EFFECT OF SINUSOIDAL MAGNETIC FIELD ON K562 AND HYBRID CELL LINES

A dissertation submitted in partial fulfilment of the requirements for the degree of Master of Technology

By

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ABSTRACT

Effect of low frequency (<500 Hz) weak magnetic fields (<1 mT) on the growth of Hybridoma and non secretory cells in culture were studied.

The effect on the growth of a non-secretory cell line was essentially the same (with some qualitative difference) as on that of the the secretory cells. Prolonged plateau phase in the growth cycle of exposed population (as compared to the control) indicates longer survival of cells in this group.

Ultrastructure of exposed secretory cell line have shown more active form of mitochondria as compared to control population.

Protein in the culture supernatants in exposed population showed some qualitative alteration suggesting, possibly, some effect(s) of low frequency weak magnetic field on the secretory function of the exposed cells compared to the control.

DISSERTATION APPROVAL SHEET

Dissertation entitled **EFFECT OF SINUSOIDAL MAGNETIC FIELD ON K562 AND HYBRID CELL LINES**, by Subrata Biswas is approved for degree of Master of Technology in Biomedical Engineering.

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CONTENTS

CHAPTER 1

INTRODUCTION

1.1 AN OVERVIEW

It has always been fascinating to study the possible results of interaction of electricity and living organisms since the first awareness of electrical phenomenon. Since then the volume of research activities in this field has been increasing rapidly. Interest in the biological and medical effects of low frequency (0-400 Hz) electric and magnetic field (MF) is increasing because of their roles in healing of fractured bone [1] and skin of experimental animals [2, 3 & 4], growth and re-generation of damaged nerve [5] increase in permeation of natural membranes (electroporation) [6], fusion of two cells' membranes (electrofusion) in forming hybrid cell [7 & 8] and different types of induced effects, on cell metabolism [9]. Most of the research activities in this field involved manipulation of important biological processes by electric and MF and measuring its responses in cells in vitro, that is, outside living body condition.

1.2 PROBLEMS ASSOCIATED WITH HYBRID ANIMAL CELL CULTURES

There are quite a few major problems which are associated with current animal cell technology involved production of valuable immuno-secretory products, like polyclonal and/or monoclonal antibodies. One of the major problems is the low level of antibodies secreted and the

frequent loss of this function in in-vitro culture [10, 11]. Magneto-electro-chemical treatment of such poorly secreting cell lines, may prove useful in (i) preventing loss of genes which code for immunoglobulin chains, (ii) in stopping malfunctioning of regulatory genes and, (iii) in correcting the defects in the synthetic and secretory machinery of the cells, thereby enhancing either the rate of secretion of cells or the life-span of the secretory cells in culture.

Quite a few different types of approaches have been undertaken to study the positive effects of static and dynamic electric and magnetic fields on enhanced DNA and RNA synthesis and other cellular changes in different types of cells e.g. human fibroblast, neuronal system, etc. [12, 13, 14, 15, 16].

1.3 OBJECTIVE

This study consists of preliminary screening experiment, conducted to identify the parameters of sinusoidal magnetic field (MF) which have some stimulatory effects on growth and secretion of Hybridoma cell lines in culture.

In order to achieve this objective, cell lines cultured inside a magnetic coil excited by a current for setting up the appropriate MF. The culture was harvested at definite intervals of time and biological responses were screened by various analytical methods.

1.4 OUT LINE OF THE DISSERTATION

Second chapter deals with a review of responses to electric and MF exposure, Chapter 3 describes the selection

of different cell lines for our study. Chapter 4 describes the material and method used for our experiments. Chapter 5 presents the analysis of experimental results and discussions. Conclusion from experimental results are given in Chapter 6. Detail on magnetic coil, coil driving amplifier, some experimental method and experimental results tables are given in appendices.

CHAPTER 2

CELL RESPONSES TO ELECTRIC AND MAGNETIC FIELD EXPOSURE

2.1 INTRODUCTION

Depending on the dose of electric field (EF) and magnetic field (MF), various effects - spanning from the killing of cells to stimulation of metabolism, have been detected. Effect has been studied for static as well as time varying fields and reported in the literature. Time varying EF and MF are coupled, therefore reporting the effect of EF or MF is purely from the point of view of mode of application of the field, that is whether in a particular experiment EF or MF was the one primarily controlled. In this chapter such well known magnetic and electric ıeld influences on different cells will be briefly discussed.

2.2 EFFECT OF MAGNETIC FIELD ON BIOLOGICAL SYSTEMS

Most of the MF parameters influence cell membranes, nerve growth and cell metabolisms. A brief summary of MF influences on cell, organ, etc. is given in Table 2.1. This clearly shows that low frequency (0-400 Hz) excitation (sinusoid and rectangular) have some definite influences on various cellular activities like growth, DNA and RNA synthesis, membrane permeability, etc.

2.2.1 Effect on DNA Synthesis

In culture, human fibroblasts (active cells, which produce white fibrous tissues under normal and pathological conditions, and are also responsible for formation of

amorphous ground substance) have exhibited enhanced DNA synthesis when exposed to sinusoidally varying magnetic fields for a wide range of frequencies (15 Hz to 4 kHz) and magnetic fields (2.3μT to 0.56 mT) [13]. Two independent series of experiments were carried out, both with human embryonic fore skin fibroblast in cell culture condition, and in both the experiments fibroblast cells were exposed to MF, produced by Helmholtz coils (0.5 m inside diameter, 0.25 m long), which provided a horizontal magnetic field with an active field uniformity to less than 3 parts out of 17 where either could be an experimental or control (no field) unit.

In the first series, cells seeded in 96 - well culture plates (at densities of approximately 1.0×10^6 cells/ml) were exposed to a frequency of 76 Hz and intensity of 0.16 mTrms for duration of 24, 48, 72 and 96 hours. At the end of magnetic field exposure for varying periods of time, radio active Thymidine [3H], nucleic acid base, was added to each well of the culture plate. The cells were incubated for 18 hours for thymidine incorporation in the newly synthesized DNA by the principle of isotope exchange. This study has shown higher amounts of incorporated thymidine [3H] in cells exposed to MF as compared to the control (See Fig.2.1).

In the second set of experiments, radio active thymidine [3H] was added to the culture media seeded with cells (5×10^4 cells/ml) at the begining of the experiment and then exposed to various frequencies (between 15 Hz and 4 kHz) and intensities (between 1.6 μTrms and 0.4 mTrms) for a limited period of 24 hours and radioactive thymidine incorporation

was studied at 18, 20, 22 and 24 hrs post exposure. The peak uptake of 3H-Thymidine were found to be high in all experiments, corresponding to the middle of the 'S' phase (specific part of interphase during which DNA synthesis occurs) of the cell cycle (Fig.2.2).

To evaluate whether induced electric field 'E' (extracellular and intracellular currents) have any influence on DNA synthesis, relative Thymidine uptake of two separate exposure periods of 20 & 24 hrs was plotted against product of frequency (ν) and root mean square of field strength (Brms) (since, ∇ x E (induced electric field) = - ∂ B/ ∂ t & ∂ B/ ∂ t = 2 π ν B) (Fig.2.3). The maximum effect of magnetic field at 'S' phase were found to be independent of the time derivative of the magnetic field suggesting thereby, an underlying mechanism other than Faradays law. From this study it seems there is a relation, albeit a weak one, between time-varying magnetic field (threshold: 0.5×10^{-5} to 2.5×10^{-5} T/sec) and DNA synthesis.

2.2.2 Effect on RNA Synthesis

Pulsed electromagnetic field (PEMF) has been reported to induce cellular transcription [12 & 14]. Such cellular responses are dependent on pulse characteristics i.e. the repetitive single pulse and repetitive pulse train (Fig.2.4). In this study, Sciara coprophila salivary gland (in which cells are in permanent interphase, thereby simplifying the detection of cellular changes) was dissected in Schneider's Drosophila medium containing [3H] uridine. The tissue in the medium was exposed to two said PEMF for various periods (5 to

90 minutes). Transcription of cells, exposed to PEMF was monitored with tritiated uridine using transcription autoradiography, cytological nick translation, and analysis of isolated RNA fractions (Fig.2.5). The investigations clearly showed, varying qualitative and quantitative responses in the patterns of RNA synthesis depending on different pulse characteristics. The single pulse increased the specific activity of messenger RNA after 15 and 45 minutes of exposure, whereas pulse train increased specific activity only after 45 minutes of exposure indicating signal specific changes in the cells.

2.2.3 Fracture Healing by Magnetic Stimulation

The use of MF as a non-invasive technique to resolve delayed unions and non-unions is now widespread and clinically established technique with success rate of 80% [15 & 16].

Different authors have used different function or pulse shape and MF (Fig.2.6). Generally the magnetic stimulator used for this purpose consists of a pair of Helmohltz coils, aligned across the wound site and is activated with monophasic 150 ms phase with repetition rate of 75 Hz (Fig.2.7). But two aspect of this technique are not clear ;

i) The mechanism of action of a MF in inducing tissue regeneration in a bone gap, where natural bone growth was absent before the application of the magnetic field.

ii) As mentioned above, MF pulses have been reported to give a success rate up to 80%. However there is no known

explanation for MF failures. Also unknown is the optimal geometry of magnetic field pulses to achieve the best efficiency with this non invasive technique.

Most of the research in this field has been focused mainly on the action of MF on biological aspects, cellular physiology [13] on vascular system [3], etc. Recently some attempts were made, to explain continuous MF technique for bone healing from the point of view of inorganic deposits i.e. calcium salt crystal formation [16]. The action of MF on calcium crystal formation explained by an increase in the crystalline perfection and density of the population of calcium crystal seeds, which have remarkable influence on the calcium deposition process in the intracellular medium. So MF control mineral bone formation at seeds on a proteineic micro-network. Theory may explain many other experimental findings. It was observed that MF treated bone were significantly more rigid than control (Fig.2.8). This can be explained if it is accepted that the MF increases the population density of crystal seeds, yields a finer calcium phosphate grain, having consequently better mechanical properties.

Similarly in another investigation on influence of MF on ligament fibroblast, it was observed that fibroblasts proliferation enhanced if the frequency were tuned to the "calcium crossing cell membranes resonant frequency" [16].

2.2.4 Wound Healing by Magnetic Stimulation

Sinusoidal MF of very low frequency (72 to 500 Hz) have been reported to have some influence on skin flaps (skin flap

model is generally accepted for the investigation on wound healing) [3]. In experimental set-up [3], dorsal skin flaps (2 cm x 6 cm) of Sprague Dawley rats were treated with sinusoidal electromagnetic field of 20 Hz/3.6 mT; 72 Hz/1.1 mT and 500 Hz/0.16 mT and area of necrosis was measured with MOP-3 digitizer on 8th day of the treatment. The result show on average up to 14.5% less necrosis in treated animals compared to match controls. This corresponds to decrease in the amount of necrosis by 38% in the group treated with 72 Hz signal. No simple relationship between results of the treatment and frequency/magnetic flux density could be derived by the author.

This indicates a clear effect of treatment with sinusoidal MF (of low frequencies) on skin flap survival and hence possibly on wound healing as such.

2.3 EFFECT OF ELECTRIC FIELD ON BIOLOGICAL SYSTEMS

Most of the electric field (EF) parameters influence cell membranes, nerve growth and regeneration, wound healing and rapid healing of fractured bone. In this section the literature, on effect of EF on biological system will be briefly reviewed.

2.3.1 Fractured Bone Healing by Electric Field Stimulation

Many investigations have demonstrated that, application of electrical energy by means of direct or alternating current can enhance and stimulate osteogenic activities [1, 15, 16]. Some of the commercially available electrical stimulators are shown in Fig.2.9a & b. Although exact

mechanisms of the stimulation are not yet fully understood. The electrical stimulation may be related to the highly electronegative nature of the fracture site due to increase in ionic and metabolic activities. The extra electrical energy input to the wound area seems to signal more osteogenic activities. One investigation [17] demonstrated that the extent of healing due to electrical stimulation is also related to the total energy consumed. One might correlate the improved healing effects with the altered amounts of materials, such as calcium ions consumed during healing and more importantly with enhanced amounts of matrix material (protein and complex carbohydrate) elaborated under the MF effects.

There are three main forms of electrical therapy in medical use - invasive, semi-invasive, and non-invasive. The invasive and semi-invasive modalities use electrodes directly into the defect site and connected to a power source, usually delivering 10-20 μA DC [1]. Typical success rates claimed varies between 70% to 90% [1].

2.3.2 Wound Healing by Electrical Field Stimulation

Promotion of wound healing, is a topic that has received considerable attention [2 and 3] and a comprehensive review is beyond the scope of this report. However, the role of epidermally generated electrical potentials in the healing of vertebrate skin, is both feasible and worthwhile to discuss. There are indications that electrical potentials can promote the healing of wounds, although the rationale as to why there

should be an electrical component to wound healing is only in the earliest stage of appreciation. Existence of wound current has been recognized 200 years ago and, recently 10-30 μA/cm2 current densities were measured at the stump surface of children's fingers whose tips had been accidentally amputated [2].

When a wound is made in the skin, an electrical leak is produced that short-circuits the skin-transepithelial (TEP) membrane potential (Fig.2.10) allowing current to flow out as long as the wound is not healed. At the wound, the potential drop from outside the skin to inside is relatively low. To either side of the wound, as the distance from the wound increases, the potential across the skin is found to be progressively greater and greater, until a point is reached a few millimeters from the wound where normal value is found. If the wound is allowed to heal, the wound resistance increases and blocks the flow of current so that there is no lateral potential drop at the edge of the wound. So it is clear that there is a field in the epidermis (100 mV/m for mammal; 50 mV/mm for amphibian) close to the edge of the wound. It is important to consider whether the electrical field of such magnitude can affect the behavior of cell in any way. It was observed in vitro that application of 100-150 mV/mm field to embryonic quailsomite fibroblast showed directed movements. Similar migration (Galvanotaxi) of leukocytes and macrophages was observed [2]. Imposed field (50 μA DC & 20 μA AC) in vivo also reported to increase capillary permeability to fibroblast, macrophage and

leukocytes. Beneficial effects on wound healing, may be due to migration of these cells to cover the wound and may also be due to increased secretion from fibroblast cells [13].

2.3.3 Nerve Repair and Regeneration and Development

Elongating nerve fibers are similar to an elongating pollen tubes, sperm acrosomes or germinating eggs of the brown algae. All these different varieties of cells share a common physiological expression. In all these apically elongating cells, a steady endogenous ionic current (Fig.2.11) enters the growing tip and this current is a cause and not consequence of growth [18]. This current is associated with redistribution of cellular macromolecules and hence with growth, regeneration and development.

It is now well established that an applied electric field comparable to endogenous ionic current profoundly affects the development and regeneration of nervous tissue [4]. Such neuronal responses include an increase in the rate of growth of fibers facing the cathode resorption of newly formed neurites by the cell body when such fibers face the anode, and inhibition of axonal resorption in fibers facing the cathode, increase in outgrowth from cultured ganglia, an increase in the rate of growth in fibers that turn toward the cathode etc.

2.3.4 Cell Membrane Responses

Some of the electric field parameters influence cell membranes in such a way that theoretical explanations can be given [7]. The discovery of the phenomenon of reversible

break down of cell membrane in response to electric pulse of high intensity and short duration paved the way for the development of new tools in bio-processing and genetic engineering. Depending on the chosen electrical parameter applied on membrane, its permeability to low and high molecular weight molecules could be altered (electroinjection) or two adjacent cell membranes may be fused to give hybridized cell (electrofusion).

Electro injection : Mouse L cells and Macrophages have been reported to be successfully electropermeabilized with a single field pulse of 5 kV/cm strength and 5 μs duration of exposure to electroinject DNA molecule in it [7].

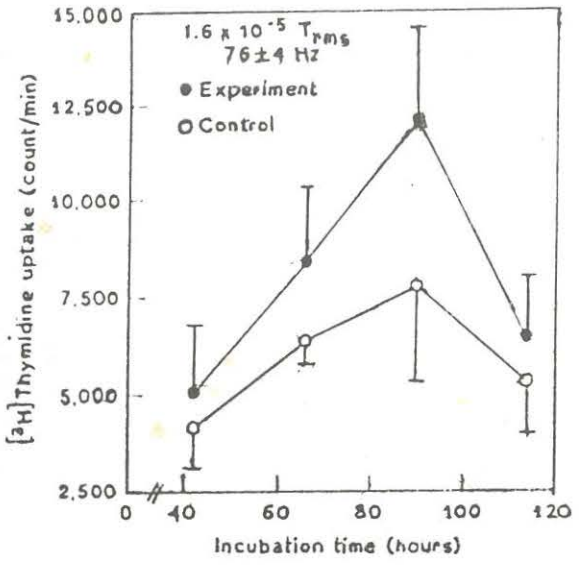
Electrofusion : Mouse myeloma cells were successfully fused with activated B cell to form secretory Hybridoma cells using Biojet CF [7]. The schematic diagram of the sequence of alignment and fusion pulses application is shown in Fig.2.12. In brief the process involved,

i) Cells alignment in an alternating heterogeneous field with frequency range between 100 kHz and 10 MHz, maximum peak amplitude 15 V (between 100 kHz and 5 MHz), 12 V (between 5 and 8 MHz) 10 V (above 8 MHz) maximum duration of 1000 s.

ii) Fusion (reversible break down pulse) of cells was done by applying strength from 1 to 400 V, for 5 to 100 μs duration, 1 to 9 pulses, (time interval between consecutive pulses are 0.1 to 999s) and alignment of time (A0T) 10 to 999 ms.

Table 2.1 : Summary of MF parameters studied by various investigators which have shown some effect on different types of cells and organisms.

MF parameters	Response of cell/organisms
1. 0-3.5 mT; rectangular pulse, 4.5 KHz, 26 Hz repetition rate.	Mutagenic effect on *Drosophila*, M.[14]
2. 0.12-12 μT; 10-1000 Hz	Abnormal development in chick embryo.[13]
3. 2.3 μT to 0.56 mT,; 76 Hz to 4 kHz.	Enhance DNA synthesis in fibroblast.[13]
4. 3.6 mT/20 Hz; 1.1 mT/72 Hz; 0.16 mT/500 Hz Sinusoidal.	Increased wound healing. [18]



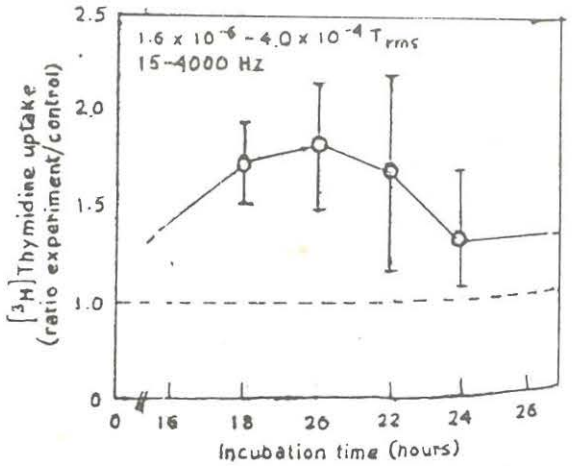


Fig. 21 (left). Tritiated thymidine uptake (mean counts per minute ± standard error) in human foreskin fibroblastic cells exposed to (magnetic field of amplitude 2.3×10^{-5} T oscillating at 76 ± 4 Hz in the horizontal plane (experiment) and uptake in unexposed cells (control). Mean levels differ at $P = 0.0001$, as determined from the pooled variances. The ambient 60-Hz magnetic field in the area of the incubators was $\leq 1 \times 10^{-7}$ T_{rms}. Note that the approximate intensity of the geomagnetic field is 5×10^{-5} T. Fig. 22 (right). Mean ratios and standard errors of [³H]thymidine uptake in exposed cells to that in control cells for ten different combinations of frequencies and fields. The dotted line indicates the expected response if there is no effect due to a magnetic field. The peak at 20 hours corresponds to the middle of the S phase of the cell cycle. The dashed lines below 18 and above 24 hours represent the expected, but inexact trends, in these data [13]

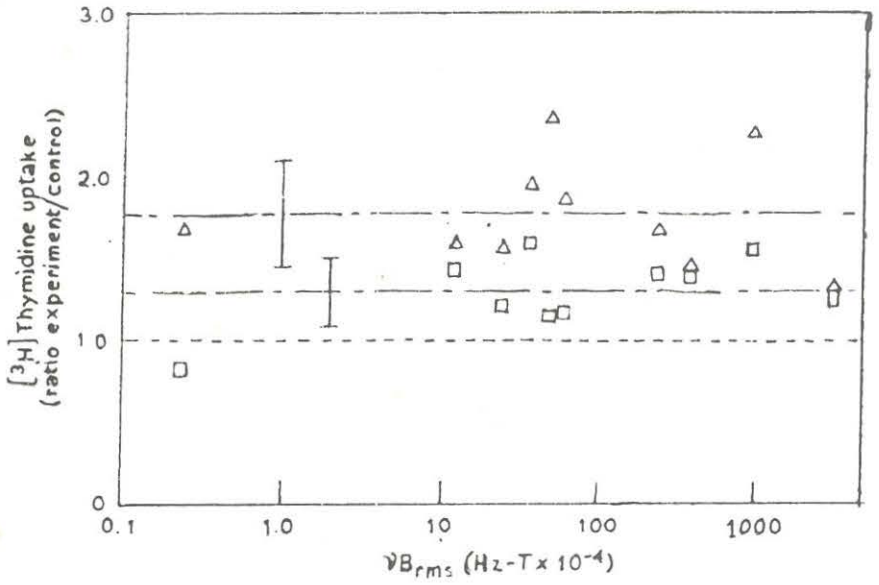


Fig. 2.3 The mean ratios (from Fig. 2.2) as a function of the product of frequency and magnetic field intensity. Exposure times of 20 hours (Δ) and 24 hours (□) are shown. Note the apparent lack of variation in response for four orders of magnitude, indicating that this interaction is independent of the time derivative of magnetic field, $\partial B/\partial t$. The two upper lines represent the mean levels for the 20- and 24-hour exposures, respectively. [13]

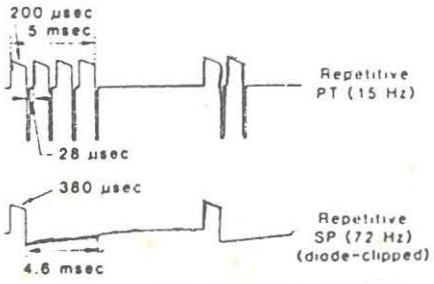
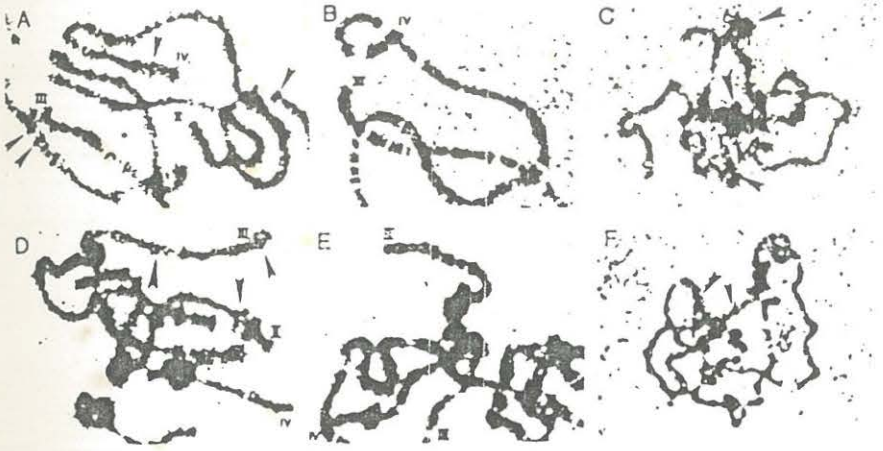


Fig 2.4 Major waveform characteristics of the SP and PT stimuli (Biosteogen system 204, Electro-Biology, Inc.). Pulse amplitude, on a calibrated coil probe of 15 mV (coupled to a Tectronix 5103N oscilloscope), was equal to 1.5 mV per centimeter of cortical bone. The glands were exposed to PEMF's in 0.5 ml of Schneider's *Drosophila* medium in petri dishes (60 by 15 mm) between a pair of 10 by 10 cm Helmholtz aiding coils, delivering an average magnetic field parallel to the floor of the dish. Coil orientation was vertical. The rate of change of the magnetic field (dB/dt) was approximately 0.1 G/µsec for PT's and 0.05 G/µsec for SP's. Frequency content of the two pulses, derived by discrete Fourier transforms, differs significantly [14]



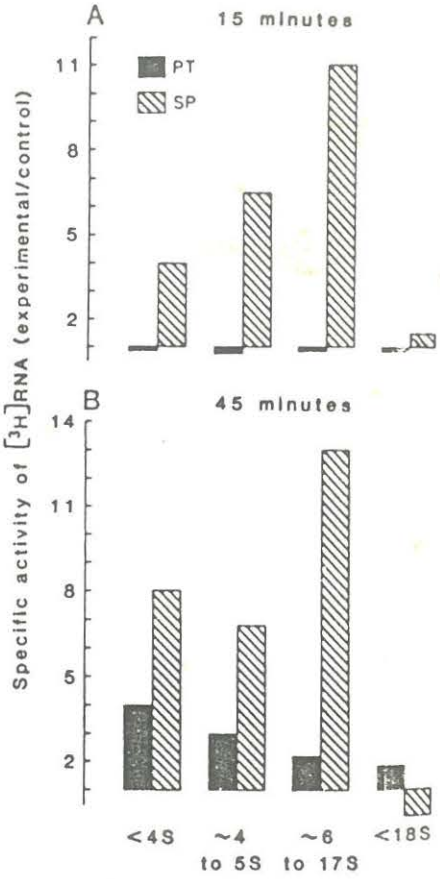


Fig. 2.5 (left). (A) Transcription autoradiogram of salivary gland chromosomes from cells incubated in the presence of SP's and [^{3}H]uridine for 45 minutes. Labeling is heavy and specific on chromosomal bands and interbands (arrowheads). Roman numerals denote chromosome numbers. Exposure time, 3 days. (B) Transcription autoradiogram of control chromosomes from salivary glands incubated in [^{3}H]uridine in the absence of SP's. The relatively few grains are distributed in a random pattern. Exposure time, 3 days. (C) Nick-translated cytological preparation of chromosomes from salivary glands stimulated with SP's for 45 minutes. DNA sites more heavily nicked by deoxyribonuclease I were labeled with [^{125}I]deoxycytidine triphosphate after repair with DNA polymerase and prepared for autoradiography. Arrowheads indicate transcriptively active regions ("hot spots"). Exposure time, 18 hours. (D) Transcription autoradiogram of salivary gland chromosomes from cells incubated in the presence of PT's and [^{3}H]uridine for 45 minutes. The general labeling pattern over the chromosomal bands and interbands is less intense and less specific than the pattern depicted in (A). Specific deviations are indicated by arrowheads. Exposure time, 3 days. (E) Transcription autoradiogram of control chromosomes from salivary glands incubated in [^{3}H]uridine in the absence of PT's. Exposure time, 3 days. (F) Nick-translated cytological preparation of chromosomes from glands stimulated with PT's for 45 minutes. DNA was labeled with [^{125}I]deoxycytidine triphosphate and prepared for autoradiography. Hot spots are denoted by arrowheads. Exposure time, 18 hours. (All magnifications ×1240). Fig. (right). Tritiated RNA isolated after exposure to SP's or PT's [illegible]. Approximately 100 salivary glands (2×10^3 cells) were used for each set of time points (four experiments each). Gradients of 5 to 30 percent sucrose were run for 17 hours at 32,000 rev/min in an IEC ultracentrifuge. The gradients were collected with an automatic fraction collector to monitor optical density. A portion was removed from each fraction and its radioactive pattern was determined. The only major difference in the radioactive pattern (compared to the profile of optical density) consisted of highly radioactive fractions of less than 4*S*, probably reflecting partially transcribed RNA. Fractions of each size class were combined on the basis of these profiles and specific activity was determined and compared to RNA of unexposed cells run in parallel gradients. *Analysis of SP's*: Specific activity of RNA in experimental samples, 10,000 to 15,000 dpm/μg. Specific activity of RNA in controls, 2000 to 4000 dpm/μg. Approximately 50 μg of the experimental and control samples was used for each gradient. Size classes greater than 18*S* were combined since relative incorporation was not significant when compared to controls. *Analysis of PT's*: Specific activity of RNA in experimental samples, 8000 to 10,000 dpm/μg. Specific activity of RNA in controls, 2000 to 4000 dpm/μg. About 50 μg of the experimental and control samples was used for each gradient. [14]

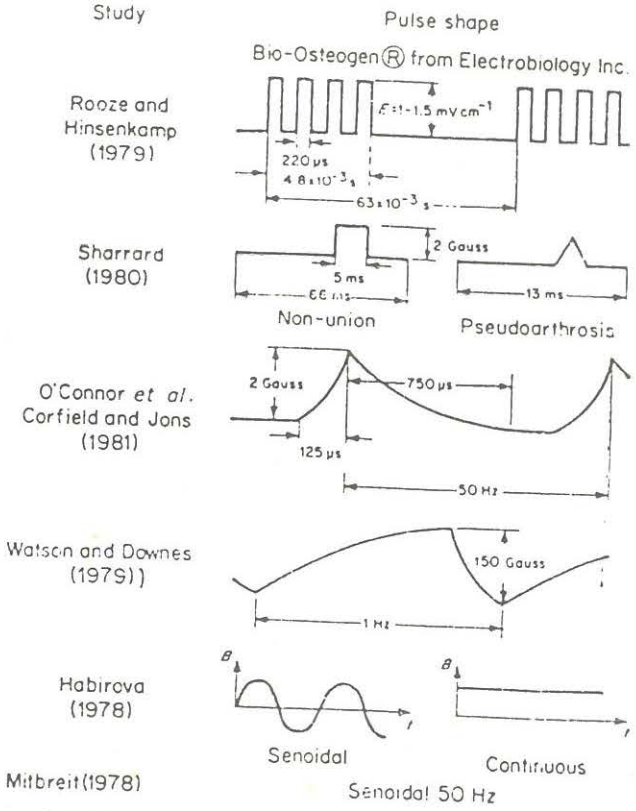


Fig. 2.6 Morphology of magnetic field pulses used in equipment for PEMF treatments [16]

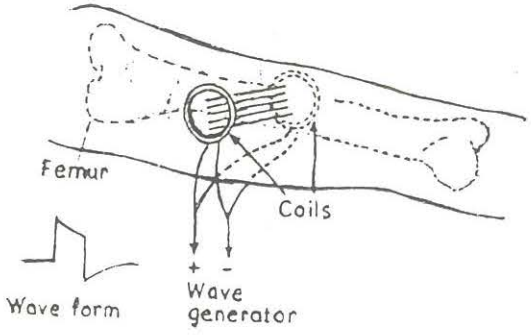


Fig. 2.7 Representation of pulsed electromagnetic field bone stimulation.[15]

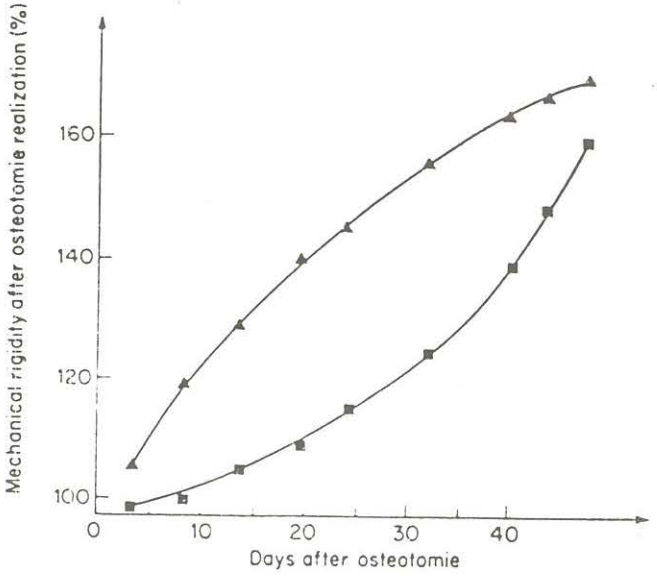


Fig 2.8 Effect of PEMF on the evolution of mechanical rigidity after an osteotomic realization. ■, control osteotomies; ▲, PEMF stimulated. From reference [16]

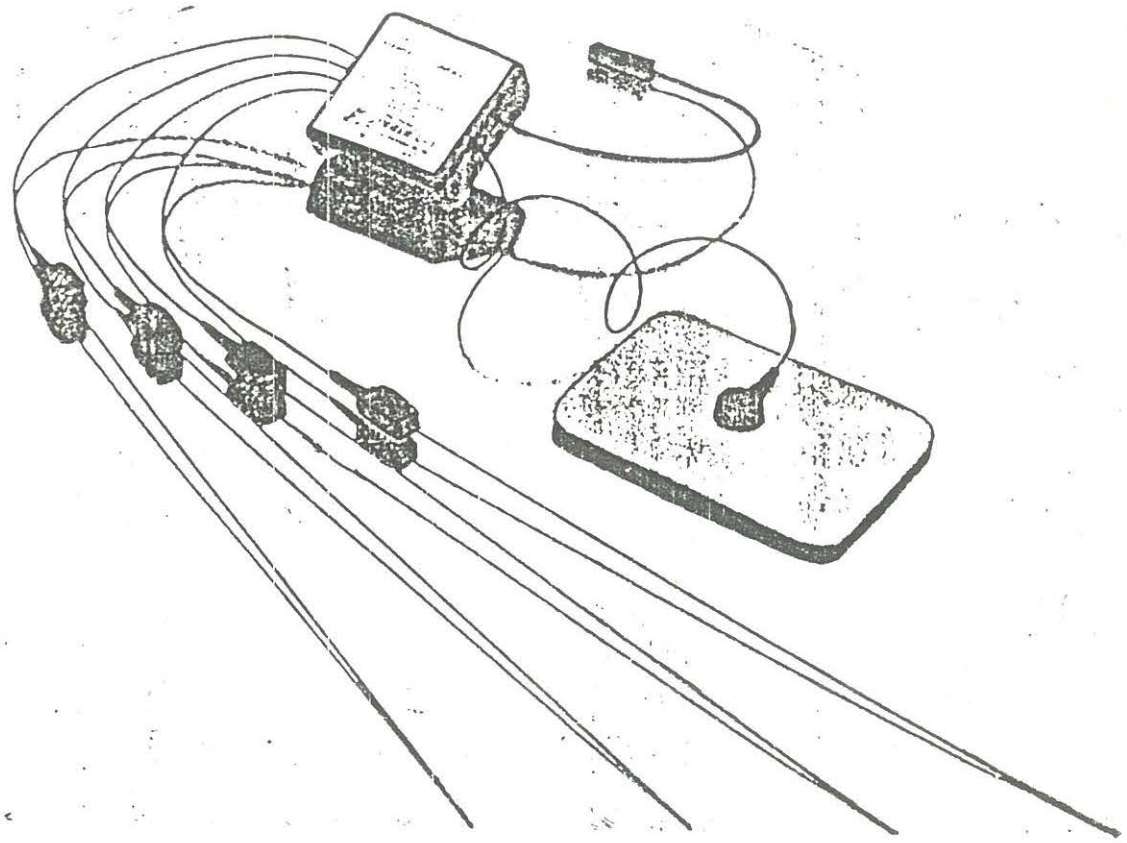


Fig. 2.9 a. A commercial electrical stimulator for bone fracture repair. [15]

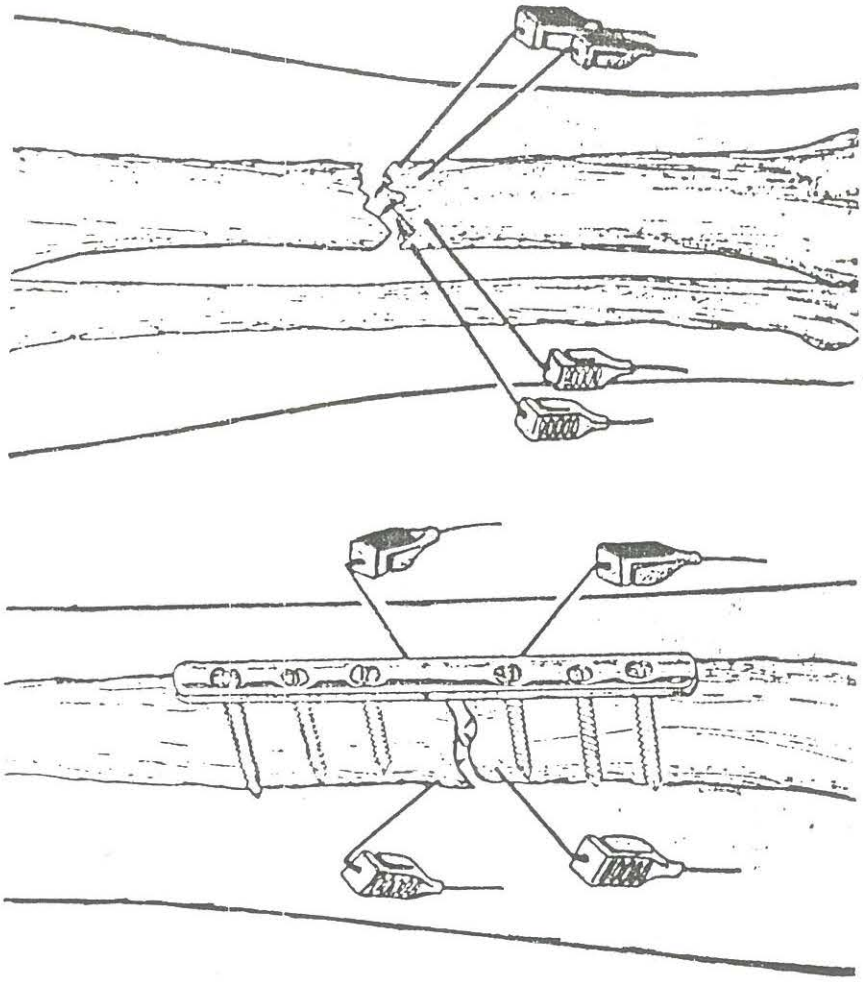


Fig. 2.9 b Schematic illustration of the use of an electrical stimulator with or without fracture fixation devices [15]

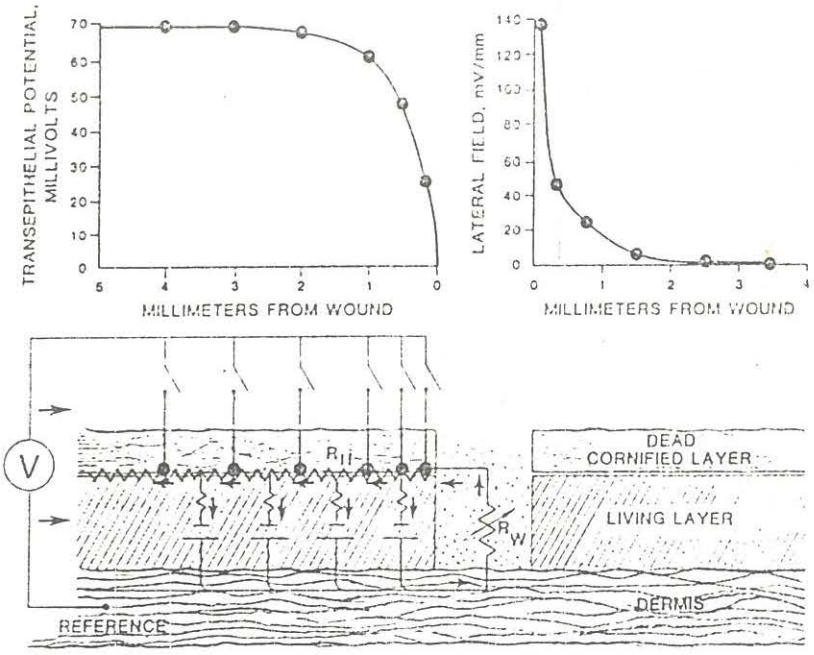


Fig. 2.10 Lateral potential in the vicinity of a wound made in mammalian skin. Left: schematic illustration of the circuit diagram of the skin, the electrodes for measuring transepithelial potentials in the vicinity of the wound, and (above), representative data obtained from such determinations of transepithelial potentials. (For actual data and more detailed information, see Barker et al., 1982. $R_{||}$, resistance of the return path of the current to the epidermal battery, along which the lateral potential drop occurs near the edge of the wound; R_W, resistance of the wound. When R_W is low, as when the wound is kept moist, current driven by the epidermal TEP flows out the wound and returns to the battery via $R_{||}$. When R_W is high, little or no current flows in the circuit, and little or no lateral field exists at the edge of the wound. Right, below: diagrammatic representation of the relevant layers of mammalian skin corresponding to the circuit on the left, above: the lateral field in the vicinity of the wound, plotted as a function of distance from the wound edge (based on the hypothetical data plotted on the left) [2]

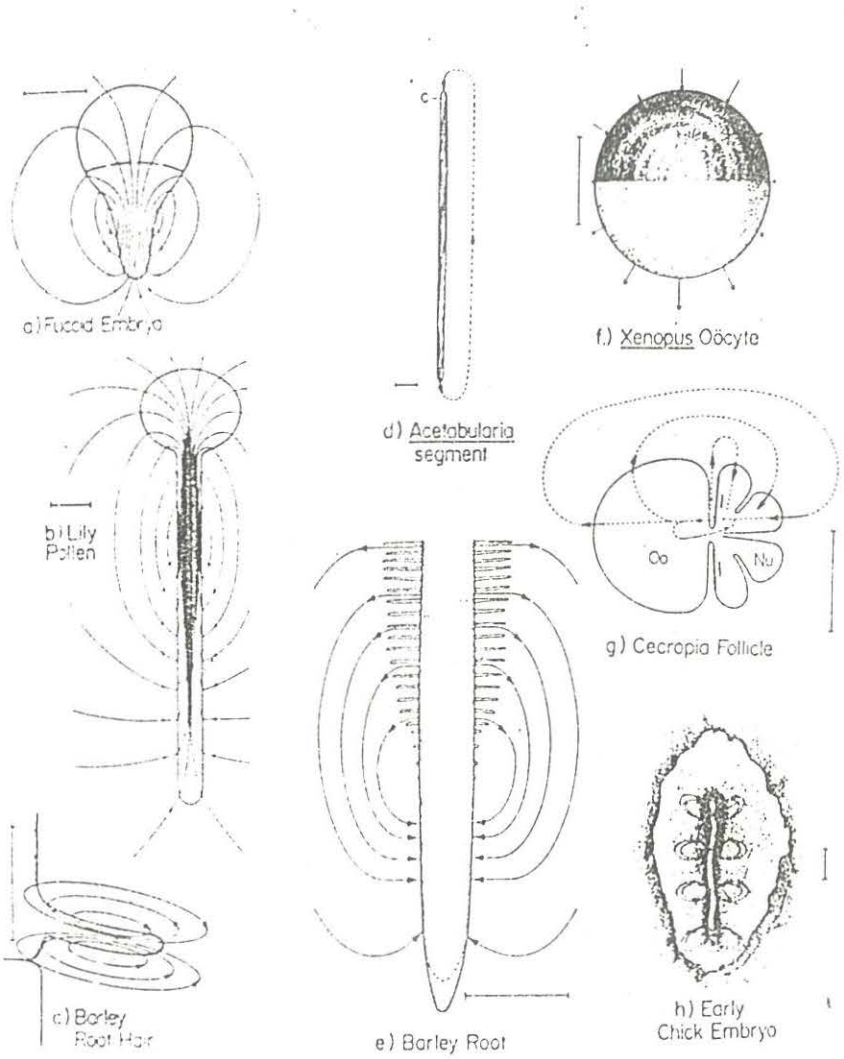
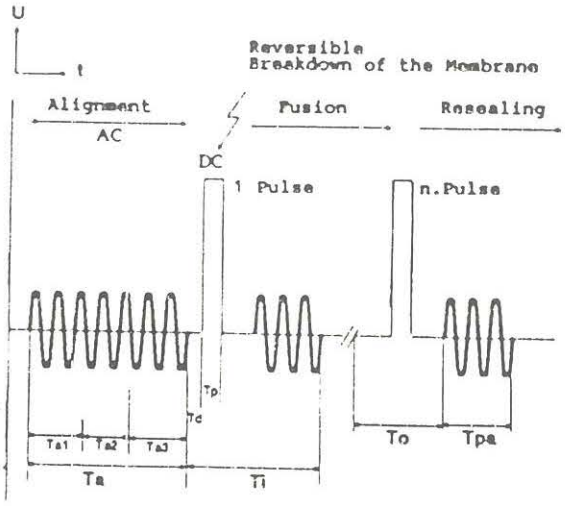


Fig 2.11 Developmental currents in animals and plants. a–e: Currents associated with five apically growing plant cell systems. Current (peaking on the order of 1 $\mu A/cm^2$) enters the growing end in a, b, and c. The scale bar = 0.05 mm. For d and e, the scale bar = 1 mm. In d, the arrows indicate the direction of 100-second-long pulses of current entering and indicating the end of the anucleate stalk segment of *Acetabularia* that will eventually regenerate a new cap structure. These pulses of current have peak densities on the order of 10–1000 $\mu A/cm^2$. In e, current enters the elongating region of the barley root; densities of current are about 1 $\mu A/cm^2$. f–h: Three developing animal systems. Current enters the animal pole of the *Xenopus* oocyte (about 1 $\mu A/cm^2$ in density) and leaves the vegetal pole in about the same density. Current enters the nurse cell end of the (Nu) Cecropia oocyte-nurse cell complex. This transcellular current traverses the narrow cytoplasmic bridge coupling the two compartments and leaves the oocyte (Oö). Current is somewhat concentrated at its entry into the nurse cell; densities can reach 20 $\mu A/cm^2$. An endogenous current (about 30 $\mu A/cm^2$) leaves the chick



Ta = Alignment Time
Ta 1,2,3 = Ramp-Function
Td = DC-Pulse Delay
Ti = Interval Time
To = AC-Off-Time
Tpa = Post-Alignment Time
T_p = Pulse Duration

Fig. 2.12 Schematic diagram of the sequence of alignment and fusion pulse(s) application. [7]

CHAPTER 3

SELECTION OF CELL LINES FOR THE STUDY

3.1 INTRODUCTION

Effect of magnetic field (MF) was studied on three different cell lines. One of them was non secretory (K562) and rest were hybrid cell lines (OKT3 and AFP) secreting monoclonal antibodies (MAb's). Study was carried out on these cell lines as they were easily available and their secretory products had application in clinical and diagnostic tests in cancer which is described in the subsequent section.

3.2 K562 CELL LINE

K562 is a multipotential erythroleukemic cell of human origin (Fig.3.1). This cell was established in 1975 [19]. This cell is usually seen in the bone marrow and peripheral blood in chronic myelogenous leukemia (CML). It has myeloid characteristics which is evident from the presence of myelogenous leukemia antigen, group specific granulocytic antigens and foetal antigens [19]. K562 blasts, in a stationary phase of growth, undergo spontaneous differentiation.

3.3 OKT3 CELL LINE

OKT3 cell line, (Fig.3.2) is an anti human T-cell hybridoma.

This hybrid cell is formed by fusion of P3X63 Ag 8 Ul mouse myeloma cells with splenocyte from CAF mice immunized with human peripheral T-lymphocyte MAb (IgG2a) reactive to human T-lymphocytes (T3Ag) [20].

3.4 AFP CELL LINE

AFP clone (Fig.3.3) is a hybrid cell line which secretes monoclonal antibody against human alpha foeto protein (AFP).This hybrid cell is formed by fusion of sp 2/0-Ag 14 myeloma cells with splenocytes from BALB/C mice immunized with partially purified AFP [21].

AFP level in serum is reported to be associated with different types of malignancies e.g. liver cancer, gastrointestinal malignancy etc. [21]. It is also a good post operative indicator of metastasis (secondary growth originating from the primary tumor growing elsewhere in the body) [21]. As AFP is a good indicator of tumor cell activity in vivo, MAb against AFP which is a secretory product of AFP clone, is an important reagent for quantitation of AFP and hence tumor cell activity.

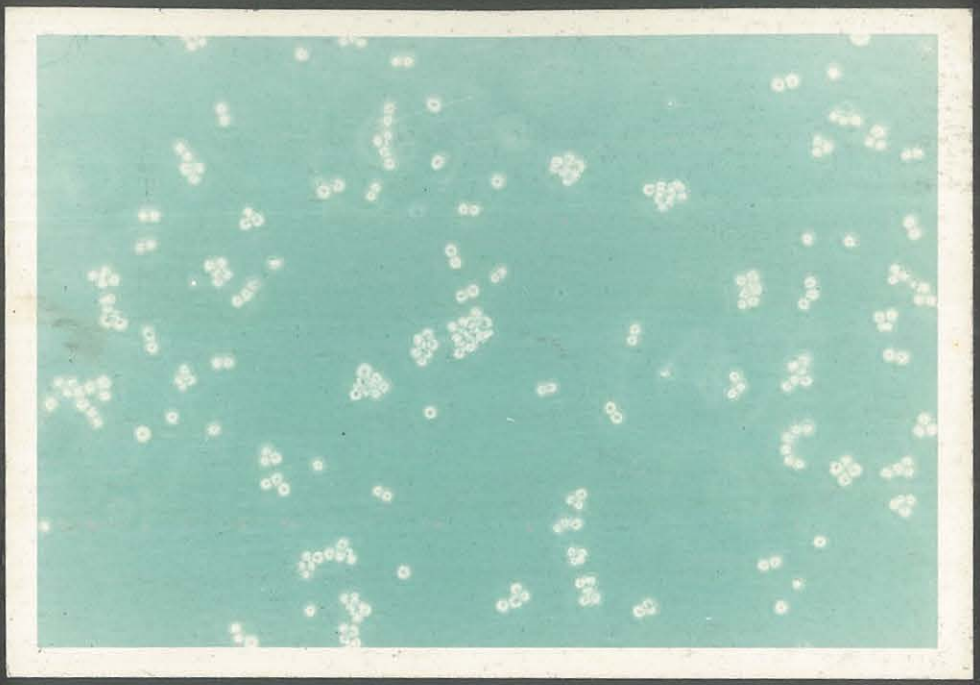


Fig. 3.1 K562 is a multipotential erythro leukemic cell line of human origin. This cell is detectable in the bone marrow and peripheral blood in chronic myelogenous leukemia (CML).

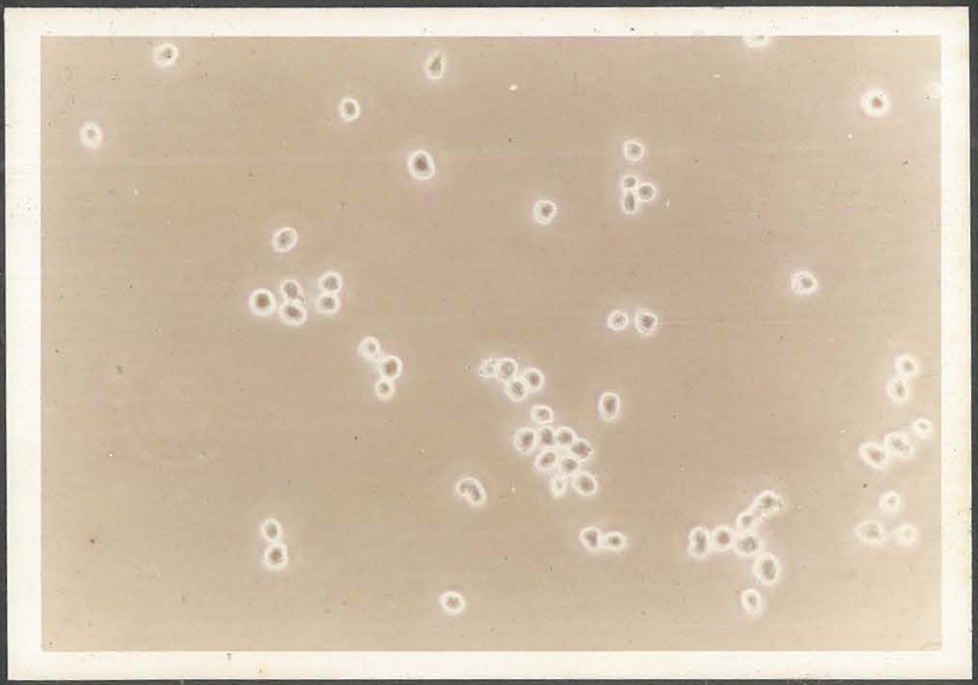


Fig. 3.2 OKT3 hybridoma. It is an anti human T cell hybridoma.

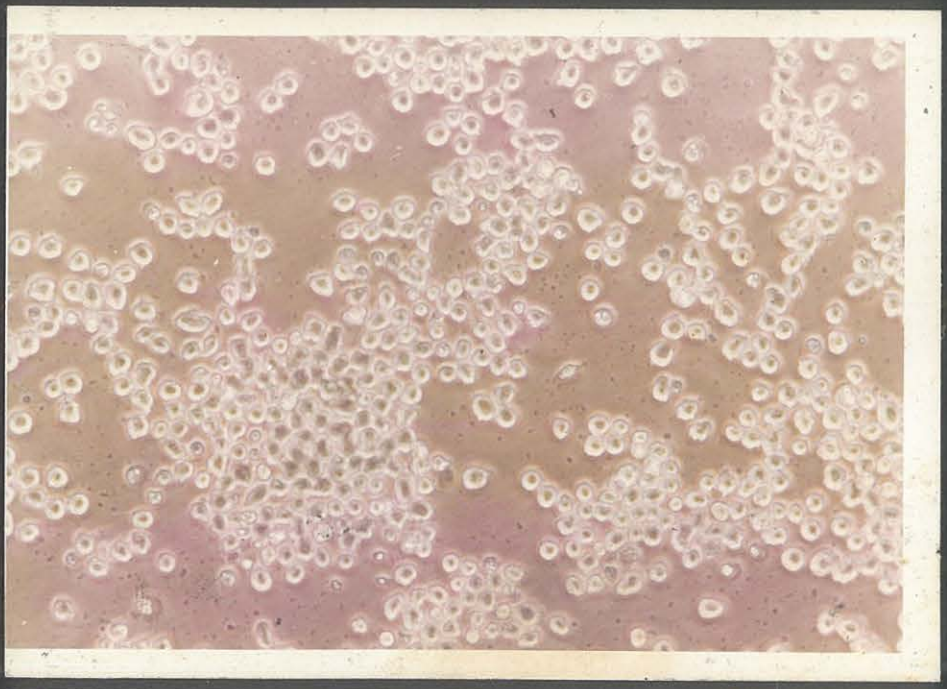


Fig. 3.3 AFP clones. This cell line secretes monoclonal antibody against human alphafoeto protein.

CHAPTER 4

EXPERIMENTAL MATERIALS AND METHODS

4.1 INTRODUCTION

This study was undertaken to determine the effect(s) of sinusoidal magnetic field (MF) on growth and secretory status of the cell lines in culture, as already discussed in Chapter 3. For this purpose cell lines were cultured inside a magnetic coil, excited by a current for setting up the appropriate magnetic field. The culture was harvested at definite intervals of time and biological responses were screened by analytical steps shwon in Fig.4.1 e.g. viable cell count, elctron microscopy, etc.

4.2 EXPERIMENTAL SET-UP FOR MF EXPOSURE

A schematic of the experimental set-up for exposing the cell-lines in culture to sinusoidal MF is shown in Fig.4.2. The main equipment of the set-up comprised of a coil, current amplifier, function generator (FC100,L & T Gould), dual DC power supply (LAVD15/1, Aplab) and the incubator (MBE4017, Metzer).

The current was powered by applying +15 and -15 V from a dual DC power supply. Input signals were given to the amplifier using a function generator. The output of the amplifier was connected to the coil as a floating load. Output current, frequency and voltage were continuously monitored by a digital multimeter in current mode and an oscilloscope (Systronic).

4.2.1 Cell Culture Vessel

Cells were grown in 96-well flat-bottom plates (Linbro, Flow Laboratories), shown in Fig.4.3 . Low density seeding ($1x10^5$ cells/ml) in this plate saves culture media.The dimensions of this plate were 126 mm x 85 mm x 18 mm, magnetic coil dimensions (inside cavity) were chosen, accordingly so that it could comfortably accommodate the plates.

4.2.2 Coil For The Generation Of Magnetic Field

An air cored coil (solenoid), as shown in Fig.4.4, was designed and fabricated. It consisted of a polyvinyl chloride (PVC) pipe of length 0.3 m and 0.136 m internal diameter. The length was so chosen that it could be comfortably accommodated inside an incubator (0.45 m x 0.45 m x 0.45 m). and similarly, its internal diameter was such that 96-well flat-bottom plate could easily fit inside it. Four layers of wires were wounded on the PVC tubing and all of them were connected in parallel so as to get an adequate magnetic field. To avoid possibility of contamination from these wires, the entire coil was encased in an acrylic box which could be easily sterilized with hot water and savlon. The design details for the coil are provided in Appendix A.

The most important design consideration was the generation of uniform horizontal MF inside the coil. The length-to-diameter ratio for the coil is approximately 2.5. The horizontal MF inside the coil was measured with and without the 96-well plate (containing tap water). The

uniformity of the field was tested by exciting the coil by 80 Hz sinusoidal currents of 97mA rms and measuring the axial field by using a guassmeter (the test method will be described later in subsection 4.2.4). The axial field strength of the coil is shown in Fig.4.5, and it is seen to be fairly constant in a segment of 0.1 m length (sufficient for exposing all cells seeded in a 96-well plate to uniform MF) around centre of the tube. At the ends, field strength declined rapidly. There was less than 6.25% variation in field strength across the diameter of the coil as compared to maximum axial MF strength.

4.2.3 Excitation Of The Coil

The coil was excited by sinusoidal waveforms from the function generator. As the signal generator produces very low current, a current boosting amplifier was used for driving the coil with adequate current so as to generate sufficient magnetic field (since MF $\propto$ current). The amplifier circuit was designed and manufactured for use in this experimental set up. The circuit detail are given in Appendix B. Special feature of this amplifier is a high output voltage swing and good efficiency. The excitation apparatus was set up on the top of the incubator itself and can be seen in Fig.4.6. Appropriate frequency and intensity was set by using the control knobs on the waveform generator.

The output voltage waveform from the amplifier to the magnetic coil was measured using a dual channel oscilloscope. The current in the coil was monitored using a multimeter used

in the mA meter mode and connected in series with the coil. Diffrent parameters chosen for exposing the cell lines to sinusoidal MF were as follows :

i) 0.6 mT and 90 Hz

ii) 0.8 mT and 45 Hz

iii) 0.8 mT and 76 Hz

iv) 1 mT and 500 Hz

All the above parameters are in the range for which studies reported in the literature [13 and 14]. All these parameters have influence on DNA and RNA synthesis of fibroblast cells in culture. Being a preliminary screening experiment, no attempt was made to find out effect of different frequency at constant field intensity or effect of different intensity at constant frequency.

4.2.4 Measurement Of Magnetic Fields

After activating the coil with proper signal, the MF inside the coil was measured by a gaussmeter (Type 3251, Yokogawa Electric Work) fitted with Hall generator probe (Fig.4.7).

After setting the appropriate instrument range, the Hall generator probe was inserted in the zero gauss chamber and then the meter reading was adjusted to zero. The Hall generator was mounted on a ruler in such a way that the surface of the Hall generator intersect the direction of MF at right angles. The ruler with hall generator was inserted inside the activated coil and the MF was then measured at different points inside the coil as shown in Fig.4.7.

4.2.5 Cell Culture Environment

There are certain optimal values of pH,tempature and RH for animal cell growth. Subsequent sections describes in brief about the culture environmental parameters.

The cells were grown in an open multiwell plate at low concentration (seeding density $10x10^4$ cells/ml). The cells were to be incubated in an atmosphere of elevated CO_2, (the concentration of which was in equilibrium with the sodium bicarbonate in the medium thereby stabilizing the pH of the medium) and 5% to 7% CO_2 in the culture environment is known to be optimum for animal cell culture [22]. Hence present studies were carried out in 5% CO_2 environment to maintain an atmosphere of relative anaerobiosis (Fig.4.8 & 4.9). Relative humidity was maintained at around 96% for carrying out the multiwell-plate-exposure cell culture experiments under ideal condition.

Selection of optimal temperature for cell cultures depend on

(i) normal body temperature of the animal from which the cells were obtained,

(ii) any regional (anatomical) variation in temperature and

(iii) incorporation of a margin factor to allow minor errors in incubator regulation [22].

This study dealt with human (K562) and mouse hybrid cell lines (AFP clone and OKT3 clone).Hence an incubation temperature of 36 $\pm$ 0.5 ^oC was chosen, which was close to the human body temperature and cells were incubated at the said temperature as shown in Fig.4.10.

4.3 EXPERIMENTAL METHODS

Erythroleukemic cell line (K562) and hybridomas (OKT3 and AFP clones) were given by the Immunology Division, Cancer Research Institute, Bombay.

The K562 cell was maintained in 90% DMEM (Dulbecco's modification of Eagle's minimum essential medium) and 10% FCS (Sera lab.).Hybrid cells (OKT3 & AFP) were propagated in 80% IMDM (Iscove's modification of Dulbecco's medium) [22] and 20% FCS.

For each set of experiment about 4 to 5 ml of each of the 3 cell lines was suspended in their respective media at a density of $10x10^4$ cells/ml. These samples were then seeded in the 96-well flat-bottom plates. Treated groups were kept inside the activated coil whereas the control was kept outside the coil (at less than 0.1 mT ambient MF).

4.4 ANALYTICAL METHODS

The biological responses of the cells due to exposure to MF were screened by measuring the cell growth by viable cell count, electron microscopy, SDS-poly acrylamide gel electrophoresis and fast protein liquid chromatography (FPLC).

4.4.1 Viable Cell Count

Cultured cells in the 96-well flat bottom plates were harvested at the end of 12, 24, 36, 45, 60, 72, 96 and 100 hours. 100 µl of each cell suspension was mixed with 100 µl of 0.4% Erythroycin-B and incubated at room temperature for 5 min. Viable cells (unstained) were counted with an improved

Neubauer ruling haemocytometer (Silverlite). The number of viable cells was computed by using the following formula [23]:

No. of viable cells/ml = No. of unstained cells x original dilution $x10^4$

Doubling time (time taken for the initial cell population to become double in the middle of exponential or log phase of cell growth) of cell lines was calculated from growth curve.

4.4.2 Electron Microscopy

For electron microscopy, AFP cells were grown in a 24-well flat-bottom plate at a density of $2x10^6$ cells/ml. Both exposed and control groups were incubated for 48 hours at 36 $\pm 0.5^{\circ}C$ in 96% humidity and 5% CO_2 approximately. At the end of 48 hours, the culture were treated as per the following steps

1. Cultures were centrifuged at 1000 r.p.m. for 5 min. and the supernatants were removed.
2. The cells were fixed in 3% glutaraldehyde for 45 min. at room temperature.
3. Fixed cells were then washed (centrifugation at 1000 r.p.m. for 5 min., removal of supernatant and resuspension in fresh buffer) 4 times in Sodium Cacodylate Buffer.
4. Cells were post-fixed in 1% Osmium tetraoxide for 1 hour at 4 $^{\circ}C$. Again washed 2 times with Na-Cacodylate buffer.
5. Dehydrated with graded ethyl alcohol (50%, 70%, 90% & 100%).

6. Ethyl alcohol was then removed (100% Ethyl alcohol + Propylene oxide; 100% propylene oxide).

7. Incubated at 60 °C for 1 hr in 50% Araldehyte 'A' (1:1 Araldihyte in propylene oxide).

8. Incubated at 60 °C for 1 hour in pure Araldihyte 'A'.

9. Freshly prepared Araldehyte-B was added and incubated at 60 °C for 1 hr and 45 min.

10. Embedded in Araldehyte B and the capsules were incubated at 60 °C for 3 days.

11. Blocks were sectioned in ultramicrotome, stained with Uranyl acetate and Lead Citrate and observed in TEM (Zeiss,109E), at an accelerating voltage of 80 kV [24].

4.4.3 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS - PAGE)

SDS-PAGE is an excellent method to identify proteins [25 and 26].SDS, being an anionic detergent, bind tightly to most of the protein at about 1.4 SDS-to-protein weight ratio, imparting a negative charge to the resultant complexes. Under applied electric field, migration of SDS derivatives (in polyacrylamide) is towards anode at rate inversely proportional to the logarithms of their molecular weights. SDS-polypeptides thus move through the gels in predictable manner, with low molecular-weight complexes migrating faster than larger one [25]. Detailed method of SDS PAGE given in the Appendix-C. In the assembled electrophoresis cell the upper and lower reservoirs were filled with buffer and comb were removed from the stacking gel. Prepared samples were

then loaded into the wells (well 2,3,4 contain 50,30, & 20 µl of 80% IMDM + 20% FCS; well 5,6,7 contain 20, 30, 50 µl of control supernatant of OKT3 at 106 hrs.; well 9,10,11 contain 10,25 and 50 µl of cell supernatant exposed to 0.8 mT & 45 Hz) in the stacking gel by lowering them under electrode buffer using a micropipette. The samples were run through the casting gel. Proteins were detected by dye staining with Coomassie Brilliant Blue-R 250 (Sigma). Gels were soaked in excess of staining solution for 30 min. then destain with large excess (at least 4 washings) of 40% methanol + 10% acetic acid to remove background stain.

4.4.4 Detection Of Secretory Products Of Hybrid Cells By Fast Protein Liquid Chromatography (FPLC)

FPLC is a link between established chromatographic methodology and the best of high pressure liquid chromatography and is dedicated to separation of protein [26]. The major components of the FPLC were buffer reservoir, peristaltic pump, column, UV analyser and a chart recorder (Fig.4.11). For this study, gel filtration column (Superose-12; Pharmacia) was used.

For each set of analysis, 100 µl sample (20% FCS; 80% Medium +20% FCS; Culture supernatant of exposed and control group) was loaded through the injection valve (one sample at a time) and was flushed into the coloumn by normal saline (no buffer was used) at 1 MPa pressure and at a flow rate of 0.5 ml/min. Absorbance of the eluted volume was screened (280 nm) in UV spectophotometer set at 0-0.1 sensitivity. Elution profile of the sample was then plotted in a chart recorder.

4.5 DATA ANALYSIS

All the graph were plotted using GRAPHER package and Student's 't' test were performed using SPSS package. By FPLC it was not possible to resolve two fused peaks of IgG (peak No.2) and albumin (peak No.3) as shown in Fig. 4.12. For a quantitative analysis it was assumed that the peak characteristics of the two unresolved compounds do not affect each other and IgG peak (labeled peak number 2) was apparently reconstructed using typical fused peak's as shown in Fig. 4.13 by the following method [26]:

i) A line CC' perpendicular to the base line B'B" was drawn through point N (junction of the peaks 2 and 3).

ii) A tangent was drawn along the peak 2 which interest base at B' and line CC' at C'.

iii) Line B" C' was drawn, such that B'C=B"C, forming isosceles triangle B'B"C'.

iv) Area under the peak was estimated as the area of the triangle B'B"C.

$$\text{Area} = 1/2 \times CC' \times B'B"$$

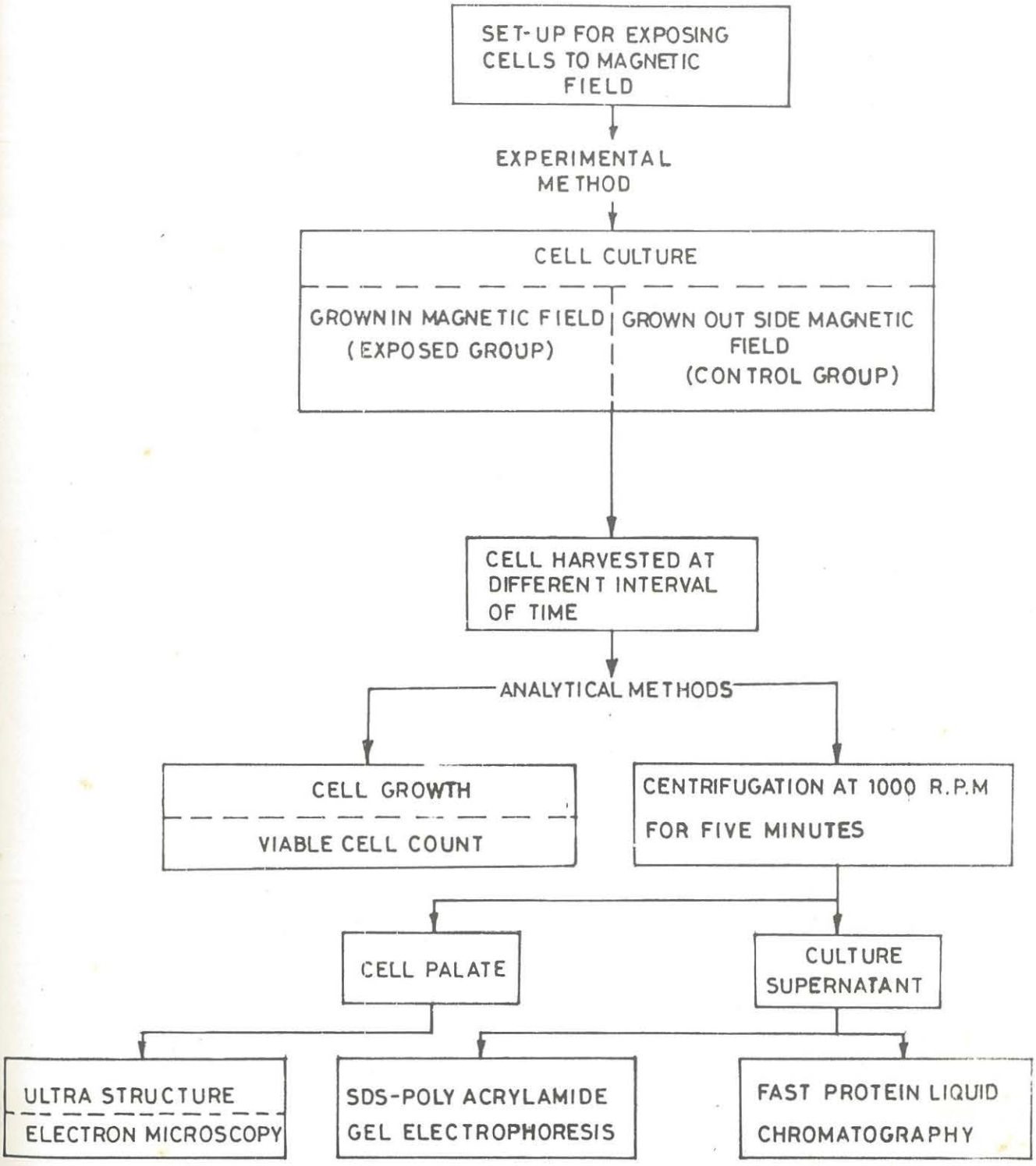


FIG. 4.1- OUT LINE OF THE EXPERIMENTAL AND ANALYTICAL METHODS.

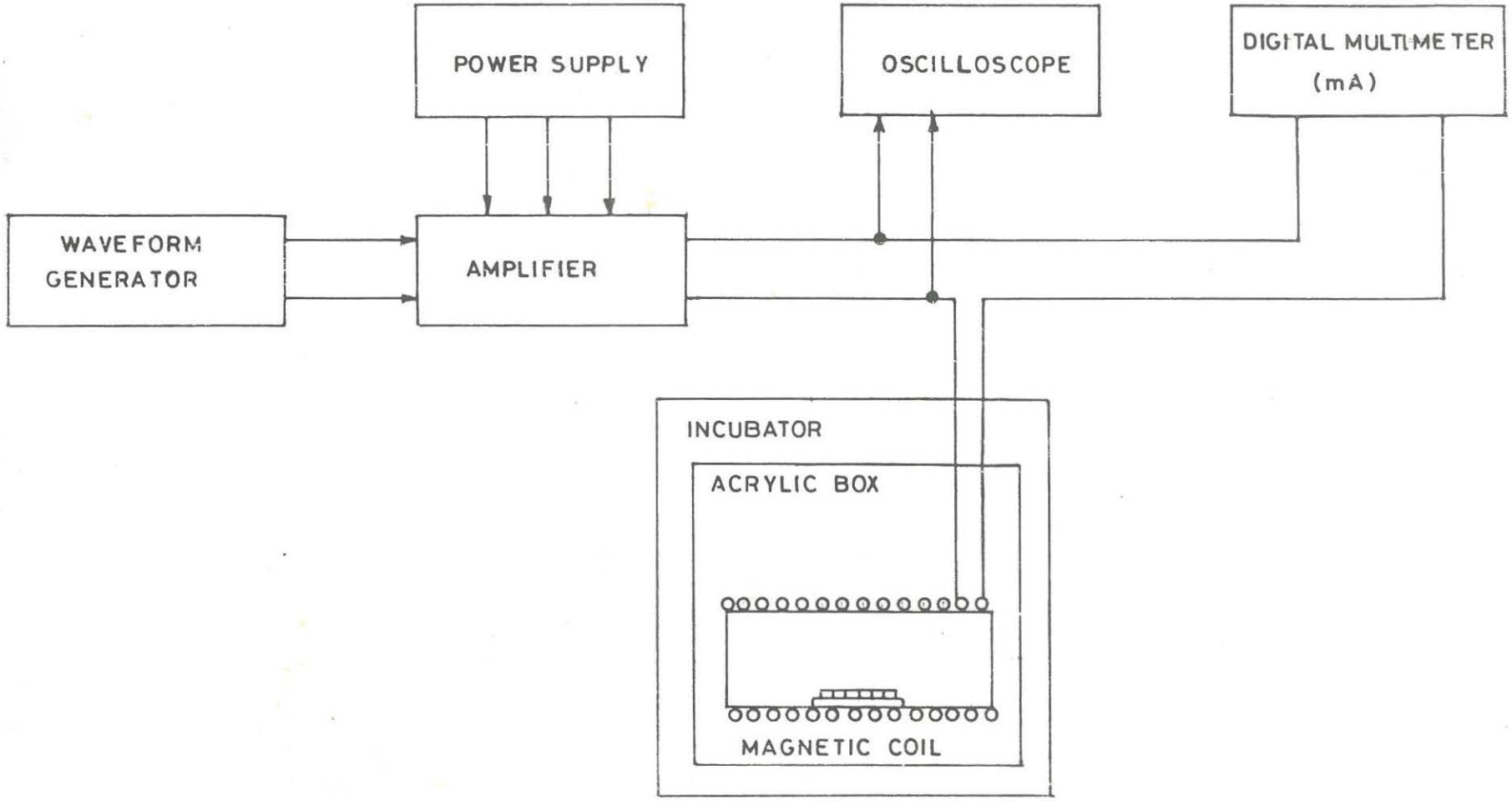


FIG. 4.2-SCHEMATIC OF THE EXPERIMENTAL SET-UP FOR EXPOSURE OF CELL-LINES TO MAGNETIC FIELDS.

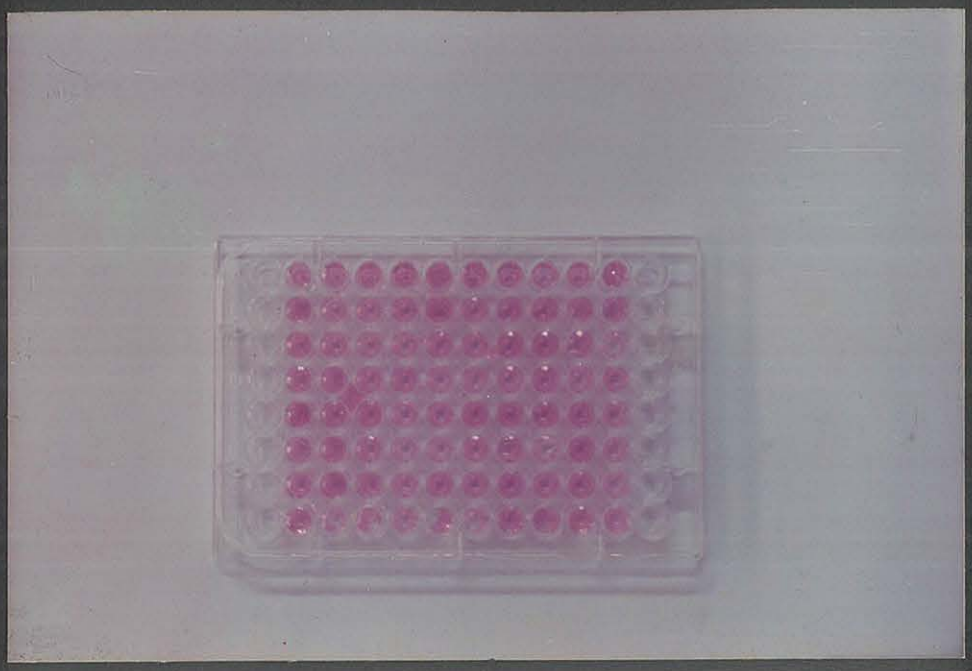


Fig. 4.3 96-well flat bottom plate. Cell were seeded in this multiwell plate (10×10^4 cells/ml) for exposing to magnetic field.

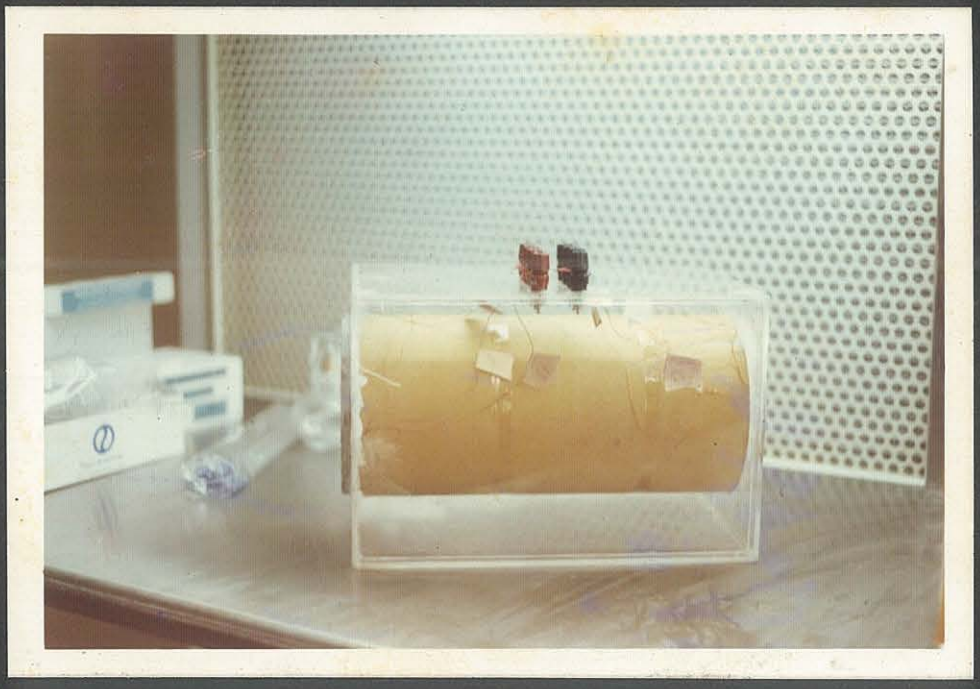


Fig. 4.4 Coil for the generation of magnetic field. It is a PVC tube (of internal diameter 0.136 m and 0.33 m length) on which four layers of wire were wounded and connected in parallel. The acrylic box prevents possibility of contamination by these wires. This coil can generate uniform MF along the axis spanning length of 0.1 m. Cells were cultured inside this coil.

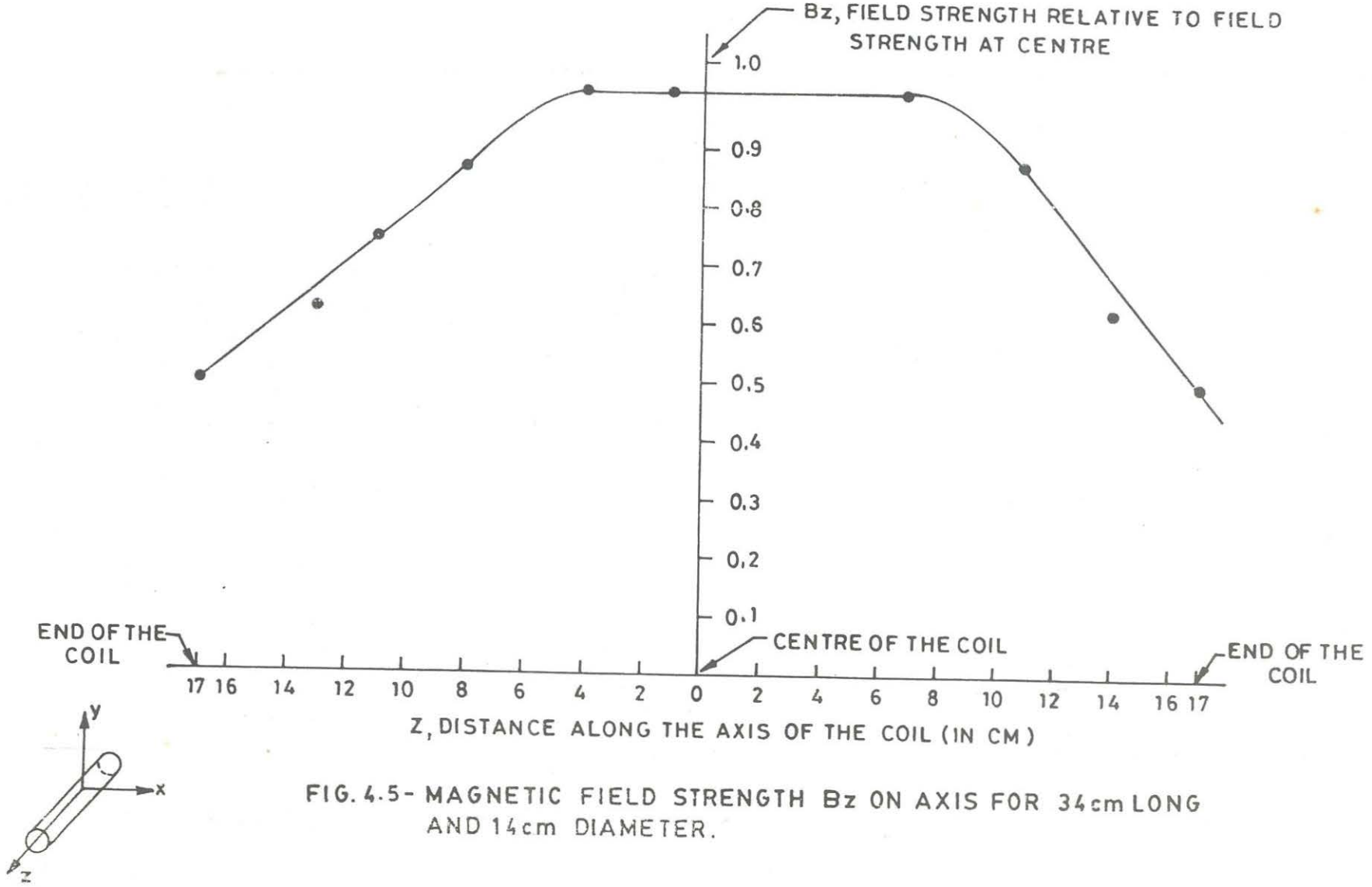


FIG. 4.5- MAGNETIC FIELD STRENGTH Bz ON AXIS FOR 34 cm LONG AND 14 cm DIAMETER.

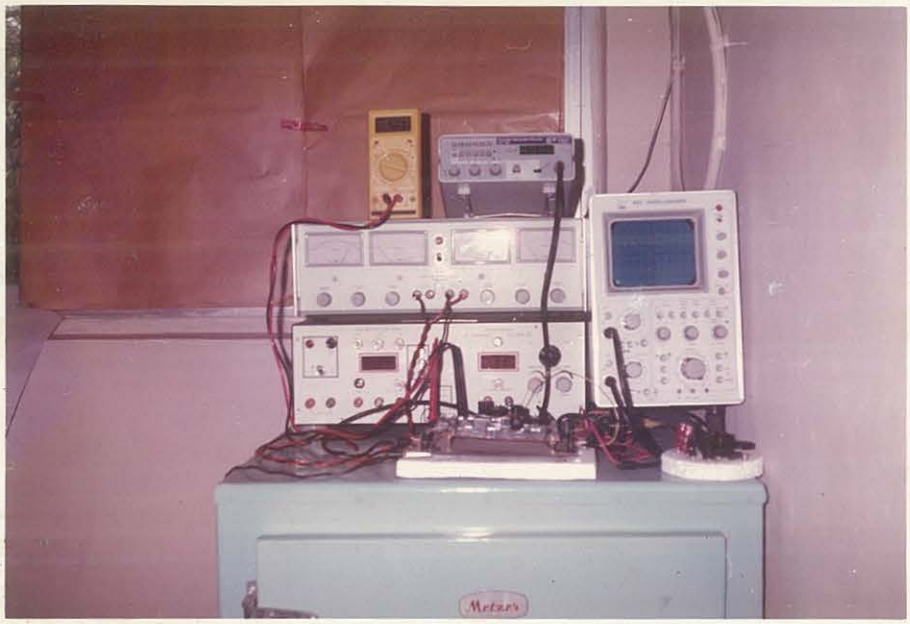


Fig. 4.6 Experimental setup for studying effect of sinusoidal MF on K562, OKT3 and AFP clones. It consist of an amplifire fed by function generator and powered by dual DC power supply. Digital multimeter (set in current mode) and oscilloscope continuously monitor current and, wave shape and voltage respectively. Activated coil accommodating culture is inside the incubator.

Fig. 4.7 Type 3251 gauss meter (Yokogawa Electric Works) with Hall generator probe. By merely inserting the probe into the magnetic field to be measured, the meter directly indicates the magnetic flux density.

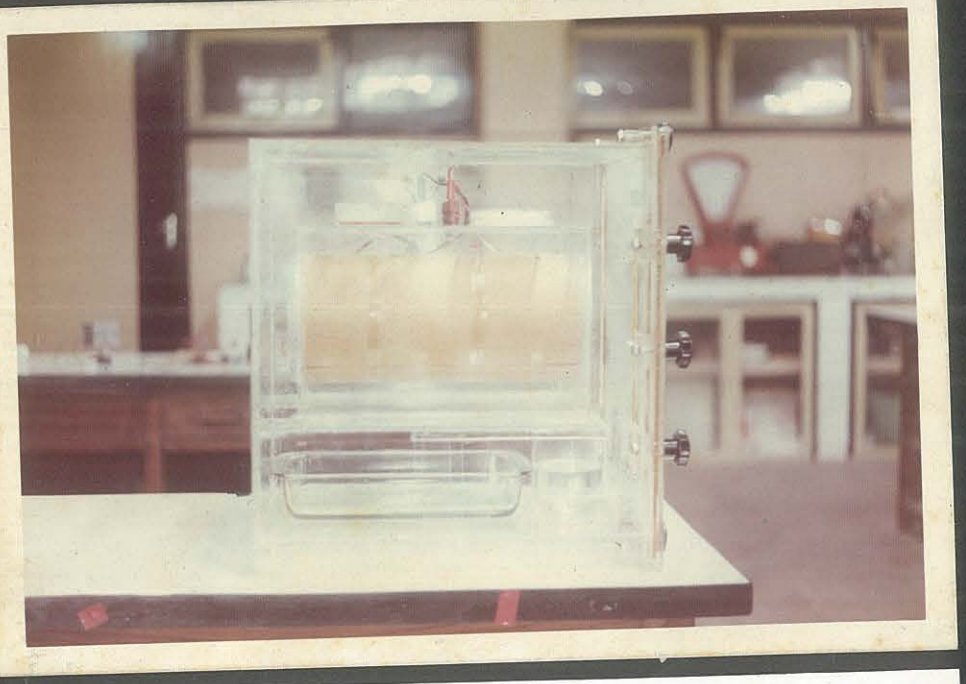


Fig. 4.8 An air tight acrylic box fabricated for cell culture at 5% (Approx.) CO_2 and 96% relative humidity.

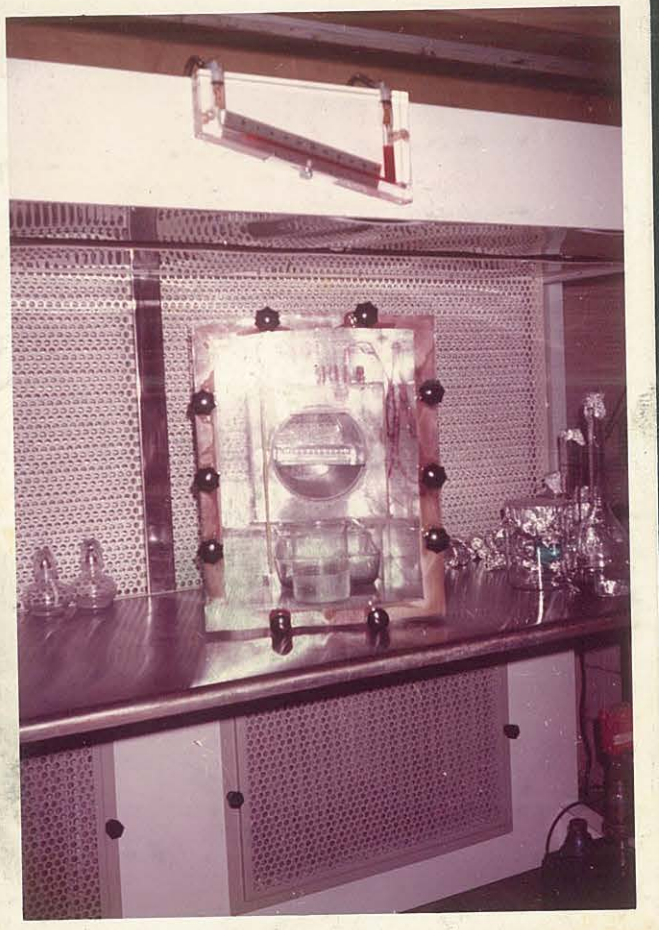


Fig. 4.9 Front view of the air tight acrylic box. Inside is the magnetic coil in which cells were grown and exposed to MF.

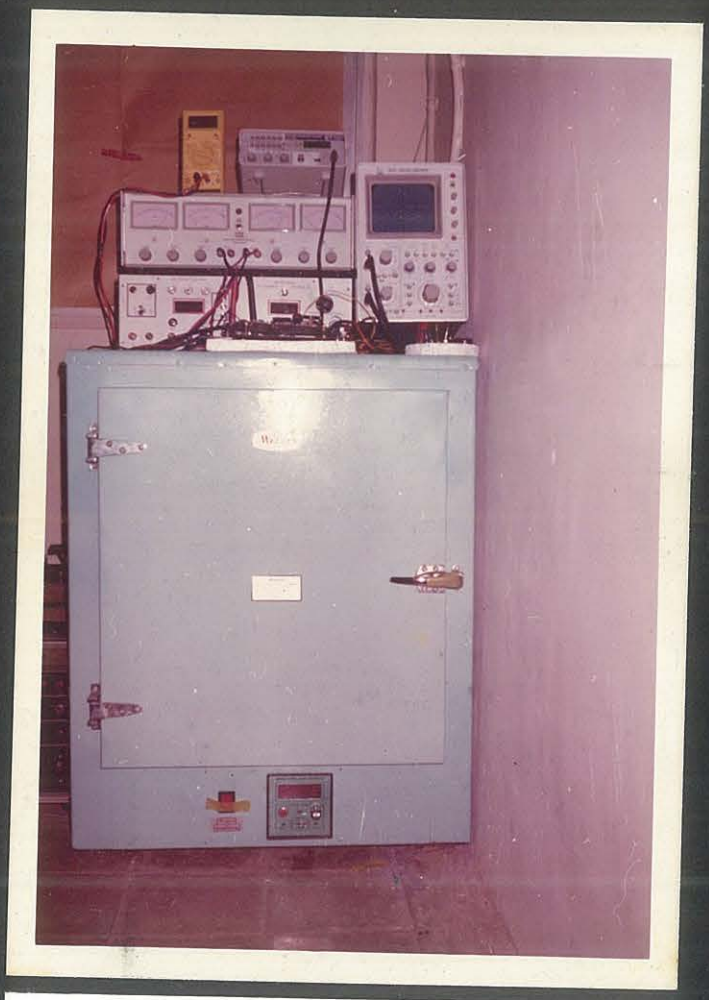


Fig. 4.10 Complete view of the setup for exposing cell lines to MF. The air tight acrylic box (in which cells were grown) is gassed with 5% CO_2 (Approx.)& kept inside the dry incubator to maintain the culture at 36 ± 0.5 ^{0}C.

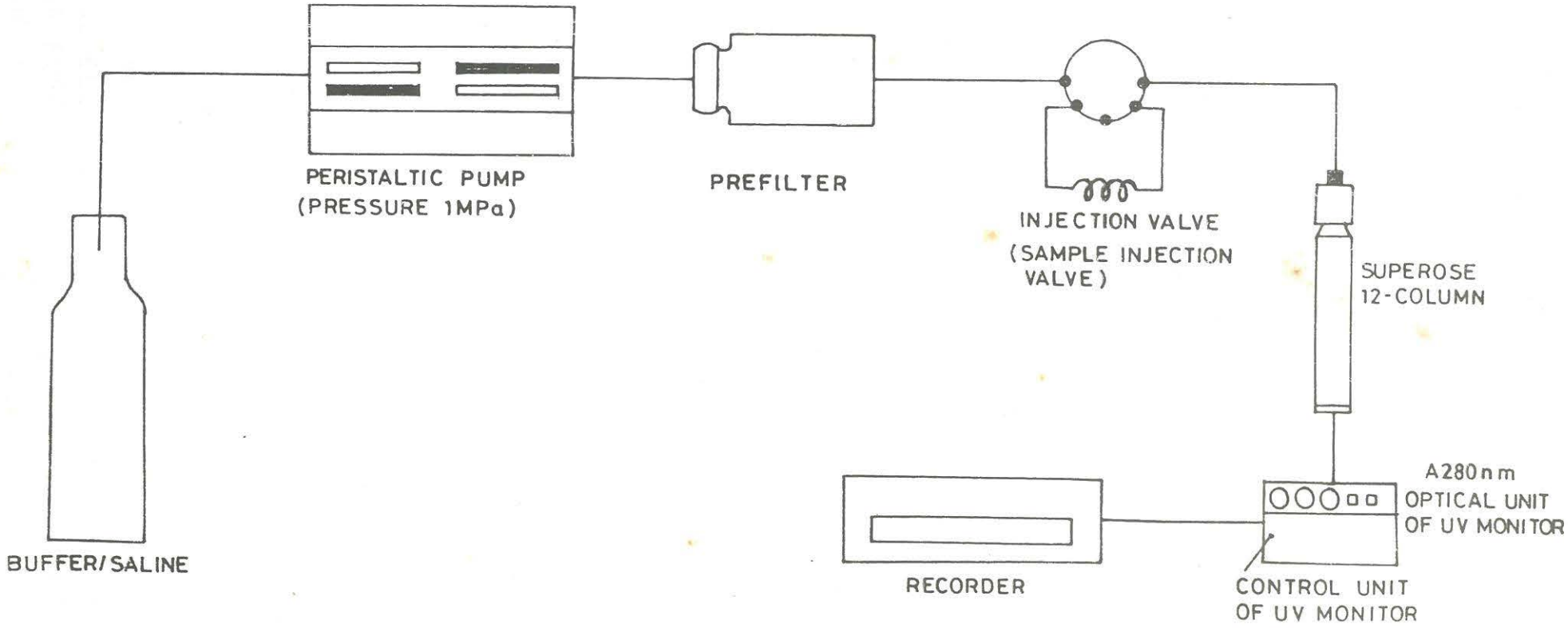


FIG. 4.11 - FAST PROTEIN LIQUID CHROMATOGRAPHY PROCESS
FLOW CHART

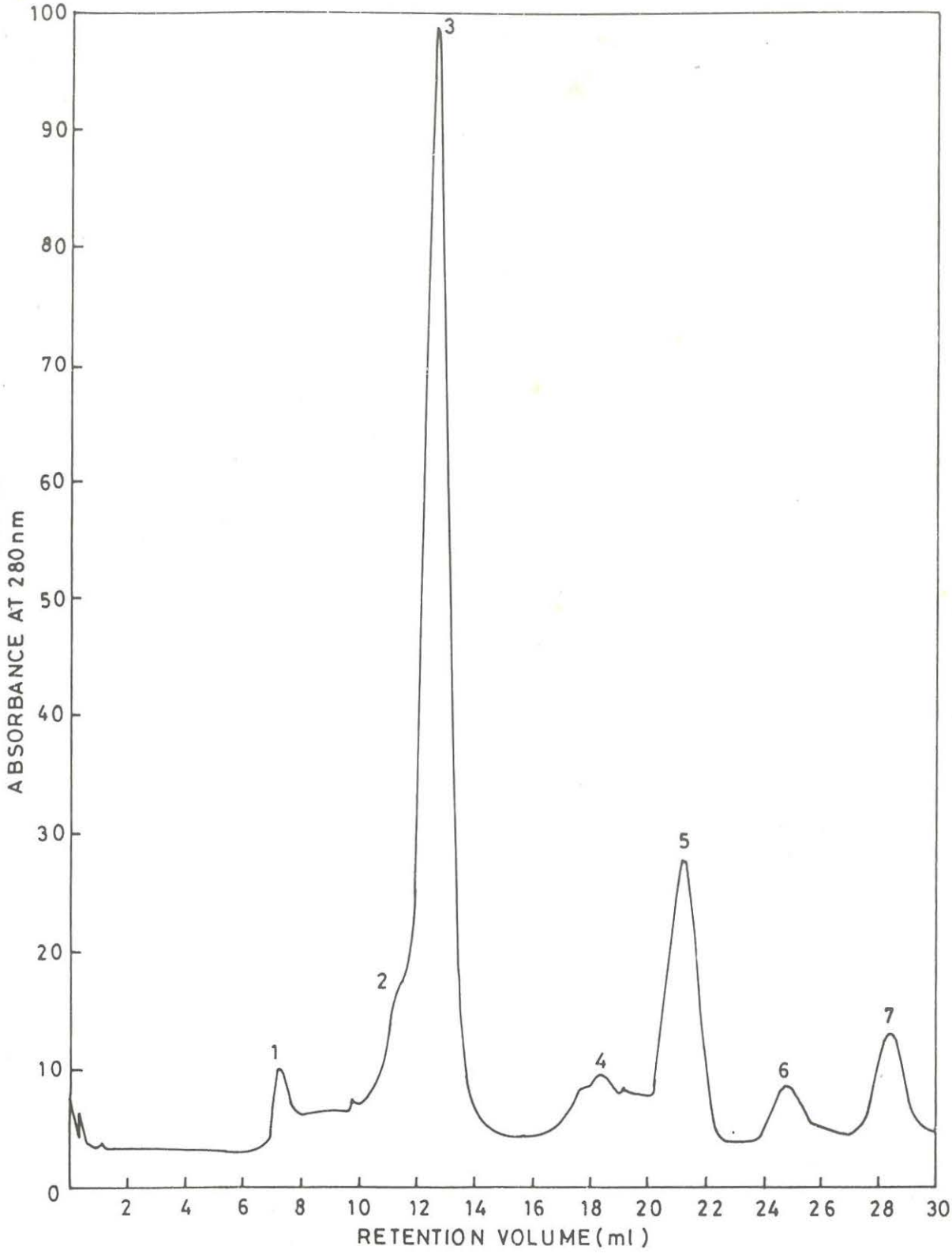


FIG. 4.12 - A TYPICAL ELUTION PROFILE OF 'OKT 3' CELL SUPERNATANT SHOWING FUSED PEAKS OF IgG (PEAK NO. 2) AND ALBUMIN (PEAK NO. 3)

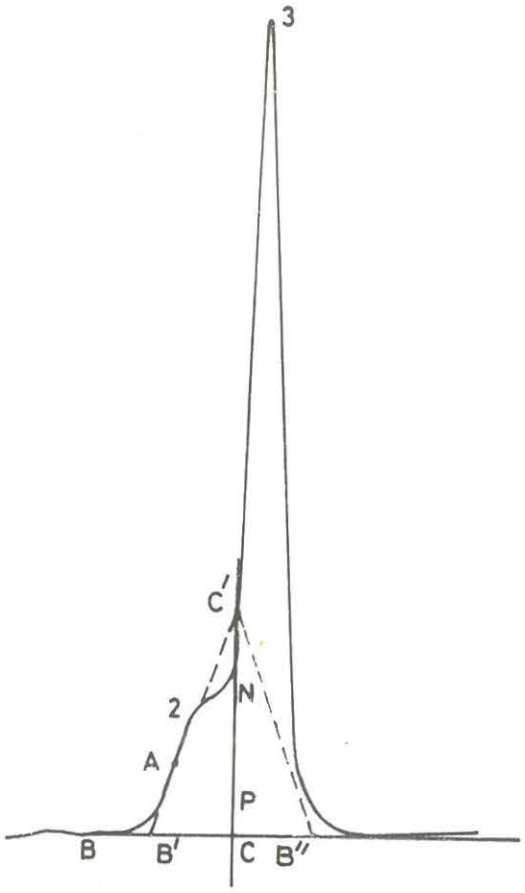


FIG. 4.13- RECONSTRUCTION OF A TYPICAL FUSED PEAKS OF IgG (PEAK NO. 2) AND ALBUMIN (PEAK NO. 3)

CHAPTER 5

RESULTS AND DISCUSSIONS

5.1 INTRODUCTION

Growth curves from viable cell counts, both in control (unexposed) and experimental group show the differential effects of magnetic field (MF) on the growth pattern of the exposed cell vis-a-vis the control. All cell lines cultured in MF had shown significantly different growth pattern. Doubling time (time taken for the initial cell population to become double in the middle of exponential or log phase of cell growth) of cells were changed when grown in MF.

Transmission electron microscopic (TEM) study of the cells showed distinct ultrastructural features both in the control and experimental cell populations and is discussed later.

Studies were undertaken to find out whether significant difference in the growth pattern of exposed population was comparably reflected in the secretory capacities of these cells compared to the controls. Secretory products were first detected by SDS-polyacrylamide gel ectrophoresis (SDS-PAGE) of the culture supernatants from both the groups and then attempt was made to identify the proteins by fast protein liquid chromatography (FPLC). FPLC had shown qualitative changes in the exposed population. Also, some indication of quantitative changes in secretory products of the treated cells was obtained as compared to control.

This chapter deals with the analysis of results of screening tests - both qualitative and quantitative.

5.2 CELL GROWTH

Results of the cellular growth studies have been expressed as number of viable cells versus the time in culture.

5.2.1 Cell Growth at 0.6 mT and 90 Hz

Number of viable OKT3 cells were high throughout the growth cycle when grown in MF of defined parameters (Fig.5.1, Table D 1). At the end of 45 hrs of in vitro culture, growth of the experimental cells were significantly higher as compared to the control ($p<0.01$).

Onset of "log phase" (period of exponential increase in cell number) was early as compared to control (where immediately after seeding there was a fall in number of viable cells) thereby indicating that 90 Hz MF of 0.6 mT shortens the 'synthetic' or 'S' phase. This 'negative' effect on the 'S' phase might be reflected in the stimulation of the 'mitotic' or 'M' phase (enzymes, such as DNA polymerase increase might have taken place followed by the synthesis of DNA). Shortening of 'S' phase might be due to

(i) some associated effects on the synthetic machinery of the cell, either qualitatively or quantitatively, or,

(ii) MF of this parameters might be having an inhibitory effect on gene expression as such.

This might be due to some other associated factors affecting the genetic expression process itself. At the end

of 144 hrs, viable OKT3 count remained high as compared to the control ($P < 0.01$). This might indicate a sustained effect of MF on cell viability in prolonging the "plateau phase" (culture becomes confluent i.e. all the available growth surface were occupied as all the cells were in contact with each other and hence growth rate is either reduced or near zero).

Doubling time of the cells grown in MF of defined parameters, vis-a-vis the unexposed population were found to be same i.e. 37 hrs (Table D5).

In the first 12 hrs of in vitro culture, AFP grown in MF, had shown significant ($P < 0.01$) increase in the number (Fig.5.2, Table D1). From 12 hours onwards viable count has gone down gradually as is evident from the plot. At 62 hrs , the viable count in exposed population shows an upward shift which is much slower and sustained compared to control. It seems interesting at this stage to investigate whether the differential viable counts in the two populations are really reflected in the secretory capacity of the cells at all, specially, in the initial phase of culture (between 10-60 hrs in AFP). Also to note, is the significant change in the doubling time of the exposed cells compared to unexposed population which, ultimately might reflect an apparent lowering of mitotic index and hence a prolonged 'S' phase. This is in sharp contrast to OKT3, where the doubling time, which is a good index of mitotic activity of cells in culture, remains exactly identical at 37 hrs for both the populations.

In accordance with the 'secretory' cell (OKT3 & AFP) growth pattern, the non-secretory cell - K562 - shows an initial 'lag' phase in the control population compared to the exposed ones, although, at around 40 hrs of culture, the effective viable counts in the two populations are almost identical (Fig.5.3 , Table D.1). This is followed by a slow and gradual fall in the viable count of exposed population, which again shows gradual upward turn, at around 60 hrs. This apparent suppression of the 'M' phase between 45 and 60 hrs is much more sustained and might reflect a simultaneous operative 'S' phase. It is again interesting to compare - both qualitatively and quantitatively- the status of apparently operative 'S' phases between 45-60 hrs in the exposed population, compared to control cells between initial seeding and first 10-12 hrs of in-vitro culture. This apparent suppression of mitotic activity in the exposed population is duly reflected in the significant prolongation of the doubling time compared to unexposed population.

5.2.2 Cell Growth at 0.8 mT and 45 Hz

Onset of the 'log phase' was rapid and no fall in initial viable count was observed when the K562 cells were exposed to MF of specified parameters. Significantly high growth rate was observed in exposed cells till around 39 hrs although the final cell population was comparable at the end of 60 hrs of in vitro culture (Fig.5.4, Table D.2). Doubling time of both the groups remained 18 hrs (Table D.5).

Apart from the initial difference in response in the two groups at around 30 hrs no significant difference in growth pattern was observed in OKT3 cells when exposed to the said MF (Fig.5.5, Table D2) , till around 60th hrs of in vitro culture when the plateau phase sets in, in the exposed population as compare to the control which still shows a weak mitotic response at around this time. This near - plateau phase in exposed population continued upto around 106 hrs, beyond which there was a significant difference (0.05) in the viable cell counts in the two groups. As two successive mitotic phases are interspersed with 'G' and 'S' phases in cell cycle, this sustained plateau of mitotic index might reflect an operative 'S' phase as discussed earlier. Further it is interesting to note the secretory status of the exposed population from around 60 hrs of in vitro culture onwards. The control shows a sharp decline in cell number beyond 106 hrs.

5.2.3 Cell Growth at 0.8 mT and 76 Hz

Specified MF had significantly ($P < 0.01$) increased growth of AFP clones till about 25 hrs of culture time and again from around 50 hrs till 70 hrs of in vitro culture (Fig 5.6,Table D3). As the cells from both the groups were maintained in unchanged media of defined composition, ultimately around 96 hrs the number of cells in the exposed population was significantly higher than the controls. As the culture was discontinued around this time, it remains to be seen whether the cell population in the exposed group

shows only qualitative deviation in the growth pattern or there is a concomitant difference in the secretory capacity compared to the control (with a change of fresh media). Interestingly, although the cell population in the exposed group does not show any quantitative increase, they could be maintained viable in the unchanged media from 50 hrs onwards whereas, the control group shows a distinct quantitative decline in cell number from that period.

76 Hz MF of 0.8 mT had apparently some stimulatory effect on the growth of K562. Exposed cells had significantly ($P<0.01$) high growth till plateau phase at around 96 hrs (Fig.5.7, Table D.3). Doubling time of the K562 had also gone down from 20 hrs in control to 12 hrs in exposed population (Table D.5).

Although K562 is an 'apparent non-secretory' cell, the first plateau (between 30-50 hrs of exposure) probably indicated an 'S' phase and needs further clarification as far as synthesis of the structural components of the cells are concerned.

5.2.4 Cell Growth at 1 mT and 500 Hz

K562 cell growth has been significantly ($P < 0.01$) more till about 72 hrs (Fig.5.8, Table D.4). As discussed earlier, the small plateau which is almost identical in duration in both the groups needs further clarification.

5.3 ULTRASTRUCTURAL FEATURES

AFP cells exposed to 76 Hz MF of 0.8 mT for 48 hrs were studied by transmission electron microscope (TEM) and

compared with the control group. Three zones were studied:

i) cell membranes;

ii) cytoplasmic components, mainly the protein synthesizing machinery; and

iii) the condensation status of chromatin.

There was no structural change in the cell membrane of the two groups (Fig.5.9 & Fig.5.10).

Rough endoplasmic reticulum in the cytosolic compartments showed no qualitative change in either of the groups (Fig.5.11). Cytosol in both the groups was full of ribosomes, indicating possibly, a comparable secretory status in the two group. Remarkably, the exposed group cell cytosol always showed a more "active" form of mitochondria which was depicted by the alternation in the mitochondrial shape (Fig.5.12). Nuclear compartment showed evidence of active replication of nuclear DNA.

5.4 SECRETORY STATUS OF THE CELLS

Secretory products of hybrid cells, in culture supernatant was detected by SDS-PAGE and FPLC. FPLC is a fairly sensitive and accurate method to detect quantity of secretory protein. Elution profiles of FPLC have detected difference in secretory status of exposed group as compared to control.

5.4.1 SDS-PAGE

SDS-PAGE pattern of exposed (90 Hz,0.6 mT) and control supernatant 0f AFP showed no qualitative change near the origin. Culture medium (80% IMDEM + 20% FCS) was compared

with the exposed and control groups. As mentioned, although high molecular weight protein bands were comparable and were present near the origin, the low molecular weight group of proteins could not be compared effectively because of very low concentration of protein in the samples loaded in each lane (96-well plate culture supernatant - exposed and control were taken for SDS-PAGE).

5.4.2 FPLC

Clear supernatants of OkT3 (exposed to 45 Hz MF of 0.8 mT for 139 hrs; exposed to 76 Hz MF of 0.8mT for 96 hrs) and AFP (exposed to 76 Hz MF 0.8mT for 96 hrs) were run vis-a-vis the control (< 0.1mT) by gel filtration technique in FPLC, elution solvent being Nacl (0.9 g/dl). Medium with FCS (80% IMDM + 20% FCS) was run for comparision.

Standard IgG (NPFC) was run to identify the retention volume (volume of mobile phase required to elute a particular solute) or the peak position of IgG which corresponds to the peak position of the secretory products of the cell lines OkT3 and AFP. Standard albumin (NPFC) was run to mark the retention volume (the peak position) which is the major protein component of FCS [22].

Elution profiles of standard IgG, albumin, and FCS showed single peaks as shown in Fig. 5.13, 5.14, and Fig. 5.15 respectively. Medium with FCS showed six peaks (Fig. 5.16). Protein composition in the control supernatant showed qualitative change compared to the exposed one. This qualitative alternation was common to both the high molecular

weight as well as low molecular weight protein components (Figs 5.17 to 5.25).

Standard IgG elution profile contains a single major peak which started at the retention volume of 11.83 ml (Fig. 5.13). Similarly standard albumin elution profile contains a single major peak which started at the retention volume of 12.83 ml. (Fig. 5.14). Culture medium elution profile (Fig. 5.16) has a small peak (Peak No. 1) at 10.93 ml (IgG position) indicating possibly the presence of trace amount of IgG in the medium. It also contains a major peak (Peak No.2) which started at 12.38 ml retention volume (at the position of albumin).

Elution profile of the OkT3 cell supernatant (culture time 139 hrs) exposed to 45 Hz MF of 0.8mT shows IgG peak (Peak No.2) which has 25.8% higher area under the peak as compared to that of control, indicating possibly an enhanced IgG secretory status when grown in the specified MF. Exposed supernatant gave a new peak (Peak No.1) at 8.88 ml. These findings are clearly shown in Fig. 5.18 and Fig. 5.19.

For further MF exposure studies, OkT3 and AFP cells were grown in 25ml culture flask (Linbro, flow laboratories) till confluency (cultured up to 96 hrs). Fig. 5.20 shows the elution profile of confluent there of OkT3 under control condition. One new peak (Peak No.1) at 7.44 ml for duration of 1.44ml was observed, which was not present in control group culture supernatant of OkT3 cells when grown in 96 flat bottom well plate (Fig. 5.17). Fig. 5.19 and Fig.5.20 showed increased secretion of IgG by OkT3 cell when exposed to 76 Hz

MF 0.8 mT as compared to control. There was about 4.1% increase in the area under peak 2 (IgG peak) as compared to control. Elution profile of confluent AFP culture supernatant at (96 hrs) exposed to 76 Hz MF at 0.8 mT shows IgG peak (Peak No.2) which has 25% higher area under the peak (Fig. 5.24 and Fig. 5.25) as compared to that of control (Fig. 5.23) indicating possibly an enchanced secretion of IgG when AFP cells were grown in specified MF. It is interesting to note the increase in height of high molecular weight peak (Peak No. 1) which started at retention volume of 7.28 ml for the duration of 1 ml. This could be due to formation of low molecular weight protein aggreates.

Low molecular weight peaks (peak No 5,6, and 7) of exposed OkT3 and AFP cell supernatants showed qualitative differences in size and shape compared to control populations of the respective cells. Possibly this indicates some basic changes in the metabolic patterns of the exposed population which might have had a reflection in the peak quality alteration. To pinpoint the changes occuring in the exposed population compared to the control, the individual peaks are to be analyzed using an elution profile of gradually increasing salt contentration (NaCl) keeping the snesitivity same. This is true specially for the overlapping peaks, where a gradual increase in the osmollality of the eluting solvent should give a better separation of proteins having molecular weights which are very close. . A combination of salt solutions should also be tried in subsequent analysis to get better separation. Also, secretory status of exposed

population of cells remains to be elucidated compared to the controls at a given instant of time by changing the media from time to time.

5.5 DISCUSSIONS

Biological materials are generally nonmagnetic, having a permeability nearly that of free space [12]. Except for some specialized magnetite-bearing cells [12] no cell or tissue interaction with low or moderate magnetic field is expected unless an oscillatory magnetic field induces significant electric field (Faraday induction). Surprisingly Faraday induction may not be sufficient to explain some of the biological effects with sinusoidal MF in the range of 0.1 to 1.0 mT [13].

Analysis of the results of this study, possibly indicates some effect(s) of sinusoidal magnetic field (MF) on growth and secretion of hybridoma cells (OKT3 and AFP clones), although the pattern of changes in the exposed populations in both the cells compared to the control group showed obvious differences.

In all cases sinuoidal MF was observed to have influence on rapid transition of cells from 'Go' (resting phase) to synthetic phase. This rapid transition from the resting phase to an active phase of mitotic division can be defined as a process of faster adaptation to the media environment under the specified MF (almost all parameters showed this initial effect) for the exposed population compared to the control. In molecular terms, this faster adaptation might reflect at the following:

(i) better permeability [16] of solute across cell membranes due to increased fluidity of membrane PLs.

(ii) there might be some effect(s) on genetic expression, which in its turn might be either at the nuclear level [13] or in the cytosolic compartment [14] in the form of altered rate of processing and/or translation of matured RNA and other nucleic acids. Since this was only an initial study in the direction of electromagnetic manipulation of established hybrid cell lines in an effort to - possibly - increase the yield of MAbs (low volume - high cost biological), the elucidation of the physical and molecular basis of changes occurring under the influence of fixed parameter MFs was beyond the scope of this study and would be attempted in subsequent studies.

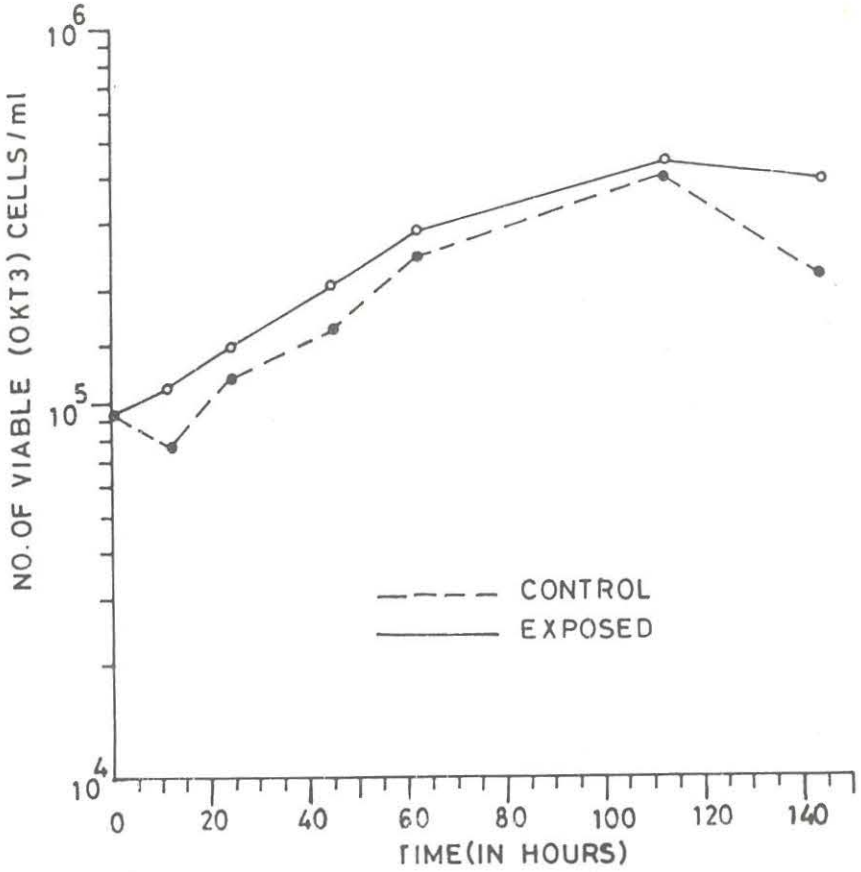


FIG.5·1 - GROWTH OF 'OKT3' EXPOSED TO SINUSOIDAL 90 Hz AND 0.6mT MAGNETIC FIELD.

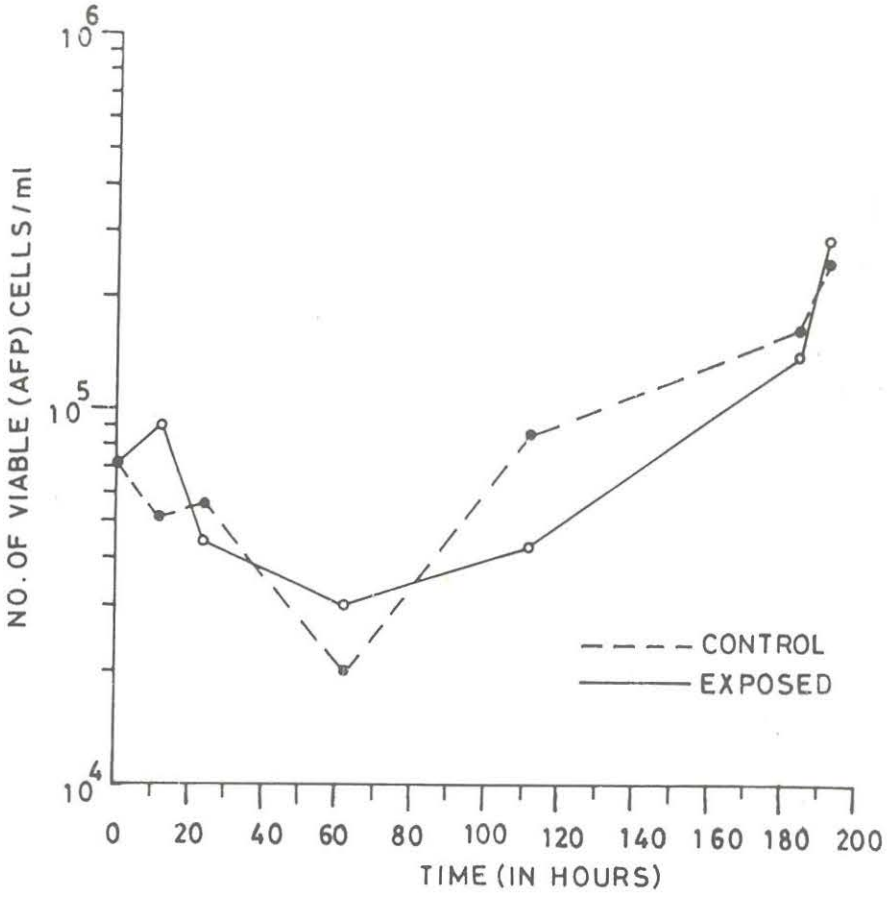


FIG. 5.2 - GROWTH OF 'AFP' EXPOSED TO SINUSOIDAL 90Hz AND 0.6 mT MAGNETIC FIELD.

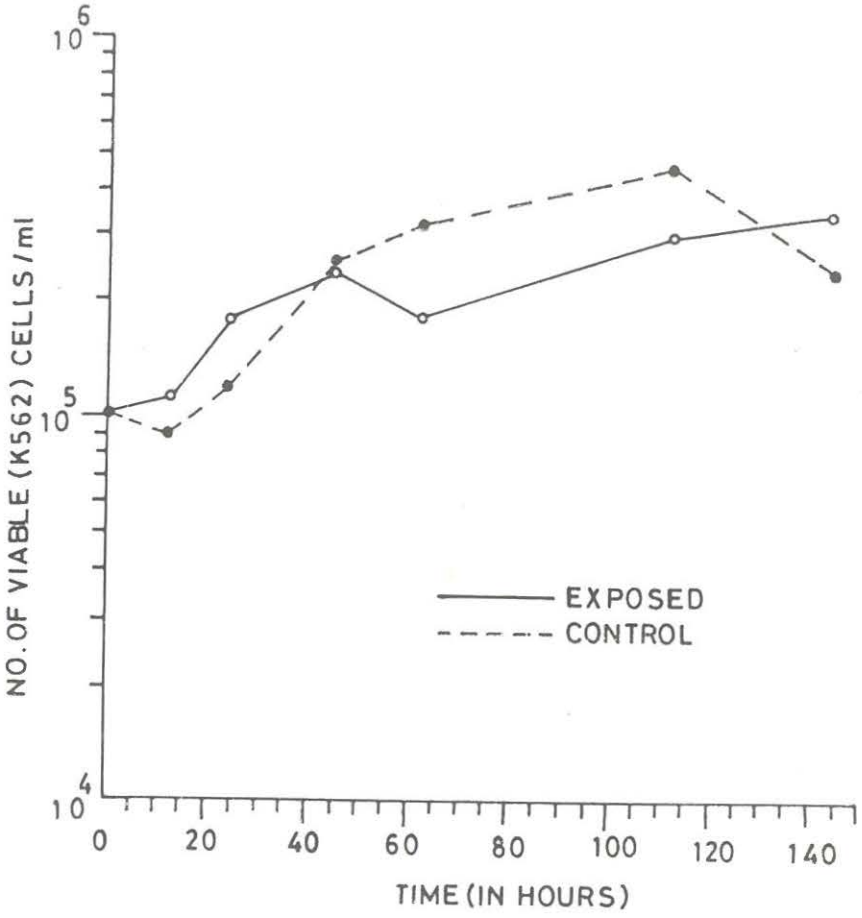


FIG.5.3 - GROWTH OF 'K562' EXPOSED TO SINUSOIDAL 90Hz AND 0.6mT MAGNETIC FIELD.

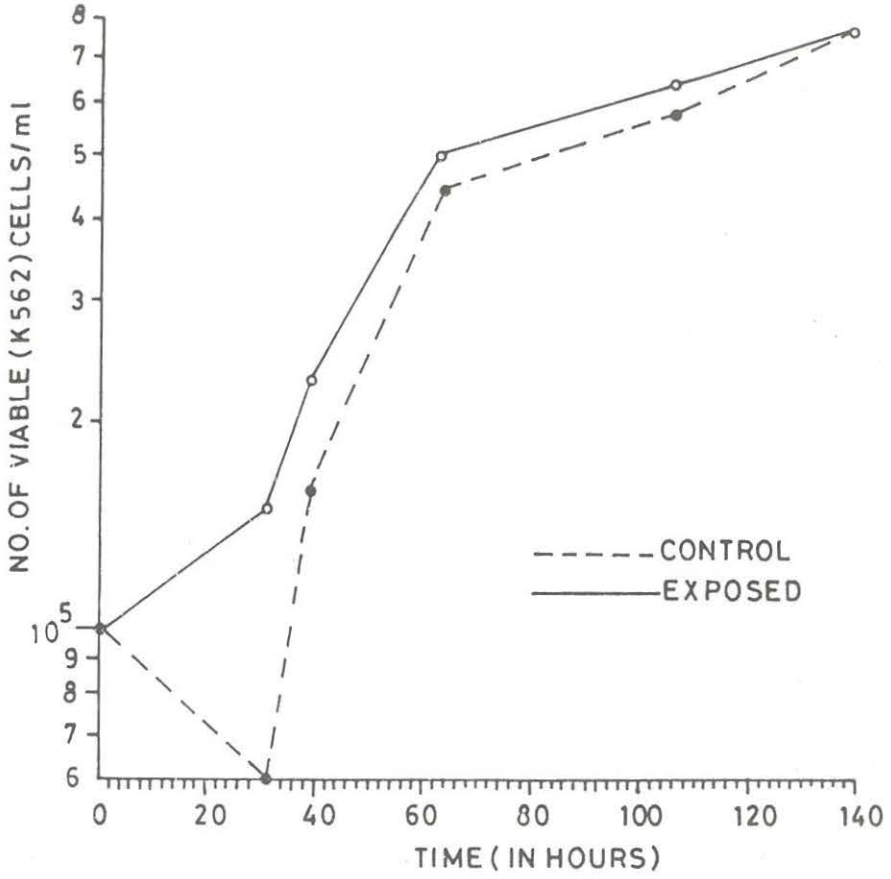


FIG.5.4 - GROWTH OF 'K562' EXPOSED TO SINUSOIDAL 45Hz AND 0.8mT MAGNETIC FIELD.

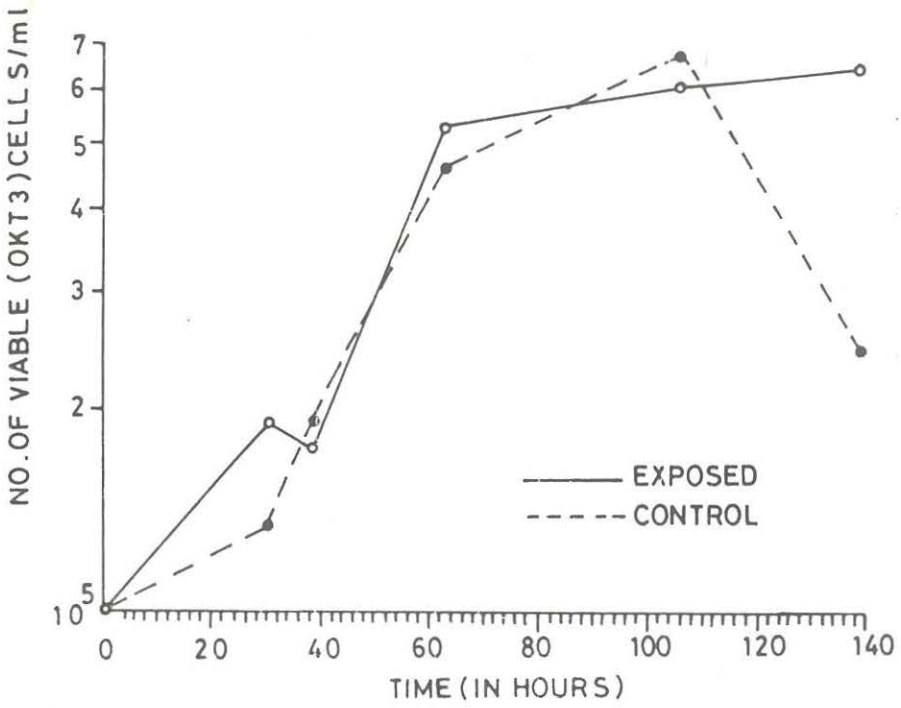


FIG. 5.5 - GROWTH OF 'OKT3' EXPOSED TO SINUSOIDAL 45Hz AND 0.8 mT MAGNETIC FIELD.

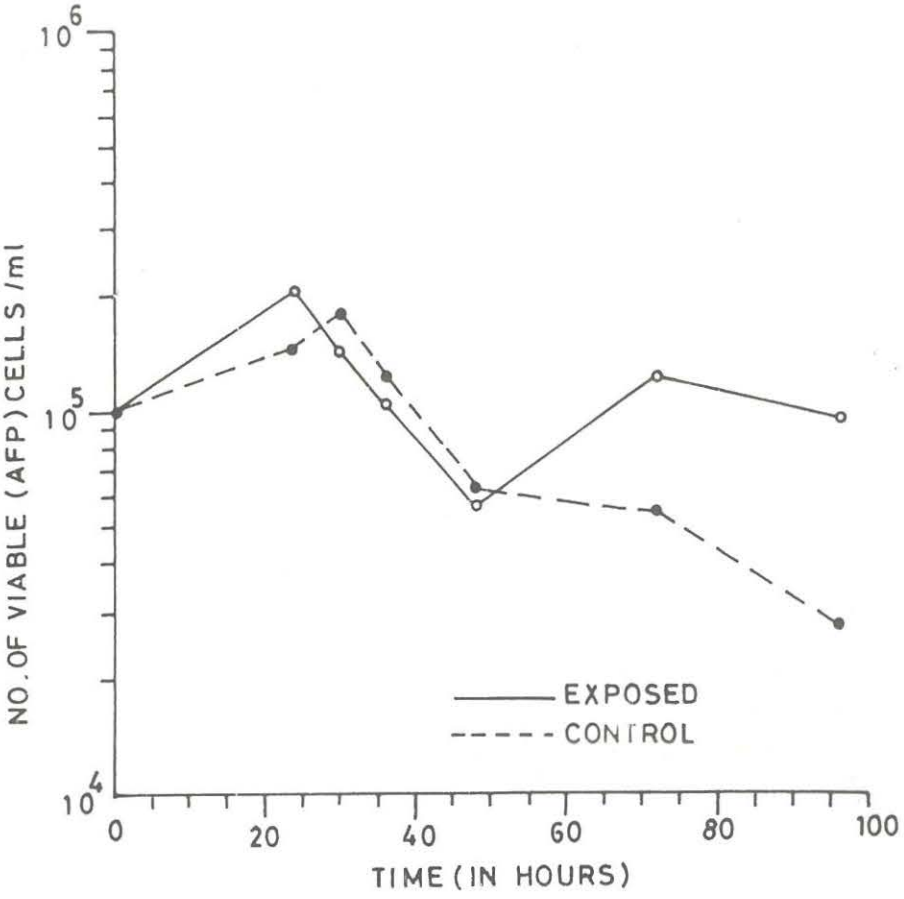


FIG. 5.6 - GROWTH OF 'AFP' EXPOSED TO SINUSOIDAL 76 Hz AND 0.8 mT MAGNETIC FIELD.

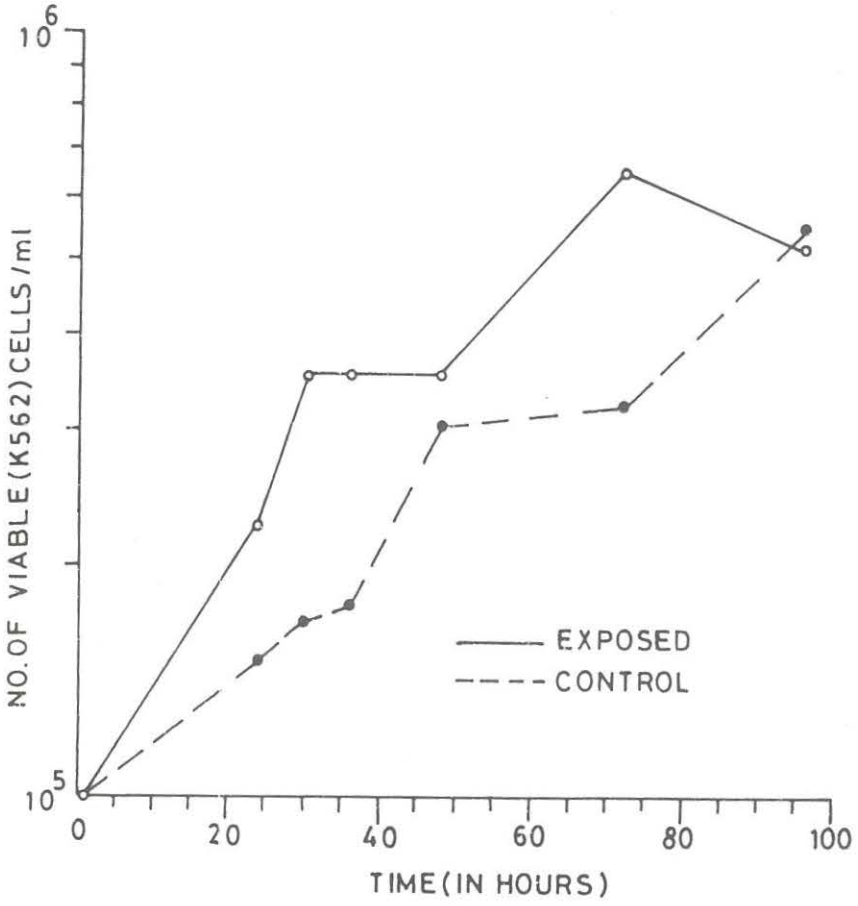


FIG.5.7 - GROWTH OF 'K562' EXPOSED TO SINUSOIDAL 76 Hz AND 0.8 mT MAGNETIC FIELD.

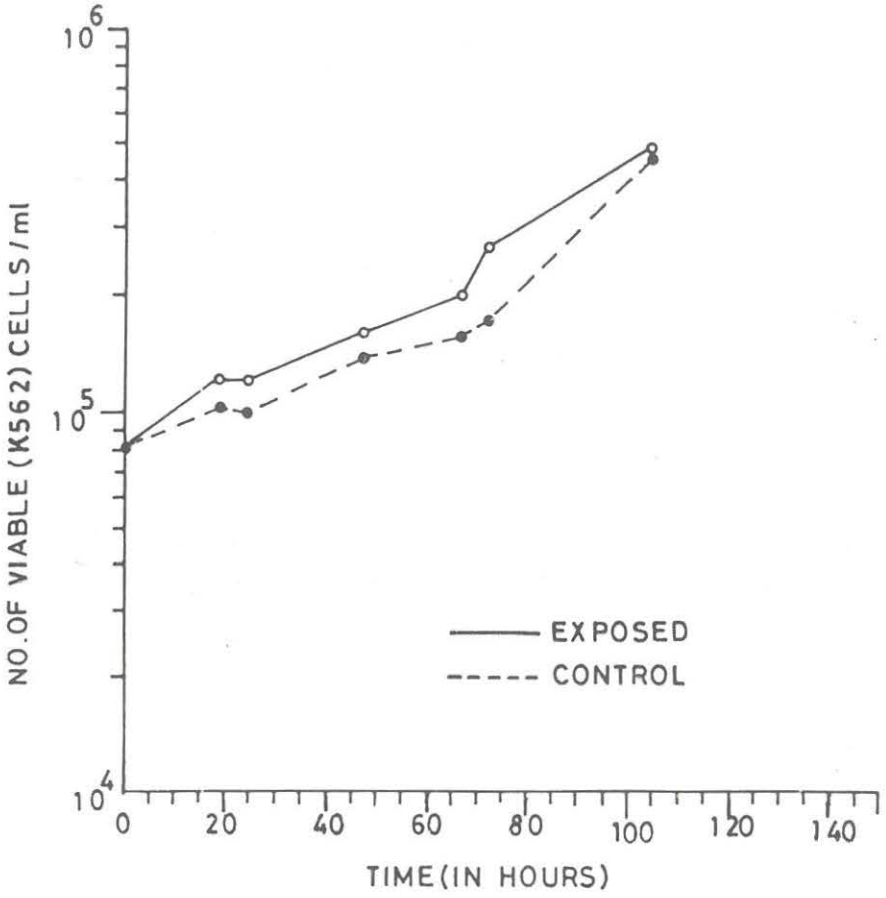


FIG.5.8 - GROWTH OF 'K562' EXPOSED TO SINUSOIDAL 500Hz AND 1 mT MAGNETIC FIELD.

Fig. 5.9 AFP cells grown under controled condition (x20000) Prominent mitochondria at resting stage and numerous ribosome in the cytosol.

Fig. 5.10 AFP cells exposed to 76 Hz and 0.8 mT MF (x12000). Lobed nucleus with chromatin condensation. Prominent mitochondria at active phase and numerous ribosomes in the cytosol.

Fig 5.11 AFP cells exposed to 76 Hz and 0.8 mT sinusoidal MF (x20000). Nuclear indentation with condensed chromatin. Prominent rough endoplasmic reticulum associated with the secretion MAb against alpha-foeto protein.

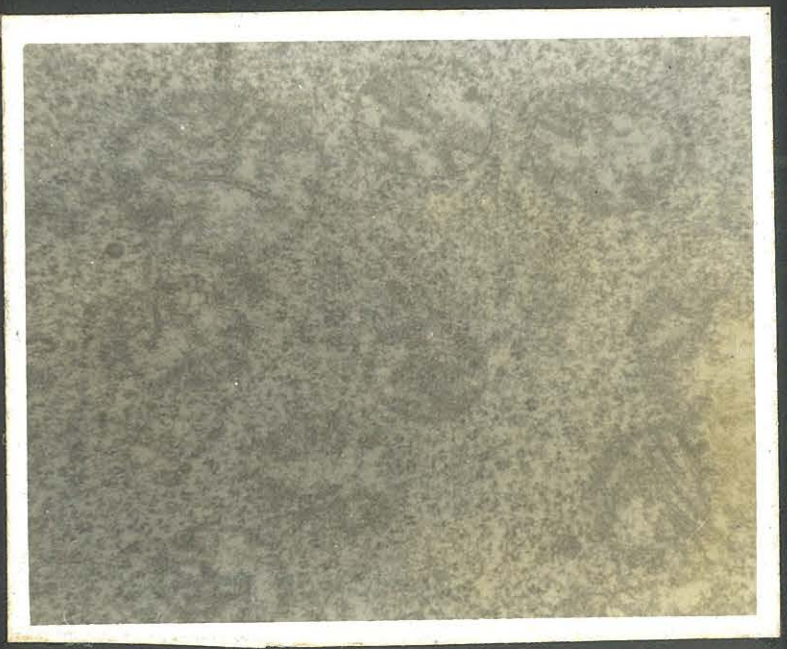


Fig. 5.12 AFP cells exposed to 76 Hz and 0.8 mT MF (x12000). Many active spherical mitochondria in the cytosol.

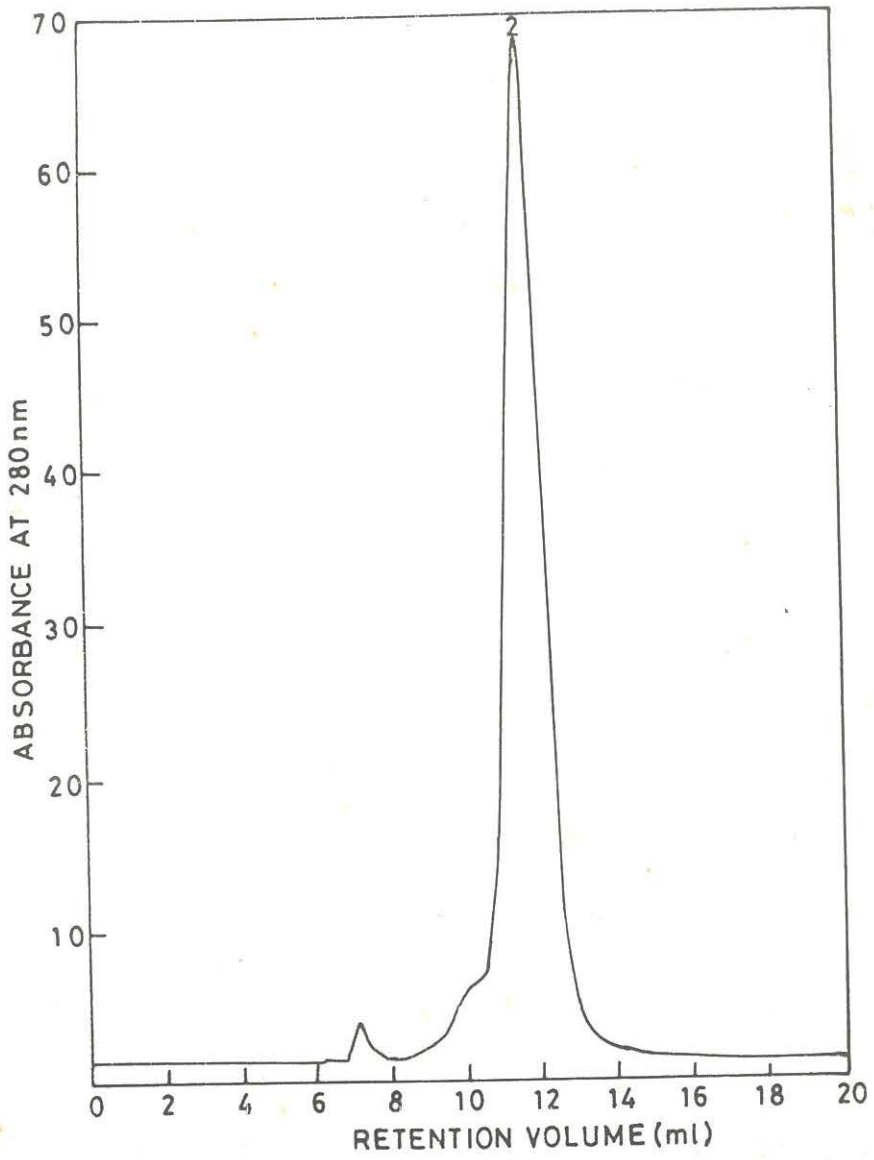


FIG.5.13-ELUTION PROFILE OF 100 µl (2·6mg) OF STANDARD IgG (NPFC) (OD:0-2; FLOW RATE: 0.5 ml/min)

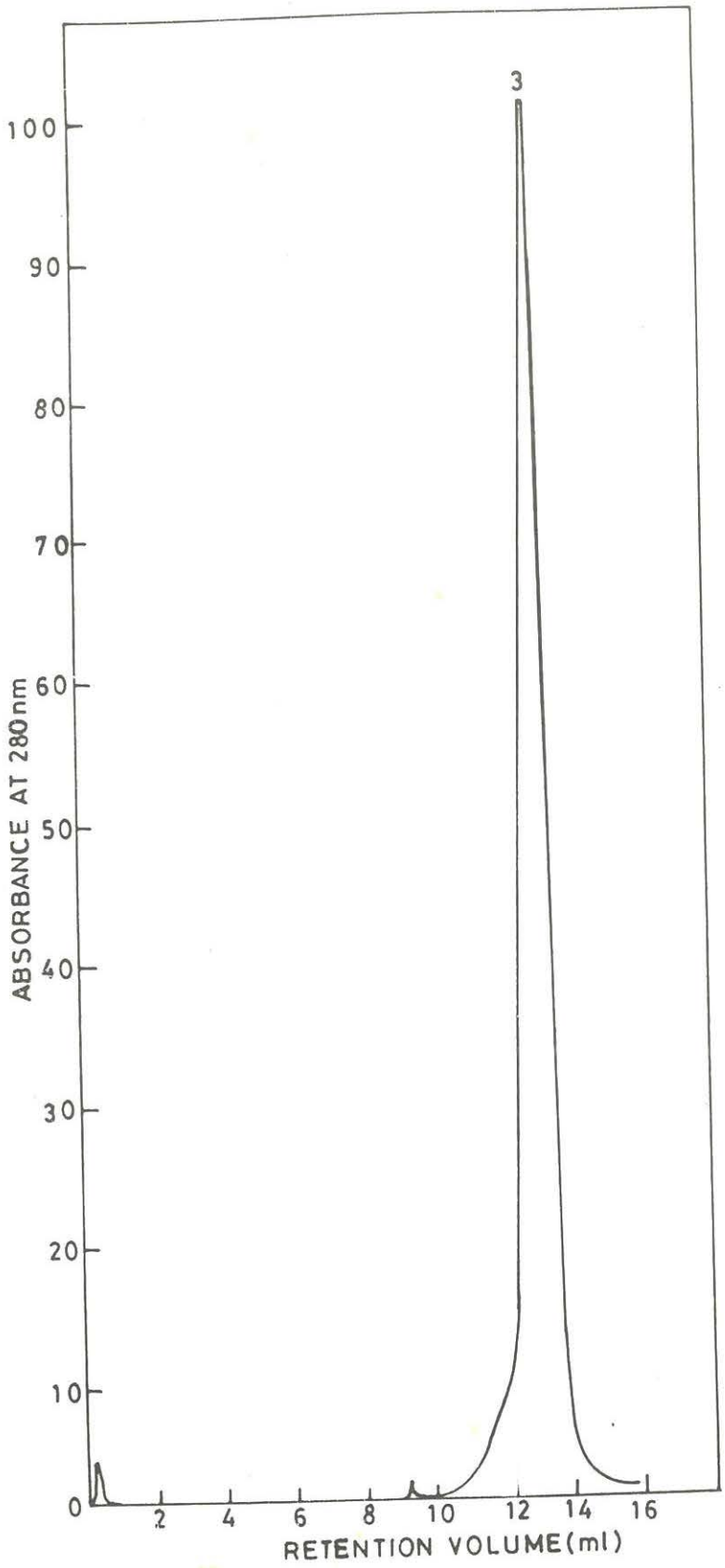


FIG.5.14-ELUTION PROFILE OF 100 µl OF ALBUMIN (1.3g/dl) (OD: 0-0.1; FLOW RATE: 0.5 ml/min)

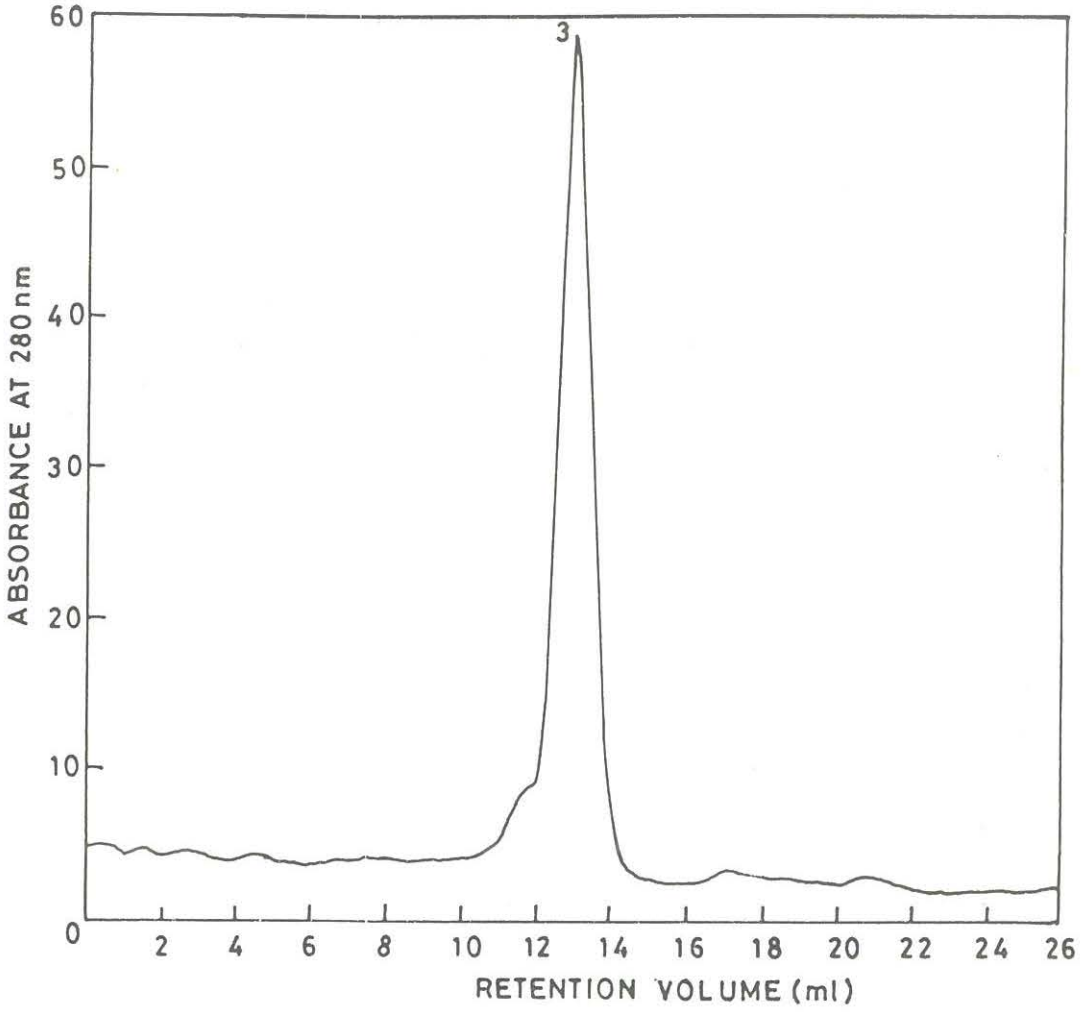


FIG.5.15 - ELUTION PROFILE OF 25 µl OF FOETAL CALF SERUM (OD: 0.5; FLOW RATE: 0.5 ml/min)

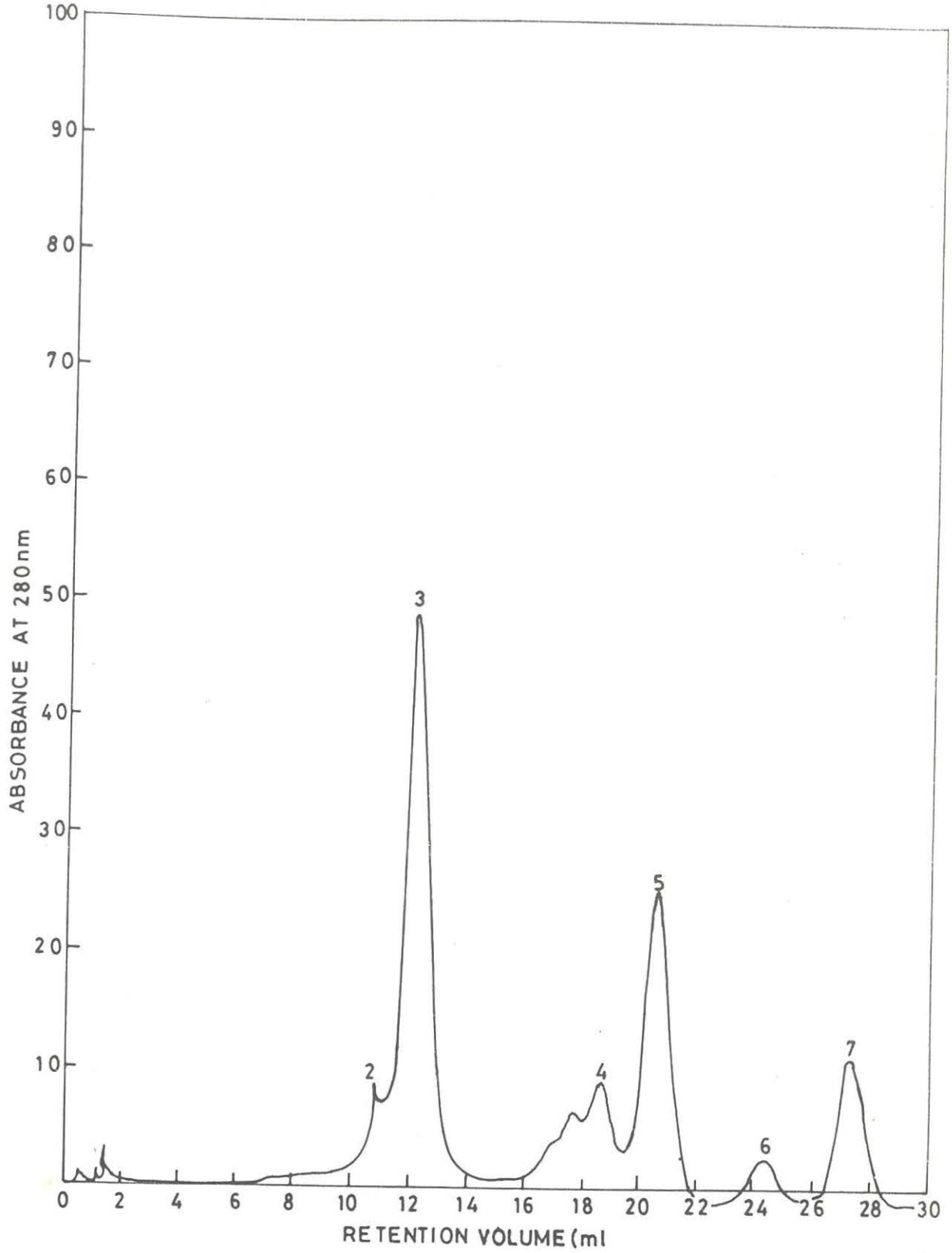


FIG. 5.16 - ELUTION PROFILE OF 100 µl CULTURE MEDIUM (80% IMDM + 20% FCS) (OD: 0-0.5; FLOW RATE: 0.5 ml/min)

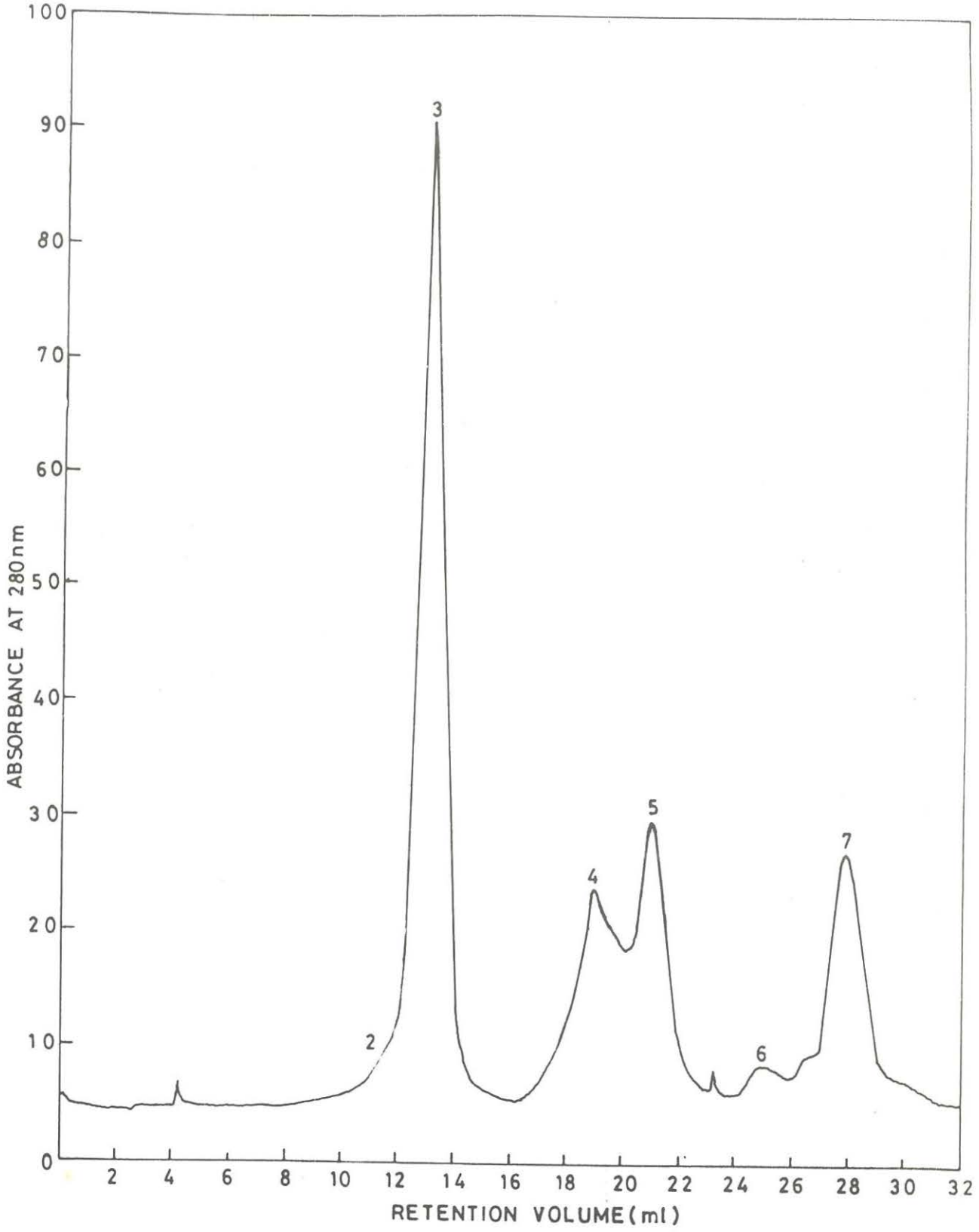


FIG.5.17 - ELUTION PROFILE OF 100 µl CLEAR SUPERNATANT OF 'OKT3' (CONTROL) (OD: 0-0.1; FLOW RATE: 0.5 ml/min)

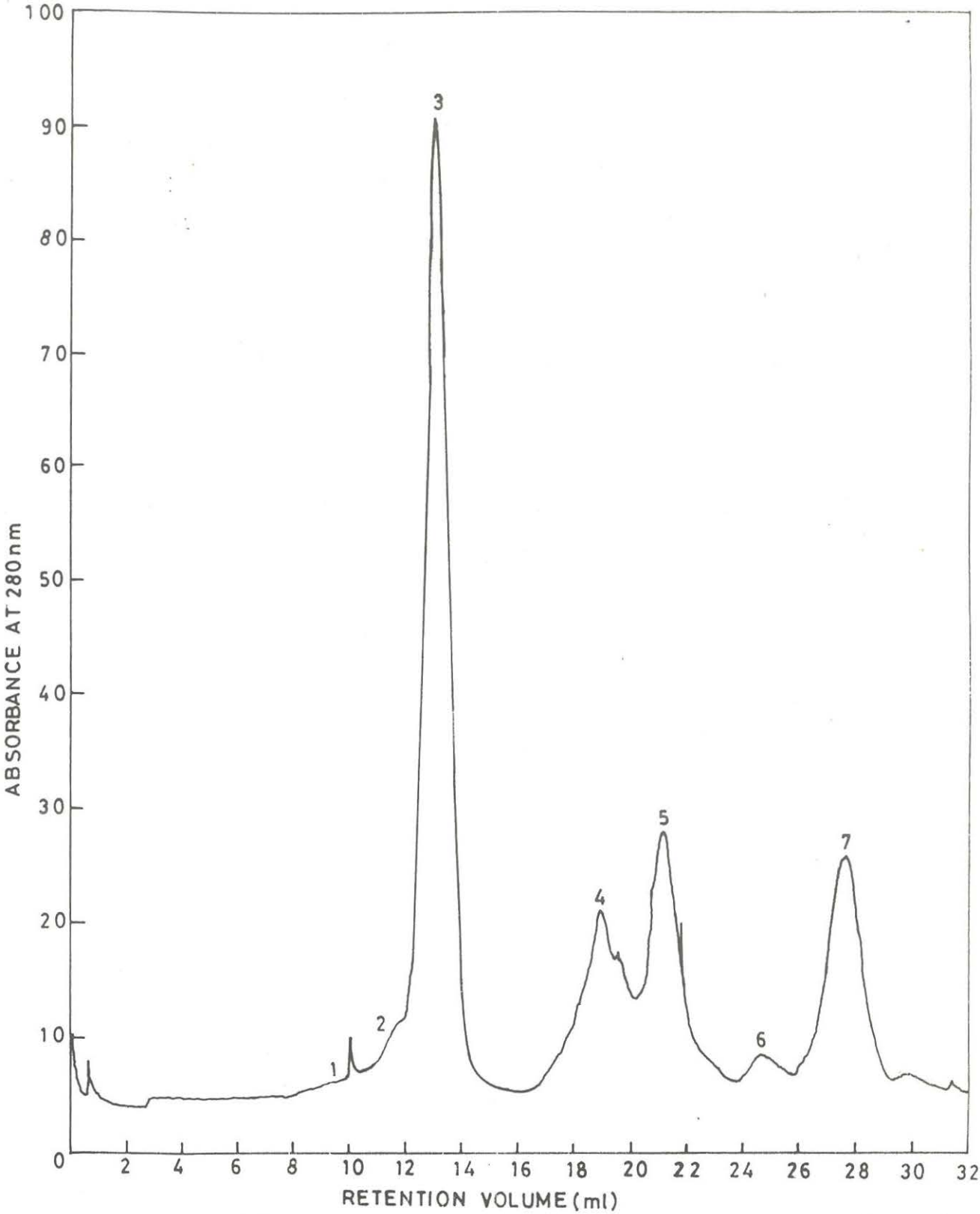


FIG.5.18 - ELUTION PROFILE OF 100 µl CLEAR SUPERNATANT OF 'OKT3' EXPOSED TO 45 Hz AND 0.8mT MF. (CULTURE TIME: 139hrs; OD: 0-0.1; FLOW RATE: 0.5ml/min)

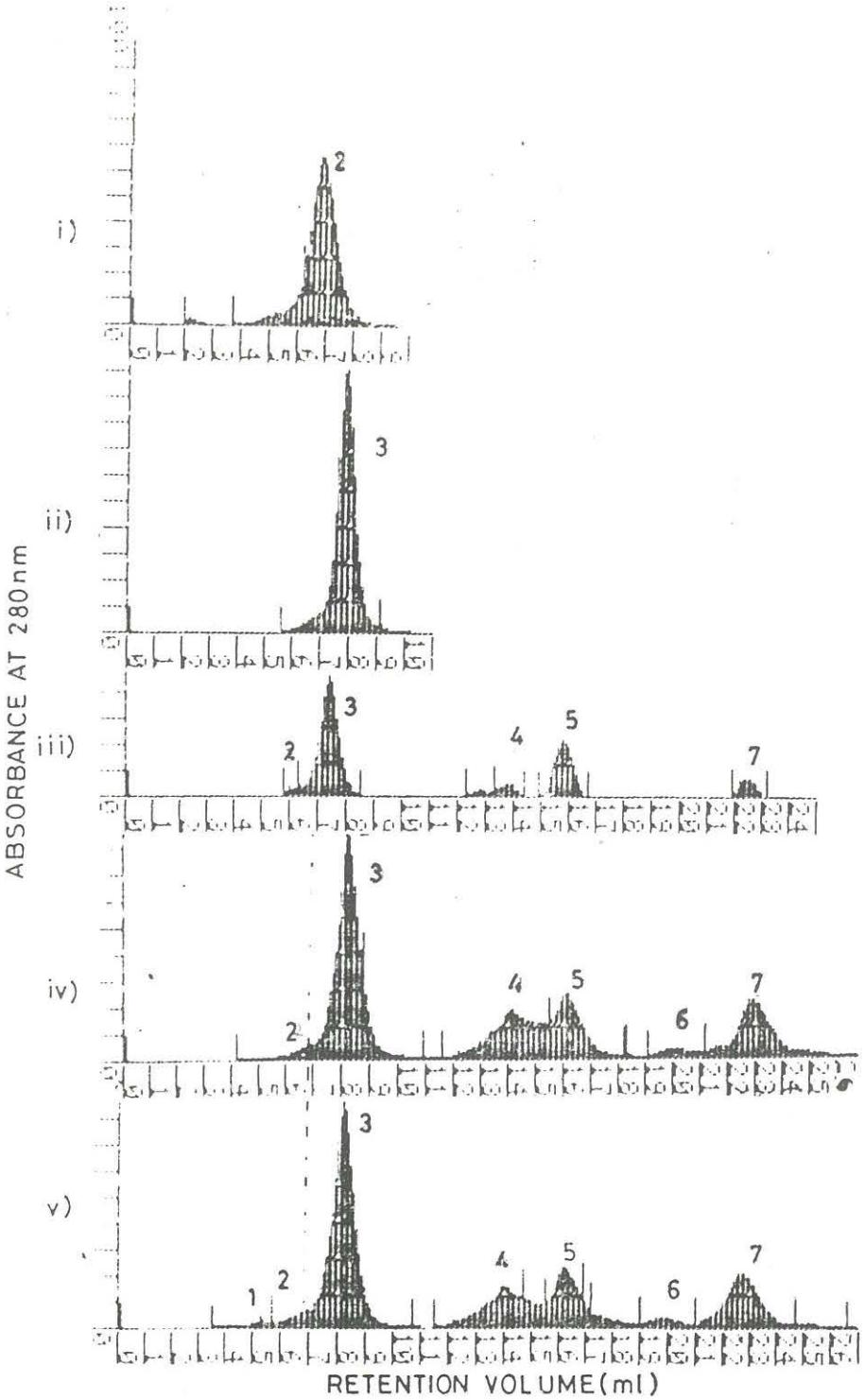


FIG.5.19- ELUTION PROFILE OF i) PURE Ig G , ii) PURE ALBUMIN iii) 80% IMDM + 20% FCS; iv) CONTROL 'OKT3' CELL SUPERNATANT AND v) EXPOSED 'OKT3' CELL (45Hz & 0.8ml) SUPERNATANT (Plot start at 5 ml)

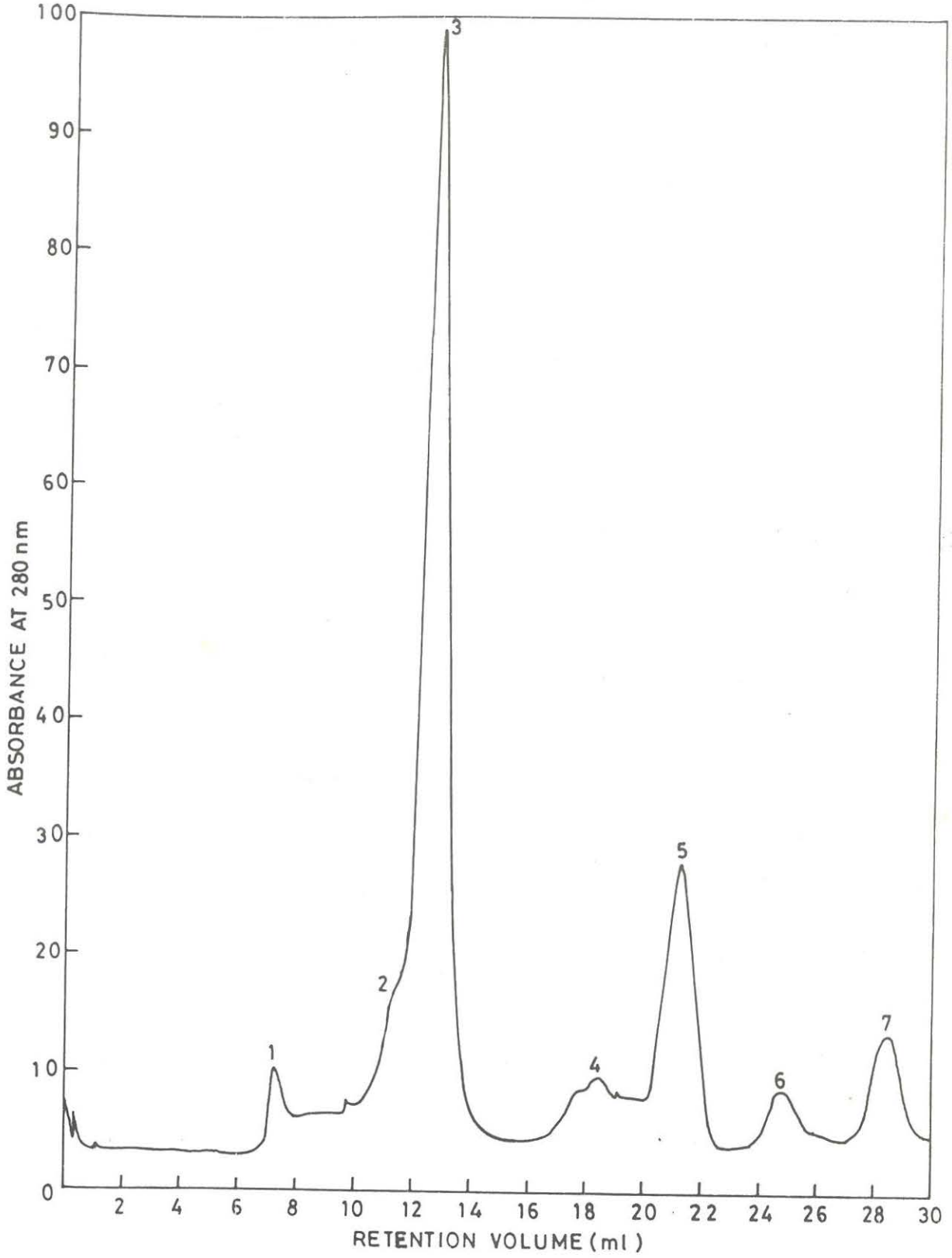


FIG.5·20 - ELUTION PROFILE OF 100 µl CONFLUENT 'OKT3' CULTURE SUPERNATANT (CONTROL) (CULTURE TIME: 96 hours; OD: 0-0.1; FLOW RATE: 0.5 ml/min)

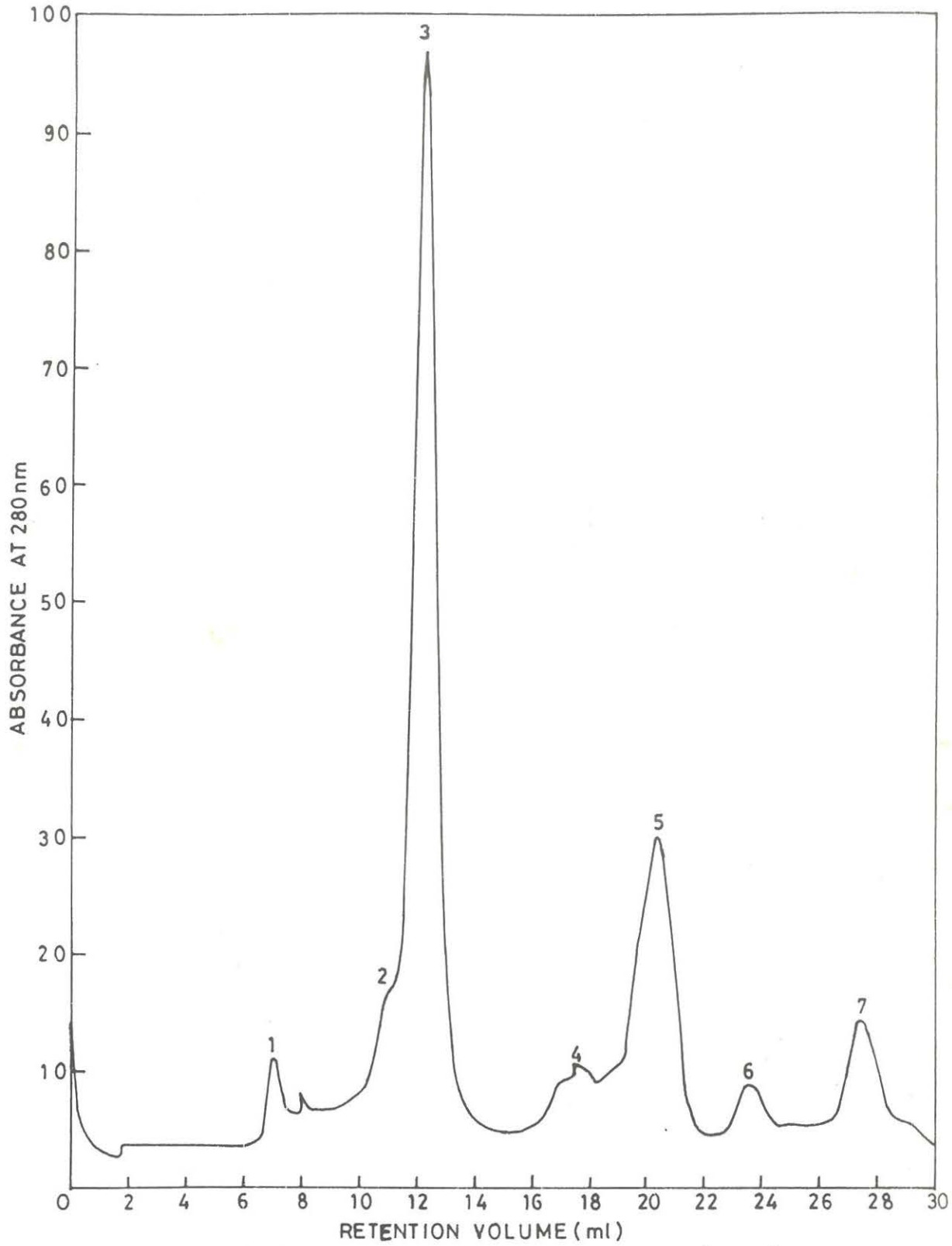


FIG.5.21-ELUTION PROFILE OF 100 µl CONFLUENT 'OKT3' CULTURE SUPERNATANT EXPOSED TO 76 Hz AND 0.8 mT MF (CULTURE TIME: 96 hrs; OD: 0-0.1; FLOW RATE: 0.5 ml/min)

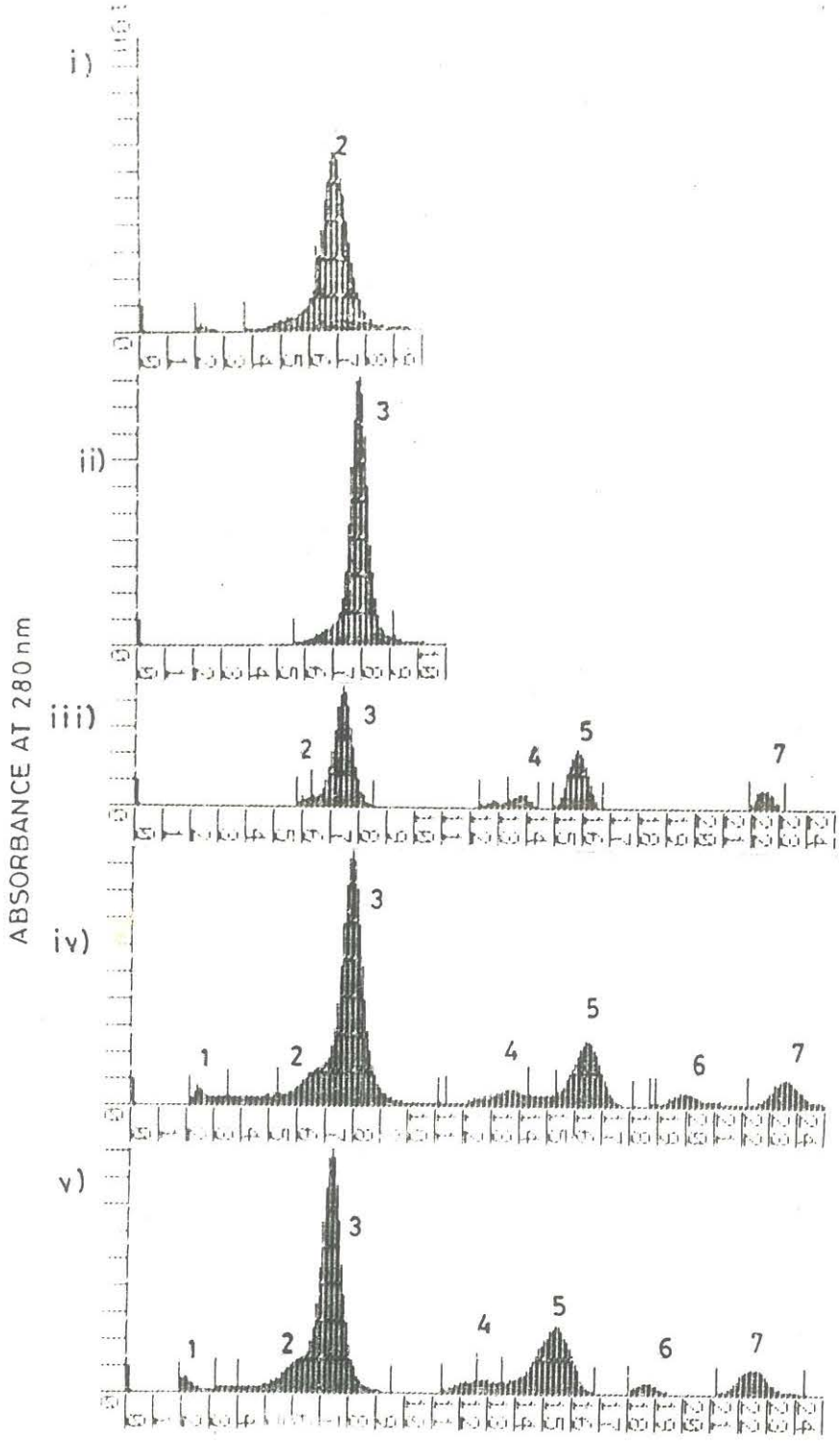


FIG. 5.22 - ELUTION PROFILE OF i) PURE IgG; ii) PURE ALBUMIN iii) 80% IMDM + 20% FCS; iv) CONTROL 'OKT 3' CELL SUPERNATANT; v) EXPOSED OKT3 CELL SUPERNATANT (76 Hz AND 0.8 mT MF) (Plot start at 5 ml)

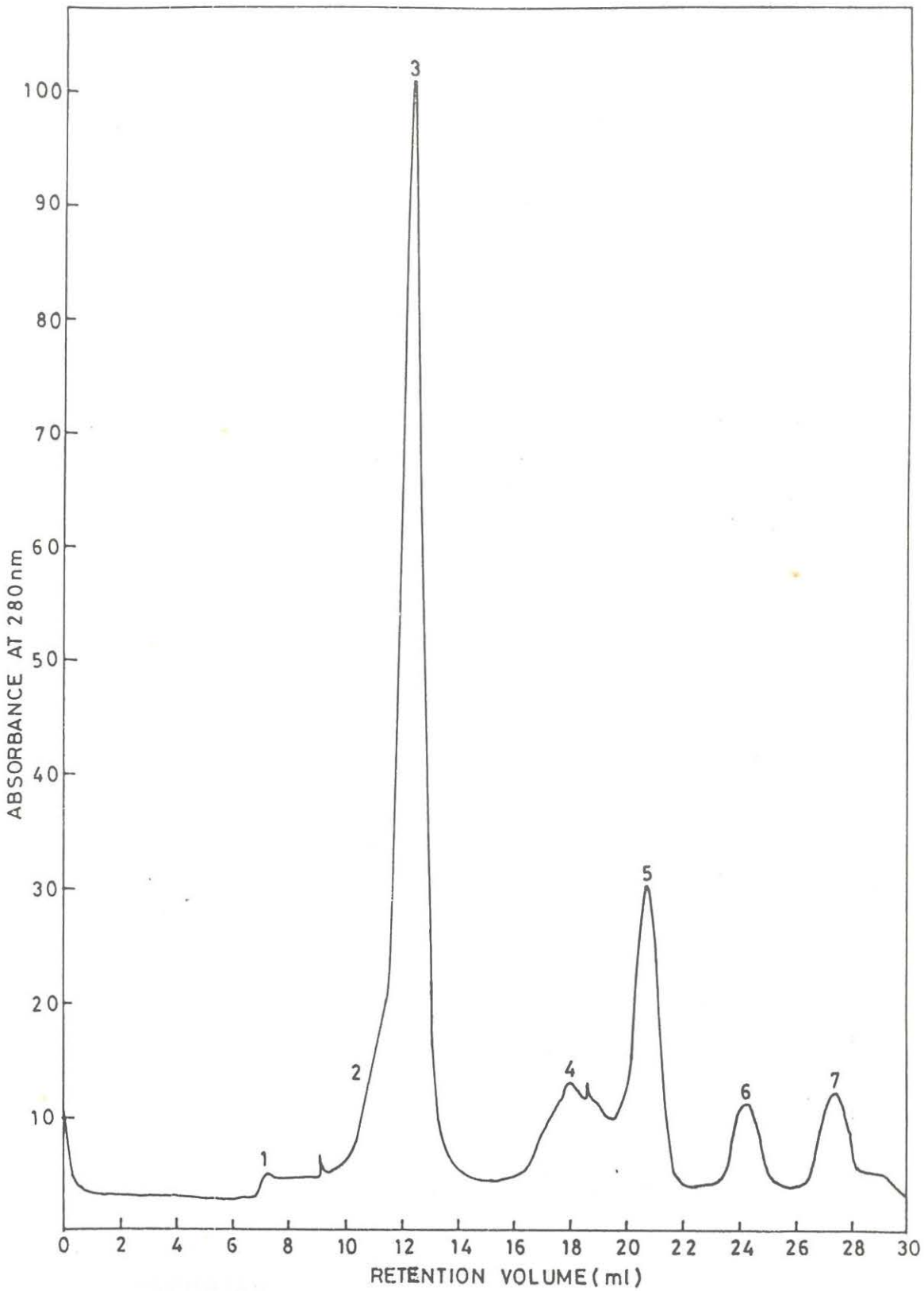


FIG.5.23- ELUTION PROFILE OF 100 µl CONFLUENT 'AFP' CULTURE SUPERNATANT (CONTROL) (CULTURE TIME 96hrs; OD: 0 - 0.1; FLOW RATE: 0.5 ml/min)

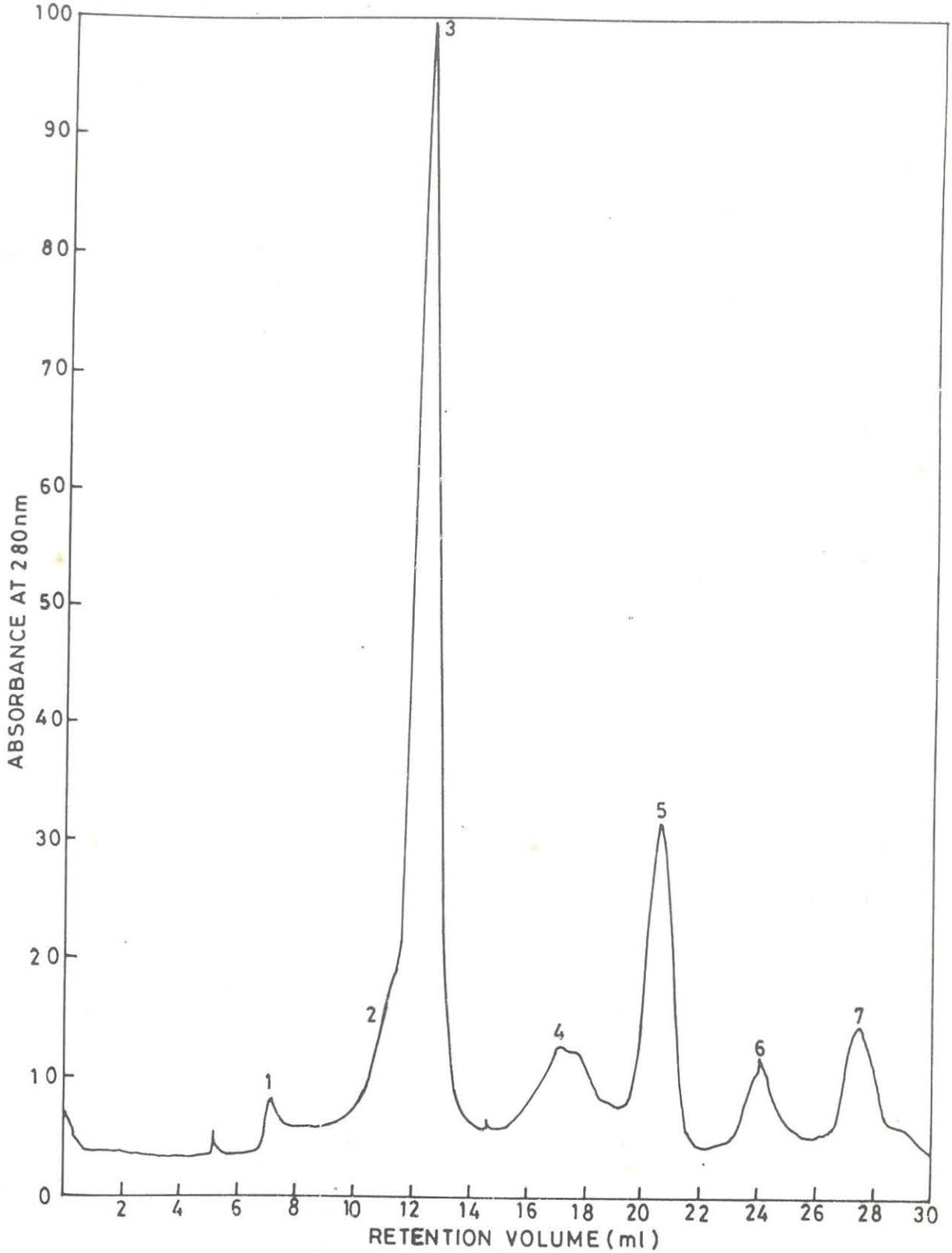


FIG. 5.24-ELUTION PROFILE OF 100 µl CONFLUENT 'AFP' CULTURE SUPERNATANT EXPOSED TO 76 Hz AND 0.8 mT MF (CULTURE TIME: 96hrs; OD: 0 - 0.1; FLOW RATE: 0.5 ml/min)

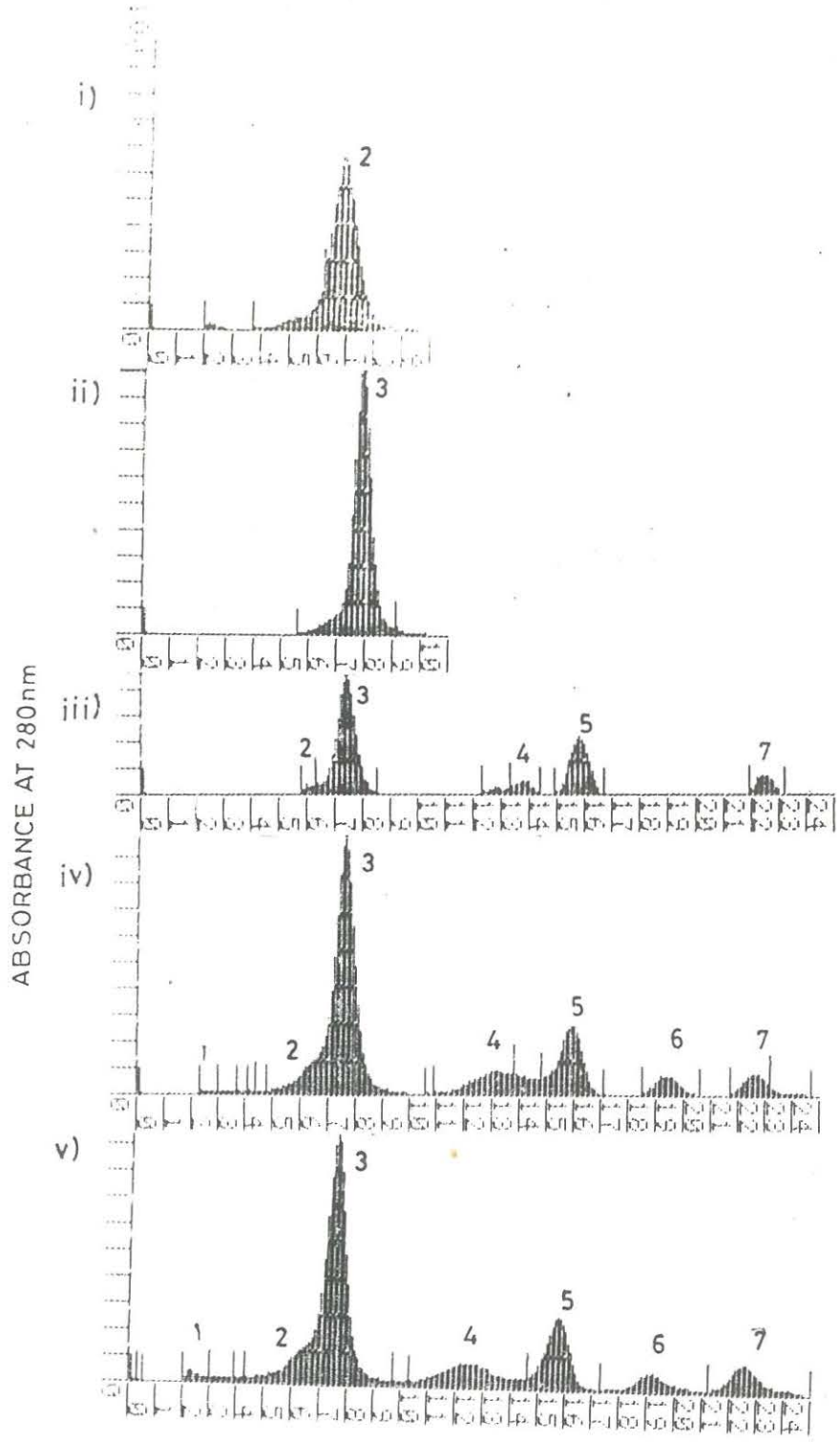


FIG.5.25 - ELUTION PROFILE OF i) PURE IgG; ii) PURE ALBUMIN; iii) 80% IMDM + 20% FCS; iv) CONTROL 'AFP' CELL SUPERNATANT AND v) EXPOSED 'AFP' CELL SUPERNATANT (76 Hz AND 0.8 mT) (Plot start at 5 ml)

CHAPTER 6

CONCLUSION

1. Hybrid (secretory) cell line AFP and OKT3 have exhibited enhanced growth and secretion characteristics when exposed to sinusoidal magnetic fields of defined parameters.
2. OKT3 exhibited enhanced growth when exposed to 90 Hz magnetic field of 0.6 mT. Similar augmentation in mitotic activity was observed at the later stage of OKT3 growth cycle when exposed to 45 Hz magnetic field of 0.8 mT and this enhanced/altered secretion characteristics was also confirmed by FPLC. Also at 76 Hz magnetic field of 0.8 mT OKT3 exhibited enhanced IgG secretion.
3. AFP clones had also exhibited enhanced growth till around 12 hours of culture time when exposed to 90 Hz magnetic field of 0.6 mT. At 76 Hz magnetic field of 0.8 mT, AFP exhibited increased growth and secretion characteristics (confirmed by FPLC) and increased survival time was reflected in prolonged plateau phase in the exposed population. However, as individual hybrid cell line has unique growth and secretory characteristics, any one parameter (which is apparently stimulatory for one cell type) may not have any desirable effect(s) on all the other cell lines.
4. K562 (erythroleukaemic non secretory cell) was included in the study to get an idea of magnetic field effects on

a non secretor vis-a-vis a secretory cell. It showed enhanced growth when exposed to 45 Hz and 76 Hz magnetic fields of 0.8 mT, and also at 500 Hz magnetic field of 1 mT. When exposed to 90Hz magnetic field of 0.6 mT, after an initial surge of significantly high growth there was a phase of prolonged growth suppression which was ultimately reflected as a extended plateau phase of cell cycle and hence reflects prolonged survival of exposed population compared to the control. Although, qualitatively somewhat different from the secretory counterpart, the initial "fast-adaptation" phenomenon of the exposed population (K562) (compared to control) is uniform, whatever be the parameters of the MF.

5. In all the studies, the experimental group was exposed to the MF of specified parameters throughout the duration of the experiment, although it is evident that the stimulatory effect on growth is intermittent. It seems interesting to study the 'in-between' phase of two adjacent mitotic phases having higher mitotic index because, this really reflects the preparatory or 'S' phase for the next division.

7. It is important to note that between a secretory and non-secretory cell line, the number of spurts in mitotic activity was usually two. The first one was around 25-30 hrs in the secretory cells compared to 30-35 hrs for a non-secretory cell. In contrast to this, the second mitotic phase in both the cell types exactly coincided with each other (around 70-75 hrs).

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APPENDIX-A

COIL FOR EXPOSING TO MAGNETIC FIELD

An air-core coil (solenoid) was used for this study (Fig.4.3). Different dimensions of the coil were chosen so that it could generate a uniform magnetic field inside the chamber and easily fit in an incubator.

The coil consisted of a PVC pipe (Finolex) of length 0.3 m and 0.136 m internal diameter. The length was so chosen that it could be comfortably accommodated inside an incubator of dimension 0.45 m x 0.45 m x 0.45 m. Similarly, its internal diameter was such that a 96-well flat-bottomed plate could easily fit inside.

Four layers (each separated from the others by means of presban paper) of enamel-coated copper wires were wound on PVC tube. Out of the four layers, two were 33 SWG whose diameter was 0.254 mm (current capacity 94 mA) and had 35.1 turns per cm. The remaining two were 36 SWG (0.193 mm dia, 54 mA current capacity) and had 45.70 turns per cm.

Resistance of each layers of the coil were 135 ,144 ,300 and 326 ohm. All four layers of the coils were connected in parallel so that the total resistance was low (50.2 Ω) and the net current capacity of the coil become 300 mA (approx.). Hence MF generated by each layer of coil was added up resulting in an adequate magnetic field inside the coil.

When the coil was activated with proper signal of 97 mA, and frequency of 80 Hz it generated a MF inside it. The field strength on the axis of the coil (the length of which was two-and-a-half times its diameter) was observed to be fairly constant in a segment of 0.1 m length (sufficient for exposing all cells seeded in a 96-well plate to uniform MF). At the ends, field strength declined rapidly (Fig.4.4). There was less than 6.25% variation in field strength across the diameter of the coil compared to maximum axial MF strength.

There was no rise in temperature inside the coil, even when the coil remained active (at 64 mA) for 7 days. This indicated that the coil can be used for cell culture in side it.

One important factor in animal cell cultures is sterility. So, materials for fabrication of the magnet were so chosen that the culture surroundings could be sterilized properly. The PVC pipe of the coil could be cleaned with alcohol. To avoid possibility of contamination from the coiled wires, the entire coil was encased in an acrylic box, which could be easily sterilized with hot water and savlon.

APPENDIX-B

MAGNETIC COIL DRIVER

The waveform for driving magnetic coil can be obtained form a waveform generator, but we need a large current drive (200 mA) and relatively large voltage swing (50 V p-p) for the coil resistance of 50 Ω. One arrangement is to have two buffer amplifier with the required output current capability, connected in voltage doubling configuration as shown in Fig.B-1.

The ckt has been realized as shown in Fig.B-2. (ckt for low current amplifier). Circuit uses LM741 opamps and medium power transistors SL100 and SK100's. A1 is configured to provide a gain of 1 and A3 is configured to provide a gain of -1.

$$Va = Vi \ , \quad Vb = - Vi$$

Complementary pair transistors Q1 (NPN type, SL100) and Q2 (PNP type, SK100) are connected as class-B push-pull output stage for opamp A2. R3 and R4 are short-circuit protection resistances. Opamp A2 and transistors Q1 and Q2 together will act as a power opamp. This opamp is connected to provide a gain of 1, and consequently this circuit exhibits negligible cross-over distortion. We get

$$V01 = Va$$

Opamp A4, and transistor Q3 and Q4 also act as a unity gain power buffer, and we get

$$V02 = - Va$$

If each buffer section has a p-p voltage swing of Vp, then

across the load we get a voltage swing of 2 Vp.

The current capacity of this circuit is limited by rating of transistors, β of the transistor and the driving capacity of the opamp A2 and A4.

This ckt was assembled on a g-p printed ckt board (Fig.B-3) and used together with a signal generator and 15V dual dc power supplies. It could be used for a current output of 45 mA.

For a higher current output, we should use output transistors with a higher current rating, however that will require increased base drive from the opamp. To avoid this problem, we can use a Darlington pair or a gm multiplier as shown in Fig.B-4. In both cases, the base drive for the current drive is greatly reduced.

$$I_B = \frac{I_C}{\beta_1 \beta_2}$$

However, in case of a Darlington pair, the base-emitter voltage drop is doubled, reducing the output voltage swing. Therefore we prefer a gm multiplier ckt. The high-current driver ckt as shown in Fig.B-5 similar to the previous ckt, except that the transistor in the output stage are replaced by composite transistors using gm multipliers. Also the first stage has a selectable gain of 1,2, and 5. Capicitors C1 and C2 (~ 68 pf) are used for frequency compensation, i.e. for suppressing high frequency oscillation in the two output buffer stages.

A PCB layout was prepared and circuit fabricated (Fig.B-6). The p-p output voltage swing across the load of this ckt (with + 15 V, - 15 V supply) is 54 V at no load and a current of 200 mA can be drawn without appreciable heating of output transistors.

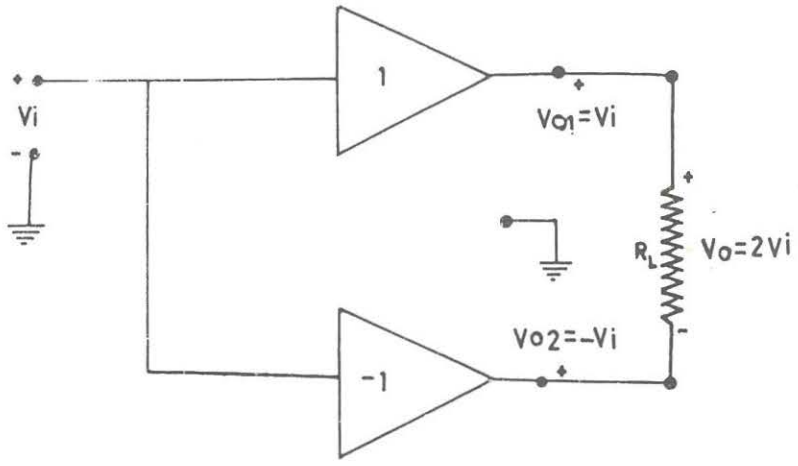
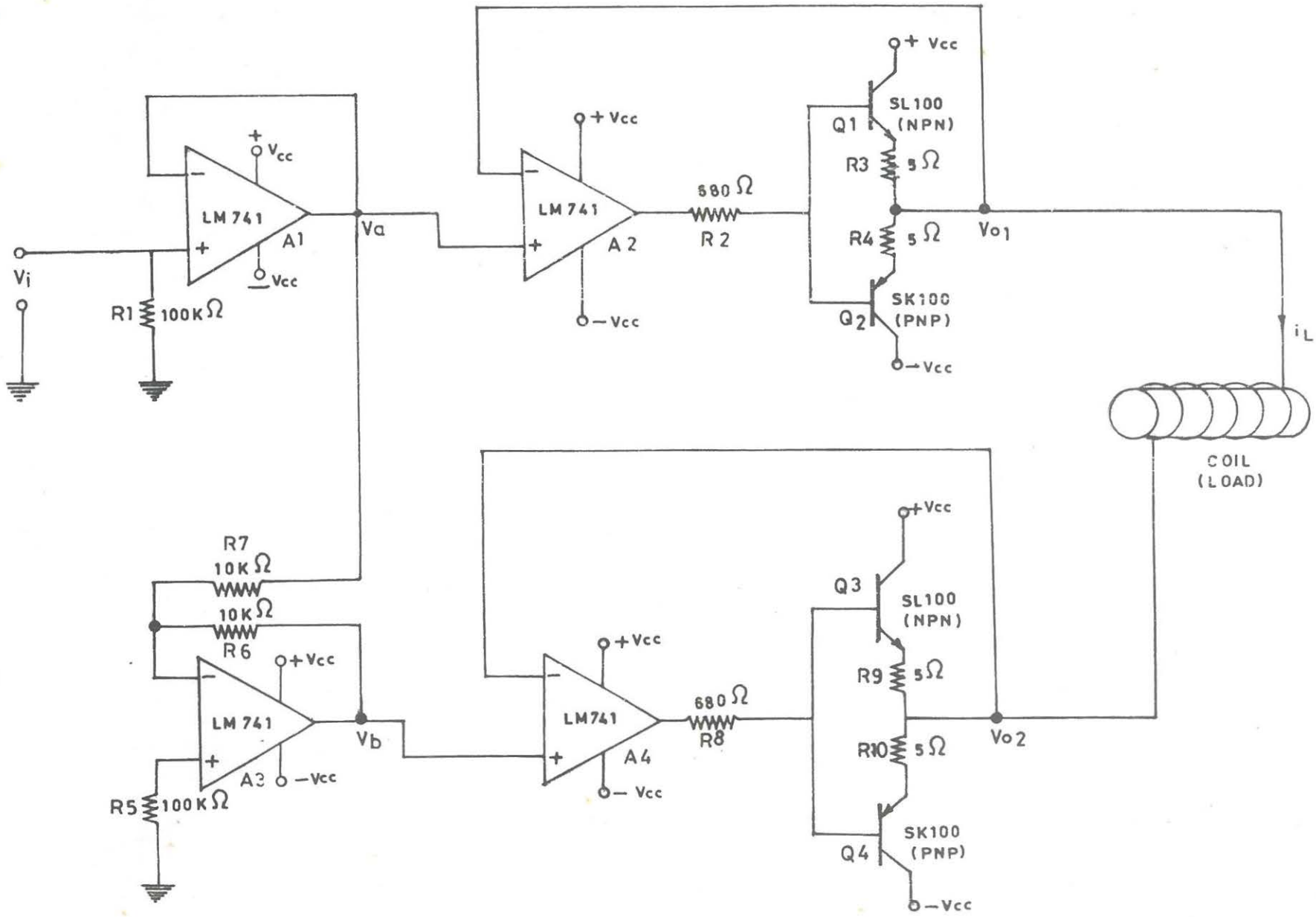


FIG. B-1 - VOLTAGE DOUBLING CONFIGURATION OF TWO BUFFER AMPLIFIERS.



B-2 LOW CURRENT (45 mA) DRIVER CIRCUIT FOR COIL

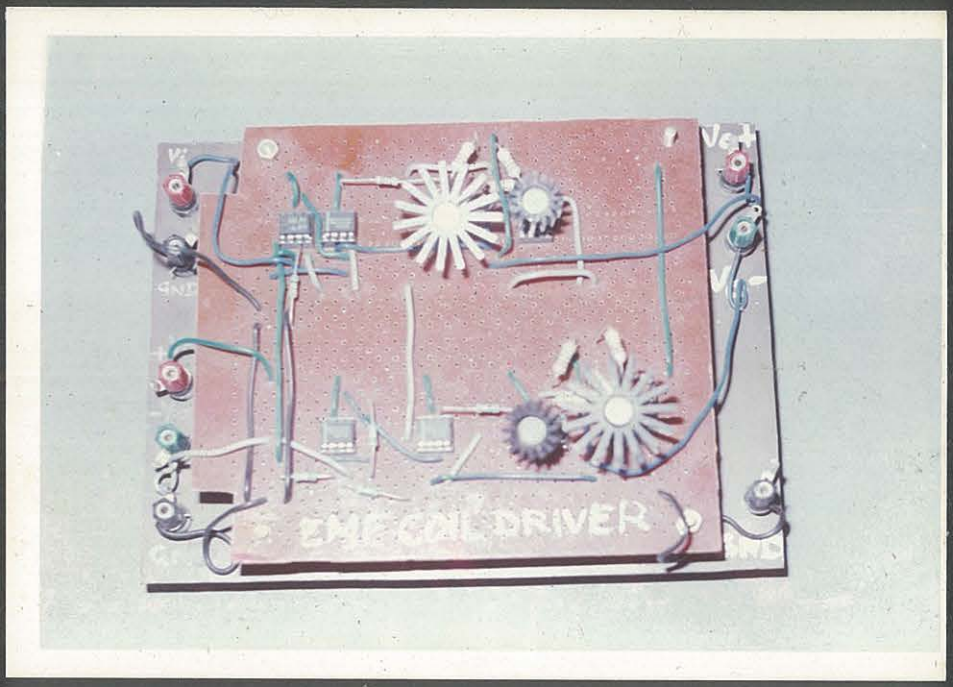
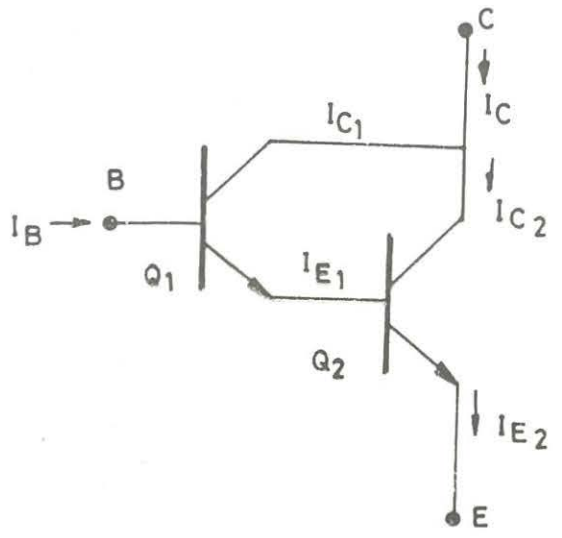


Fig. B - 3 Magnatic coil driver. This is a push pull current amplifier used for activating coil to generate up to 0.6 mT MF.

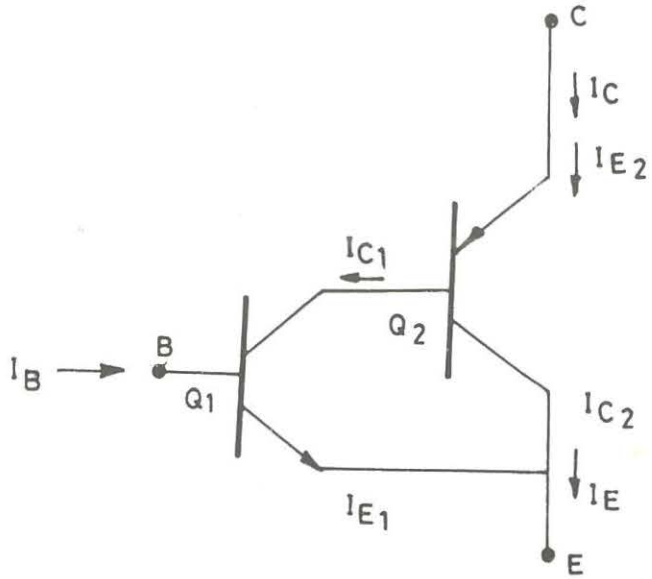


a)

$$I_C = I_{C_1} + I_{C_2}$$
$$= \beta_1 I_B + \beta_2 I_{E_1}$$
$$= \beta_1 I_B + \beta_2 [(\beta_1 + 1) I_B]$$
$$= (\beta_1 + \beta_2 + \beta_1 \beta_2) I_B$$

$$I_B = \frac{I_C}{\beta_1 + \beta_2 + \beta_1 \beta_2}$$

$$V_{BE} = V_{BE1} + V_{BE2}$$



b)

$$I_C = I_{E_2}$$
$$= (\beta_2 + 1) I_{C_1}$$
$$= (\beta_2 + 1) \beta_1 I_B$$

$$I_B = \frac{I_C}{\beta_1 + \beta_1 \beta_2}$$

FIG. B-4 - COMPOSITE TRANSISTORS.
a) DARLINGTON PAIR
b) g_m MULTIPLIER

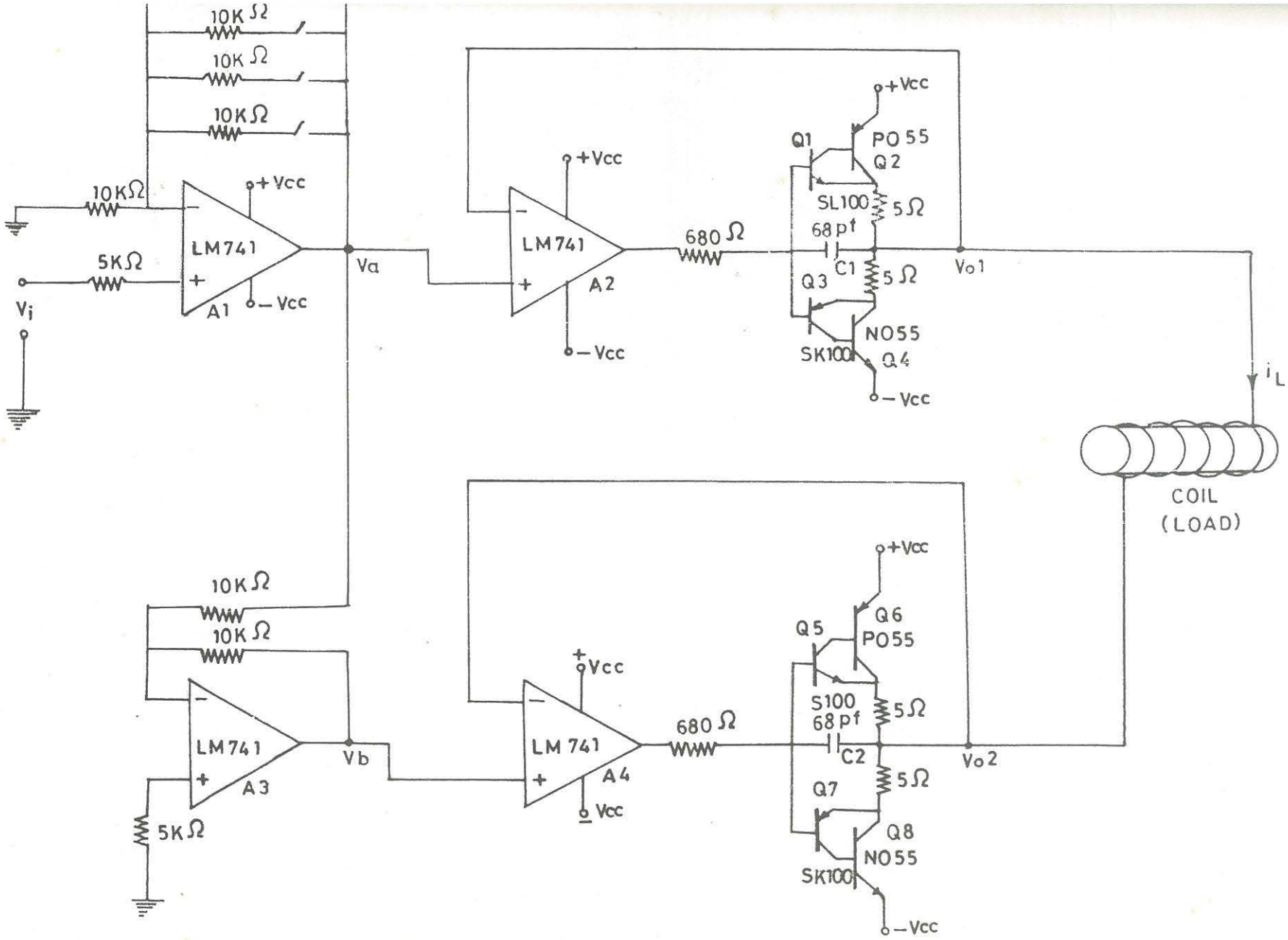


FIG.B5-HIGH CURRENT (200mA) DRIVER CIRCUIT FOR THE COIL

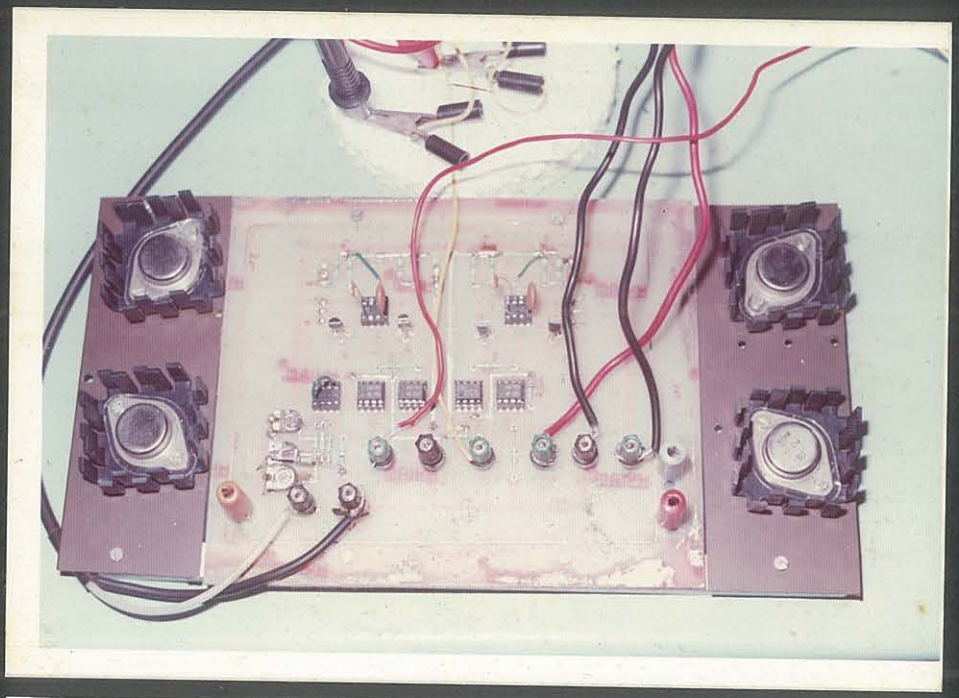


Fig. B - 6 High current magnetic coil driver.

APPENDIX-C

SODIUM DODECYL SULFATE-POLY ACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

1. INTRODUCTION

SDS-PAGE is an excellent method to identify protein. SDS an anionic detergent, bind tightly to protein at about 1.4 of SDS/mg of protein, imparting a negative charge to the resultant complexes. So under applied electric field migration of SDS derivative in polyacrylamide gel is toward the anode at rate inversely proportional to the logarithms of their molecular weights. SDS-Polypeptide thus move through the gels in predictable manner, with low molecular-weight complexes migrating faster than large one.

2. COMPONENTS OF "SDS-PAGE"

SDS-PAGE has four component i.e. the electrode buffer, the stacking gels, the resolving gels and the sample.

2.1 Acrylamide Concentrate

29.2 g of acrylamide dissolved in 0.8 g of bis-acrylamide in 100 ml of water (deionized double distilled water) and resultant was filtered through 0.45 µm membrane. 12% Acrylamide solution was used in this study.

2.2 1.5M Tris-Cl, pH 8.8, Concentrated Resolving Gel Buffer

18.2 g Tris base in 80 ml of distill water, adjusted pH 8.8 with HCl and distilled water added to a final volume of 100 ml.

2.3 0.5 M Tris-Cl, pH 6.8, Concentrated Stacking Gel Buffer

6.1 g of Tris base in 80 ml of distill water pH adjusted to pH 6.8 with HCl, and added water to a final volume of 100 ml.

2.4 10% (W/V) SDS

10 g SDS dissolved in 100 ml water.

2.5 Stock Sample Buffer

Mixed following in 4.8 ml distilled water.

0.5 M Tris-Cl, pH 6.8 - 1.2 ml

10% SDS - 2.0 ml

Glycerol - 1.0 ml

0.5% Bromophenol blue (W/V water) - 0.5 ml

SDS buffer was prepared by adding 50 µl of 2-mercaptoethanol to each 0.95 ml of stock sample buffer before use.

2.6 Catalyst

10% Ammonium persulfate (APS) prepared by dissolving 0.1 g APS in 1 ml of water.

TEMED (N, N, N′, N′ - Tetramethylene diamine)

Undiluted TEMED was used.

2.7 Electrode Buffer

0.3 g Tris base, 1.4 glycine, 1 ml of 10% SDS dissolved in 80 ml of distilled water and then finally volume made to 100 ml by adding distilled water.

2.8 Resolving Gel

Formulation of SDS-PAGE resolving gel (12%)

i) Distilled water - 3.25 ml

ii) 1.5 M Tris-Cl, pH 8.8 - 2.5 ml

iii) 10% SDS - 0.1 ml

iv) Acrylamide - 4.0 ml

v) APS - 50 µl (0.05%)

vi) TEMED - 5 µl (0.05%)

First four items was combined and deaerated under vacuum for 15 min. Then gently APS and TEMED were mixed into the deaerated monomer solution. This monomer solution was then added between the gel plates.

2.9 Stacking Gel

Formulation of stacking gel (10 ml) is as follows :

i) 0.5 M Tris-Cl, pH 6.8 - 2.5 ml

ii) Acrylamide stock solution - 1.3 ml

iii) 10% SDS - 0.1 ml

iv) Distilled water - 0.1 ml

All these solution was mixed and degassed under vacuum for 15 min. The 50 µl 10% APS and 10 µl of TEMED were added to form stacking gel.

2.10 Sample Preparation

Samples were diluted with 4 volume of SDS-reducing buffer and then heated at 95 ^{o}C for 4 min. in hot water bath.

2.11 Stain Solution

i) Coomassie Brilliant Blue- R 250 - 0.1%

ii) Methanol - 40%

iii) Acetic acid - 10%

Resultant solution was filtered through Whatman filter paper No.1.

2.12 Destaining Solution

i) Methanol - 40%

ii) Acetic acid - 10%.

3. CASTING THE GEL

Freshly prepared resolving gel aqueous isobutanol (butan-3-01 saturated with distilled water) overlayed between the glass plates and allowed to polymerized for about 30 min. Butanol was then washed off.

Freshly prepare stacking gel was poured in to the mold over resolving gel and the plastic comb was inserted to form sample wells and it was allowed to polymerized.

4. RUNNING THE GEL

Comb was removed. In the well, samples were added as follows.

Well No.2,3,4 - 50, 30 and 20 µl medium 80% IMDM with 20% FCS.

Well No.5,6,7 - 20, 30 and 50 µl of supernatant of control group.

Well No.8,9,10- 20, 30 and 50 µl of supernatant of exposed group.

To run the samples through the separating gels, upper (cathode) and lower anode were connected to a DC power supply. 24 mA/mm of thickness of gel or 30 V/cm length of gel was applied to the gel.

APPENDIX - D

EXPERIMENTAL RESULTS TABLE

Table D.1 : No. of viable OKT3, AFP & K562 cells under control condition and exposed to 0.6 mT and 90 Hz sinusoidal MF at different culture time. (Values: Mean ± Std.dev.) (n = 10)

Cell Lines	No. of viable cells in ($x10^4$/ml) at different culture time							
	0 hrs	12 hrs	24 hrs	45 hrs	62 hrs	112 hrs	144 hrs	192 hrs
1. OKT3 Control	9.3±1.0	7.6±1.0	11.7±2.0	15.7±1.0	24.5±1.0	40.4±1.0	21.6±10	-
2. OKT3 Exposed	9.3±1.0	11.0±1.0**	14.2±2.0*	20.5±1.0**	28.5±3.0**	43.6±1.0**	38.4±1.0**	-
3. AFP Control	7.1±2.0	5.1±1.0	2.0±0.0	No count	2.0±0.0	8.6±3.0	16.3±1.0#	22.5±2.0
4. AFP Exposed	7.1±2.0	9.0±1.0**	3.0±0.9**	No count	3.0±0.9**	4.2±1.0**	14.0±1.0**#	28.8±1.0
5. K562 Control	10.0±0.0	8.8±0.8	17.1±1.0	25.0±1.0	31.5±1.0	45.0±2.0	23.0±1.0	-
6. K562 Exposed	10.0±0.0	10.7±1.0**	17.5±1.0	23.4±0.8**	17.8±0.9**	29.2±1.9**	34.0±2.0**	-

* P < 0.05 # Culture time : 185 hrs.
** P < 0.01

Table D.2 : No. of viable OKT3 and K562 cells under control condition and when exposed to 0.8 mT and 45 Hz sinusoidal MF at different culture time.(Values:Mean +/- Std.dev.) (n = 10)

Cell lines	No. of viable cells in ($x10^4$/ml) at different culture time					
	0 hrs	31 hrs	39 hrs	63 hrs	106 hrs	139 hrs
1. K562 Control	10.0±0.0	6.0±1.0	16.0±6.0	44.5±4.0	58.2±26.0	78.1±11.0
2. K562 Exposed	10.0±0.0	15.6±3.0 **	23.0±4.0 **	50.2±9.0	64.0±12.0	76.3±5.0
3. OKT3 Control	10.0±0.0	13.4±6.0	19.2±5.0	46.3±8.0	67.4+19.0	24.4±7.0
4. OKT3 Exposed	10.0±0.0	19.0±4.0	17.8±3.0	52.5±7.0	60.8±8.0 *	64.4±12.0 **

* $p < 0.05$

** $p < 0.01$

Table D.3 : No. of viable cells AFP and K562 cells under control condition and when exposed to 0.8 mT and 76 Hz sinusoidal MF at different culture time. (Values:Mean +/- Std.dev.) (n = 10)

Cell lines	No. of viable cells in ($x10^4$/ml) at different culture time						
	0 hrs	24 hrs	30 hrs	36 hrs	48 hrs	72 hrs	96 hrs
1. AFP Control	10.0±0.0	14.6±0.8	12.3±1.0	12.3±1.0	6.0±1.0	5.5±1.0	2.5±1.0
		**	**	*	**	**	**
2. AFP Exposed	10.0±0.0	20.6±2.0	14.3±1.0	10.5±2.0	5.7±1.0	12.2±2.0	9.5±1.0
3. K562 Control	10.0±0.0	15.0±1.0	17.0±4.0	17.7±1.0	30.8±3.0	32.0±2.0	54.0±8.0
		**	**	**	**	**	
4. K562 Exposed	10.0±0.0	22.3±2.0	35.2±1.0	35.6±6.0	35.3±1.0	65.0±7.0	51.6±1.0

* $p < 0.05$

** $p < 0.01$

Table D.4 : No. of viable K562 cells under control condition and when exposed to sinusoidal MF of 1 mT & 500 Hz at different culture time.(Values:Mean ± Std.dev.) (n=10)

Cell line	No. of viable cells in ($x10^4$/ml) at different culture time						
	0 hrs	19 hrs	24 hrs	47 hrs	67 hrs	72 hrs	102 hrs
1. K562 Control	8.2±1.0	10.25±1.0	10.0±1.0	14.0±1.0	15.7±2.0	17.3±1.0	46.0±3.0
2. K562 Exposed	8.2±1.0	12.0±1.0**	12.2±1.0**	16.2±1.0**	20.0±3.0**	27.0±1.0**	49.0±2.0*

* $p < 0.05$

** $p < 0.01$

Table D.5 : Doubling time of the cell lines when exposed to different sinusoidal magnetic field.

	Physical Parameters		Type of cell lines	Doubling Time (Hrs)	
	Field strength (mT)	Frequency (Hz)		Control	Exposed
1.	0·6	90	OKT3	37	37
2.	0·6	90	AFP	25	68
3.	0·6	90	K562	22	32
4.	0·8	45	OKT3	20	14
5.	0·8	45	K562	18	18
6.	0·8	76	AFP	-	25
7.	0·8	76	K562	20	12
8.	1	500	K562	23	25