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TO MY PARENTS

UNIVERSITY OF DURHAM

Department of Biological Sciences

A study of antibody secretion from hybridoma cells

by

P.N. Holland B.Sc. H.D.App.Sc. (N.U.I.)

A thesis submitted in partial fulfilment of the requirements for the degree of Master of Science in Biotechnology at the University of Durham

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Durham, England

September 1988

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21 SEP 1992

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To my parents, for their constant kindness and encouragement, and for financially supporting me during my past five years at University. Bufochas.

Abstract

The secretion of monoclonal antibody in both batch and continuous cultures was studied. It was found that when hybridoma cells were continuously cultivated in a simple lab scale fermenter, they secreted less antibody than cells grown in stationary culture, even though the cell densities were the same. It appeared that monoclonal antibody was retained by the hybridoma cells in the fermenter, and in order to investigate this, membrane work was carried out. The effects of three ionophores on the hybridoma cells was studied. It was found that Valinomycin and Gramicidin were very toxic and killed the cells within a short period of time. Monensin was found to be less toxic, and although cell growth was inhibited, it appeared that this ionophore increased the secretion of monoclonal antibody from the cells.

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CHAPTER 1

INTRODUCTION

1.1 ANTIBODIES

The antibody fraction of serum consists predominately of one group of proteins with molecular weight around 150,000 of which the major component is IgG, and another of molecular weight of about 900,000 which is IgM. The IgG antibodies can be split by papain into three fragments. Two of these are identical and are able to combine with antigen to form a soluble complex which will not precipitate. These are univalent antibody fragments, and are given the nomenclature Fab (Fragment antibody binding). The third has no antigen binding ability and is called the Fc fragment (Fragment crystallizable). Antibodies can be broken down into their constituent polypeptide chains, by breaking the disulphide bonds linking the different chains using an excess of a sulphydryl reagent. The reduced molecule can be separated into two sizes of peptide chain termed light and heavy chains by lowering the pH. On the basis of these findings, Porter(1973)put forward a symmetrical four peptide model for antibody consisting of two heavy and two light chains linked together by intrachain disulphide bonds.

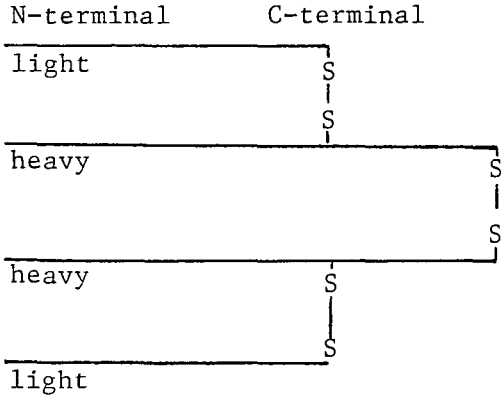


Figure 1

Antibody model proposed by R R Porter with two heavy and two light polypeptide chains held together by intrachain disulphide bonds.

When visualised in the electron microscope by negative staining, the purified IgG antibodies are Y shaped molecules whose arms can swing out to an angle of 180°, through the papain and pepsin sensitive region acting as a hinge.

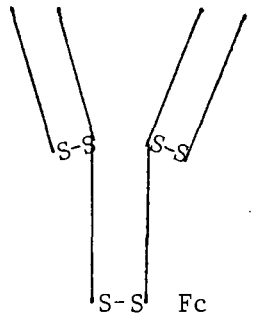


Figure 2

Illustration of the flexibility of the Immunoglobulin molecule at the hinge region.

Any attempt to analyse the amino acid structure of the immunoglobulins in normal serum is complicated by the different number of molecules present. A study of the myeloma proteins has provided the answer to the problem of analysing the amino acid structure. Amino acid analysis of a number of purified myeloma proteins has revealed that within a major immunoglobulin class such as IgG, the N terminal portions of both heavy and light chains show variations , wheareas the remaining parts of the chains are constant in structure. Each variable region has a basic overall amino acid structure which is common to a number of antibodies with differing specificities. They are said to belong to the same subgroup. Antigenic analysis of IgG myelomas showed that they could be grouped into four subclasses, now termed IgG_1, IgG_3 and IgG_4. The differences all lie in the heavy chains which have been labelled γ_1 γ_2 and γ_3 respectively.

1.1.1 Monoclonal Antibodies

A monoclonal antibody is obtained by fusing an antibody producing cell which secretes a single species of antibody molecule, with a myeloma tumour cell. The fused cell secretes the desired antibody, but has the characteristic immortality of the tumour cell line. The fused cell line is called a hybridoma. Hybridomas are now a major source of homogeneous antibodies, which can have almost any desired antigen binding specificity. Monoclonal antibodies from hybridomas have an advantage over those prepared from myeloma tumours in that they can be made to order against the desired antigen.

Monoclonal antibodies have many applications. They can be used for the purification of human interferon, for classification of bacteria, viruses and parasitic organisms, or in tumour therapy where they are used in an effort to kill tumour cells by attacking them with an antibody which is conjugated to a highly toxic molecule. The antibody would, in principle, attach to the tumour in vivo and the toxic agent would then kill the tumour cells.

Production of monoclonal antibodies in mouse ascites is accomplished by injecting hybridomas into the peritoneal cavity of mice.

The injected cells proliferate and produce monoclonal antibody, which is regularly withdrawn within the ascites fluid until the mouse dies. Theoretically, any amount of monoclonal antibody can be produced in mouse ascites by simply maintaining sufficiently large mouse colonies, but in practice there are several technical and economic limitations.

(1) Scale up of the ascites approach is basically linear (i.e. to double the amount of antibody, the number of mice must also be doubled.) and there is little economy of scale.
(2) Contaminating mouse immunoglobulins may result in purification problems.
(3) Contamination by mouse viruses may occur.
(4) Batch to batch reproducibility is not achievable.

An alternative method to monoclonal antibody production in mouse ascites is suspension culture of hybridoma cells. In this process, cells are suspended in culture medium and allowed to replicate. Low cell densities, typically 1.0×10^6 cells/ml, result in low concentrations of antibody, and places a severe constraint on the suitability of this method for large scale production.

Method of Manufacture	Achievable Cell Density/ml	Antibody % Total Protein	Process Volume (litres) to Produce 10g Antibody
Ascites	$> 10^8$	5-20	2-10
Conventional Culture	2×10^6	1	100-1000
Airlift Culture	2.5×10^6	1	100-1000

Table 1
Major methods for manufacturing monoclonal antibodies

1.1.2 Monoclonal Antibody Purification.

Mouse serum normally contains appreciable quantities of IgG_1, IgG_{2a} and IgG_{2b} and lesser amounts of IgG_3 (Grey et al., 1971). However, these proteins, especially IgG_{2a} and IgG_{2b}, overlap extensively in their properties, and no method had been developed by which each could be isolated pure and in good yield from serum. Nisonoff et al., and Dissanayake and Hay reported in 1975, that mouse IgG could be purified from serum by chromatography on diethylamino-ethyl cellulose (DEAE) but IgG_{2a} and IgG_{2b} are not resolved, and remain contaminated with a significant proportion of the original IgG_1. Several years ago, Protein A was shown to interact with mouse IgG_{2a} and IgG_{2b} and IgG_3 myeloma proteins, but not with IgG_1, IgM, or IgA. IgG_2 has since been reported to bind firmly to Protein A Sepharose, but IgG_1, whose passage through Protein A is retarded, seemed to bind weakly.

Kronvall et al., in 1970 made the first observation suggesting that Protein A could be used to separate a subclass of IgG from the other subclasses. An important improvement in subclass isolation was achieved by Ey et al.,(1978), Goding (1978), and MacKenzie et al., (1978) who bound IgG to Protein A Sepharose and eluted different subclasses by buffers of different pH. The major subclasses IgG_1, IgG_{2a} and IgG_{2b} came off with pH values 6.0, 4.5, and 3.5 respectively. Seppala et al., confirmed this method in 1981, and discovered that the elution characteristics of IgG_{2a} were dependent on the allotype. If the allotype was b, IgG_{2a} came off at pH. 4.5, but if the allotype was a or j, the pH. 5.0 buffer eluted it. Some of the fractions that were obtained by the acid elution approached physical purity, since the amounts of IgG_1, IgG_{2a} and IgG_{2b} in the fractions roughly corresponded to the protein concentrations indicated by the U.V. light absorption data. Therefore, the protein concentration of the Protein A eluates can be used to determine the concentrations of the immunoglobulin subclasses.

1.2 FERMENTATION

The aims of this project were to study the secretion of monoclonal antibody from hybridoma cells in both batch and continuous cultures. Batch cultivation was achieved by growing the cells in petri dishes in an incubator, testing for monoclonal antibody by Enzyme Linked Immunosorbent Assay (ELISA) and comparing the results to that of cells grown in a simple lab scale fermenter which was constructed so that hybridoma cells could be cultivated on a continuous basis. Continuous cultivation of hybridoma cells may be more favourable because once steady state is achieved, a steady supply of monoclonal antibody is ensured. The rates of absorption of limiting nutrient, excretion of toxic products, cell growth and loss of cells from the fermenter are balanced and constant with time. Therefore, the environment of the cell is constant, and this constant environment leads to a constant physiology.

The simplest type of continuous culture, the chemostat, relies on a set constant flow rate (F), which together with a constant volume (V) defines the dilution rate (D)

$$D = \frac{F}{V}$$

which at steady state is equal to the growth rate of the population μ. Therefore, the growth rate of the population is defined by manually adjusting the flow rate of the fresh medium into the culture vessel.

The population density is controlled by the concentration of the limiting nutrient in the inflowing medium, and consequently controlled by the operator.

The dilution rate (D) is set at a value less than the maximum growth rate (μmax) of the population, to avoid cell washout. The chemostat is a self regulating system. A temporary decrease in the steady state cell concentration will cause a corresponding increase in the cell growth rate, which will restore steady state conditions. The mean generation time or the doubling time (td) of the cells is controlled directly by the flow rate, according to the following relationship:

$$\mu = \frac{0.693}{td} = (D)$$

$$td = \frac{0.693}{(D)}$$

$$F = D \times V$$

Köhler and Milstein published the first report on the continuous production of mouse monoclonal antibodies by a hybridoma cell line in 1975. In 1982, Fitzgerald *et al.*, studied the pattern of immunoglobulin secretion in a continuous culture of human/mouse lymphoid hybridomas.

In 1983, de St. Groth worked on the automated production of monoclonal antibodies in a cytostat. In 1983, Marcipar et al., immobilised hybridoma cells on ceramic supports so that monoclonal antibodies could be produced continuously in a thermostated fixed bed reactor. In 1985, Pankratov et al., worked on the continuous cultivation of hybridoma cells grown on the surface of glass disks in a cell reactor. In 1986, Altshuler et al., grew hybridomas in the extracapillary space of hollow fibre reactors and produced monoclonal antibodies on a continuous basis.

1.2.1 Serum-Free Medium

During the course of this project, two different methods were used to adapt the hybridoma cells to serum-free medium. It is generally accepted that the growth of virtually all types of cells in culture requires the presence of serum in the medium. Serum is a complex mixture, and many serum components are poorly characterised, or completely unstudied. In addition, the concentration of some of the components vary drastically among different serum batches. Investigators in the field have long recognised the problems associated with the complexity and undefined nature of serum (Kawamuto et al., 1983, Yabe et al., 1986, Long et al., 1988) and a number of different approaches have been taken to eliminate the requirement for a serum supplement in the culture medium (Barnes and Sato 1980, Yamane et al., 1975, Chang et al., 1980)

The elimination of serum from the culture medium allows the simple design and interpretation of experiments which would be difficult or impossible to carry out in serum containing medium. Experiments which examine the release of cellular products, such as secreted products of differentiating cell lines into the medium are much easier in the absence of large amounts of serum protein.

There are several advantages in using serum free medium. These include the ability to perform detailed studies on cell physiology and metabolism, the facilitation of product recovery by elimination of animal proteins present in serum, and the application of *in vitro* techniques for the production of monoclonal antibodies for human therapeutic applications. The economy of the medium represents an additional advantage, particularly in the case of large scale cell production.

Hybridoma cell cultivation in defined serum-free medium requires the presence of certain growth promoting substances (McHugh *et al.*, 1983). Kovar (1985,1986,1988) has carried out extensive research in this area and concludes that substances like ethanolamine, insulin, hydrocortisone, selenium, manganese, linoleic acid, cholesterol etc are essential for promoting the growth of cells in serum-free medium.

In 1986, Tharakan and Chau successfully attempted the fed batch cultivation of a murine hybridoma secreting IgM in a serum-free medium. They found that the average specific secretion rates of antibody in serum supplemented medium and serum-free medium were the same. They also studied IgG production kinetics in serum supplemented medium and concluded that serum supplemented medium supported a higher cell growth rate and a higher IgG titre. However, the antibody secretion rate on a per cell basis was higher in the serum-free medium.

1.2.2 Viable Cells

All cultivated cells in this project were counted using trypan blue staining. Schrek (1936) suggested that an intact cell membrane was necessary for the exclusion of certain dyes. Trypan blue is the most commonly used procedure, where unstained cells are counted as viable.

1.2.3 Mycoplasma Contamination

Contamination of cell cultures can cause the loss of rare cultures, and the waste of valuable time and expensive media. Unwanted organisms may introduce multiple unmeasurable variables by producing metabolites, consuming nutrients, or altering the metabolism or the morphology of the other cells and cause the investigator to draw erroneous conclusions.

Cell cultures are prone to contamination by mycoplasma. Mycoplasma are the smallest, simplest self-replicating procaryotes. Unlike other procaryotes, they have no cell walls, and are therefore placed in a separate class: Mollicutes.

There is no one simple way to tell if a culture is contaminated with mycoplasma. There are several species of mycoplasma, and a method that will detect some, will not detect others. Another reason is the limited sensitivity of the tests. Most will not detect the low levels of mycoplasma which can affect host cells. The easiest, fastest, cost effective method of maintaining contamination free cultures is prevention.

42% of all mycoplasma in the monoclonal antibody laboratory are of human origin. Strict aseptic technique and the elimination of mouth pipetting reduces the risk of contamination. The highest incidence of bovine mycoplasma implicates the bovine serum. The serum used throughout this project was obtained as mycoplasma free from Flow laboratories. Strict aseptic technique was also employed in the laboratory. Since mycoplasma are known to interfere with the specificity of a monoclonal antibody, an ELISA determining the specificity of the monoclonal antibody to legumin was carried out at regular intervals, and the readings showed that this specificity was constant.

1.3 IONOPHORES

The secretion of monoclonal antibody from hybridoma cells obviously involves the transport of the antibody across the cell membrane. The growth of the hybridoma cells in a steady state system may very well show some light on such transport mechanism.

This mechanism was investigated by studying the effects of ionophores on the antibody secretion process. Recent work by Keevil et al., (1984) and West et al., (1984) relates the secretion of protein from bacteria to protonmotive force. Ionophores are protonmotive force modifiers (Pressman 1976). Three different ionophores were studied, Valinomycin, Gramicidin D and Monensin A.

The ionophores were first recognised through their effect of stimulating energy linked transport in mitochondria by Moore et al.,(1964) and Pressman (1965). This not only provided a valuable tool for studies on the linkage between metabolism and transport, but also prompted extensive in vitro studies to provide insight into the molecular basis of ionophore action. Ionophores are compounds of moderate molecular weight (about 200-2000) that form lipid soluble complexes with polar cations, of which K^+, Na^+, Ca^+, Mg^+ and the biogenic amines are the most significant biologically.

While the first recognised ionophores were metabolites of microorganisms, several synthetic compounds were later discovered to have equivalent molecular properties. Physical studies indicate that the complexation-decomplexation kinetics and diffusion rates of ionophores and their complexes across lipid barriers are so favourable, that their transport turnover numbers across biological membranes attain values of thousand per second, exceeding the turnover of most macromolecular enzymes Haynes et al., (1969). This, along with their high cation selectivity, inspired the consideration of ionophores as model biological carriers. The ion carrying ionophores fall into two main classes, the Valinomycin group, and the Nigericin group. Members of the Valinomycin group are electrically neutral and form a charged complex alkali metal cations, wheareas those of the Nigericin group have a carboxylic acid grouping which dissociates over the physiological pH range, leading to electrically neutral complexes.

1.3.1 Valinomycin

Valinomycin is an antibiotic produced by certain strains of Streptomyces, and was first isolated by Brockman and Schmidt-Kastner (1955) from the mycelium of cultures of S.fulvissimus strain Ihr 582.

In 1963, Brockman et al., identified the constituents of Valinomycin as D and L-valine, L-lactate and D-hydroxyisovalerate, but concluded that the sequence repeated only twice. In 1965, Shemyakin et al., reported Valinomycin to be a cyclododecadepsipeptide with a molecular weight of 1111, consisting of three repetitions of the sequence D-valine, L-lactate, and L-valine and D-hydroxyisovalerate.

In 1959, Mc Murray and Begg, found that a concentration of Valinomycin as low as 10^{-7} M completely uncoupled oxidative phosphorylation in rat liver mitochondria. In 1964. Moore and Pressman discovered that the ability of Valinomycin to uncouple oxidative phosphorylation was dependent upon the presence of potassium. This led to the recognition that Valinomycin forms a lipid soluble positively charged complex with k^+ which diffuses rapidly across any lipid phase including both natural and artificial membranes.

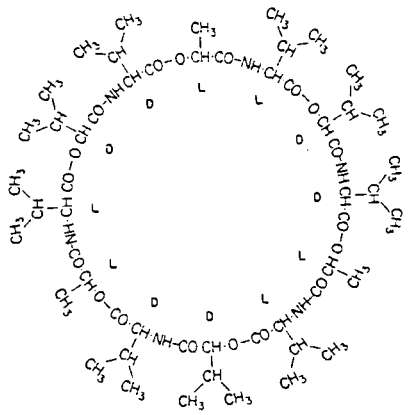


Figure 3

The structure of Valinomycin.

1.3.2 Gramicidin

The Dubos strain of Bacillus brevis produces all 6 linear Gramicidins. Gramicidins are linear polypeptides of molecular weight of about 1800. Each gramicidin is a mixture of two molecules, one having the formylated amino acid as valine, the other having isoleucine. They have low solubility in water, as there are no ionizable groups in the molecule, except for Gramicidin C which contains tyrosine. Gramicidins A, B, C are pure Gramicidins. Gramicidin D is the name given to a group of Gramicidins isolated from the Dubos strain of B.brevis. Gramicidin J is named so because it was first isolated in Japan from the Nagano strain of B. brevis. It is identical with Gramicidin S, which is not a cyclic polypeptide, and therefore not a true Gramicidin. Most studies have been carried out using Gramicidin A, but all Gramicidin molecules may be assumed to act similarly. Gramicidin A binds irreversibly to the surfaces of lipid membranes and produces a pore which spans the membrane. The pore conducts Na^+ and K^+ with a selectivity of K^+ over Na^+ of of approx six fold (Mueller and Rudin 1967).

Figure 4:

Different structures of Gramicidin

A HCO-L-Val-Gly-L-Ala-D-Leu-L-Ala-D-Val-L-Val-
D-Val-L-Trp-D-Leu-L-Trp-D-Leu-L-Trp-D-Leu-L-Trp-NH.CH_2CH_2OH

B. HCO-L-Val-Gly-L-Ala-D-Leu-L-Ala-D-Val-L-Val-
D-Val-L-Trp-D-Leu-L-Phe-D-Leu-L-Trp-D-Leu-L-Trp-NH.CH_2CH_2OH

C. HCO-L-Val-Gly-L-Ala-D-Leu-L-Ala-D-Val-L-Val-
D-Val-L-Trp-D-Leu-L-Tyr-D-Leu-L-Trp-D-Leu-L-Trp-NH.CH_2CH_2OH

Gramicidins A, B. and C. (A similar series with L-isoleucine replacing the valine at the left-handside also occurs)

1.3.3 Monensin

Monensin is a member of the Nigericin group of ionophores. This compund consists of a series of linked heterocyclic rings having a carboxyl group at one end, and a hydroxyl group at the other (Walbu et al., 1987). Monensin is produced by a strain of Streptomyces cinnamonensis, and is perhaps the best known, most historical example from among a group of about 40 naturally occurring polyether antibiotics. Monensin gains additional attractiveness as a target due to its novel Na^+ selectivity. Methods developed for the construction of Monensin may in principle find utility for preparation of more highly Na^+ selective analogues which could prove useful for biochemical probes, and even as cardiovascular drugs.

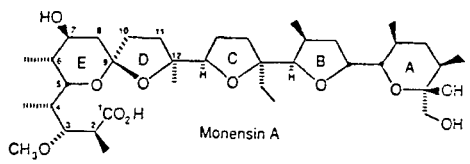


Figure 5
The structure of Monensin

1.4 AIMS

The aims of this project were to study the secretion of monoclonal antibody from hybridoma cells in both batch and continuous cultures.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

All laboratory reagents were obtained from BDH

All immunological reagents were obtained from Sigma

All chemicals were obtained from Sigma, with the following exceptions:

RPMI 1640 (Flow labs)

L-Glutamine (Flow labs)

Fetal Bovine Serum (Flow labs)

Serum Free Medium (Northumbria Biologicals)

2.2 HYBRIDOMA CELLS

The hybridoma cell line used (GD5) was obtained by fusion of Myeloma SP2 with Balb/c spleen lymphocytes from mice immunised with legumin, and was provided by Mrs C Munday.

2.2.1 Culture Conditions

Basic medium was RPMI 1640, supplemented with 2 mM L-Glutamine, 10% Fetal Bovine Serum (F.B.S), Penicillin G 560 μg/ml, and Streptomycin Sulphate 112μg/ml. All constituents of the medium were obtained filter-sterilised by the manufacturers.

The cells were cultivated in petri dishes in an incubator at 37°C in an humidified atmosphere of 5% CO_2 in air. Each petri dish contained between 10 and 20 ml of medium.

The maximum viable cell density was approximately 7 X 10^5 cells/ml. Cells were counted by using a haemocytometer slide.

2.2.2 Trypan Blue Staining

A 0.9 ml sample of cell suspension was taken and mixed with 0.1 ml of Trypan Blue stain. After 5 minutes, the cell suspension was added to both chamber sides of the haemocytometer using a pipette. The chamber coverslip was then placed on the slide. A visual count of the unstained live cells was recorded on a hand counter.

2.3 DESIGN OF THE FERMENTATION SYSTEM

The components responsible for nutrient supply may be divided into two parts, a reservoir which contains sterile medium, and a peristaltic pump to meter the medium to the culture vessel. The sterile medium reservoir was a pyrex vessel of one litre capacity (Fig 6 a). The medium was maintained at 4.^{o}C, by standing the vessel in ice. The reservoir was fitted with a medium inflow point. It was also fitted with a sterile filter, (Gelman Sciences Acro 37 TF, for the removal of all particles from gases which are larger than its rated pore size of 0.2 μm). The filter had a hydrophobic PTFE membrane which acted as a barrier to aqueous solutions and aerosols, allowing only sterile air to pass.

The culture vessel was glass and had an 80 ml capacity (Fig 7). A weir exit tube just above the 80 ml mark, facilitated the removal of spent medium and cells, and maintained the culture volume. Fresh medium was added to the vessel drop by drop, as controlled by the pump. The vessel had a water jacket, which maintained the culture at 37^{o}C, which corresponded to the temperature of the incubator. The vessel was connected to a compressed gas supply containing 5% CO_2 in Oxygen and Nitrogen. This created a similar environment to that of the humidified incubator.

The gas inflow tube was fitted with two filters (Fig 8). This helped to prevent contamination of the culture. The spent cells and medium were collected in a two litre glass vessel. This collecting vessel (Fig 6 b) was kept in ice, which maintained the material at 4°C. The vessel was fitted with a sample point, and a sterile filter (Gelman Sciences).

2.3.1 Procedure

The medium used was described under Culture Conditions (2.2.1).

2.3.2 Sterilisation

The apparatus was fully assembled and sterilised in a Baird and Tatlock 300 series autoclave, for 15 minutes at 121°C. This is the standard autoclaving time for all fermentation equipment.

2.3.3 Inoculation

The culture vessel was filled with medium, and both the stirrer, and the gas flow were turned on. From studies of the growth of GD5 cells in batch cultures, a maximum live cell density of approximately 7 X 10^5 cells/ml was reached.

An inoculum of approximately 8.0 x 10^5 cells/ml in a total volume of 10 ml was used to inoculate the fermenter. The cells were counted, and the 10 ml volume containing the appropriate number of cells was taken and centrifuged at 12000 g for 5 minutes. The supernatant was drawn off by using a pipette and retained to test for antibody. The pellet was resuspended in a known volume of medium with 10% fetal bovine serum. This was used as an inoculum. The entire procedure was carried out in the sterile cabinet.

It was essential that proper sterile techniques were employed to avoid contamination. The inoculum was injected from a syringe through the culture vessel inoculation port (Fig 7). The first cell sample was taken approximately 12 to 15 hours after inoculation. A clamp was placed on the tubing between the culture vessel and the collecting vessel. The sample could then be taken up through the stainless steel tubing. The sample came off automatically under the pressure of the CO_2/air flow. The cells were counted and fresh samples were taken every 5 to 6 hours until three exponential points were obtained on a plot of $\log_{10}$ cell density versus number of hours after inoculation. When the period of exponential growth was achieved (Fig 10 a), the pump was turned on and the medium was allowed to flow continuously.

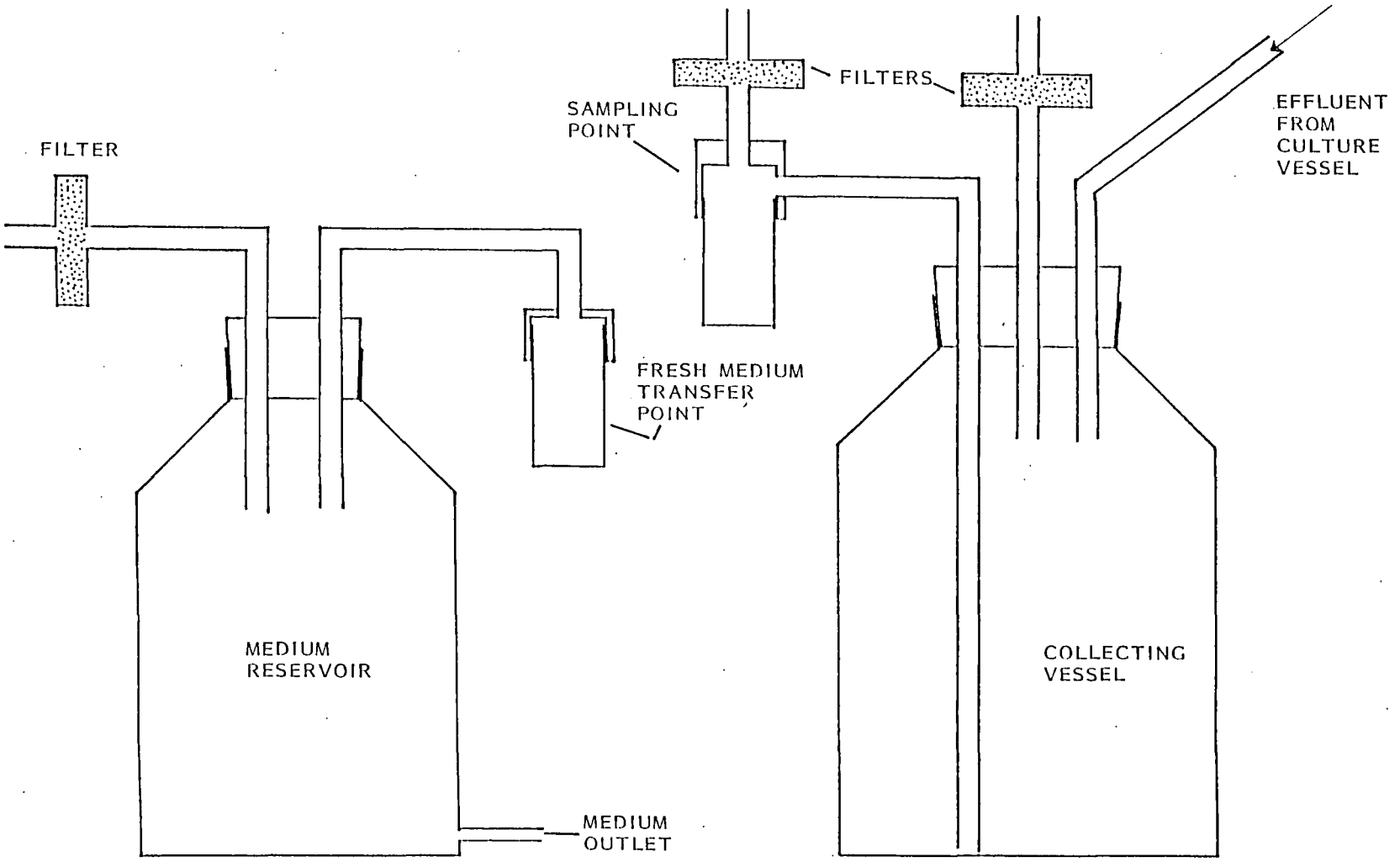


Fig 6 a - Medium Reservoir

Fig 6 b - Collecting Vessel

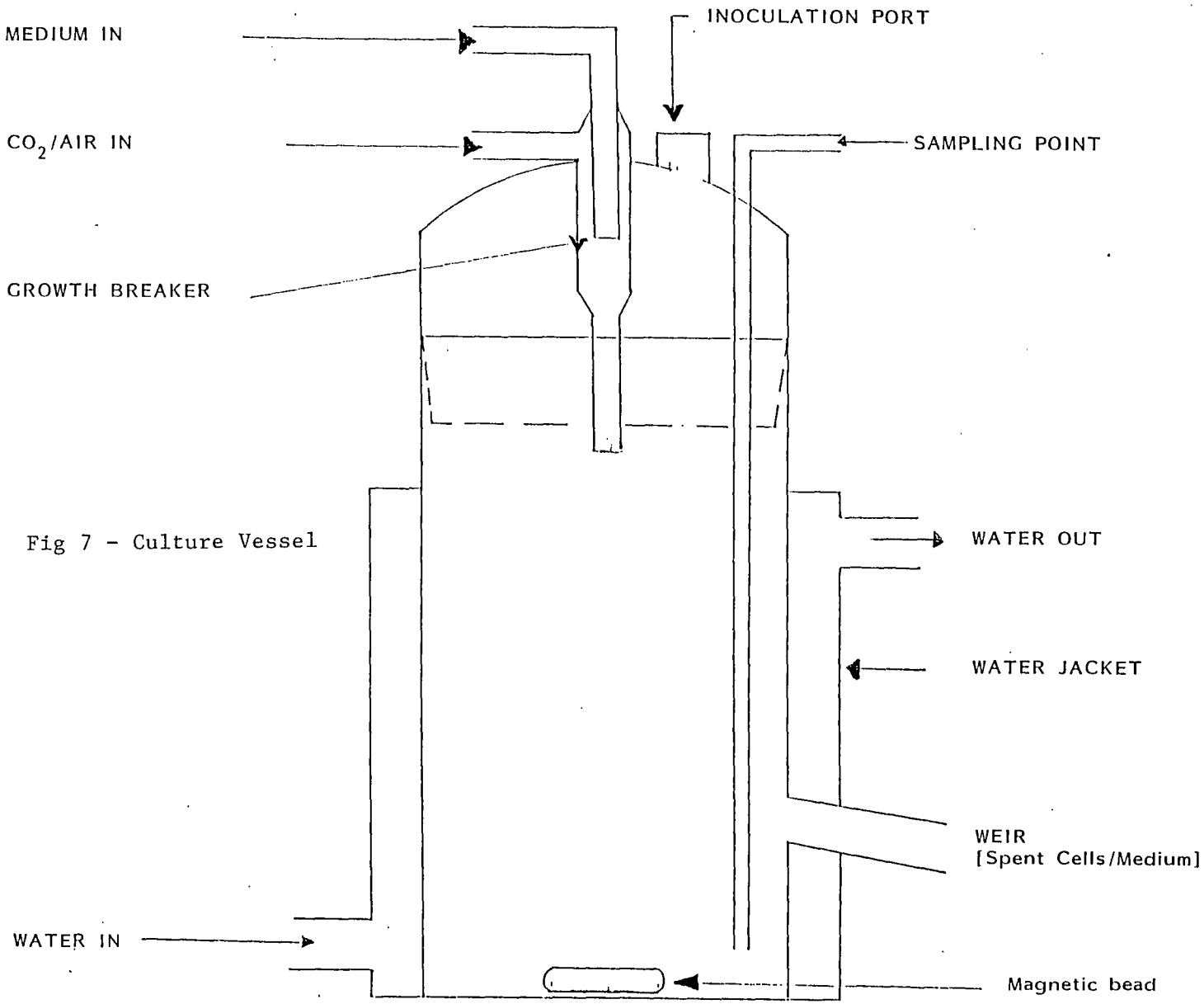


Fig 7 - Culture Vessel

Fig 8 - A SCHEMATIC DIAGRAM OF THE CONTINUOUS CULTURE APPARATUS

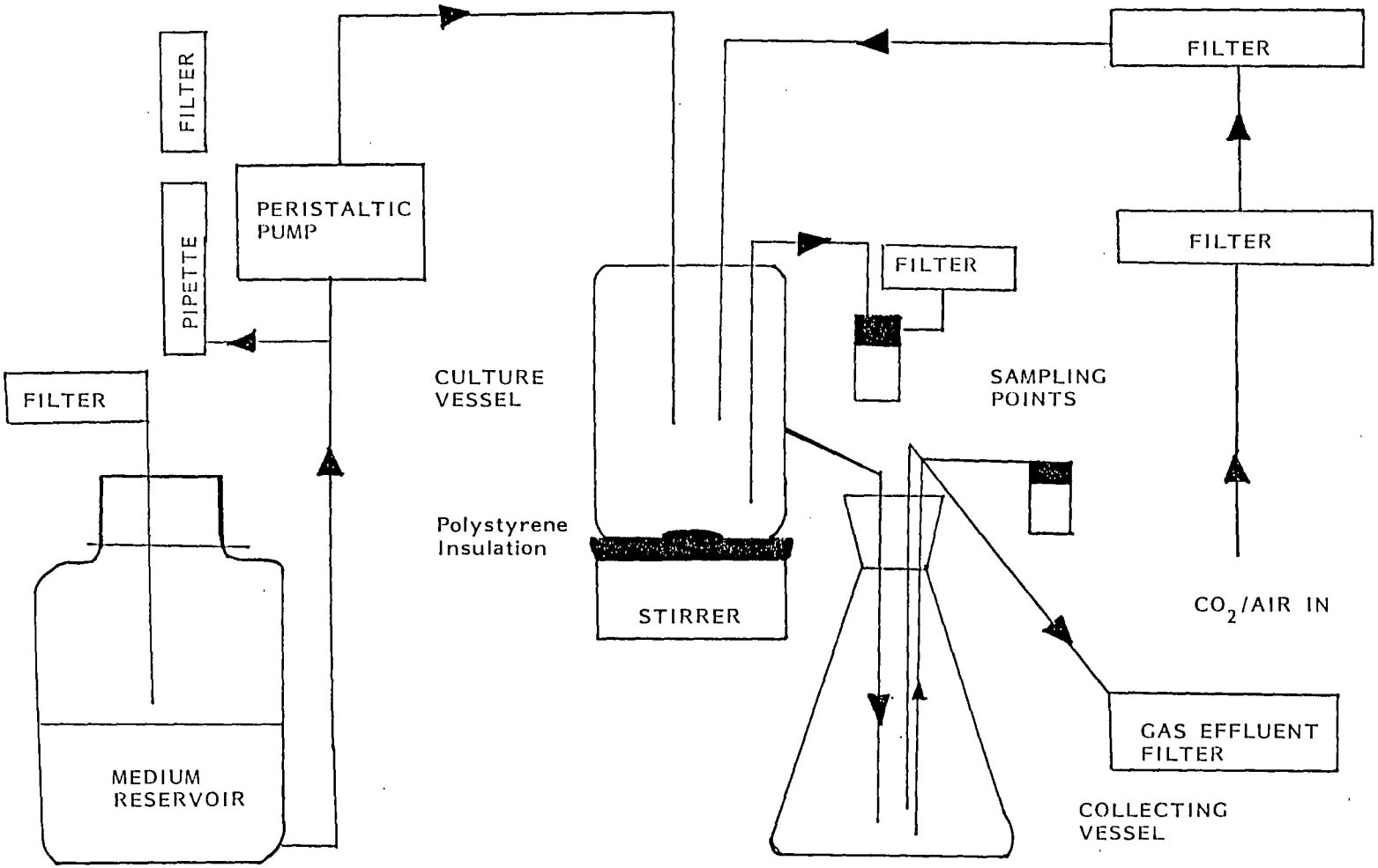


Figure (9) showing the continuous culture apparatus.

(1) Sterile Medium Reservoir

(2) Incubation Water Bath

(3) Glass Pipette

(4) Peristaltic Pump

(5) Culture Vessel

(6) Polystyrene Insulation

(7) Magnetic Stirrer

(8) CO_2/Air Supply

(9) Collecting Vessel

2.4. FERMENTATION

Daily Procedure

Once steady state was achieved, one sample from the culture vessel was taken aseptically each day. A fermentation record sheet was kept, noting the following procedures which were carried out on a daily basis.

(1) The medium flow rate was checked, using the 1.0 ml glass pipette (Fig 8). A clamp was placed on the tubing between the pipette and the pump. Medium was pumped along the tubing until it reached a point just above the 0.00 ml mark on the pipette. A clamp was then placed on the tubing between the reservoir and the pipette. The pump was set at the required flow rate, and the clamp between the reservoir and the pump was removed. The stopwatch was started when the medium reached the 0.00 ml mark, and stopped when it reached 0.80 ml. The time was recorded. The flow rate was calculated as follows:

0.80 ml corresponded to 1370 seconds, therefore

$$\frac{0.80 \text{ ml} \times 3600}{1370} = 2.1 \text{ml/Hr}$$

(2) The gas flow rate was measured using a rotameter. The rate was maintained between 50 and 70 cc/min. It is recommended that approximately 1 cc/min. of gas should correspond to 1 ml volume of the culture vessel.

(3) The water incubation temperature was maintained constant and was checked using a thermometer.

(4) Trypan blue stain was used to distinguish between viable cells and non-viable cells.

(5) The sample was checked for contamination by lawn plating a small amount on a nutrient agar plate (oxoid) which is a general purpose culture medium used to detect a wide variety of contaminating bacteria, viruses and yeasts. The sample was also tested on MacConkey agar No. 2 plates (oxoid) which detect contaminating microbes of human origin, such as staphylococci.

2.4.1 Weaning of Hybridoma Cells from Medium Containing 10% F.B.S. to Serum-Free Medium

Culture Conditions - the cells were cultivated as previously described.

Two methods were used to adapt the cells to serum-free medium.

2.4.1.1 Method 1

Inoculum - 2 ml of approx 5.0 x 10^4 cells/ml was used for inoculation.

The following petri dishes were set up.

Petri Dish Number	F.B.S. Medium	Inoculum	Serum-Free Medium	% F.B.S. Present
1	8.0 ml	2.0 ml	Nil	10
2	6.4 ml	2.0 ml	1.6 ml	8
3	4.0 ml	2.0 ml	4.0 ml	5
4	2.0 ml	2.0 ml	6.0 ml	2.5
5	Nil	2.0 ml	8.0 ml	0

Table 2

The cells in each petri dish were counted daily over a period of six days. A 0.4 ml sample was also taken from each dish daily, placed in an Eppendorf tube, and centrifuged for 5 minutes at high speed using the MSE Microcentaur. The supernatant was removed and tested for antibody.

2.4.1.2 Method 2

This method allowed the hybridoma cells to adapt gradually to the serum-free medium.

Culture Conditions - the cells were cultivated as previously described.

Inoculum - 2.0 ml of approx 1.0 x 10^5 cells/ml was used as an inoculum.

At Day 2, a 2.0 ml sample was taken from the petri dish and was used to inoculate a new petri dish containing 6.4 ml of 10% F.B.S. in the medium, and 1.6 ml of Serum-Free Medium.

At Day 4, a 2.0 ml sample was taken from this petri dish and used to inoculate a new dish containing 4.0 ml of medium containing 10% F.B.S. and 4.0 ml Serum-Free Medium, and so on.

Day	Inoculum	F.B.S. Medium	Serum Free Medium	Cell Density x 10^5/ml
1	2.0 ml	8.0 ml	Nil	2.5
2				8
3	2.0 ml of 10%	6.4 ml	1.6 ml	
4				7
5	2.0 ml of 8%	4.0 ml	4.0 ml	
6				5
7	2.0 ml of 5%	2.0 ml	6.0 ml	
8				5
9	2.0 ml of 2.5%	Nil	8.0 ml	
10				6
11	2.0 ml of 0%	Nil	8.0 ml	
12				6

Table 3

2.5 ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

Materials

(1) Multi well polystyrene strips (12 Titertek)

(2) Strip holder

(3) ELISA recorder (Titertek)

(4) Phosphate buffered saline (PBS) pH 7.4 0.15 M

0.137 M Sodium chloride

0.027 M Potassium chloride

0.080 M di-Sodium hydrogen phosphate

0.015 M Potassium di-hydrogen phosphate

Dissolved in 1000 ml of distilled water

(5) PBST-Phosphate buffered saline with 0.05% Tween 20

(6) Goat anti-mouse IgG

(7) Hybridoma supernatant

(8) Sheep anti-mouse IgG horseradish peroxidase conjugate

(9) Substrate buffer -

10.0 ml Sodium acetate (0.2 M)

0.20 ml Citric acid (0.2 M)

0.025 ml 10% Hydrogen peroxide

(10) Substrate stock -

0.025 g tetramethyl benzidene/10 ml of dimethyl sulfoxide (DMSO)

(11) Working substrate -

9.0 ml Substrate buffer

1.0 ml Stock substrate solution

Method

(a) The surface of the polystyrene wells were coated with 50.0 µl of 10 µg/ml of Goat anti-mouse in PBS. The strips were incubated overnight at 4°C.

(b) The residual antigenic material was flicked out, and 100 µl of 100% BSA in PBS was added to each well and left at room temperature for 1 hour.

(c) The strips were then flicked dry and used immediately.

(d) 50 µl of undiluted hybridoma supernatant was added to each well, and left to incubate at room temperature for 2 hours.

(e) The strips were washed 4 times with PBST.

(f) 50 µl of Sheep anti-mouse IgG horseradish peroxidase conjugate, diluted 1:1000 in PBST was added to each well, and left at room temperature for 30 minutes.

(g) The strips were washed 4 times with PBST.

(h) The substrate for enzyme was added (9.0 ml substrate buffer, 1.0 ml of TMB) and was left at room temperature for 30 minutes.

(i) 50 µl of 0.2 M H_2SO_4 was added to stop the reaction.

(j) The yellow colour was read at 450 nm using the ELISA strip reader.

2.6 PROTEIN PURIFICATION

A C10 glass column, 10 cm in length was used (Pharmacia). A flow rate of 0.8 ml/minute was provided by a peristaltic pump (Pharmacia).

Buffers

(A)	1.5 M Glycine/3M NaCl	pH 8.90
(B)	0.1 M Citric acid	pH 6.00
(C)	0.1 M Citric acid	pH 5.00
(D)	0.1 M Citric acid	pH 4.00
(E)	0.1 M Citric acid	pH 3.00

5.0 M Sodium Hydroxide was used to correct for pH.

0.3 g of Protein A Sepharose was suspended in 10 ml of buffer A and allowed to swell for 15 minutes. The solution was then poured down the column and allowed to settle. The final volume of the Protein A solution was approximately 1.0 ml. Ten bed volumes of buffer A was allowed flow through the column. Buffer A was then drawn off the column using a 1.0 ml pipette. 2.0 ml of hybridoma supernatant was diluted in an equal volume of Buffer A, and was then loaded slowly on top of the Protein A bed.

The sample flowed under gravity through the gel. Buffer A was then applied and allowed to flow under the control of the pump for 15 minutes, and fractions were collected in Eppendorf tubes at minute intervals. The buffer was then drawn off, Buffer B was applied, and fractions were collected for 20 minutes (as before). Remaining buffers were applied in the same way, for appropriate lengths of time. The collected samples were tested for antibody by ELISA. The samples were also read in a Gilford Spectrometer at 280 nm and at 260 nm, so that the protein content could be estimated.

2.6.1 SDS-PAGE Gel Electrophoresis

Dissociating polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulphate (SDS, 0.1% w/v) was performed on 10% (w/v) acrylamide slabs (with a 5% w/v stacking gel according to the method of Laemmli (1970) with a discontinuous buffer system (Tris-HCl, pH 6.8, stacking; Tris-HCL, pH 8.8, separating). Gel preparation is extensively discussed by Ames (1974)

Separating and stacking gels were prepared from a stock acrylamide solution (30% w/v), containing bis-acrylamide (0.08% w/v). The final concentrations in the separating gel were:

0.375 M Tris- HCl (pH 8.8), SDS 0.1% w/v., and 0.125 M Tris-HCl (pH 6.8), SDS 0.1% w/v in the stacking gel.

The gels were polymerised chemically by the addition of tetramethylethylenediamine (TEMED, .033% v/v) and freshly prepared ammonium persulphate (1.5 ml of a .015% w/v solution) per 60 ml of unpolymerised gel solution. The electrophoresis buffer (pH 8.3) contained Tris (0.025 M), Glycine (0.2 M) and SDS (0.1% w/v). Bromophenol blue marker dye (0.1 ml) was added to the upper reservoir, which contained 500 ml of electrophoresis buffer.

50ul of the collecting vessel sample was diluted with an equal volume of sample buffer containing Tris-HCl, pH 6.8 (0.2 M Tris), SDS (2% w/v) and sucrose (10% w/v), and the sample 50 µl was loaded into the wells of the stacking gel using a Gilson pipette. The gel was electrophoresed at 60 volts overnight, until the bromophenol blue marker dye reached the bottom of the gel.

Protein bands were visualised using the staining method of Morrissey (1981).

2.6.2 Silver Staining of Polyacrylamide Gels.

Polypeptides in the acrylamide gel were detected by the silver staining method of Morrissey (1981) which is a modification of the original method by Switzer et al, (1979).

(1) The gel was prefixed in methanol (50% v/v), acetic acid (10% v/v) for 30 minutes, followed by methanol (5% w/v), acetic acid (7% v/v) for a further 30 minutes.

(2) The gel was fixed in glutaraldehyde (100 ml, 10% v/v) for 30 minutes.

(3) The gel was then rinsed in a large volume of distilled water overnight, followed by a fresh water rinse the next morning for 30 minutes.

(4) The gel was soaked in dithiothreitol (200 ml, 5 µg/ml) for 30 minutes.

(5) The dithiothreitol solution was poured off, and the silver nitrate (100 ml, 0.1% w/v) was added, and the gel was left for 30 minutes.

(6) The gel was rapidly rinsed once with distilled water (100 ml) and then twice with developer (50 ml) containing sodium carbonate (3% w/v) and formaldehyde (50 µl of 37% solution). The gel was soaked in developer (100 ml) until the desired amount of staining was achieved. Staining was terminated by the addition of citric acid (5.0 ml, 2.3 M) directly to the developer. After 10 minutes, the solution was discarded.

(7) The gel was rapidly rinsed once with distilled water (100 ml) and then twice with with developer (50 ml) containing sodium carbonate (3% w/v) and formaldehyde (50 µl of 37% solution). The gel was soaked in developer (100 ml) until the desired amount of staining was achieved. Staining was terminated by the addition of citric acid (5 ml, 2.3 M) directly to the developer. The solution was then discarded.

2.7 THE USE OF IONOPHORES IN HYBRIDOMA CELL CULTURES

Cell Cultivation - the cells were cultivated as previously described.

Inoculum - 2.0 ml of approx 1 x 10^5 cells/ml was used as an inoculum.

2.7.1 5 μM stock solutions of Valinomycin, Gramicidin D, and Monensin in 100 ml of 50% EtOH/dH_2O were prepared.

The solutions were then filter sterilised using 0.2 μm filters (Sterilin).

Petri Dish Number	Inoculum	10% F.B.S. Medium	Ionophore Added
1	2.0 ml	8.0 ml	0.005 μM (10 μl)
2	2.0 ml	8.0 ml	0.010 μM (20 μl)
3	2.0 ml	8.0 ml	0.025 μM (50 μl)
4	2.0 ml	8.0 ml	0.050 μM (100 μl)
5	2.0 ml	8.0 ml	EtOH/dH_2O (100 μl)
6	2.0 ml	8.0 ml	Blank

Table 4.

2.7.2 Monensin

The following concentrations were used for Monensin

Petri Dish Number	Inoculum	10% F.B.S. Medium	Monensin Added
1	2.0 ml	8.0 ml	Blank
2	2.0 ml	8.0 ml	EtOH/dH_2O (500 µl)
3	2.0 ml	8.0 ml	0.0025 µM (50 µl)
4	2.0 ml	8.0 ml	0.0050 µM (10 µl)
5	2.0 ml	8.0 ml	0.0125 µM (25 µl)
6	2.0 ml	8.0 ml	0.025 µM (50 µl)
7	2.0 ml	8.0 ml	0.050 µM (100 µl)
8	2.0 ml	8.0 ml	0.250 µM (500 µl)

Table 5

Live cells from all the ionophore treated cultures were counted daily for five days, using trypan blue staining. 0.4 ml samples were taken from each petri dish, placed in Eppendorf tubes and were then centrifuged for five minutes at high speed using the MSE Microcentaur. The supernatant was retained to test for antibody.

CHAPTER 3

RESULTS

3.1 FERMENTATION

Since mammalian cells divide approximately once daily a mean generation time in the region of 24 hours was required. According to the relationship:

$$\mu = \frac{0.693}{td}$$

$$\mu = \frac{0.693}{24} = 0.028875 = D$$

Flow rate = Dilution rate x Volume of culture vessel

(F) = (D) x (V)

Flow rate = 0.028875 x 80 = 2.31 ml/ hr

F = 2.31ml/hr

The most convenient flow rate setting was 2.1ml/hr. This yielded a doubling time of 26.4 hours.

When the fermenter was inoculated, there was a lag period of 36 hours, before the cells began to grow exponentially. Once three exponential points were obtained on a graph of cell density vs time (Fig 10 a), the pump was turned on and the medium was allowed to flow continuously.

Once steady state was achieved, samples were taken from both the culture and collecting vessels, a cell count was taken, and the supernatant was retained to test for antibody.

The bar graph (Fig 11), shows that the collecting vessel contained the highest level of monoclonal antibody, and both the collecting vessel and the batch sample contained more antibody than the culture vessel, during the first week at steady state.

The pie chart (Fig 12) shows that the level of monoclonal antibody in the culture vessel was less than that present in the collecting vessel and that an uncentrifuged culture vessel sample left standing at room temperature for approximately two hours, yielded a higher ELISA reading than the same sample taken and centrifuged immediately.

Figure 10 (a), Plot of $\log_{10}$ cell density versus time (hours), showing a period of exponential growth which occurred approximately 36 hours after inoculation. When three exponential points were obtained, the medium was allowed to flow continuously.

Fig 10 a - Log Cell Density vs Time

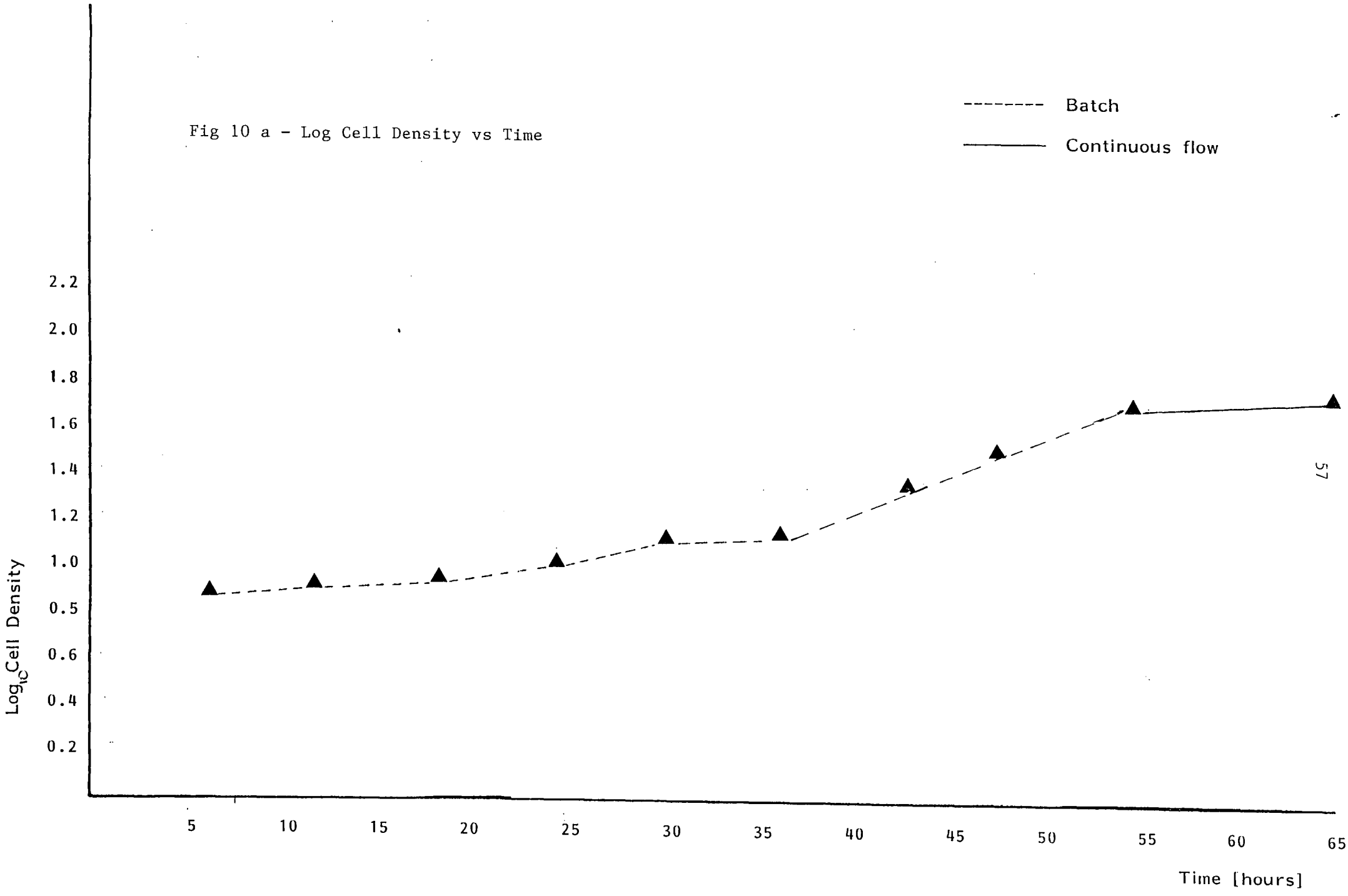


Fig 10 (b), Plot of cell density x 10^5/ml versus time (hours), showing the steady state period.

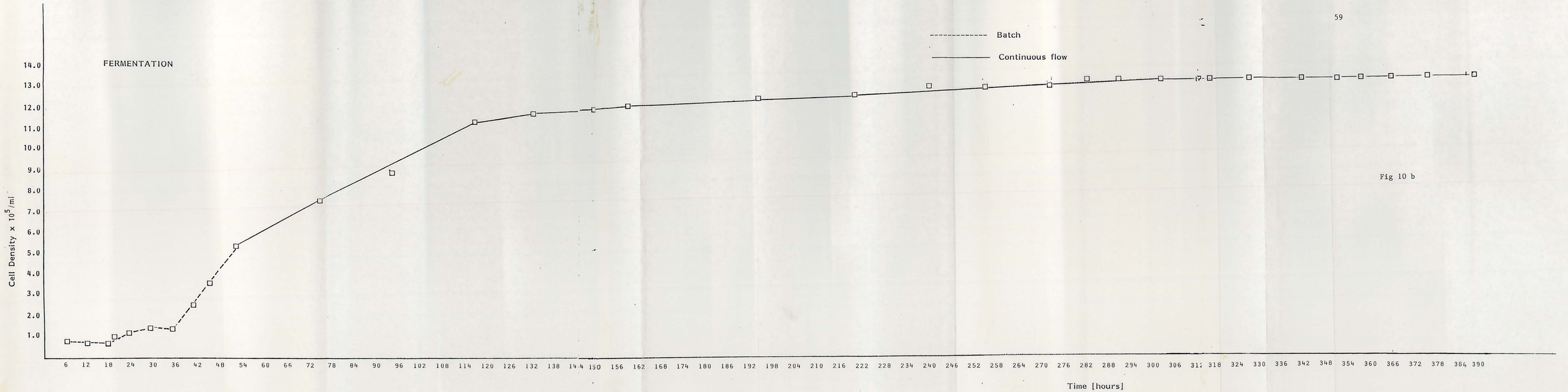

FERMENTATION
Batch
Continuous flow
Cell Density x 10^5/ml
14.0
13.0
12.0
11.0
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
6 12 18 24 30 36 42 48 54 60 66 72 78 84 90 96 102 108 114 120 126 132 138 144 150 156 162 168 174 180 186 192 198 204 210 216 222 228 234 240 246 252 258 264 270 276 282 288 294 300 306 312 318 324 330 336 342 348 354 360 366 372 378 384 390
Time [hours]

Fig 10 b

Figure 11, Bar graph showing a higher ELISA reading for both the collecting vessel and batch sample as compared to the culture vessel sample, during week 1 at steady state.

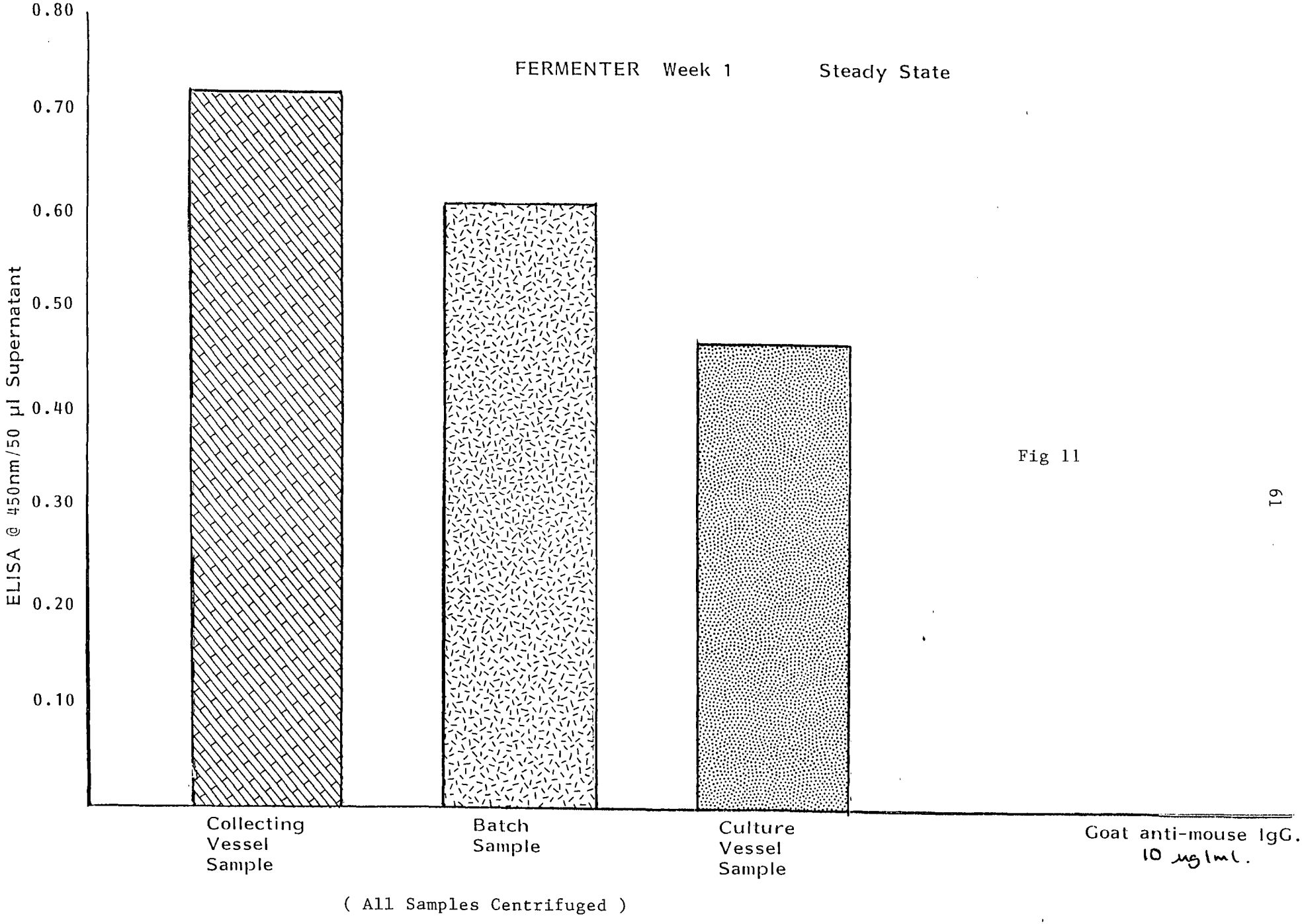
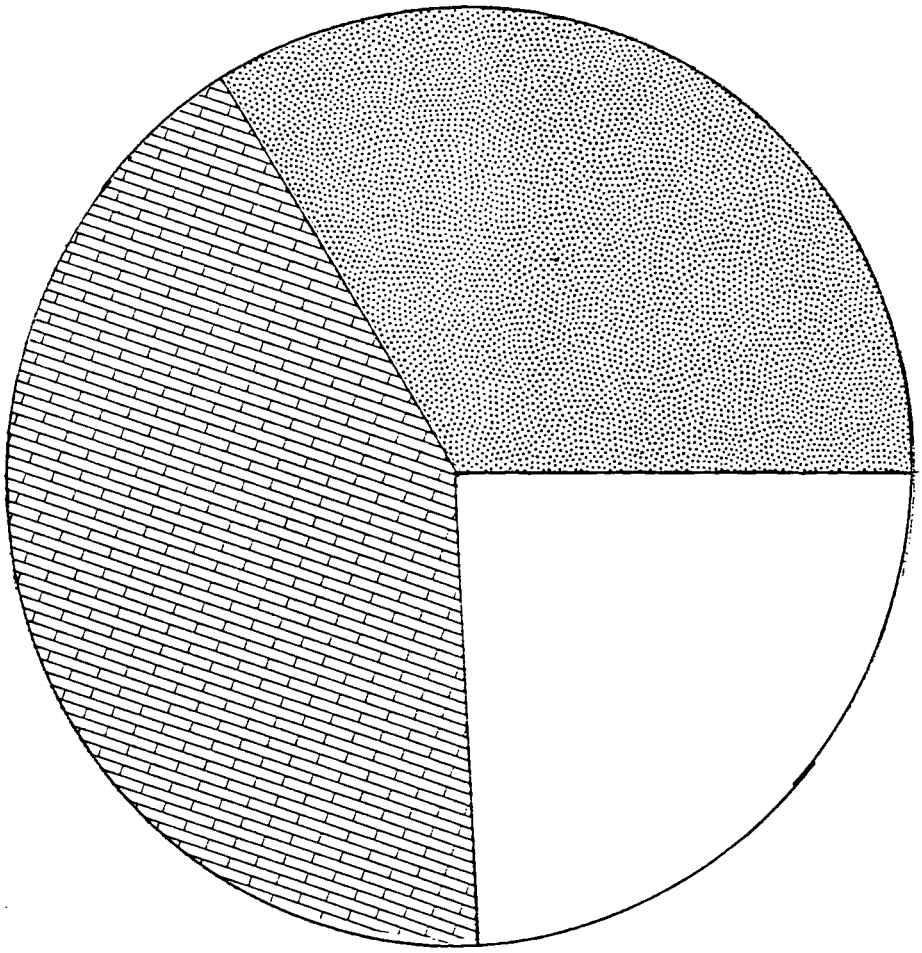

FERMENTER Week 1 Steady State
ELISA @ 450nm/50 µl Supernatant
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
Collecting Vessel Sample
Batch Sample
Culture Vessel Sample
Goat anti-mouse IgG. 10 µg/ml.
(All Samples Centrifuged)

Fig 11

Figure 12, Pie-chart showing the high ELISA reading for the collecting vessel, and also the high reading for a sample uncentrifuged and left at room temperature for 2 hours, and the lower reading for a sample taken at the same time and centrifuged immediately.

FERMENTER Week 2 Steady State



	ELISA Reading @ 450nm
Culture Vessel Sample [Centrifuged]	.439
Culture Vessel Sample [Uncentrifuged] Left room temperature for two hours	.612
Collecting Vessel Sample [Centrifuged]	.745

Fig 12

(Positive and Negative Controls were within acceptable limits)

3.2 SERUM FREE MEDIUM

As described in method (1), (Table 2) the cells were placed directly into medium containing lower concentrations of fetal bovine serum. The plots of cell density vs time (Fig 13 a) and ELISA vs time (Fig 13 b) show that a decrease in the concentration of serum supplement . resulted in a concomitant reduction in cell density and antibody secretion. The hybridoma cells survived in medium containing 5 and 8 % fetal serum, but the cell density was reduced by 50%. Cells placed directly into medium containing 2.5 and 0% fetal bovine serum resulted in cell death within a short period of time.

The second method (Table 3) allowed the cells to adapt gradually to the serum-free medium. An inoculum was taken from a plate containing 10% F.B.S. and used to inoculate another plate containing only 8% F.B.S. Again, once the cells were growing exponentially, an inoculum was taken from this plate, and was used to inoculate a new plate that contained 5% F.B.S., and so on, until the medium contained no serum supplement.

The results show that this method is the best way to adapt the cells to serum-free medium. Although there is a decrease in cell density as the concentration of serum-free medium is increased the cell density stabilised once the cells were placed in medium containing 5% F.B.S., and when placed in serum-free medium, a cell density of 6.3×10^5 cells/ml was achieved (Fig 14 a). Antibody present is a function of cell density (Fig 14 b). These cells were then cultivated in serum-free medium for a further week, and a cell density of 1.1×10^6 cells/ml was recorded.

Figure 13 a, Plot of ELISA at 450nm versus time for method (1) of the serum-free medium.

Figure 13 b, Plot of cell density x 10^5cells/ml versus time for method (1) of the serum-free medium.

METHOD 1

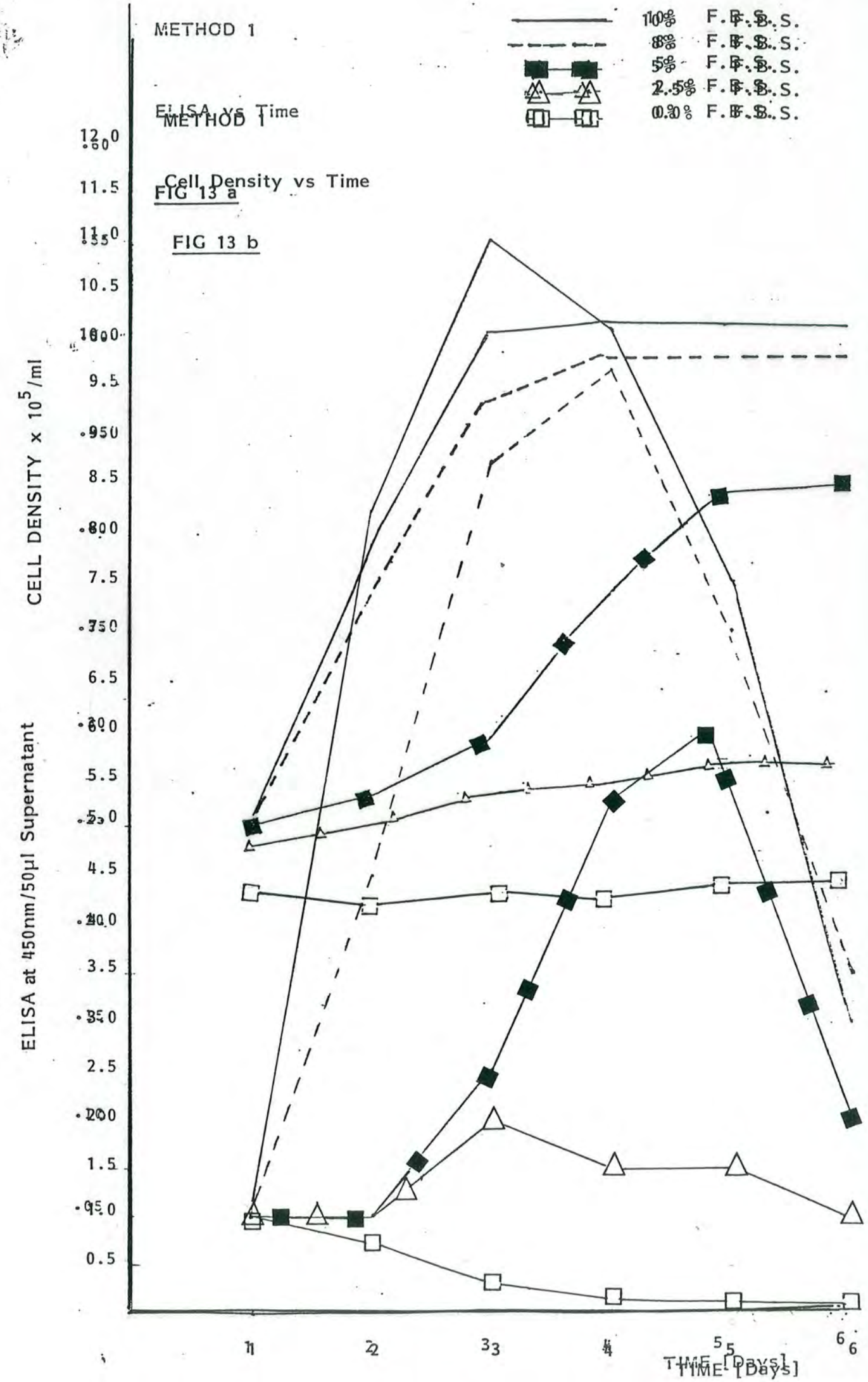
ELISA vs Time

METHOD 1

Cell Density vs Time

FIG 13 a

FIG 13 b



METHOD 22

Elisa vs Time

Cell Density vs Time

FIG 14 a

10% F.B.S.
8% F.B.S.
5% F.B.S.
2.5% F.B.S.
0% F.B.S.

FIG 14 b

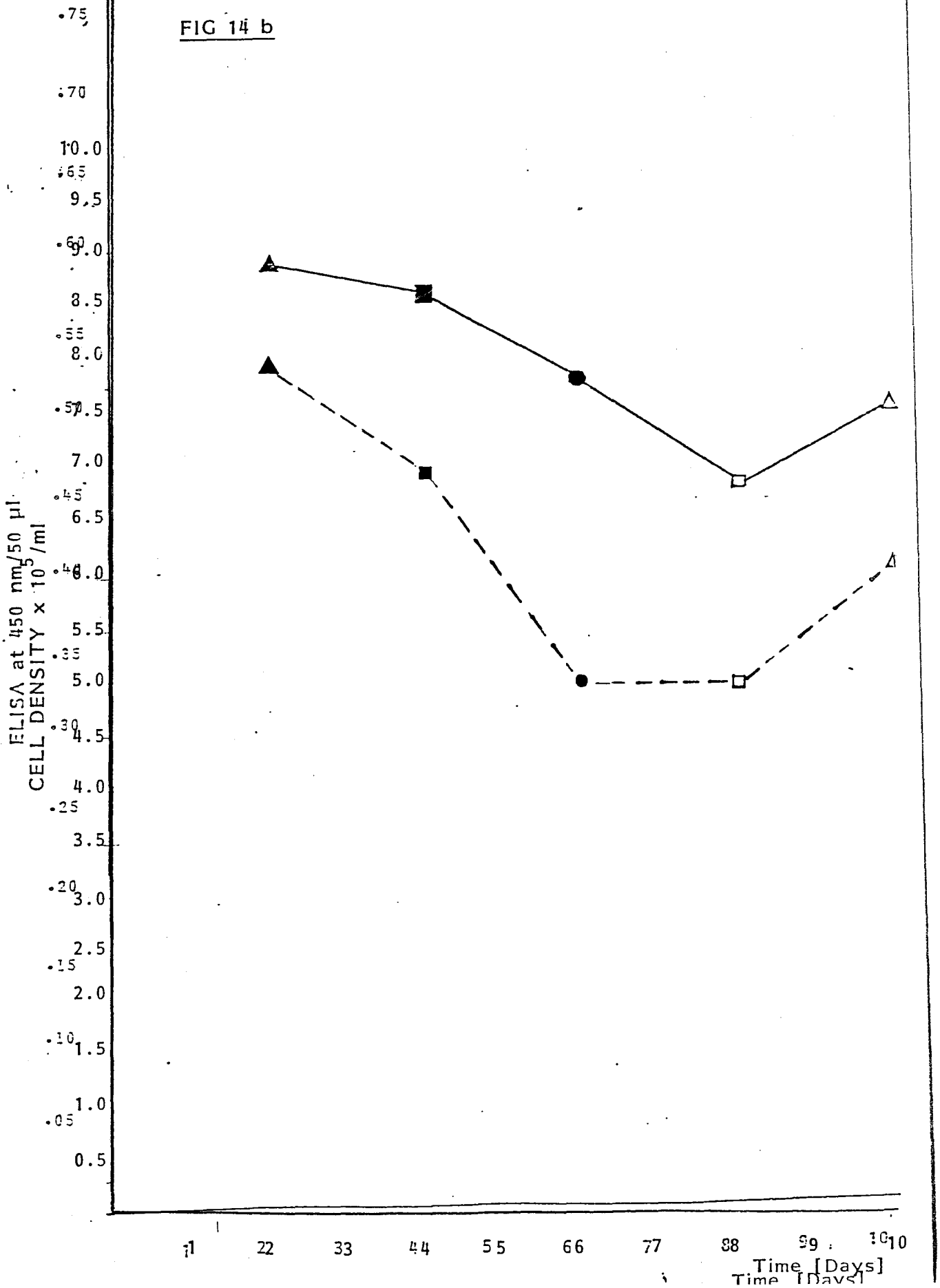


Figure 14 a, Plot of ELISA at 450 nm versus time for the second method of weaning cells to serum-free medium.

Figure 14 b, Plot of cell density versus time for the second method of weaning cells to the serum-free medium.

3.3 ANTIBODY PURIFICATION

Supernatants from batch culture, collecting vessel and culture vessel were purified on a Protein A column. Fractions from the column were tested for antibody by ELISA.

Three plots of ELISA vs time were constructed for samples from batch culture (Fig 15) collecting vessel (Fig 16) and the culture vessel (Fig 17). In all three plots, there is a peak present between 18 and 30 minutes elution time. These peaks represent the IgG_1 subclass. Since the peak is present in all three samples, no antibody switching has occurred. Therefore, the low antibody level present in the culture vessel was not due to switching off of the antibody during continuous fermentation.

The fractions were also read at A_{280} and A_{260}, so that the protein concentration could be estimated. It was found that the collecting vessel contained the highest concentration of monoclonal antibody, approximately 65 ug/ml, and that the culture vessel contained approximately 36 ug/ml protein. These results confirm the earlier ELISA results.

Figure 15, Plot of ELISA at 450 nm versus time (minutes) for the sample of batch supernatant, showing the presence of an IgG peak at approximately 27 minutes elution time.

BATCH

Fig 15

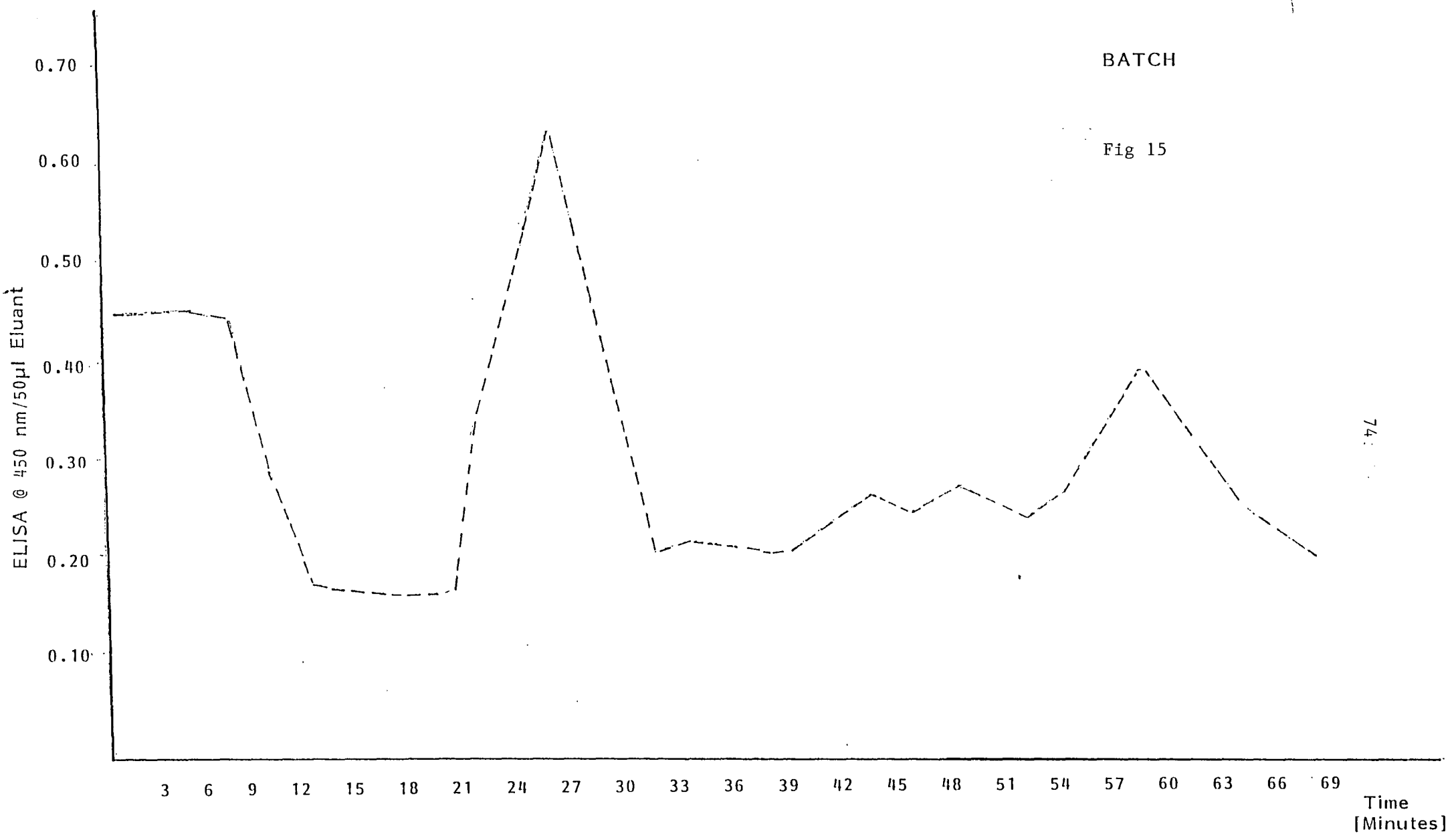

0.70
0.60
0.50
0.40
0.30
0.20
0.10
ELISA @ 450 nm/50µl Eluant
3 6 9 12 15 18 21 24 27 30 33 36 39 42 45 48 51 54 57 60 63 66 69
Time
[Minutes]

Figure 16, Plot of ELISA at 450 nm versus time (minutes) for the collecting vessel sample, showing the presence of the IgG peak at approximately 24 minutes.

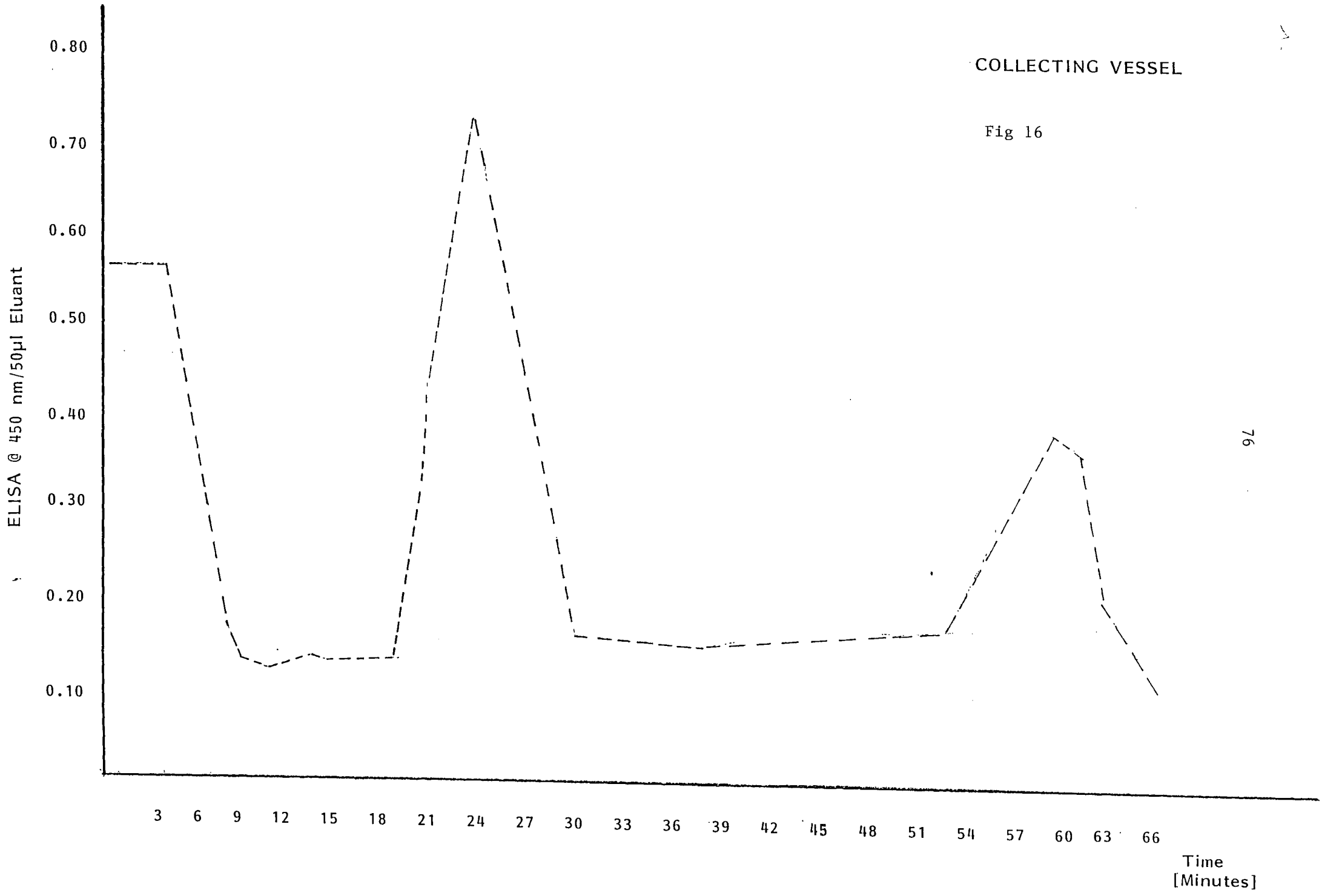
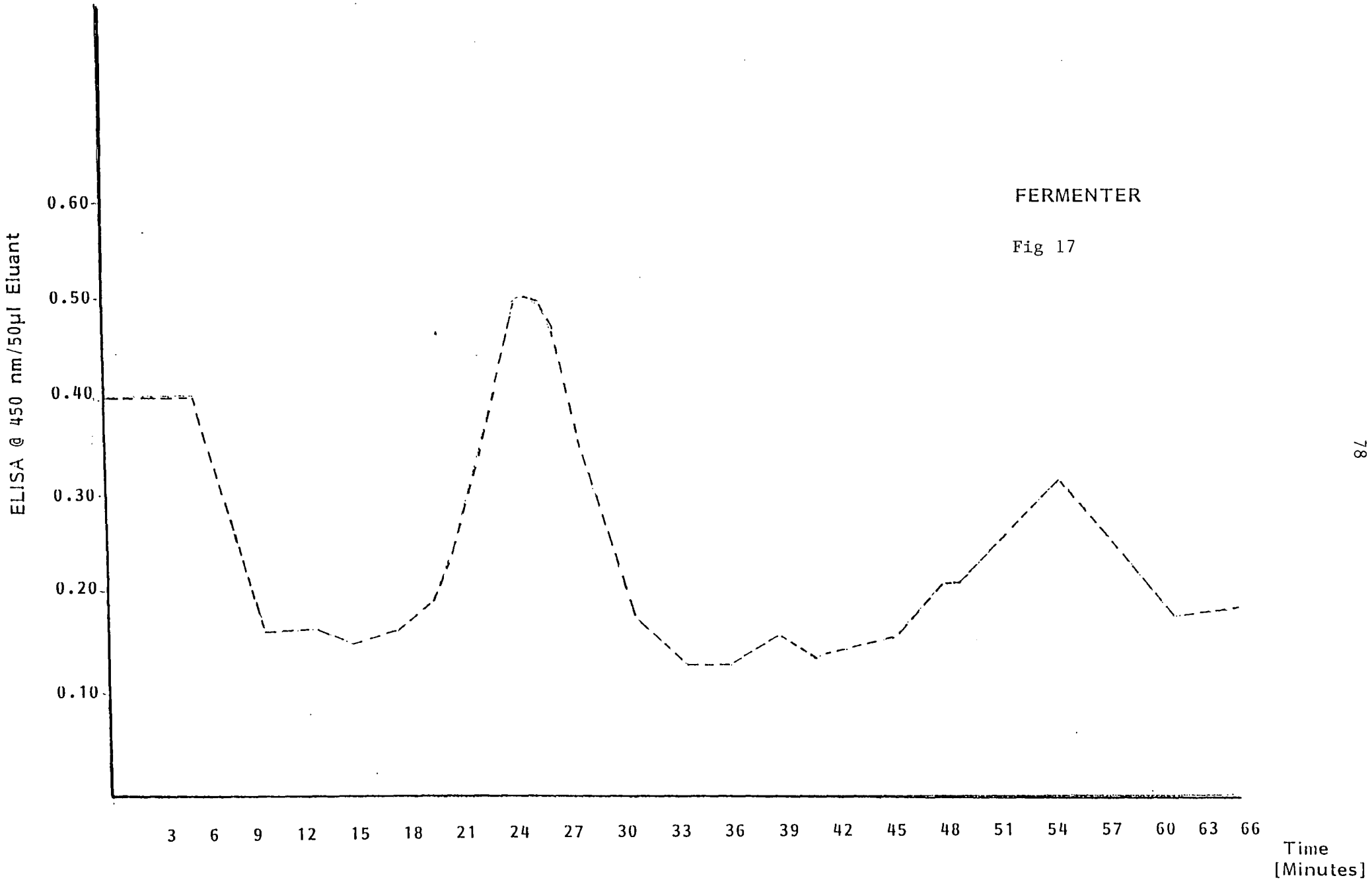

COLLECTING VESSEL
Fig 16
ELISA @ 450 nm/50µl Eluant
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
3 6 9 12 15 18 21 24 27 30 33 36 39 42 45 48 51 54 57 60 63 66
Time
[Minutes]
76

Figure 17, Plot of ELISA at 450 nm versus time for the Fermenter (culture vessel) sample,showing the presence of the IgG peak at approximately 24 minutes elution time.

FERMENTER

Fig 17



3.3.1 Protein Estimations

Eluants from the Protein A column were read at 280 nm and at 260 nm using the Gilford spectrometer, so that the protein concentration could be estimated according to the method of Warburg and Christian (1946).

Collecting Vessel

$$A_{260} = 0.0812 \qquad \frac{A_{280}}{A_{260}} = 1.013 \quad \text{Factor} = 0.794$$
$$A_{280} = 0.0823$$

$0.794 \times 0.0823 = 0.065$ mg/ml = 65.0 µg/ml protein.

Culture Vessel

$$A_{260} = 0.0618 \qquad \frac{A_{280}}{A_{260}} = 0.892 \quad \text{Factor} = 0.680$$
$$A_{280} = 0.0539$$

$0.0539 \times 0.68 = .036$ mg/ml

= 36 µg/ml protein.

Batch supernatant

$$A_{260} = 0.0770 \quad \frac{A_{280}}{A_{260}} = 0.930 \quad \text{Factor} = 0.930$$

$$A_{280} = 0.0712$$

$0.733 \times 0.0712 = .052$ mg/ml

$= 52\ \mu g/ml$ protein.

3.3.2 SDS-Gel Electrophoresis

Supernatant from the collecting vessel was purified on the Protein A column, and the heavy and light chains of the antibody were isolated by SDS-Gel Electrophoresis (Fig 18). Track(1) represents the purified supernatant. Track (2) represents the molecular weight markers. In Track (1), Two bands are present, one at 49,500 M_r and the other at 21,500 M_r. These bands represent the heavy and light chains of the antibody.

Fig 18

Track (2)

Molecular Weight Markers

66,000 Albumin, bovine

45,000 Albumin, egg

36,000 Glyceraldehyde 3-Phosphate Dehydrogenase

29,000 Carbonic Anhydrase

24,000 Trypsinogen, bovine pancreas

21,100 Trypsin inhibitor, soybean

14,200 Lactalbumin, bovine milk

Track (1)

49,500 Heavy chain

21,500 Light chain

3.4. IONOPHORES

Three Ionophores were tested, so that their effects on the hybridoma cells and antibody secretion could be studied. All three Ionophores were dissolved in 100% ethanol, and then diluted by 50% in water. 50% EtOH/dH20 was not found to be toxic to the cells at a volume at or less than 500 ul. Plots of cell density vs time, and ELISA vs time were constructed for all three ionophore treated cultures. The plots show that Valinomycin (Fig 19 a and b) and Gramicidin (Fig 20 a and b) were both found to very toxic. The hybridoma cells died within a short period of time. There is a direct relationship between the ELISA results and the cell densities present. Since there are low cell densities present, the ELISA results are also low, except for the control samples where cell densities and ELISA readings are high.

Monensin treated cultures show a different result. Monensin was found to be less toxic. Although cell growth was inhibited (Fig 21 a) the ELISA results were found to be in the range of the control cultures (Fig 21 b). Monensin, therefore appeared to have increased the secretion of monoclonal antibody.

All ELISA tests were carried out at the same time. Positive and negative controls were used, and were found to be within acceptable limits.

Figure 19 a, Plot of ELISA at 450 nm versus time for cells treated with Valinomycin

Figure 19 b, Plot of cell density x 10^5 cells/ml versus time for cells treated with valinomycin

VALINOMYCIN

Cell Density vs Time

FIG 19 b

VALINOMYCIN

Elisa vs Time

FIG 19 a

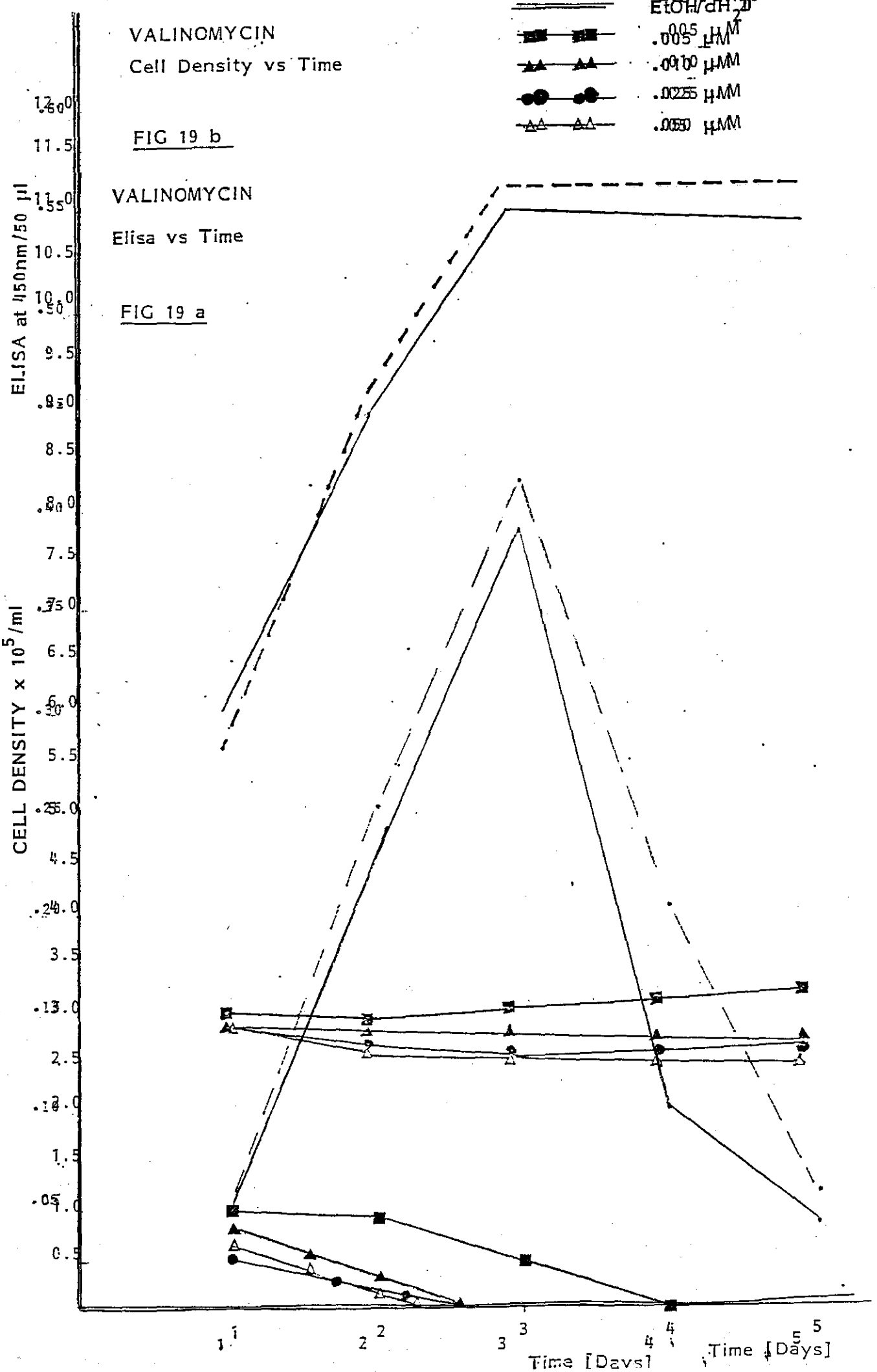


Figure 20 a, Plot of ELISA at 450 nm versus time for cells treated with Gramicidin.

Figure 20 b, Plot of cell density x 105 cells/ml versus time for cells treated with Gramicidin.

GRAMICIDIN

Cell Density vs Time

Elisa vs Time

FIG 20 b

FIG 20 a

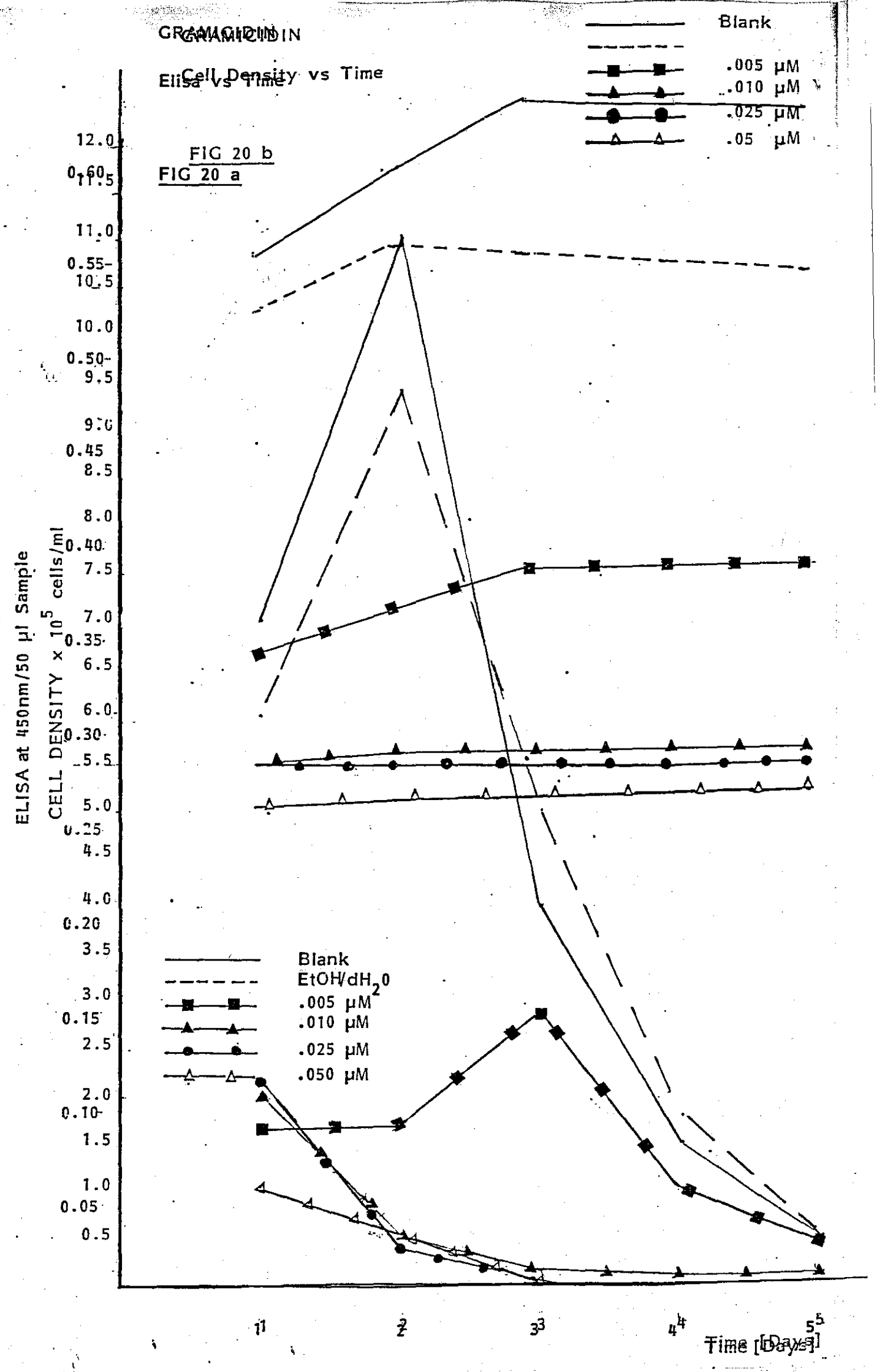
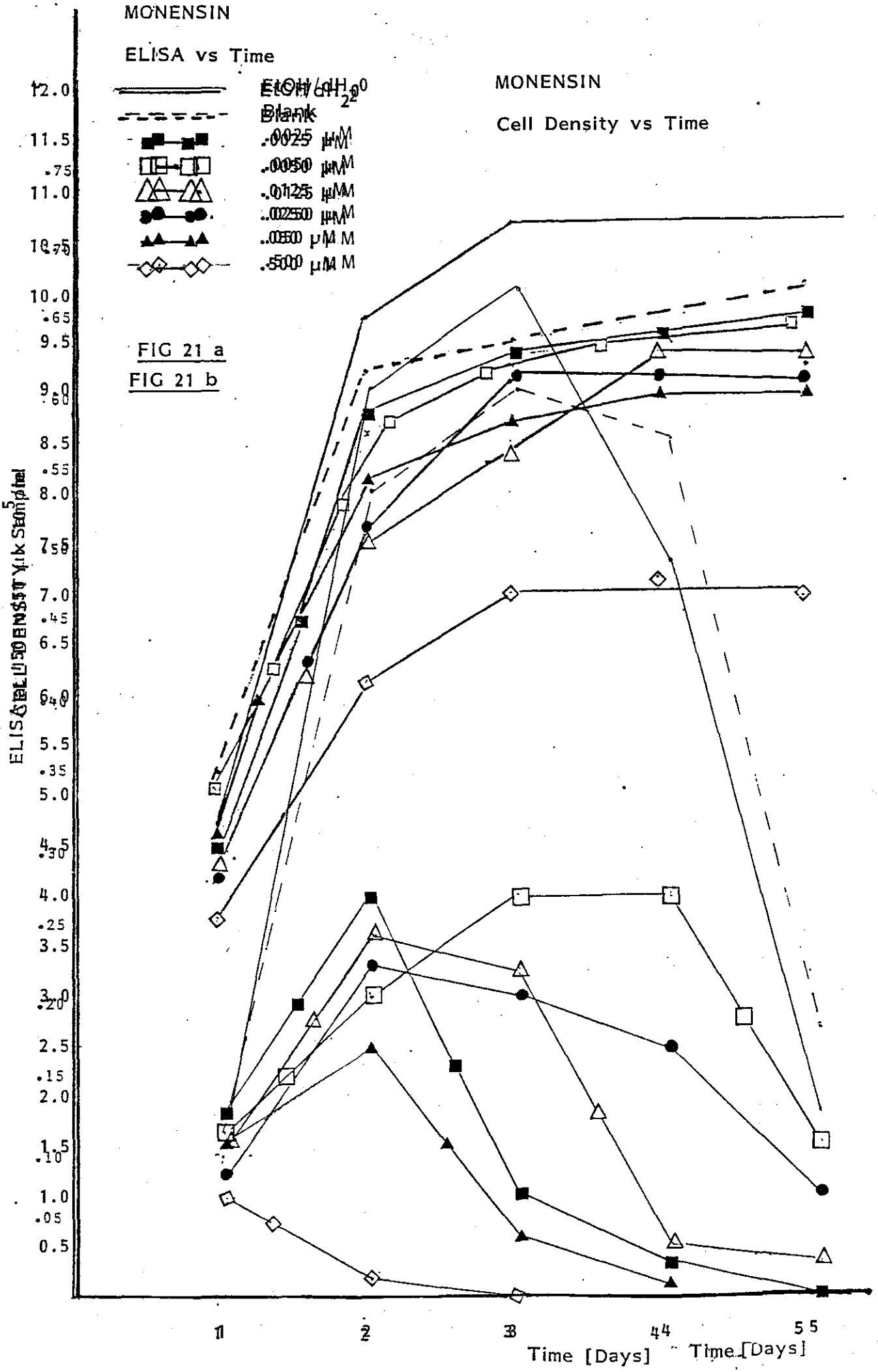


Figure 21 a, Plot of ELISA at 450 nm versus time for Monensin treated cells

Figure 21 b, Plot of cell density x 10^5 cells/ml versus time for Monensin treated cells


MONENSIN
ELISA vs Time
EtOH/dH2O
Blank
.0025 µM
.0050 µM
.0125 µM
.0250 µM
.0500 µM
.5000 µM
MONENSIN
Cell Density vs Time
FIG 21 a
FIG 21 b
ELISA
Time [Days]
Time [Days]

CHAPTER 4

DISCUSSION

4.1 FERMENTATION

When the fermenter was first set up, the hybridoma cells were successfully grown for a period of two weeks on a continuous basis. It was found that the level of monoclonal antibody in the culture vessel was less than that present in batch cultures of hybridoma cells, and that the collecting vessel contained the highest amount of antibody.

It was also found that when a sample of cells and supernatant was left standing undisturbed at room temperature for approx. two hours, the ELISA reading for this sample was considerably higher than that of the same sample taken and centrifuged immediately. It appeared that monoclonal antibody was being retained by the cells in the culture vessel and, in order to understand this, it was decided to concentrate on membrane work. While this work was being carried out, problems were encountered in re-establishing the fermenter. It was found, after inoculation, that the cells grew slowly for 2-3 days, and then died. In some cases death of the inoculum occurred within 12 hours of being introduced into the culture vessel. It appeared that the cells were being damaged when injected through a needle into the inoculation port. It was therefore decided to pour the inoculum into the culture vessel.

The entire apparatus was placed in the sterile cabinet, where the septum was removed from the culture vessel, the inoculum was poured in gently using a pipette, and the septum was replaced. Cell counts taken 12 hours after the addition of the inoculum by this method confirmed the survival of the inoculum. In some cases, a cell density of 7.0×10^5 cell/ml was achieved while the culture was stationary, but once stirring commenced the cells died. This indicates the sensitivity of these cells to shear forces. In order to process the cells it is often necessary to provide sufficient agitation to maintain the cells in suspension. During such handling cells inevitably encounter stresses caused by fluid dynamic effects.

In 1971, Augenstein et al., studied the effect of shear on the death of two strains of mammalian tissue cells. Human and mouse cells were harvested and suspensions were pumped through various lengths and diameters of stainless steel tubing. Cell deaths occurred, and could be correlated with either average wall shear, or power dissipitation within the capillary tube. Mouse cells were found to be more sensitive to shear forces than human cells. In 1987, Dodge and Hu studied the growth of hybridoma cells under different agitation conditions. They concluded that an agitation rate four times that required to maintain the cells in suspension, resulted in both reduced growth rate and growth extent.

In 1983, de St. Groth reported that the growth rate of hybridoma cells was 15% lower in a stirred vessel than in stationary culture.

Another problem encountered was ensuring that the oxygen transfer rate was correct, otherwise the cells died. The importance of the gas phase oxygen composition for the optimal growth of animal cells has been noted (Kilburn et al., 1968, Bradley et al., 1978, Boraston et al., 1972, Radlett et al., 1972). Various hybridoma cell lines have been observed to grow best at normal atmospheric oxygen concentrations (Birch et al., 1985), or at less than atmospheric oxygen concentrations (Reuveny et al., 1986). Since the rate of oxygen uptake by the cells is low, the liquid phase oxygen concentration could be assumed to be close to equilibrium with the inlet gas in most hybridoma culture systems. In 1986, Reuveny et al., observed that the highest titres of monoclonal antibodies occurred at oxygen partial pressures below those required for optimal growth in perfusion reactors. In 1987, Phillips et al., examined the effect of oxygen on antibody productivity in hybridoma cultures and concluded that maximum viable cell concentrations of 1.2 x 106 cells/ml were obtained in split flow bioreactors mixed with a gas containing 10% oxygen. However, maximum antibody concentration was achieved at a suboptimal level of growth with an oxygen concentration of 2.5%.

Specific studies on the effect of dissolved oxygen partial pressure on animal cell growth or metabolism have been made, both in static and suspension cultures (Danes et al., 1963, and Fleischaker et al., 1961). It has been found that anaerobic conditions generally halt or severely depress cell growth (Clark 1964). At the other extreme, high oxygen partial pressure inhibits replication (Brosemar and Rutter 1961). Between these limits, an optimum pO_2 range for cell growth must exist. Although several workers have demonstrated a beneficial effect of moderately low pO_2 (Cooper and Burt 1958), most information on the influence of pO_2 on cellular growth and metabolism have been obtained at extreme pO_2 values.

Processes employing pneumatically mixed bioreactors have been designed and applied successfully for producing commercial quantities of monoclonal antibody. Pneumatically mixed bioreactors have been proven to provide a balance of adequate mass transfer and mixing without injury to the sheer sensitive hybridoma cells. However, the culture conditions for optimising monoclonal antibody in these reactors have not been described. On some occasions the fermenter was contaminated by yeasts. This illustrates another of the difficulties encountered while attempting to grow hybridoma cells continuously. A comprehensive study of these problems is required, so that monoclonal antibodies can be produced more efficiently on a laboratory scale.

4.2 SERUM-FREE MEDIUM

It is necessary to distinguish between cultivation of cells not adapted to growth in serum-free medium, and adapted cells. Non-adapted cells are cells that are transferred directly from serum supplemented medium into serum-free medium as described in the first method. Adaptation takes approximately five passages of medium, over a period of approximately twelve days. Adapted cells grow significantly faster than non-adapted ones (Kovar and Franek 1986). Once hybridoma cells are adapted, they can be propagated in serum- free medium as long term cultures without any special care (Cleveland et al., 1984). The second method (Method 2) represents cells that have adapted to serum-free medium. This method is the best way of adapting the hybridoma cells to the serum-free medium.

Manipulations with cells cultivated in serum-free medium are not entirely the same as in the case of cells grown in serum supplemented medium. Serum-free culture is more sensitive to environmental variations i.e. changes in pH due to changes in CO_2 concentration or evaporation of the medium, due to insufficient humidity. It is recommended that hybridoma cells are passaged every two to three days to avoid rapid cell degeneration and death.

4.3 ANTIBODY PURIFICATION

The IgG_1 subclass was isolated from the supernatants using Protein A Sepharose. There was no subclass switching observed, which rules out the possibility that the low level of antibody in the culture vessel was due to antibody switching. Protein estimations were carried out on the supernatants from the culture vessel, collecting vessel and batch culture, according to the method of Warburg and Christian (1946). The results show that a protein concentration of 65 μg/ml was obtained for the supernatant from the collecting vessel, 52 μg/ml for batch supernatant, and 36 μg/ml for the culture vessel samples, thereby confirming the ELISA results obtained. SDS- Gel Electrophoresis was carried out on the purified immunoglobulin. There is a protein band of 49,500 M_r and another of 21,500 M_r, which correspond to the heavy and light chains of the antibody.

4.4 IONOPHORES

Three ionophores were studied so that their effects on the hybridoma cells and antibody secretion could be studied. Valinomycin and Gramicidin were found to be very toxic to the cells. Monensin, at the concentration that resulted in cell death with the other two ionophores, did not kill the cells, but inhibited cell growth.

The plots of cell density vs time and ELISA vs time for both Gramicidin and Valinomycin show that the level of antibody present is a function of cell density, and that these ionophores did not have any effect on monoclonal antibody secretion. In the case of Monensin, the plots of cell density vs time and ELISA vs Time indicate that there is an increased secretion of antibody in the cultures, even though the cell densitities are low, i.e. a cell density of 4.0×10^5 yielded the same antibody reading as the control cultures with a cell density in the region of 1.0×10^6 cells/ml.

In order to explain these observations, it is important to understand how these ionophores exert their effects.

Valinomycin is a neutral ionophore, and is a cyclic depsipeptide which forms a lipid soluble complex with monovalent cations (Pressman 1967). The ionophore is highly specific for K^+ and creates an insulating cage around the ion, enabling its transport across the lipid phase of both artificial and biological membranes. Valinomycin dissipitates K^+ gradients and abolishes the electrical potential difference $\Delta\varphi$ of the cell membrane.

The electrochemical proton potential (Δp) as defined by Mitchell (1961) is composed of electrical and concentration components according to the relation:

$$\Delta p = \frac{-2.3RT\ pH}{F}$$

Where Δp = Electrical potential difference across the membrane.

pH = pH Gradient

R = Gas constant

T = Absolute Temperature

F = Caloric equivalent of the Faraday.

In the intact cell, the ionophore exerts its effects at two sites at least,

(1) By energy uncoupling in the mitochondria (Mueller and Rudin 1967), or by

(2) Its actions on the electrical properties of the cell membrane (Spector and Palfrey 1975, Lerner et al., 1982)

It seems probable that Valinomycin at the concentrations used in this study, exerts its effect at the cell membrane, by altering its electrical properties. The increase in K^+ permeability results in the hyperpolarization or an impairment of the electrical behaviour of the membrane.

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Gramicidin binds irreversibly to the surfaces of lipid membranes and produces a pore which spans the membrane. This pore conducts Na^+ and K^+ with a selectivity of K^+ over Na^+ of about six fold. There is a rapid loss of K^+ from the cells in exchange for external Na^+ or H^+ ions. Gramicidin reduces the pH of the cell membrane.

Monensin increases the internal concentration of Na^+, by increasing the supply of sodium to the sodium- potassium pump. Monensin is a Na^+ carrier. Serum accelerates the Na^+/K^+ pump by enhancing Na^+ entry and availability to the Na^+ transport site of the pump, which is on the cytoplasmic side of the lipid bilayer. There is a quantitative relationship between pump activity and internal cations. A small increase in internal Na^+ enhances the activity of the pump.

Monensin was found to be the least toxic of the three ionophores, and appeared to increase the secretion of monoclonal antibody. Since Monensin is a sodium carrier, traffic of sodium into the cell will be balanced by reverse traffic of sodium extracellularly. It is proposed that this reverse transport of sodium allows increased secretion of the monoclonal antibody out of the cells.

4.5 SUMMARY

A study of the secretion of monoclonal antibody from hybridoma cells in both batch and continuous cultures has been carried out. It has been found that there was less monoclonal antibody present in the culture vessel during continuous cultivation of the cells (Fig 11 and Fig 12). Since the cell density achieved was the same as that achieved in stationary culture, there appeared to be retention of monoclonal antibody during continuous cultivation.

In order to investigate this retention, membrane work was carried out. Since the secretion of monoclonal antibody from a hyridoma cell obviously involves some sort of transport mechanism, this transport mechanism was investigated by studying the effects of ionophores on the hybridoma cells. Three ionophores were chosen, Valinomycin, Gramicidin and Monensin, and all three exert their effects in different ways. Valinomycin and Gramicidin were found to be toxic (Fig 19 a, 19 b) (Fig 20 a, 20 b), and killed the cells within a short period of time. Monensin was found to be less toxic, and although cell growth was inhibited, it appeared that this ionophore increased the secretion of monoclonal antibody from the cells (Figure 21 a, 21 b).

Various problems were encountered in re-establishing the fermenter. Death of the inoculum often resulted when it was injected through a syringe into the culture vessel. It was decided to pour the inoculum into the culture vessel, by placing the entire fermentation apparatus into the sterile cabinet. It was found that the inoculum survived, but once stirring commenced, the cells died. This illustrates the sensitivity of the hybridoma cells to shear forces. Problems were also encountered with ensuring that the oxygen transfer rate was correct otherwise the cells died.

4.5.1 Conclusion

Monoclonal antibody secretion in both batch and continuous cultures has been studied. It was found that monoclonal antibody was retained by the cells in the continuous system, and in order to investigate this retention, membrane work was carried out. Three ionophores were tested, and Monensin appeared to have increased the secretion of monoclonal antibody out of the cells. It will be left to the next student to investigate whether or not Monensin can increase the production of monoclonal antibody during continuous cultivation, or when added to a culture containing a high cell density. The problems encountered during the continuous cultivation of the hybridoma cells were too comprehensive and could not be solved within the time scale of this project.

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