"The Effects of Viral Inactivation Agents on the Activities of Monoclonal Antibodies"

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A thesis submitted to fulfill the requirements of the
Masters of Science in Medicine,
for the University of Sydney, 1993.

"But I shall let the little I learnt go forth into the day in order that someone better than I may guess the truth, and in his work may prove and rebuke my error. At this I shall rejoice that I was yet the means whereby this truth has come to light."

Albrecht Dürer

ACKNOWLEDGMENTS

I wish to acknowledge Colin Harbour and John Barford for their excellent supervision throughout the course of this project.

I am indebted to many other people, without whose help this project would not have been possible. In particular I thank:

Ray Kearney for allowing me the use of his anti-pneumococcal SIII hybridoma cell-line (IgM F11/A3)

Anne Fletcher for allowing me the use of her human anti-RhD B-lymphoblastoid cell-line.

Candace Gabelish and Peter Pavlovic' for hardware and software contributions.

My parents who have given me the wisdom and the enthusiasm for the pursuit of knowledge which has brought me this far.

My collegues and friends at CSIRO who have given generously in support and encouragement; in particular I thank Bruce Cornell, Peter Osman and Frances Separovic'.

Friends associated with the department of Chemical Engineering: Chris Marquis, John Delich, Peter Phillips, Jenny Quan and Rosemary Dever.

A special thanks must go to Rita Steffé whose technical expertise and friendship have been of great value to me.

I thank John Barford for his financial support through the ARC of which I was extremely appreciative.

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ABSTRACT

The aim of this thesis was to determine whether or not two viral inactivation methods which had been developed for use with blood products, affected either antigen binding or secondary biological functions of two selected model antibody molecules. It was postulated that at least one of the viral inactivants, ßPL, would affect the functional integrity of antibody molecules since it is reactive with proteins and has been used to has been used to block the complement fixing properties of gamma-globulins.

As one of the most important functions of antibodies is opsonization, which occurs through binding of the Fc region to the Fc receptors in the cell membrane and binds complement, an *in vitro* model for this process such as haemolysis, serves as a useful assay. Because haemagglutination and haemolysis serve as useful models for determining the functional integrity of antibodies *in situ*, these were the initial assays chosen to detect whether either of the two chemical viral inactivants, ßPL and TNBP, had a detrimental effect on the variable (antigen-binding) region or Fc region (effector function) of the two model monoclonal antibodies. However, the results from these assays were not clear cut and difficult to interpret due to both the inherent difficulty in reading the assays, i.e. the absence of an electronic component to provide sensitivity in measurement and the sensitivity of the antibody reaction to its physicochemical environment. The ELISA provides a useful comparison to haemagglutination and haemolysis assays as it overcomes these limitations. ELISAs can be used as a comparison but not a substitute for haemagglutination and haemolysis since the proteins are fixed onto a solid substrate so their mobility is restricted and the Fc is not assessed for its effector functions.
It is therefore proposed that a series of assays is required to determine the functionality of the variable and Fc regions of antibodies. In the future NMR will have a large part to play in the analysis of antibody purity but so far its use is limited to observing the conformation of variable domains. For the present, the assays chosen for this thesis were seen as the appropriate option to obtain data of greatest significance.

By ELISA it has been determined that the viral inactivation treatment using ßPL with an IgM monoclonal antibody preparation resulted in a 25 % reduction in antigen-binding activity of the variable region, i.e. a 25 % reduction of antigen-antibody complex was detected. The results obtained from haemagglutination and haemolysis assays were less clear cut for reasons discussed previously. Initially, a reduction in titre for both haemagglutination and haemolysis was observed so that, as with the ELISA, a reduction in antigen binding activity followed treatment with ßPL. Because an antigen-antibody complex has a greater affinity for an Fc receptor than an unbound antibody, the reduction in haemolytic activity does necessarily indicate a chemical modification of the Fc region. Subsequently, when the assays were repeated in the presence of 5 % BSA, a reduction in neither the haemagglutination nor the haemolytic titres were observed. It is conceivable that while the affinity constant and hence detectability was increased using BSA, the precision of the measurement was reduced to such a degree that the differences in avidity of the antibody molecules could not be detected.
No significant alteration in overall biological activity, as measured by ELISA, haemagglutination or haemolysis resulted when the same IgM monoclonal antibody preparation was subjected to the viral inactivation procedure using TNBP.
No significant alteration in the antigen-binding activity, measured by haemagglutination resulted when a model human IgG1 monoclonal antibody preparation was subjected to the viral inactivation procedures using either TNBP or ßPL; however, this assay was carried out in the presence of 5 % BSA and, as been concluded from the previous experiments, further assays may be required to detect an alteration.

It may be concluded that although the use of ßPL as an viral inactivant is the only chemical treatment method available to inactivate all viruses for which it has been tested, it significantly reduces the antigen-binding ability of a mouse IgM molecule; also, it is likely that ßPL modifies the Fc region to reduce its functional integrity (115). Procedures such as filtration or lyophilisation have advantages in that they have been demonstrated to yield a safe product; however, filters available cannot remove very small viruses and the use of lyophilisation for inactivation has not been substantially investigated.

ABBREVIATIONS

ADCC	Antibody-dependent cell-mediated cytotoxity
ßPL	Beta-propiolactone
BRV	Bovine Rhinotracheitis Virus
BSA	Bovine Serum Albumin
BSE	Bovine Spongiform Encephalopathy
BVD	Bovine Viral Diarrhoea
CFF	Cross-Flow Filtration
CHO	Chinese Hamster Ovary
CMV	Cytomegalovirus
CPV	Canine Parvo Virus
dAb	Single domain antibody
DMEM	Dulbeccos Modified Eagles' Medium
ELISA	Enzyme linked immunosorbent assay
EBV	Epstein Barr Virus
Fab	Fragment antibody binding
Fc	Fragment crystallizable
FcR	Fc Receptor
FCS	Foetal Calf Serum
FPLV	Feline Panleukopenia Virus
HA	Haemagglutination
HL	Haemolysis
HAMA	Human Anti-Mouse Antibody
HBV	Hepatitis B Virus
HCV	Hepatitis C Virus
HIV	Human Immunodeficiency Virus
HPLC	High Performance Liquid Chromatography
Ig	Immunoglobulin
LCM	Lymphocytic Choriomeningitis Virus
NMR	Nuclear Magnetic Resonance Spectrometry

MAb	Monoclonal Antibody
PCR	Polymerase Chain Reaction
PFM	Protein Free Medium
SFM	Serum Free Medium
SpA	Staphlococcal Protein-A
STR	Stirred Tank Reactor
TNBP	Tri(n-butyl)phosphate
TPAS	Two-Phase Aqueous System

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INTRODUCTION

1 INTRODUCTION

The literature review, which follows this section, provides ample evidence that the use of biologicals for therapeutic purposes, particularly blood products, has been associated with the transmission of viral diseases. The evidence of human immunodeficiency virus (HIV), hepatitis B virus (HBV), hepatitis C virus (HCV) and human parvovirus infections among many haemophiliacs who require regular infusions of coagulation factors, either Factor VIII or Factor IX, derived from human plasma is a sad testament to this fact. In order to address this problem, the manufacturers of blood products have had to develop strategies for either the removal or inactivation of contaminating viruses during the production of plasma proteins. As a result a number of different procedures have been developed which appear to have successfully eliminated the risks of HIV and HBV transmission via the use of plasma proteins as therapeutic biologicals and these methods are described in more detail in the literature review.

In addition some manufacturers have taken an alternative approach in which the tremendous advances that have been made in genetic engineering and cell-culture technology have been combined to generate recombinant proteins in cell culture. Thus the problems of viral contamination associated with the use of human blood and plasma are avoided by producing the plasma protein as recombinant proteins in cell culture. Unfortunately, as described in the literature review, the use of cell culture technology does not guarantee that the products are free from viral contamination.

Cells used in the production process are derived from animal or human cells which could be contaminated with a number of viruses. Thus the manufacturers of cell culture derived biologicals, such as monoclonal antibodies and recombinant proteins, should also be concerned about viral inactivation during the production and purification of such products. At the start of the work described in this thesis in 1989, these problems had not been addressed in any detail and the project sought to determine whether or not some of the inactivation strategies developed for use with plasma proteins could be employed for cell culture derived biologicals.

1.1 Aims and Scope of Project

The major premise underlying this research project was that it is highly desirable to incorporate a viral inactivation procedure into the downstream processing operations of cell culture derived biologicals. If such a strategy is adopted then the major challenge facing biotechnologists involved in the production process is how to develop a procedure which will inactivate viruses with minimal effect on the physical structure and biological function of the protein products.

In order to investigate this problem two chemical inactivation methods, which had been developed for use with blood products, were chosen for use with two monoclonal antibodies. The latter were chosen as model proteins because they are complex proteins with different activities associated with different region of the molecules, i.e. a variable region which binds with antigen, the so-called Fab region, and another region associated with secondary biological functions such as complement binding and attatchment to receptors on lymphoid cells, the Fc region. The major aim of the study was to determine whether or not the inactivation methods, which when used according to the prescribed protocols inactivated a range of viruses, affected either the antigen binding or secondary biological functions of the antibody molecules.

In order to achieve this aim the following preliminary experimental tasks had to be carried out:

* Choice of suitable antibody models.
* Production of sufficient antibody to carry out required tests.
* Processing of antibody to give appropriate purity for tests.
* Choice or development of appropriate assays to determine functional activity of antibodies.
* Confirmation that chemicals inactivate appropriate model viruses.
* Suitability of treatment methods to scale-up.

A survey of the appropriate literature was carried out to address these areas.

LITERATURE REVIEW

2 LITERATURE REVIEW

The literature review has been divided into two major sections. The first of which, in giving a general background to the monoclonal antibody industry, aims to illustrate the rapid growth of monoclonal antibodies in certain markets, particularly as therapeutics. This has resulted from improvements in: the understanding of immunology, techniques in molecular biology and protein engineering, production of mammalian cells at large scale and techniques in separation methods to obtain proteins of very high purity.
The second section reviews the problem of viral contamination in monoclonal antibody preparations and the potential sources of contamination in their production. It then describes methods available for the removal of viruses from biological material, methods which have been used to inactivate viruses in biological materials and their possible applications to large-scale cell-culture processes.

2.1 BACKGROUND

2.1.1 APPLICATIONS OF MONOCLONAL ANTIBODIES

Monoclonal antibodies have had a great impact on the growth of the biotechnology industry. Soon after Köhler and Milstein (1975) published details of their technique for producing cells secreting antibodies specifically to sheep erythrocytes (1), investigators worldwide realised potential applications for monoclonal antibodies, i.e. antibodies of predetermined specificity, in the research laboratory and in the marketplace. The uses of monoclonal antibodies have been well reviewed as general tools in biotechnology (2) and as pharmacological agents (3).

A. DIAGNOSTICS

The first major application resulting from hybridoma technology was in diagnostics, i.e. the development of immunoassay systems for the detection and measurement of drugs, hormones, proteins or micro-organisms (4,5). By labelling the monoclonal antibody with an appropriate signal, rapid and sensitive detection systems can be obtained.
The methods used for the development of these *in vitro* test kits have been taken a step further and applied to *in vivo* diagnostic

imaging, i.e. detection of antigens within the body using a labelled monoclonal (6,7). Over the past ten years much effort has been put into raising antibodies against tumour cells for diagnosis and treatment (8). Problems in this area have been the human anti-mouse antibody (HAMA) response of the patient to the monoclonal used in the treatment and the inability to identify tumour specific antigens. However, recent advances in the areas of immunology and oncology have led to the development of improved techniques for producing human antibodies which are increasingly more compatible with the human immune system meaning that great advances in this area have at last occurred. Some of these techniques are described in section 2.2.1. of this thesis.

B. IMMUNOPURIFICATION

Immunopurification is a powerful and selective purification procedure which allows a high degree of purity, approx. 95 %, to be obtained in a single step, giving a yield of about 90 %. The method involves immobilising monoclonal antibodies of desired specificity to a polysaccharide matrix at up to 10 g antibody per litre of matrix. A substance is then separated from a mixture by adsorbtion of the antigen to the antibody. The antibody-antigen complex can be broken by changing ionic or pH conditions so that the adsorbed substance is eluted. As methods for producing monoclonals are becoming more economical this method is being used more widely in the production of biologicals, for example: in the purification of coagulation factors such as FVIII from human plasma (9) and purification of recombinant hepatitis surface antigen vaccine (Smith-Kline Beecham). At industrial scale 5 l columns are commonly used giving a yield of up to 50 g product per batch.

C. VACCINES

There are two ways in which antibodies can be used as vaccines, either as passive vaccines or as active vaccines. Passive immunity can be acquired by maternal antibodies or from homologous pooled gamma-globulin. Active immunization provides a protective state through contact with a harmless form of the disease organism or its antigen.
Antibodies have been generated for the use of active vaccines (10,11), in the form of anti-idiotypes. The theoretical basis of this was described in Jerne's idiotype-anti-idiotype network

theory (12).

Antibodies can themselves act as antigens, to be recognised by other antibodies. Antibodies which bind to the variable regions (idiotypes) of other antibodies are called anti-idiotypes and carry the internal image of the original antigen and so therefore can replace the antigen to elicit an immune response. Anti-idiotypic vaccines could replace viruses, microbes or their toxins which could be hazardous to the patient.

The response which is obtained to an anti-idiotypic antibody is dependent on a number of factors including: the extent to which the antibody causes cross-reactions, how foreign the antibody is to the recipient and the amount of antibody which is injected. Some anti-idiotypes seem to induce suppressive rather than protective immunity in the vaccinated animal even though they elicit both B and T-cell responses against the antigen (13). At present, caution must be taken regarding anti-idiotypic antibodies, as their exact effects on the immune system are not fully understood. However, they do offer exciting possibilities.

D. THERAPEUTICS

As with the use of *in vivo* diagnostics the use of monoclonal antibodies in therapy has been limited to date due to the lack of appropriate antibodies. There are at present very few monoclonals, e.g. (OKT3 - an anti-T lymphocyte antigen murine monoclonal used in organ transplantations, Centotoxin - an anti-endotoxin monoclonal and an anti-dioxin monoclonal) licensed as therapeutics. This is mainly due to the foreign nature of murine antibodies which may induce an immune reponse, i.e. the generation of human anti-mouse antibodies (as the HAMA response). In well over half the patients treated to date with murine monoclonals, the HAMA response has been observed (14). Human monoclonals are more likely to have species specific carbohydrates which may be important in a number of effector functions including: Fc receptor-mediated antibody-dependent cellular cytotoxicity, complement activation and phagocytosis (15).

Because of the adverse response caused by murine antibodies in a human host, much work has been directed towards the production of human monoclonal antibodies and the humanising of murine monoclonal antibodies. Various methods have been used to produce cell-lines for the continuous production of human monoclonals,

however, problems have arisen in producing stable cell-lines. Recent progress has shown that the immortalization of human B-cells from an immunized donor, with the Epstein-Barr Virus (EBV) can result in stable cell-lines secreting human monoclonal antibodies (16)(see section 2.2.1 B). Other methods for producing human-like antibodies have recently been developed, including genetic-engineering and protein-engineering (these are described in section 2.2.1) and with these developments it is likely that monoclonal antibodies will find a routine place in patient therapy and diagnostics. The prospects for using antibodies for therapy have been very well reviewed (17).

2.1.2 PRODUCTION OF MONOCLONAL ANTIBODIES FROM CELL-LINES

A number of cell vehicles have been developed for the production of monoclonal antibodies, these are commonly hybridomas: a cell resulting from the fusion of a spleen cell of an immunised mouse, rat or human with a myeloma (tumour of B-lymphocyte) cell. Because human spleen cells are often unavailable, human monoclonal antibodies are sometimes produced by lymphoblastoid cell-lines (B-lymphocytes which have been transfected using Epstein-Barr Virus). Human cell-lines, however, have the disadvantage that they are less productive than mouse cell-lines, so that mouse hybridomas, which have been genetically modified to secrete "humanised antibodies" (see sect. 2.2.1 C), may ultimately be more preferable. Animal cells are the only vehicles available for the production and glycosylation of these large complex proteins at scale. Although fragments of antibodies have been synthesised in bacterial and phage systems (see section 2.2.1 D), the assembly of the complete molecule has not been achieved.

The production of hybridomas which secrete monoclonal antibodies is a very well documented task and many methods have been described in publications. A flow diagram, **figure 2.1** shows the steps involved in producing a stable cell-line which produces the required antibody over a given period of time.

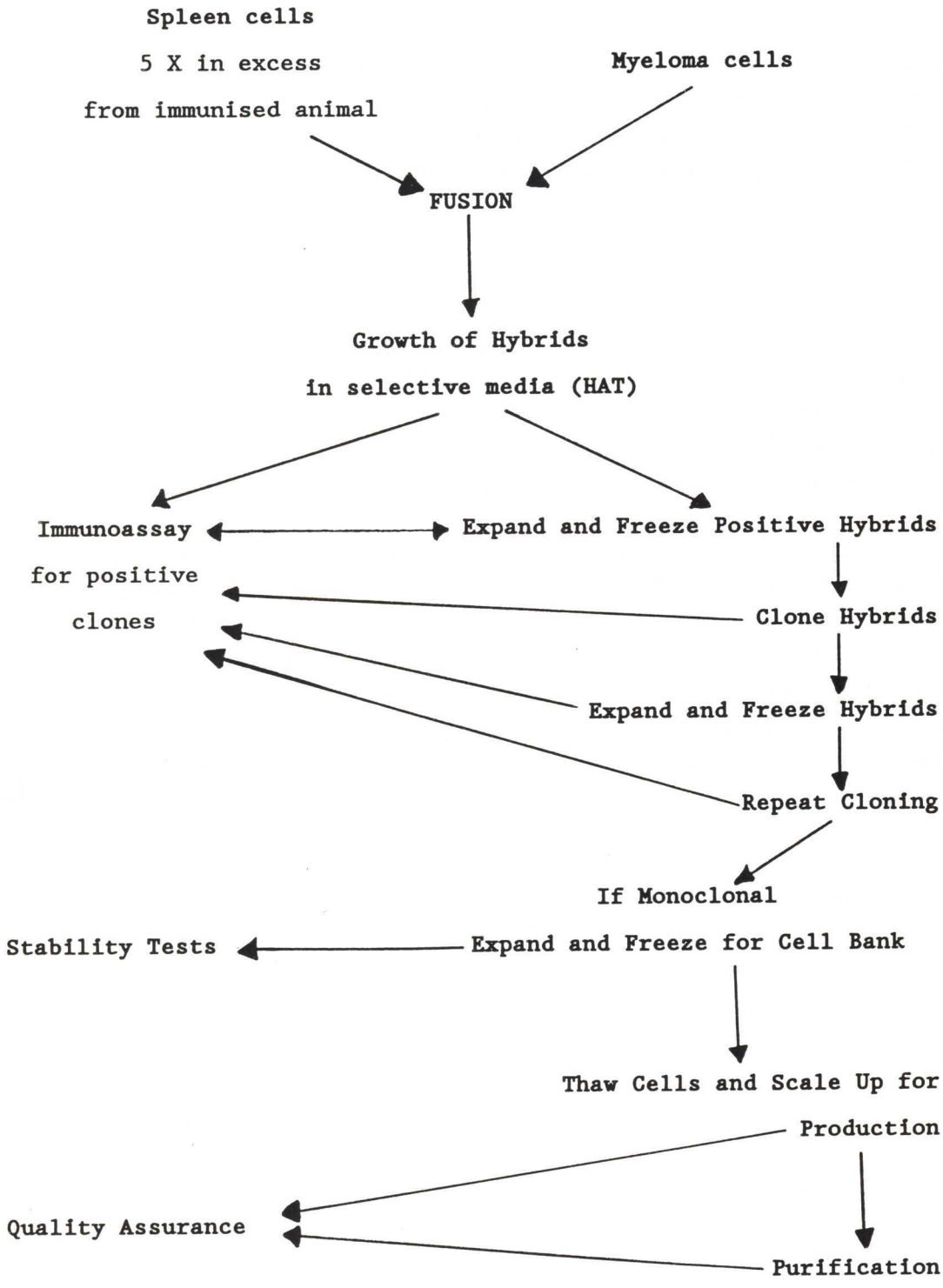


Figure 2.1: Flow diagram to show the production of a stable cell-line secreting a MAb of pre-defined specificity.

The quantities of monoclonal antibodies required to meet present and future markets have led to increased and optimised scale of production and optimisation of production methods. For therapeutic applications many kilograms of antibody are required per year. Two approaches have been followed for antibody production, these are *in vivo* and *in vitro* production.

A. *IN VIVO* PRODUCTION

In vivo production of monoclonal antibodies involves growing hybridomas as ascites in the peritoneal cavity of mice. This method produces monoclonals at high concentrations (approx. 50 mg per mouse), but in small volumes which may include a large number of contaminants, largely mouse immunoglobulins and murine viruses (see section 1.2.1 A).

In vivo production methods are more useful for production of monoclonals for research purposes; industrially these methods have generally been used by immunologists who have not had experience in scale-up of *in vitro* methods. Despite considerable disadvantages, (i.e. presence of contaminants mentioned above and difficulty in scale up) *in vivo* production methods are still being used for commercially available monoclonals, although they are becoming less common.

B. *IN VITRO* PRODUCTION

Cell culture has distinct advantages over ascites in monoclonal antibody production.

These include:

- -Economy in scale.
- -Process control and automation
  (leading to improved consistency of the process).
- -Reduced contamination
  (e.g. mouse immunoglobulin, protein and infectious material)
- -Compatibility with upstream and downstream processing.

(i). BIOREACTOR DESIGN

Many bioreactors have been designed for the large scale production of monoclonals and these have been well reviewed (18). Broadly they they fall into two groups: simple air lift

and stirred tank reactors, in which cells grow at relatively low population densities in a homogeneous suspension and immobilized or entrapped systems which encompass hollow fibre perfusion, micro-capsules, agarose microbeads and ceramic cartridges, in which cells grow at a population which is at least ten times higher.

The former group of bioreactors are larger, more conventional and occupy more space. They are used commonly in industries with experience in fermentation where they have been accepted mainly because they are familiar and have been well characterised. Because of simplicity of operation and ease of monitoring consistent batches can be obtained (important for quality assurance), sterilization of stainless steel reactors is straightforward and mixing is easily achieved. Air-lift fermentation was an option chosen by some companies in the early days of hybridoma cultivation as high mass transfer rates can be achieved at large scale without generating high shear forces which could damage the fragile cells. However, where serum is present in media, antifoam needs to be included to avoid excessive foaming in these systems and it has been proposed that cell-damage is caused by the motion and disengagement of sparged air-bubbles, so that cell protecting agents are required if air-lift reactors are used (19). However, it is now thought that in most cases gaseous and nutrient transfer can be achieved at low impeller rates without cell-damage unless a particularly sensitive cell-line is being used; so conventional stirred tank reactors (STRs) have gained popularity. Low aeration and agitation requirements and the use of lower concentrations of FCS mean that foaming problems are negligible.

The second group of reactors include many novel processes which have been developed with the aim of production at higher concentrations. A major advantage with these systems is that laboratories or small companies which do not have the space or expertise to operate large reactors, can produce monoclonals in reactors one tenth of the volume of conventional types, since cells are cultured at 10 times the concentration obtained in conventional reactors (max. cell density of approx. 10^8 cells ml^{-1}). Because these systems are still considered novel, companies have mostly opted for the use of the larger type of reactor. The hollow fibre membrane perfusion reactors have had some place though. The main disadvantage is that despite many

modifications mass transfer limitations occur due to insufficient mixing. They will probably always have a place though for groups which have limited space or which do not have existing fermentation facilities.

There have been a great many adaptations of the reactors described over the last decade or so, and as the demand for monoclonals has increased so have the size of the bioreactors, with 2000 l air-lift (Celltech) and 100 l immobilized-cell bioreactors having been developed. Fed-batch reactors also have been used commercially with great success, as low glucose levels can be maintained which results in reduced levels of lactate, which is inhibitory to cell growth (see following section). An important route being currently followed for the optimisation of antibody production is the understanding of hybridoma metabolism in order that metabolic pathways and reaction kinetics may be optimised. An improved understanding in this area should lead to significant increase in product yields by obtaining the best use of medium (20).

(ii). NUTRIENT REQUIREMENTS

Nutrient requirements for animal cells are complex and there is much work being carried out in this area. Briefly, their energy source comes from glutamine and glucose and the major toxic metabolic byproducts are lactate and ammonia. Glacken (21) and Miller (22) have shown that lactate concentrations of up to 40 mM can inhibit cell growth and the production of antibody. This results from pH inhibition due to high concentrations of lactate which exceed the buffering capacity of the medium. Glacken (21) and Hu (23) have maintained glucose at low levels in the culture media using fed-batch fermentation which results in reduced production of lactate. Miller (24,25) has shown that the specific glutamine consumption rate of murine hybridomas is independent of the initial glucose concentration but is increased with increasing glutamine concentrations. Initial glutamine concentration has no effect on the glucose metabolic quotient of the same cell-line.

Components of cell-culture media are vitamins (26,27), essential amino acids (28), salts and trace elements as well as glucose and glutamine. The basal media is supplemented with either foetal calf serum (FSC) or serum free media which contains other growth factors. The amount of serum required to satisfy the

poorly defined nutrient requirements must be minimised and if necessary supplemented with other defined nutrients so as to reduce contamination with other proteins. Many of the growth factors in serum have been isolated and their functions have been identified. Attempts have been made to produce monoclonal antibodies in serum free media to simplify downstream-processing and to reduce cost of process. These have not always been satisfactory and serum is still sometimes found to be neccessary in low quantities, however, many cell-lines may be weaned onto serum free media and it has been reported (29,30,31) that serum free media could be replaced with protein free media.

Oxygen has 7 ppm air saturation and poor solubility in water. It must therefore be monitored and controlled so it does not become limiting. Spier and Whiteside, 1990 (32) estimated that for cell densities of between 10^6 - 10^9 viable organisms ml^{-1}, oxygen demand rates can vary from 10^{-3} - 10 mg ml^{-1}, so the oxygen level in media must be optimised for each cell-line.

(iii). GROWTH KINETICS OF MAMMALIAN CELLS

Traditionally batch-culture has been used for the production of monoclonal antibodies. Batch culture is an example of a closed culture system, it is characterised by an initial inoculum and a limited amount of nutrients which become limiting during cell growth. In batch culture the cells exhibit the following phases of cell growth: lag phase, accelerating phase, exponential phase, decelaration phase, stationary phase, decline phase.

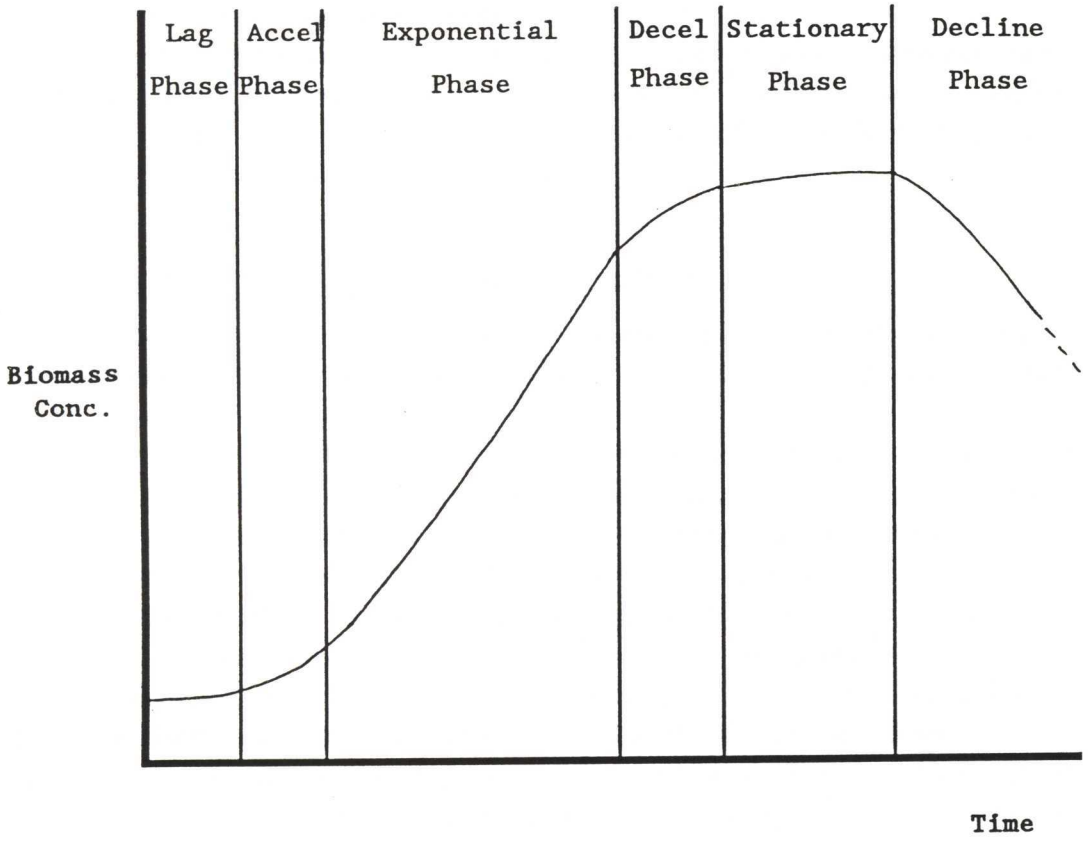


Figure 2.2: Growth of a typical hybridoma cell-line in batch culture.

This method of cell cultivation has been chosen as it was observed that for many cell-lines antibody continues to be produced in the stationary and decline phases; this may be attributed to antibody secretion into the media by dying cells. However, further studies have shown that antibody productivity appears to vary with different cell-lines used. Some cells lose antibody synthesis ability due to genetic mutations and immunoglobulin genes in myelomas are particularly prone to mutation (33). Because antibody production is not essential for cell viability cells may gain a growth advantage by losing their antibody producing genes. Chuck and Palsson (1992)(34) have shown that the competition between immunoglobulin producing and non-producing cells is very dependent on the the state of the

inoculum and media composition in batch culture.
Batch or fed-batch culture is still used by most manufacturers. Fed-batch cultures have been shown to be useful in animal cell-culture by stepwise dilution of inhibitor, while pulse-feeding essential nutrients.

2.1.3 PURIFICATION OF MONOCLONAL ANTIBODIES

The objective of purification is to obtain the required antibody in the desired form while maintaining the activity of the paratope and effector function.
It is of enormous commercial interest to optimise the downstream processing of monoclonal antibodies, as with such valuable products small losses in yields are costly. Protein is lost at each step of a purification procedure, therefore to maximise yield, the number of steps used should be kept to a minimum. However, by reducing the number of steps, the purity (i.e. the percentage monoclonal antibody of total protein), is also reduced. Some methods of purification give higher yields than others, depending on the starting material used and the product to be obtained. Hence, often a purification strategy must be designed for an individual protein. This section discusses the steps involved in obtaining product purity, the choices of operations which may be used in each step and the need to select appropriate combinations of operations to obtain optimal purity.
The design of downstream processes for protein purification and for monoclonals in particular, has been recently reviewed (35,36). However, traditionally these processes have not included operations which deal specifically with removal or inactivation of viruses and DNA; processes which could be used to address these saftey issues will be discussed in later sections.

A. FACTORS WHICH INFLUENCE CHOICE OF A PURIFICATION STRATEGY

(i). PHYSIOCHEMISTRY OF THE IMMUNOGLOBULIN

Knowledge of some of the physicochemical properties of the monoclonal antibody aids the choice of a purification process, as the most effective process would be one which differentiates most between the physicochemical properties of the end product and other proteins, especially other immunoglobulins (Igs). When

high purity products are required, i.e. therapeutics, it is necessary to use a combination of steps which separate using different characteristics.

The characteristics most often exploited in protein separation are:

1. Molecular weight (gel filtration)
2. Isoelectric point (ion-exchange chromatography)
3. Solubility (precipitation, chromatography)
4. Hydrophobicity (hydrophobic interaction chromatography)
5. Affinity to a specific ligand (affinity chromatography)

Although immunoglobulins within the same class have similar molecular weights the other characteristics listed vary even within the same sub-class. Different variable and hypervariable regions lead to differences in the iso-electric point of a protein and its solubility, hydrophobicity and charge density. Studies have shown that the iso-electric point of murine monoclonal antibodies ranges from 4.9-8.2 (37).

(ii). CONTAMINANTS IN THE STARTING MATERIAL

To differentiate between the product immunoglobulin and contaminants to be removed, it is useful to know as much about the contaminants as possible.
The starting materials may be categorised as follows:

***in vitro:* Fermenter Broth**

Hybridomas are grown in tissue culture medium in a wide range of vessels and conditions, so that the concentrations of cells, monoclonals and contaminants vary, but the nature of the major contaminants will remain similar and depend on the growth supplement in the media which can be serum, chemically defined serum free media or low serum media.

Major Contaminants in Cell-Culture Broth:

1. *In Fetal Calf Serum (FCS):*
   - Albumin
   - Bovine Immunoglobulins
   - Transferrin
   - Pyrogens

2. *In Serum Free Media (SFM):*
   Transferrin
   Insulin

In vivo: **Ascites**
Monoclonal antibodies can be produced in the peritoneal cavity of mice as ascites, which is an exudate of plasma.
Monoclonal antibody is present in concentrations which range from 3-15g l^{-1} quantities in mice, approximately 16-30% of the total protein.
The major contaminants associated with ascities are:
lipids, albumin, host non-hybridoma Igs, nucleic acids and viruses.

(iii). DEGREE OF PURITY REQUIRED

The degree of purity is attributed to the percentage of active monoclonal antibody of the total protein. Because of difficulty in measuring activity, purity is usually quantified as the ratio of the concentration of monoclonal antibody to the concentration of the total protein present. To maintain activity, harsh treatments such as extreme elution conditions must be avoided as antibodies are proteins and easily denatured.
The degree of purity required depends on the intended use of the final product. For example, diagnostic kits require crude purification and the use of expensive chromatography separations may not be required. However, therapeutics require a high degree of purification and guidelines have been issued, by several different government health agencies (38,39,40), which suggest the various quality control procedures that should be adopted in testing for contaminants and the level of contaminants which are acceptable. These guidelines are disscussed in more detail in section 2.2.1. The major potential contaminants to be considered include: viruses, nucleic acids, proteins, process chemicals.

B. COMMON STRATEGIES USED IN PURIFICATION OF MONOCLONAL ANTIBODIES FOR THERAPEUTIC USE

Many methods for the purification of monoclonal antibodies intended for therapeutic applications have been reported (41-47). These are based on the following sequence of events:

(i). CELL SEPARATION/ CLARIFICATION

Clarification is used to separate insolubles from the surrounding fluids. The most common operation used for this process is centrifugation. Cross-flow filtration (CFF) is being used increasingly as an alternative to centrifugation. In CFF, developed from conventional dead-end ultrafiltration, the cell suspension flows parallel to the membrane at such a velocity that the deposition of cells on the filter medium is kept to a minimum. The advantage of this system is that the filtrate may be recovered with cell containment and avoidance of aerosols, also, it is easy to scale-up and is a cheaper alternative to centrifugation. The main problem to date is that proteins tend to adsorb to the materials currently used as filters. Centrifugation which results in a much higher yield is harsher on the cell and disruption of these sensitive animal cells may cause contamination of the supernatant with intracellular material. Although analysis of the cells prior to and after centifugation shows no loss in viability undoubtedly some cell lysis occurs.

(ii). CONCENTRATION

A concentration step usually follows clarification. The main contaminant, i.e. water, is removed in order to aid the subsequent purification steps. Concentration results in a decrease in culture volume as well as an increase in purity. This step may not be necessary if a process has been used which involves high density cell culture and continuous removal of concentrated product, or if the starting material is ascitic fluid. Ultrafiltration is usually the process of choice. A membrane retains larger molecules and removes smaller molecules such as water and salts. Products can be concentrated up to 50 times giving high yields.

This process can incoporate a conditioning process, such as buffer exchange, to prepare the fluid for subsequent purification steps if required.

At small scale ammonium sulphate precipitation is commonly used as an initial concentration/ purification step. This involves the precipitation of protein from the starting material. This process is not suitable at large-scale as large amounts of salt are required, creating waste disposal problems. Also it can be difficult to remove the salt completely from the protein.

An alternative and novel process which can be used as a combined concentration and purification step is the use of an aqueous two-phase system; i.e. the use of two polymers or a polymer and a salt that when mixed together form two phases in an aqueous solution. This system provides mild conditions for bioseparation. The distribution of a substance between the phases is described by the partition coefficient which can be manipulated by altering various physical parameters or by binding a ligand to one of the polymers to achieve affinity partitioning. Aqueous two-phase systems have been used at both research and industrial levels to separate proteins from cell debris, to purify, to concentrate, or a combination of these processes (48).

(iii). PURIFICATION

Ion exchange chromatography separates proteins from a mixture according to their iso-electric point and charge density.
Anion exchange has been used largely at research scale and has been adapted for HPLC with typical yields of 95 %.
Cation exchange is more suitable for large scale since buffer conditions can be manipulated so that the amount of contaminants initially bound to the matrix are minimised so that high resolution chromatography is not necessary.

Gel filtration separates proteins according to their size. This method is commonly used for separating IgM from other globular proteins as IgM is significantly larger than other immunoglobulins and albumin.

Affinity chromatography involves the purification of a protein which has an affinity for a ligand which is immobilized to a solid matrix. This type of separation is used widely in antibody purification. Protein A, a protein which can be readily extracted from the cell walls of *Staphylococcus aureus*, binds specifically to the Fc region (effector function) of human immunoglobulin class IgG1, 2, 4, some IgM and IgAs; it binds to murine immunoglobulin class IgG2a, 2b and 3 and weakly to murine IgA and IgMs (49). Protein A has therefore become a useful tool in antibody purification. Protein G, a cell surface protein of group G *Streptococci* complements protein A in that it can bind human IgG3, rat IgG2a and 2b (Protein A does not bind to these

classes) as well as some other immunoglobulins. Protein G does not have an advantage over protein A for separating murine immunoglobulins.
An alternative ligand which can be used in affinity chromatography is the antigen of the corresponding antibody, although in some circumstances this may be too difficult or expensive to obtain.
The antibody is bound to the immobilized ligand, contaminating proteins are washed through the column and finally the antibody is eluted, from the column by reducing the pH of buffer.
Affinity chromatography has the advantages of resulting in both high purity (>98 %) and high yield (>95 %).
Classically, protein A follows an initial purification step (often ion-exchange chromatography), which cleans the broth up sufficiently so as not to foul the expensive affinity column.

The solid-phase matrix used for affinity chromatography is generally an agarose gel with cyanogen bromide as the common reagent for coupling ligands. Recently ligands such as protein-A, have been immobilized onto a cellulose and acrylic matrix (50). The advantages of such matrices are their mechanical stability and fast flow characteristics compared with agarose gels.

(iv). FINAL POLISHING

This step is to ensure that the product is of the appropriate quality for its required application. This may involve the use of an additional purification step, (gel-filtration is often used for this purpose), although many procedures involving two step purifications have been described so theoretically this should not be necessary.

In recent times the definition of a therapeutically pure monoclonal antibody has changed as saftey issues have been taken into consideration. The reasons for these saftey considerations and how they may be dealt with are discussed in the second half of this literature review. The conditions which dictate whether a monoclonal antibody is of sufficient purity to be used as a therapeutic are described by various governmental health authorities (38,39,40). Cell-line characterisation both before and after representative production and control of viral entry

from raw materials by routine testing are included in recommendations for prevention of viral contamination. Impurities which must be tested for in the final product include: DNA residues, pyrogens, protein A and other leachable components of affinity columns, aggregates, BSA and other non-Ig proteins and viruses.

2.1.4 IN SUMMARY

It must be emphasized that the steps involved in the production and purification of monoclonal antibodies have tended to be viewed in isolation; there are many options available for carrying out each unit process, each of which is complex and offers certain advantages over the alternatives, but to optimize an overall process the stages must be integrated and the number of stages to produce the desired product must be minimised.

In the following section the potential sources of viral contamination are reviewed and processes for inactivation of viruses and their application to large-scale processes are discussed. From guidelines published describing quality control procedures recommended in the preparation of monoclonal antibodies for therapeutic use, it is felt that manufacturers can bypass essential inactivation processes and yet demonstrate a safe product, by viral removal. From the manufacturers point of view virus removal is a more preferable option than viral inactivation using chemicals, since the main disadvantage of using chemicals as inactivatants is that they require subsequent removal. From the literature reviewed it would seem that the only way to be certain of a safe product, i.e. virus free, is to incorporate a step which inactivates all known viruses. It is the role of the biotechnologist to design such a step to integrate into the overall production process and it is the responsibility of the manufacturer to implement it.

2.2 VIRAL CONTAMINATION IN MONOCLONAL ANTIBODY PRODUCTION

Stringent purification procedures are required if monoclonal antibodies are to be administered for therapy, *in vivo* diagnosis or vaccination. The contamination of blood products, e.g. coagulation factors such as factor VIII preparations used for treating

haemophilia with the human immunodeficiency virus (HIV) and transmission of hepatitis C virus (HCV) to some patients receiving intravenous immunoglobulin, has focused attention on the risks inherent in obtaining therapeutics from biological sources; also the presence of retroviral particles in many hybridoma cell-lines has raised concern regarding the safety of monoclonal antibody preparations.

The first biologicals to be administered extensively for *in vivo* applications were viruses for vaccination and blood products for therapy.
Infectious diseases which have resulted from some of these products, as a consequence of either incomplete inactivation of viral vaccines or of contaminating viruses in the biological product include:

a) Outbreaks of polio and foot and mouth disease resulting from inadequate inactivation of viral vaccines (51).

b) Outbreak of allergic encephalitis caused by contamination of rabies vaccine with the adventitious virus from the cell substrate used in manufacture (51).

c) Transmission of infectious viruses, i.e. HIV, HBV and HCV to recipients of contaminated whole blood or its' products used for transfusion or therapy (52).

Although in these cases, use of complete inactivation procedures has meant that the products are now safely administered routinely, if this action had been taken previously the epidemics would not have occured.

Contamination of a new host cell with an infectious agent which has transferred from another species is a process which has increased in recent decades (53). There have been several examples of cross-species transfer of infectious agents this century, which have been well documented. The most recent of these has been **Bovine Spongiform Encephalopathy (BSE)**: a disease which appeared in cattle, on farms throughout Britain in 1985. Because the source and mode of transmission of the agent was not immediately realised, a wide-spread epidemic occurred causing a disease widely known as "mad-cow disease", because of its effect on the brain. The agent is similar to those which cause Scrapie in sheep and kuru and Creutzfeldt-Jakob in humans. It causes similar symptoms: infecting the brain it leads to progressive dementure and inevitable death

and no immune reaction is produced. It is widely believed that BSE was transmitted to cattle via scapie-infected sheep feed (54,55). If BSE was transfected to humans it not unlikely that a disease similar to Creutzfelt-Jakob disease would result.
Canine Parvovirus (CPV) appeared in 1977 and caused worldwide enteritis and myocarditis in dogs within two years. CPV is genetically very similar to feline panleukopenia virus (FPLV). A modified live FPLV vaccine has been used to inoculate cats, however CPV is more similar to the modified FPLV vaccine than the original virus, leading many virologists to believe that the modified virus could adapt itself to replicate in canines.
These examples go to illustrate the devastating effects that a "new virus" may have on an animal population.

The spread of BSE in cattle in Great Britain has been attributed to the contamination of cattle feed contaminated with prions which cause scapie in sheep; it is not known whether the agent causing BSE in cattle is the same as that causing scrapie or whether the infection has resulted from the selection of a mutant scrapie strain crossing the species barrier. However, it has been demonstrated, in the laboratory, that BSE can infect other species.
Cell culture involves the use of fetal calf serum (FCS) or serum free media (SFM) as a growth supplement. Bovine Viral Diarrhoea Virus (BVDV) is the primary contaminant of FCS, some claim that it contaminates 100 % of FCS batches. It is not easily detected because it is not cytopathic and it is neutralised by antibody present in FCS. Components of serum-free media are of bovine origin, so both of these products carry the risk of being infected with bovine viruses such as the very infectious Bovine Rhinotracheitis Virus (BRV). BVDV and mycoplasma have been found to be contaminants of transferrin although not specified by the manufacturer (56). Although all FCS used for cell-culture is now carefully screened for possible contaminating viruses, there will always be a risk of contamination of cell-culture products (through either FCS or other substrates) with "unknown viruses" unless a complete viral inactivation process is incorporated into the overall production process.

The final sections of this chapter reviews the cell-subtrates used for monoclonal antibody production with particular reference to

their viral contaminants. Methods for removing and inactivating viruses and the application of these methods to monoclonal antibody preparations are discussed.

2.2.1 CELL SUBSTRATES USED IN MONOCLONAL ANTIBODY PRODUCTION

Continuous cell-lines are increasingly being used to produce biologicals for a variety of therapeutic purposes (e.g. anti-tumour therapy, immunomodulation, immunization, *in vivo* diagnostics).
Examples of products which have been emerged from the use of animal cell technology are: tissue plasminogen activator, growth factors, erythropoietin and monoclonal antbodies.

The two commonly used technologies which involve the use of animal cell culture for the production of proteins are: monoclonal antibody production by cell hybrids and genetic-engineering which involves transfecting animal cells with manipulated DNA sequences.

The cell-lines resulting from these technologies either originate from tumour cells (hybridomas) or acquire tumour cell characteristics upon establishment (genetically transformed cells), so raising major safety concerns (57,58), i.e.:

a) the possibility of infecting patients with cellular DNA which may code for tumour producing proteins

b) the presence of retroviral particles or viral nucleic acids which have been shown to infect some cell-lines (including hybridomas)

Table 2.1, on the following page, lists viruses which should be considered as potential contaminants in the manufacture of monoclonal antibodies. Viruses for which evidence exists of a capacity to infect man or primates are to be found in Group I. Those viruses for which there is no evidence of infection in man but which could nevertheless pose a potential danger, for example in immunosuppressed patients, are listed in Group II. The table was obtained from the European guidelines for the production of therapeutic monoclonal antibodies (39).

Group	Virus	Species affected
I	Hanta virus (haemorrhagic fever with renal syndrome)*	M, R
	Lymphocytic choriomeningitis virus (LCMV)*	M
	Rat rotavirus*	R
	Reovirus type 3 (reo 3)*	M, R
	Sendai virus*	M, R
II	Ectromelia virus*	M
	K virus (K)	M
	Kilham rat virus (KRV)*	R
	Lactic dehydrogenase virus (LDH)	M
	Minute virus of mice (MVM)	M, R
	Mouse adenovirus (MAV)*	M
	Mouse cytomegalovirus (MCMV)	M
	Mouse encephalomyelitis (MEV, Theiler's or GDVII)	M
	Mouse hepatitis virus (MHV)	M
	Mouse rotavirus (EDIM)	M
	Pneumonia virus of mice (PVM)*	M, R
	Polyoma virus	M
	Rat coronavirus (RCV)	R
	Retroviruses*	M, R
	Sialodacryoadenitis virus (SDA)	R
	Thymic virus	M
	Toolan virus (HI)*	R

Table 2.1: Potential contaminants in the manufacture of monoclonal antibodies (39).

M: mouse; R: rat; *Known to be capable of replicating *in vitro* in cells of human and monkey origin.

A. MURINE HYBRIDOMAS

It was not until 1982 that the presence of retroviruses in mouse hybridomas was first reported (59,60). At that time monoclonal antibody preparations were already being used to treat cancers in patients (61,62,63). Since then many investigators (64-68) have raised warnings about hazards of cross-species infections involved in the use of monoclonal preparations.

The viral hazards involved in the use of rodent monoclonal antibody preparations have been well reviewed by Carthew (66), who described the potential contaminating viruses, including those which may be found in the peritoneal cavity of mice and so contaminate ascitic fluid. Problems have been recognised by various health authorities who have issued guidelines for the production of antibodies intended for therapeutic use, e.g. *"Points to consider in the manufacture and testing of monoclonal products for human use"*, issued by Office of Biologics, FDA, June 1987; *"Guidelines on the production and quality control of monoclonal antibodies of murine origin intended for use in man"*, Commission of the European Communities Committee for Propriety Medicinal Products, 1988; *"Guidelines for the production of monoclonal antibodies intended for therapeutic use"*, the Australian national standards laboratory, Department of Community Services and Health, 1988 (38,39,40). These guidelines discuss safety concerns including viral contamination of cell-lines as well as contamination of extraneous DNA (which may contain oncogenic sequences) and proteins (which may be antigenic).

In the European Guidelines, 22 murine viruses are listed as potential contaminants and 24 are listed in the Australian Guidelines. They include: oncogenic retroviruses, e.g. murine sarcoma virus, infectious zoonotic viruses, such as Hantaan virus and Lymphocytic Choriomeningitis Virus (LCM) and murine viruses, which, although are not known to infect human cells, could be health risks to unhealthy and particularly immunocompromised recipients.

Reports of hybridomas containing retroviruses (viruses which induce tumours in animals) (59,60,69,70) and, more specifically, the infectious retroviral type C particles (71,72), have been published. Bartal and Hirshaut (73) have reviewed the nature of retroviruses and retroviral type particles and their abundance in many cell-lines studied. Retroviruses are spherical structures

about 100 nm in diameter which contain a core electron-dense region enclosed in an outer envelope or unit membrane. Some retrovirus surfaces are characterised by spike-like projections consisting of glycoproteins. Retroviral morphology is classified as A-type, B-type and C-type particles, as termed by Bernhard (74). The main properties of A-type and B-type particles is their lack of infectivity while C-type particles are infectious. Bartal and Hirshaut found that about two thirds of all cell-lines studied, including a myeloma (NS-1) cell-line, contained retroviruses resembling A-type and C-type.

Weiss (75) observed that cells derived from fusions of NS-1 were full of A-type particles but released no detectable C-type particles, whereas 12 out of 17 hybridomas released large quantities of C-type particles.

Lubinieki (76) has reported that at least twenty percent of murine hybridoma cell-lines express one or more families of retroviruses and similar findings have been found by Rozman (69).

B. HUMAN MONOCLONALS

Conventionally monoclonal antibodies have been produced from rat or more commonly mouse hybridomas. Hybridomas are produced by the fusion of mouse myeloma cells and B-lymphocytes from the spleen cells of an immunized mouse. Many monoclonals have been produced in this way, largely for diagnostics and immunoaffinity chromatography. The use of rodent monoclonals in therapy is limited due to the foreign nature of the antibody which causes an allergic response in the recipient. Also rodents do not synthesize effective antibodies to some human antigens, such as Rh blood group antigens. For this reason there has been great interest in the production of human monoclonals.

A variety of techniques have been employed for the production of human monoclonals (77-80). The methods used to date include: fusion of human myeloma cells with the spleen cells from an immunized donor, fusions using heteromyelomas, i.e. cells resulting from the fusion of human and mouse myeloma lines to improve fusion characteristics, fusion of mouse myeloma cells with spleen cells from immunized donors and finally immortalization of antigen specific human lymphocytes using the Epstein-Barr Virus (EBV) (81). The latter method has been the most successful in producing stable antibody secreting cell-lines, this method also carries the obvious advantage of

using more readily available human cells. The product of the transformed lymphocyte is a human lymphoblastoid cell-line.
Viruses can contribute to the development of human tumours by a variety of mechanisms and immunosupression, (e.g. as a consequence of HIV), clearly increases the risk for specific tumours as an indirect result of viral infection. Zur Hausen (82) describes the viruses which cause human tumours and the mechanisms of infection; he notes that EBV has been linked to at least four different types of cancers and postulated that it is linked with others. Other tumour causing viruses which may be present in human cell-lines include a number of adventitious viruses which are known to be capable of replicating in human lymphocytes, i.e., Herpes 6, Cytomegalovirus (CMV), Human T-Cell Lymphotropic Virus (HTLV) as well as Epstein Barr Virus (EBV). In addition sequences of HIV-1 and -2, and HBV could be present in the genome of a lymphblastoid cell-line and these should be tested for.
Regulatory bodies require that the viruses are tested for in the final product and some authorities, e.g. the European Economic Community (EEC) and Australian health authorities recommend that viral inactivation steps should be included in the processing of products derived from continuous cell-lines. However, the risks associated with products from cells which have been transfected with viruses (83) and alternatives have been explored (84).

C. GENETICALLY ENGINEERED MONOCLONAL ANTIBODIES

Increased knowledge of the immune response and immunoglobulin structure has led to an interest in "engineering" antibodies to improve activity and to reduce adverse responses in the recipient of such products.
Because of difficulty in dictating the constant region of human monoclonals, recombinant methods used have involved linking human constant region genes to mouse variable domains and, taking this process further, "humanised" variable domains can be created by transplanting the hypervariable region of a mouse antibody into the framework of a human antibody (85,86). These novel chimaeric antibodies have one obvious major advantage over other monoclonal antibodies for therapy, i.e. reducing the HAMA response in patients.
This technology could also be adapted to create novel effector functions by replacing the Fc region with an enzyme for

diagnostics or toxins for "magic bullets" in cancer therapy, so increasing the potential applications for monoclonal antibodies significantly. The rat anti-CAMPATH-1 mab (87) has been the most successful therapeutic mab humanised to date; it has been used successfully for the therapeutic control of graft versus host disease in patients receiving bone marrow transplants.
These recombinant products can be produced by human cells, however they have genes of murine origin so that, with respect to viral contamination, they have problems associated with both murine and human derived monoclonal antibodies. Chinese Hamster Ovarian cells (CHO) have also been used as expression vehicles, there is no data available comparing the production efficiency of these cell-lines. Virus-like particles resembling type A and type C have been observed in CHO cells (88,89,90). Lubiniecki has noted (91) that although about 20 % of the hybridomas tested in one series expressed infectious endogenous retroviruses, many studies of CHO cells used for many different constructions of recombinant DNA products have failed to demonstrate any biological or biochemical activity in a battery of tests, despite the presence of retrovirus-like particles; he nevertheless states the importance of virus inactivation of removal steps in downstream processing of biologicals derived from cell-culture.

D. ANTIBODIES PRODUCED IN PROKARYOTES

Traditionally monoclonal antibodies have been produced in mammalian cells of lymphoid origin because of their inherent ability to assemble and secrete the active protein. It has been known for sometime that *Escherichia coli* and yeast cells can produce small functional immunoglobulins, but their inability to secrete, modify (e.g. glycosylate) and fold the protein into its complete structure has inhibited their use for routine production of antibodies.
Recent work carried out by McCafferty *et. al.* (92) has involved the construction of an artificial immune system gene library from which human antibodies of virtually any specificity can be made without the need of using an immunised animal. The procedure uses a phage selection system. Fragments of antibodies are displayed on the surface of bacteriophage and these fragments fold correctly and bind antigen. Repertoires of antibody genes are amplified using PCR and cloned into phage so creating a large library of phage each displaying a specific antibody. Each phage

expresses an individual heavy- and light-chain combination (fab). Antibodies are selected using antigen ligand affinity chromatography and by using the polymerase chain reaction (PCR) to produce libraries of variable heavy chain (VH) domain genes, VH domains can be expressed and secreted by *E.coli* (93). Human constant regions can be linked to the fabs using the technology described previously (section 2.2.1 C), using mammalian cells to express selected antibody. Mammalian cells are used since bacterial systems lack the ability to fold and glycosylate the polypeptides which are responsible for giving the Fc region its functionality; also incomplete glycosylation is known to induce auto-antigenicity. Given the success of the CAMPATH-1 mab (87), expressed in CHO cells (section 2.2.1 C), there is no reason to suppose that therapeutic mabs obtained using this technology would be any less functional; their main advantage being the speed of raising and screening antibodies. Because the prokaryotic cell lacks the ability to express the whole antibody in a form which could be used therapeutically, its use is limited to the production of single domain antibodies which have no applications unless modified synthetically. Therefore CHO cells will not be replaced as cell vehicles for genetically-engineered antibodies in the foreseeable future.

2.3 VIRAL INACTIVATION AND ITS EFFECTS ON THE BIOLOGICAL ACTIVITY OF ANTIBODIES

2.3.1 THE STRUCTURE OF IMMUNOGLOBULINS

A viral inactivation procedure used in monoclonal antibody preparations must not only be a complete one but must have minimal denaturing effects on the antibody molecule.

The primary function of antibodies is to recognize and bind foreign components; the components of antibody responsible for this function are known as fab's (fragment antigen binding). In addition, many antibodies have secondary biological activities which are usually mediated through the Fc region of the molecule, by mechanisms which are not fully understood. The antibody-antigen complex binds to a cell carrying the corresponding Fc receptor on its membrane usually stimulating phagocytosis and cytotoxic events but also certain secretory responses. Specificity for the Fc receptor occurs within class and subclass.

Immunoglobulins are multichain glycoproteins, basically of tetrapolypeptide structure. They are of five classes: IgG, IgM,

IgD, IgA, IgE; there is a certain amount of homogeneity within each class but variations within the two reactive parts of the molecule, ie the parotope (the region which complements the epitope) and the effector function or Fc region. They consist of two identical halves which are stabilized predominantly by di-sulphide bonds. Each half is composed of two polypeptide chains, designated as heavy and light chains because of their relative molecular weights. The light chain consists of a variable domain (V_L) and a constant domain (C_L). The function of V_L is to bind antigen, no function has been attributed to the C_L domain. Certain regions of the variable domain have greater variations than the remainder which are termed hypervariable domains, these regions are the basis of of the diversity of immunoglobulins. The constant region consists of a variable domain (V_H) and three constant domains: C_H1, C_H2 and C_H3.

The locations for the Fc receptor binding sites within the Fc region have not been found and there is a considerable amount of data presenting conflicting evidence regarding this region. However, evidence suggests that different Fc receptors recognize distinct sites on Fc and that recognition is dependent on the presence of the C_H2 and C_H3 domains together, as the conformation of one is dependent on the presence of the other.

IgG accounts for over 70 % of the total immunoglobulin present in normal human sera. It is a monomeric protein with four sub-classes (IgG1-IgG4) and is the major antibody of secondary immune responses and the exclusive anti-toxin class.

IgM accounts for about 10 % of the total immunoglobulin present in normal sera. It forms a closed pentameric ring structure, it is considered to have two sub-classes which have not been characterized and it is the primary response to immunization. It is able to fix complement more efficiently than IgG. Heat aggregation destroys its ability to fix complement C1, whereas chemical cross-linking does not.

IgA is a dimeric protein found mostly in endocrine secretions. Its major role is to inhibit penetration of organisms into the epithelium and it is also very efficient at neutralizing viruses.

IgD and IgE are trace immunoglobulins and the role of these immunoglobulins is not completely clear.

The vast majority of monoclonal antibodies produced are of IgG and IgM classes. Retention of active sites within the antibody

molecule is necessary during its processing, this includes activity specific to a particular immunoglobulin and also activity common to its class or subclass.

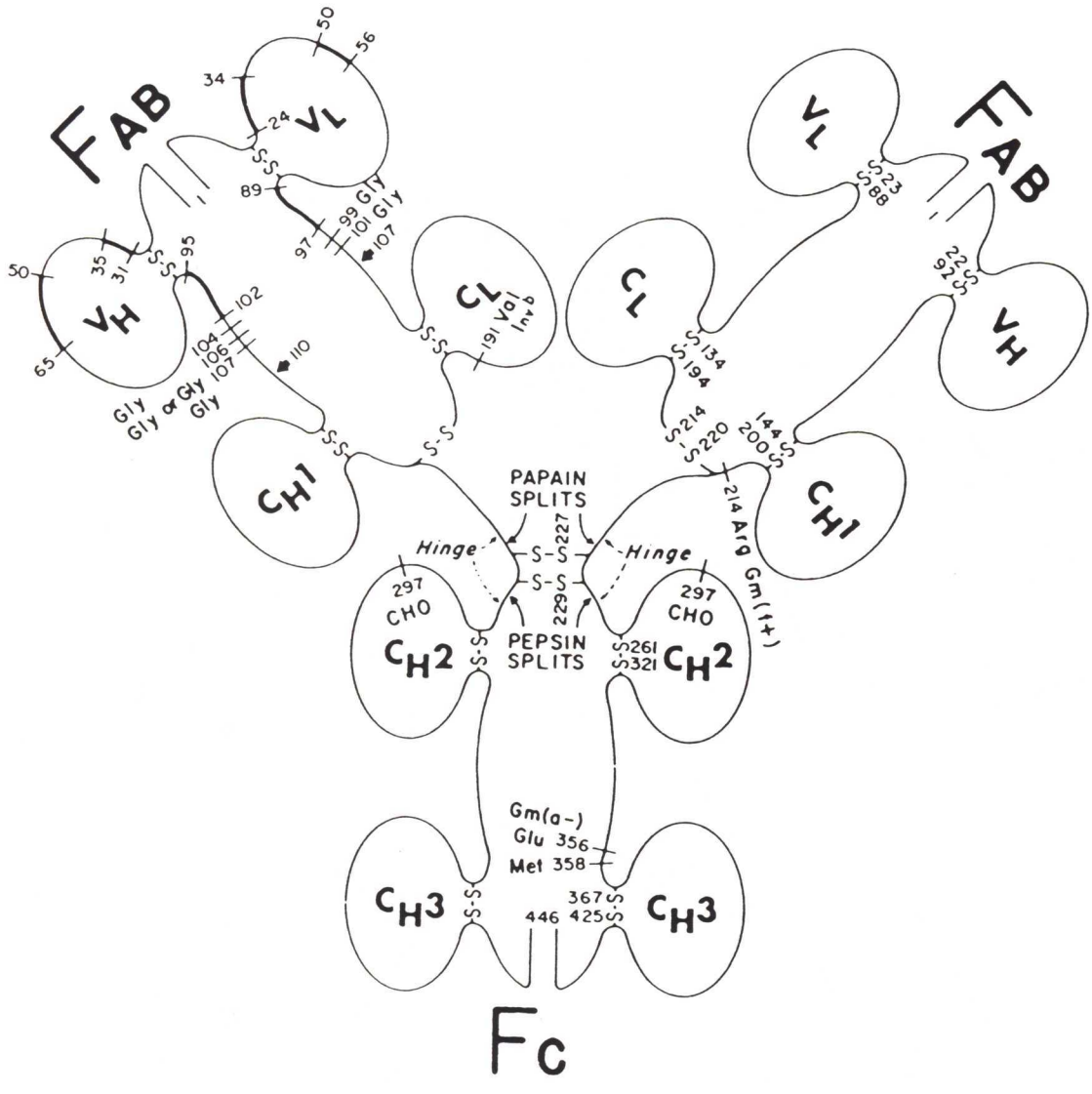


Figure 2.3: Schematic representation of a human IgG1 antibody (94). The numbers on the right-hand side denote the actual residues of protein Eu. Hypervariable or complimentary-determining regions are shown by heavy lines. The hinge region, in which two heavy chains are linked by di-sulphide bonds, is indicated approximately. The attatchment of carbohydrate is at positon 297. Arrows at positions 107 (in the light chain) and 110 (in the heavy chain) denote transition from variable to constant region.

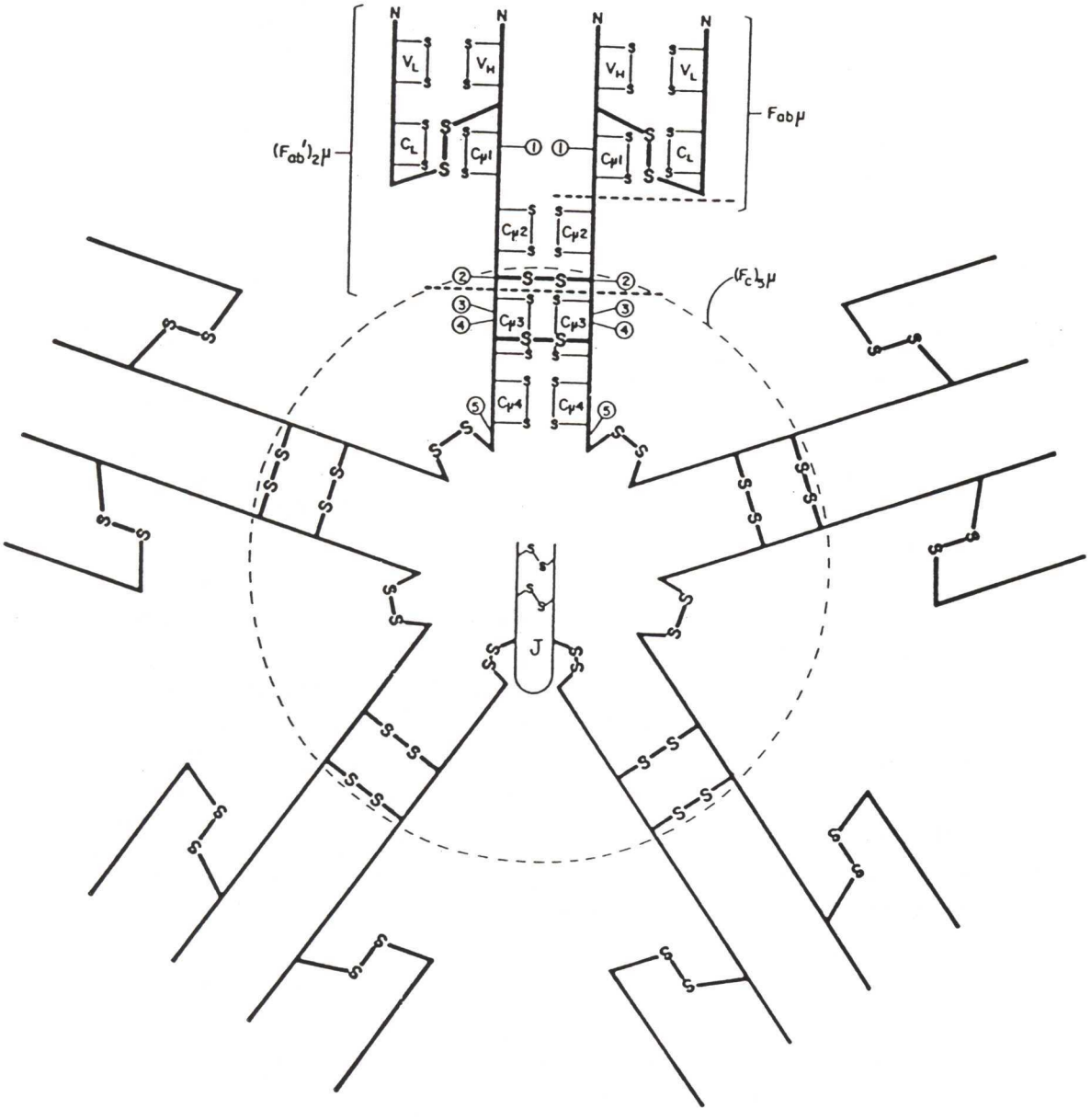


Figure 2.4: Schematic representation of the pentameric structure of IgM antibody (95). Linkage between IgMs and J chain involve the penultimate cysteine on the μ-chain. Positions of the five oligosaccarides (circled numbers) and the five u-chain domains are shown. Cleavage by trypsin at elevated temperatures yields Fab μ and $(Fc\mu)_5$ as indicated, whereas at 37 °C only $F(ab')_2$ is obtained.

2.3.2 ASSESSMENT OF ANTIBODY ACTIVITY

Affinity is defined by the strength of antigen binding to a single antibody combining site (Fab) and it is described by the equilibrium constant (K)

of the reaction:

$$K = Ab + Ag \rightarrow AbAg$$

given by the mass action equation:

$$K = \frac{[AbAg]}{[Ab][Ag]}$$

where:

$[Ab]$ = concentration of free antibody

and:

$[Ag]$ = concentration of free antigen

The term avidity describes the ability of antibodies to bind antigens and depends on not only affinity but also multivalency and other non-specific factors, e.g. fluctuations in temperature, ionic strength and composition.
Because of the multivalent nature of IgM it is common that the avidity of this molecule is 10^2 - 10^4 higher than the affinity of the isolated sites (its Fab fragments). Slight fluctuations in temperature, ionic strength and composition can cause changes in antibody avidity.

There are a plethora of assays available to test for the purity of antibodies in terms of functional activity; these include Enzyme linked immunoassays (EIA), Radio-immunoassays (RIA), haemagglutination and haemolysis assays. It is unfortunate that none of the assays answer directly the questions posed with respect to product purity. The fact that the antibody-antigen interaction is due to a virtually unlimited variety in the composition of the paratope leads to a variety of different physiological forces which are involved in binding, so that a large number of different affinities and cross-reactions result.

The assays do have limitations with respect to reproducibility of the actual physiological antibody-antigen interaction as immobilization of the ligand to the solid substrate is likely to result in steric hindrance of binding sites and reduced avidity of antibody. Despite these limitations immunoassays have been widely used as the most useful models of antibody reactions.

2.3.3 METHODS FOR INACTIVATING OR REMOVING VIRUSES

A. REMOVAL OF VIRUSES

Separation of viruses would be an ideal process to incorporate into a procedure for antibody purification.
Chromatograhic operations such as gel-filtration, ion-exchange and affinity chromatography have been used and demonstrated to remove viruses by 3 logs (96, 97).
Cross-flow microfiltration processes using polyamide membranes have been used successfully for concentration of viruses in biotechnology. However, because proteins tend to adsorb to these membranes, such a process, when feeds of high protein concentrations are applied, results in reduced flow rates due to gradual contamination of the membrane. Hence, these operations have been unfavourable due to loss in yield during the process and inability to maintain a sufficiently high flow rate across the membrane.
Recently reports from Millipore, Amicon and Pall of the use of UF membranes to remove viruses have emerged (98,99,100). The most successful system has been reported very recently (101), it uses a composite membrane composed of a pre-formed microporous membrane with a thin finely porous retentive layer; clearance of 4 to 6 logs removal of virus particles in the range of 30-70 nm has been demonstrated. This is sufficient to remove all but very small viruses, such as the parvoviruses (18-26 nm).

B. INACTIVATION OF VIRUSES

An ideal process for the inactivation of viruses in monoclonal antibody preparations would inactivate all types of viral contaminants. The process chosen must inactivate the broadest spectrum of known viruses. The products from an inactivation procedure must be non-toxic and easily removed. The process must not effect the overall biological activity of the end-product. In addition the process must be easily incoporated into the

manufacturing process and be compatible with it. This section describes the processes which have been used for viral inactivation and their suitability for the preparation of therapeutic monoclonal antibodies.

(i). PHYSICAL INACTIVATION OF VIRUSES

Heat has traditionally been a very effective means of sterilization and methods of heat treatment for the inactivation of viruses in blood products have been developed (102,103). Pasteurisation procedures have been used successfully to inactivate HBV in albumin preparations, but more recently heat inactivation processes applied to more labile blood derivatives resulted in incomplete activation (104).

However, it has recently been shown that by increasing the stability of a monoclonal antibody preparation, ie by lyophilisation, it is possible to inactivate a particularly heat resistant virus (vaccina) by heating to 80 °C for 72 hours, while retaining its activity (105).

Low doses of ultraviolet light have been used in combination with chemicals (106), as mentioned in the following section, e.g. use of ßPL as chemical inactivant. Complete sterilization cannot be achieved by UV alone.

(ii). CHEMICAL INACTIVATION OF VIRUSES

1. FORMALDEHYDES

Formaldehyde has been used to inactivate a wide range of viruses in the preparation of vaccines. Its use led to tragic consequences in the 1950's due to incomplete or reversible inactivation of polio and foot-and-mouth disease viruses which were used for vaccination (107). These accidents led researchers to increase their attention on the mechanism of the inactivation of viruses.

Formaldehyde reacts with almost all kinds of organic groups including amino groups. For complete inactivation to occur the inactivant must disrupt the nucleic acid. Gard proposed in 1960 (108) that over a period of time the polio virus becomes resistant to inactivation because of decreased permeability of the protein coat due to interactions of formaldehyde with the protein. Other explanations around that time, included aggregation of virus particles into resistant clumps and adsorption of virus onto the walls of the vessel. It is now

assumed that viral inactivation is usually characterised by two phases, with a fast first phase and a slow second phase and it is considered that the slow phase takes the form of a resistant fraction of virus aggregates (109); however little is known of the actual mechanisms operating. It is highly likely that no chemical inactivants (except enzymes) can react specifically with nucleic acids without affecting proteins, however, it is well known that formaldehyde is very reactive with amino acids, which is why it has historically been used as a fixing agent. It would therefore not be the inactivant of choice for the treatment of complex, labile proteins like monoclonal antibody preparations.

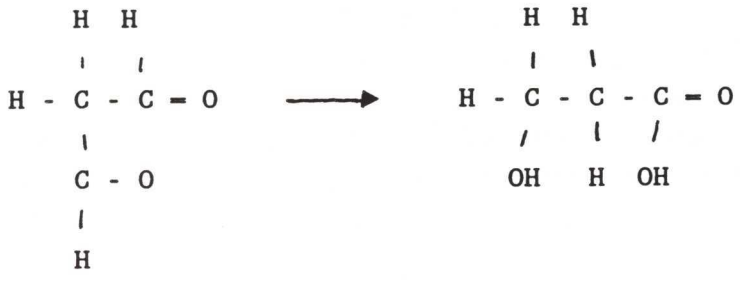
2. AZIRIDINES

The use of aziridines as an inactivating agent was initiated by Brown (109), as a consequence of incomplete inactivation of FMDV by formaldehyde. Acetylethyleneimine was an effective inactivant under certain conditions, when formaldehyde was not. Since that time aziridines have been used to inactivate other viruses including rabies and parvovirus (111, 112).

The advantage of their use is that the aziridines do predominantly attack nucleic acids and so apparently, would not react with amino acid residues or other components of the antibody. Also, a tailing effect has not been observed. However it is less effective with double stranded RNA viruses, where complete inactivation has not been achieved.

3. BETA-PROPIOLACTONE (ßPL)

Lo Grippo (113) introduced the use of ßPL as an inactivating agent, primarily for the inactivation of hepatitis B in blood and plasma. Since then, it has been used very widely and has successfully inactivated every virus tested to date. It was chosen by Lo Grippo's group out of 23 chemicals screened to be the most effective for inactivation and was considered safe for intravenous use. This is despite the fact that it is a considered a carcinogen in its pure form. Based on a ring structure, ßPL reacts strongly with nucleic acids as an alkylating agent. In water it breaks down to the non-reactive, non-toxic beta-hydroxypropionic acid:



The acidic product of this reaction needs to be converted to its sodium salt and the pH level altered.

ßPL is reactive with proteins however and has been used to block the complement fixing properties of gamma-globulins (114,115). It therefore can not be used for the treatment of blood products without affecting their functional activity. Lo Grippo proposed the combined use of ßPL with ultra-violet light. Each of these, when used independently, denature proteins, but their combined use is not so severe on these molecules although labile proteins can still be denatured. Furthermore, there is a marked decrease in the amount of ßPL required for sterilisation and an elimination of the "tailing effect" (see section 2.2.4) was observed when using UV light with ßPL. Stephan (116), Horowitz (117) and their colleagues have since used modified procedures which have been applied to the production of plasma products from blood.

It is important that proteins are not altered by inactivants as not only does this result in a loss in purity and hence yield but also immunogenic products may result.

4. TRI(N-BUTYL)PHOSPHATE (TNBP)

TNBP has been used in conjunction with a non-ionic detergent, tween 80, to inactivate viruses associated with the contamination of blood (118). However, TNBP only inactivates lipid-enveloped viruses, such as HIV, HBV and HCV, but it has no reported effect on the activity of proteins, which is why it was chosen for study in this project. This method has been used for the inactivation of viruses in preparations of Factor VIII, a large complex protein used for treating haemophilia.

5. UNSATURATED FATTY ACIDS

The use of two unsaturated fatty acids with sodium oleate has been reported for the inactivation of lipid-enveloped viruses (119). These agents were chosen because of their natural

occurrence and lack of toxicity. Although retention of the activity of a range of labile proteins (including immunoglobulin) was attained, inactivation was incomplete. The authors comment that a favourable balance between viral inactivation and retention of protein activity was achieved; however, inactivation must be absolute to be effective.

2.3.4 KINETICS OF INACTIVATION

There is little data describing specifically the kinetics of viral inactivation and there is little known about the mechanisms involved. The rate of death of organisms follows first order rate kinetics, so that rate of destruction is proportional to the exponent of time.

i.e.

$$\frac{N_t}{N_0} = e^{-kt}$$

where:

N_t = number of active organisms after treatment

N_0 = number of organisms present before treatment
t = time of treatment
k = reaction rate constant or specific death rate

Because this description implies that an infinite time period is required for complete inactivation to be achieved, it is usual to designate a sufficiently small number for N_t, so that the probability of contamination is considered negligible. For example if $N_t = 0.001$, the probability of an organism surviving is one in one thousand, so the probability of contamination is also one in one thousand. This is standard value commonly used for N_t when determining time taken for destruction of organisms.
Although thermal death rates do follow the above description, in the early days of vaccine inactivation, investigators found there were deviations when chemicals were used. These were first noticed when formaldehyde was used to inactivate preparations of foot-and-mouth disease virus (FMDV) and polio virus. A "tailing effect" was observed indicating incomplete inactivation of the

viruses and Gards' theory of reduced permeability (refer to section 2.2.3.2) is a widely accepted explanation for this phenomenon (108). The tailing effect has also been observed with the use of ßPL, however, when using a combination of ßPL and UV for inactivation the tailing phenomenon is dramatically shortened and the viral activity is reduced to zero (119).
A second deviation is the appearance of a shoulder prior to exponential inactivation. This can be explained by the "multiple-hit target theory", when a series of sub-lethal hits are required before the organism is killed (120), the mechanism by which the organism is killed is unknown. Ideal inactivation kinetics, characterized by linear death curves, describe the "single-hit mechanism", when modification of a single base within the nucleic acid is sufficient to completely inactivate the virus. Today it is generally accepted that virus inactivation is not a simple first-order reaction but is usually characterised by a fast "phase 1" and a slow "phase 2" and that incomplete inactivation occurs if the virus escaping the first phase of inactivation is resistant to subsequent steps (109). The resistant fraction may take the form of virus aggregates, however, little is known in detail of the mechanisms involved in viral inactivation.

ßPL and TNBP were selected for study in this thesis because they are the most commonly used viral inactivants in the manufacture of blood products and there is some literature associated with their use. However, their suitability for use with complex glycoproteins derived from cell-culture has not been previously studied and the work that follows was carried out to address this issue.

METHODS

3 METHODS

3.1 CHOICE OF MODEL CELL-LINES

The following cell-lines were chosen as models for inactivation studies as they secrete monoclonal antibodies of different sub-classes. They were raised by groups who kindly gave their permission for our group to use them for certain studies.

3.1.1 MOUSE IgM: IgM 1A(K) F11

A mouse hybridoma cell-line which secretes a mouse IgM class monoclonal antibody was raised by injecting capsular polysaccaride from Type III *S.pneumoniae* into balb/C mice (1 µg i.p.) and fusing the spleenocytes (4 days after immunization) with NS-1 myeloma cells. The cell-line had been cloned once. This work was led by Associate Professor Ray Kearney, Dept. Infectious Diseases, University of Sydney, (personal communication).

3.1.2 HUMAN IgG1: ALL BR1

A human cell-line has been raised by the immortalisation of anti-RhD lymphocytes from a human donor by the Epstein-Barr Virus. This cell-line secreted human IgG1 class immunoglobulin and was raised by Anne Fletcher, Red Cross Blood Bank, Sydney, (personal communication).

3.2 CLONING OF HYBRIDOMA CELL-LINE

The hybridoma cell-line donated for use in this project had been cloned once. The rapid loss of chromosomes by hybridomas after fusion and over a given period in cell-culture results in non-producing and producing variants from the same heterokaryon. As Goding observed (118), antibody production may take up 30-50 % of the cellular activity and cells not producing antibody divide more rapidly and overgrow the useful clones. So the objective of the cloning procedure is to obtain a population of cells which have derived from a single hybridoma to ensure that the antibodies secreted from the cell-line obtained are indeed monoclonal. It is generally considered that a very stable antibody secreting cell-line can be obtained by cloning a cell-line three times.
Cloning was achieved by "limiting dilution", hybridomas were seeded so that there would be one cell per two wells of a 96-well micro-titre plate. The viability of the cells before seeding was

>90 % and the growth medium used was DMEM + 20 % FCS (appendix I). The plates were incubated at 37 °C in a 7 % CO_2 humidified atmosphere. The plates were examined for cell-growth and viability regularly using an inverted microscope; in this way the cells could be distiguished easily. After 10 days supernatant was taken from the wells of the microtitre trays containing cells and assayed for antibody by ELISA. Cell-lines were frozen soon after cloning at -196 °C (liquid nitrogen).

3.3 CRYOPRESERVATION OF CELLS

Cells in the early exponential growth phase were taken and suspended in a cryopreservation media (growth media supplemented with 10 % dimethylsulphoxide) at a concentration of 5.10^6 - 2.10^7 cells ml^{-1}. The cells were dispensed into freezing vials in 1 ml aliquots. The vials were insulated (with cotton wool or polystyrene) to control cooling rate (approx. -1 °C.hr^{-1}), and placed at -18 °C for 2 hr and -70 °C for two hours before storage at -196 °C (liquid nitrogen).

3.4 PRODUCTION OF ANTIBODY FROM CELL-LINES

3.4.1 PRODUCTION OF MOUSE IgM ANTIBODY

Mouse IgM was produced using the following methods from the cloned hybridoma cell-line named: 1A/F11/D12/A3. The purity and yield of antibody produced by each method was assessed so that the best method for producing a sufficient amount of antibody for further experiments could be determined.

A. ASCITES

The hybridoma cell-line was grown as ascities tumours in Balb/C mice. Hybridomas were grown initially in cell culture. Cells were taken from tissue culture in their late logarithmic growth phase and injected into the peritoneal cavity of mice primed with Freund's incomplete adjuvant (10^7 cells.$mouse^{-1}$). After about ten days the mice were sacrificed; the skin of the peritoneum was swabbed with 70 % ethanol and then stripped back from the peritoneum. A small incision was made in the peritoneal wall and as much ascitic fluid as possible was withdrawn from the peritoneum. The ascitic fluid was centrifuged to remove cell

debris, red blood cells and blood clots; the supernatant was separated and stored at -20 °C for later analysis and purification.

B. BATCH FERMENTATION IN STIRRED TANK REACTOR.

Batch cultivation was carried out using a 1 litre (working volume) stirred tank reactor (LH fermentation, 500 series).

Preparation of Inoculum.

Cells were taken from a cell-bank, stored in liquid nitrogen (frozen at 3.10^6 viable cells ml^{-1}), and thawed in DMEM supplemented with 10 % FCS. The cells were scaled up from a 10 ml tissue culture flask to a 50 ml and then 100 ml and 200 ml Techne stirred batch vessel, (Corning). 10^8 cells were taken from the final vessel at > 90 % viability, and suspended in 100 ml of fresh medium.

Cleaning and Sterilization of the Bioreactor.

The vessel was cleaned thoroughly with pyroneg detergent and rinsed thoroughly with tap water and finally with cell-culture quality, pyrogen-free (Milli-Q) water. This procedure was repeated for the head-plate, impeller and ports.

All air-filters, silicone tubing, O-rings and seals were checked and damaged ones replaced.

Oxygen and pH probes (Ingold: polarographic p/322336110 O_2 and 9816 pH probes) were thoroughly rinsed with Milli-Q water. The oxygen probe was calibrated against distilled water, DOT 0% (water sparged with 100 % N_2 for 20 min.) and distilled water DOT 100% (water sparged with air for 20 min.). The pH probe was calibrated against pH buffer solutions of pH 4 and 7.

The oxygen and pH probes were fitted into the bioreactor headplate which was then fitted on to the reactor vessel. A clean, sterile 5 ml bijou sample bottle was fitted to the sample port, all unused ports were sealed and nuts were loosely tightened.

The bioreactor was autoclaved for 20 minutes at 121 °C (15 p.s.i.). The reactor was removed from the autoclave when cool and the nuts were secured tightly.

Addition of Medium and Inoculum.
The bioreactor was placed in a laminar flow cabinet and using aseptic techniques, 900 ml sterile DMEM with 5 % FCS (see appendix I) was introduced into the reactor through an addition port. 100 ml of concentrated suspended cells at 10^6 cells ml^{-1} @ >95 % viability was introduced into the reactor through an inoculum addition port.
The reactor was set on the bench and appropriate probe and impeller leads were connected. Water, thermostatically maintained at 37 °C using on/off control (± 1 °C), was introduced into the water jacket of the bioreactor. The impeller speed was set to 50 r.p.m.. DOT and pH was monitored via an FC-3 data system monitor, (Real Time Engineering Ltd.). The pH was maintained at 7.0 and the DOT at 10-40 %.

Monitoring the Cultivation.
Cell growth was monitored by aseptic sampling of the culture via the sampling port at 6 hr intervals. The samples were analysed for cell concentration and viability (using a Neubauser haemocytometer and trypan blue staining) and antibody (through ELISA, section 3.9.1 C).

Termination of Fermentation.
The batch fermentation was terminated after a period of 140 hours; at this point cell growth is in decline phase. The fermenter broth was collected aseptically in a laminar flow cabinet and then centrifuged at 2 000 r.p.m. @ 4 °C. The supernatant was separated from the cellular matter and stored at 4 °C.

3.4.2 PRODUCTION OF HUMAN IgG1 ANTIBODY

Since human cell-lines can not be grown in mice because of host incompatibility, the only method of production is by cell-culture. The following methods were used.

A. BATCH FERMENTAION IN STIRRED TANK REACTOR.

The human cell-line was grown in batch culture as for the mouse IgM cell-line (section 3.4.1 B). The growth medium used was Iscove's Modified Dulbecco's Medium (IMDM) suppplemented with 10 % FCS and 200 mM glutamine (see appendix I). IMDM is a modification

of DMEM containing additional amino acids and vitamins, selenium, sodium pyruvate, Hepes buffer and potassium nitrate instead of ferric nitrate. This medium has been used widely to support the growth of hybridomas.

B. FED-BATCH FERMENTAION IN STIRRED TANK REACTOR.

The lymphoblastoid cell-line was grown in cell-culture to produce monoclonal antibody against Rhesus D antigen. The medium used was IMDM supplemented with 10 % FCS, 200 mM glutamine and 10 mM glucose. The cells were taken from a cell-bank, stored in liquid nitrogen (frozen at 3.10^6 viable cells ml^{-1}), and thawed in the above medium with 0.5 % Condimed, a conditioned media containing cytokines for cell growth enhancement, (Boehringer). The cells were scaled up from a 10 ml tissue culture flask to a 50 ml and then to 100 ml and 200 ml stirred vessels (Techne). The cells were scaled up further and inoculated into a 1.5 l stirred vessel (Belco) at an approximate viable cell density of 30.10^4 cells ml^{-1}. The inoculum was incubated at 37 °C. Agitation was achieved using a magnetic stirrer. Cell broth was periodically withdrawn from the vessel and replaced with fresh media; a cell density of $200\text{-}230.10^4$ viable cells ml^{-1} was maintained and viability of 80-85 % of total cells.
The cells were separated from the broth by centifugation at 2 000 rpm (Beckman Superspeed, J2.22ME) and the supernatant was kept at 4 °C until required for analysis and further processing.

C. EXPRESSION OF GROWTH DATA

The growth of cells in the exponential phase of batch culture is given as:

$$N = N_0 . 2^x$$

where:

N = final cell number
No = initial cell number
x = number of generations of cell growth

The time taken for a population of cells to double in number during the exponential phase of cell growth is known as the "doubling time" and this is a common way of describing

exponential growth. The exponential growth phase of animal cells usually exibits a doubling time of 15-25 hrs and occurs between cell densities of approximately 10^5-10^6 cells ml^{-1}.

Specific growth rate describes the relationship between instantaneous rate of biomass increase at a given biomass concentration :

$$\mu = \frac{dN}{dt} \cdot \frac{1}{N}$$

Integrating:

$$\ln N = \ln N_0 + \mu_t$$

The specific growth rate μ(hr-1) is the gradient of the plot of $\ln N$ against t.

3.5 PURIFICATION OF MONOCLONAL ANTIBODIES

3.5.1 PURIFICATION OF MOUSE IgM FROM ASCITES

After a preliminary pre-treatment, the ascites containing IgM was applied to two purification procedures: gel filtration followed by protein-A affinity chromatography. Gel filtration was chosen as an initial step as it separates the large mouse IgM antibodies from smaller immunoglobulins and other proteins. Protein-A affinity chromatography was chosen as a second step as it separates Protein-A binding immunoglobulins (such as the monoclonal antibody IgM 1A F11) from a mixture, resulting in high purity and high yield.

A. DELIPIDATION

The lipids were removed from ascitic fluid by centrifugation; this was acheived using a Beckman superspeed centrifuge (model: J2.22ME) at 16 000 r.p.m., 5 °C for 20 mins. (9 000 g). After the centrifugation the clarified ascites was carefully removed from beneath the lipid layer and refrigerated until subsequent purification.

B. GEL FILTRATION: SEPHACRYL-300 COLUMN CHROMATOGRAPHY

Sephacryl-300 (Pharmacia) is a gel matrix consisting of a mixture of dextran and bisacrylamide which is used for high resolution separation of proteins between the range of 10^4-10^6 KDaltons.
This gel was obtained in a pre-swollen form. A slurry of about 80 % gel and 20 % tris (0.05 M)-buffered saline (0.5 M, pH 8) was degassed; this was used to fill 2 x 50 ml columns. The gel was allowed to settle and the columns were washed with approximately 3 column volumes of tris-buffered saline, pH 8.
The columns were used in series to improve resolution and were connected to a U.V. monitor and pen recorder (Gilson, series 202), as shown in figure 3.1.
2.5 ml of ascites was loaded on top of the column, the column was flushed through with buffer and 2 ml fractions containing proteins of similar size were collected using a fraction collecter (Gilson).
The fractions containing IgM were pooled and applied to a protein-A affinity column.

C. AFFINITY PURIFICATION: PROTEIN A-SEPHAROSE COLUMN CHROMATOGRAPHY

Protein A-Sepharose CL-4B is a preparation of a cross-linked agarose gel to which protein-A has been covalently bound by the cyanogen bromide method (121).
This preparation was obtained from Pharmacia in a freeze dried form, it was swollen in Tris-buffered saline, pH 8.6 and packed in a 6 ml column.
The column was washed with Tris-buffered saline, pH 8.6. 2ml ascites was loaded onto the column and a step elution procedure was carried out using Tris-buffered saline (0.1 M) at pH 7.0, 5.5, 4.3, 2.3 until the antibody was eluted. A UV monitor was used in conjunction with a pen recorder to measure absorbance of eluent at 280 nm and to detect antibody elution (Gilson, series 202).
Eluents containing antibody were pooled and dialyzed (see following section) against NaCl (0.98% w/v). The fractions were assayed for antibody and total protein, their purity was checked using gel electrophoresis. They were aliquoted and stored at -70 °C.
The column was washed with glycine-HCl-buffered saline, pH 2.3 and equilibriated with Tris-buffered saline, pH 8.6 containing 0.02 %

sodium azide.

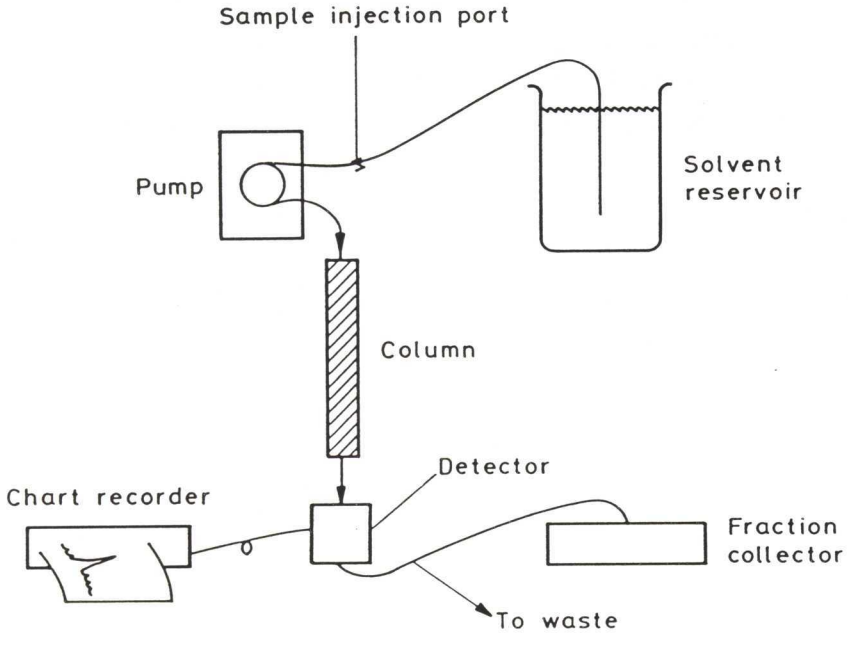


Figure 3.1: Components of low pressure chromatography.

3.6 EXCHANGE OF BUFFER BY DIALYSIS

1. Dialysis tubing was cut into lengths and submerged in a solution of 2 % sodium bicarbonate and 0.05 % EDTA. The solution was boiled for 10 mins ensuring that the tubing remained submerged.
2. The tubing was rinsed with distilled water and sealed at one end. The antibody solution to be dialysed was poured into the dialysis bag. The excess air was expelled and then the bag was sealed using a clamp.
3. The bag was placed in a large volume (approximately 2 l) of exchange buffer: saline (0.85 %). The buffer was agitated gently using a magnetic bar and stirrer at 4 °C, overnight.

3.7 ASSESSMENT OF ANTIBODY PURITY USING SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

Electrophoresis was used under denaturing conditions to determine the purity of immunoglobulin. Sodium Dodecyl Sulphate (SDS) is an anionic detergent which is an effective solubilising agent for many proteins and effectively masks the intrinsic charge on proteins so that the net charge becomes constant. Electrophoretic separation of proteins which have been treated in this way occurs solely on the basis of the size of the molecule and results from molecular sieving through the polyacrylamide gel.
Prior to electrophoresis the samples were treated with 2-mercaptoethanol, to cleave disulphide bonds and so reduce the immunoglobulins to heavy and light chains.

3.7.1 PROCEDURE FOR SDS-PAGE

1. Very clean glass plates were assembled in accordance with the manufacturers instructions (Biorad mini-protean II dual slab cell). Sufficient separating gel was prepared (see appendix II) and this solution was degassed using a vacuum pump to remove oxygen (oxygen inhibits polymerisation). The appropriate volumes of ammonium persulphate and TEMED (polymerisation initiaters) were added to the separating gel mixture. The solution was then mixed gently and poured between the gel cassettes to a distance of about 1 cm below the space to be occupied by the comb.

2. The gels were overlayed with distilled water and left to polymerise for at least 1 hr.

3. The required amount of stacking gel was prepared (see appendix II) and degassed using a vacuum pump. The appropriate volumes of ammonium persulphate and TEMED were added. The water was poured off the top of the polymerised separating gel and the stacking gel solution was poured to fill the glass cassettes. The combs were immediately put in place and the stacking gel was left for at least 0.5 hr for polmerisation.

4. Standards (low molecular weight, Biorad) and samples for electrophoresis were prepared by adding an equal volume of treatment buffer (see appendix II) and heating in boiling water for 5-10 mins. The treated samples and standards were then placed on ice.

5. The gels were installed into the electrophoresis apparatus, the wells of the stacking gel and the electrode chamber was filled with electrode buffer (see appendix II).
6. The samples were loaded into the wells of the stacking gel using a hamilton syringe.
7. The electrophoresis unit was connected to a power pack. A 200 V potential was applied to the gel in accordance to the manufacturer's recommended running conditions, for optimal resolution with minimum band distortion.
8. The gels were removed from the gel and stained to show the protein bands.

3.7.2 PROCEDURE FOR STAINING PROTEINS WITHIN GEL

1. Gels were submerged in a solution of Coomassie Brilliant Blue R-250 for 2 hrs (appendix II).
2. Destaining was accomplished by gentle agitation in the same acid-methanol solution but in the absence of the dye. Destaining was achieved overnight and was halted when the blue background in the gel had been eliminated.

3.7.3 DRYING THE GELS

After destaining the gels were soaked in an aqueous solution of 7 % acetic acid and 10 % glycerol for at least 2 hrs.
The gels were then dryed using a slab gel dryer (Pharmacia).

3.8 DETERMINATION OF TOTAL PROTEIN CONCENTRATION

3.8.1 ULTRAVIOLET SPECTROPHOTOMETRY

Most proteins exibit a maximum absorption of light at 280 nm, this is due to the presence of phenolic groups of tyrosine and the indolic groups of tryptophan. The extinction coefficient, $E_{280}^{1\%}$ varies depending on the presence of these amino groups in the protein. To estimate protein content accurately using this method it is necessary to obtain the protein in a pure form and to calibrate its extinction coefficient against dry weight. This method was used to estimate the protein concentration of purified immunoglobulin using extinction coefficients previously obtained for immunoglobulins of the same class. It is a rapid assay which provides a good comparison for Bradfords' assay (3.8.2)

The absoption of the immunoglobulin and solvent were taken at 280 nm (using quartz cuvettes and a Varian Techtron spectrophotometer), the absorbance of the latter was subtracted from that of the former; the value obtained was divided by the extinction coeficient giving a result which was the total protein, mg ml^{-1}.

3.8.2 BRADFORD'S REACTION

This assay exploits the binding capacity of organic dyes to the acidic and basic groups of proteins. Bradford developed the assay using Coomassie Brilliant Blue G-250 (122); the binding of this dye to protein causes the absorption of the dye to change from 465 nm (red) to 595 nm (blue). The assay is very sensitive in the range of 0.2-1.4 g protein l^{-1}, but interference has been observed with a number of laboratory chemicals, including Tris (123).

The method used a commercially available preparation of the dye, Coomassie Brilliant Blue G-250 (Biorad).
Protein standards were prepared using bovine plasma gamma globulin (Biorad), dilutions were in the range of 0.2 to 1.4 mg ml^{-1}. 0.1 ml of each standard and sample was placed in a clean test-tube, 5 ml of diluted dye reagent was added to each tube. The mixtures were mixed and after a time period of 5 minutes to one hour the optical density at 595 nm was measured versus the reagent blank. The optical densities of the standards were plotted versus their concentrations and the values for the unknowns were calculated from the standard curve obtained.

3.9 ANTIBODY ASSAYS

3.9.1 ASSAYS FOR MOUSE IgM: A1 F11

A. HAEMAGGLUTINATION

Agglutination has been used widely to detect immune responses *in vitro*. Antigenic determinants on cells are destabilised by cross-linking them to antibodies directed to the antigenic determinants. Passive agglutination describes the reaction when antigens have been directly adsorbed or covalently attatched to cells as they do not occur naturally as part of the cell wall.
Sheep Red Blood Cells (SRBC) were coated with the antigen SIII (a capsular polysaccharide from type III *S.pneumoniae*) by direct adsorption.

This assay was used to test for the activity of the Fab region of the immunoglobulin, as haemagglutination is dependent on the formation of an antibody-antigen complex.

Procedure for coating sheep blood cells with SIII antigen

1. Sheep red blood cells (SRBC) were washed three times with saline.
2. One volume of SIII was added to one volume of packed SRBC and the saline was added to make up to 5 volumes.
3. Following an incubation of 45 mins at 37 °C the SBRC were washed three times with saline.
   The SIII coated red blood cells were stored at 4 °C and used within one week of preparation.

Passive haemagglutination

1. Cell culture supernatant (or antibody preparation) was diluted 2-fold in saline[1], using a 96 U-well microtitre plate (25 μl well^{-1}).
2. 25 μl of SIII-coated SRBC (0.5% suspension of packed cells), were added to each well.
3. The plate was incubated at room temperature for 30 minutes.
4. The plate was left to stand at an angle for a few minutes and the wells were read by eye for presence or absence of haemagglutination and an end-point was determined. The end-point was taken to be the last well in which haemagglutination was observed.

B. PASSIVE HAEMOLYSIS

Once haemmaglutination is achieved, the activity of the Fc region of this IgM can be tested by its ability to bind complement. The binding of complement to the antibody-antigen complex results in lysis of the red blood cells (haemolysis).

---

1 NOTE: In latter experiments 5 % BSA (w/v) was used as a diluent in place of saline for both titration of antibody and for SIII-coated SRBC. Incubation was carried out at 4 °C instead of room temperature. These alterations in the experimental procedure resulted in an enhanced haemagglutination reaction.

Preparation of guinea-pig complement

SRBC were coated with the SIII antigen as described in section 3.9.1.A.
The hapten of SIII polysaccharide is phosphorylcholine (PC); because guinea-pig serum has normal anti-PC activity, it is adsorbed with an equal volume of SIII coated red blood cells for 30 minutes on ice.

Assay for haemolysis

1. The assay for haemagglutination (above) was followed up to step 3. After an incubation period of 30 minutes at room temperature, 25 μl of pre-adsorbed guinea pig serum (CSL), diluted 1:7 (distilled water), was added to each well.
2. The plate was incubated at 37 °C for 30 minutes.
3. The wells were read for presence or absence of haemolytic activity, i.e. lysis of red blood cells, and an end-point was determined. The end-point was taken to be the last well in which haemolysis was observed.

C. **ELISA**

1. 10 μg ml^{-1} antigen (affinity-purified mouse IgM + IgG, KLP) was made up in coating buffer, pH 9.6 (appendix III). 100 ul was added to each well and the plate was incubated overnight in a moist container.
2. The plates were washed with 3 times with wash buffer (0.05 % tween 20 in PBS, appendix III) and blotted dry. 100 μl of diluent (1% BSA in PBS) was placed in each well and the plate was incubated for one hour at 37 °C.
3. The plates were washed three times with wash buffer and blotted dry.Standards and samples were serially diluted with diluent. Standard concentrations were used to give an appropriate standard curve. Samples were either used neat or diluted with diluent samples and were tested in triplicate. 100 μl was added to each well.
4. The plates were washed twice using wash buffer and twice using PBS alone. 100 μl of conjugate, (peroxidase-labelled goat anti-mouse IgM + IgG (H+L), KPL), diluted 1:400, was added to each well and the plate was incubated for one hour at 37 °C in a humid container.

5. The plates were washed three times using wash buffer, followed by three times using distilled water, each time shaking and blotting dry.
   The substrate was prepared by mixing equal volumes of solutions A and B (Peroxidase substrate and hydrogen peroxide, KPL), 10 ml/plate. 100 μl of the substrate was added to each well. The optical densities were read using a Titertek multiscan at 405 nm, at 5 minute intervals from 0-20 mins. The optical densities of the standards were plotted versus their concentrations, and the concentrations of the samples were determined from the standard curve obtained. The optical densities should lie in the range of 0.2-0.8.

D. ANTIGEN SPECIFIC ELISA

The above ELISA was adapted to be antigen specific for the model IgM. For this assay, the ELISA plate was coated with a 1:100 dilution of SIII polysaccharide in coating buffer. The remaining part of the assay was carried out as above.
A chequerboard type assay, in which the antigen was titrated down the plate and the conjugate was titrated across the plate, was performed to establish optimal dilutions of the reagents to get a good standard curve of optical densities between 0.2 and 0.8 when read at 405 nm.

3.9.2 ASSAY FOR HUMAN IgG1: ALL

A. MICROPLATE PAPAIN AGGLUTINATION TEST

Haemagglutination is generally achieved more readily with IgM class immunoglobulins than with IgG classes. This is because the blood cells have to be brought closer together to achieve agglutination using IgG as they are smaller molecules. This requires more energy to overcome the electrostatic repulsion of the cells.
In the following assay the red blood cells are treated with papain to lower their surface potential. The use of an albumin solution as a diluent also facilitates haemagglutination as it decreases the thickness of the ionic layer around the cell by increasing the colloid-osmotic pressure.

A. RED BLOOD CELL PAPAINISATION

1. 200 μl of packed red cells (which have been washed three times with saline), were dispensed into a 10 ml centrifuge tube.
2. 200 μl of papain solution, prediluted prior to use with 2 volumes of glucose citrate, were added to the red cells.
3. The contents of the tube was mixed thoroughly and incubated at 37 °C for 10 minutes.
4. The volume was diluted with glucose citrate to make a 10 ml solution.
   The cells were used within 24 hours.

B. ASSAY

1. 25 μl of supernatant to be assayed was placed in each well of a U-well microtitre plate. To assay quantitatively, supernatants were diluted by 1:1 along the plate using 5 % (w/v) bovine albumin in saline as a diluent.
2. 25 μl of 2 % papainised red cells were added to each test well. O R_2R_2 were used for the detection of anti-D, and O Rh(D) positive and O Rh(D) negative were added to control wells.
3. The tray was mixed gently by tapping, covered and incubated for 10 minutes at 37 °C.
4. Plates were left to stand at an angle for a few minutes and the wells read for the presence or absence of agglutination and an end-point was determined.

3.10 CHEMICAL VIRAL INACTIVATION METHODS

3.10.1 BETA-PROPIOLACTONE TREATMENT

The treatment was carried out according to the recommendation of the Australian Drug Evaluation Committee (38).
18 parts of the antibody preparation were mixed with 1 part of disodium hydrogen orthophosphate (0.5M) and 1 part beta-propiolactone (ßPL)(2 % v/v in distilled water). The mixture was incubated for one hour at 37 °C, in a glass container, with frequent mixing and avoiding mixing air and water at interface. The mixture was then transferred to a fresh glass container and incubated for one further hour at 37 °C with conditions as before.

The pH was adjusted to 7.3-7.4 and the chemically treated antibody preparations were stored overnight at 4 °C and then frozen in aliquots at -70 °C to avoid repeated freezing and thawing.

3.10.2 TRI(N-BUTYL)PHOSPHATE

The treatment was carried out as described by Horowitz (117). The antibody preparation was mixed with 1 % Tween 80 and 0.1 % Tri(n-butyl) phosphate and incubated at room temperature for 6 hours.
The chemically treated antibody samples were frozen in aliquots at -70 °C to avoid repeated freezing and thawing.

3.11 ASSESSMENT OF BIOLOGICAL ACTIVITY OF ANTIBODIES FOLLOWING INACTIVATION TREATMENTS

Antibody was obtained, purified and analysed using the procedures described. The buffer solvent was exchanged with physiological saline (0.85 % w/v) by dialysis and concentrated to 0.8 mg ml^{-1} using Amicon concentrating cones (cut off mw 100 kD).
Viral inactivation procedures (sections 3.10.1 and 3.10.2) were followed using:

a) Crude cell-culture supernatant containing mab.
b) Purified mab.

Post-treated samples were aliquoted out and frozen at -70 °C (to avoid repeated freezing and thawing) and assayed for specificity and biological activity using the assays described in section 3.9.

RESULTS AND DISCUSSION

4 RESULTS AND DISCUSSION

The objectives of this work were described on pages 1 and 2 of this thesis. They are to investigate the effects of two commonly used chemical inactivation treatments (ßPL and TNBP) on the biological activity of 2 model monoclonal antibody preparations (a mouse IgM and a human IgG1 class antibody).
In order that the effect of inactivation procedures on the model monoclonal antibodies could be investigated it was necessary to develop appropriate assays. In addition, preparative experiments were necessary to produce and purify sufficient antibody for the tests.

4.1 ASSESSMENT OF THE EFFECT OF INACTIVATION STRATEGIES ON A MOUSE IgM CLASS ANTIBODY

The preparative work involved in preparation of monoclonals prior to the inactivation treatments is described in the first part of this section. The following procedures were used: cloning of cell-line to obtain monoclonal-secreting hybridomas, production of sufficient monoclonal antibody for tests (aiming to use 1 mg ml^{-1} antibody per inactivation test), purification of antibody to >98 % (ie that of therapeutic monoclonal), assesment of purity and quantitation of final antibody product.

4.1.1 CLONING OF HYBRIDOMA CELL-LINE IgM 1A/F11

The hybridoma cell-line IgM 1A, raised against the capsular polysaccharide from Type III *S.pneumoniae* (section 3.1.1), was cloned three times by "limiting dilution" (section 3.2).

Table 4.1 shows how many colonies were observed at each cloning in one 96 micro-well plate. The method used for cloning (described in section 3.2) resulted in aliquoting cell-suspensions containing 96 cells and 48 cells into the wells of two separate 96 multi-well plates to give average cell concentrations of 1 cell/well and 0.5 cell/well respectively.
A clone was selected after one week of incubation and named according to the well from which it was selected, a standard method for naming clones. Following each cloning, the clone was assayed for antibody productivity by haemolysis (3.10.1 B), frozen and stored at -196 °C.

			No. colonies per plate	Clone no. selected
Cloning 1	1	cell/well	95	
	0.5	cell/well	55	F11
Cloning 2	1	cell/well	90	
	0.5	cell/well	53	D12
Cloning 3	1	cell/well	91	
	0.5	cell/well	43	A3

Table 4.1: Table to show the cloning efficiency of cell-line IgM 1A

After each cloning the number of wells showing positive growth, in 0.5 cell/well plate was less than 66.6 %. This indicates that the cells are probably monoclonal in each well (124).
The final cloned cell-line was termed IgM 1A/F11/D12/A3, it was expanded and aliquots of the cells were frozen at -196 °C.

4.1.2 PRODUCTION OF IgM

The model IgM was required in sufficient quantity to carry out the series of proposed experiments (section 3.11). It was calculated that for the experiments which were to be performed using purified IgM, a concentration of 1 mg ml^{-1} should be used; in order to relate the process to conditions of processing therapeutic proteins.

A. ASCITES

Ascitic fluid was obtained from the peritoneal cavity of mice as described in section 3.4.1 A.
It was found that the IgM 1A/F11/D12/A3 hybridoma cell-line did not grow particularly well in the peritoneal cavity of mice and only a small amount of ascitic fluid was recovered (approx. 0.5 ml) while a large amount of solid tumour could be observed in the intestine. The volume of ascites obtained from one mouse was increased slightly by taking the solid tumours and passaging them into the peritoneal cavitiy of new mice. By carrying out this procedure, yields of between 0.5 and 2 ml of ascites per mouse

could be obtained.
The amount of biologically active IgM present was quantified by haemolysis, the concentration of IgM was found to be constant and low in ascites obtained either by passaging tumours or the original cell-line into the mouse.
The ascitic fluid was pooled and the titre obtained was: <1/256 and >1/512, i.e. haemolysis was observed when the ascitic fluid was diluted 1/256 but not when it was diluted 1/512.
As it was found that the cell-culture supernatant (section 3.2.3) had a haemolytic titre of <1/16 and >1/32 and that this is equivalent to 20 $\mu g\ ml^{-1}$, (determined by ELISA, section 3.2.3) it was estimated that the concentration of monoclonal antibody in ascitic fluid is approximately 320 $\mu g\ ml^{-1}$. This is a lower concentration than expected as commonly 3-15 $mg\ ml^{-1}$ of monoclonal antibody is produced in ascites.

B. SERUM

Sera obtained from mice injected with hybridoma cells, were assayed by haemolysis and found to have a titre of 1/2048. It can be estimated that this titre is equivalant to approximately 1 $mg\ ml^{-1}$ by comparing it to the titre of cell-culture supernatant (section 4.2.3) and thus is much higher than that obtained in asitic fluid.

C. CELL-CULTURE SUPERNATANT

The profiles for cell growth and for antibody production are illustrated in **Figure** 4.1. The doubling time was 20 hours, the maximum number of viable cells was 98.10^4 viable cells ml^{-1} and the maximum antibody concentration was 24 $\mu g\ ml^{-1}$. Antibody production appeared to be growth associated. The antibody concentration was measured using a non-specific ELISA assay for the detection of mouse IgM (H and L), the standard curve for which is given in **figure**: 4.1'. The final antibody titre was 20 $\mu g\ ml^{-1}$ and although antibody concentrations of up to 100 $\mu g\ ml^{-1}$ have been reported in the supernatants of batch culture broths, the figure obtained is not unusually low from experience in our lab.

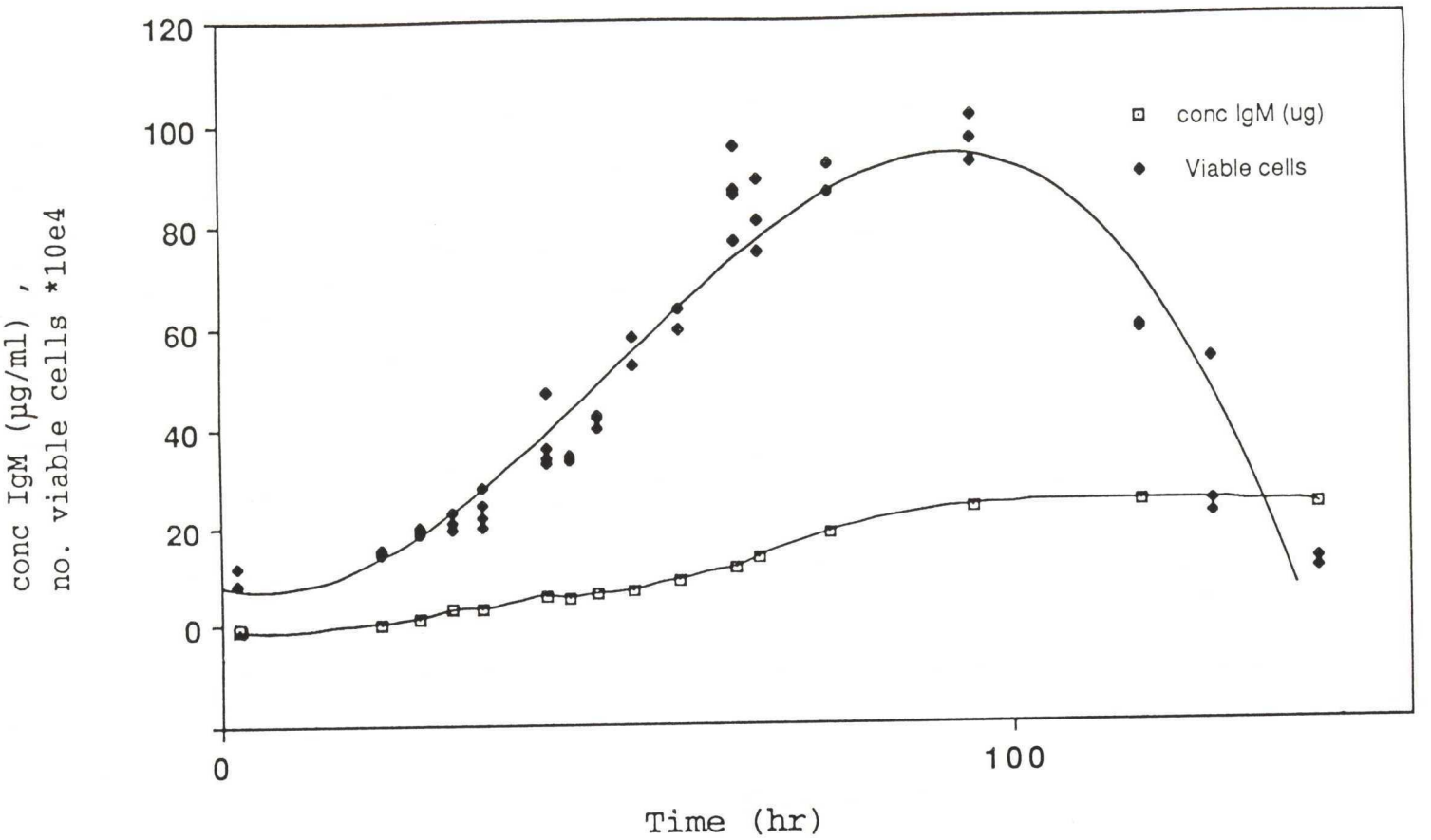


Figure 4.1: Rate of cell growth and antibody production of IgM 1A..A3 in batch conditions, with DMEM supplemented with 5 % FCS media.

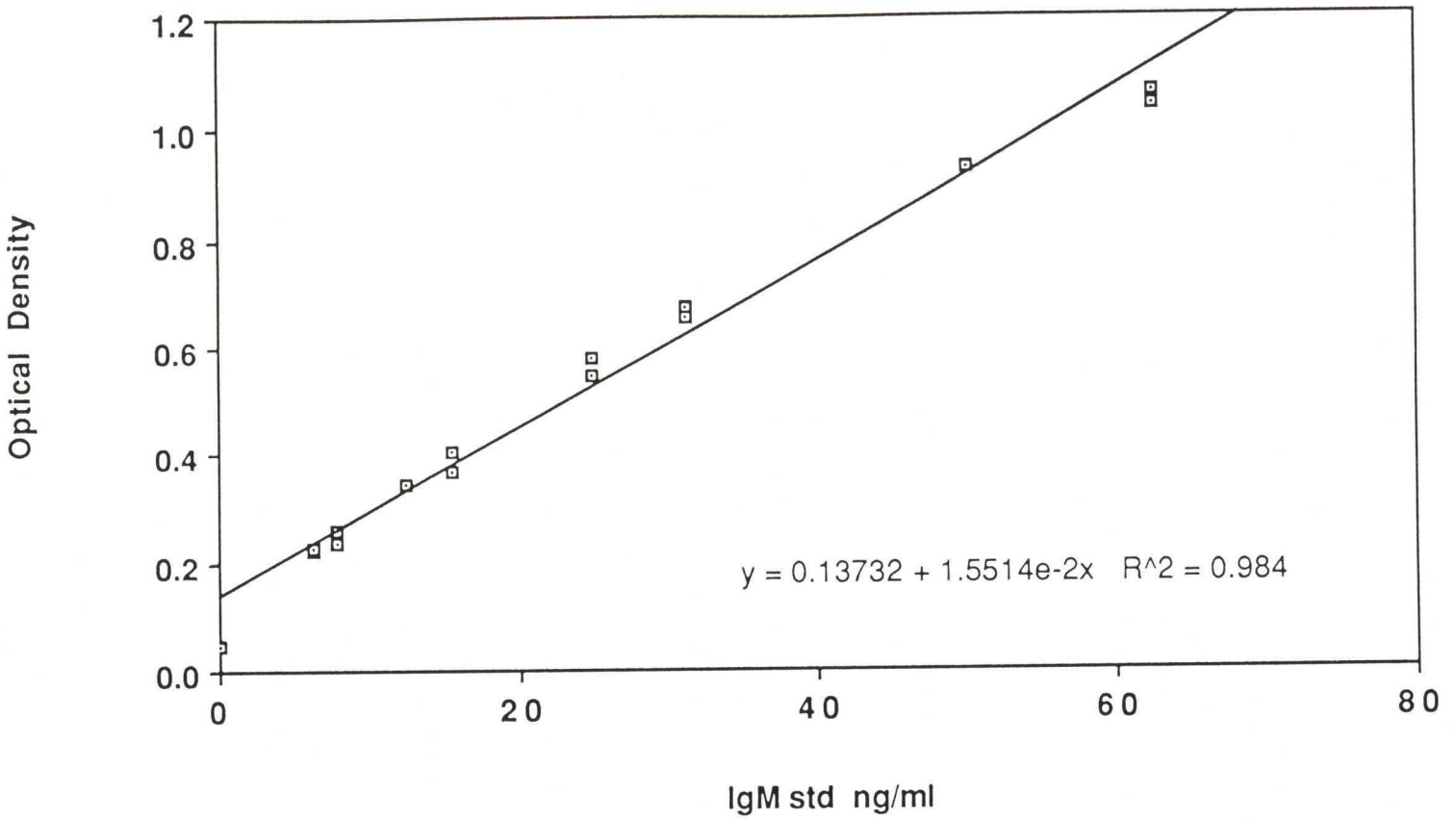


Figure 4.1': Standard curve of IgM standards (affinity-purified polyclonal mouse-IgM, KPL) used in ELISA assay to determine rate of antibody production in batch culture.

4.1.3 PURIFICATION OF IgM

The aim of the purification procedure was to obtain IgM of very high purity, (i.e. to the level of purity required for therapeutics), and to obtain a quantity which would be sufficient for use in later experiments.

IgM was purified from mouse sera because it contained higher concentrations of IgM than ascites from this cell-line. On analysis it was found that the serum had a haemolytic titre which was ten times greater than obtained in the ascitic fluid.

IgM was also purified from ascites because of the comparitively low level of contaminating proteins. The separation characteristics of IgM from each of these sources were compared and are described below.

A. GEL FILTRATION

Profile of Protein Fractions in Serum (Figure 4.2)

This profile shows the optical densities of the various fractions following the gel filtration. The peaks seen are those of IgM, IgA, IgGs and albumin elutions respectively as these are the major proteins expected to be present in the mixture. The fractions collected were tested for specific activity, i.e. anti-phosphorylcholine activity by haemolysis (method 3.10.1.2), so that they could be collected for further processing. The major contaminants in this pooled fraction were likely to be IgA and other mouse IgMs; fractions showing haemolytic activity may contain depolymerised IgM which, because of their size, would be eluted just prior to or with IgA.

Profile of Protein Fractions in Ascites (Figure 4.3)

This profile of the optical densities of the eluted protein fractions, shows a much larger proportion of IgM elution compared with other proteins in the ascites mixture. Again the fractions were assayed for anti-phosphorylcholine activity and the positive samples were collected for further processing.

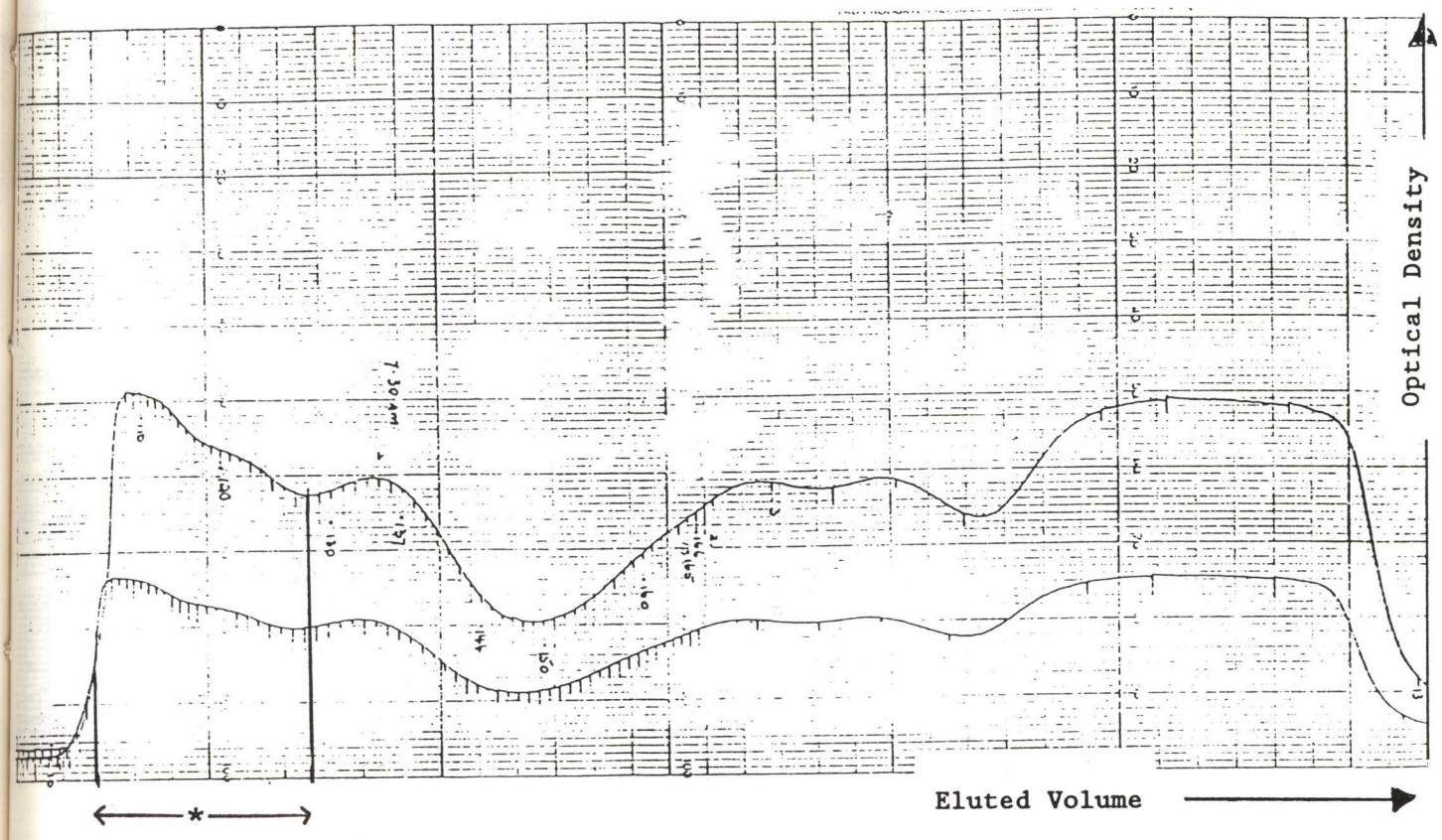
Assessment of Presence of IgM and Maintenance of Activity During Purification Procedure.

Presence of IgM and anti-phosphorylcholine activity in eluted fractions was measured using assays for haemolysis. The fractions which gave arise to haemolysis are indicated on the purification profiles and were tested for purity using SDS-PAGE (section 4.4).

B. PROTEIN-A CHROMATOGRAPHY

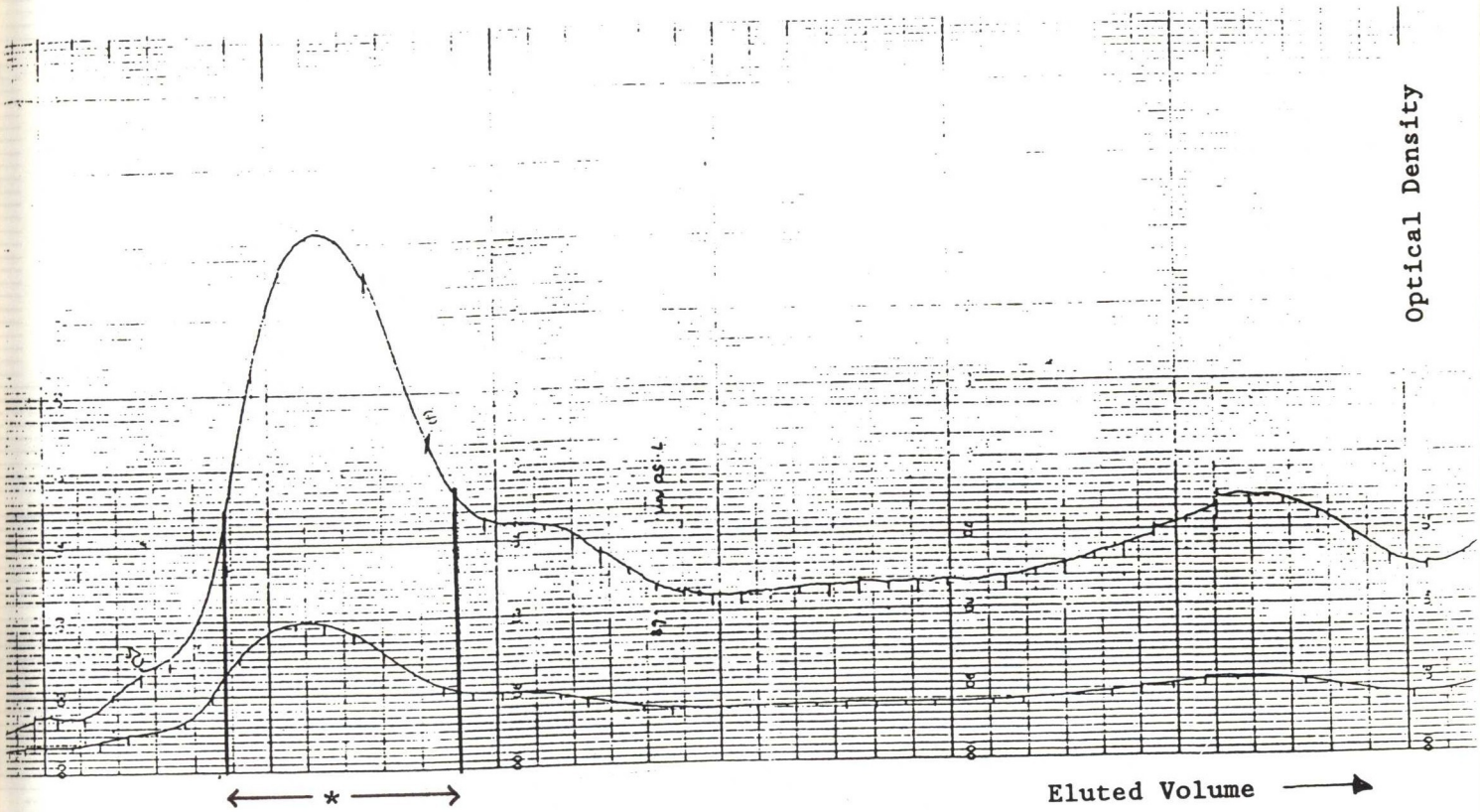
Absorbance Profile of Antibody Elution.

Figure 4.4 clearly shows the elution of the antibody at pH 2.4. SDS-PAGE electrophoresis was carried out to determine the purity of the antibody, the results of which are described in the following section.



Sample: 10 ml Serum
Equilibration Buffer: Tris (0.05 M) buffered saline (0.5 M pH8)
Elution Buffer: Tris (0.05 M) buffered saline (0.5 M pH8)
Gel: Sephacryl-300
Column: 2 columns of 1 x 50 cm
Flow: 1.5 ml/min
Detection: 280 nm
Fraction: 10 ml/fraction

Figure 4.2: Chart recorder output showing the fractionation of serum components by gel filtration. Optical densities of different fractions are shown as they are eluted from a S300 Sephacryl gel filtration column.



Sample: 3ml Ascites
Equilibration Buffer: Tris (0.05 M) buffered saline (0.5 M pH8)
Elution Buffer: Tris (0.05 M) buffered saline (0.5 M pH8)
Gel: Sephacryl-300
Column: 2 columns of 1 x 50 cm
Flow: 1.5 ml/min
Detection: 280 nm
Fraction: 10 ml/fraction

Figure 4.3: Chart recorder output showing the fractionation of the components of ascites by gel filtration. Optical densities of different fractions are shown as they are eluted from a S300 Sephracryl gel filtration column.

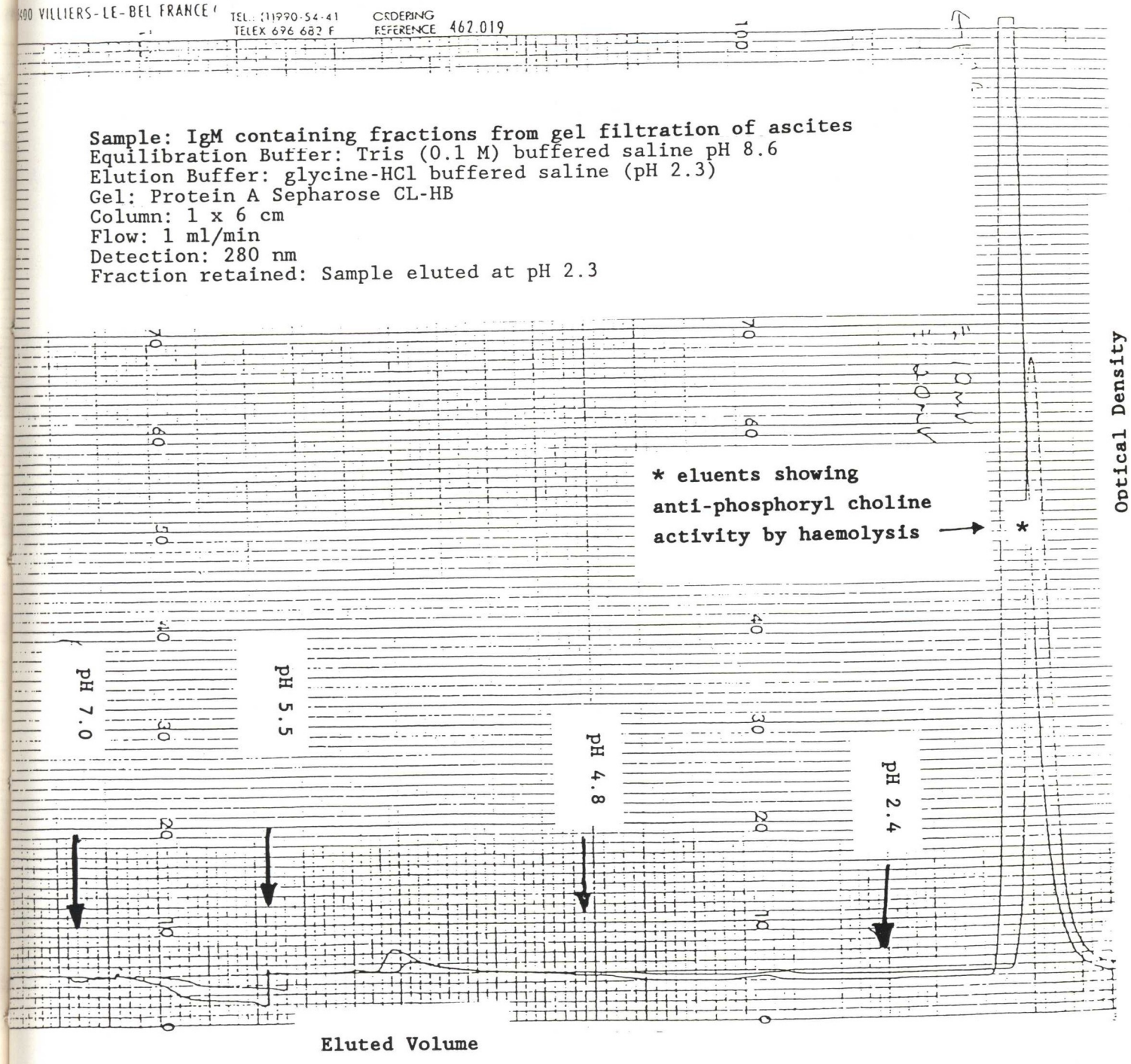


Figure 4.4: Chart recorder output showing the fractionation of IgM following gel filtration using a protein-A affinity column. The IgM was eluted from the column at pH 2.4.

4.1.4 USE OF SDS-PAGE ELECTROPHORESIS TO ASSESS ANTIBODY PURITY

From **Figure 4.5** it can be seen that there are many contaminants in the separated serum. These include many mouse immunoglobulins and it is difficult to distinguish the contaminating IgM from the monoclonal IgM in this fraction. In figure 4.6, however, the purity of IgM derived from ascitic fluid was much greater; it was therefore decided to use these fractions for further purification despite the fact that the monoclonal titre was much lower than in serum.

In **Figure 4.6** it can be seen that the IgM derived from ascitic fluid is of very high purity indeed following gel-filtration and protein-A chromatography, with no other protein bands visible on the gel. Following purification the buffer was exchanged with saline by dialysis, it was aliquoted and stored at -70 °C.

The figures of SDS-PAGE gels show the purity of different fractions collected during the purification stages. The fraction numbers correspond to the fraction numbers shown in the related purification profile.

It is clear from these profiles that a much better separation of the monoclonal was achieved from ascitic fluid than from serum. Although this was expected (since sera contains many more contaminating immunoglobulins) it was hoped that a higher yield could have been achieved because of the difficulty in obtaining ascitic fluid of high titres compared with sera.

From these gels and the haemolytic titres, it was decided to select fractions 53-62 obtained from the gel filtration of ascitic fluid and to further process these pooled fractions using protein-A chromatography.

Figure 4.7 shows the purity of the IgM after gel-filtration and affinity chromatography compared that after gel-filtration only. **Figure 4.8** shows the final purity of IgM is greater than that of a commercially prepared IgM standard of quoted purity of > 98 %.

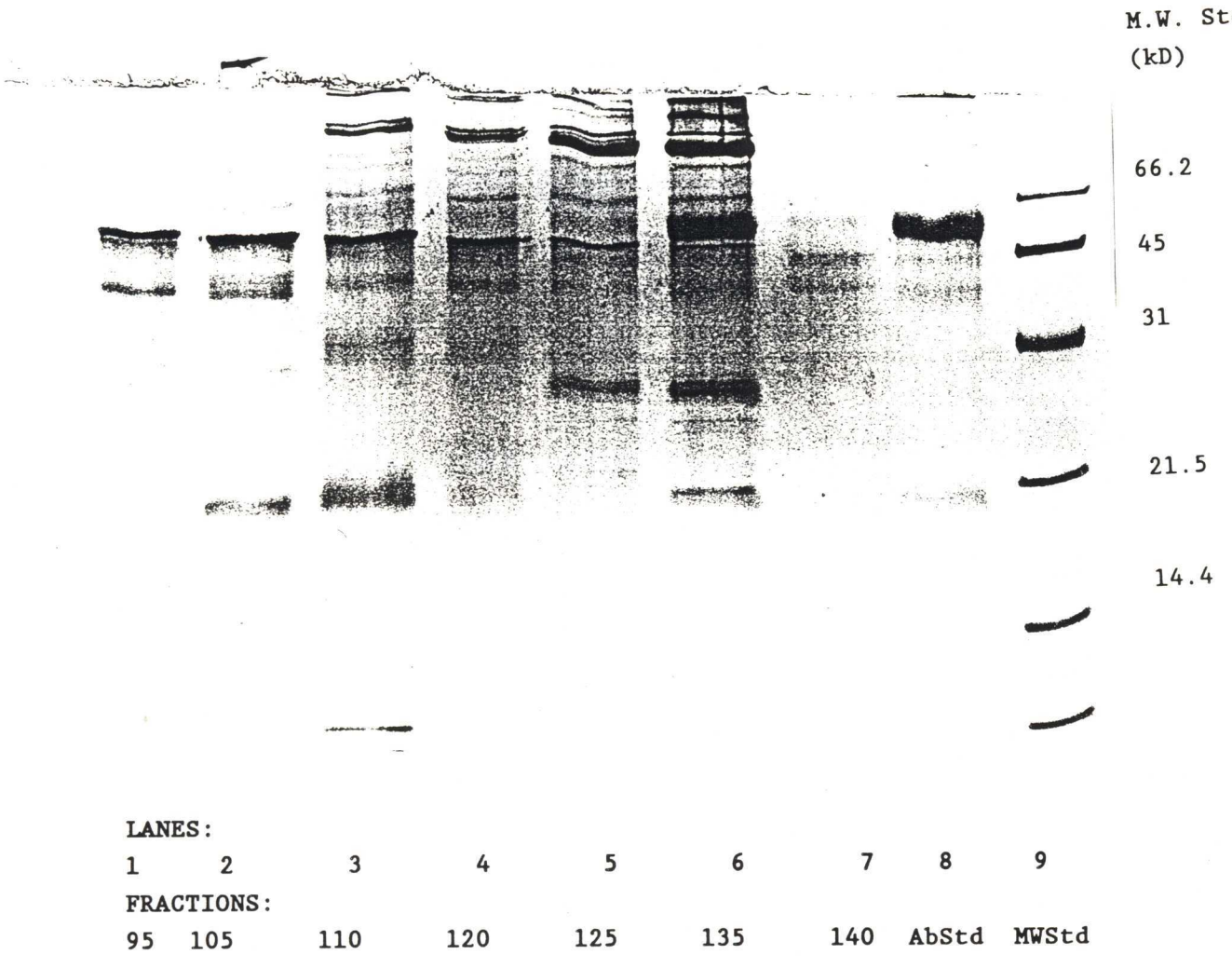
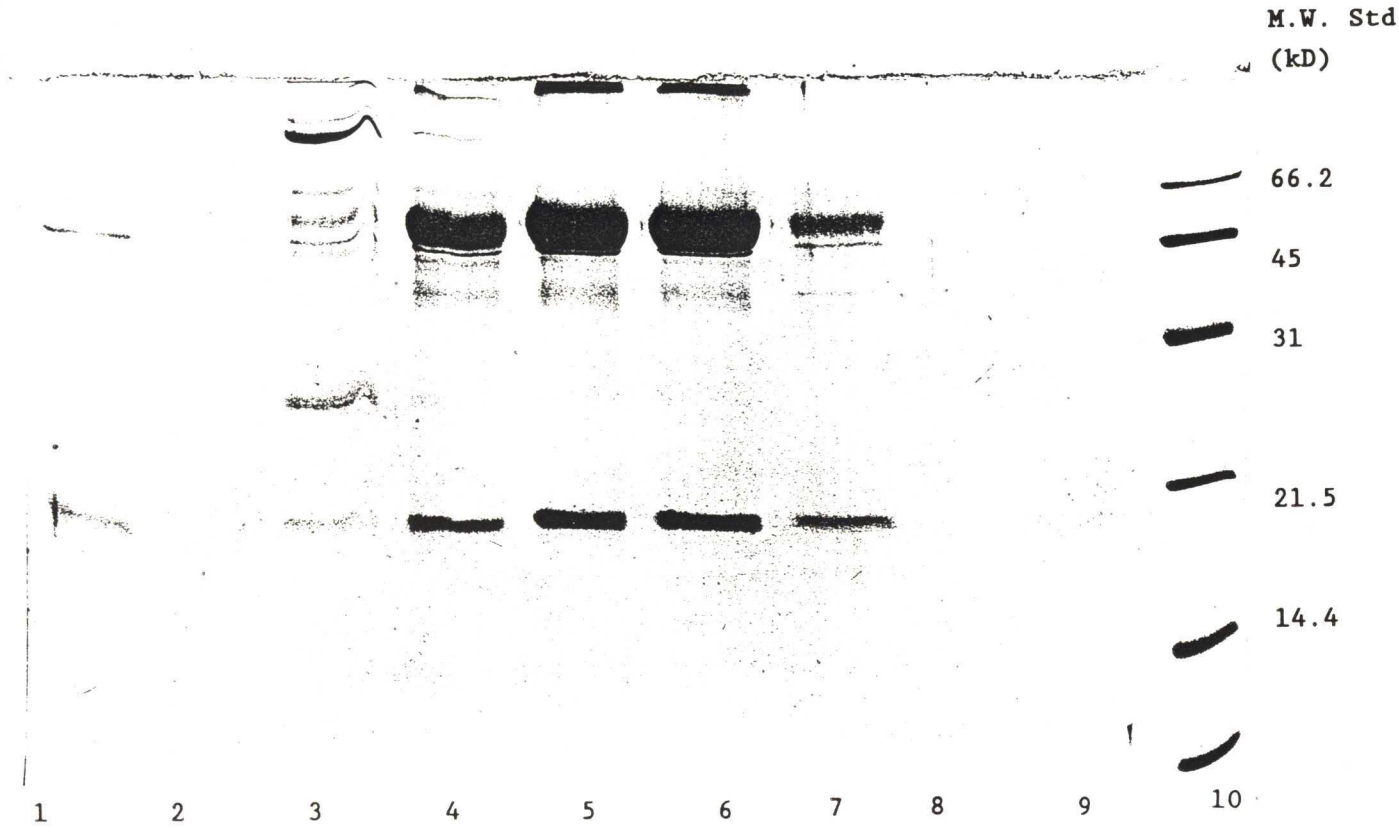


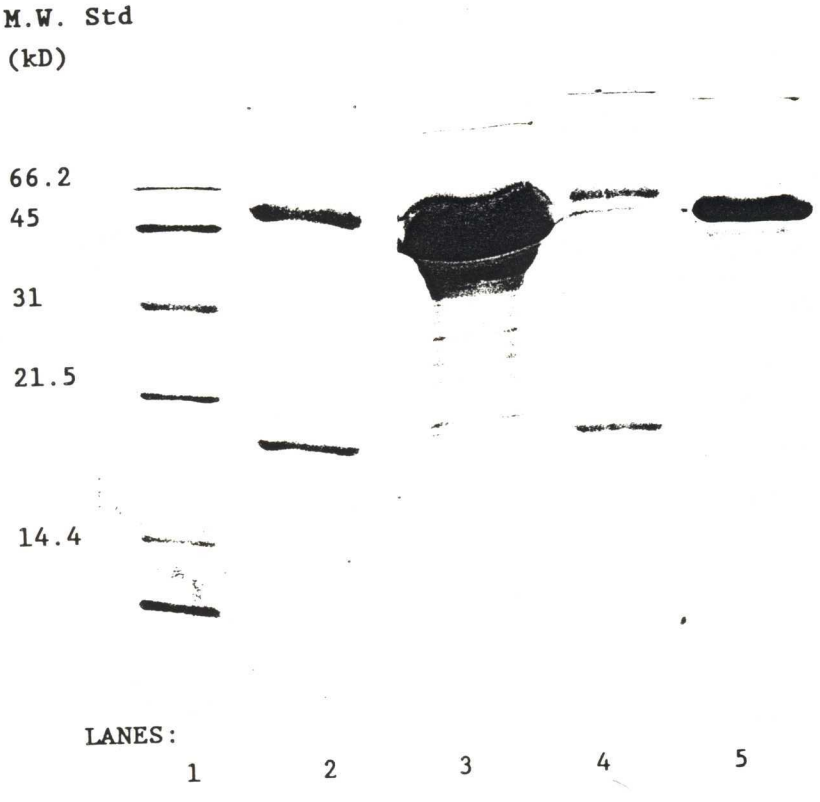
Figure 4.5: Photograph of a SDS-page gel to show the purity of antibody in different fractions of protein peaks separated from serum using gel filtration. From left to right: Lanes 1-9 fractions corresponding to fractionations obtained (figure: 4.2), lane 10 protein standards.



LANES:

1	2	3	4	5	6	7	8	9	10
FRACTIONS:									
80	70	64	60	58	56	53	50	45	MW Std

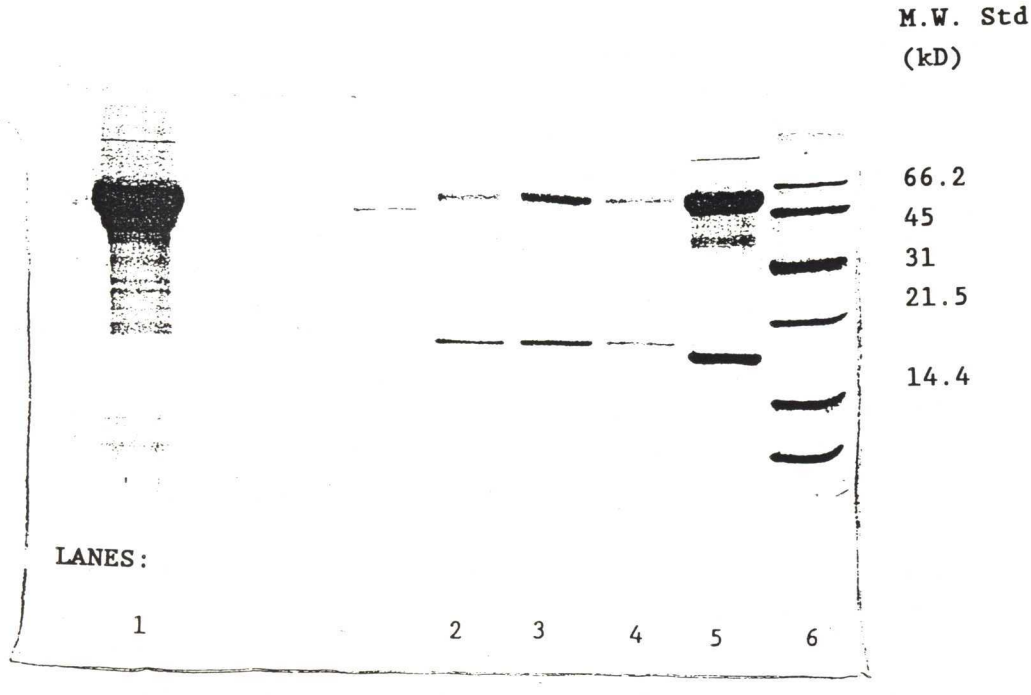
Figure 4.6: Photograph of a SDS-page gel to show the purity of antibody in different fractions of protein peaks separated from ascities using gel filtration: From left to right: Different lanes corresponding to fractionations obtained (figure: 4.3), lane 10 protein standards.



LANES:

1. Low molecular weight standards.
2. IgM antibody, gel-filtered then affinity purified.
3. Cell-Culture supernatant in FCS.
4. IgM antibody, gel-filtered.
5. Cell-Culture supernatant in SFM.

Figure 4.7: Photograph of a SDS-page gel to show the purity of antibody separated from ascites following protein-A chromatography: From left to right: molecular weight standards, purified IgM crude supernatant containing IgM, IgM following gel-filtration (prior to protein-A purification).



LANES:

1. Cell-Culture supernatant.
2. IgM antibody affinity purified.
3. IgM antibody affinity purified.
4. IgM antibody affinity purified.
5. IgM std (KPL), affinity purified.
6. Low molecular weight standards.

Figure 4.8: Figure shows the purity of the monoclonal following protein-A purification. No contaminating proteins were observed so it can be assumed that the purity is >98% as it is appears to be of greater purity than the commercial affinity purified Ig standard of 98% purity.

4.1.5 DETERMINATION OF TOTAL PROTEIN IN PURIFIED ANTIBODY ELUENT

Bradford's reaction

The purified stock of IgM was assumed to approach 100 % purity and was measured for total protein by Bradford's reaction, (section 3.8.2). The protein was measured against Biorad's gamma-immunoglobulin standards, and was found to have a concentration of 1.4 mg ml^{-1}. The standard curve for this assay is given in Appendix IV.

UV Spectrophotometry

The absorption of the purified stock, diluted 1:30, at 280 nm was 0.449. Using the extinction coefficient for IgM as a general subclass, $E_{280}^{1\%} = 13.3$, the concentration was calculated to be 1.03 mg ml^{-1}

The extinction coefficient varies from protein to protein depending on the precise amino-acid composition and so the extinction coefficient of immunoglobulins even within the same sub-class varies. Consequently this method of protein estimation was considered to be the least accurate of those used unless an extinction coefficient of the pure protein had been found and calibrated against dry weight. This would have been time consuming and used up too much of the purified protein.

So for the purpose of the following studies the protein concentration was taken to be 1.4 mg ml^{-1}, as measured by the Bradford reaction. In this case the exact protein concentration was not as important as having a reference standard.

ELISA

The purified IgM was compared to an IgM standard for ELISA (affinity-purified polyclonal mouse-IgM, KPL), by ELISA (section 3.10.1 C) and the ODs compared, **figure 4.9**. It can be seen from the graph that the calibration curve for the purified monoclonal standard compares very well with the curve for the polyclonal IgM standard. Since the accuracy of the ELISA is taken to be ± 5 %, the affinity of both of these standards for the antigen, i.e., affinity-purified mouse IgM standard (KPL) can be considered equivalent.

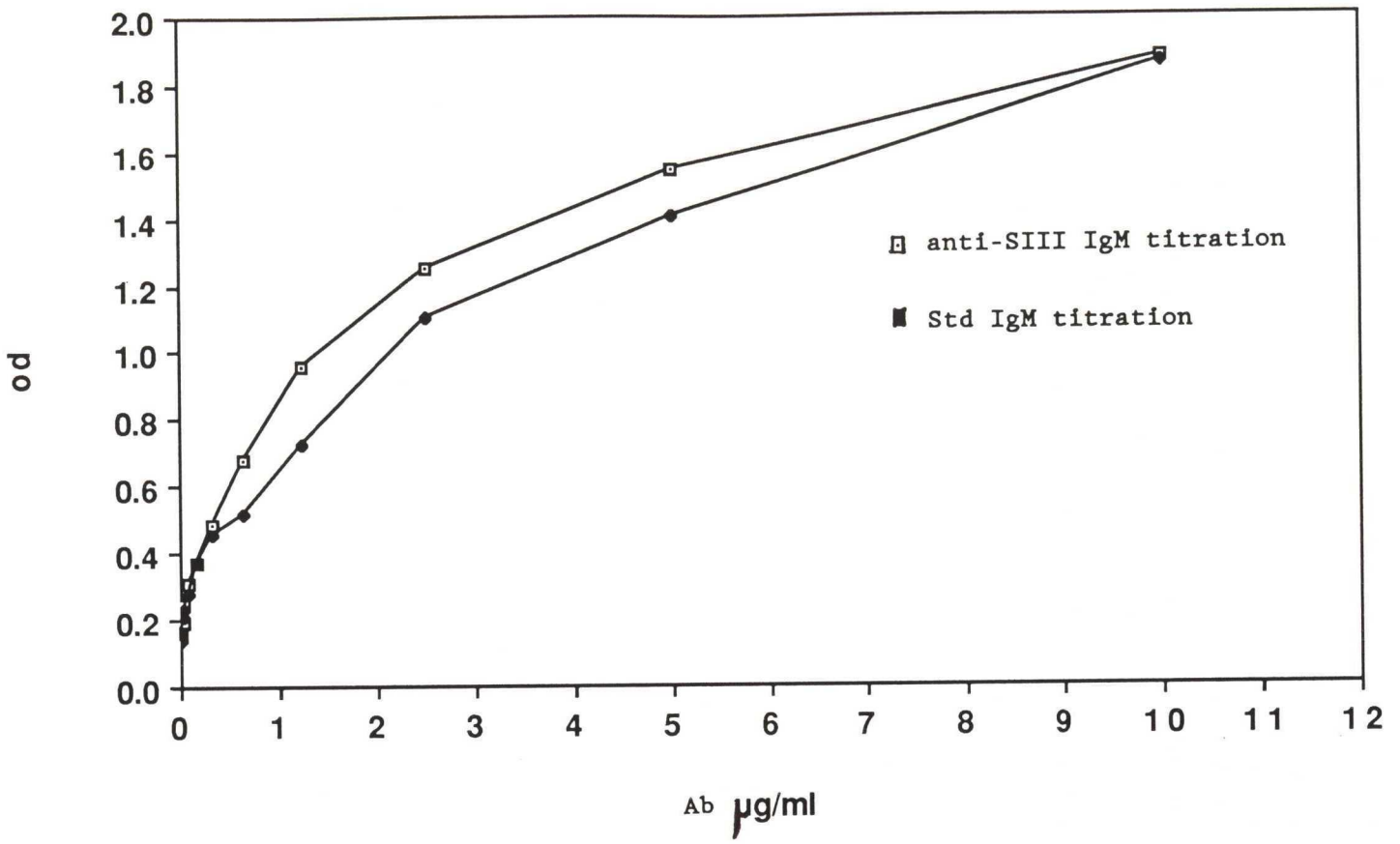


Figure 4.9: Comparison of the relative affinities of purified anti-SIII IgM with IgM standard (affinity-purified polyclonal mouse-IgM, KPL) in ELISA assay.

4.1.6 CHEQUERBOARD ELISA ASSAY TO DETERMINE OPTIMUM ANTIGEN CONCENTRATION TO COAT PLATES.

The method was carried out as described in section 3.9.1 D. The optical density was plotted against antibody concentration for each of the different antigen concentrations used to coat the plates, **figure 4.10**. The antigen concentration corresponding to the plot which was most linear over the widest range of optical densities between the values of 0.2 and 1.0 was chosen for use in the ELISA assay to be used specifically for the model IgM monoclonal antibody.
The value chosen from the graph was a 1/64 dilution of the SIII polysaccharide antigen preparation.

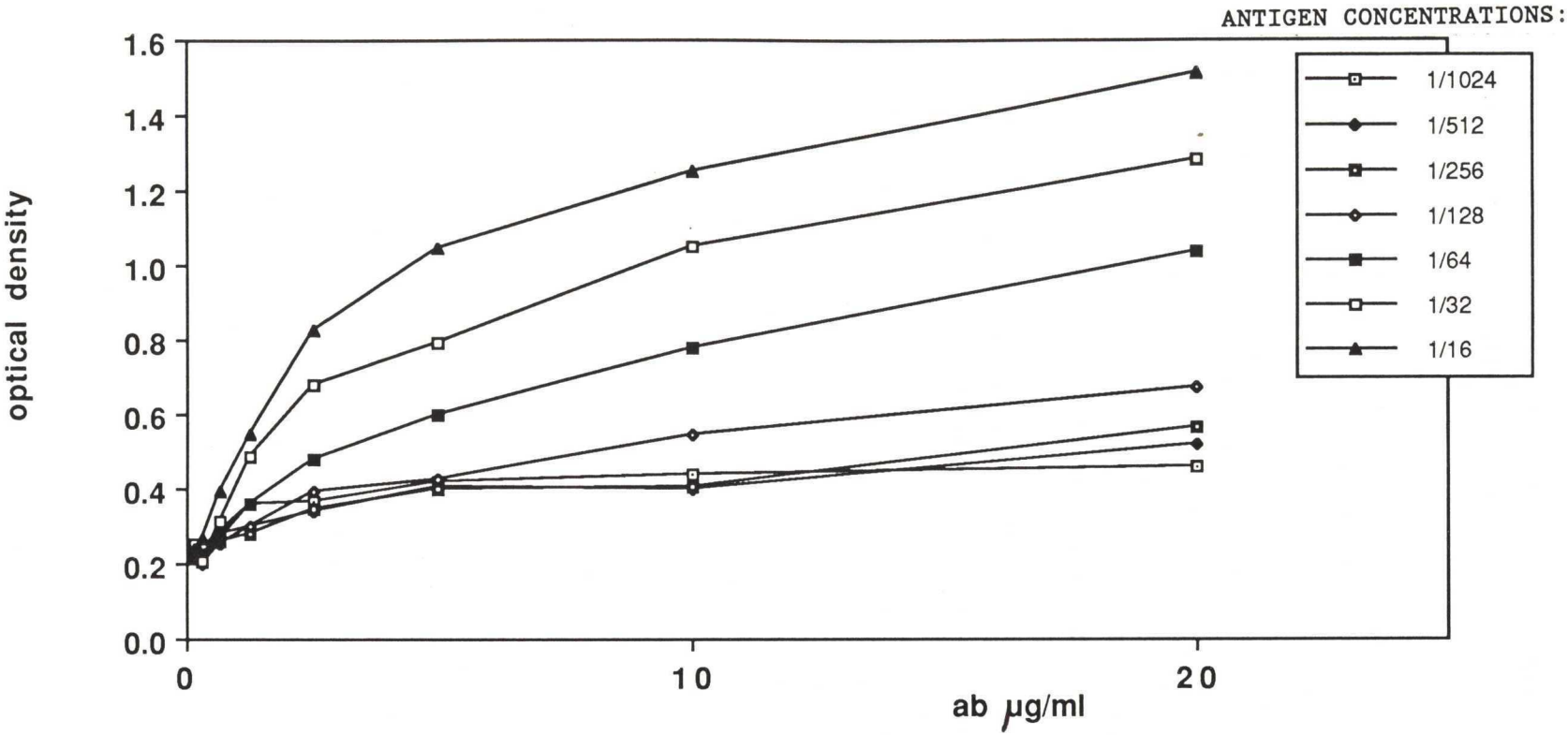


Figure 4.10: Determination of optimal antigen concentration for anti-SIII ELISA assay.

4.1.7 ASSESSMENT OF BIOLOGICAL ACTIVITY OF ANTIBODIES FOLLOWING CHEMICAL INACTIVATION TREATMENTS

A. HAEMAGGLUTINATION AND HAEMOLYSIS

Unpurified monoclonal IgM in the form of cell-culture supernatant and the affinity purified monoclonal IgM were treated independently with ßPL and TNBP as described in section 3.11. The treated IgM samples were assayed for overall biological activity pre- and post-treatment. The assays used tested for haemagglutination (section 3.10.1 A), which tests for Fab activity and haemolytic activity (section 3.10.1 B), which tests for activity of the Fab and the Fc regions of the immunoglobulin. The supernatant was diluted 2 fold across a microtitre plate.

The results are expressed as $\log_2$ titre which represents the lowest concentration of supernatant in which haemolysis was observed. The actual end-point falls between this dilution factor and the next one, in which the supernatant contains half the concentration of antibody.

The results shown in the table represent six separate analyses carried out on the same treated supernatants using different batches of reagents (i.e. coated red blood cells and complement).

(i) THE EFFECT OF ßPL ON CELL-CULTURE SUPERNATANT

HAEMOLYSIS:

	TEST SAMPLE / CONTROL	LOG_2 TITRE[1]						MEAN TITRE	SD
	SAMPLE:								
1	Mab + Na_2HPO_4 + ßPL	0	8	3	4	0	3	3.0	2.7
	CONTROLS:								
2	Mab + dH_2O + ßPL	0	5	8	3	4	4	4.0	2.4
3	Mab alone	8	5	10	7	7	9	7.0	1.6
4	Saline + Na_2HPO_4 + ßPL	0	0	0	0	0	0	0	0

Table 4.2: Effect of ßPL on the Haemolytic Activity of IgM in Cell-Culture Supernatant, expressed as log_2 haemolytic titre.

Haemolysis was easily observed in this assay so that the end-point was well defined. It was concluded, from these results, that the ßPL treatment clearly reduced the activity of IgM. It is apparent, however, that there is distinct variation in the end point titre from one experiment to the next. Due to the nature of these assays, slight fluctuations in temperature, ionic strength and composition of samples cause changes in the affinity of the antibody to antigen. It was therefore concluded that the standardisation of reagents in this assay is of great importance to improve the consistency of the data. Variation in data is most likely to be due to inconsistency of prepared

1 Titres represent titrations of experiments carried out on different occasions under the same conditions using different batches of reagents.

reagents, such as SIII(antigen)-adsorbed red blood cells and complement pre-adsorbed with these blood cells. The problems arising from the preparation of these reagents could be due to different batches of sheep red blood cells having different adsorption characteristics and also the nature of preparation involving variables which are difficult to accurately standardise from one preparation to another; such as packed volume of red cells and removal of complement from adsorbed red cells.

Due to the weak agglutination reactions obtained using this assay (section 3.10.1 A) the titres were not recorded as the end-points were difficult to determine by eye and it was decided that the assay should be developed to enhance the agglutination of red blood cells so that the end-points could be more well defined.

(ii) THE EFFECT OF ßPL ON PURIFIED IgM

IgM was purified from ascitic fluid as described (section 3.5.1). The supernatant was treated with ßPL (section 3.11.1). The treated IgM was assayed for both haemagglutination (section 3.10.1.1) and haemolytic activity (section 3.10.1.2). The supernatant was diluted 2 fold across a microtitre plate and the results are recorded as a log_2 titre end-point; i.e. the last well in which haemolysis or haemagglutination was observed, as in section 4.2.1.

HAEMAGGLUTINATION:

	TEST SAMPLE / CONTROL	LOG_2 TITRE[2]				MEAN TITRE	SD
	SAMPLE:						
1	Mab + Na_2HPO_4 + ßPL	13	11	19	1	11.0	3.1
	CONTROLS:						
2	Mab + dH_2O + ßPL	1	7	0	4	3.0	3.5
3	Mab alone	12	15	21	5	13.25	5.7
4	Saline + Na_2HPO_4 + ßPL	0	0	0	0	0	0

Table 4.3: Effect of ßPL on the HA of high purity IgM, expressed as log_2 HA titre.

It can be seen from the data above that the titre is markedly reduced when the antibody was treated with ßPL in the absense of di-sodium hydrogen orthophosphate (Na_2HPO_4); HPO_4 neutralises the acidity of ßPL, so that the antibody could have undergone

2 Titres represent titrations of experiments carried out on different occasions under the same conditions using different batches of reagents.

partial acid denaturation in the absence of HPO_4. It is unlikely that Fab tolerates conditons below pH 2 and will not maintain optimal affinity at low pH. The agglutination reaction was weak so that the end-titre was not clear and hence difficult to read. These results are not defined well enough to make a conclusion on the effect of ßPL treatment on the Fab region of the immunoglobulin.

HAEMOLYSIS:

	TEST SAMPLE / CONTROL	LOG_2 TITRE	MEAN TITRE	SD
	SAMPLE:			
1	Mab + Na_2HPO_4 + ßPL	0		
	CONTROLS:		Not	
2	Mab + dH_2O + ßPL	4	Applicable	
3	Mab alone	5		
4	Saline + Na_2HPO_4 + ßPL	0		

Table 4.4: Effect of ßPL on the Haemolytic Activity of High Purity IgM, expressed as log_2 haemolytic titre.

The haemolytic titre was reduced to zero after ßPL treatment in the presence of HPO_4, indicating that ßPL has destoyed the activity of the immunoglobulin molecule during the treatment process. However, in the absence of HPO_4, the activity was comparable to that of the control. These results did not correlate with the haemagglutination data and suggest that the assay was not properly standardised.

(iii) THE EFFECT OF TNBP ON IgM CELL CULTURE SUPERNATANT

The experiment with ßPL was repeated using TNBP as an inactivant (section 3.10.2) instead of ßPL, to determine whether TNBP has a detrimental effect on antibody activity.

HAEMOLYSIS:

	TEST SAMPLE / CONTROL	LOG_2 TITRE			MEAN TITRE	SD
	SAMPLE:					
1	Mab + 1% Tween 80 + 0.1% TNBP	3	4	6	4.3	1.25
	CONTROLS:					
2	Mab + 1% Tween 80	4	6	6	5.3	0.94
3	Mab alone	4	5	8	5.7	1.70
4	Saline + 1% Tween 80	0	0	0	0	0

Table 4.5: Effect of TNBP on the Haemolytic Activity of IgM in Cell-Culture Supernatant, expressed as log_2 haemolytic titre.

The results obtained were inconsistent. The presence of tween 80 in the samples seemed to affect the haemolytic assay. The unlysed pellets of blood cells appeared to be larger when tween 80 was present and the end-point was not well defined in the presence of tween 80 as partial lysis was observed in several wells. This could be due to tween affecting the properties of the cell-membrane or due to the tween interfering with haemolytic activity of the antibody. Tween is present in very

low quantities and, because of its viscosity, is difficult to measure volumetrically; it would be reasonable to assume that this has contributed to the variabilty of the results obtained.

(iv) THE EFFECT OF TNBP ON PURIFIED IgM

Purified IgM was also treated with TNBP.

HAEMAGGLUTINATION:

	TEST SAMPLE / CONTROL	LOG_2 TITRE		MEAN TITRE	SD
	SAMPLE:				
1	Mab + 1% Tween 80 + 0.1% TNBP	4	4	4	0
	CONTROLS:				
2	Mab + 1% Tween 80	3	3	3	0
3	Mab alone	11	10	10.5	0.5
4	Saline + 1% Tween 80	0	0	0	0

Table 4.6: Effect of TNBP on the HA of High Purity IgM, expressed as log_2 HA titre.

As for experiment (ii), the agglutination reaction obtained in this assay is weak. The presence of tween 80 in the samples has a notable affect on the haemagglutination reaction; the agglutination of the red blood cells in the initial wells was stronger in the presence of tween 80 even though the titres read are lower, the pellets of red blood cells appeared to be larger in the presence of tween 80. However, the end-point is notably lower for the antibody samples which have been treated with

TNBP, indicating that the chemical may have some effect on the Fab region of the antibody which is responsible for antigen binding.

HAEMOLYSIS:

	TEST SAMPLE / CONTROL	LOG_2 TITRE[3]					MEAN TITRE	SD
	SAMPLE:							
1	Mab + 1% Tween 80 + 0.1% TNBP	3	6	10	4	4	5.4	2.5
	CONTROLS:							
2	Mab + 1% Tween 80	5	5	7	3	3	4.6	1.5
3	Mab alone	7	7	9	11	10	8.8	1.6
4	Saline + 1% Tween 80	0	0	0	0	0	0	0

Table 4.7: Effect of TNBP on the Haemolytic Activity of high purity IgM, expressed as log_2 haemolytic titre.

From the results given in **Table 4.7** it can be seen that the treatment of the antibody with tween reduces the activity of the antibody 8 fold.

3 Titres represent titrations of experiments carried out on different occasions under the same conditions using different batches of reagents.

From these preliminary experiments, it was concluded that the assay methods in the presence of tween were inappropriate for the determination of the effect of the inactivating agent on the activity of monoclonal antibodies.
Possibilities for using an alternative assay or enhancing the agglutination in the existing assay were considered. It was thought that removing tween from the samples would introduce significant error to the experiment and that diluting the tween to a level where it no longer interferes with the assay used would be a more satisfactory approach to validating the effect of TNBP and tween 80 on the Fab region of the immunoglobulin more precisely. Hence, further experiments were to be carried out using the purified antibody only.

The results from this section of work have been summarised below. Difficulties involved in obtaining experimental data and action undertaken in an attempt to overcome the problems which arose to provide more conclusive data, are subsequently discussed in detail.

Table 4.8: Summary Table of Effect of ßPL Treatment on Mab: The Significance of the Data.

		MEAN TITRE		
		Supernatant	Purified	
	TEST SAMPLE/ CONTROL	HL Titre	HA Titre	HL Titre [4]
	SAMPLE:			
1	Mab + Na_2HPO_4 + ßPL	3	11	0
	Significance:	+++	-	
	CONTROLS:			
2	Mab + dH_2O + ßPL	4	3	4
	Significance:	+++	+++	
3	Mab alone	7	13.25	5

Significance, (see appendix IV, does this value differ significantly from Mab alone?):

+++ is >99 % significant
++ is 95 - 99 % significant
+ is 90 - 95 % significant
- is <90 % significant

4 Not enough data collected for significance evaluation.

Table 4.9: Summary Table of Effect of TNBP Treatment on Mab: The Significance of the Data.

		MEAN TITRE		
		Supernatant	Purified	
	TEST SAMPLE/ CONTROL	HL Titre	HA Titre	HL Titre
	SAMPLE:			
1	Mab + 1% Tween 80 + 0.1% TNBP	4.3	4	5.4
	Significance:	+	+++	+++
	CONTROLS:			
2	Mab + 1% Tween 80	5.3	3	4.6
	Significance:	-	+++	+++
3	Mab alone	5.7	10.5	8.8
4	Saline + 1% Tween 80	0	0	0

Significance, (see appendix IV, does this value differ significantly from Mab alone?):

+++ is >99 % significant
++ is 95 - 99 % significant
+ is 90 - 95 % significant
- is <90 % significant

The t-distribution has been used for determination of the significance between two mean values, (i.e. the untreated Mab control and the chemically treated Mab), (appendix V).

From the results shown in **Table 4.8** it can be seen that the mean haemolytic titre has been reduced significantly when the Mab in supernatant and in its purified form is treated with ßPL.
The mean haemagglutination titre has not been reduced significantly when the Mab has been given treatment with ßPL and orthophosphate. However the haemagglutination titre was reduced when the purified Mab was treated with ßPL in the absence of disodium hydrogen orthophosphate.
ßPL degrades into ß-hydroxypropionic acid in aqueous solution. Disodium hydrogen orthophosphate is included in the treatment process so that ß-hyroxypropionic acid is converted into its sodium salt. In the absence of Na_2HPO_4 it is likely that the low pH conditions led to acid degradation of the IgM during the treatment process; hence, with careful pH control of the inactivation process this degradation maybe overcome.

From **Table 4.9**, showing the effect of TNBP on the Mab, the mean titres for haemolysis and for haemagglutination were reduced significantly when purified MAb was treated with TNBP and tween 80; however, the haemolytic titres for treated and untreated cell culture supernatant are not significantly different.
Difficulties arose in reading the end points of these assays. The pellets of red blood cells were notably larger in diameter in the presence of tween; also some autolysis of the cells was noted in the presence of tween, e.g. in the negative control where no MAb was present but tween was, although complete lysis of a pellet did not occur pigment was observed in solution, indicating presence of haemoglobin in solution and hence lysis of red blood cells.
Because tween is a powerful ionic surfactant, its presence in solution causes a reduction in surface tension leading to a reduction in flocculation of red blood cells. Its affinity for the polar heads of the cells lipid membrane may be such that lysis occurs in older and more fragile cells.
However, because of the apparent effect of tween 80 on the assays used and the difficulty in removing it from samples, it

was decided that an ELISA assay should be used as an alternative method for determining the antigen binding ability of the antibody after treatment with chemicals.

Problems arose from the haemagglutination assay used, in that agglutination of the sheep red blood cells was weak so that it was difficult to establish the end-points. It was therefore decided, that for the purpose of these experiments, the assay agglutination needed to be improved to obtain clear-cut end-points.

Because the ßPL treatment process involves pH changes, it is critical to adjust the pH so that it is equal in all the samples that are tested; pH significantly affects antibody binding and function. Due to limitations of the amount of antibody that could be practically purified at research scale, small amounts of antibody samples were treated with the inactivating chemicals; the adjustment of pH could have contributed to experimental errors in these assays because of the small volumes involved. To overcome this problem, more antibody was obtained and purified so that the experiments could be repeated using larger volumes and so minimising experimental error.

The nature of the reagents used, ie SIII-adsorbed sheep red blood cells and complement, could also contribute to experimental error. Different batches and age of red blood cells used could affect the adsorption properties of the SIII polysaccharide to them and hence the adsorption of both antibody and complement.

Furthermore, the use of standard reagents, by preparation of appropriate reagents in bulk and by carrying out a large number of experiments at one time was seen to be important. It was decided that to produce statistically sound data, a number of experiments should be carried out at one time and the data analysed.

Because of the apparent effect of tween 80 on the assays used and the difficulty in removing it from the samples, it was decided that an ELISA assay should be used as an alternative method for determining the antigen binding ability of the antibody after treatment with the chemicals.

Thus the following measures were taken in an attempt to improve experimental data and the previous experiments were repeated.
By changing the diluent of the titrated samples and the red blood cells from physiological saline (0.95 % NaCl) to 5 % bovine serum albumin (BSA), haemagglutination was visibly much stronger (section 3.10.1 A).
More antibody was purified and larger volumes of antibody preparations were treated with the inactivating chemicals in an attempt to avoid experimental error due to change in final volumes in post-treatment pH adjustment.

It was decided that each sample should be assayed 8 times and that all the assays should be carried out at the same time. This number was chosen in part as it is a feasible and practical number of assays to carry out one time. It was decided that if the ratio of the standard deviation to the mean titre, i.e. coefficient of variance, was less than 70 % that the results should be discarded. The samples were taken from antibody preparations which had been treated with inactivating chemicals at one time and they were assayed with reagents which had been obtained from the same source and prepared appropriately at one time.

B. HAEMAGGLUTINATION ACHIEVED AFTER ASSAY MODIFICATION

Agglutination of red blood cells in the original assay (section 3.10.1) was improved by using Bovine Serum Albumin (BSA) at 5% (w/v) in saline as a diluent (instead of saline) and by incubating the cells at 4 °C (instead of room temperature).
Experiments 4.2.1 and 4.2.3 were repeated and the samples were assayed by haemagglutination to determine the effects of ßPL and TNBP on the specificity of the IgM in cell culture supernatant. The supernatant was titrated 2 fold across a microtitre plate and the end-point was taken as the last well which showed haemagglutination; the reaction was read by eye. Because of much improved haemagglutination giving a much clearer end point, it was decided that the dilutions should be of a tighter range; the assay was repeated using 3:1 dilutions (antibody: diluent). However, the assays were not sensitive enough to detect end-points, by visual inspection at these dilutions.
The assays performed in each of the following sections were performed simultaneously with standardised reagents.

(i) THE EFFECT OF ßPL ON PURIFIED IgM

HAEMAGGLUTINATION:

	TEST SAMPLE / CONTROL	LOG_2 TITRE	MEAN TITRE	SD
	SAMPLE:			
1	Mab + Na_2HPO_4 + ßPL	12 11 12 11 11 11 12 11	11.37	0.48
	CONTROLS:			
2	Mab + dH_2O + ßPL	4 4 4 4 4 4 4 4	4.0	0
3	Mab alone	11 11 11 11 11 11 11 12	11.12	0.33
4	Saline + Na_2HPO_4 + ßPL	0 0 0 0 0 0 0 0	0	0

Table 4.10: Effect of ßPL on the Haemagglutination of Purified IgM, expressed as log_2 haemagglutination titre.

HAEMOLYSIS:

	TEST SAMPLE / CONTROL	LOG_2 TITRE	MEAN TITRE	SD
	SAMPLE:	8 9 9 8		
1	Mab + Na_2HPO_4 + ßPL	9 9 9 8	8.63	0.48
	CONTROLS:	0 0 0 0		
2	Mab + dH_2O + ßPL	0 0 0 0	0	0
		8 8 8 8		
3	Mab alone	9 9 8 8	8.13	0.43
		0 0 0 0		
4	Saline + Na_2HPO_4 + ßPL	0 0 0 0	0	0

Table 4.11: Effect of ßPL on the Haemolytic Activity of Purified IgM, expressed as log_2 haemolytic titre.

From the results in the above two tables it would appear that ßPL has no effect on the functional activity of the immunoglobulin.

(ii) THE EFFECT OF TNBP ON PURIFIED IgM

HAEMAGGLUTINATION:

	TEST SAMPLE / CONTROL	LOG_2 TITRE	MEAN TITRE	SD
	SAMPLE:			
1	Mab + 1% Tween 80 + 0.1% TNBP	11 11 11 11 11 11 11 11	11.0	0
	CONTROLS:			
2	Mab + 1% Tween 80	11 11 11 12 12 11 11 11	11.25	0.43
3	Mab alone	10 10 10 10 10 9 10 9	9.75	0.43
4	Saline + 1% Tween 80	0 0 0 0 0 0 0 0	0	0

Table 4.12: Effect of TNBP on the Haemagglutination of Purified IgM, expressed as log_2 haemagglutination titre.

From the **table 4.12** it can be seen that the titres where tween is present is higher than they are in the Mab control. This indicates that tween enhances agglutination.

HAEMOLYSIS:

	TEST SAMPLE / CONTROL	LOG_2 TITRE	MEAN TITRE	SD
	SAMPLE:			
1	Mab + 1% Tween 80 + 0.1% TNBP	6 6 7 6 7 7 7 8	6.6	0.66
	CONTROLS:			
2	Mab + 1% Tween 80	7 7 7 7 7 7 7 7	7.0	0
3	Mab alone	7 7 8 7 7 7 7 7	7.1	0.33
4	Mab + TNBP	7 7 7 7 7 7 7 7	7.0	0
5	Saline + 1% Tween 80	0 0 0 0 0 0 0 0	0	0

Table 4.13: Effect of TNBP on the Haemolytic Activity of Purified IgM, expressed as log_2 haemolytic titre.

From the above table it can be seen that the titres where tween and TNBP are present are slightly lower than the controls. However the amount is only small and the t-test was applied to determine the significance of these differences, **table 4.15.**

Table 4.14: Summary Table of Effect of ßPL Treatment on Purified Mab: The Significance of the Data.

		MEAN TITRE	
		Purified	
	TEST SAMPLE/ CONTROL	HA Titre	HL Titre
	SAMPLE:		
1	Mab + Na_2HPO_4 + ßPL	11.37	8.63
	CONTROLS:		
2	Mab + dH_2O + ßPL	4.0	0
3	Mab alone	11.12	8.13
4	Saline + Na_2HPO_4 + ßPL	0	0

All results are > 95 % significant.
(see appendix IV)

Table 4.15: Summary of the Effect of TNBP on Purified Mab: The Significance of the Data.

		MEAN TITRE	
		Purified	
	TEST SAMPLE/ CONTROL	HA Titre	HL Titre
	SAMPLE:		
1	Mab + 1% Tween 80 + 0.1% TNBP	11	6.6
	CONTROLS:		
2	Mab + 1% Tween 80	11.25	7.0
3	Mab alone	9.75	7.1
4	Saline + 1% Tween 80	0	0

All results are > 95 % significant.
(see appendix IV)

C. THE USE OF ELISA AS A COMPARISON TO HAEMAGGLUTINATION

An ELISA was adapted (section 3.9.1 C) to detect the specificity of IgM 1A/F11/D12/A3. A conventional ELISA technique was used, using an antigen coated ELISA plate. The antigen used was the SIII polysaccharide which had been extracted from the cell wall of type III *S.pneumoniae*.

A chequerboard type assay, where the antigen was titrated down the plate and the conjugate was titrated across the plate, was performed to establish optimal dilutions of these reagents to obtain a good standard curve, (section 4.1.6).

Experiments 4.1.7 B was repeated and the samples were assayed by ELISA to quantify the effects of ßPL and TNBP on the specificity of the IgM in cell culture supernatant. Purified IgM which had been assayed for total protein and therefore of known concentration, was used as a standard in the assay.

(i) THE EFFECT OF ßPL ON CELL-CULTURE SUPERNATANT

RESULTS:

	TEST SAMPLE / CONTROL	ACTIVITY[5] (µg/ml)	MEAN ACTIVITY (µg/ml)	SD
	SAMPLE:			
1	Mab + HPO_4 + ßPL	66 52 61 53	58.0	5.78
	CONTROLS:			
2	Mab + dH_2O + ßPL	60 60 61.2 58.4	60.0	0.99
3	Mab alone	88 66 80 89.6	80.9	9.34
4	Saline + HPO_4 + ßPL	0 0 0 0	0	0

Table 4.15: Effect of ßPL on the Activity of IgM measured by ELISA in Cell-Culture Supernatant, expressed as mean activity.

It is clear from the results tabulated above, **table 4.15** and the graph, **figure 4.11** that ßPL distinctly reduces the activity of the Fab region of this monoclonal antibody by 25%. It is interesting that these results, obtained by ELISA, are significantly different from the results obtained by haemagglutination, although these assays are designed to measure the same property of the antibody. Although this method, where the biological activity is measured by transduction, is undoubtedly more reproducible than the former in which the

5 mean activity is the concentration of antibody measured against a purified antibody standard which had been assayed for total protein by Bradford's reaction.

end-point is read by eye, one would not expect to obtain results so different.

Other differences between the two assays which may have an effect on the antibody-antigen binding are:

Differences in antigen adsorption in each assay; it is not known which part of the antigen binds to the red blood cell or to the surface of the ELISA plate and whether the same sites are involved in each case. Adsorption to the ELISA plate may result in steric hindrance of the antigenic site so that although an interaction with the antibody may occur, it may not occur with full affinity and if some other part of the IgM molecule has been denatured by chemical treatment the molecule may not be able to fold adequately to utilise all its antigenic sites.

In the haemagglutination assay the antigen is adsorbed onto red blood cells which are freely suspended in solution, whereas in ELISA the antigen is immobilised onto a fixed surface. This could mean that the antibody binds more easily to the antigen adsorbed onto the red blood cells as they are free to move in space so that they may be attracted towards the antibody, allowing the antibody to maintain its natural conformation, whereas in an ELISA, because the antigen is fixed, the five antigen binding sites of the IgM molecule may not be sufficiently accessible to be saturated due to the inability of the molecule to fold adequately.

(ii) THE EFFECT OF ßPL ON PURIFIED IgM

	TEST SAMPLE / CONTROL	ACTIVITY (µg/ml)[6]	MEAN ACTIVITY (µg/ml)	SD
	SAMPLE:			
1	Mab + Na_2HPO_4 + ßPL	45 61 46 53	51.0	6.4
	CONTROLS:			
2	Mab + dH_2O + ßPL	18 17 15 15	16.25	1.3
3	Mab alone	66 57 93 62	69.5	13.9
4	Saline + Na_2HPO_4 + ßPL	0 0 0 0	0	0

Table 4.16: Effect of ßPL on the Activity of Purified IgM, measured by ELISA, expressed as mean activity.

From the results tabulated above and the histogram, **figure 4.12**, it can be seen that ßPL has reduced the activity of the Fab region of the antibody molecule by 26 %. This correlates with the results obtained in section A. However there is also a further reduction in activity that can be seen where Na_2HPO4 is not used to neutralise the acidity of the ßPL. It is possible that the buffer in the cell-culture supernatant prevented acid denaturation of the antibody molecule in the previous experiment, or that other proteins present in the cell-culture supernatant were more readily denatured than the immunoglobulin at this pH.

6 mean activity is the concentration of antibody measured against a purified antibody standard which had been assayed for total protein by Bradford's reaction.

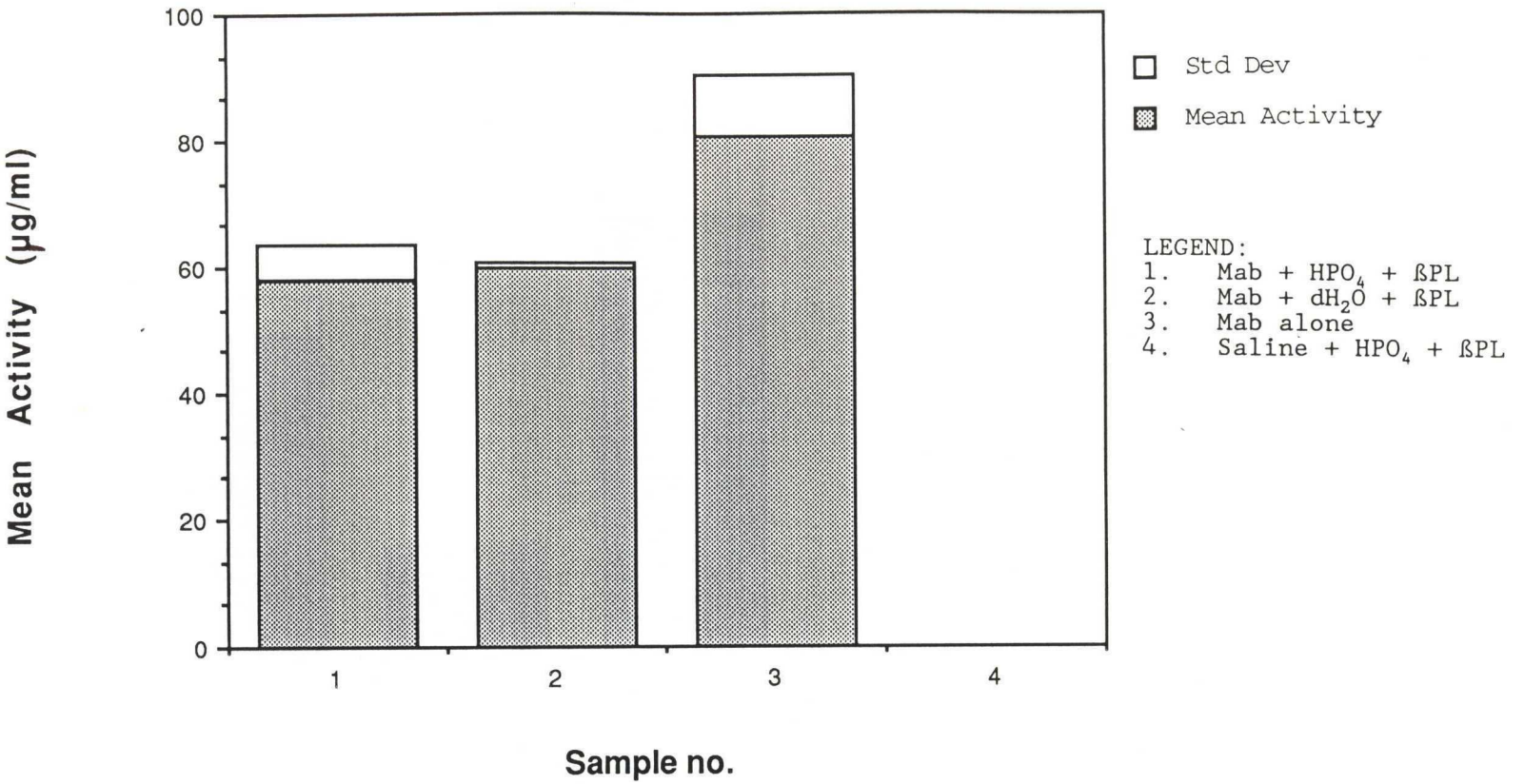


Figure 4.11: Histogram showing the effect of ßPL on IgM in cell-culture supernatant, expressed as mean activity by ELISA.

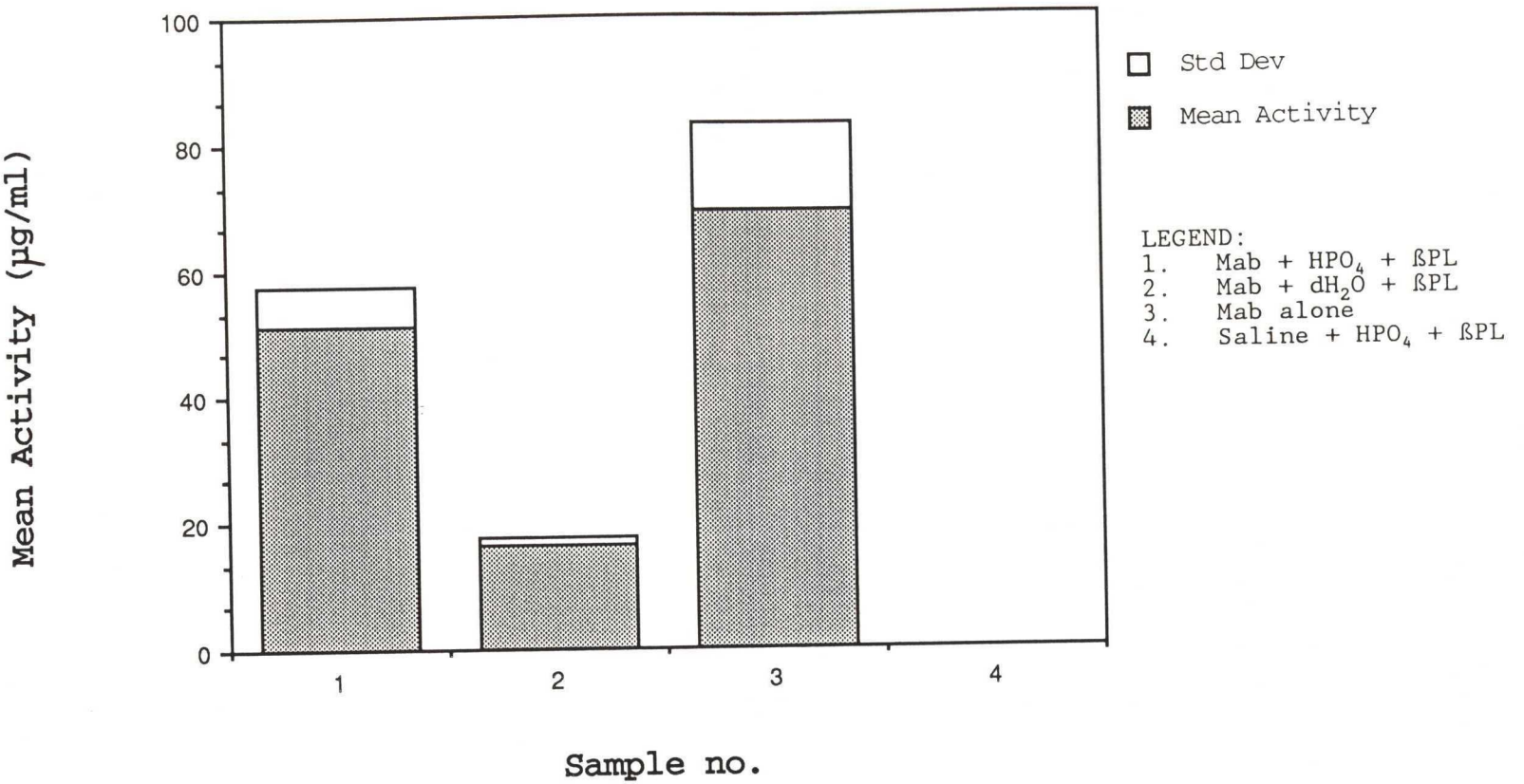


Figure 4.12: Histogram showing the effect of ßPL on purified IgM, expressed as mean activity by ELISA.

(iii) THE EFFECT OF TNBP ON CELL-CULTURE SUPERNATANT

	TEST SAMPLE / CONTROL	MEAN ACTIVITY (μg/ml)[7]				MEAN ACTIVITY (μg/ml)
	SAMPLE:					
1	Mab + 1% Tween 80 + 0.1% TNBP	96	80	49	23	62
	CONTROLS:					
2	Mab + 1% Tween 80	96	70	41	26	58
3	Mab alone	62	31	16	8	29
4	Mab + 1% TNBP	53	23	12		29

Table 4.17: Effect of TNBP on the Activity of IgM in Cell-Culture Supernatant, measured by ELISA, expressed as mean activity.

7 mean activity is the concentration of antibody measured against a purified antibody standard which had been assayed for total protein by Bradford's reaction.

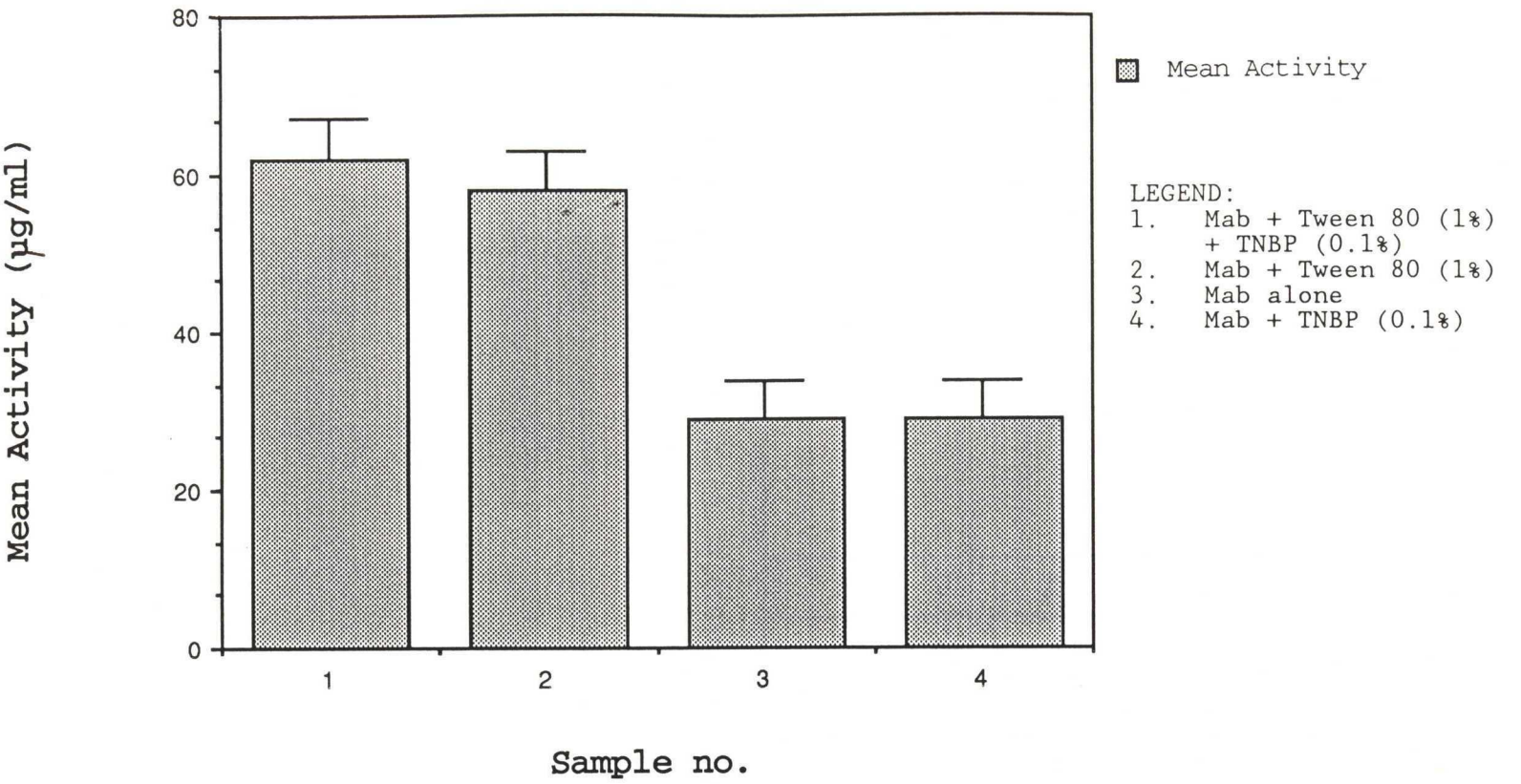


Figure 4.13: Histogram showing the effect of TNBP on IgM in cell-culture supernatant, expressed as mean activity by ELISA. Error bars are given at 5 % indicating limitations of assay.

(iv) THE EFFECT OF TNBP ON PURIFIED IgM

	TEST SAMPLE / CONTROL	MEAN ACTIVITY (μg/ml)[8]				MEAN ACTIVITY (μg/ml)
	SAMPLE:					
1	Mab + 1% Tween 80 + 0.1% TNBP	96	80	49	23	58
	CONTROLS:					
2	Mab + 1% Tween 80	96	70	41	26	60
3	Mab alone	62	31	16	8	51
4	Mab + 1% TNBP	53	23	12		55

Table 4.18:Effect of TNBP on the Activity of purified IgM, measured by ELISA, expressed as mean activity.

8 mean activity is the concentration of antibody measured against a purified antibody standard which had been assayed for total protein by Biorad.

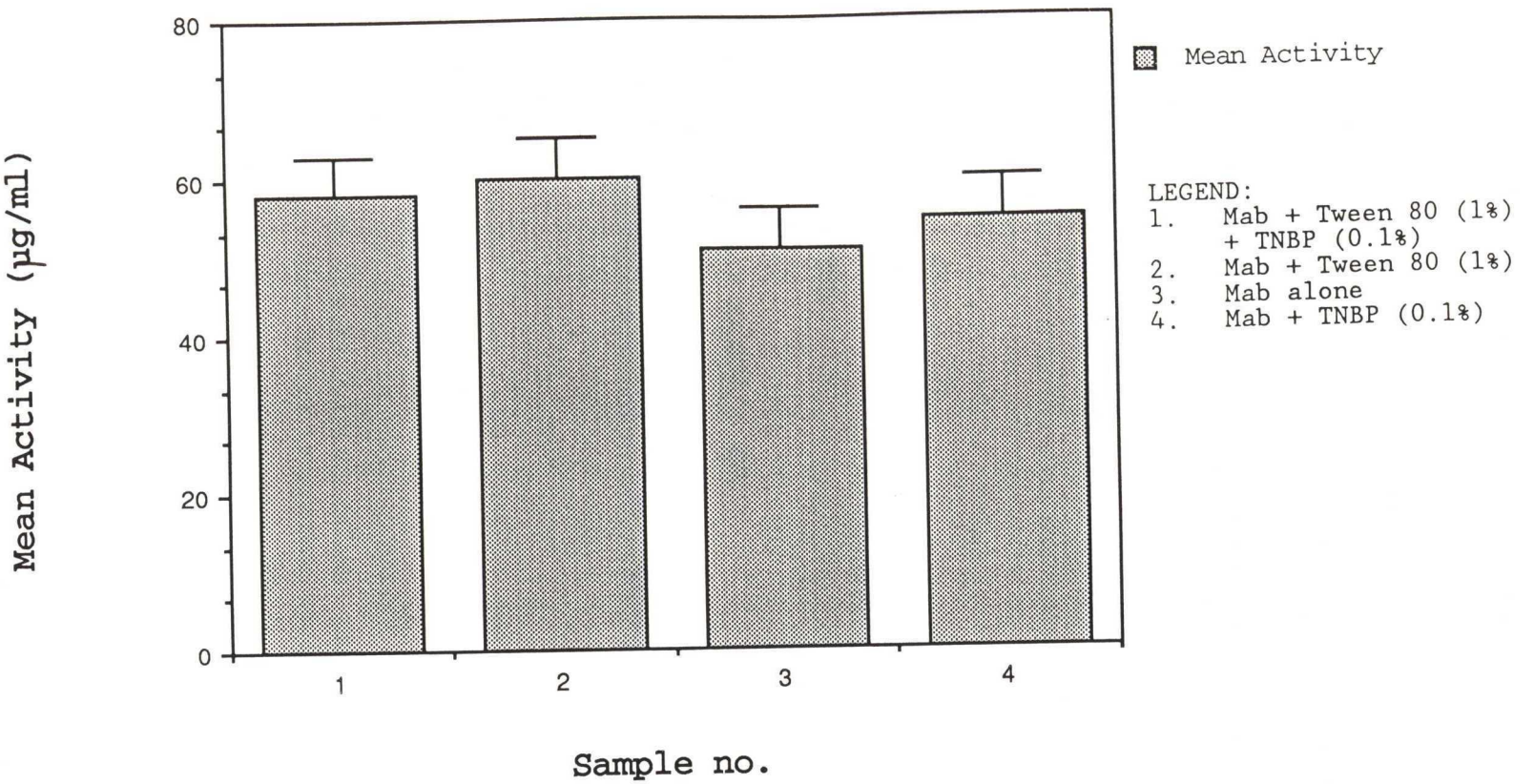


Figure 4.14: Histogram showing the effect of TNBP on purified IgM, expressed as mean activity by ELISA. Error bars are given at 5 % indicating limitations of assay.

Table 4.19: Summary Table of Effect of ßPL Treatment on Purified Mab, measured by ELISA: The Significance of the Data.

		MEAN ACTIVITY[9]	
	Mab treatment/ Control	Supernatant	Purified
	SAMPLE:		
1	Mab + Na_2HPO_4 + ßPL	0.72	0.73
	CONTROLS:		
2	Mab + dH_2O + ßPL	0.74	0.23
3	Mab alone	1.0	1.0
4	Saline + Na_2HPO_4 + ßPL	0	0

9 Mean activity has been normalised so that the monoclonal antibody control is 1.

Table 4.20: Summary Table of Effect of ßPL Treatment on Purified Mab, measured by ELISA: The Significance of the Data.

	Mab treatment/ Control	MEAN ACTIVITY[10] Supernatant	Purified
	SAMPLE:		
1	Mab + 1% Tween 80 + 0.1% TNBP	2.14	1.14
2	CONTROLS:		
	Mab + 1% Tween 80	2.0	1.18
3	Mab alone	1.0	1.00
4	Saline + 1% Tween 80	1.0	1.08

It is apparent from these results that ßPL distinctly reduces the activity of the antibody binding region of this monoclonal antibody by 25 %.

The effect of TNBP on this antibody is not so well defined however. From **figure 4.13** it can be seen that in the first two columns, i.e. when tween is present with antibody, the signal is stronger than in the third and fourth columns, where tween is absent. This would indicate that tween enhances the optical signal in this reaction. It can be seen from **figure 4.14** that the difference between the optical signals in the first and second columns compared with those in the third and fourth columns is insignificant when taking into account the error of

10 Mean activity has been normalised so that the monoclonal antibody control is 1.

the assay; this results from the fact that the chemically treated purified antibody is 40 times as concentrated as the chemically treated cell-culture supernatant (1 mg ml^{-1} versus 0.024 mg ml^{-1}), so that the tween has been diluted 1:40 by virtue of the assay. Hence the difference between the signals read for the samples in the presence and absence of tween diminishes with increasing dilution of the samples. This observation supports the previous comment that tween may enhance the optical signal. To ensure that tween does not affect the assay it would be necessary to remove it completely from the samples.

However, it does appear from **figure 4.14** that tween has been diluted out sufficiently so as not affect the assay and that TNBP alone does not effect the antigen binding capacity of this monoclonal antibody during the treatment process.

4.2 ASSESSMENT OF THE EFFECT OF INACTIVATION STRATEGIES ON A HUMAN IgG1 CLASS ANTIBODY

4.2.1 PRODUCTION OF HUMAN IgG1

Figures 4.15 and **4.16** describe the profiles for cell growth of the anti-RhD lymphoblastoid cell-line (all clone 1) in stirred controlled and unstirred, uncontrolled batch conditions, repectively.
In the stirred vessel (STR) the growth profile shows faster growth throughout the exponential growth phase (doubling time = approx. 15 hrs) than in the unstirred tissue culture flask (doubling time = approx. 30 hrs); also the maximum cell density achieved in the STR (approx. 200.10^4) was over double that of the flask (approx. 85.10^4). The inoculum used was the same for each vessel and was characterised by large aggregates of cells. It is likely that the differences in cell yields results from the fact that the aggregates were broken up by shear forces generated in the STR; consequently mass transfer limitations (due to starvation of the cells in centre of aggregates) were greatly reduced. The cell aggregates were larger in the unstirred vessel and account for the error in cell counts seen on the graph.

Cell-culture supernatant generated by fed batch fermentation was found to have a haemagglutination titre of between 1/2848 and 1/5696. The agglutination reaction was much stronger using this IgG1 antibody than when using the IgM antibody.
A preliminary study was undertaken to investigate the effects of TNBP and ßPL on the haemagglutination titre of this antibody.

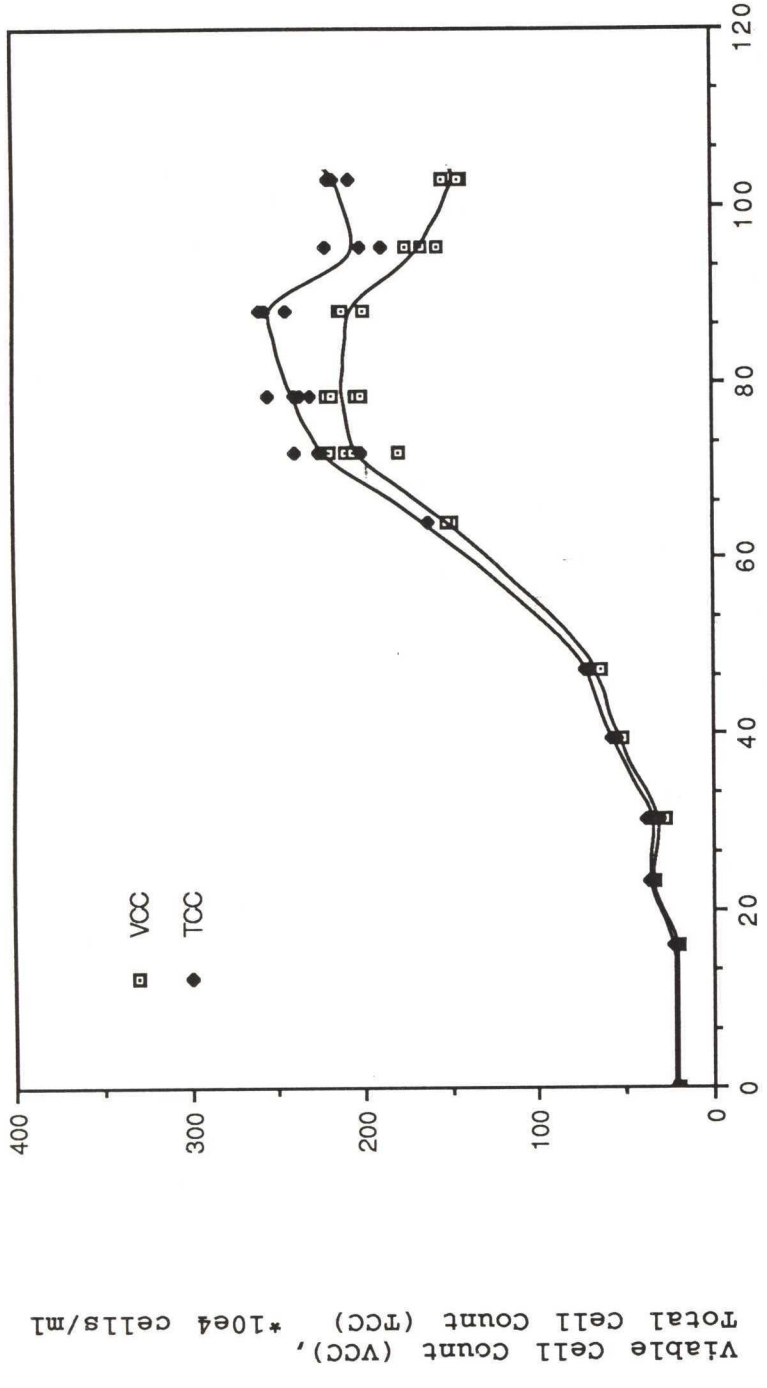


Figure 4.15: Rate of cell growth of human anti-RhD IgG1 secreting cell line, (all clone 1) in stirred batch conditions (STR).

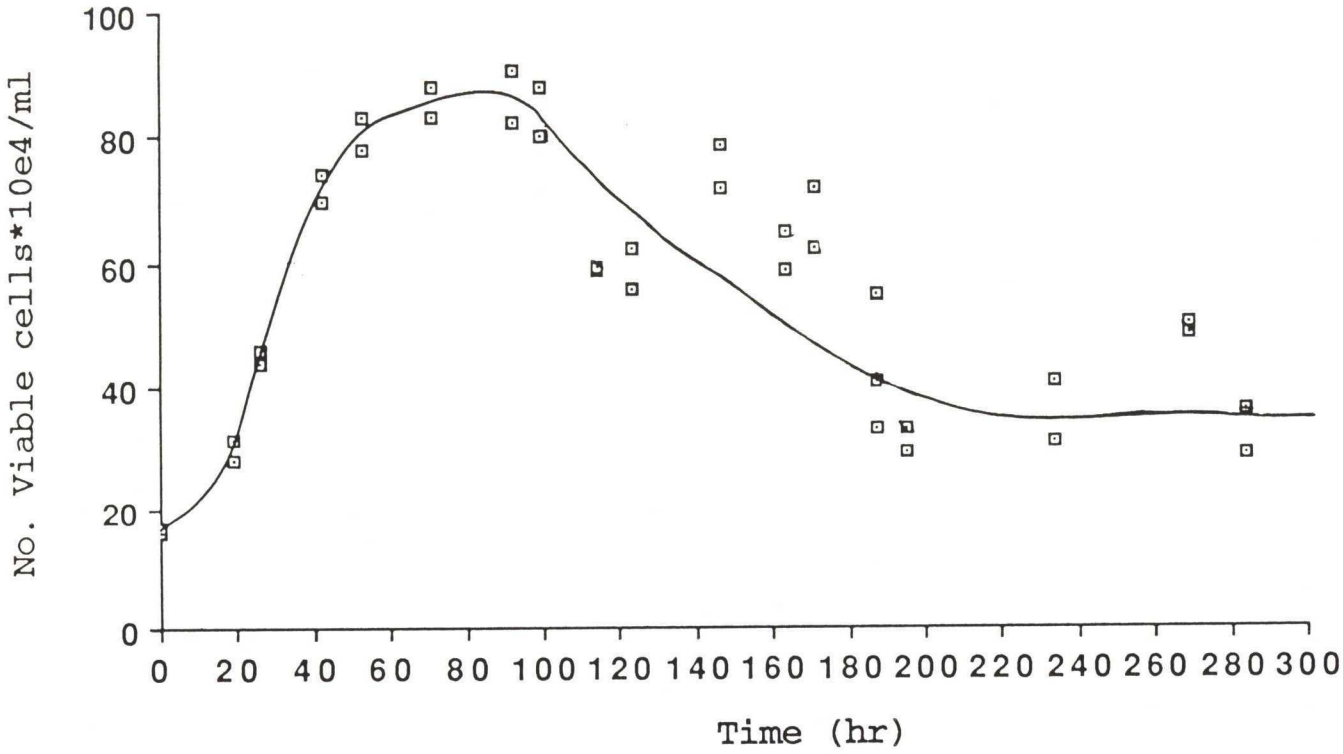


Figure 4.16: Rate of cell growth of human anti-RhD IgG1 secreting cell line, (all clone 1) in unstirred batch conditions (flask).

4.2.2 ASSESSMENT OF Fab ACTIVITY OF ANTIBODIES FOLLOWING CHEMICAL INACTIVATION TREATMENTS

A. HAEMAGGLUTINATION

(i) THE EFFECT OF ßPL ON HUMAN IgG1 IN CELL-CULTURE SUPERNATANT

HAEMAGGLUTINATION:

	TEST SAMPLE / CONTROL	LOG_2 TITRE	MEAN TITRE	SD
	SAMPLE:			
1	Mab + Na_2HPO_4 + ßPL	12 11 12 11 11 11 12 11	11.37	0.48
	CONTROLS:			
2	Mab + dH_2O + ßPL	4 4 4 4 4 4 4 4	4.0	0
3	Mab alone	11 11 11 11 11 11 11 12	11.12	0.33
4	Saline + Na_2HPO_4 + ßPL	0 0 0 0 0 0 0 0	0	0

Table 4.21: Effect of ßPL on the Fab Activity of IgG1 in Cell-Culture Supernatant, expressed as log_2 haemolytic titre.

(ii) THE EFFECT OF TNBP ON HUMAN IgG1 IN CELL-CULTURE SUPERNATANT

HAEMAGGLUTINATION:

	TEST SAMPLE / CONTROL	LOG_2 TITRE	MEAN TITRE	SD
	SAMPLE:			
1	Mab + 1% Tween 80 + 0.1% TNBP	11 11 11 11 11 11 11 11	11.0	0
	CONTROLS:			
2	Mab + 1% Tween 80	11 11 11 12 12 11 11 11	11.25	0.43
3	Mab alone	10 10 10 10 10 9 10 9	9.75	0.43
4	Saline + 1% Tween 80	0 0 0 0 0 0 0 0	0	0

Table 4.22: Effect of TNBP on the Fab Activity of IgG1 in Cell-Culture Supernatant, expressed as log_2 haemolytic titre.

Preliminary investigations demonstrated no change in the antigen binding capacity of the human IgG1 molecule following treatment with either ßPL or TNBP, tables 4.21 and 4.22. The agglutination reaction with this antibody was noted to be very strong, as a large network of agglutinated red blood cells was observed when the reaction was positive and the end-point could be easily determined.

CONCLUSION

5 CONCLUSION

The monoclonal antibody, IgM 1A/F11/D12/A3, which reacts with the capsular polysaccaride material of *S.pneumonae*, was produced in ascites and *in vitro* cell culture. The antibody yield in ascites was 2 mg. The yield in animal cell-culture was 24 µg Mab ml^{-1}. The antibody was successfully purified to a purity estimated to be greater than 98 % using gel filtration followed by affinity chromatography using protein A. The purified antibody was concentrated for use at 1 mg ml^{-1}.

The monoclonal antibodies were treated using two protocols for inactivation of viruses using the chemicals TNBP and ßPL. Immunoassays were used to determine the effect of these chemicals on the specific activity (structure of Fab) and the functional activity (structure of Fc) of the model monoclonal antibody.

The findings regarding the effect of TNBP and ßPL on the mouse IgM antibody can be summarised in the following way:

a) In the haemagglutination and haemolysis assays in the absence of BSA, the titres were significantly reduced after the monoclonal antibody (purified and cell-culture supernatant) had been treated with ßPL, **table 4.8**. However, the agglutination in these assays was weak and difficult to interpret, the assay was subsequently modified using 5 % BSA diluent to enhance agglutination. In the presence of 5 % BSA there was no difference seen in activity of the monoclonal antibody following treatment in either assays, **table 4.14**.

b) In the absence of BSA from the assay, the haemolytic titre of antibody was reduced when it had been treated with TNBP and tween 80. The titres for haemagglutination and for haemolysis were reduced when the purified antibody had been subjected to either TNBP and tween 80 or tween 80 alone, **table 4.9**. The initial concentration of purified antibody was greater than that of the supernatant; at low concentration of antibody the accuracy of assays is poor due to the law of mass action: i.e. the rate of complex formation is proportional to the concentration of reagants, so that at low antibody concentrations the reaction may not go to completion. Again, agglutination was weak in these assays; in addition tween was seen to cause auto-lysis of agglutinated cells. Hence the assays were repeated using 5 % BSA diluent to enhance agglutination. In the

presence of 5 % BSA the titres for haemagglutination and haemolysis were not significantly different for untreated and treated antibody, **table 4.15**. It was concluded that TNBP does not significantly alter the activity of the monoclonal antibody in the presence of 5 % BSA.

c) From the ELISA data it can be seen that ßPL reduced the activity of the IgM supernatant preparation by 25 %, **table 4.19**. The activity of purified antibody was reduced by 75 % in presence of ßPL and the absence of hydrogen orthophosphate. This was almost certainly due to acid denaturation, the buffer present in the cell-culture media could have buffered this pH change enough to prevent denaturation.

d) The ELISA data shows no alteration of activity of the monoclonal antibody following treatment with TNBP, **table 4.20**, when assuming such assays are subject to error of ± 5 % (from experience in our lab.). It was noted that at certain concentrations tween 80 enhances specific binding.

e) Haemagglutination assays show no alteration in the activity of human IgG1 subsequent to treatment with either ßPL or TNBP, **table 4.21** and **table 4.22**. However, these were preliminary studies and investigations using alternative assays must be used to draw conclusive evidence of the effect of the inactivants on this antibody.

When drawing conclusions from the results of immunoassays it is important to differentiate between differences in avidity and affinity of the antibodies participating in the reaction (section 2.2.2 B). As the reactions were carried out under different conditions, they must be viewed independently since the affinity constant will be different for each reaction. However, the change in activity of the antibody following a chemical treatment may be compared to the antibody control in that test to determine the change of antibody avidity following that chemical treatment protocol.
Because of the multivalent nature of IgM it is common that the avidity of this molecule is 10^2 - 10^4 higher than the affinity of the isolated sites (its Fab fragments). In the absence of BSA it was difficult to determine the end-point of the haemagglutination assay

because of the weak avidity of the molecule; because of this, the assay was repeated in the presence of 5 % BSA. While increasing the BSA concentration increases affinity and hence detectability of the antibody, the precision of the measurement avidity is reduced.
Although there are inherent inaccuracies in these type of assays, since there is no electronic component to provide sensitivity in measurement, the analysis of the results gives a degree of confidence in the data. If the difference in end-point between treated and untreated antibody was ± 2 titres, then the difference is taken to be significant.

Therefore, it is apparent that both chemicals affect the activity of the monoclonal antibody as specificity is more accurately determined in the absence of BSA. However, by increasing affinity, i.e. in the presence of BSA, no significant alteration in the biological activity of the antibody can be detected using the haemagglutination and haemolysis assays described. Because the model is applicable to antibodies for therapeutic application and 5 % BSA is closer to the concentration of proteins in human sera, the assay using this diluent serves as a better model. Assessment of the functional integrity of Ig is difficult as an antigen-antibody complex has a greater affinity for an Fc receptor than an unbound antibody, therefore, since it is apparent that the activity of the antigen-binding region has been reduced, it would be useful to assay the Fc region of unbound antibody. Approaches which could be taken include assessment of ability of Fc to bind to Protein A or use of an anti-Fc antibody in an ELISA.

ELISAs have the advantage that sample constituents are removed in dilution and washing of samples so that errors are likely to be smaller than in the previous assays described. The ELISA was carried out in the presence of 1 % BSA so that the precision of measurement of specificity is higher than when 5 % BSA is used. However, it would be interesting to repeat the assay in the presence of 5 % BSA so that the results could be more comparable to those of the haemagglutination and haemolysis assays; the sensitivity and accuracy of ELISA assays is obviously greater than for haemagglutination and haemolysis.

There is little work which has been published regarding the effect of ßPL and TNBP on immunoglobulins. The application of these chemicals as viral inactivants has been in the preparation of biologicals from blood products. The intravenous administration of gammaglobulin can provoke severe anaphylactic reactions in the recipient due to its spontaneous anticomplementary activity. ßPL has been used to chemically modify IgG to abrogate the anticomplementary activity of IgG (114), however Jungi (115), has reported that ßPL treatment severely interferes with the ability of IgG, at 20 ug ml^{-1}, by interacting with monocyte Fc receptors.

Further Experimentation:

Because of nature of these tests, slight fluctuations in temperature, ionic strength and composition can cause changes in antibody avidity; experimental errors caused by these parameters have been discussed in the latter part of section 4.17. For the purpose of this thesis it would have been timely and costly to remove contaminants without introducing a greater amount of error due to alteration of sample concentrations. However, further work could be carried out to improve the understanding of the effect of the chemical inactivation treatments used on the biological activity of monoclonal antibodies.

There are a plethora of assays available to test for the purity of antibodies. It is unfortunate that none of the assays available answer directly the questions posed with respect to product purity. While the problem of potential viral contamination remains unquestionably large, a related industrial problem imposed by regulatory requirements is that of product characterization i.e. purity. It has been reported that 750 separate assays were applied to Protropin - the recombinant human growth hormone manufactured by Genentech (125); few monoclonal antibodies for therapy have been FDA approved, but the analysis of these proteins for purity is almost certainly as difficult and contributes to difficulty in obtaining FDA approval. The methods most suitable for characterization are immunoassays, mass spectrometry and nuclear mass spectrometry (NMR).

Enzyme linked immunoassays (EIA) were developed in the mid-sixties and many different designs have been described since in hundreds of publications which have appeared annually. They have proved to be

the most popular method for assaying antibodies, however they do have limitations. As one of the most important functions of antibodies is opsonization, which occurs through binding of the effector function to the Fc receptors in the cell membrane, an *in vivo* model for this process (such as haemolysis) serves as a useful assay. Other disadvantages of such assays have been seen in the work described for this thesis. They result from the fact that the antibody-antigen interaction is due to a virtually unlimited variety in the composition of the paratope leading to a variety of different physiological forces invloved in binding, which results in a large number of different affinities and cross-reactions. The assays do have limitations with respect to reproducibility of the actual physiological antibody-antigen interaction as immobilization of the ligand to the solid substrate is likely to result in steric hindrance of binding sites and reduced avidity of antibody. Despite these limitations immunoassays have been widely used as useful models of antibody reactions.

Over the last several years advances have been made in NMR which have resulted in the emergence of a tool for the high resolution structure determination of biopolymers. The use of NMR to study antigen-antibody reations has recently been reviewed by Wright, (126). The advantages of using NMR to look at comformations are obvious, molecules can be observed at the molecular level, the distance estimation of individual protons on amino-acid side chains being in the range of 3-20 Å. Kinetics of hapten-antibody reactions can be observed as on a s-ms time-scale so fleeting intermediates can be probed. It has been recently suggested (127) that NMR has reached such a stage of development that it should be used to demonstrate confidence in product identity and quality before the costs of developing further analytical technologies become self-defeating. However its use has so far been limited to observing the conformation of variable domains, as the technology has restricted to analysing low molecular weight proteins.

Suitability of Chemical Inactivation Process for Scale-Up

Work related to this thesis has demonstrated that the use of ßPL in an inactivation process reduced the infectious viral titre of Canine Parvo Virus (CPV), a virus reputed to be very resistant to a range

of physicochemical conditions, by 5 logs (128). More recently (Tao and Harbour, personal communication) have demonstrated that the same conditions do completely inactivate CPV. The evidence from work carried out in this thesis is that ßPL affects the specific activity of one of the antibodies tested. Whether the inactivants affect non-functional parts of the antibody which may cause an immune response in humans was not determined. The evidence is that the use of ßPL as a viral inactivant is the best choice to date for complete inactivation although it can reduce the specific activity of the antibody; a decrease in the amount of ßPL required for inactivation has been reported when used with low doses of ultraviolet light (116), the effect of this treatment on antibodies has not been reported. The use of ßPL must be treated with caution as in its pure state it is considered carcinogenic, although it does break down into non-toxic components in aqueous solution. With regard to scale-up, chemical inactivation is unfavourable to the manufacturer as after antibody treatment, the inactivants must subsequently be removed form the product, e.g. by ultrafiltration or precipitation, and disposed of.

Lyophilisation followed by heating has been demonstrated to be a suitable process for viral inactivation for one particular monoclonal antibody preparation, however unless the product is required in a freeze-dried form lyophilisation could be an expensive alternative.

The use of filters to remove viruses involves the addition of only one step to a downstream processing operation, hence there has been much interest in developing novel filters for this purpose. The most recent of these developments has shown to successfully remove viruses of 30-70 nm (101). However, until such a filter can demonstate removal of small viruses, such as the parvovirus (18-26 nm) a chemical inactivation procedure will remain essential. Even, if a filter capable of removing very small viruses is developed, a risk of contamination of viral DNA or very small unknown viruses remains unless a chemical inactivation procedure is used.

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APPENDICES

APPENDIX I: PREPARATION OF MEDIA FOR CELL-CULTURE

Preparation of DMEM

Add powdered medium to 90 % of the final required volume of sterile distilled water into a flask, stir thoroughly. Add 3.4 g $NaHCO_3$, stir until dissolved. Adjust pH of the medium to 6.8 with 1M HCl. The pH of the medium rises slightly during filtration so the pH is adjusted 0.2 - 0.3 units below that of the final desired pH. Add sufficient distilled water to bring the medium to the required volume.

Sterilize medium immediately by filtration through a cellulose acetate membrane of 0.22 ^{u}m porosity. To reduce loss of carbon dioxide, positive pressure is applied during filtration.

The medium is collected in sterile glass bottles and stored at 4 °C. An aliquot of 100 ml is incubated at 37 °C for 3 days as a sterility test.

Preparation of IMDM

IMDM is prepared as DMEM except 3 g of $NaHCO_3$ is used per litre of medium. The pH of the medium should not need to be adjusted.

The shelf-life of media made up with glutamine is 2 months at 4 °C. Glutamine is a labile amino-acid and should be used within 4 weeks of preparation.

Feotal Calf Serum

Batches of FCS (Hybriserum, Commonwealth Serum Laboratories) were obtained in 500 ml glass bottles and stored at -20 °C until required. Before use FCS is thawed at room temperature and heated for 2 hours at 56°C to inactivate complement and other serum factors which may inactivate and lyse cells. Following inactivation FCS is stored at 4°C.

APPENDIX II: PREPARATION OF REAGENTS AND GELS FOR SDS-PAGE

Separating Gel Preparation (12%) - 0.375 M Tris, pH 8.8

Distilled water	3.35 ml
1.5 M Tris-HCl, pH 8.8	2.5 ml
10% (w/v) SDS stock	100 µl
Acrylamide/Bis (30% stock)	4.0 ml
10% ammonium persulphate (fresh daily)	50 µl
TMED	5 µl
TOTAL MONOMER	10 ml

Stacking Gel Preparation (4.0%) - 0.125 M Tris, pH 6.8

Distilled water	6.1 ml
1.5 M Tris-HCl, pH 8.8	2.5 ml
10% (w/v) SDS stock	100 µl
Acrylamide/Bis (30% stock)	1.3 ml
10% ammonium persulphate (fresh daily)	50 µl
TMED	10 µl
TOTAL MONOMER	10 ml

Enough for two gels of 0.75 mm thickness

Electode Buffer - 0.025% M Tris, pH 8.3

Tris	
Glycine	15 g l^{-1}
SDS	72 g l^{-1}
H_2O	5 g l^{-1}

Treatment Buffer

dist. water	4.0 ml
0.5 M Tris-HCl, pH 6.8	1.0 ml
Glycerol	0.8 ml
10% (w/v) SDS	1.6 ml
2 beta-mercaptoethanol	0.4 ml
0.05% (w/v) bromophenol blue	0.2 ml

Dilute the sample at least 1:4 with sample buffer, and heat at 95 °C for 4 minutes.

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Preparation of Reagents or Staining Gels with Coomassie Blue

Stain:

0.125% Coomassie Blue R-250, 50% Methanol, 10% Acetic acid

Destaining solution 1:

50% Methanol, 10% Acetic acid

Destaining solution 2:

7% Acetic acid, 5% Methanol

APPENDIX III: PREPARATION OF BUFFERS USED IN ELISA ASSAY

Coating Buffer:

8 ml, 0.2 M Na_2CO_3)
22 ml, 0.2 M $NaHCO_3$)

Make up to 100 ml with dist. water.
Check pH is 9.6

Phosphate Buffered Saline (PBS):

8.50 g NaCl)
1.07 g Na_2HPO_4)
0.39 g NaH_2PO_4)

Make up to 1 L with dist. water.

Washing Buffer:

0.05% Tween 20 in PBS

Diluent:

1% BSA in PBS - made fresh each time.

APPENDIX IV: STATISTICAL METHODS USED FOR DATA ANALYSIS

The use of t-distribution to test whether the means of two samples differ significantly.

For n_A items in sample A and n_B items in sample B the sample means are respectively x_A and x_B and the sample variances are s_A^2 and s_B^2. s_A^2 and s_B^2 do not differ significantly.

The experimental values of t are calculated using the formula:

$$t = \frac{|\bar{x}_A - \bar{x}_B|}{\sqrt{\frac{n_A - 1 s_A^2 + n_B - 1 s_A^2}{n_A + n_B - 2} \frac{1}{n_A} + \frac{1}{n_B}}}$$

The degrees of freedom associated with t (i.e. v) = $n_A - 1 + n_B - 1 = n_A + n_B - 2$

The critical values of t are obtained from statistical tables using the appropriate value of the degrees of freedom v, and the experimental t-value compared with these to establish the significance of any difference of the sample means $\bar{x}_A$ and $\bar{x}_B$

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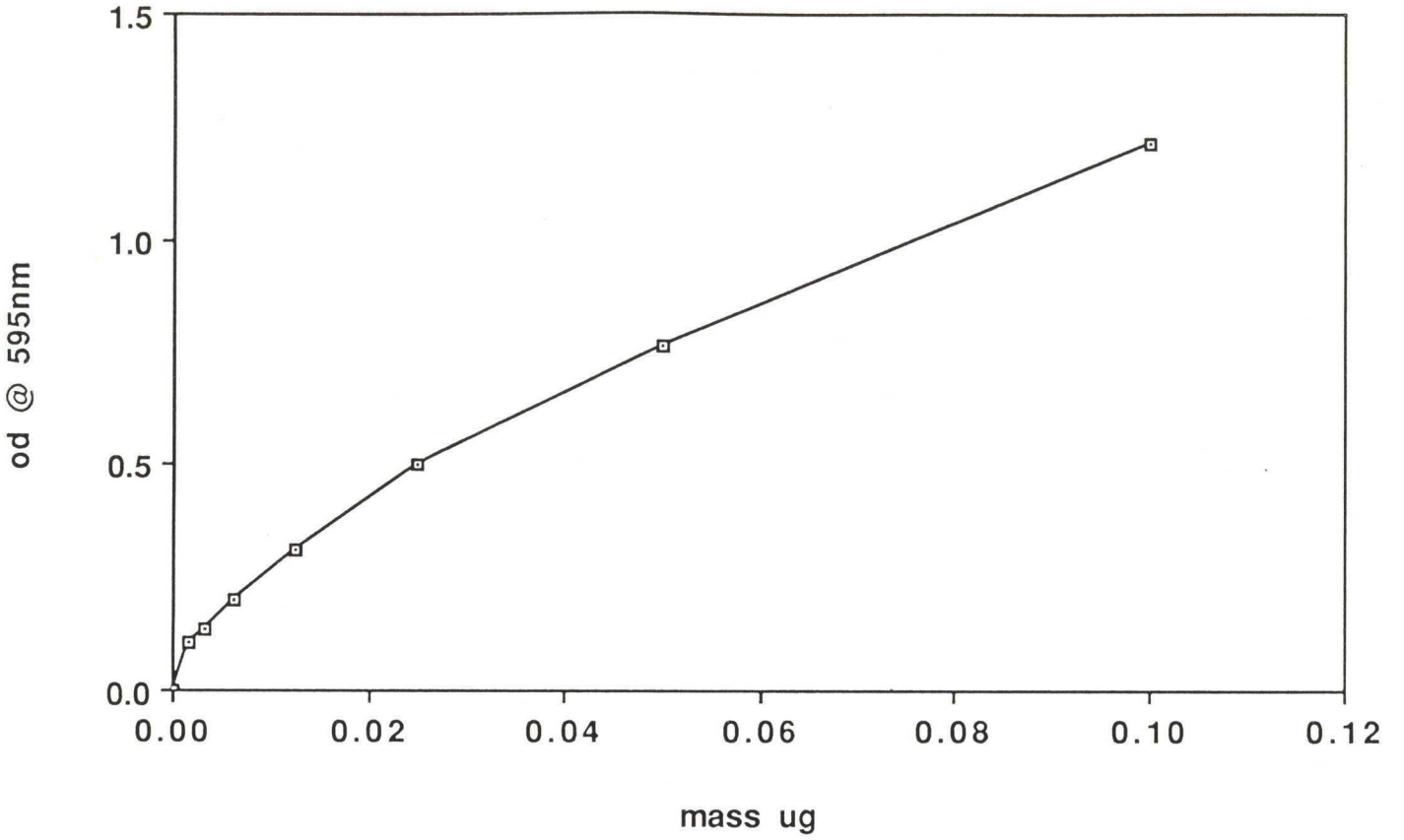
APPENDIX V: STANDARD CURVE FOR BIORAD REACTION



Concentration of protein standard (bovine Ig), $\mu g\ ml^{-1}$