is based on the formation of a diffraction pattern when a laser beam hits a particle. An average of the product of the signal from the photomultiplier and a delayed version of the signal, as a function of the delay time, leads to a diffusion coefficient, via an autocorrelation function. This method was used to establish the diameter in nanometers of the various molecular weights of polyethyleneimine (PEI) used (Bulmer, 1989). It takes into account the water associated with the molecule.
The Laser Doppler Velocimetry principle is a method which uses an electric charge to make the particles move towards electrodes. An interference fringe is generated by splitting and recombining an illuminating beam, and the intensity fluctuations caused by the particles moving through the fringe are monitored. The fluctuations are proportional to particle velocity, which in turn is proportional to particle size. A photomultiplier is used to detect the fluctuations, which are then processed using a spectrum analyser in the same way as for photon correlation spectroscopy.
The Coulter principle (Resistive pulse technique) states that "...if a nonconducting particle suspended in a conducting medium is placed within a small aperture, an increase in the resistance across the orifice relative to that of the medium

alone is produced" (Barth, 1984).

$$\Delta R = \frac{8\rho_f d}{3\pi D^4}\left[1 + \frac{4}{5}\frac{d^2}{D} + \frac{24}{35}\frac{d^4}{D} + \ldots\right]$$

where:-
ΔR = increase in resistance (Ω)
ρ_f = resistivity of conducting medium (Ω m)
d = particle diameter (spherical) (m)
D = aperture diameter (m)

The resistance pulse, ΔR can be converted to a voltage pulse, $I\Delta R$ where I is the current across the aperture. The system needs calibration with monodisperse particles of known diameter before being used experimentally. The results can be interpreted to give particle volume (m^3 aggregate:m^3 suspension) or mass (mass protein:m^3 aggregate) as well as the equivalent diameter of the particle (average diameter, or that which the particle would have if it were spherical). The main advantages of the technique are the flexibility with regard to particle size given by the ability to change the orifice size, and the fact that no other property of the particle is used in the calculation of size. However, care must be taken with the interpretation of results as the method assumes all particles are solid and nonconducting. The presence of water in loose aggregates will cause an erroneously low reading of particle diameter or volume. Also, free electrolyte in totally open pores within particles will be conductive, while that in a pore closed at one or both ends will not. Any particles that do conduct electricity will have their size underestimated (Horák *et al*, 1982). The assumption that the particles are perfectly spherical in the calculation of diameter can also cause inaccuracies. However, these were considered to be minor problems when the technique was used to determine the diameter of mammalian cells, as they can be considered to be spherical, and the orifice selected meant that flocs or aggregates could not pass through.
The Elzone (Particle Data Limited, Cheltenham, Gloucs.) was

used to perform these measurements, fitted with either a 48 μm or 38 μm diameter orifice. Standard techniques used included the use of a phosphate buffered saline solution to ensure good medium conductivity, filtered to 0.2 μm and degassed. Good dispersion during sample dilution is vital, as is the exclusion of foreign particles. Latex standards were used for calibration at 5 and 20 μm (Polysciences, Moulton Park, Northampton). The Elzone was blanked on the buffer, then samples were added, taking care not to allow the solid level to get too high (less than 0.2 kg m^{-3}) to allow for good dispersal and minimise interference between particles or clogging of the orifice. As well as mean values of the diameter, L50 and L90 values were calculated, at which there are 50 or 90% by volume oversize (Glatz *et al*, 1986). The former value is useful in designing crossflow membrane processes, while the latter measures the fine end of the size distribution spectrum and is useful for centrifugation specification. No orifices were available that were able to admit even the smaller flocs formed on addition of certain flocculants, and the irregular shapes, sizes and densities would have impaired the accuracy of this technique. Section 8.1.6 describes an alternative method of monitoring the aggregation of particles based on their diameter, and would be of benefit in any future studies.

6.11 Measurement of PEI molecular weight.

The PEIs used were of the following molecular weights:
600 Daltons (7,500)
10,000 Daltons (30,000)
70,000 Daltons (450,000)
50-100,000 Daltons (350,000)
500,000 Daltons (550,000)
The initial figures show the values advertised by the commercial suppliers. The bracketed figures show the values calculated within the Department (Bulmer, 1989) using size exclusion HPLC. The work was performed using a TSK G4000SW column and pre-column supplied by Pharmacia LKB, with 0.5 M

acetic acid and 0.5 M sodium acetate buffers at pH 4.5. The absorbance was read at 230 nm and the results were analysed using Perkin-Elmer Nelson software.

6.12 Flocculant removal.

The ability to detect residual levels of flocculant in the centrifuged supernatant would be useful in determining whether or not a flocculant removal stage would be necessary. It is possible to detect levels of PEG as low as 0.01% by the addition of one drop of the sample to 5 mls of Nesslers reagent. A white precipitate forms in the presence of PEG. This work was done on PEG with a molecular weight of 6,000 Daltons (Polson *et al*, 1964).
Work has been carried out on an assay for the detection of PEI (Bulmer, 1989) using radio-labelling with ^{32}P. This method has been shown to detect levels as low as 0.005% w/v PEI, and it is anticipated that its sensitivity can be increased. Stocks of PEI were labelled with a known concentration of $^{32}PO_4^{2-}$, resulting in a ratio of approximately 5 nM of $^{32}PO_4^{2-}$ to each PEI molecule. After the flocculation, performed in the usual manner, the samples were spun in an Eppendorf bench-top centrifuge at 3000 g for five minutes. 50 μl of the supernatant was placed in a scintillation vial and counted for one minute on the tritium channel of a Phillips 4700 Scintillation Counter using a technique known as 'Cerenkov counting' which uses no added scintillation cocktail. The method is only 10% efficient, but the samples examined to date have been so radioactive that this is enough to get strong readings. Should more sensitivity be required, the technique can be modified to include a scintillation cocktail.

6.13 Small-scale simulation of flocculant addition to disc-stack centrifuge.

The aim of this study was to predict the effectiveness of adding flocculant to a large-scale process to aid the removal of cells and debris. Having ascertained the optimum dosage of

PEI to cause aggregation, an experiment was devised to simulate the addition of PEI to a large-scale cell culture by reproducing, on a small scale, the conditions the broth would encounter during cell separation by disc-stack centrifugation. Cells (cell line #2) were cultured in roller bottles for several days until the viable cell count had increased fifteen to twenty-fold from inoculation. A daily sample was then taken and treated in the following manner: the sample was divided into six 20 ml measures, each of which had a volume of either 0.01% v/v PEI or distilled water added to it, as shown in Table 2. The samples were inverted ten times to mix them thoroughly, then examined under a microscope. Where possible, cell counts were made. Certain samples were then exposed to conditions of shear as described in Section 6.13.1, to mimic the forces cells would encounter in the feed zone of a disc-stack centrifuge. Other samples were not sheared and acted as controls for the experiment. After shearing the samples were again examined microscopically and cell counts were made where possible. Solids were then removed from the samples by centrifugation in 14 ml test-tubes (MSE (Cat. #34411-122, Code 1286), MSE Scientific Instruments Ltd., Crawley, Sussex.) in a bench-top centrifuge with a swing-out rotor (MSE Centaur II). The revolutions per minute and centrifugation time were calculated to give the same separation capability as the disc-stack centrifuge, as described in Section 6.13.2. At all stages, cell counts were made and photographs of the samples were taken, and the final supernatant was assayed for extracellular IgM, DNA and protein.

6.13.1 Mimic of centrifuge feed zone.

Cells entering the disc-stack centrifuge encounter a shear force as they are accelerated to the speed of the centrifuge. Hoare and Dunnill (1986) note that the effects of such forces on protein precipitates is significant, and it is reasonable to assume that flocs of cells and debris could also be affected. The aim of this part of the experiment was to simulate on a small scale the shear experienced by the cells,

to ascertain whether or not the flocs, or indeed the cells themselves, were strong enough to survive the shear without breaking. This was done by shearing the samples in a capillary rheometer system based on an Instron Food Testing Instrument, Table Model 1140 (Instron Ltd.).

It was difficult to quantify the shear forces experienced by the cells in the feed zone of the disc-stack centrifuge, as little information was available on the subject. However, the diameter of the feed line to the centrifuge was much larger than the largest capillary diameter for the Instron, which was 0.575 mm, approximately forty times the diameter of a viable mammalian cell. Since clumps of one hundred or more cells can form on the addition of PEI, there was deemed to be a risk of a smaller capillary becoming blocked and the flocs being broken by this mechanism rather than by applied shear, a situation that would not arise in a large-scale situation because of the larger diameter feed line used. The level of shear in this experiment was therefore limited to that attainable with the largest bore capillary. By varying the length of capillary used the residence time of the samples could be altered. Two lengths of capillary were used in this study; Table 2 shows the capillary length used on each sample. Shear was applied to both flocculated and non-flocculated cells. Capillaries of varying lengths and internal diameters could be affixed to the barrel of the Instron. The sample to be sheared was then placed in the barrel and driven through the capillary of choice by the action of a piston travelling through the barrel at a fixed speed. The sheared sample was collected from the end of the capillary. The sample size used was 20 ml.

The shear applied was calculated using the following equation:-

$$\gamma = \frac{64}{15} \frac{VB^2}{d^3}$$

(Thomas, 1977)

where:-
γ = average shear rate (s^{-1})
V = crosshead speed ($m\ s^{-1}$) (fixed at 8.33×10^{-3} $m\ s^{-1}$)
B = barrel internal diameter (m) (fixed at 9.54×10^{-3} m)
d = internal bore of capillary (m) (fixed at 5.75×10^{-4} m)

From this, a shear rate of 1.70×10^{4} s^{-1} was calculated. The mean residence time was calculated according to the following equation:-

$$\theta = \frac{d^2 L}{VB^2}$$

(Thomas, 1977)

where:-
θ = mean residence time (s)
d = internal bore of capillary (m) (fixed at 5.75×10^{-4} m)
L = capillary length (m) (set at either 0.075 m or 0.15 m)
V = crosshead speed ($m\ s^{-1}$) (fixed at 8.33×10^{-3} $m\ s^{-1}$)
B = barrel internal diameter (m) (fixed at 9.54×10^{-3} m)

From this, the residence time was calculated as either 0.033 seconds (for 0.075 m capillary) or 0.065 seconds (for 0.15 m capillary).
Problems with this method arose due to the use of a large bore capillary; unless the bottom of the capillary was blocked off during sample loading, the sample flowed out before shear could be applied.

6.13.2 Comparison of sigma values.

In order to compare centrifuges of different scales, the following relationship is used:-

$$\frac{Q_1}{\Sigma_1} = \frac{Q_2}{\Sigma_2}$$

where:-
Q_1 = flowrate through centrifuge 1 ($m^3\ s^{-1}$)

Q_2 = flowrate through centrifuge 2 ($m^3\ s^{-1}$)
Σ_1 = sigma value for centrifuge 1 (m^2)
Σ_2 = sigma value for centrifuge 2 (m^2)

The sigma value is calculated from several parameters of a centrifuge and denotes the area of a gravitational settling tank with the same separation performance as the centrifuge. Different centrifuge types require different equations to calculate this value, and it is not recommended that scale-up is carried out between different types of centrifuge. In this case however, no disc-stack centrifuge small enough for use on a bench scale was available, so there was no option but to attempt to compare different types of centrifuge.
For a disc-stack centrifuge, the equation for calculating the sigma value is as follows:-

$$\Sigma = \frac{2\pi\omega^2(s-1)(r_2^3-r_1^3)}{3\ g\ \tan.\Omega}$$

(Ambler, 1952)

where:-
Σ = area of equivalent settling in a gravitational field(m^2)
ω = angular velocity (rads s^{-1}) = 2π(rps) = $\frac{\pi(rpm)}{30}$
s = number of discs in stack
r_1 = inner radius of discs (m)
r_2 = outer radius of discs (m)
Ω = half angle of discs

For the disc-stack centrifuge used in the large-scale studies, the following information was used in the calculation:-
rpm = 9650; therefore ω = 1010.55 rads s^{-1}
s = 80
r_1 = 4.0 x 10^{-2} m
r_2 = 8.45 x 10^{-2} m
Ω = 40°

From this, a sigma value of 11,073.20 m^2 was calculated. The flowrate through the centrifuge was an average of 600 L h^{-1},

or 1.67×10^{-4} m^3 s^{-1}, so the ratio Q/Σ was calculated as 1.51×10^{-8} m s^{-1}.

For a swing-out bottle centrifuge, the equation used to calculate the sigma value is as follows:-

$$\Sigma = \frac{\omega^2 V}{4.6\ g\ \log.\left(\frac{2r_2}{r_1 + r_2}\right)}$$

(Ambler, 1959)

where:-

Σ = area of equivalent settling in a gravitational field(m^2)

ω = angular velocity (rads s^{-1}) = 2π(rps) = $\frac{\pi(\text{rpm})}{30}$

V = volume of liquid in bottle (m^3)

r_1 = radius of liquid surface in bottle from axis of rotation (m)

r_2 = radius of surface of sedimented cake from axis of rotation (m)

Using the following information a sigma value was calculated:-

rpm = 4000; therefore ω = 418.88 rads s^{-1}

$V = 1 \times 10^{-5}$ m^3

r_1 = 0.093 m

r_2 = 0.167 m

The sigma value calculated was 0.36 m^2. Using the ratio:-

$$\frac{Q_1}{\Sigma_1} = \frac{Q_2}{\Sigma_2}$$

and knowing that the ratio for the disc-stack centrifuge is 1.51×10^{-8} m s^{-1}, the effective 'flowrate' through the bottles in the swing-out centrifuge can be calculated as 5.44×10^{-9} m^3 s^{-1}. Given the following equation:-

$$t = \frac{V}{Q}$$

where:-
t = length of time samples are centrifuged (s)
V = volume of samples (m^3)
Q = effective 'flowrate' through bottle (m^3 s^{-1})

then the length of centrifugation necessary to give the small-scale samples the same Q/Σ value as the large-scale samples was calculated as 1838.24 seconds, or 30.64 minutes at 4000 rpm.

6.14 Cell disruption.

For the purpose of determining the quantity of intracellular DNA in a sample containing intact cells a method had to be found to cause complete lysis of those cells. Several possibilities were examined including osmotic shock, sonication, freeze/thawing, shear and exposure to organic solvents.
Osmotic shock was inducing by sedimenting the cells centrifugally and resuspending them in 1 mM phosphate buffer. When this did not lyse the cells the procedure was repeated using distilled water to resuspend the cells. Although the cells swelled slightly, no lysis was caused.
Sonication, using a probe sonicator, took so long to lyse the cells that considerable heating of the sample occurred, even when packed in ice.
Freeze/thawing repeatedly at both -20 °C and -70 °C was found to be ineffectual at lysing the cells. The method is also known to degrade DNA.
The use of shear to lyse the cells was limited by the size of the samples; a homogeniser was not a viable option. Shear was applied to the cells by passaging the samples through a narrow bore needle three times. This did not lyse the cells.
Octanol, butanol and n-amylamine were tested to see if they lysed the cells; octanol was the only one that had an effect on the cells, and that was limited to fragmenting some cells. Furthermore, the effect of such solvents on the DNA assay would be difficult to take into account.

A method was found that lysed the cells effectively by addition of sodium dodecyl sulphate (SDS) in the following manner (Majumdar *et al*, 1991):-

5 ml samples were spun at 2000 rpm (75 g) for 5 minutes to sediment the cells, then the supernatant was discarded. The cells were resuspended in 5 ml ice-cold phosphate-buffered saline solution to wash them, then the samples were respun. The supernatant was again discarded and the cells were resuspended in 2 ml SDS-EDTA solution (17.5 mM SDS in 10 mM EDTA, pH 8). This lysed the cells completely, as was verified by examination under a microscope. The samples were then buffer-exchanged and assayed for DNA in the manner described in Section 6.6.1.

7. EXPERIMENTAL RESULTS AND DISCUSSION.

7.1 Large-scale mammalian cell culture and product recovery.

A series of studies were undertaken with large-scale cultures of mammalian cells during their industrial production in order to assess the effectiveness of the currently-used techniques and to establish a datum with which later flocculation results could be compared. This study covered a range of cell lines and products, and extended from analysis of inocula to the fermenter through early downstream processing to sterile filtration or azide addition. The extent of the study is shown in Figure 2, which portrays a generalised layout of the downstream processing of the cell lines and indicates the points at which samples were taken.

7.1.1 Characterisation of large-scale cell culture.

The cell lines studied were cultured in 1000 L or 2000 L airlift fermenters and were inoculated with 10% of their volume. Some cell lines were grown in serum-free media, while others had 5% foetal calf serum added. pH control was by the addition of CO_2 gas. Each run lasted between nine and fourteen days, and was harvested at a cell viability of less than 10% as determined by trypan blue dye exclusion.

Throughout each cell culture, samples were taken and the total and viable cell counts determined as described in Sections 6.1.3 and 6.1.4. Readings of the absorbance of the samples at 670 nm were also taken where possible. The solids content was measured according to the method described in Section 6.1.2 and the supernatant from this assay was analysed further to give extracellular immunoglobulin, total protein and DNA levels.

The following sections give examples of the trends observed during the study of several cell lines.

7.1.1.1 Cell line #1.

The majority of the studies were done on one cell line; a rodent hybridoma producing IgM in serum-containing media. For the purposes of this thesis, it is designated cell line #1. Figure 3 shows the cell counts from one of the runs sampled, as an example of the trends observed.

The viable cell count rose to a maximum, then fell. This trend was mirrored by that of the solid content, including a fall towards the end of the run. This drop in solids content is due to the fact that the cells shrink as they lose their viability, and some lyse to give small fragments. Both occurrences aid closer packing of the solids during centrifugation.

The total cell count rose throughout the run, and was mirrored by the rising A_{670} readings. This is because the smaller non-viable cells and debris are still present in the sample and thus will still affect the absorbance of the sample.

Figure 4 shows the viable cell and IgM profiles from a different run. The IgM levels continued to increase after peak cell viability had been reached and the cells had entered the decline phase. This is commonly observed in batch and fed batch processes (Birch & Arathoon, 1990; Marx *et al*, 1990). Several theories exist to explain the phenomenon. It has been established that antibody is not stored in the cells for longer than two hours (Walker & Davison, unpublished), so the increased production rate is not due to the release of antibody by cell lysis. Therefore the overall antibody production rate changes during a culture period. Work at the Harwell Laboratory in Oxfordshire has led to the hypothesis that several sub-cultures of cells exist in any culture, each with its own growth and production rates. The changes in ratio of these sub-cultures are thought to cause the changes in overall production rate. A more likely explanation is that the entire culture alters its production rate due to more cells being in the production phase of the cell cycle towards the end of the culture. Antibody production is associated with the G1 phase of the cell cycle and is almost completely halted

during the DNA synthesis and cell replication stages of the cycle. As cell replication slows towards the end of a batch culture, more cells switch to antibody production alone, thus increasing the rate of antibody production (McCullough & Spier, 1990). Work by Leno *et al* (1992) on semicontinuous cultures confirm that slower-growing cells, which would spend a longer period of time in the G1 phase, produce antibody at a higher rate, perhaps due to the fact that less of their energy source is spent on cell replication. Hayter *et al* (1992) observed a higher specific rate of antibody production in cells arrested by the addition of 2 mM thymidine, suggesting that cell proliferation and antibody production can be uncoupled.

When observing this phenomenon, it should be noted that the antibodies produced towards the end of a culture may not be fully active. Increased proteolytic activity in the culture medium (Harbour *et al*, 1988), deamidation and oxidation (Marx *et al*, 1990) all affect the activity of the antibodies. This has been observed in other cell lines with different products, and results suggest that the physiological state of the cells also affects the accuracy of protein processing (Hayter *et al*, 1990).

Figure 5 shows that the total extracellular protein level in the culture increased towards the end of the run. The point at which the level started to increase corresponds well with the beginning of the drop in the percentage viability of the culture, measured as the ratio of viable to total cells. The more permeable membranes of the non-viable cells will release protein into the culture, as will lysing cells.

Figure 6 compares the extracellular DNA profile with that of non-viable cells. It was expected that extracellular DNA would be a good marker for cell death and, as shown in the figure, the trends are similar. However, an increase in DNA was observed early in the run, prior to any increase in the non-viable cell count. This was unexpected, as it was thought that the cells would allow the small dye molecules through their increasingly permeable membranes before the much larger nucleic acid escaped into the media. There are several

possible explanations for this event; one is that the cells have an active system for exporting DNA. Another is that the trypan blue dye is oxidised by viable cells in a manner similar to methylene blue, and that the oxidation system survives the changing membrane permeability. Finally, it is possible that the cells which released the DNA did so by lysis, leaving no intact cell to count. Particle sizing of the early stages of a cell culture would perhaps be able to confirm or deny this hypothesis, although the results of particle size studies presented in Section 7.2.1 are not clear enough to do so. The release of nucleic acid from intact bacterial cells is not unknown. McGregor & Finn (1969) note the release of RNA from *Escherichia coli* without the shock of warming or shear.
The DNA trend also deviated from that of the non-viable cells at the end of the culture, flattening out while the non-viable cell count was still increasing.

7.1.1.2 Cell line #2.

This cell line is a rodent hybridoma producing an IgM. When cultured on a large scale, media containing serum is used, though the cells grow well in serum-free media on a small scale. This is the cell line that was used for all small-scale work and flocculant screening for this thesis.
Figure 7 shows the viable cell and IgM profiles of a typical cell #2 culture. The trend was similar to that observed in cell line #1.
Figure 8 shows the viable cell count and the DNA profile. The drop in DNA at the end of the run is often seen, and is thought to be due to the breakdown of the nucleic acid by nucleases into fragments too small to be detected by the fluorescence assay used.

7.1.1.3 Cell line #3.

This is a human cell line, grown in serum-containing media. Figure 9 shows the non-viable cell and DNA profiles across the

cell culture. As before, the DNA rose before the cell count. No drop in DNA at the end of the culture was seen in this example.

7.1.1.4 Cell line #4.

This cell line is a recombinant mouse hybridoma producing humanised IgG_1 in serum-free media for therapeutic applications.
Figure 10 shows the viable cell and IgG profiles. Again, IgG production continued to increase after peak cell viability had been passed.
Figure 11 shows the DNA and non-viable cell profiles. In this case, the two curves were very similar; regression analysis produces an R_2 value of 95.56%. As this was the only cell line #4 run to which access was given it was not possible to determine whether this was an unusual case, or whether this cell line does not exhibit the same trend of rising DNA in the early stages of the cell culture. It is important to note that the release of DNA into the media corresponds closely to IgG production, as shown in Figure 12. This means that any benefit gained in the reduction of free DNA by harvesting the culture early would have to be balanced carefully against the correspondingly lower IgG level. This is equally true for the other cell lines examined.
Figure 13 shows the expected trend of total protein rising as the cell viability started to drop. The initial level of just above 1 mg ml^{-1} protein is consistent with what is expected in a serum-free culture to which bovine serum albumin (BSA) has been added.

7.1.1.5 Cell line #5.

This cell line is a rodent hybridoma producing IgG_1 in serum-free media.
Figure 14 shows the expected profiles of viable cells and IgG, with the immunoglobulin level continuing to rise after peak cell viability had been passed. The level of the product in

this cell run was much higher than has been observed in any other cell line in the course of these studies.
The DNA profile for this cell line was unusual, as is shown in Figure 15. It rose sharply midway through the culture, then fell. Repeated assaying of the samples produced the same trend. It is possible that some mistreatment of the samples occurred immediately after they were taken, resulting in these abnormal readings. Possibilities include the dilution of the samples with water prior to cell counting.
Figure 16 shows the protein and percentage viability trends, with the extracellular protein level rising as the viability dropped.

7.1.1.6 Summary of findings.

Although each cell line differed slightly in its profiles and the duration of its cell cultures, there were many similarities. All the cell lines studied continued to produce immunoglobulin well into the decline phase of the culture, and released DNA into the media throughout the run. This supports the practice of harvesting the culture at low viability, and restricts any attempt to harvest at such a time as to reduce DNA levels while retaining a high level of immunoglobulin. Many of the examples looked at exhibited a rise in DNA early in the culture, and some also showed a drop at the end of the culture. Possible reasons for this are discussed in Section 7.1.1.1. Total protein levels generally rose toward the end of a run. This rise coincided with a fall in the viability of the culture. For serum-containing media, the extracellular protein level at the start of a culture was around 1.5 mg ml^{-1} and rose to 2.0 mg ml^{-1} or higher. For serum-free media, the level was around 1.0 mg ml^{-1} initially, rising to 1.5 mg ml^{-1} or more. Given that the levels of immunoglobulin produced were around 60 μg ml^{-1}, and never higher than 100 μg ml^{-1}, the increase in total protein cannot be attributed simply to product formation. Calculations show that 10^6 mammalian cells yield approximately 0.3 mg of total protein when dried cell paste is analysed (Pers. comm: C. Hill, Celltech Ltd., Slough,

Berkshire). The increase seen in these cell runs was not as high as this, which was to be expected as the protein being assayed was that found in the supernatant after the samples had been spun to remove solids, rather than the total protein content of the cells.

7.1.2 Mass balance of early downstream processing.

This section covers the studies of the harvesting of the various cell lines, from centrifugation through to sterile filtration or azide addition. Some cell lines are purified by slightly different sequences, but most follow the pattern laid out in Figure 2 as mentioned earlier, and which is described more fully here.

The broth from the fermenter, which was aerated until harvesting commenced to ensure good mixing, was fed to a disc-stack centrifuge. This was run at its maximum speed of 9650 rpm and with its full complement of 80 discs. Samples were taken from ports on both the feed and supernatant lines. An intermittent discharge system was used to periodically remove solids from the bowl. This sludge passed through a cyclone and into a collecting chamber from which sampling was possible. The sludge in this chamber was collected in its entirety as far as was possible, with the sludge from each discharge being collected and analysed separately.

The clarified supernatant passed to a collecting vessel, from which it was circulated through an ultrafiltration rig until its volume had been reduced to approximately 9%. The retentate was then transferred to a smaller vessel and some of the permeate retained from the ultrafilter was used to make up the volume of the retentate to 10% of the original culture volume. This method allows for more accurate measurement of the final volume of the retentate. The pH of the retentate was then reduced to 5.5 as a virus-inactivating measure. This method is used on some of the older cell lines; newer cell lines either use different methods of virus inactivation or include a pH drop at a later processing stage.

A series of three dead-end sterile filters with a pore size

of 0.2 μm were then used, followed by the addition of sodium azide to diagnostic products.
All samples were assayed using the methods laid out in Chapter 6.
The following sections give examples of the results obtained.

7.1.2.1 Cell line #1.

This cell line was extensively sampled throughout centrifugation and later downstream processing steps. Figures 17 to 21 show a profile of such a centrifugation, a process which generally lasted about two hours. The vertical lines marked on all the centrifuge profiles indicate points at which solids were discharged. The feed stream analysis is shown in Figure 17 and indicates that the levels of extracellular immunoglobulin, total protein and DNA remained constant throughout the centrifugation process. Exceptions to this occurred when the contents of the fermenter were left to stand for 24 hours, awaiting the availability of the harvesting equipment. Figure 18 shows such a profile, in which the IgM levels rose as the centrifugation progressed. Although the air supply to the fermenter remained connected during the waiting period, ensuring that the culture was well-mixed, it would appear that some separation of the IgM occurred. This trend was observed in another run involving this cell line, the harvest of which was also delayed by 24 hours. The fact that no other component was similarly affected in either case is surprising.
Figure 19 profiles the supernatant stream from the centrifuge. The results shown here were unusual in that the IgM level fell as the centrifugation proceeded; all the other runs showed constant levels of IgM, total protein and DNA. This exception may have been due to technical difficulties on this particular occasion, such as insufficient flow of cooling water to the centrifuge, which would have allowed the centrifuge bowl to gradually heat up, possibly reaching unacceptably high temperatures.
A study was performed on the feed and supernatant streams of

one harvest of this particular cell line to ascertain whether or not the cells reaching the centrifuge were completely lysed. The method described in Section 6.14 was used to wash and lyse the cells in the feed sample, and the supernatant samples were treated in a similar manner in case any intact cells were present in them. The samples were then assayed for DNA. The results are shown in Table 3 along with the extracellular DNA levels of the samples prior to cell lysis. From these results it can be seen that there are a significant number of intact cells in the feed samples, and a measurable number in the supernatant.

Figure 20 shows the solids content of the samples, and indicates that while the solids found in the feed remained constant, those in the supernatant fluctuated. The samples were taken prior to and immediately after a discharge, and as a rule, a peak in solids content was observed following each discharge. This was due to the disturbance of the solid:liquid interface within the centrifuge during discharge, causing more solids to be carried into the supernatant. Figure 21 shows the A_{670} of the samples and confirms the trends displayed by solids determination.

Taking an overview of the downstream processing of this cell line a mass balance of the various components can be attempted, as laid out in Figure 22. This is one of several mass balances performed on cell line #1. It indicates a certain amount of protein and DNA release during the harvesting of the broth, followed by reductions in all components. The IgM level fell to about 75% of its original value. Given that no large loss was seen in the volumes removed from the product stream (ie. the sludge and UF permeate), within the limits of accuracy of the assays used, the loss must be due either to adsorption of the IgM to the process equipment, such as UF or sterile filter membranes, or inactivation of the IgM by thermal alteration or enzymic action. If the latter is true, this has implications for the centrifugation step, with which excessive heat or cell lysis and thus enzyme release may be associated. Physical examination of the sludge confirms that lysis does occur.

Quantification of the degree of this by particle sizing was not possible as the sludge was difficult to resuspend.
Figure 22 also shows a mass balance of the solids (v/v) over the centrifugation step. The 1.15 L of solids not accounted for in this balance were probably retained in the centrifuge itself, or in the cyclone attached to the discharge port, neither of which were accessible.

7.1.2.2 Cell line #3.

The results obtained from this cell line were similar to those for cell line #1, but were limited to one run. More extensive samples were taken of the centrifugation, and the results are shown in Figures 23 and 24. Because the results from cell line #1 showed a trend in the solids content and absorbance at 670 nm of the supernatant, further samples were taken to determine the extent of the effect of discharging solids. As before, samples were taken immediately prior to and after each discharge, then a further sample was taken three minutes after the discharge. As shown in Figure 23, the solid levels fall rapidly to a lower level. The trends in A_{670} shown in Figure 24 confirm this.
No further samples were available during this harvest, so a mass balance of the early downstream processing was not possible. However, a balance of the centrifugation step is shown in Figure 25. An increase in both protein and DNA levels indicate cell lysis, and physical examination of the sludge confirms this.

7.1.2.3 Cell line #5.

Figure 26 shows a mass balance of the harvest of this cell line. Again, conclusions are difficult to draw from such limited samples.
An observation of interest from these results is the marked decrease in the DNA level after the pH drop from 7.1 to 5.5, thought to be due to activation of nucleases by the changing pH. This cell line differs from the others in that the pH drop

was implemented before centrifugation, with the subsequent pH rise in conjunction with ultrafiltration.
The increased DNA levels after centrifugation indicate that cell lysis occurred, as has been observed in other cell lines. There was also a drop in IgG level during centrifugation.

7.1.2.4 Cell line #6.

Figure 27 shows the profile of the harvest from which the subsequent mass balance, shown in Figure 28, was calculated. Again, increases in component levels indicate cell lysis during centrifugation. The initially high DNA level at the start of the harvest is probably due to the fact that the harvest of this run was delayed for 48 hours until such time as it was convenient to process the broth. During this time the air supply was switched off and the temperature was dropped to some unspecified level. Samples of the centrifuge feed and supernatant from this harvest were unavailable, so the immunoglobulin profiles observed in the two cell line #1 runs in which the harvest was delayed (Section 7.1.2.1) could not be verified in this cell line.

7.1.2.5 Summary of findings.

Overall, cell lysis is indicated during the centrifugation of the broth, despite the fact that the centrifuge has been designed to reduce the shear normally associated with the inlet zone (Whittington, 1990). The removal of solids is otherwise efficient, with minor fluctuations caused by the discharge of solids from the centrifuge. The liquid volume of the centrifuge bowl is 3.1 L; with an average feed flowrate of 600 L h^{-1}, the minimum residence time of the broth is 18.6 seconds. It is worth noting the effect the method of centrifuge operation has on the supernatant. Most harvests were performed with the discharge times controlled automatically, spaced at twenty minute intervals, though these timed intervals varied from fifteen to thirty minutes depending on the decision of the operator. Some runs, however,

were harvested with manual discharges, with the timing of these discharges determined by the visual comparison of feed and supernatant clarity by the operator. Under these circumstances the intervals between discharges varied greatly, extending to anything up to an hour. The effect of this was to allow a certain amount of solids through into the supernatant. Although isolated events are unlikely to have a major effect on subsequent processes, repeated occurrence could cause fouling of the ultrafiltration membrane, leading to reduced efficiency and a shorter membrane lifespan. A calculation of the optimum timing of discharges can be performed:-

$$T_1 = \frac{V_1 \times 60 \times 100}{Q \times V_2}$$

(Whittington, 1990)

where:-
T_1 = interval between discharges (mins)
V_1 = volume of solids collecting space (L)
Q = feed flowrate (L h^{-1})
V_2 = percentage solids in feed suspension

From this the optimum time between discharges for the centrifuge used in these studies, given values of V_1 = 1.2 L; Q = 600 L h^{-1}; V_2 = 0.38%, is approximately thirty minutes.
As stated in Section 7.1.2.1, the apparent loss of immunoglobulin during the early stages of downstream processing could reflect the severity of the conditions encountered during centrifugation. If the decrease is indeed due to inactivation of the immunoglobulin by enzymes, there is a case for including some method to minimise the amount of lysis caused during centrifugation. One possibility would be an aggregative step, to clump the cells and debris and thus decrease the centrifugal force needed to separate solid and liquid. Another method of reducing the inactivation of IgM during harvesting would be to crash-cool the broth in the fermenter prior to centrifugation, thus inhibiting the action

of proteases. It should be noted, however, that the scope of these studies did not include testing for proteolysis of the antibodies, and the above is a hypothesis. Future studies of this subject could usefully include research into this area.

7.2 Small-scale cell culture.

A series of experiments were performed to ascertain that the growth and production characteristics of cell line #2 at a larger scale were reproducible at a small scale. It should be noted, however, that the small-scale cultures were grown in serum-free media, while 5% foetal calf serum was added to the media for large-scale culture. On the small scale, cells were revived from frozen storage into 75 cm^2 flasks, then used to inoculate roller bottles. When the cell count approached 10 x 10^5 cells ml^{-1}, 25 ml of this was used to inoculate spinner flasks, with 225 ml of fresh media added. Samples were taken at intervals, and total and viable cell counts and percentage solids determined according to the methods described in Section 6.1. The size of the cells were also determined using the Coulter principle described in Section 6.10. The sample supernatants were assayed for IgM, DNA and total protein.
The results of one such small scale culture are shown in the following figures. Figure 29 shows the viable cell counts, IgM levels and percentage solids (v/v) throughout a small-scale run. The trends are similar to those seen on a large scale in all the cell lines studied (see for example Figures 3 and 7). However, the levels are considerably lower than those observed on a large scale, with a maximum viable cell count of only 15.07 x 10^5 ml^{-1} compared with 29.45 x 10^5 ml^{-1} for a large-scale run. The IgM level attained is also lower, reaching only 11.72 μg ml^{-1} instead of 58.19 μg ml^{-1}. This is due to the fact that large-scale cultures are run as fed-batch fermentations to boost cell counts and product levels. The cultures performed on a small scale were run as simple batch cultures. Other differences between the two methods, such as the presence of serum in the large-scale runs or better oxygenation in an airlift fermenter, would also affect final

cell counts and product levels.
Figure 30 shows the expected trend of increasing extracellular protein as the culture viability drops. Figure 31 shows the extracellular DNA levels with the non-viable cell count. The level of DNA attained by the end of the cell culture is much higher than that observed in the large-scale runs. This could be due to the ability of serum components to bind nucleic acids, or due to low oxygenation levels causing high levels of cell death. Shear is not a factor; the spinner flasks are stirred at 60 rpm by low-shear magnetically-impelled glass stirrers.

7.2.1 Particle sizing.

Figure 32 shows the mean diameter of the cells during the culture period. Although the trend is unclear, an overall reduction in the cell diameter was seen as the percentage viability of the cells fell. A viable hybridoma has a diameter of 12-14 μm, while a non-viable cell is approximately 8 μm according to Batt *et al* (1990), so as the proportion of non-viable to viable cells within the culture changes, so the mean diameter should fall.
Further work was undertaken to ascertain whether or not particle sizing techniques could differentiate between viable and non-viable cells, with a view to establishing an accurate alternative method of counting viable and non-viable cells. A mercury switch was fitted to the Elzone, allowing the counting of small, accurately-measured sample volumes. The volume used for this work was 50 μl. For each sample, a known volume of cell suspension was added to a known volume of buffer, and up to ten 50 μl volumes were counted. The total cell population was recorded, and a chart was printed out showing the particle distribution by diameter. Figure 33 shows a typical profile of the particle distribution of a sample, with the peaks attributable to viable cells, non-viable cells and debris marked accordingly. The profile seen here corresponds with that observed by Batt *et al* (1990) using forward-angle light scattering measurement.

Using the data given by the Elzone software, it is possible to calculate the cell number in each peak in Figure 33. Knowing the volume counted and the dilution of the original sample, the viable and non-viable cell counts can be calculated. This was done for samples from four spinner flasks over a four-day period. The results of one such experiment, along with conventional dye exclusion cell counts, are shown in Table 4 with the viable cell counts shown in Figure 34. The non-viable cells proved more difficult to count, as the corresponding peak soon merged with the debris. The Elzone tended to overestimate the cell count as the percentage viability dropped. This was due to the fact that cells that had started to allow trypan blue to enter but had not yet started to shrink were counted as non-viable in the dye exclusion test but not in the Elzone. Unfortunately, the mercury switch in the Elzone developed a fault, so further readings as the cultures declined were not possible.
It was not possible to determine whether or not there was an increase in debris corresponding to the appearance of extracellular DNA in the culture, so the hypothesis suggested in Section 7.1.1.1 that the early increase in extracellular DNA is due to cell lysis remains unproven.

7.3 Cell flocculation.

This section describes the results of a series of experiments on a range of different flocculants. In each case the protocol used to examine the effectiveness of the flocculant was that described in Section 6.9. Measurement of total and viable cell numbers was performed using the methods described in Sections 6.1.3 and 6.1.4.1 and measurements of extracellular IgM, total protein and DNA was performed using those described in Sections 6.4, 6.5 and 6.6. The range of molecular weights of PEI used had their diameter measured by photon correlation spectroscopy, as described in Section 6.10.

7.3.1 Polyethyleneimine (PEI) flocculation.

7.3.1.1 Initial studies.

Initial work on PEI showed that it was capable of flocculating mammalian cells to some degree at a level of 0.001% v/v, but that the most effective concentration was at 0.01% v/v. Higher levels lysed the cells completely, while lower levels failed to produce the necessary degree of cell flocculation or contaminant removal. Extracellular IgM, DNA and protein levels were all affected by the amount of PEI added, as was the packed volume of cells.
Further work was undertaken to determine the effect of pH and PEI molecular weight variations on the cells and their product and contaminant levels.

7.3.1.2 Influence of pH.

The results of this experiment are displayed graphically in Figures 35 to 37.
At high levels of PEI (0.1% v/v) most of the cells were lysed, and the lysate was aggregated. The PEI concentration above which cell lysis occurs is marked on Figure 35. At 0.01% v/v PEI there was an increase in the number of flocs, though none were larger than about 10 cells. There was also an increase in the amount of debris in the culture. At 0.001%, there were fewer flocs and the amount of debris was less. At the lowest concentration of PEI, 0.0001% v/v, there was no discernible difference from the control containing no PEI.
As is shown in Figure 35, the effect of the highest PEI concentration at all pH values was an increase in the recorded level of IgM. This could be due to cell lysis causing the release of IgM not yet secreted by the cells, or it could be due to the effect of the PEI on the ELISA used to assay the antibody levels. Two further studies arose from this result; the use of a known concentration of pure IgM in a flocculation experiment, to determine whether or not the presence of PEI interfered with the ELISA, and the determination of the

activity of the antibody after exposure to PEI. Both studies are discussed in Section 7.4.1, and the first confirms that the cause of the increased measured IgM level in the presence of PEI is due to interference in the assay by PEI. At a level of 0.01% v/v the PEI did not appreciably affect the IgM concentration, according to the assay. This confirms the observations recorded in this pH study.

At each concentration of PEI studied, the results for all five pH values fell within the 20-25% tolerance levels of the assay, showing pH to have no effect on the binding of IgM to PEI.

Figure 36 shows the removal of DNA from the supernatant by PEI. The initially low level at pH 8 is likely to be an inaccuracy, possibly due to over-dilution of the sample in the buffer-exchange columns used in the preparation of the samples for DNA analysis, or due to sample degradation. This graph shows that initial DNA levels were proportional to pH even though DNA is a strongly acidic molecule, and subsequent removal was affected. The DNA released from the cells into the supernatant would still have proteins and histones associated with it. Histones have a very positive charge, facilitating bonding to the negative backbone of the DNA molecule. This makes it difficult to draw any conclusions regarding the isoelectric point of the DNA, and whether it is affecting the measured value. An alternative possibility is that nucleases were activated by the drop in pH, and broke down the DNA into fragments too small to detect by means of this assay. However, even the highest initial levels of DNA were reduced to virtually zero by PEI levels of 0.001% v/v.

The trends in Figure 37 show a possible decrease in the level of protein at a PEI concentration of 0.01%, the optimum level for cell flocculation. The return of the protein concentrations to their original levels following the addition of 0.1% PEI was probably due to saturation of the solution with PEI and its subsequent restabilisation. Such a phenomenon occurs when excess polymer is absorbed, causing surface saturation and a change in the overall charge of the molecule (Gregory, 1987). Alternatively, it could be due to the

excessive levels of cell debris and protein in the culture following cell lysis. The lowered levels of protein at low pH could be due to denaturation by the acid conditions. Again, the initial sample in the pH 8 series was low. It seems likely that the sample denatured.
It was expected that lower pH levels would enhance the flocculation ability of PEI due to an increase in the positive charge density of the molecule by protonation of the amino groups. Such an increase in charge density would also cause the expansion of the polymer through electrostatic repulsion. Eriksson and Härdin (1987) state that a high charge density is important for effective flocculation. However, Akers (1975) states that the adsorption of a polymer onto a surface can largely be due to non-specific van der Waals forces and may not be strongly influenced by charge, and thus polymers may be effective over a wide range of conditions. The studies undertaken indicate that although pH does affect the condition of the samples themselves, it has little effect on the aggregative abilities of PEI.

7.3.1.3 Influence of PEI molecular weight.

The experiment described in section 6.9 was repeated using PEIs of different molecular weight. The results were then compared to assess the effectiveness of the different molecular weights. Since the experiments were performed over a period of several weeks using material drawn from different cell culture runs, all values are expressed as a percentage of the control sample of that particular experiment.
Another factor to be taken into account is the actual size of the molecule, including the water associated with it. This is a function of the degree of branching of the polymer, and also the degree of protonation. Photon correlation spectroscopy was used to measure the size in nm of the different PEIs (Bulmer, 1989).
600 Daltons: Not measurable -- too small.
10,000 Daltons: Not measurable -- too small.
70,000 Daltons: 71.5 nm

50-100,000 Daltons: 66.0 nm
500,000 Daltons: 66.7 nm
These results would seem to corroborate Bulmer's calculations of molecular weight, described in Section 6.11 (Bulmer, 1989), and could explain some of the following results.
In all but one case, the highest concentration of PEI caused cell lysis (as marked on Figure 38). The exception was the 70,000 M.W., where lysis was incomplete. Some protein was released, which adhered to the intact cells, and the culture became highly alkaline. At 0.01% v/v PEI, all samples showed some lysis, with protein and debris both free in the media and adhering to the cells. At 0.001%, the samples contained some debris, except in the case of 600 M.W. where the sample was visually identical to the control. At the lowest level of PEI no samples showed any sign of lysis or flocculation.
At all molecular weights the highest concentration of PEI caused an increased IgM reading, with the most marked effect seen in the low molecular weight examples (Figure 38). It is possible that the more effective binding of the lower molecular weight PEIs is due to their simpler structure; the larger molecules are highly branched, which could interfere with binding. Alternatively, the trend could be due to competition between the components of the cell broth to bind with the PEI molecules. Section 7.4.1 examines this possibility more fully by examining the effect of PEI on pure IgM.
As shown by Figure 39, DNA removal was accomplished more readily by the lower molecular weight PEIs. This could be explained by competition for binding sites from other components in the cell culture. The larger molecular weight PEIs will be more capable of forming stable polymer bridges between cells and debris than the smaller ones, perhaps leaving less PEI free to bind to DNA. PEI 70,000 actually has a larger diameter than PEI 50-100,000 or PEI 500,000, indicating the possibility of a higher charge density. This would facilitate its binding to cell surfaces. Given that the DNA molecules are expected to carry a higher charge than the cells and debris, however, this result is still surprising.

As mentioned in Section 7.3.1.2 it is possible that the DNA is still associated with positively charged histones, which would reduce its overall charge.

Figure 40 shows that the higher molecular weight PEIs were more effective at removing protein from solution, though none removed more than 50%. Again, the fact that PEI 70,000 is the largest molecule (thus probably offering the largest number of binding sites) could explain why it was the most effective at removing protein from solution. The lowest molecular weight PEI (600 Daltons) had virtually no effect on protein levels at any concentration. Studies on the effect of PEI on pure protein are discussed in Section 7.4.2, and confirm the results observed here.

Work done within the department (Bulmer, 1989), studying the PEI remaining in solutions of mixed molecular weight after flocculation indicates that the higher mobility of the smaller PEI molecules allow them to bind more quickly to debris and other components. In cases where affinity is strong, such as negatively charged DNA, or immunoglobulin, the low molecular weight PEI remains bound, but if the affinity is weak, as with cell debris or protein, the larger PEI molecules displace the smaller ones. Although the PEI molecular weights used in the experiment described above were applied separately, this would appear to explain the observed results. Akers (1975) states that in the case of low molecular weight polymers, where the chain length is insufficient for bridging to occur to any extent, alternative mechanisms such as zeta potential reduction must be used. Other studies in this field confirm that while higher molecular weight polymers tend to be more effective flocculants, giving stronger flocs, a higher flocculant:solid ratio is required for optimum solids removal (Akers, 1975). However, it is also acknowledged that in the case of the flocculation of dilute suspensions of negative particles by cationic polymers, as in this study, the charge density is of more importance than the molecular weight of the polymer (Gregory, 1989). The higher the charge density of the polymer, the more effective its action as a flocculant.

7.3.2 Polygalacturonic acid (PGA) flocculation.

7.3.2.1 Initial studies.

Initial work on PGA at pH 7 was not promising, with even the highest concentration of flocculant showing no ability to aggregate mammalian cells or remove contaminants. The use of this polymer was suggested by Celltech Ltd., of Slough, in Berkshire, who had studied it previously (European Patent Application No. 0 160 520 A2, 1985; Wood, 1985). Because no effective flocculation was observed under the conditions initially tried, and the previous work recorded the most successful results under mildly acidic conditions (pH 5-6) the scope of the experiment was broadened to include other pH conditions.

7.3.2.2 Influence of pH.

This study was undertaken in the same manner as the pH study on PEI, following the techniques set out in Section 6.9. Results are shown in Figures 41-44.

Physical examination of the samples under a microscope showed no evidence of cell aggregation at any pH. Measurement of the percentage 'solids' using Westfalia solids determination tubes (Section 6.1.2) showed a rapid increase with rising PGA concentration, caused by the formation of a gel. At 0.1% PGA, centrifugation did not compress the gel at all (Figure 41), making the measurement of its volume in the solids determination tubes used impossible. An arbitrary value of 2 was given to the gels at 0.1% PEI for the purpose of drawing Figure 41.

Figure 42 shows the IgM levels throughout the experiment. It indicates that PGA at pH 6-8 had no effect on the antibody at any concentration. The two extremes of pH examined, pH 5 and 9, both showed increased IgM levels in the control samples. Both showed a further increase at low PGA concentrations, then declined steadily until the pH 5 sample reached the same levels as the other pH values at 0.1% PGA. The cause of the

enhanced IgM readings at low PGA concentration could be a mechanism similar to that which PEI achieves at high concentrations, with the subsequent decline due to gel formation, early signs of which could be seen from concentrations of 0.001% PGA upwards. Extremes of pH may render the antibody more susceptible to bonding with the polymer.

Figure 43 shows the DNA levels. The increasing levels in the control samples with increasing pH observed in the PEI studies were not seen here. This could be due to the fact that the cells for the PGA experiment were harvested at a lower viability than those for the PEI experiment, allowing more time for the release and action of nucleases. The DNA levels increased at intermediate concentrations of PGA at pH 5, 6 and 7, a result not seen in any other flocculation experiment. The negative charge of PGA will be reduced in this pH range, making bonding to the negative 'backbone' of DNA more likely, though this should not increase the DNA readings. Under no conditions was a reduction in DNA level seen.

Figure 44 shows that gel formation at 0.1% v/v PEI aggregated some extracellular protein at all pH values tested, with pH 9 affecting protein levels the least. The lower overall charge of protein compared with DNA or cell debris makes aggregation with PGA more likely, as the force of repulsion between the two would be smaller.

Overall, the results obtained did not verify those observed by Wood or recorded in Celltech's patent application, even though the cell line used was the same. Aunins (1989) also failed to duplicate the results on other cell lines.

7.3.3 Polyethylene glycol (PEG) flocculation.

This polymer was first studied at a limited range of concentrations, then looked at in more detail when the early results showed signs of contaminant removal. The results are shown in Figure 45.

No aggregates were found in any of the samples, and no

increase in the percentage solids was observed.
There was a slight increase in the IgM level in the higher concentrations of PEG, and a drop in the DNA concentration at the highest PEG level. Taken in conjunction with a small drop in protein level at the highest PEG level, this seems to indicate some binding of the flocculant to the components. No visual signs of precipitation were seen. It is possible that higher levels of PEG would have induced protein precipitation, and possibly cell aggregation.

7.3.4 Poly-L-histidine (PLH) flocculation.

Two molecular weight ranges were studied; 5,000-15,000 Daltons and 15,000-50,000 Daltons.

7.3.4.1 Poly-L-histidine 5-15,000.

The results of this study are shown in Figure 46.
None of the samples showed any signs of cell flocculation. The increase in the percentage solids of the higher concentration PLH samples was due to the presence of precipitated PLH, in the form of large 'flakes'. In the highest concentration, the division of the precipitate into two clear bands confirmed this; the lower, red band of cells and phenol red indicator was compressed past the control level of 0.2% by the presence of a further 0.5% of white solid; this was the precipitated PLH.
The IgM level fell in the highest concentration of polymer, possibly due to some binding with the PLH. If the concentration of PLH required to bind IgM is high, due to the low solubility and low charge of the polymer at pH 7, the antibody will be associated with the higher concentrations found near the solid PLH, and will be removed along with the solids.
The DNA level also dropped at the highest concentration of PLH, possibly due to the same reasons as the IgM.
No removal of protein was recorded.

7.3.4.2 Poly-L-histidine 15-50,000.

The results of this study are shown in Figure 47.
At the highest concentration of PLH some cell lysis occurred, although this was minimal. No flocculation of the cells or debris was seen. At the two highest concentrations of PLH, white 'flakes' were observed, which again caused an increase in the measured percentage solids levels.
IgM was removed from the supernatant at the highest concentration of PLH. This was more marked than in the lower molecular weight PLH study, and could again be due to the association of IgM with the high concentration of the polyamino-acid near the solid flakes.
The DNA concentration also fell in the highest two PLH concentrations, as in the previous study. The DNA removal at 0.001% PLH was slightly more marked than in the lower molecular weight study.
No effect was seen on protein levels across the range of concentrations examined.

The use of poly-L-histidine at neutral pH was suggested by Aunins (1989) as a method of aggregating mammalian cells without reducing viability. The reduced solubility and charge of the molecule gave mild conditions for aggregation. Aunins' work was performed on cell lines other than the one used here, and the fact that this one was not aggregated indicates the difficulty of locating a flocculant capable of affecting all cell lines. It is likely that different pH conditions would have given better results.

7.3.5 Dextran sulphate flocculation.

Two molecular weights of dextran sulphate were studied; 500,000 Daltons and 5,000 Daltons. It is reported that the presence of multivalent cations such as calcium are necessary for the aggregation of cells by dextran sulphate. No extra calcium was added to the samples, as such cations are already present in the culture medium at concentrations which promote

these mechanisms (Aunins, 1989).

7.3.5.1 Dextran sulphate 500,000.

Figure 48 shows the results of this study. It indicates that while IgM levels rose with increasing dextran sulphate concentrations, the highest concentration of flocculant reduced the total protein level to zero. The results indicate that anionic polymers can cause aggregation of negatively-charged molecules, despite the disappointing results with PGA. DNA removal was most effective at 0.001% dextran sulphate, with higher concentration causing restabilisation. No cell flocs were observed.

7.3.5.2 Dextran sulphate 5,000.

As shown in Figure 49, the IgM and DNA levels were unaffected by this molecular weight of dextran sulphate, but there was some removal of protein at 0.01% and complete removal at 0.1% flocculant. Again, no cell flocculation was observed.

7.3.6 Borax flocculation.

The ability of borax to flocculate yeast cells and debris has been extensively studied within the Department. (Bonnerjea *et al*, 1988; Milburn *et al*, 1990) The borax selectively cross-links specific carbohydrates found in the yeast cell walls. For this reason, no such reaction was expected when borax was added to mammalian cells, and a single study was undertaken to confirm this. Figure 50 shows the findings of the experiment.

The levels of protein and DNA remained relatively constant throughout the range of borax concentrations added, though IgM levels dropped as the borax concentration increased. The slight lowering of all component levels at the highest concentration of borax are likely to be due to precipitation of the borax and physical entrapment of the components. After addition of borax to a final concentration of 0.1% w/v, and

centrifugation, it was not possible to resuspend the pellet.

7.3.7 Summary of findings.

Overall, PEI was found to be the most effective flocculant of the hybridoma cell line studied. The other flocculants examined did not cause any visible cell aggregation although some removed other components, such as nucleic acid and extracellular protein. The range of molecular weights of PEI examined all aggregated cells to a degree, at all values of the pH range examined.
Although expansion of the conditions examined (eg. higher PEG concentration, lower pH for PLH application) might have yielded results, it was decided that further studies should examine PEI only, at neutral pH. This was because it was felt that the ideal flocculant for use on an industrial scale would be one which required minimum preparation of the culture prior to its addition, and one capable of effective aggregation at low concentrations. Of the flocculants studied, PEI best fulfilled these requirements.

7.4 Assay validation.

This section covers the series of studies that arose from the interaction of PEI with the components being measured. The studies were designed to show whether or not the polymer was interfering with the assays and thus giving false results.

7.4.1 ELISA validation.

Early flocculation results indicated that high levels of PEI caused an increase in the measured levels of extracellular IgM (Section 7.3.1.1). This could have been caused either by lysis of the cells and the subsequent release of intracellular IgM, or by the effect of the polymer on the assay itself. To determine the correct hypothesis, the effect of PEI on pure IgM was studied.
The IgM standard used in all ELISAs in these studies was made

up to give a final concentration of 10 μg ml^{-1} and was mixed with 0.1% or 0.01% v/v of a range of molecular weights of PEI. The samples were centrifuged for twenty minutes at 4000 rpm (300 g) and assayed in the usual manner (Section 6.4) alongside IgM standard to which no PEI had been added. The assay setup is shown in Table 5, with Figure 51 showing the results. From this, it can be seen that PEI does indeed affect the accuracy of the ELISA when present at the higher concentration of 0.1% v/v, but not at the lower concentration of 0.01%. The reason for this high IgM reading is thought to be due to the binding of several IgM molecules to PEI molecules. If any IgM molecules bound in this fashion then bind in the normal manner to the antigens coating the ELISA plate wells, the other IgM molecules attached to the same polymer are effectively bound as well, giving many extra immobilised antibodies. The conjugate and substrate added subsequently to the wells have many more IgM molecules to bind to, causing an amplification effect. This unfortunately means that the ELISA is not a valid method of quantifying IgM in the presence of high levels of PEI. The amplification effect seen in the presence of 0.1% PEI increased with molecular weight. This contrasts with results obtained in studies on cell suspensions (Section 7.3.1.3), in which the lower molecular weight PEIs gave greater amplification of IgM readings. It would seem logical that larger PEI molecules would give greater amplification as they offer more binding sites than the smaller molecules. It is possible that of other components in the cell suspensions, including other proteins, nucleic acids and solids, competed for binding sites on the PEI molecules. Results (Section 7.3.1.3) indicate that high molecular weight PEIs bind extracellular proteins such as albumin, and cells and debris, more readily than do the smaller PEI molecules; if this occurred in the cell cultures, the larger PEI molecules would be prevented from binding to IgM molecules, or at least partially blinded. There is no such competition in the study of pure IgM, and it seem likely that this accounts for the difference in observed trends in the two situations.

A limited number of samples were assayed by the Scottish National Blood Transfusion Agency to determine the ability of IgM to aggregate human blood group B cells. The method is discussed at the end of Section 6.4. The results are shown in Figure 52, and indicate that the aggregative ability of IgM is impaired by exposure to PEI. The maximum loss of activity is 25% according to this assay, though it is difficult to assess how much of the cell aggregation observed is due to IgM and how much to PEI. A blank included in the assay, consisting of 0.1% PEI in culture medium, with no IgM present, gave an activity count 43% of the control (IgM with no PEI added).

7.4.2 Protein assay validation.

The object of this study was to examine the ability of PEI to remove pure BSA from solution. The BSA was diluted to give a final concentration of 100 $\mu g\ ml^{-1}$, and was mixed with a range of concentrations of PEI, as shown in Table 6. Several molecular weights of PEI were included in the assay. The samples were then centrifuged at 4000 rpm (300 g) for twenty minutes and assayed in the manner described in Section 6.5. Figure 53 shows the results of this study. In the case of PEI with a molecular weight of 600 Daltons, there was little or no effect on the protein level on addition of the polymer, although it dropped slightly at the highest PEI concentration. In all other cases there was a marked decrease in total extracellular protein at a PEI concentration of 0.001% v/v. At higher PEI concentrations the protein levels returned to their original amount due to restabilisation of the protein. The levels of total protein found in cell cultures are ten to twenty times that examined here; accordingly, one would expect a PEI concentration correspondingly higher to give optimum precipitation of protein in cell cultures. Results presented elsewhere in this thesis (Section 7.3.1.3) show this to be correct; a level of 0.01% PEI removes protein from such solutions. Less protein is removed in proportion to the PEI concentration than is shown here, probably because there is competition for binding sites from cells, debris, nucleic acid

and IgM.

7.4.3 DNA assay validation.

As described in Section 6.6.1, the preparation of samples for DNA analysis included a buffer-exchange step using disposable NAP-5 columns. Given that the DNA was thought to have bound to the PEI, it was possible that the complexes formed were physically removed by this preparative step, thus giving false results. Two studies were undertaken to ascertain whether or not this was happening. The first compared buffer-exchanged samples with untreated ones, to ensure that there was no adverse effect on the recorded DNA levels by such a step. Samples were set up to contain a final concentration of 20 μg ml^{-1} DNA standard. Table 7 shows the setup and results of the study, which was conducted on a range of concentrations and molecular weights of PEI. In all cases, levels of PEI equal to or higher than 0.001% removed all DNA present, whether or not the samples had been buffer-exchanged, with the exception of the samples treated with 0.1% PEI. The low levels of DNA observed in nearly all these samples are probably due to restabilisation of the DNA by the high levels of PEI. Figure 54 shows a comparison of DNA concentrations at PEI levels of 0% and 0.0001% v/v. From this it can be seen that, within experimental error, the buffer-exchanging of samples prior to assaying them has little or no effect on the final DNA concentrations measured.
The second study assumed that if the NAP-5 columns were capable of removing DNA-PEI complexes, they would also remove complexes of PEI with IgM or protein. Accordingly, samples to which PEI had been added at a level of 0.01% v/v were assayed for total protein and IgM before and after buffer-exchanging. The results of this study are presented in Table 8, and show that little or no IgM or protein is removed by buffer-exchanging through NAP-5 columns.

7.4.4 Interactions of components.

The assay validation studies described so far were undertaken on individual components, rather than on the interactive systems encountered under cell culture conditions. A further experiment was devised with the dual purpose of studying the effects of PEI addition on a mixture of the three major components assayed in the course of this thesis, and the effects of changing the salt concentration of the samples. At higher salt concentrations the effectiveness of the polymer might be impaired by the screening of its charges (Katchalsky, 1964; Baran, 1988). Increasing ionic strength also causes a reduction in interparticle or intermolecular repulsion, and may enhance the adsorption of polyelectrolytes on similarly charged surfaces (Gregory, 1987). A balance must be found between these opposing effects of ionic strength.

A series of samples were set up as shown in Table 9. The standards were all prepared in distilled water to avoid the addition of salt and protein to the samples, and PEI with a molecular weight of 500,000 Daltons was applied to give final concentrations of 0, 0.01 and 0.1% v/v. On addition of PEI the samples went cloudy. Each sample was split into two and 75 μl of 5 M sodium chloride solution was added to the replicates, giving a final salt concentration of 0.5 M. The samples that had been cloudy on addition of PEI cleared on addition of the salt. The samples were then spun at 4000 rpm (300 g) for twenty minutes, after which a precipitate was visible in the sample containing 0.1% PEI and no salt.

The results of assaying the samples are shown in Figures 55 to 57. Looking at IgM first, it is seen that the control sample, with no PEI or salt, had an IgM concentration only two-thirds as high as it should have been. This was possibly a result of making up the samples in distilled water instead of the usual buffers, which might affect the binding capability of the IgM molecules. Upon addition of PEI the measured IgM level was unaffected. Elsewhere in this study it has been observed that high concentrations of PEI cause an amplification of measured IgM levels. The absence of such an

effect could also be due to the lack of the usual buffers in the preparation of the samples. On addition of salt to the samples all IgM levels rose, with the most marked effect seen in the sample containing the highest level of PEI. The IgM standard is normally prepared in buffer containing 0.1 M sodium chloride and it is possible that the addition of salt, even at such high levels, reactivated IgM molecules in the control and low PEI samples, allowing increased binding and thus giving measured IgM concentrations closer to the expected level. The effect of salt on the high PEI sample must be due to enhanced binding of multiple IgM molecules to PEI molecules, causing an increase in the amplification effect described earlier.

The protein levels are shown in Figure 56. The salt-free samples exhibit a trend of increasing protein removal by PEI with increasing PEI concentration. This differs from the study of pure BSA described in Section 7.4.2 but, as mentioned in that section, the trends seen in cell cultures are different, probably due to the other components competing to bind with the PEI. The results seen here would seem to confirm this, with protein being only partially removed even at the highest concentration of PEI. On addition of salt to the samples, it appears that the binding of protein to PEI is reversed, with no observed protein removal at the PEI concentrations examined. This is in agreement with published studies in this field. Jendrisak (1987) found that at a sodium chloride concentration of 0.5 M less than 10% of protein was precipitated on addition of 0.4% PEI, compared with 50% in the absence of salt. The cloudiness observed in the samples on addition of PEI was probably caused by protein precipitation.

Figure 57 shows the DNA levels with and without salt, and indicates that salt addition has no effect on PEI-DNA complexes. These results are in agreement with published data on the subject; Kim and Rha (1989) note that while concentrations of sodium chloride or potassium chloride above 0.6 M would cause partial desorption of DNA from cationic polymeric particles, with complete desorption occurring at 1.2 M salt, concentrations below 0.6 M would not. They suggest

that the binding of DNA to the polymeric particles is due to the high overall negative charge on the DNA molecules caused by the phosphate residues; this is in contrast to typical proteins, in which most residues are uncharged and in which positive and negative residues are present in comparable numbers. The high charge of nucleic acids allows them to form strong precipitation complexes with extensive cross bridge formation (Jendrisak, 1987).
The three components (immunoglobulin, albumin and DNA) compete for binding sites on the PEI molecules. Parker *et al* (1990) noted that by first removing nucleic acids from *Escherichia coli* extracts, the purification factor attainable by precipitating a β-galactosidase-aspartate fusion protein with PEI was greatly increased. Agerkvist *et al* (1990) note that the presence of polymeric nucleic acids strongly affects the optimal dosage of the flocculant chitosan in the aggregation of *Escherichia coli*: a minimum level of chitosan is required to neutralise nucleic acids in a cell suspension before any cell aggregation occurs. They observed that if the DNA was degraded to oligonucleotides prior to flocculation it no longer bound to the chitosan, remaining in solution instead. It has been observed (McGregor & Finn, 1969; Eriksson & Härdin, 1987) that the release of proteins and polysaccharides from the cells also adversely affects the level of cell aggregation.

Aunins (1989) noted the use of increased ionic strength of mammalian cell culture medium to induce aggregation of cells. He also noted the sensitivity of mammalian cells to changes in osmotic pressure, and the change in salt concentration used in this study was restricted accordingly to avoid the risk of cell lysis.

7.5 Small-scale simulation of flocculant addition to disc-stack centrifuge.

The objective of this study was to simulate on a small scale the conditions cells and flocs would encounter if processed

on a large scale.

Samples were taken daily from roller bottles, and were treated in the manner described in Section 6.13. The study lasted for four days and covered a range of percentage viabilities of the cells.

Figure 58 shows that within the limits of this study, the percentage viability of the samples was unaffected by shear, regardless of residence time. The samples that had been sheared retained a viability that matched the control, which was not exposed to shear. The results shown in this figure are for samples to which PEI had not been added; it was not possible to accurately count the cells in the flocculated samples.

Physical examination of the samples before and after exposure to shear added further information. The samples taken on Day 1 of the study, when the cell viability was higher than 95%, flocculated well on addition of PEI. Following exposure to shear, examination revealed that the larger flocs had been broken down into smaller ones. As the study progressed, the initial flocculation seen was reduced and the flocs formed were more effectively broken up by the shear forces, with those on Day 3 broken back into single cells. On Day 4 the cells, by this time 0% viable, would not flocculate at all. Such results are at variance with other studies of the effects of shear on flocs, in that no re-formation of flocs was observed after the shear forces had been removed. Gregory (1989) found that flocs broken by shear could reform when the shear rate was reduced, although PEI is unusual in that it is a highly-branched, spherical molecule, unlike other linear polymers examined.

Plate 1 shows the control sample from Day 2, with no added PEI and no shear applied. The clear cells are viable, the blue ones are non-viable. Plate 2 shows the same sample after being sheared in the 75 mm capillary (corresponding to a residence time of 0.033 seconds) in the absence of PEI, confirming that the shear forces involved did not disrupt single cells or affect their viability. Plate 3 shows one of the larger flocs in the sample with 0.01% PEI added and no shear applied. The

effects of the 75 mm capillary tube on this sample is shown in Plate 4. A few intact flocs remain, but they are very small compared with those seen prior to shearing the sample.

The situation is different for non-viable cell cultures. Plate 5 shows the control sample for Day 4, with no PEI and no shear. Plate 6 shows the sample after addition of PEI. The flocculation that does occur is extremely limited. Some protein precipitation has occurred; this is seen adhering to the cells. Shearing the sample in the 75 mm capillary has little effect, as seen in Plate 7.

The effects of shear on IgM levels are shown in Figures 59 and 60. These show the results for samples without and with PEI respectively, and indicate that, within the limitations of the ELISA used to quantify the IgM levels, shear has no effect on extracellular IgM concentration either in the presence or absence of PEI.

The effects of shear on total extracellular protein are shown in Figures 61 and 62. The samples gave no clear trends even when repeatedly assayed, and only general conclusions can be drawn from this data. In the absence of PEI (Figure 61) the highest protein level is seen in the control sample, while the lowest level is seen in that sheared in the 75 mm capillary. The lower protein values are perhaps due to the action of shear denaturing the protein. By Day 4, however, all the samples have similar protein levels. Figure 62 shows the protein levels in the samples to which PEI was added. In this case, the control had the lowest level of protein; this level was significantly lower than the control sample to which no PEI had been added. This was expected, as PEI causes protein precipitation. The sample sheared in the 75 mm tube had the highest protein level of the samples to which PEI had been added.

Extracellular DNA levels are shown in Figures 63 and 64. The former shows the samples without PEI. Initially all values are similar, confirming that no cells are lysed by the shear forces. Towards the end of the study the sample sheared in the 75 mm tube showed a higher level of DNA than the others. Figure 64 shows much reduced DNA levels in the samples

containing PEI, with the sheared samples containing very slightly higher levels.

Given that the use of PEI at large scale would involve its addition to a culture of less than 10% viability, its usefulness would be reduced by the high proportion of non-viable cells, which aggregate less effectively. Also, the relatively low shear forces used in this study were sufficient to disrupt the flocs, so the higher forces encountered in the feed zone of the disc-stack centrifuge would certainly do so. No sign of re-aggregation of flocs was observed when the samples were removed from the shear environment, despite published results suggesting that this should happen (Horn, 1980). Such results also cast doubt upon the ability of the cells to form flocs under large-scale culture conditions. It was initially envisaged that the air sparging used in the airlift fermenters might be enough to cause aggregation; given the results, it is possible that the localised shear caused by the air bubbles might break the flocs as they formed. Further studies would have to be undertaken to determine the optimum method of mixing the culture and flocculant.
However, the ability of the PEI to bind and remove DNA was unimpaired by exposure to shear. Whether this benefit is enough to outweigh the inability of the flocs to survive shear and the problems associated with the removal of PEI from the system is debatable.

7.6 Problems encountered during the studies.

Problems throughout the studies included the small sample sizes available, both on small and large scales. This limited the number of assays that could be performed, as well as the number of replicates. Regarding equipment, the drive unit in the Gallenkamp incubator was particularly unreliable when first purchased, and frequently caused the loss of cell cultures due to lack of aeration by holding the cultures static. The effect of PEI on the ELISA meant that the measurement of IgM concentrations was inaccurate. It was

difficult to identify an alternative assay, as IgM does not bind to Protein A. Thin layer chromatography was considered, but was not used because of the difficulty of quantifying the results. Also, the presence of PEI bound to the IgM molecules would almost certainly affect other assay techniques as it does the ELISA.

Contamination was a particular problem with the 2 L fermenters, as well as the presence of substances toxic to the cells in the seals, septa, and other vessel components.

8. SUMMARY

A set of conclusions can be reached from the studies contained in this thesis. These have been sub-divided into the work categories, and are as follows:-

LARGE-SCALE CELL CULTURE

-- Although similar overall trends are observed for the different cell lines, the final component levels vary according to both cell line and media composition. For example, the extracellular DNA level observed at the end of a culture of cell line #1 is much higher than that seen in cell line #4 (Figures 6 and 11).

-- Harvesting profiles are similar for all cell lines provided they are treated in an identical way (ie. they are not left to stand for 24 hours before being harvested).

-- Studying a typical mass balance of a cell harvest (eg. Figure 22) the figures indicate that the centrifuge is efficient at removing most solids, although a significant amount remains in the supernatant. Unfortunately, access to samples after ultrafiltration was limited, and a measurement of the solids after this process was not possible.

In this case, limited cell lysis during centrifugation seems to have occurred, judging by the relatively unchanged levels of the components. However, the mass balances shown in Figures 25 and 26 clearly show an increase in component levels, leading to the hypothesis that cell lysis is associated with the centrifugal removal of solids. This indicates a potential application for methods such as flocculation, in an effort to reduce the g forces necessary to achieve solids removal.

Returning to Figure 22, the ultrafiltration step used to concentrate the product stream ten-fold shows some protein and DNA loss. Analysis of permeate streams from this and other cell lines has shown that there is little or no loss of components to this stream, leading to the possibility that they are adhering to the ultrafiltration membrane. The increase in IgM level shown in Figure 22 is not statistically significant.

Sterile filtration would remove any solids that remained in the product stream following centrifugation and ultrafiltration. Again, measurement of the solids before and after the step to confirm this would have been beneficial. The extracellular protein and DNA levels are unchanged by this process, although the reduction in IgM level is significant.

FLOCCULATION

-- Of the range of flocculants and conditions examined it was found that the addition of polyethylene imine to a final concentration of 0.01% v/v at pH 7.0 was the most effective method for aggregating line #2 cells. This accords well with the initial aim of the project; that is, to identify an agent that can be applied to the culture in a small amount, with minimal preparation of the culture beforehand. However, its efficiency at flocculating cells and debris is compromised by decreasing cell viability. It also binds to the product, affecting its ability to perform its designated task.

-- At this concentration, PEI accords maximum extracellular DNA and protein removal without cell lysis or excessive interference with the ELISA antibody assay.

-- Methods of proving the removal of PEI from the product stream still need to be perfected.

-- At levels lower than the concentration mentioned above, DNA can still be removed very effectively. The ability of PEI to remove DNA is unimpaired by shear or by the growth stage of the culture to which it is applied. A variant on this work has already been patented by Celltech (European Patent Application, 24th May, 1991) involving the use of an immobilised form of the polymer as a method of removing DNA. The affinity of PEI for the product would need to be reduced, or a method of reversing it developed, before such a technique could be applied commercially. It should be noted, however, that the flocculation studies undertaken were restricted to one cell line and thus one product only (IgM), and such a problem might not arise with other products.

-- Regarding the selection of a flocculant capable of aggregating all mammalian cell lines regardless of their

origin or physiological state, a greater knowledge of mammalian membrane structures is required.

8.1 Future work.

Further studies in the field of mammalian cell flocculation might usefully include the following subjects.

8.1.1 Measurement of zeta potential.

By studying the zeta potential of cells before and after polymer addition, the effectiveness of the polymers could be quantified. An aspect of flocculant screening could be undertaken in this manner.
Zeta potential is calculated from microelectrophoretic measurements (Ives, 1978). Particles are observed travelling in an electric field. The polarity is reversed and the particles are again observed. Such double readings eliminate asymmetric effects and reduce polarisation effects. The glass walls of the cell in which the measurements are carried out also have a potential relative to the liquid, but the fact that the cell is a closed environment means that the overall liquid flow is zero. Two stationary layers exist where osmotic flow cancels out to zero, and where true particle mobility can be observed.

$$\zeta = \frac{\mu u}{\varepsilon}$$

where:-
ζ = zeta potential (V)
μ = dynamic viscosity (kg m^{-1} s^{-1})
ε = static permittivity of liquid (80 x 8.85 x 10^{-12} C^2 N^{-1} m^{-2} for water.)
u = electrophoretic mobility = $\frac{V_e}{F}$ (m^2 s^{-1} V^{-1})

where:-
V_e = velocity of particle migration (m s^{-1})
F = dc voltage gradient (V m^{-1})

It has been observed, however, that the release of cellular polymers such as nucleic acids and polysaccharides can be an important factor in the flocculation of cells, and the presence of such polymers on the surface of the cells may affect their electrophoretic mobility. Cells with little or no mobility may not necessarily be susceptible to flocculation (McGregor & Finn, 1969).

8.1.2 Scale-down of disc-stack centrifuges for pilot studies.

The technique of scaling down large-scale centrifuges for use in pilot studies where the supply of material to be centrifuged is limited, while retaining the separation characteristics of a full-scale model, is a useful skill. It can also be used in the calculation and testing of scale-up equations. In the case of mammalian cell flocculation, such a technique would allow the use of a 30 L culture vessel, to which flocculant could be applied in a manner identical to that which would be used on a large scale, and a study made of the effects of passage through a disc-stack centrifuge. Research undertaken to date on the disc-stack centrifuge shows that, given the correct positioning of the residual discs and the addition of a cooling system, the separation characteristics for the reduced disc stack are comparable to those of the full-scale stack. Deviations occur only at the separation limit of the reduced stack, where efficiency falls (Mannweiler *et al*, 1989; Mannweiler *et al*, 1989a). The technique involves the replacement of some of the discs in the disc stack with blank inserts which have no channels through which the liquid stream could flow, effectively reducing the separation area. To best approximate ideal conditions, the active discs in a reduced stack are placed on top of blank inserts, rather than beneath them. This is in acknowledgement of the fact that settling of solids occurs to some extent in the sediment holding space, and this particular arrangement, as in a fully-active stack, minimises such settling. A good compromise is to place the discs midway in the stack, between blank inserts, as this causes a slight underestimation of the

performance of a full-scale stack. Such caution during process development and scale-up is always advisable.
A disc-stack centrifuge can be scaled down to as low as 10% of its total available separation area, and still give accurate predictions of performance at full-scale. "If the centrifuge is to be used for almost complete (>95%) particle recovery, as is normally the case, then the prediction of the required throughput will be only slightly (<1%) underestimated"...in scale-up (Mannweiler *et al*, 1989a). However, for any predictions of centrifugal efficiency it is necessary to take into account the amount of shear-related break-up of biological particles in the feed zone of a large-scale continuous centrifuge.

8.1.3 Measurement of the extent of cell lysis.

The extent of release of the cytoplasmic enzyme lactate dehydrogenase (LDH) into the medium can be measured. A standard assay kit is available, based on the following reaction:-

$$\text{Pyruvic acid} + \text{NADH} \xrightarrow{\text{LDH}} \text{lactic acid} + \text{NAD}^+$$

The conversion of NADH to NAD^+ can be followed by monitoring the absorbance of the sample at a wavelength of 340 nm and a temperature of 37°C. The extent of lysis is calculated as follows:-

$$\text{Extent of lysis} = \frac{\text{LDH activity of sample}}{\text{LDH activity after sonication}}$$

It must be assumed that all the cells are lysed by sonication. Such an assay may also be affected by changes in membrane permeability, as LDH is a cytosolic enzyme. However, it has a higher molecular weight than trypan blue (140,000 Daltons, compared with 960.8 Daltons) so a functional membrane will be less likely to allow it to leak out (Petersen *et al*, 1988). However, results show that cells do lose LDH prior to lysis

(Marc *et al*, 1990), so the technique does not correlate exactly with cell lysis.

It proved very difficult to establish a working assay capable of detecting the very low levels of LDH released in the course of a mammalian cell culture, as the standard assay kit was not designed to be used in this range. Problems were also encountered in storing the LDH standard for any length of time without degradation, and in achieving the complete lysis of mammalian cells. It was decided that, as DNA levels were being monitored (see Section 6.6), this would provide adequate backup to the calculation of cell viability by haemocytometer (see Section 6.1.4.1). It is felt, however, that this method warrants further study, as it would be interesting to compare the results with those obtained from the DNA assay, to confirm the method of release of nucleic acid in the early stages of cell cultures.

8.1.4 Measurement of ribonucleic acid.

Ribonucleic acids can be assayed by means of the orcinol reaction:-

$$\text{Pentose (eg.RNA)} + HCl_{(l)} \xrightarrow{\text{heat}} \text{furfural}$$

$$\text{Furfural} + \text{orcinol} \xrightarrow{\text{ferric chloride}} \text{green colour}$$

This can be assayed at 665 nm, using water as a blank (Plummer, 1971). However, as this assay cannot detect the low levels of RNA found in the samples generated, and given the lack of emphasis placed on the proof of removal of RNA from therapeutic products, it was decided not to assay for RNA. As with the enzyme lactate dehydrogenase, in the light of the observation of DNA release in the early stages of cell culture, it would be interesting if any future studies also monitored RNA levels.

8.1.5 Measurement of lipids.

Oku *et al* (1986) reported the aggregation of liposomes composed of phosphatidyl serine and phosphatidyl choline, and suggested that this was due to the binding of amine residues on the PEI molecules to the head groups of the phospholipids. This suggests that PEI could effectively remove at least some of the lipid contaminants from a cell culture during a flocculation step, thus protecting further purification steps from lipid fouling.

The easiest way to assay total lipids is to perform a chloroform/methanol extraction, as the definition of a lipid is a substance which is extracted into chloroform/methanol. Such assays are well-documented (Folch *et al*, 1957) but need large samples (in the Folch paper, 60 mls are used) and are reported to lose up to 15% of the lipids during the sample washing process.

An alternative method is the use of thin layer chromatography with Coomassie Brilliant Blue staining. The range that can be assayed in this way is 0.05-0.5 mg, and the amounts can be quantified using a densitometer (Nakamura *et al*, 1984). However, the samples involved in these studies do not generate enough lipid even for this assay, and the choice of standard is also difficult, given the lack of documentation on the lipid composition of the cell line being used. Assays were found for separate lipid classes (Phospholipids: Goppelt & Resch, 1984; Kolarovic & Fournier, 1986; Nagata *et al*, 1988; Cholesterol: Omodeo Salé *et al*, 1984; Yao *et al*, 1985; Phosphoglycerides and sphingolipids: Smith *et al*, 1983). However, given that the amounts and ratios of these lipid classes vary greatly within different cell types, even from the same animal (Stryer, 1981; Lehninger, 1975) and the lack of documentation on hybridoma cells, the decision of which lipid class to assay was impossible to make. If such a decision could be made, and standards identified, the use of HPLC would greatly facilitate the assay. A reverse phase column could give rapid resolution and determination of phospholipids, glycolipids and so on from a small sample.

8.1.6 Monitoring of flocculation processes.

Turbidity measurements, defined in terms of the reduction in intensity of a beam of light passing through a suspension, are frequently used to estimate solids content or monitor flocculation processes. The turbidity of a suspension depends on the number concentration and light scattering properties of the suspended particles (Gregory, 1985). It also depends on the size of the particles and thus should change measurably as particles aggregate.

Modification of such a method to monitor a flow-through cell where the measured sample is continuously renewed by flow of suspension can lead to measurable fluctuations in turbidity due to random variations in particle concentration in a suspension. The root mean square value of these fluctuations is proportional to the diameter and concentration of the particles in the suspension, but in a very different manner than direct turbidity measurements. Such measurements can give information on particle number concentration and size in monodisperse suspensions, and therefore on the degree of flocculation, without requiring information the optical properties of the particles. For heterodisperse suspensions, a sensitive qualitative indication of aggregate formation is given. The technique could also be used to monitor the break-up of aggregates in conditions of shear. "Because of the flow-through nature of the test and the very minor amount of signal processing required, on-line measurements and process control could be easily achieved. In this respect, the very wide range of [dilute] suspension concentrations that can be directly monitored ... is a considerable advantage over conventional particle counting techniques" (Gregory, 1985). Such a monitor can easily be combined with a method for mixing varying concentrations of suspension and flocculant, thus providing an effective technique for determining the optimum conditions for flocculation (Gregory, 1987a).

However, such a method is limited in its usefulness by the low particle densities that must be used to avoid multiple scattering effects and by the maximum particle size attainable

before forward scattering of light causes inaccurately reduced readings. It is also not possible to quantify the range of particle sizes present, and thus partial and total flocculation can give similar readings.

FIGURES AND TABLES.

This section contains the figures, tables and plates referred to in the text of the thesis.

Notation:
-- Where ug or um are used as units in the figures, μg and μm respectively are intended.
-- In the figures drawn with logarithmic x-axes the initial value of the axis has been set to zero, and indicates the control value.
-- The notation used for the PEI concentration on the logarithmic x-axes is as represented in the following example; 1.0E-03 indicates 1×10^{-3}, and thus 0.001% v/v PEI.
-- Plates 1 to 7 on pages 191-194 were taken at a magnification of x200, as stated in Section 6.1.3.
-- Nomenclature for the large-scale cell cultures and harvests is as follows; during the course of these studies a number of cultures and harvests for each cell line were monitored and sampled. Thus 'Cell line #1 harvest #6' indicates that the results shown were derived from the sixth studied harvest of cell line #1. Each Figure presented is derived from a discrete run or harvest.

Further points:
-- Graphs in this thesis were produced using Harvard Graphics release 2.1.2, with the exception of Figure 33, which was produced using Quattro Pro. Harvard Graphics fits trend lines according to root mean square analysis. The curves fitted by the package are not ideal; the fact that the curves do not pass through some data points is a limitation of the software, and does not indicate a lack of faith in those points.
-- The number of samples and replicates used to generate each data point in the graphs is outlined in the Materials and Methods chapter of this thesis, beginning on page 79. The number of significant figures used are derived from this.

% flocculant	Volume 1% v/v stock (μl)	Water (μl)
0	0	1000
0.0001	100*	900
0.001	1000*	0
0.01	100	900
0.1	1000	0

* The stock flocculant solution was diluted 1:100 prior to addition to these samples, to avoid the inaccuracy of working with small volumes.

Table 1. Sample preparation for flocculation studies.

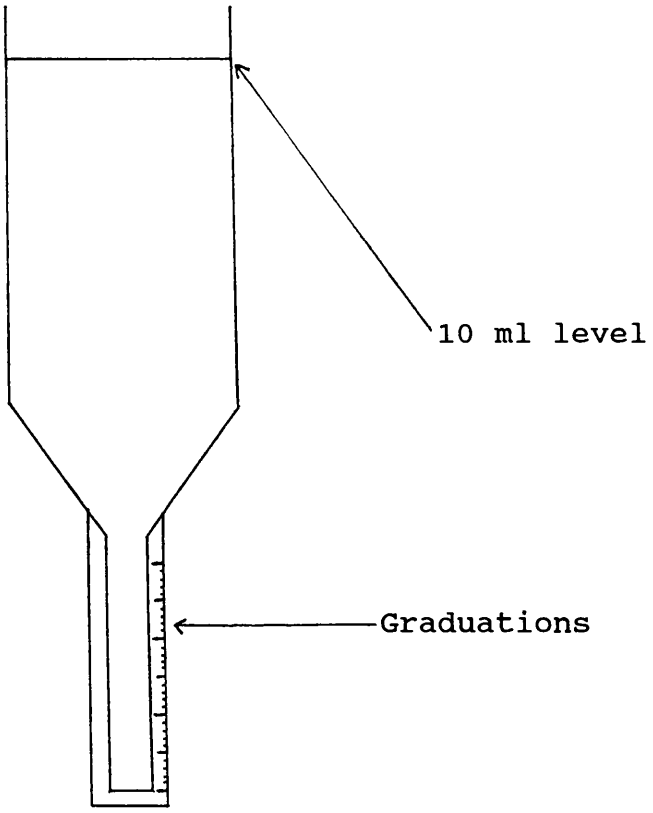


Figure 1. Solids determination tube (Westfalia Separator Ltd.), used in packed solids volume determination.

Sample	Volume of cells (ml)	Volume of 1.0% PEI (μl)	Volume of water (μl)	Length of shear tube (mm)
1	20	--	220	--
2	20	--	220	75
3	20	--	220	150
4	20	220	--	75
5	20	220	--	150
6	20	220	--	--

Table 2. Sample preparation for small-scale simulation of flocculant addition.

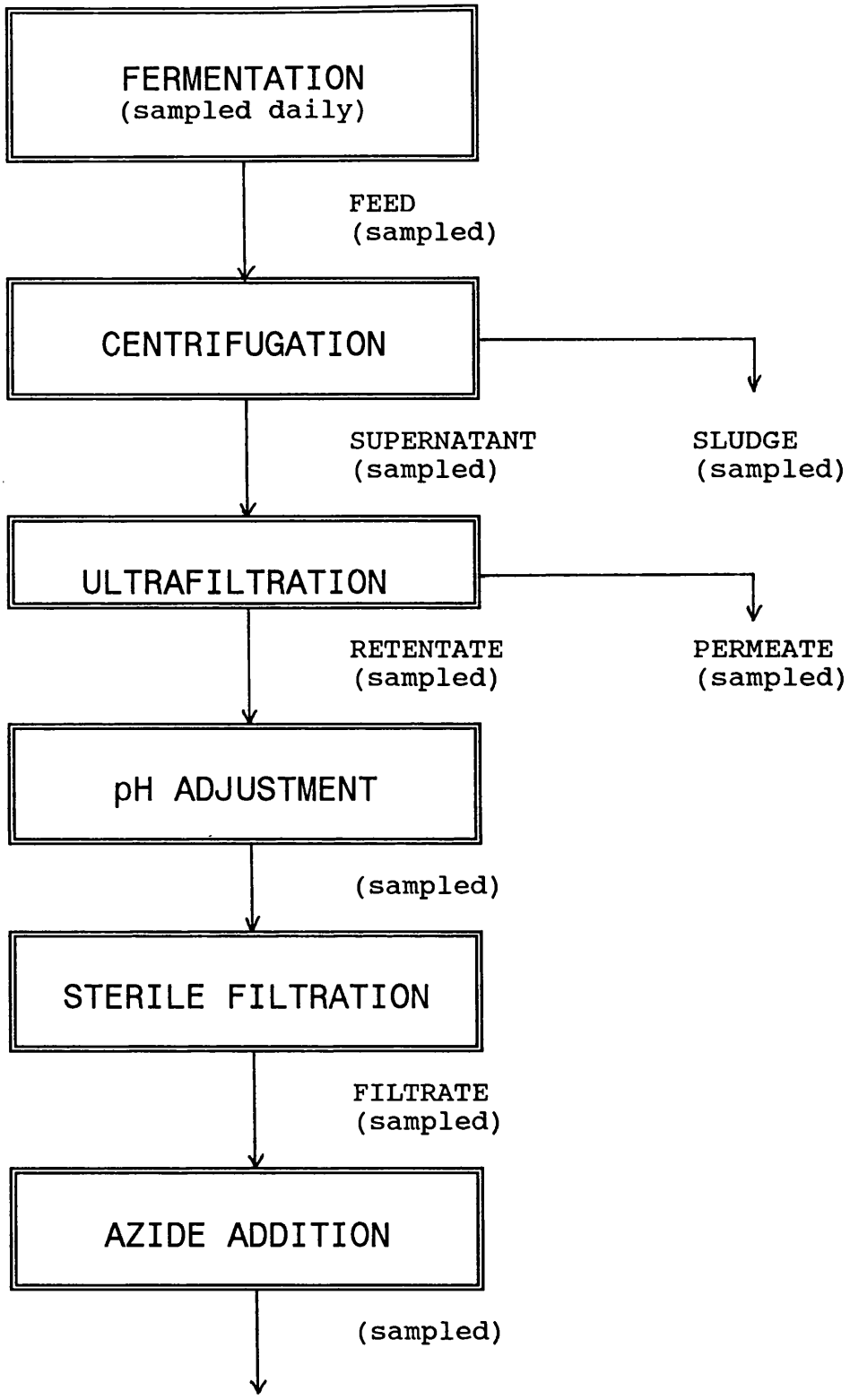


Figure 2. Early downstream processing sequence of mammalian cell cultures.

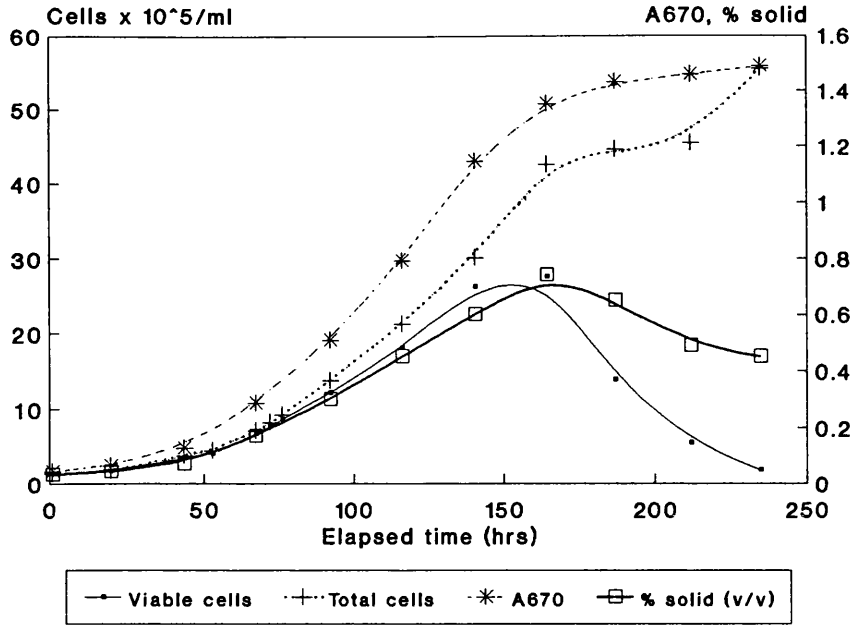


Figure 3. Profile of cell line #1 culture; run #4.

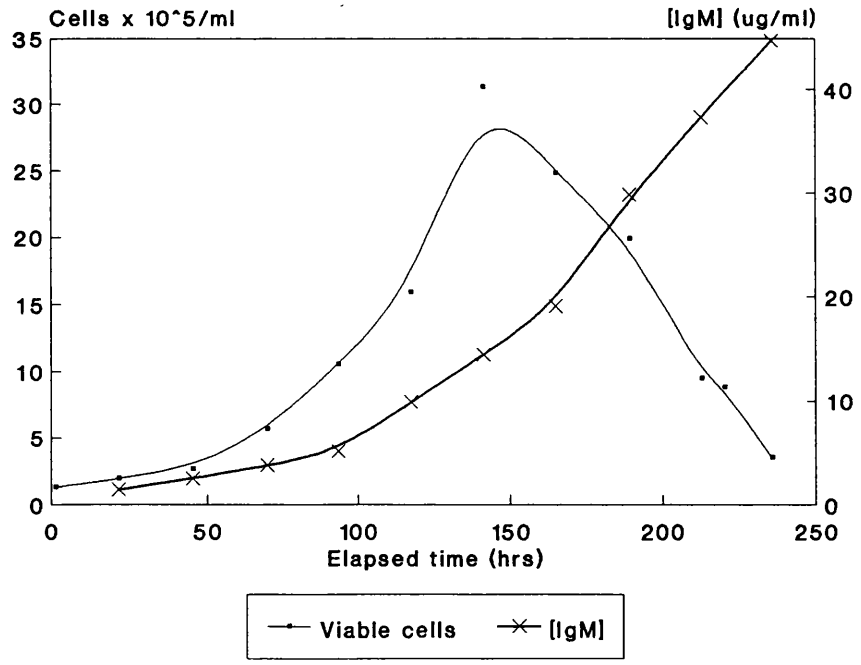


Figure 4. Profile of cell line #1 culture, run #5 showing the relationship between viable cell count and IgM level.

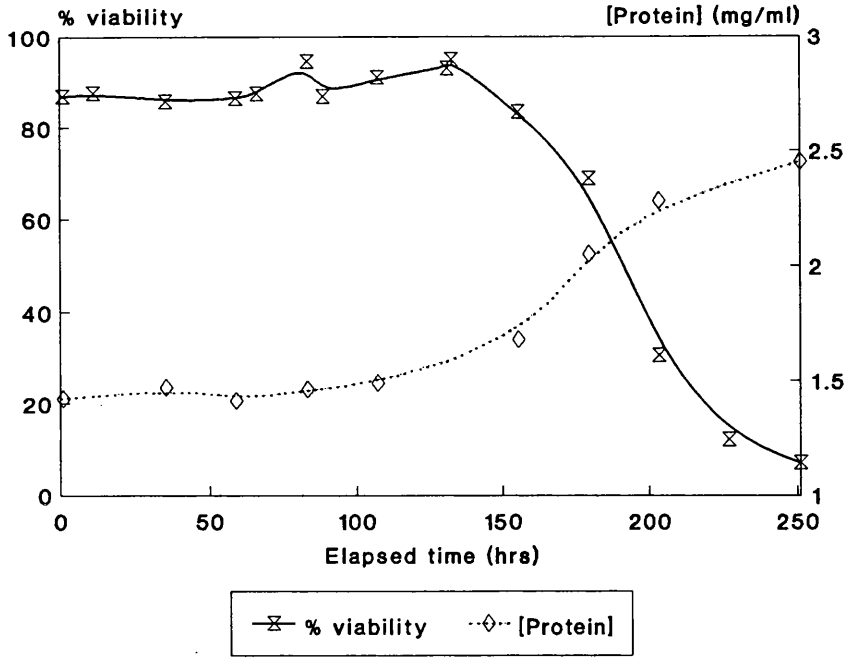


Figure 5. Profile of cell line #1 culture, run #6 showing the relationship between cell viability and protein level.

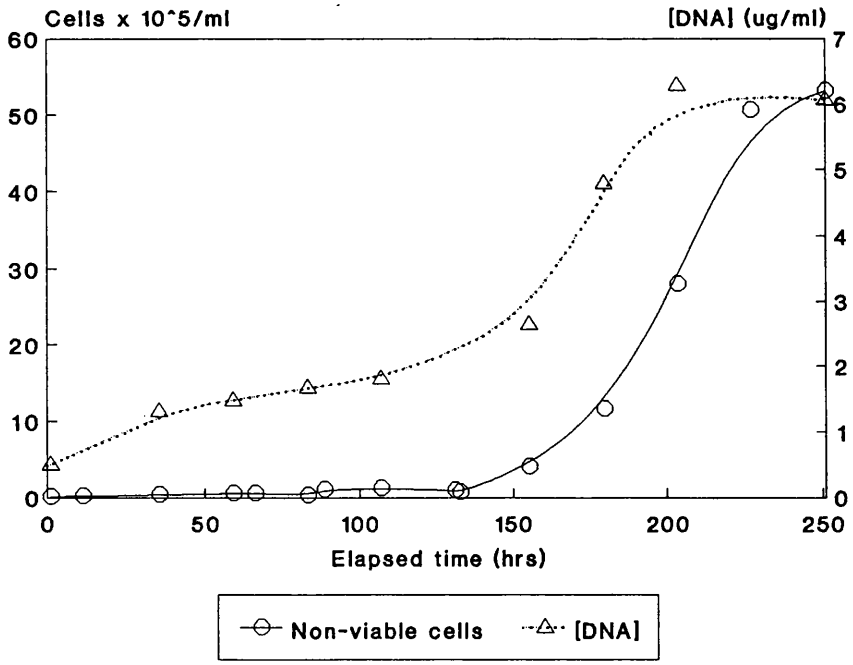


Figure 6. Profile of cell line #1 culture, run #6 showing the relationship between non-viable cells and DNA level.

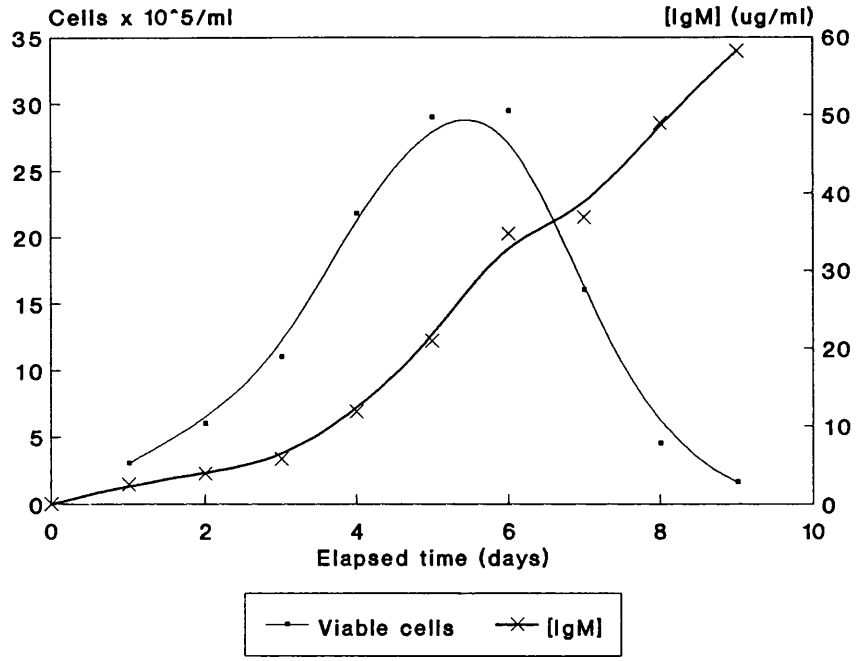


Figure 7. Profile of cell line #2 culture showing the relationship between viable cell count and IgM level.

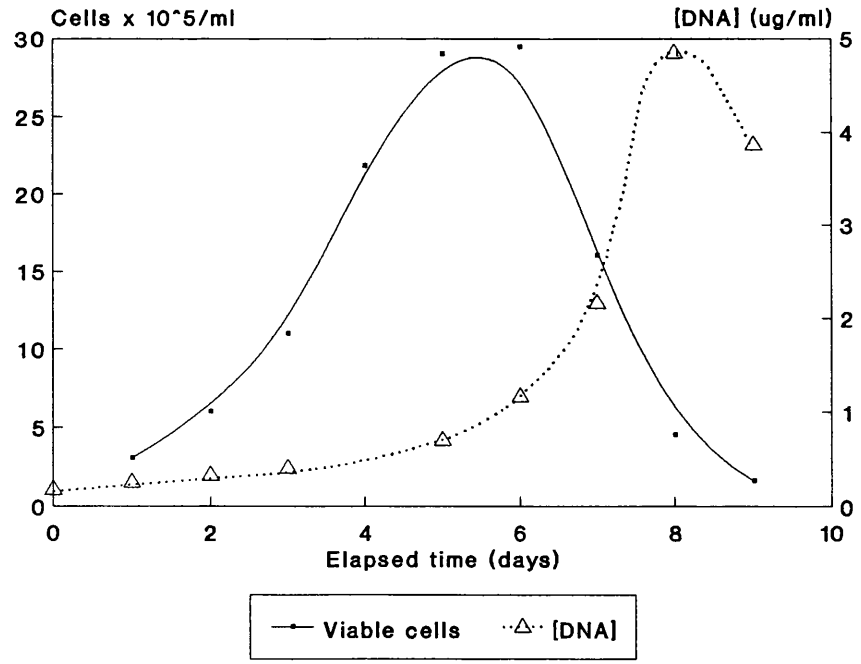


Figure 8. profile of cell line #2 culture showing the relationship between viable cell count and DNA level.

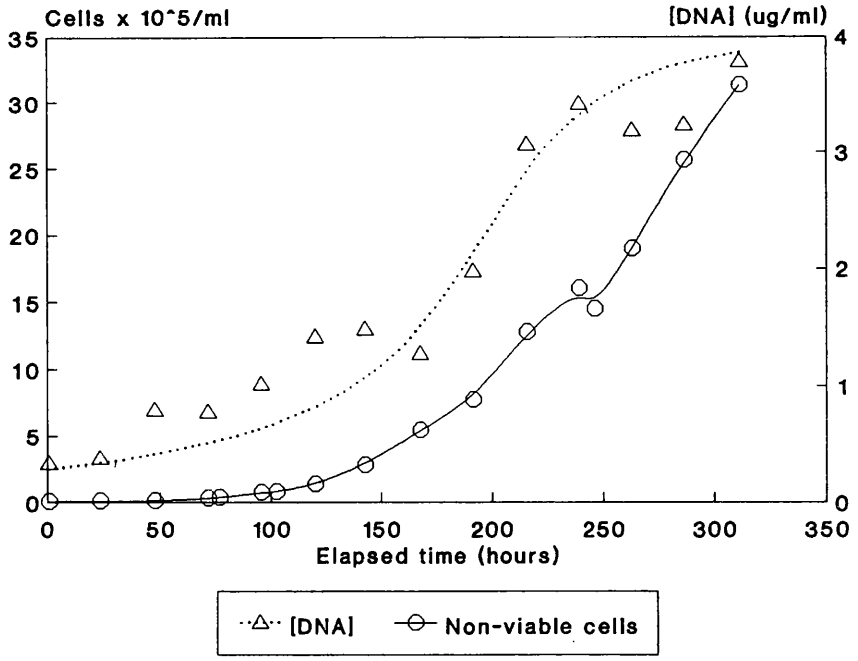


Figure 9. Profile of cell line #3 culture showing the relationship between non-viable cells and DNA level.

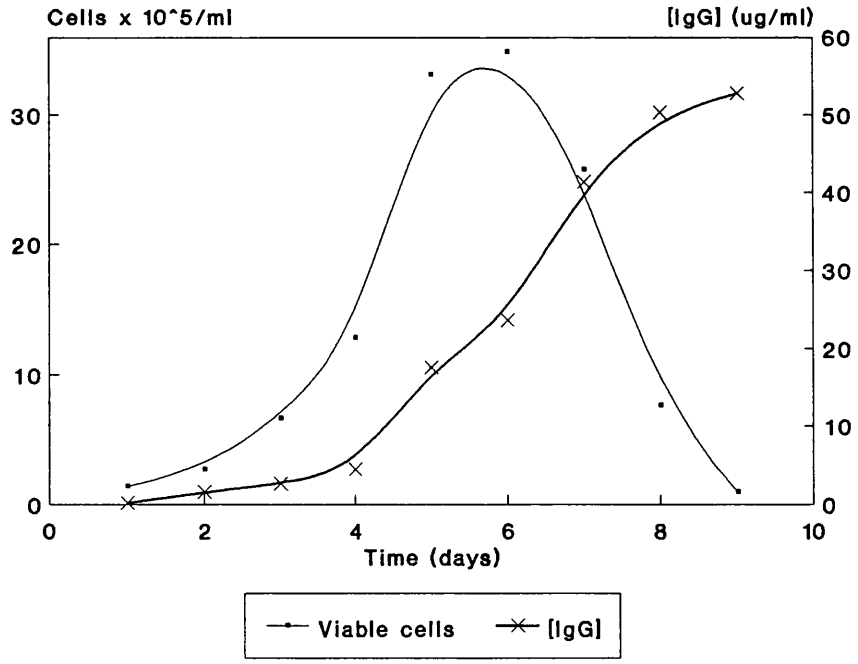
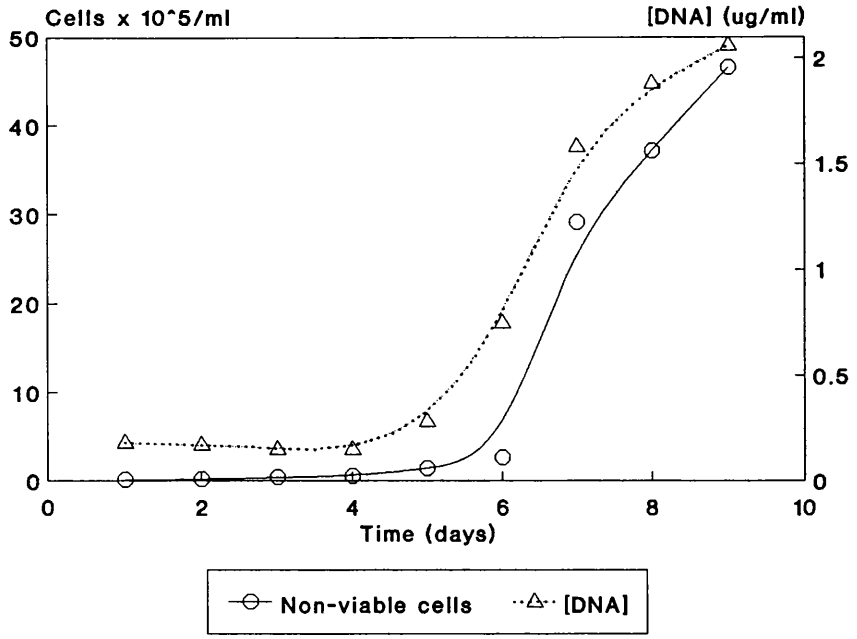


Figure 10. Profile of cell line #4 culture showing the relationship between viable cell count and IgG level.



Regression analysis: R2=95.56%

Figure 11. Profile of cell line #4 showing the relationship between non-viable cells and DNA level.

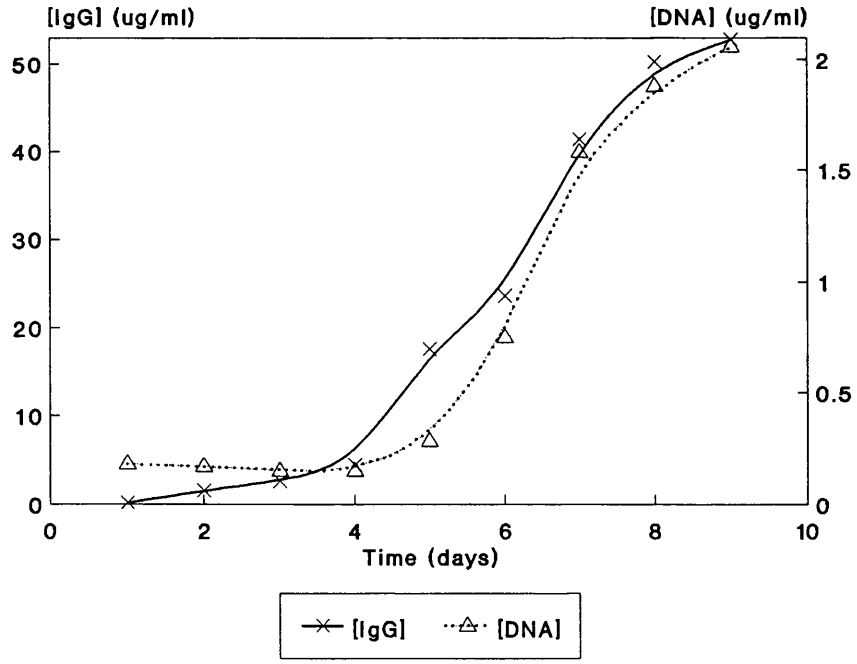


Figure 12. Profile of cell line #4 showing the relationship between IgG and DNA levels.

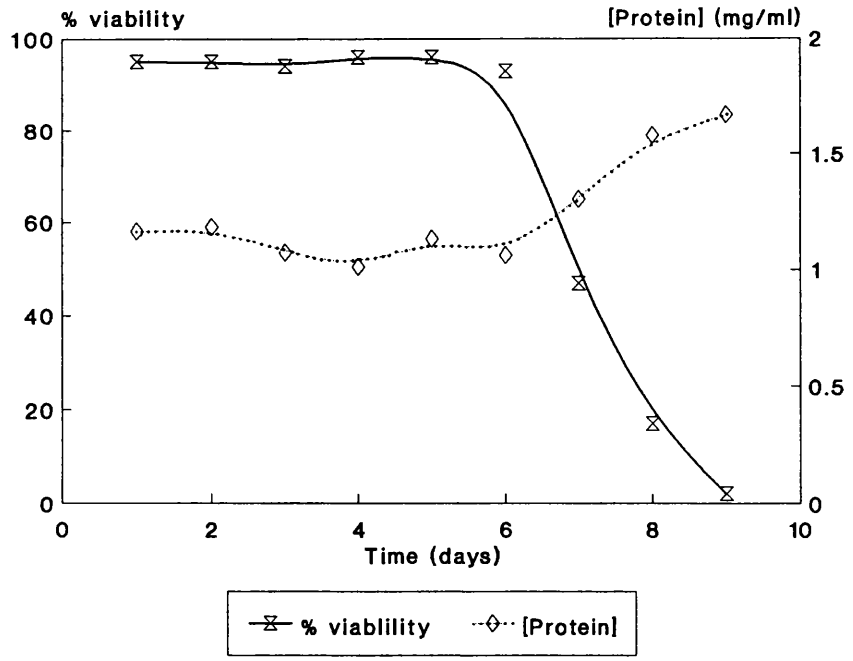


Figure 13. Profile of cell line #4 culture showing the relationship between cell viability and protein level.

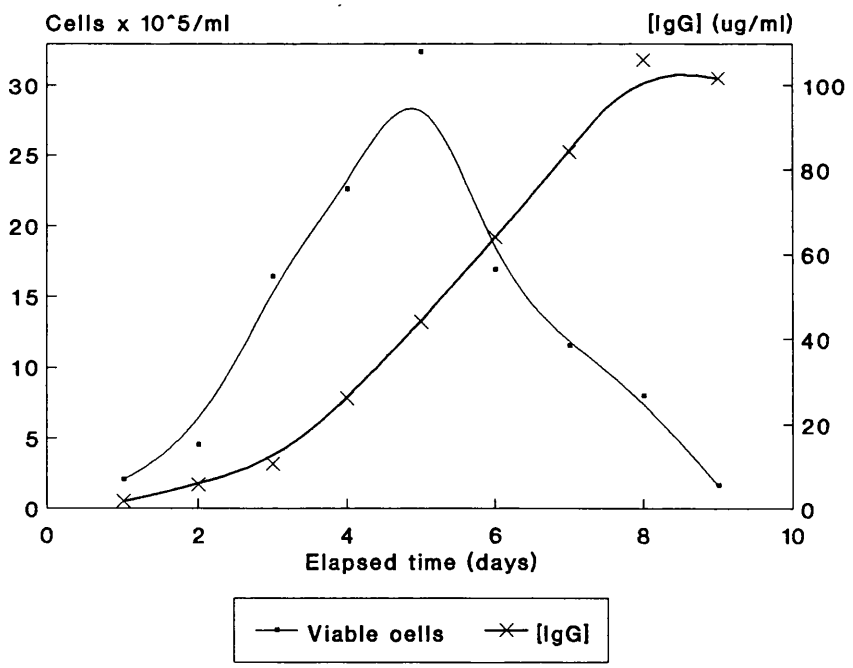


Figure 14. Profile of cell line #5 culture showing the relationship between viable cell count and IgG level.

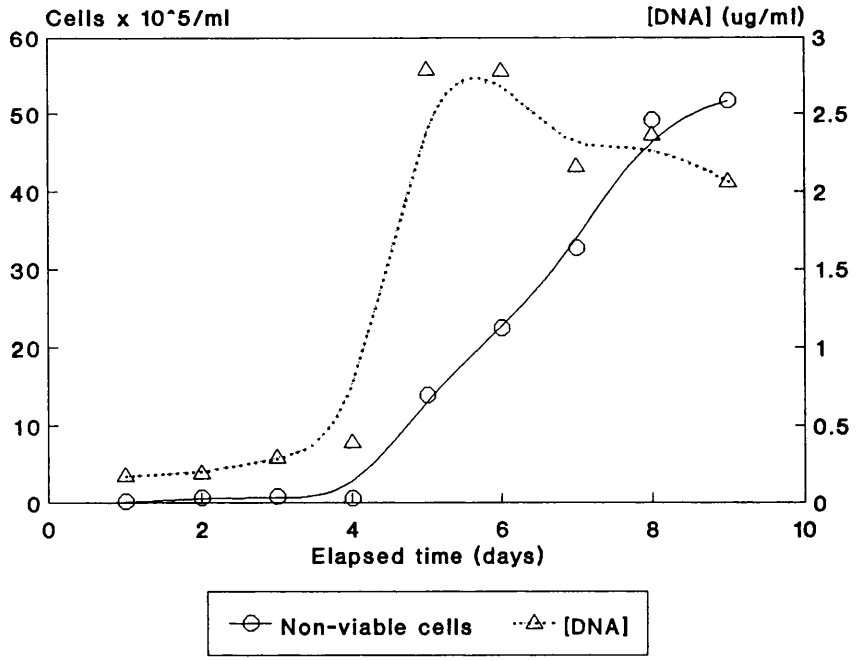


Figure 15. Profile of cell line #5 culture showing the relationship between non-viable cells and DNA level.

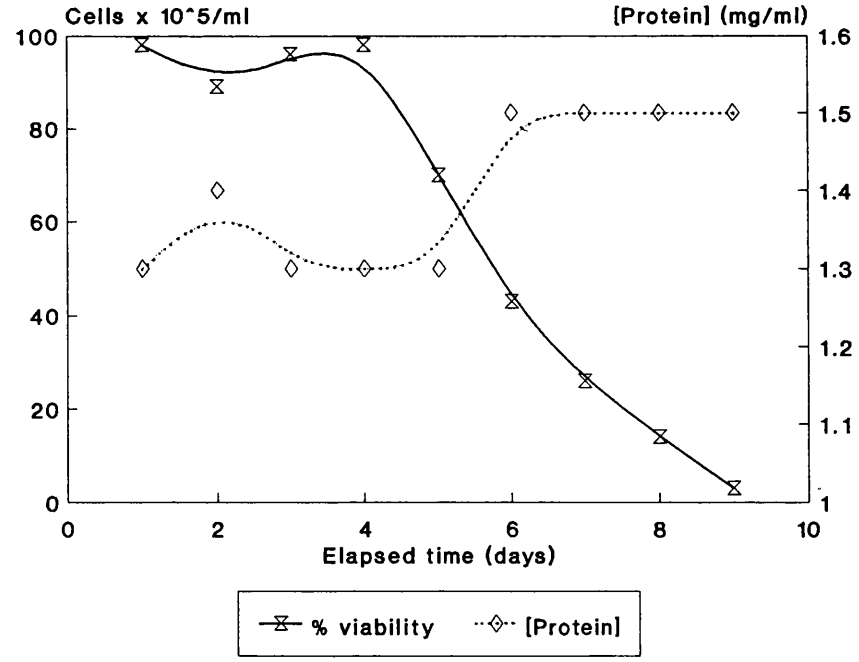


Figure 16. Profile of cell line #5 culture showing the relationship between cell viability and protein level.

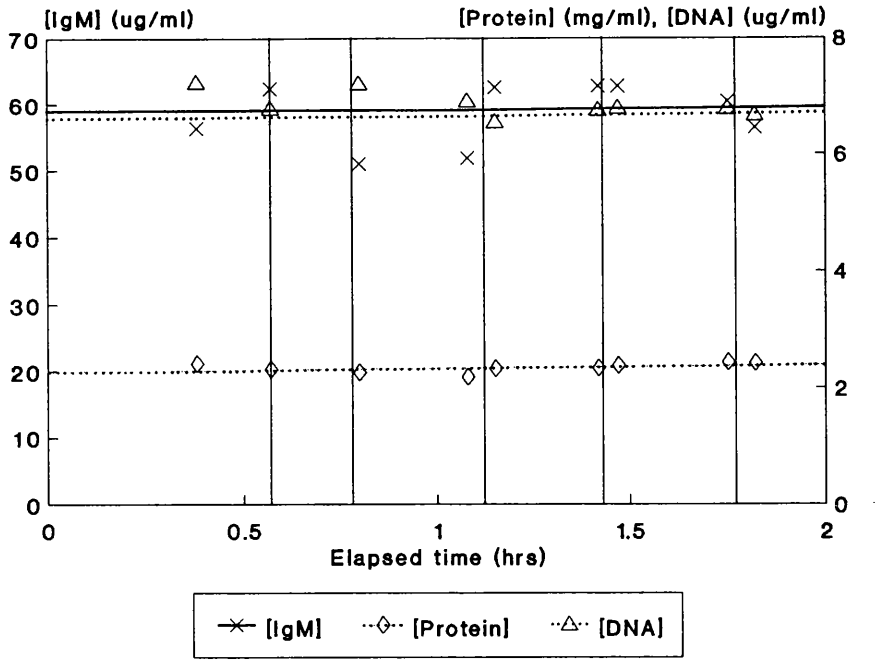


Figure 17. Profile of cell line #1 harvest #6 centrifugation showing IgM, DNA and protein levels in the feedstream.

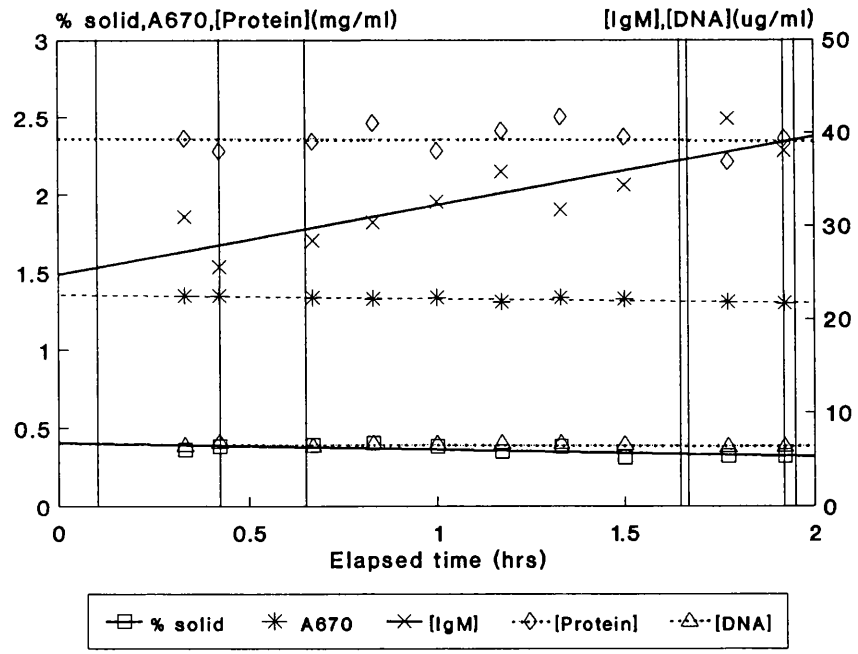


Figure 18. Profile of cell line #1 harvest #3 centrifugation showing attributes of the feedstream.

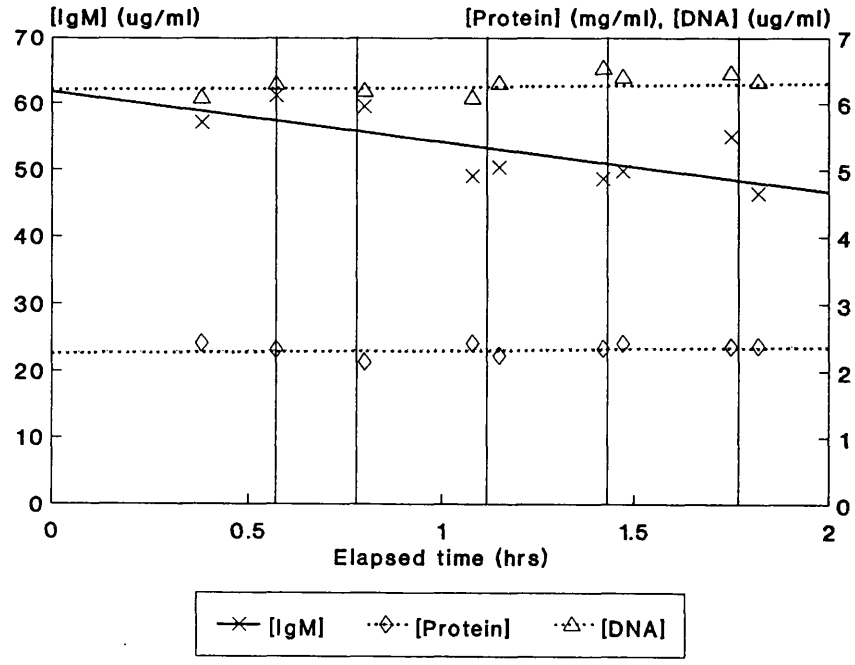


Figure 19. Profile of cell line #1 harvest #6 centrifugation showing IgM, DNA and protein levels in the supernatant.

DNA LEVELS PRIOR TO LYSIS.

Sample	[DNA] ($\mu g\ ml^{-1}$)	
	Feed	Supernatant
1	7.22	6.08
2	6.77	6.29
3	7.20	6.18
4	6.90	6.08
5	6.54	6.30
6	6.75	6.53
7	6.78	6.39
8	6.77	6.45
9	6.66	6.33

DNA LEVELS AFTER WASHING AND LYSIS OF CELLS.

Sample	[DNA] ($\mu g\ ml^{-1}$)	
	Feed	Supernatant
1	73.08	0.02
2	49.20	0.05
3	75.00	0.07
4	80.52	0.07
5	73.92	0.08
6	71.40	0.13
7	70.80	0.34
8	56.28	0.86
9	65.28	0.74

Table 3. Lysis of harvest samples; cell line #1.

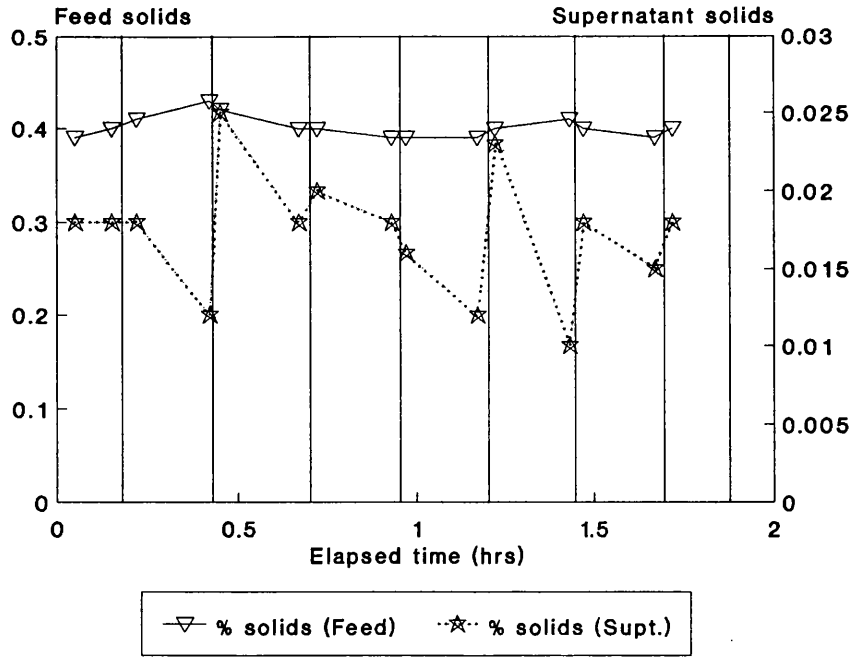


Figure 20. Profile of cell line #1 harvest #5 centrifugation comparing % solids (v/v) in feed and supernatant streams.

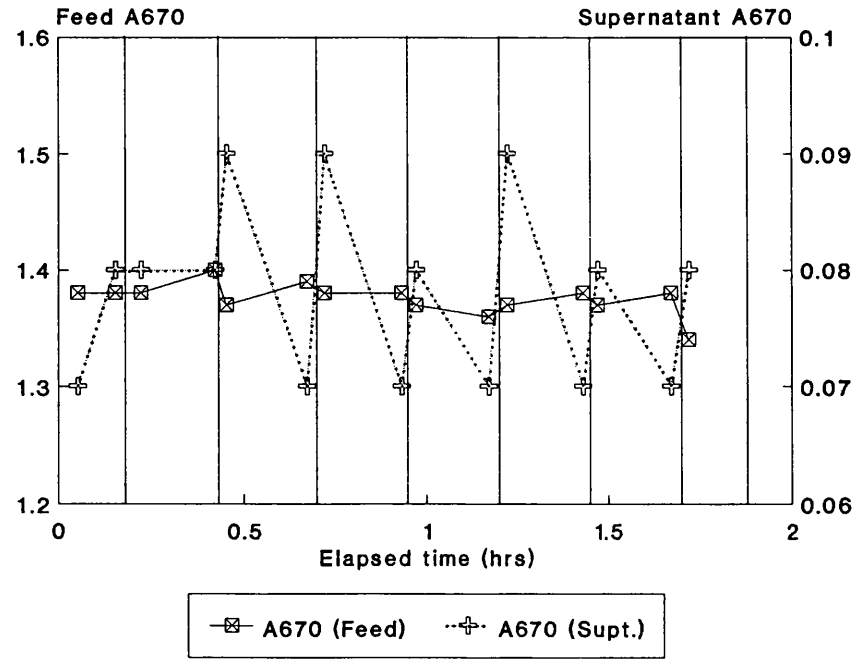


Figure 21. Profile of cell line #1 harvest #5 centrifugation comparing A670 of the feed and supernatant streams.

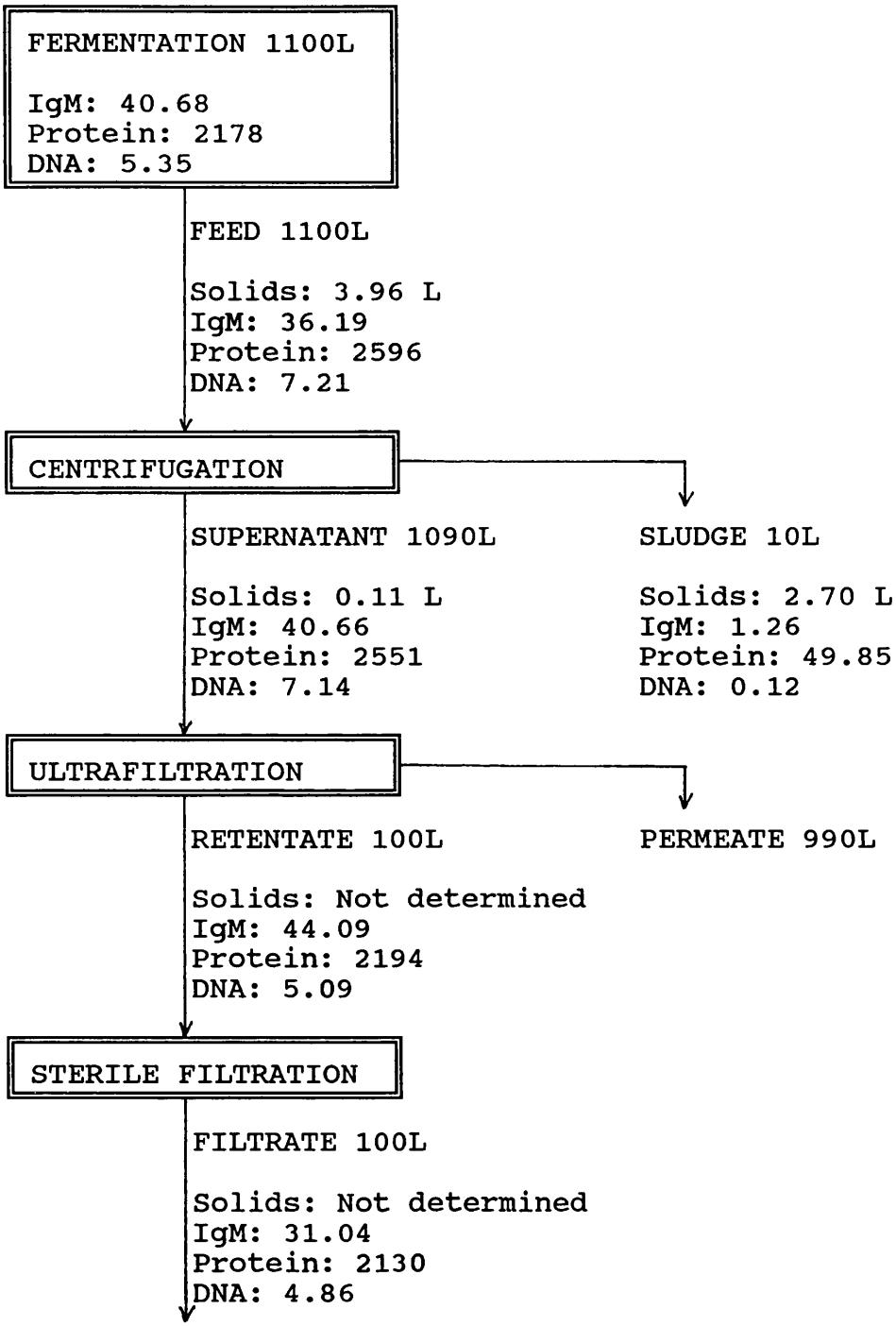


Figure 22. Mass balance of cell line #1, harvest #3.

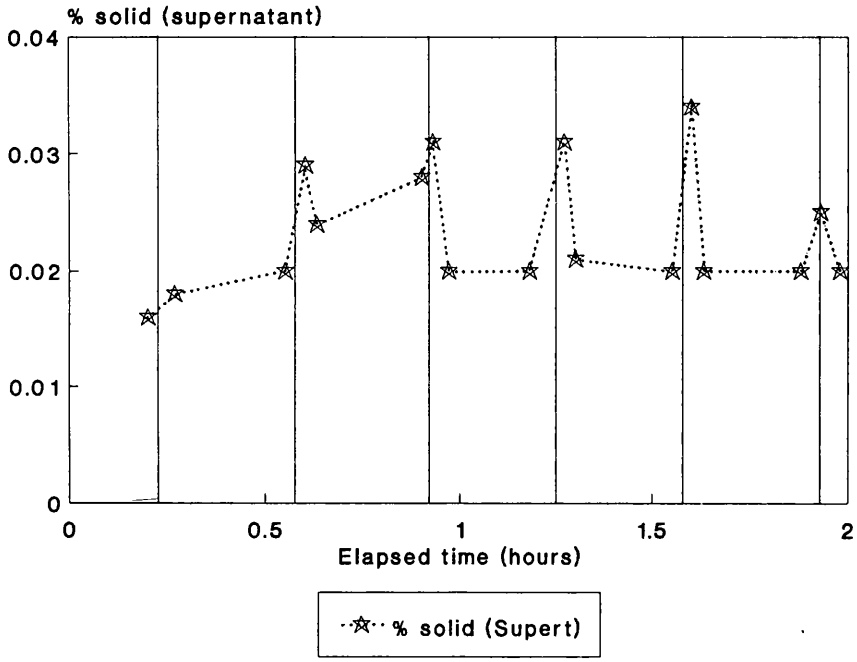


Figure 23. Profile of cell line #3 centrifugation, showing % solid (v/v) of supernatant.

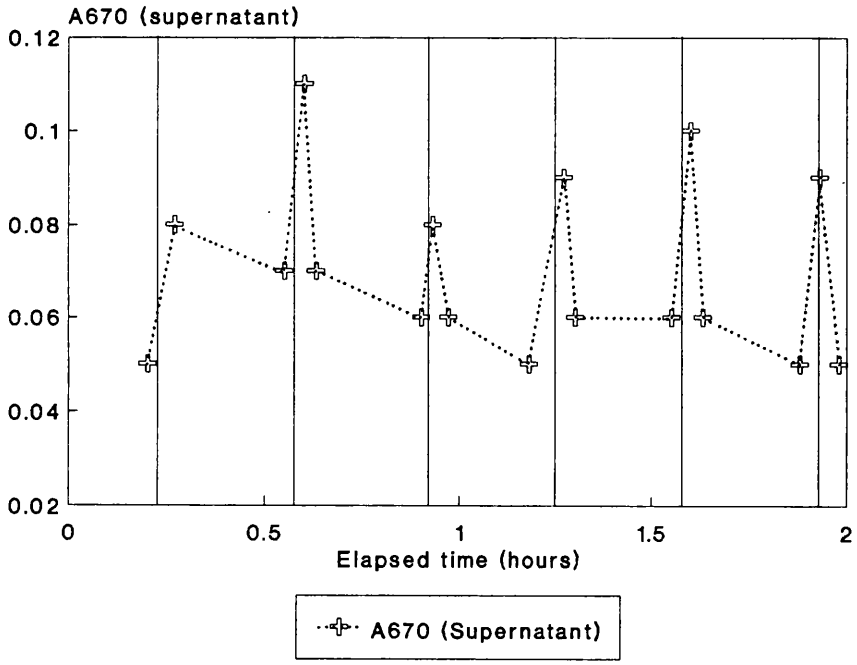


Figure 24. Profile of cell line #3 centrifugation, showing A670 of supernatant.

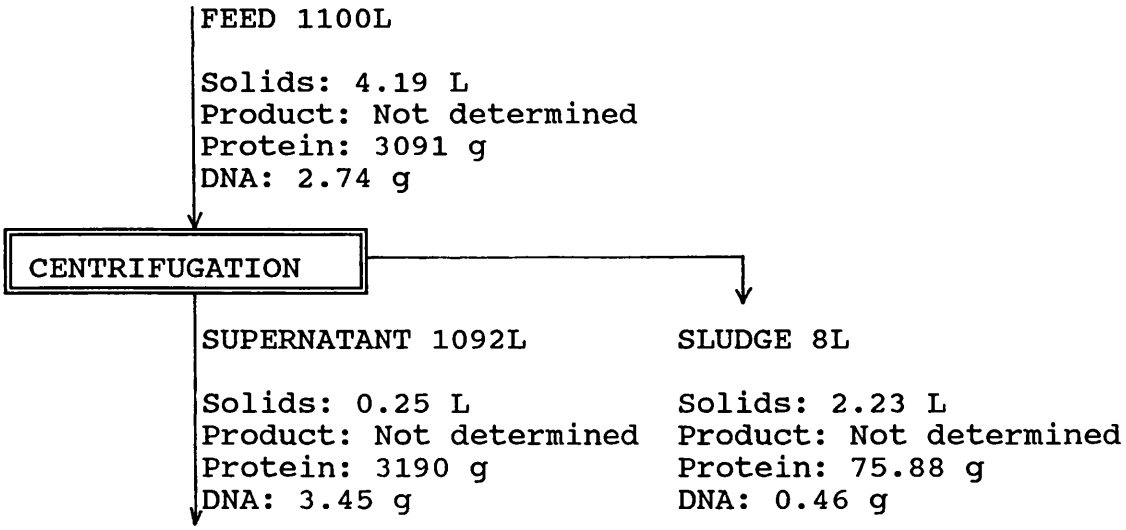
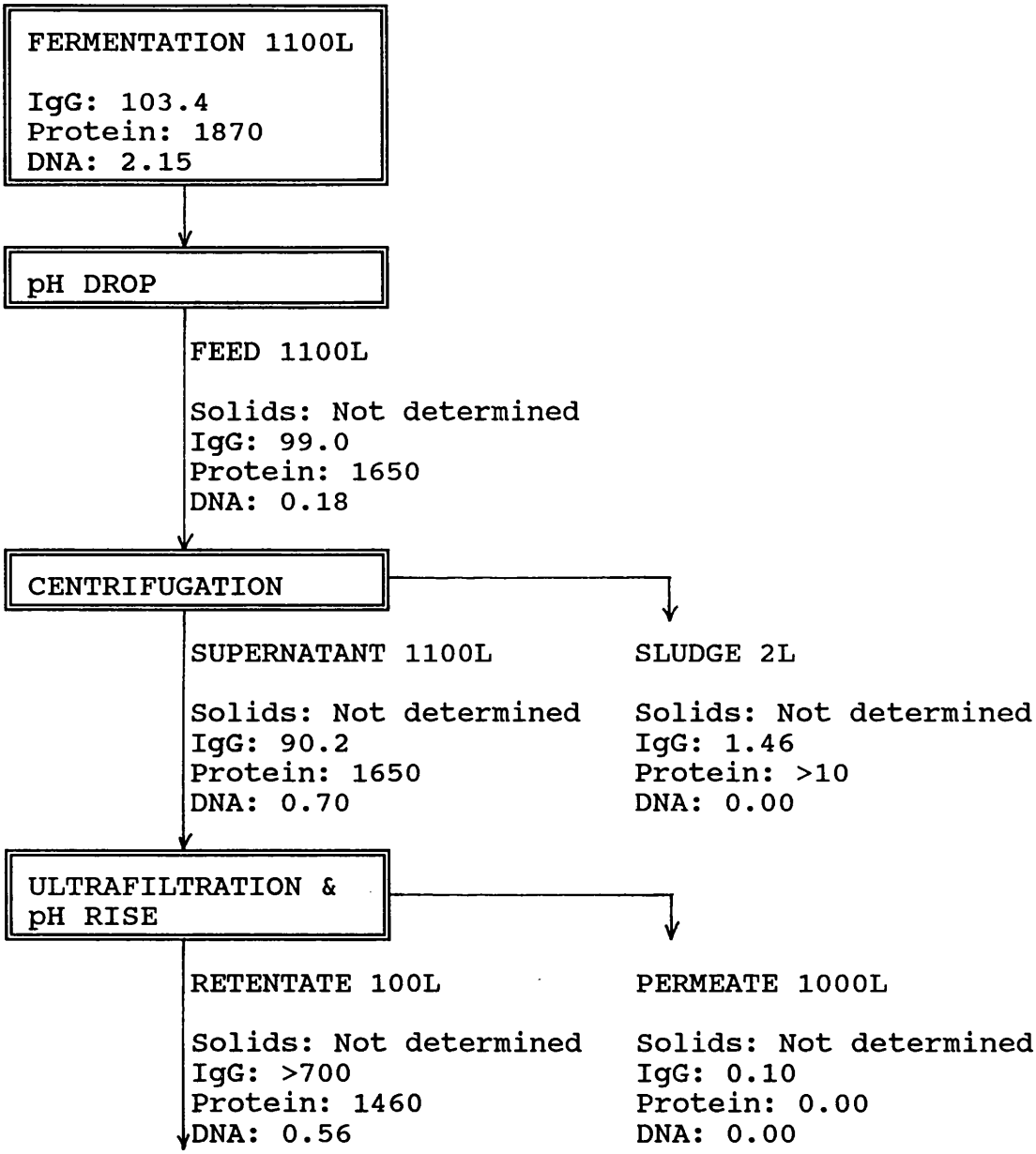
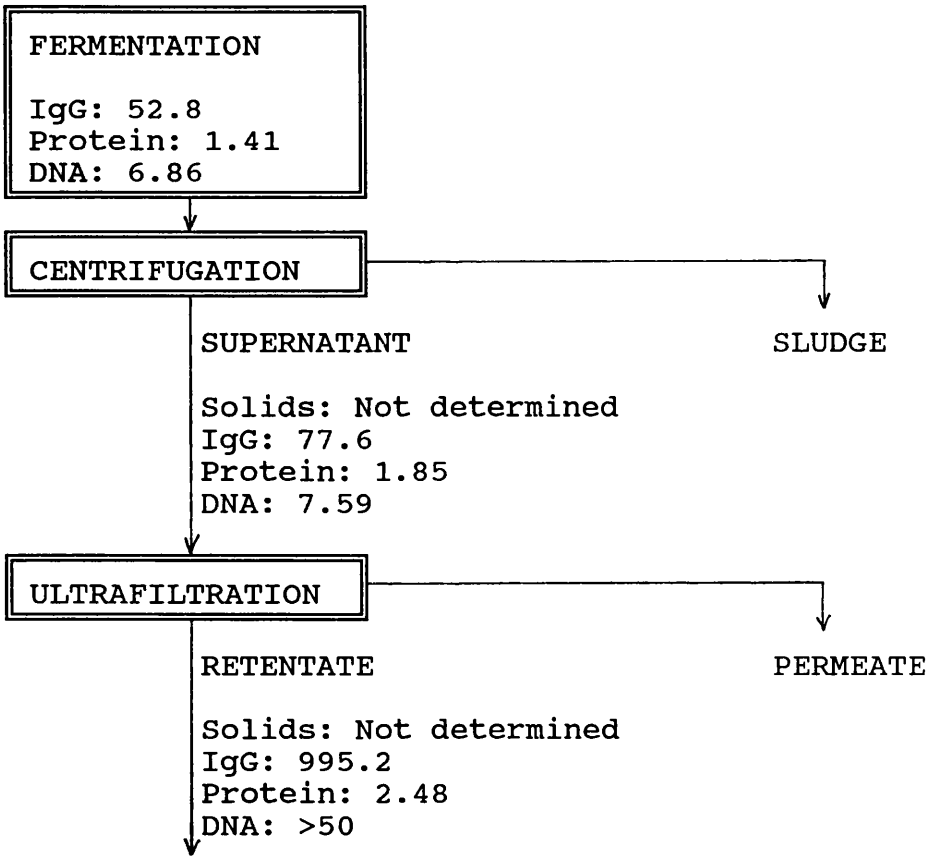


Figure 25. Mass balance of centrifugation step; cell line #3.



All units in grams.

Figure 26. Mass balance of harvest of cell line #5.



UNITS:

IgG: $\mu g\ ml^{-1}$
Protein: $mg\ ml^{-1}$
DNA: $\mu g\ ml^{-1}$

Figure 27. Profile of harvest of cell line #6.

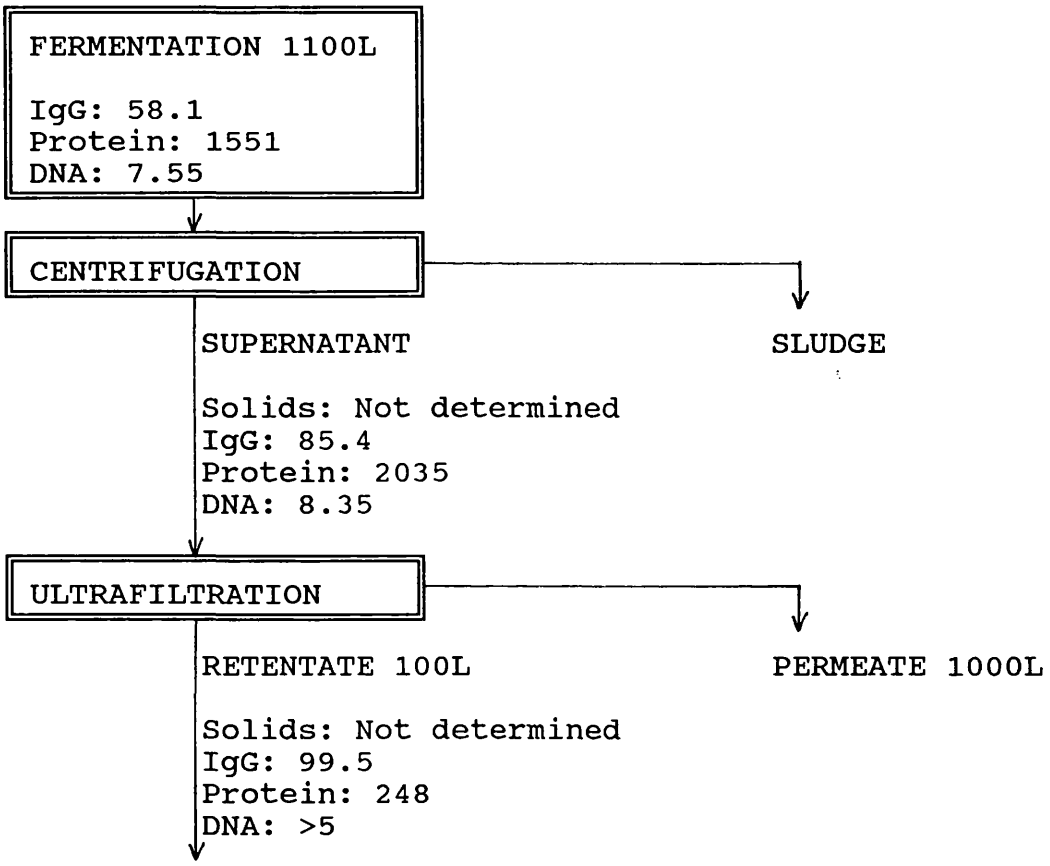


Figure 28. Mass balance of harvest of cell line #6.

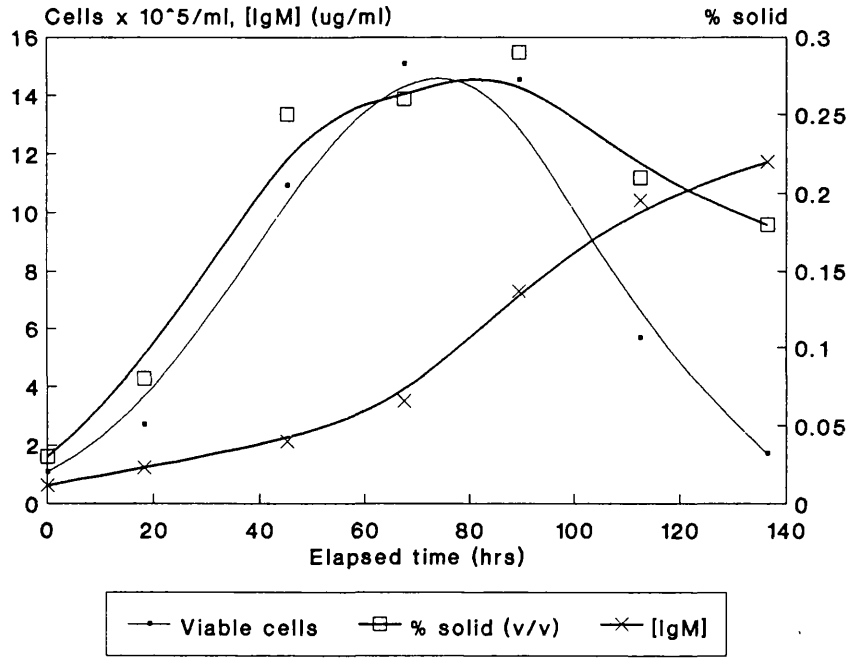


Figure 29. Profile of small-scale cell culture showing viable cell count, % solid (v/v) and IgM levels.

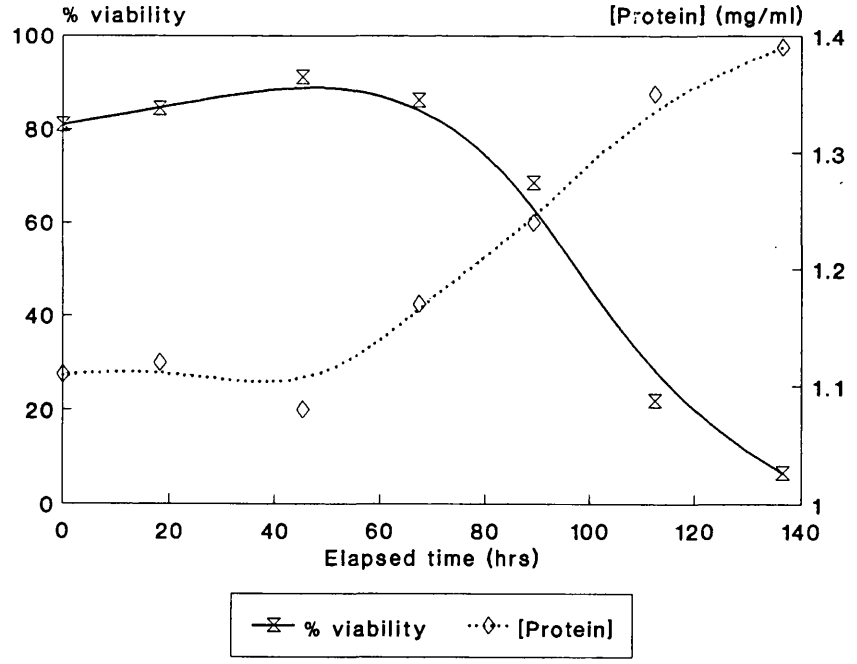


Figure 30. Profile of small-scale cell culture showing the relationship between cell viability and protein level.

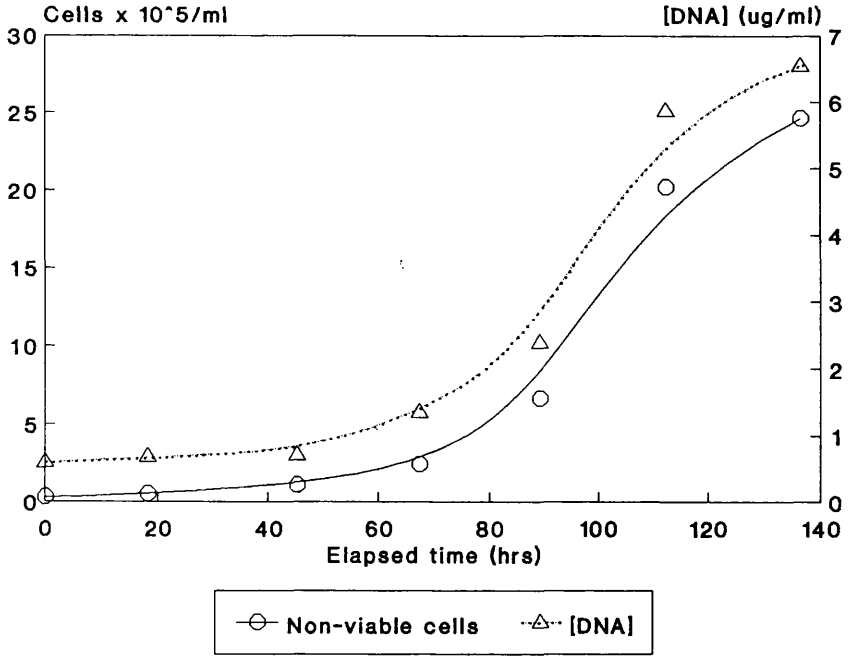


Figure 31. Profile of small-scale cell culture showing the relationship between non-viable cells and DNA level.

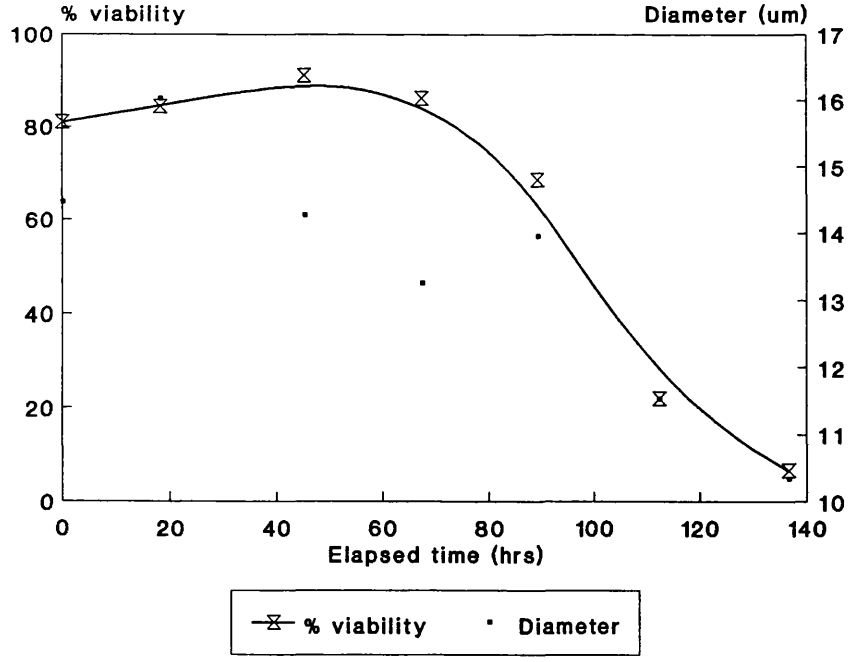


Figure 32. Profile of small-scale cell culture showing the relationship between cell viability and cell diameter.

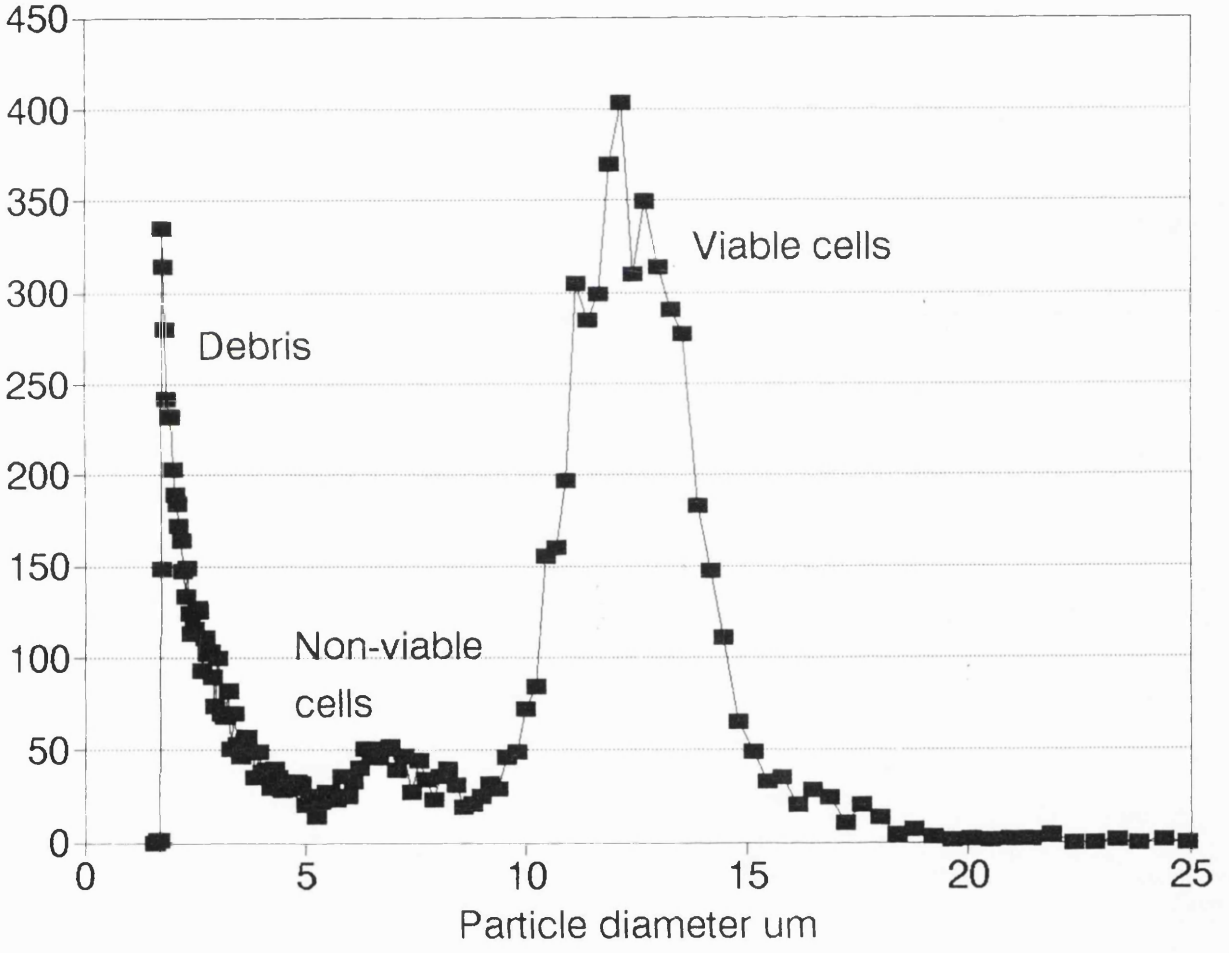


Figure 33. Profile of the particle diameter of a typical small-scale mammalian cell culture.

PARTICLE SIZING DATA.

Day	Sample volume (ml)	Volume counted (μl)	Total particle population
1	2.0	500	33292
2	1.5	500	11143
3	1.0	500	11689
4	0.5	500	11619

CELL COUNTS.

Day	Viable cells x 10^5 ml^{-1}		Non-viable cells x 10^5 ml^{-1}	
	Dye exclusion	Elzone	Dye exclusion	Elzone
1	2.03	2.05	0.14	--
2	4.50	4.58	0.43	0.83
3	9.97	9.22	1.73	0.71
4	10.43	14.07	4.64	--

Table 4. Results of particle sizing study; cell line #2.

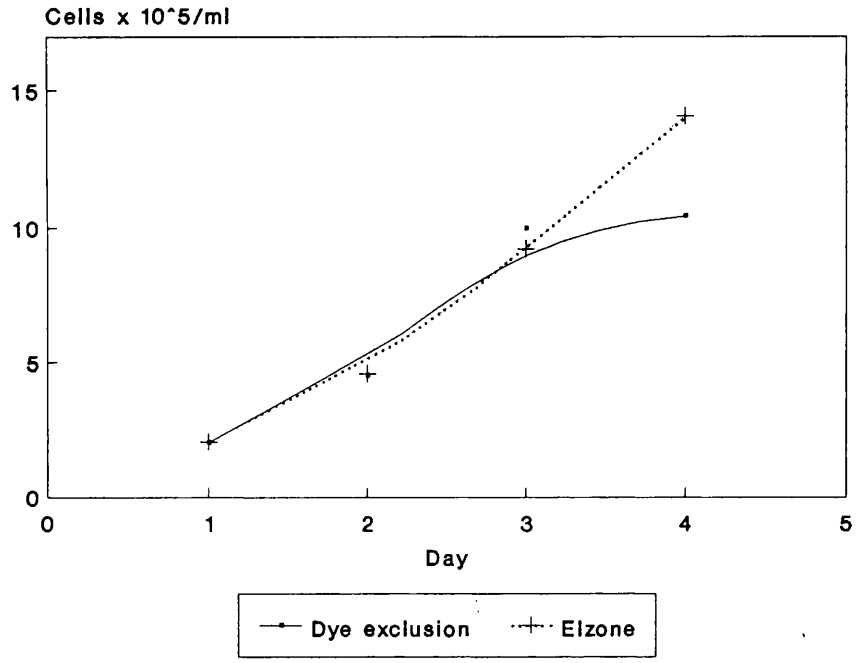


Figure 34. A comparison of viable cell counts as determined by dye exclusion and by Elzone particle sizing apparatus.

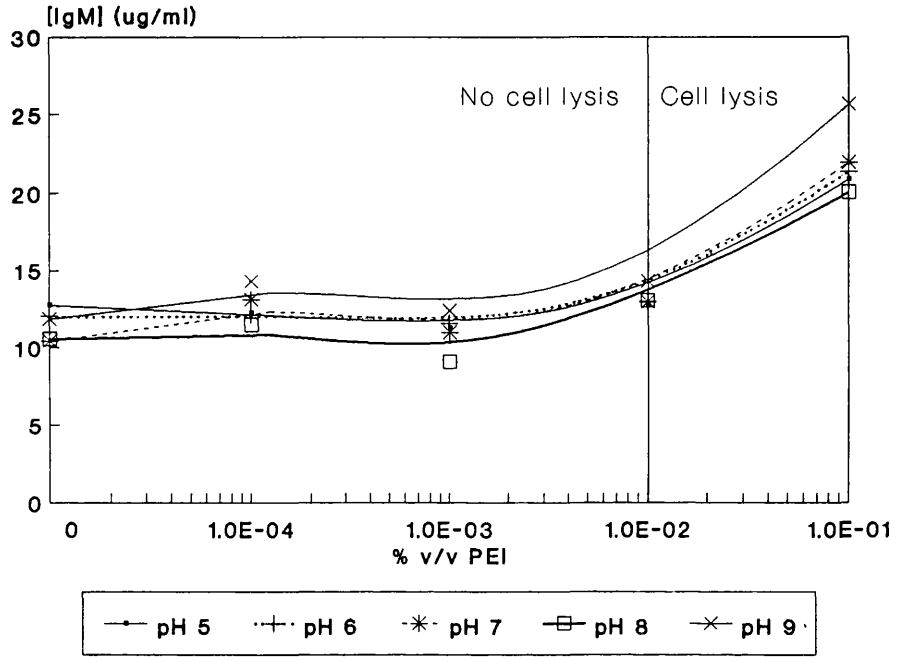


Figure 35. Profile of IgM levels over a range of PEI concentrations, under different pH conditions.

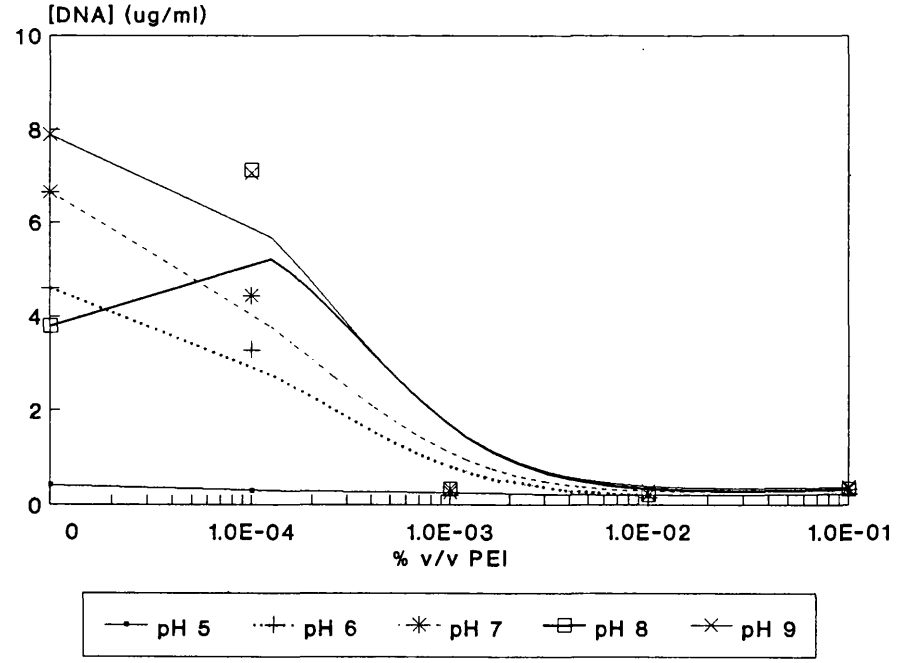


Figure 36. Profile of DNA levels over a range of PEI concentrations, under different pH conditions.

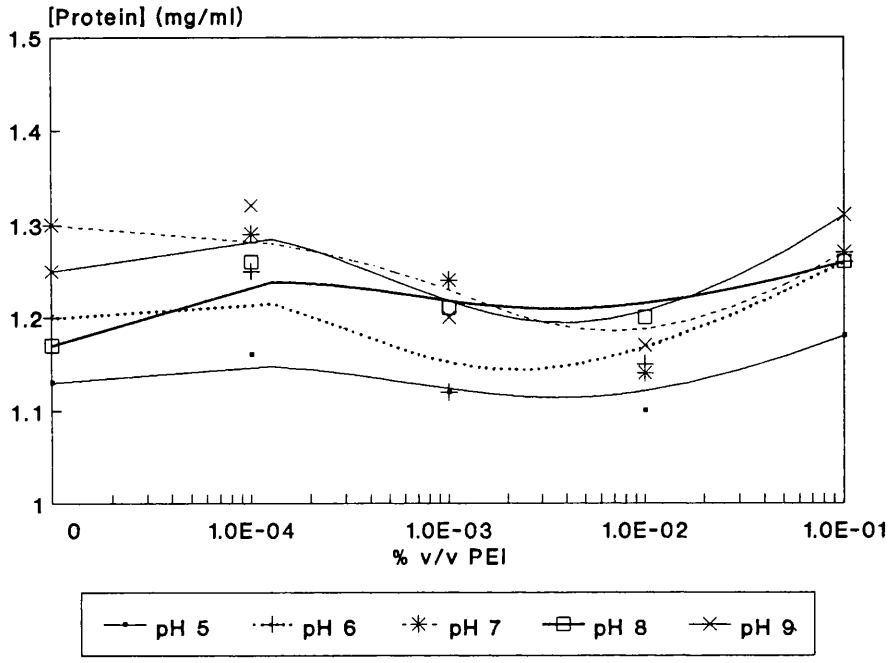
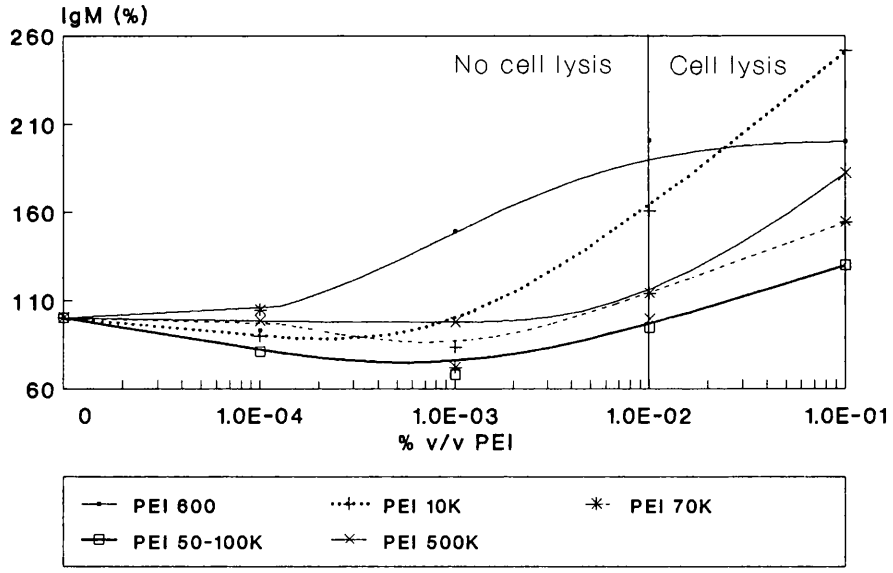
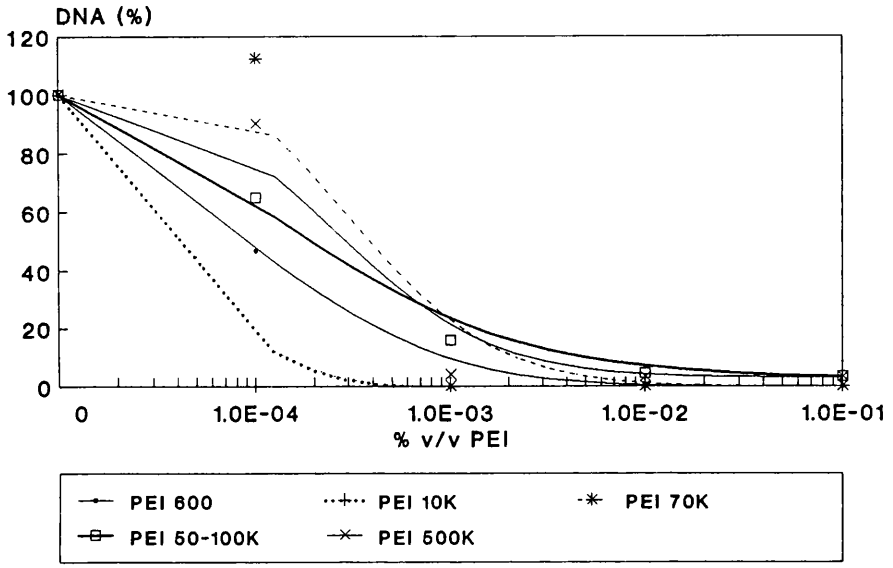


Figure 37. Profile of protein levels over a range of PEI concentrations, under different pH conditions.



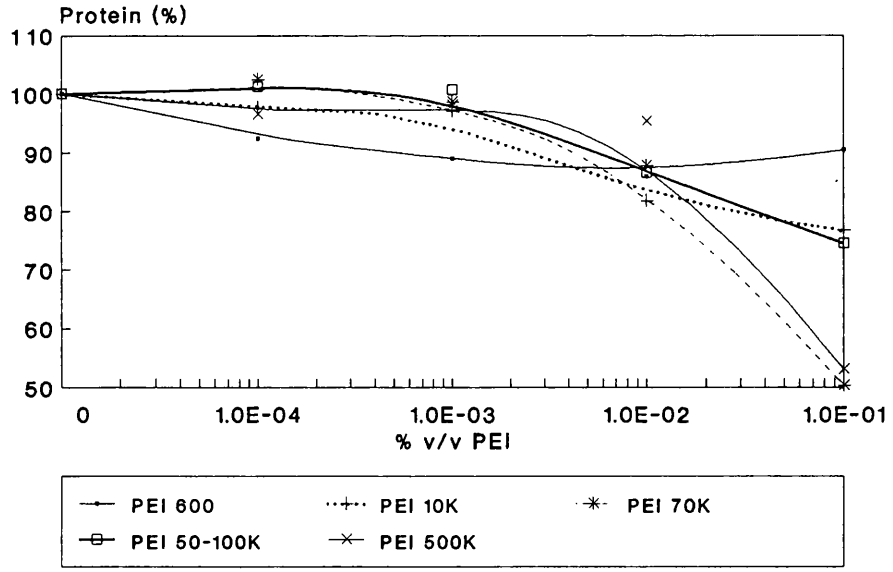
IgM levels measured as a percentage of initial level, for comparison between runs.

Figure 38. Profile of IgM levels over a range of PEI concentrations, for different PEI molecular weights.



DNA levels measured as a percentage of initial level, for comparison between runs.

Figure 39. Profile of DNA levels over a range of PEI concentrations, for different PEI molecular weights.



Protein levels measured as a percentage of initial level, for comparison between runs.

Figure 40. Profile of protein levels over a range of PEI concentrations, for different PEI molecular weights.

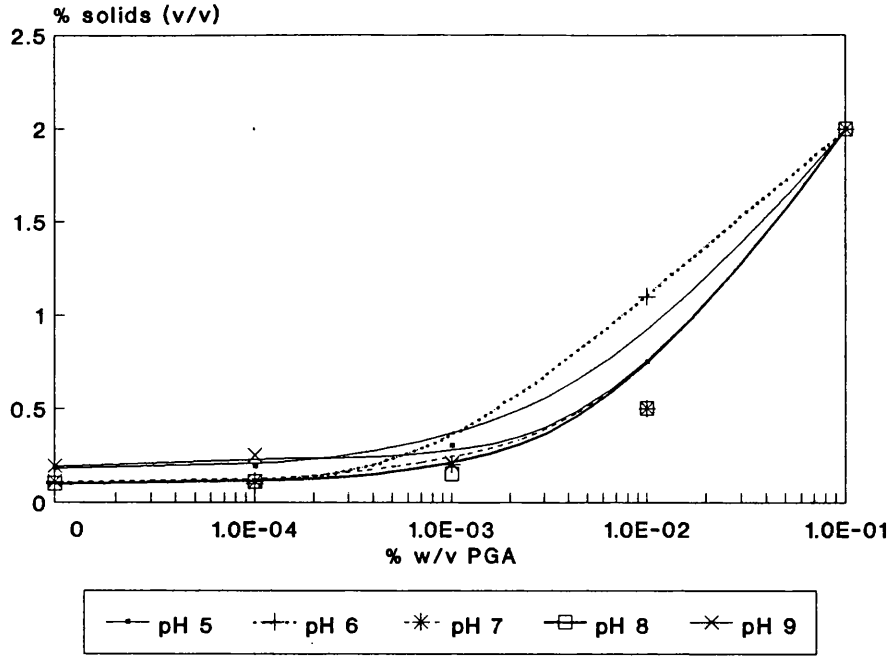


Figure 41. Profile of % solids (v/v) over a range of PGA concentrations, under different pH conditions.

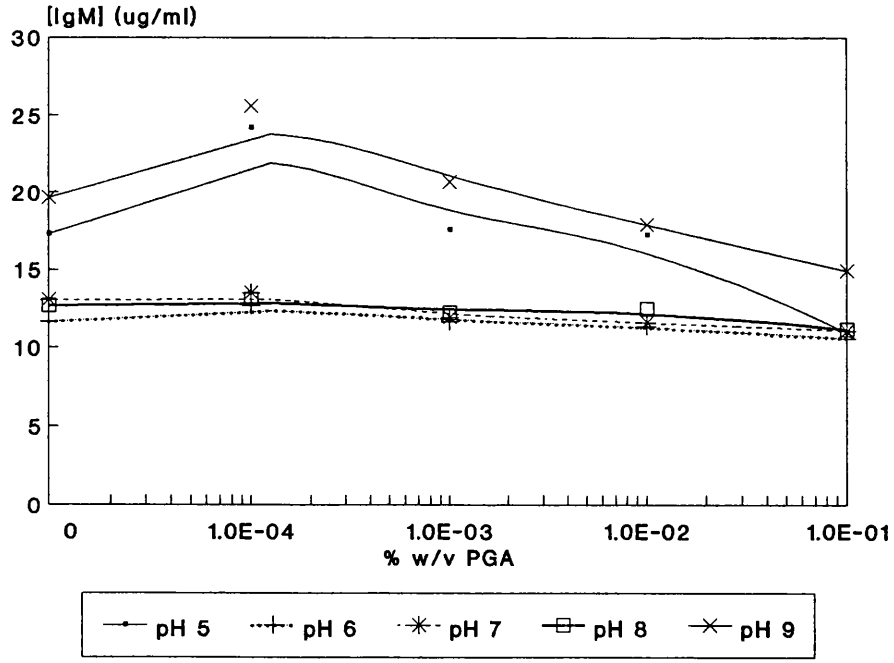


Figure 42. Profile of IgM levels over a range of PGA concentrations, under different pH conditions.

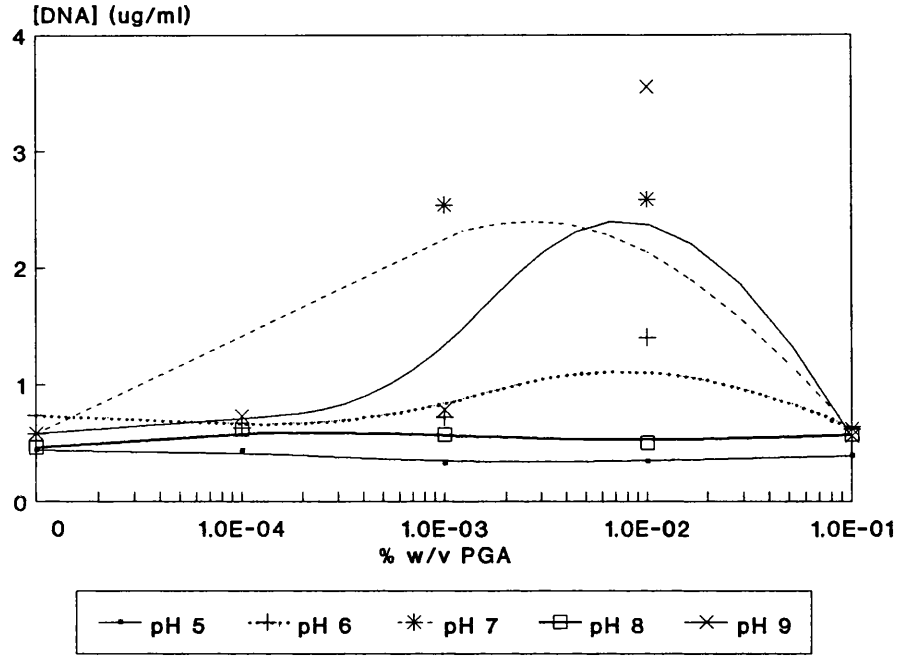


Figure 43. Profile of DNA levels over a range of PGA concentrations, under different pH conditions.

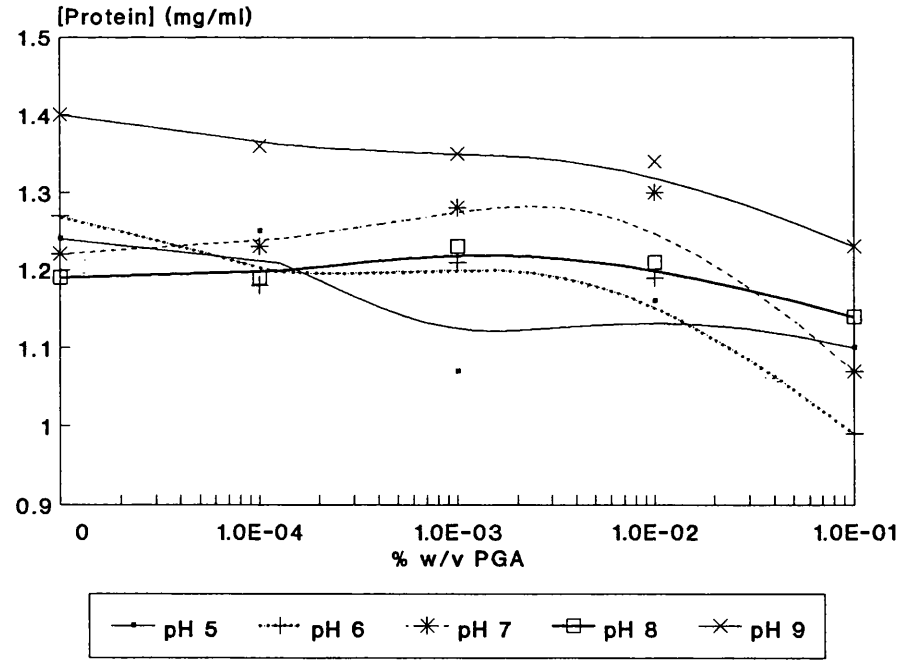


Figure 44. Profile of protein levels over a range of PGA concentrations, under different pH conditions.

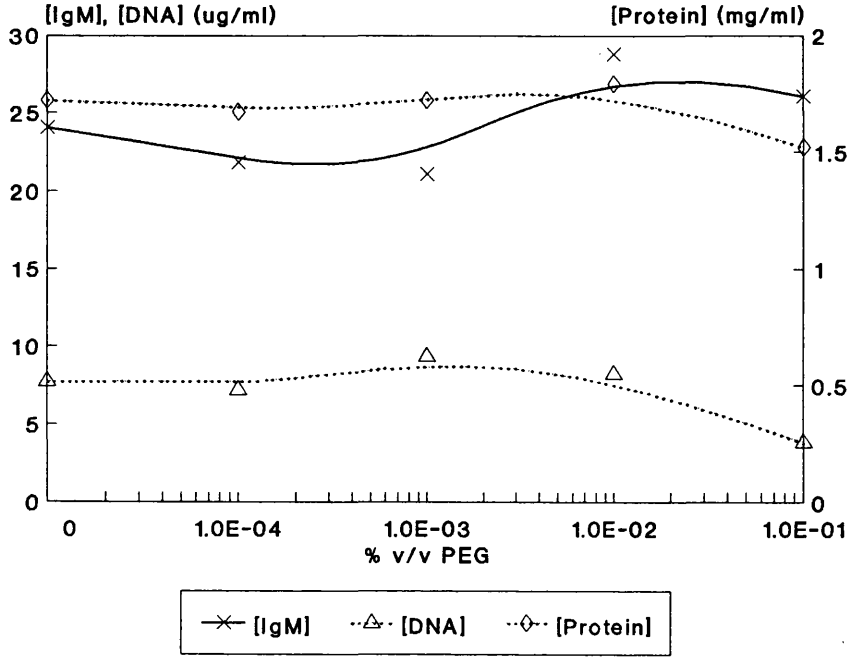


Figure 45. Profile of IgM, DNA and protein levels over a range of PEG concentrations.

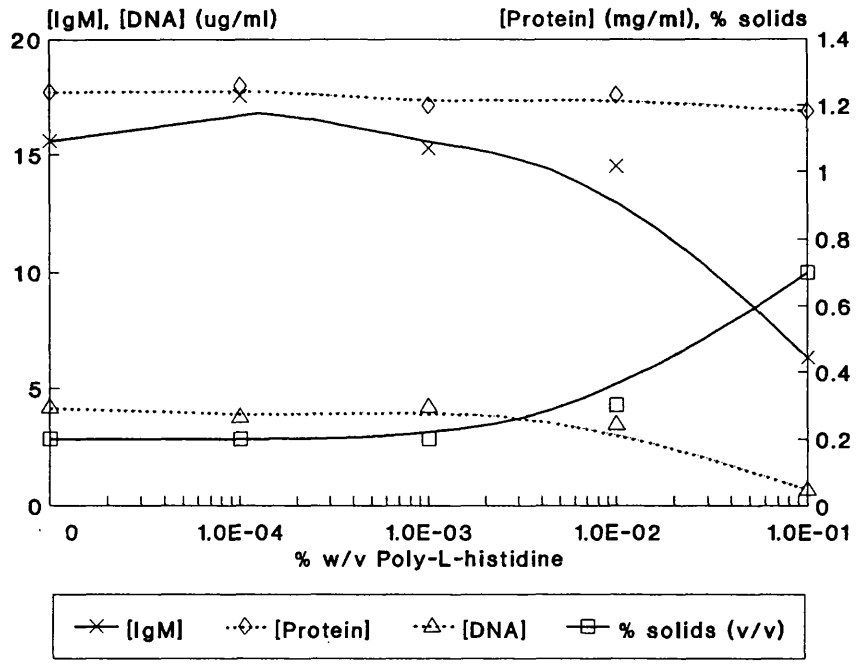


Figure 46. Profile of component levels over a range of PLH 5-15,000 concentrations.

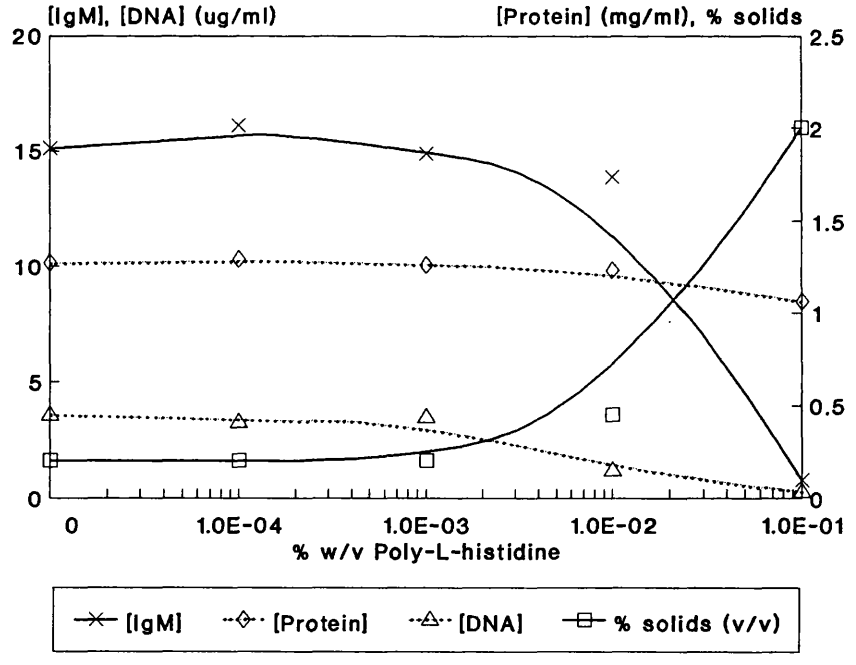


Figure 47. Profile of component levels over a range of PLH 15-50,000 concentrations.

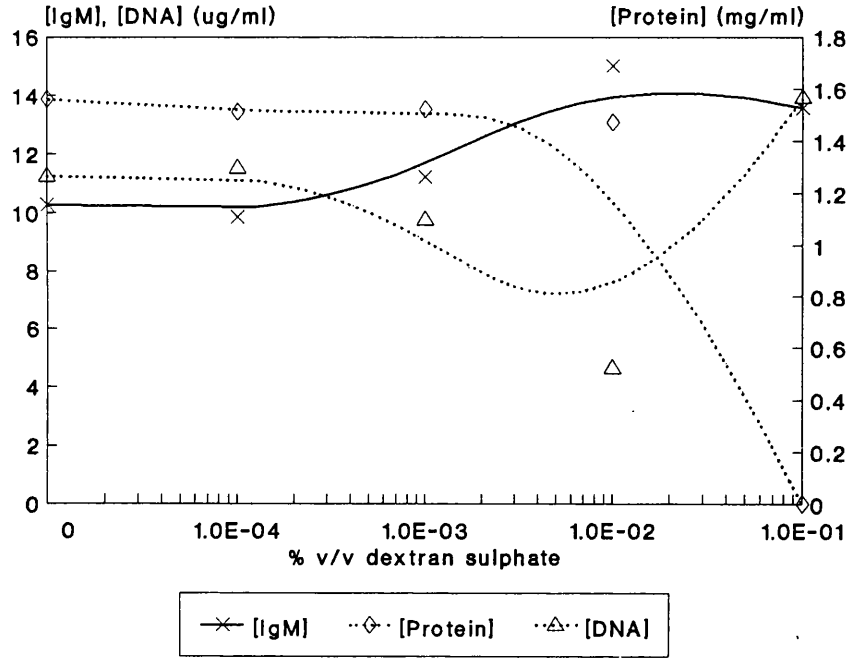


Figure 48. Profile of component levels over a range of dextran sulphate 500,000 concentrations.

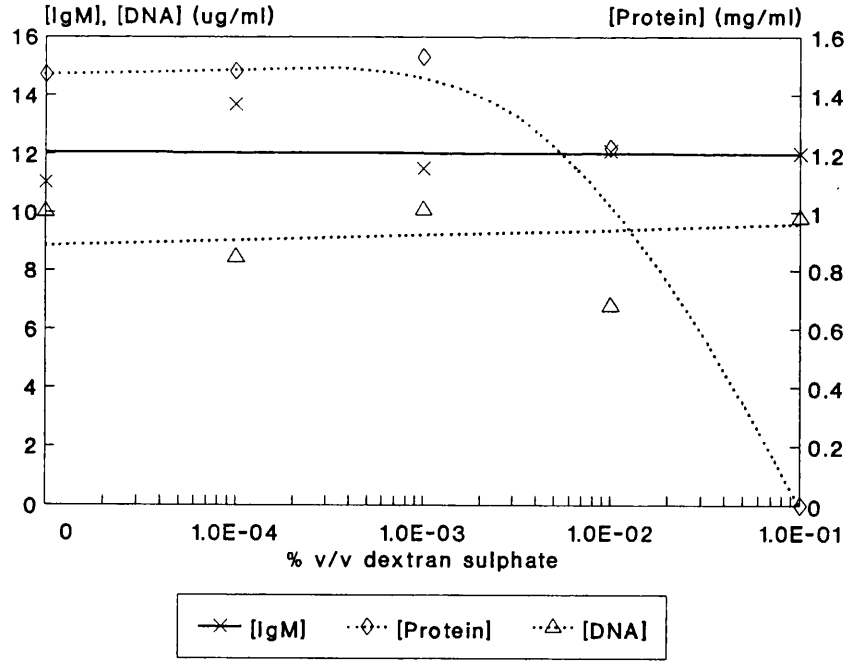


Figure 49. Profile of component levels over a range of dextran sulphate 5,000 concentrations.

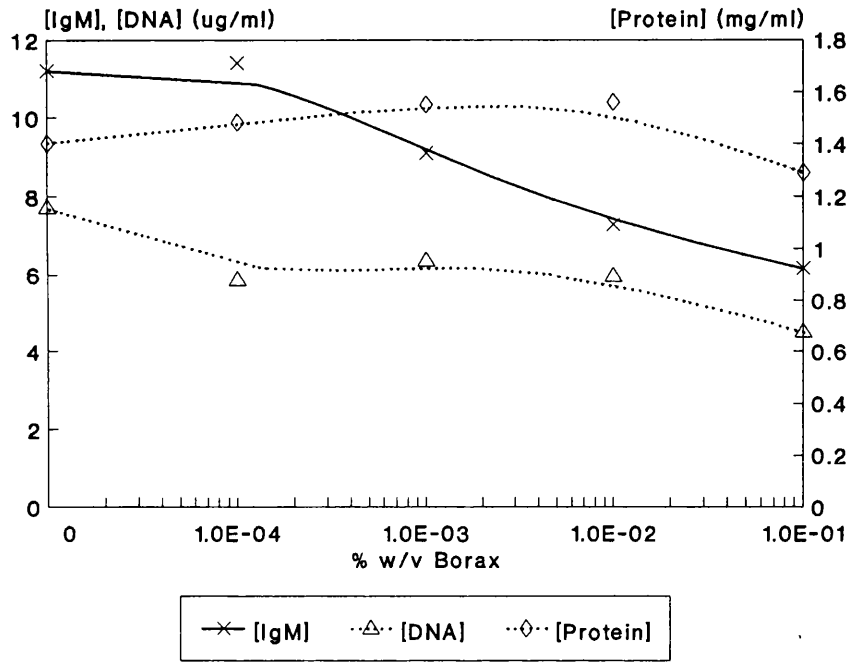


Figure 50. Profile of component levels over a range of borax concentrations.

% PEI	µl PEI (1% v/v)	µl IgM (10 µg ml^{-1})	µl buffer
0.01	10	100	890
0.1	100	100	800

This assay was performed on a range of five molecular weights of PEI.

Table 5. Setup of ELISA assay validation.

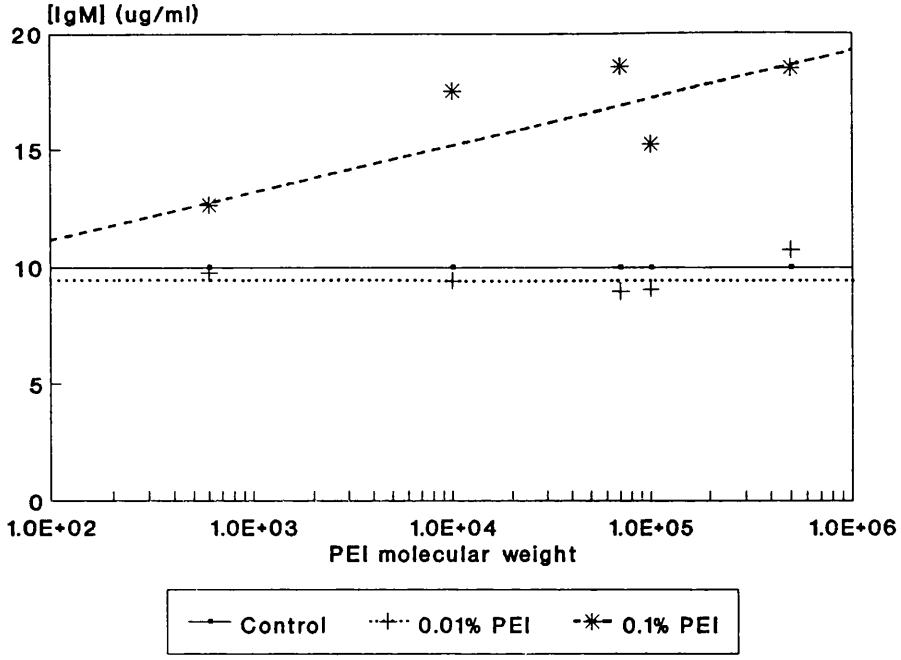


Figure 51. Profile of IgM levels over a range of PEI molecular weights at different concentrations.

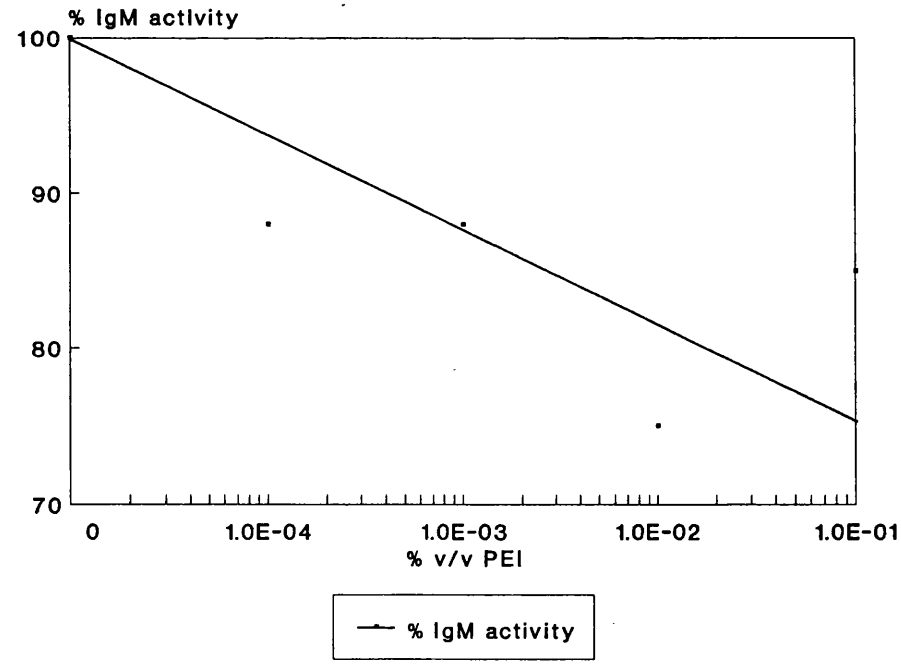


Figure 52. Results of 'titre/immediate spin' assay, showing relationship between IgM activity and PEI concentration.

% PEI	µl BSA (2 mg ml^{-1})	µl PEI (1% v/v)	µl water
0.0	50	0	950
0.0001	50	10*	940
0.001	50	100*	850
0.01	50	10	940
0.1	50	100	850

* The stock PEI solution was diluted 1:100 prior to addition to these samples, to avoid the inaccuracy of working with small volumes.

Table 6. Setup of protein assay validation.

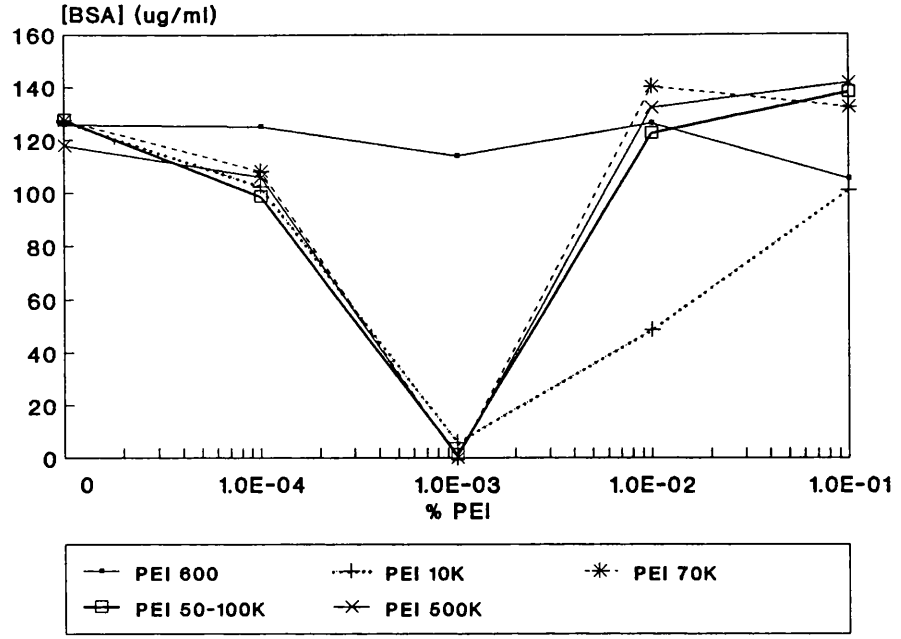


Figure 53. Profile of BSA levels over a range of PEI concentrations, for different PEI molecular weights.

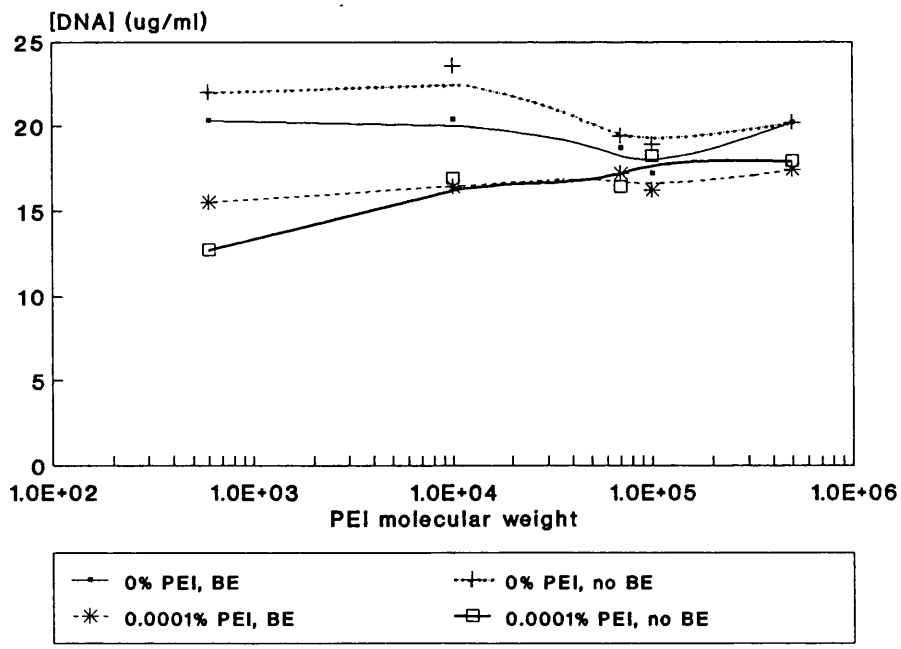


Figure 54. Profile of DNA levels over a range of PEI molecular weights and concentrations, comparing buffer-exchanged (BE) samples with untreated samples.

ASSAY SETUP.

% PEI	μl DNA (50 μg ml^{-1})	μl PEI (1% v/v)	μl buffer
0.0	400	0	600
0.0001	400	10*	590
0.001	400	100*	500
0.01	400	10	590
0.1	400	100	500

* The stock PEI solution was diluted 1:100 prior to addition to these samples, to avoid the inaccuracy of working with small volumes.

This assay was performed on a range of five molecular weights of PEI.

ASSAY RESULTS.

PEI Mol.Wt.	% PEI				
	0	0.0001	0.001	0.01	0.1
600	22.0	12.7	0.0	0.0	0.1
600 BE	20.3	15.5	0.0	0.0	0.0
10K	23.6	16.9	0.0	0.0	0.0
10K BE	20.4	16.4	0.0	0.0	0.0
70K	19.4	16.4	0.0	0.0	0.2
70K BE	18.7	17.2	0.0	0.0	0.2
50-100K	18.9	18.2	0.0	0.0	0.1
50-100K BE	17.2	16.2	0.0	0.0	0.1
500K	20.2	17.9	0.0	0.02	0.2
500K BE	20.2	17.4	0.02	0.0	0.2

The letters BE after the molecular weight indicate that the sample was buffer-exchanged prior to assaying.

Sample	IgM (μg ml^{-1})		Protein (mg ml^{-1})	
	Pre-buffer exchange	Post-buffer exchange	Pre-buffer exchange	Post-buffer exchange
1	9.87	9.99	1.33	1.25
2	9.74	9.95	1.31	1.18
3	10.56	9.45	1.25	1.14
4	9.89	10.62	1.25	1.12

Table 8. Results of study on the effects of using NAP-5 columns.

Component	Stock conc. (μg ml^{-1})	Final % PEI (v/v)			Final conc. (μg ml^{-1})
		0	0.01	0.1	
IgM	100	300	300	300	20
Protein	2000	750	750	750	1000
DNA	50	300	300	300	10
Water	--	150	135	--	--
PEI	1% (v/v)	--	15	150	--

Table 9. Setup of study on the interaction of components. (Volumes are μl throughout).

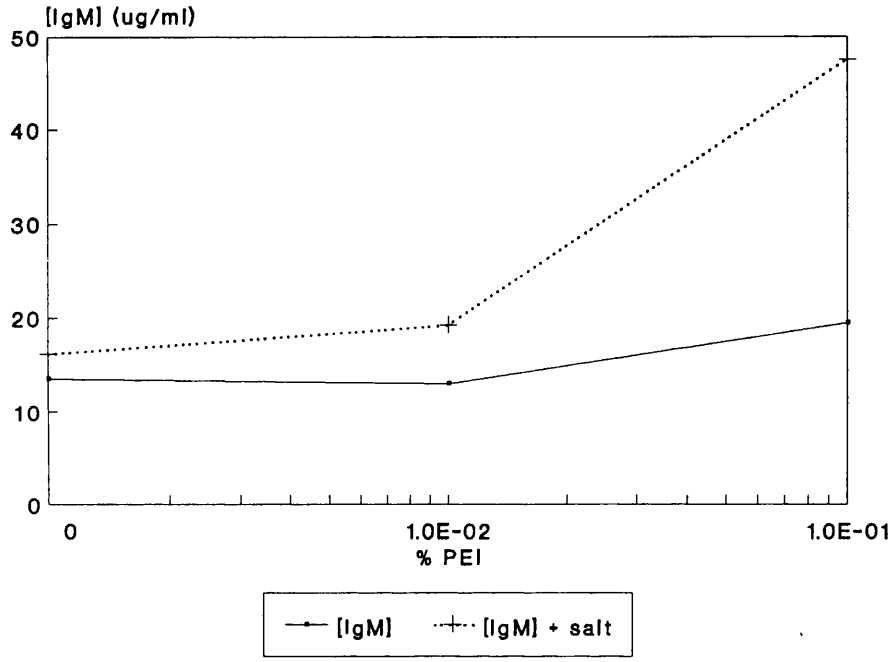


Figure 55. Profile of IgM levels over a range of PEI concentrations comparing samples with and without 0.5M salt.

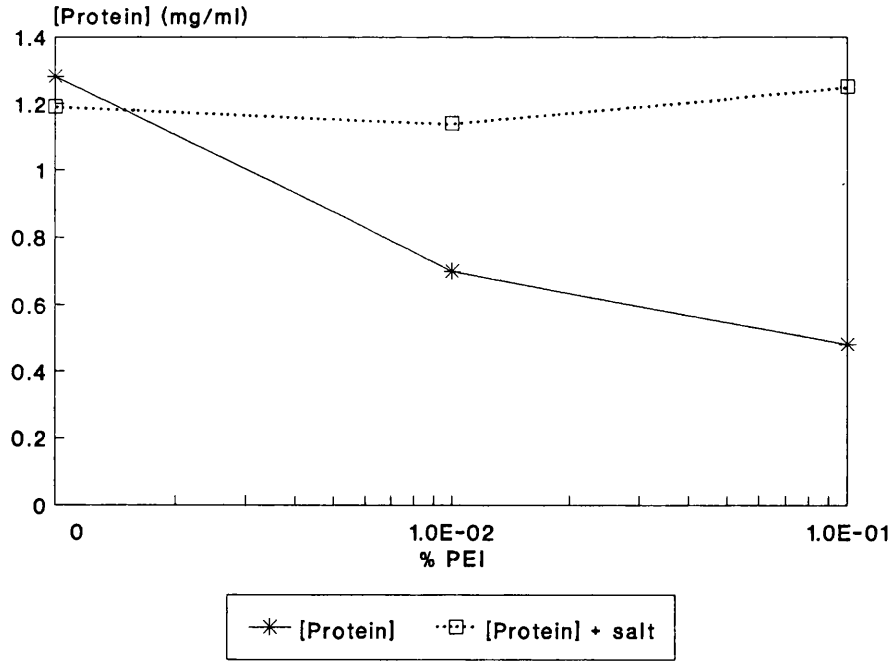


Figure 56. Profile of protein levels over a range of PEI concentrations comparing samples with and without 0.5M salt.

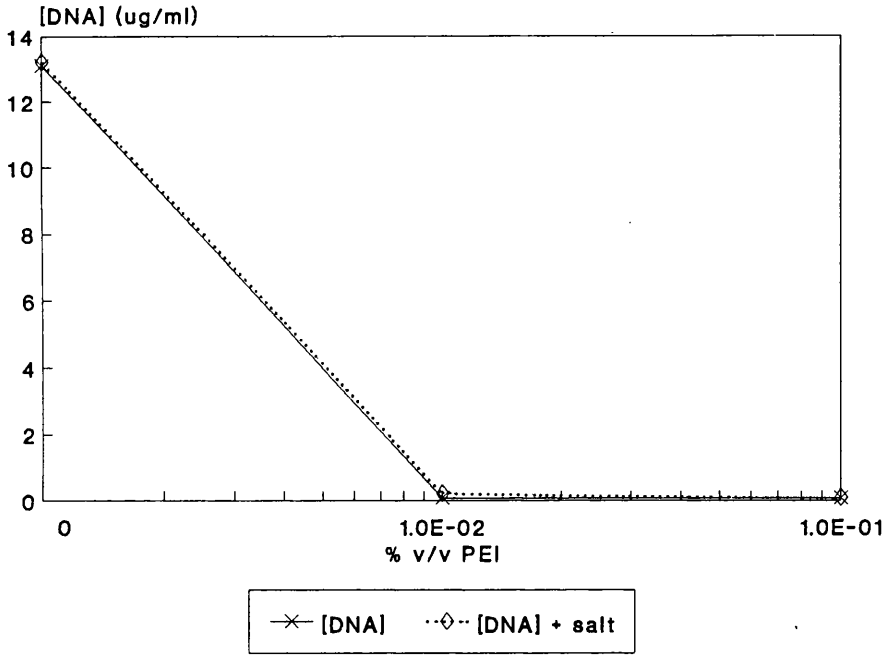


Figure 57. Profile of DNA levels over a range of PEI concentrations comparing samples with and without 0.5M salt.

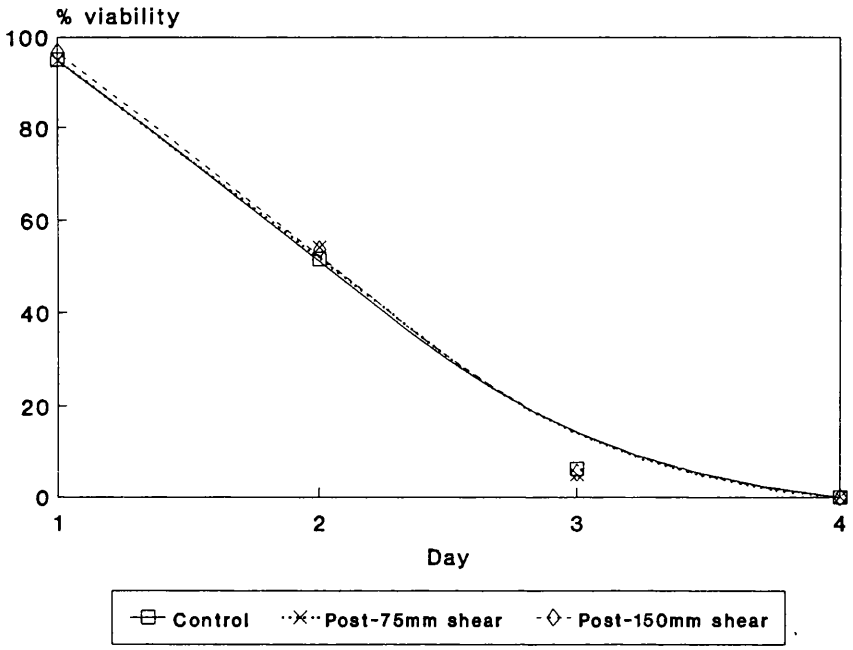


Figure 58. Scale-down simulation, showing the effect of shear on cell viability.

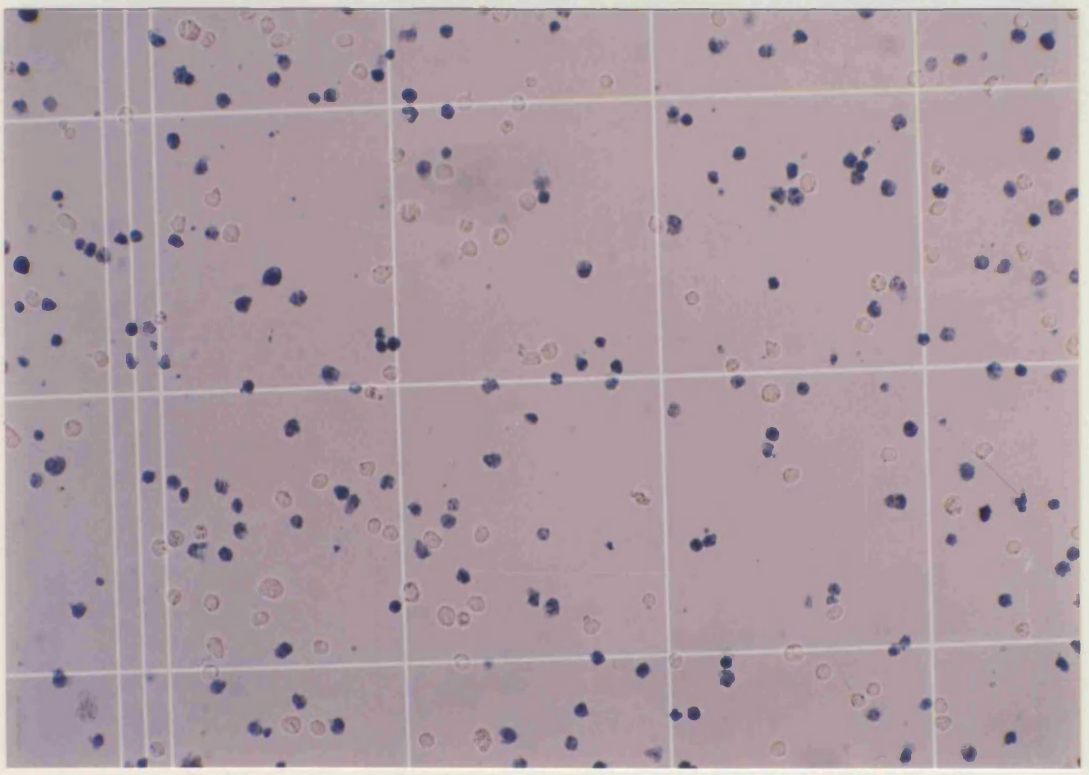


Plate 1. Control sample from Day 2 of scale-down study, with no PEI and no shear.

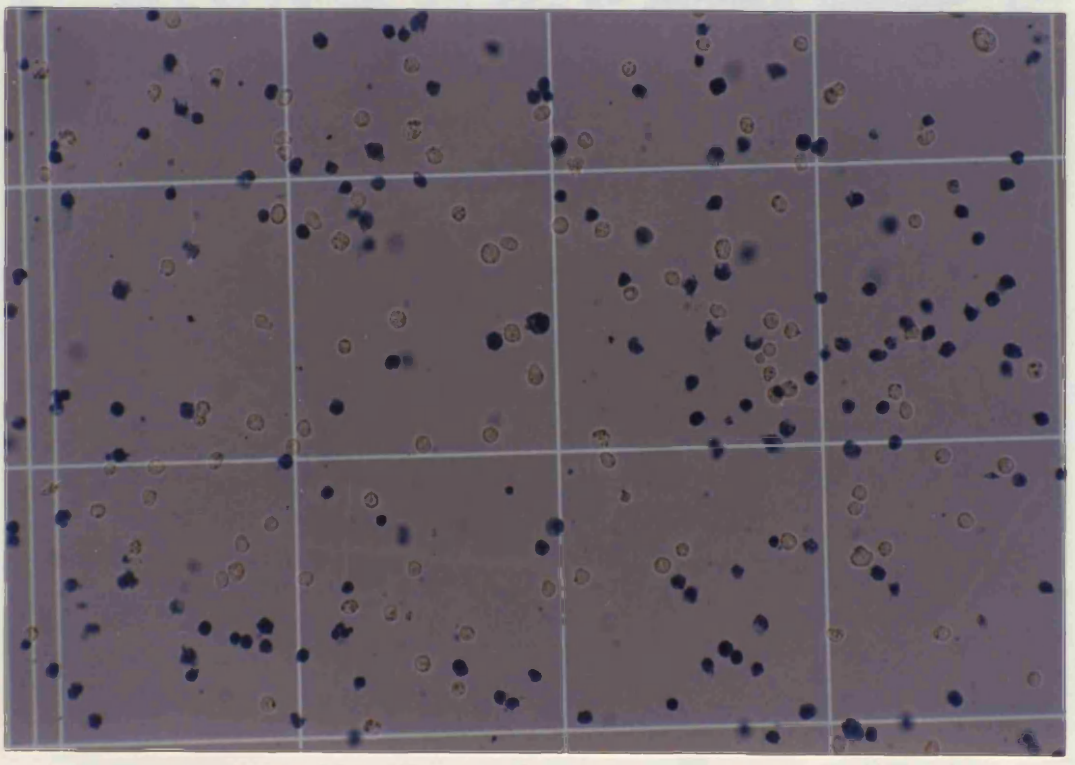


Plate 2. Control sample from Day 2 of scale-down study, with no PEI, after exposure to shear in 75 mm Instron capillary.

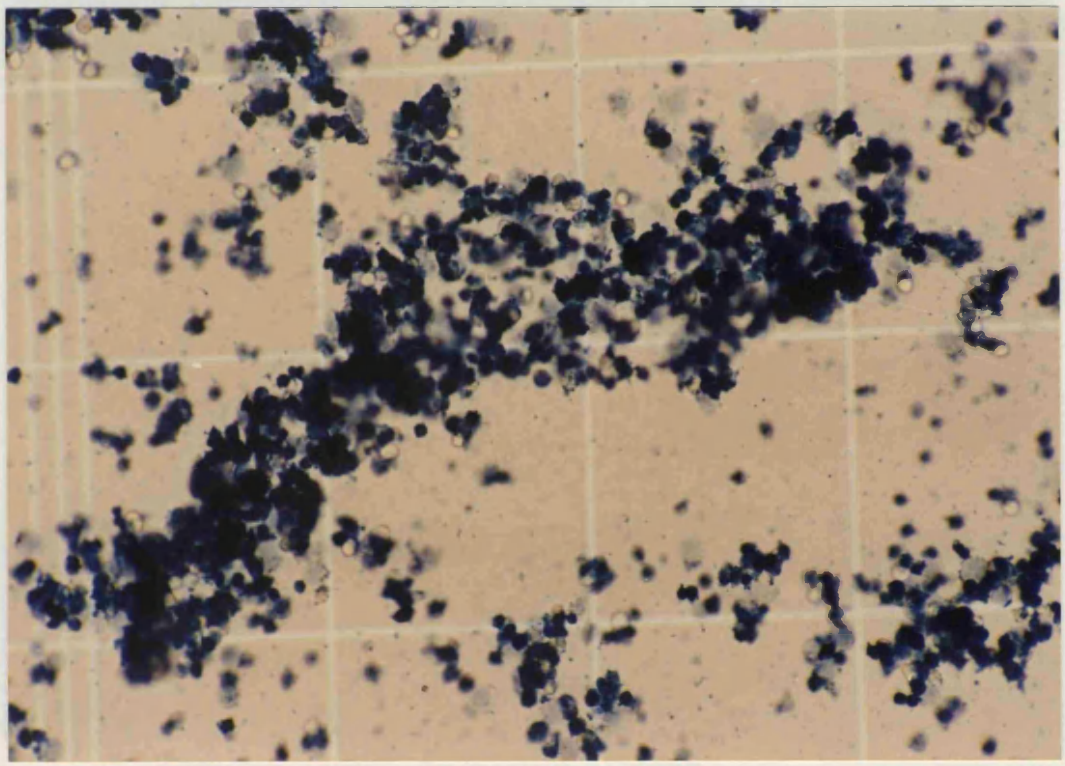


Plate 3. Sample from Day 2 of scale-down study, with 0.01% PEI and no shear.

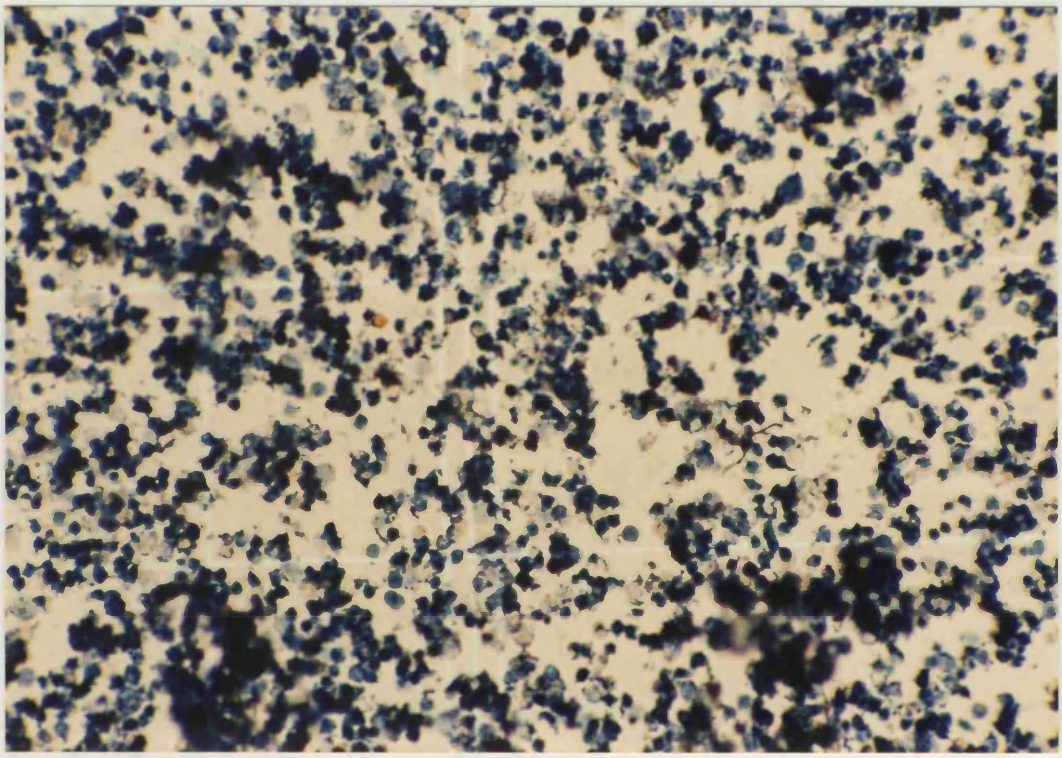


Plate 4. Sample from Day 2 of scale-down study, with 0.01% PEI, after exposure to shear in 75 mm Instron capillary.

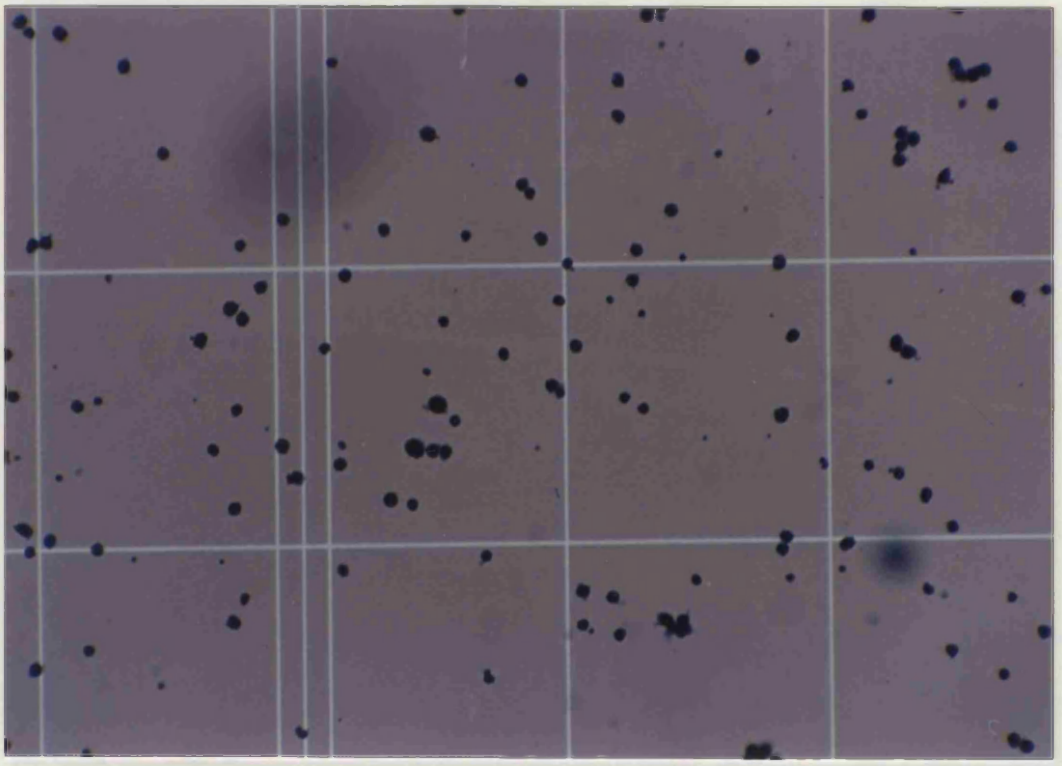


Plate 5. Control sample from Day 4 of scale-down study, with no PEI and no shear.

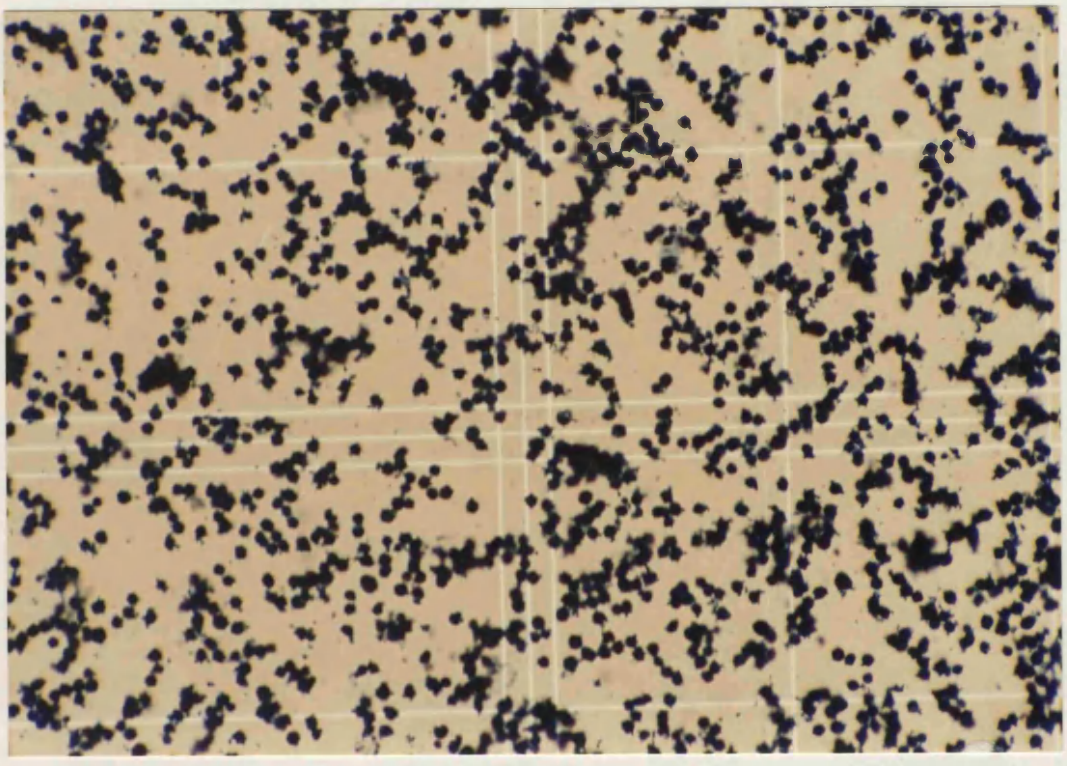


Plate 6. Sample from Day 4 of scale-down study, with 0.01% PEI and no shear.

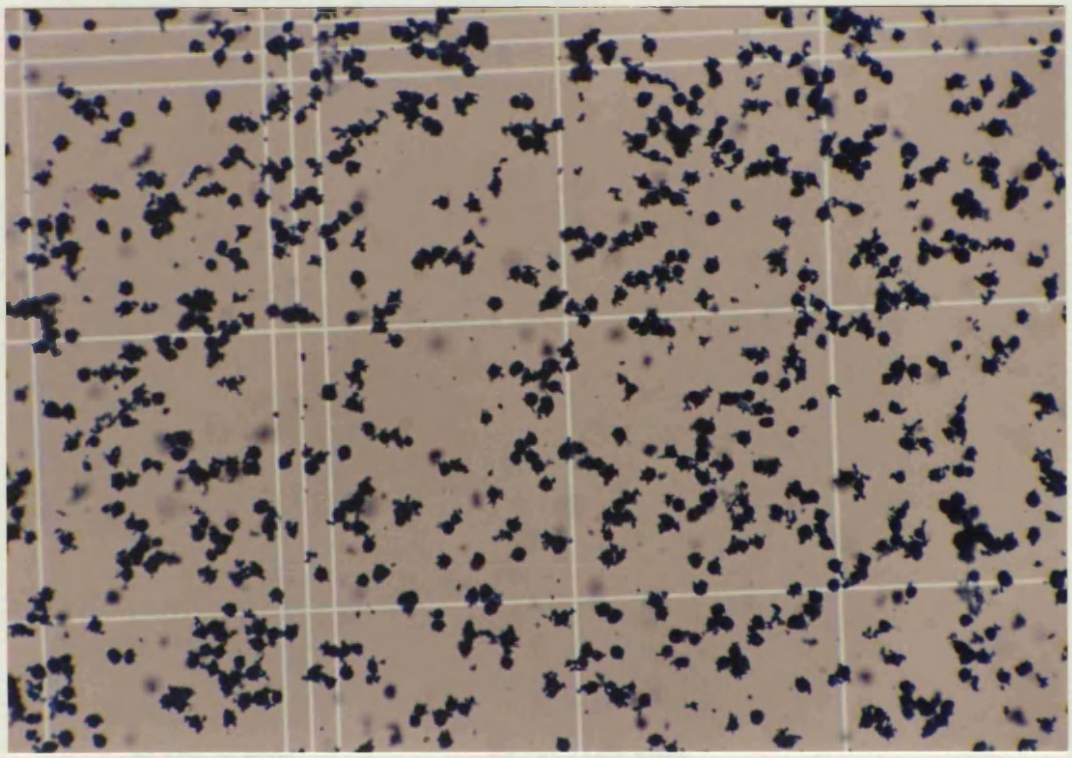


Plate 7. Sample from Day 4 of scale-down study, with 0.01% PEI, after exposure to shear in 75 mm Instron capillary.

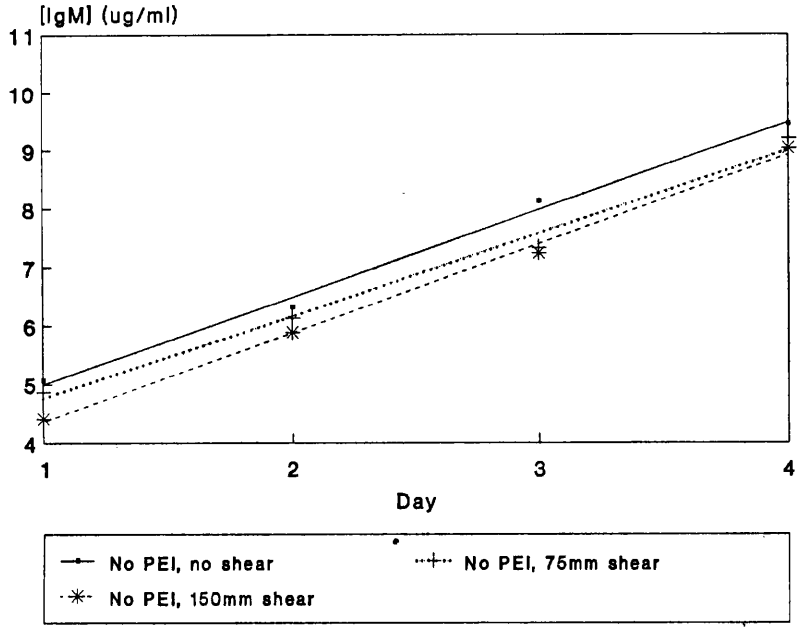


Figure 59. Comparison of IgM levels in samples exposed to different shear conditions, with no PEI added.

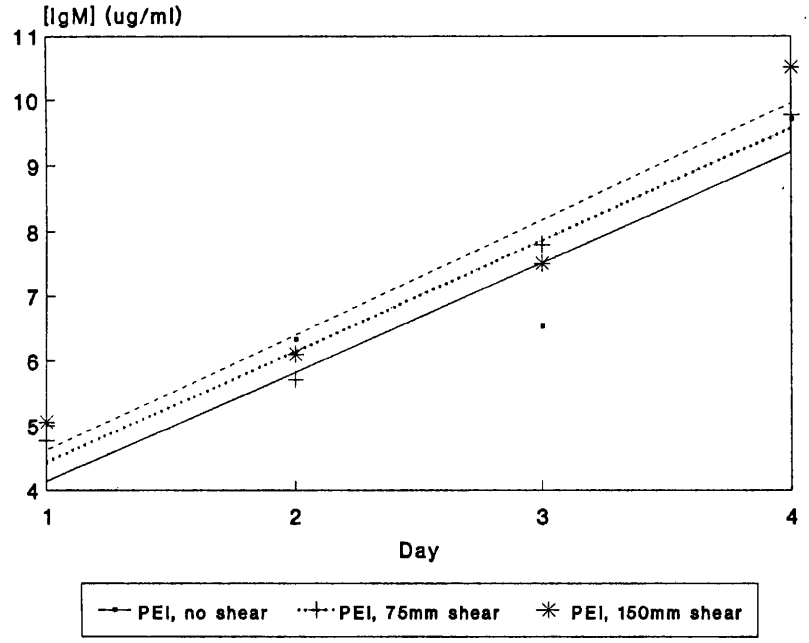


Figure 60. Comparison of IgM levels in samples exposed to different shear conditions, with 0.01% v/v PEI added.

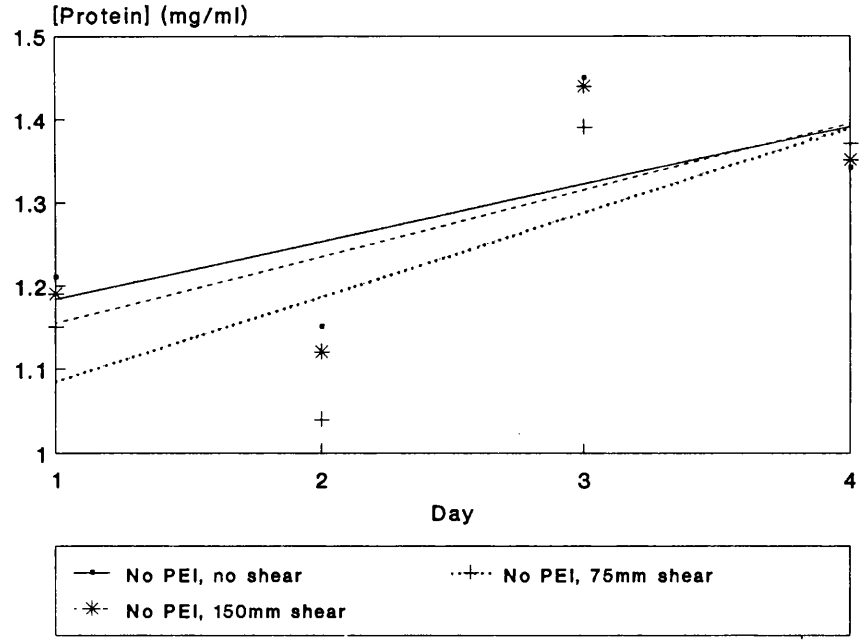


Figure 61. Comparison of protein levels in samples exposed to different shear conditions, with no PEI added.

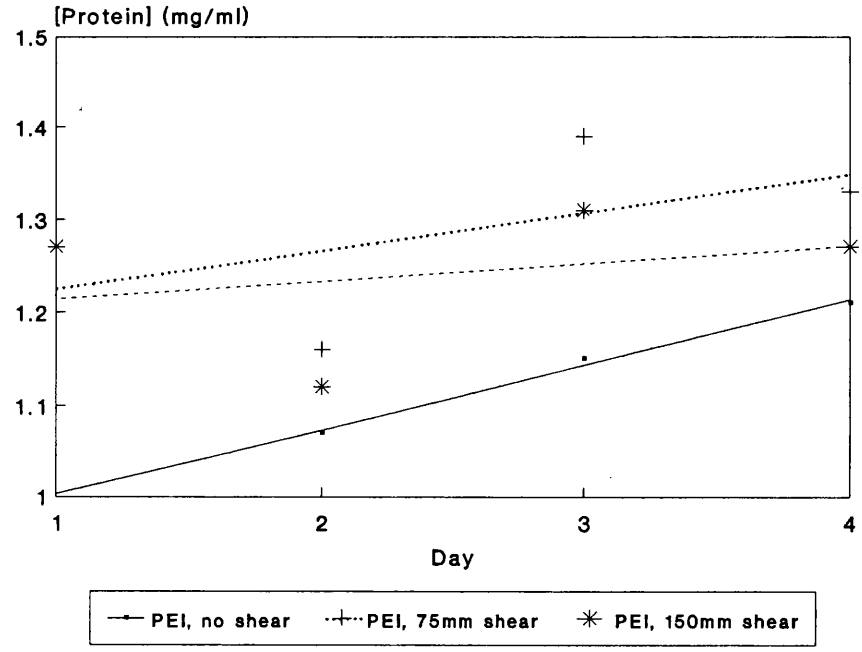


Figure 62. Comparison of protein levels in samples exposed to different shear conditions, with 0.01% v/v PEI added.

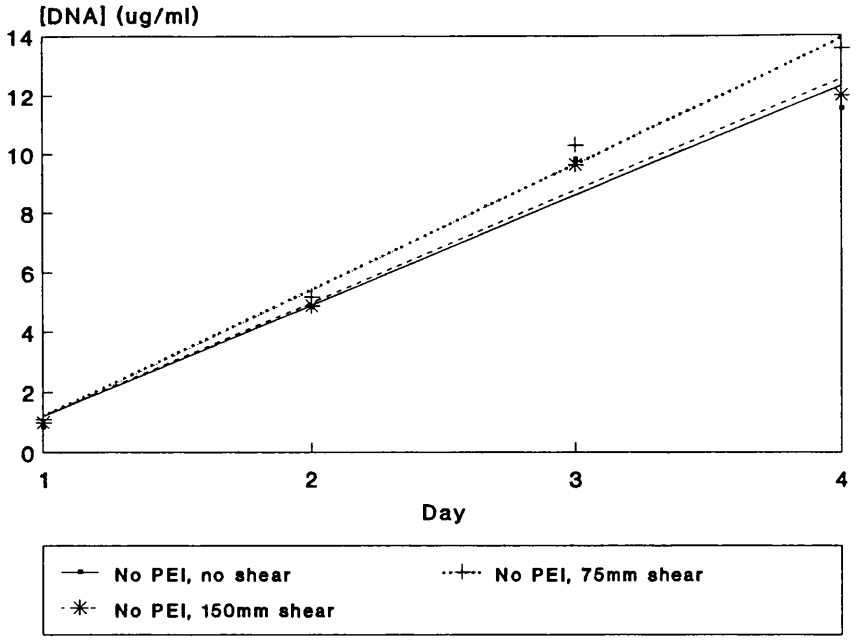


Figure 63. Comparison of DNA levels in samples exposed to different shear conditions, with no PEI added.

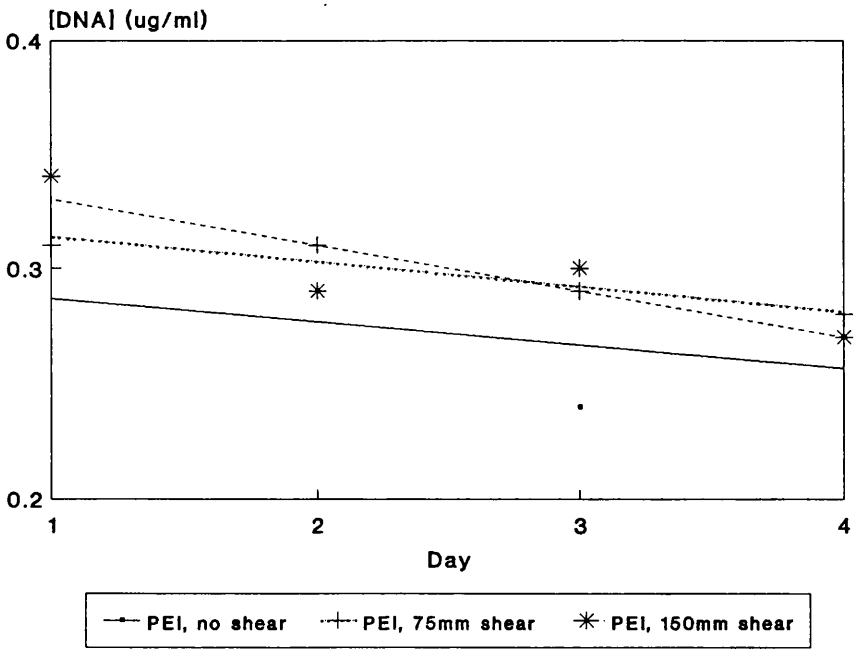


Figure 64. Comparison of DNA levels in samples exposed to different shear conditions, with 0.01% v/v PEI added.

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