DIELECTROPHORETIC INVESTIGATIONS OF HAEMATOLOGICAL CELLS

PROCEDURES AND APPLICATIONS

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DEDICATION

This thesis is dedicated to my family and friends for all their support.

ABSTRACT

The dielectrophoretic phenomenon describes the translational motion of a particle such as a biological cell, in response to a non-uniform electric field. Both the magnitude and the direction of the induced movement are dependent upon the electrical properties of the cell with respect to its surroundings and thus are a function of cell composition, and vary according to the alternation frequency of the electric field. Quantification of the response in terms of the number of cells which exhibit positive dielectrophoretic behaviour, i.e. towards greatest field intensity , as a function of field frequency, enables characteristic spectra to be compiled.

Exploitation of a dielectrophoretic technique for biological analysis offers several advantages; notably that measurements are non-invasive and require no pre-modification of the cell, thus potentially permitting the separation of populations of viable cells. The clinical applications of this phenomena have to date been restricted, since methods for investigating dielectrophoretic response have relied upon manual quantification and been limited by cell sample size. Such difficulties have been minimised by the development of automated detection and analysis systems, enabling a typical collection spectra to be generated within an hour.

The development of a dielectrophoretic technique for rapid analysis of haematological cells has been described. A computer-based system was used to control voltage application to a micro-electrode chamber through which a cell suspension was circulated. The response of the cells to the electric field was detected by various optical methods and the proficiency of each experimental system evaluated. Optimal quantification of positive dielectrophoretic behaviour exhibited by haematological cells was achieved using an image-analysis system to detect cell release from electrodes subsequent to electric field application. This system permitted parameters affecting the dielectrophoretic response of physiological samples to be identified; these included electric field properties, suspension composition and the method of treatment of cells prior to experimentation. A standard protocol for investigating the dielectrophoretic response of haematological cells was defined.

Investigations were undertaken using the image-analysis experimental system to determine the dependence of the dielectrophoretic response upon bio-electrical characteristics of the cell suspension. Various treatments were carried out using the human erythrocyte as a simple membrane system. Conductivity of the suspension medium was observed to be proportional to the minimum characteristic frequency of the dielectrophoretic response, in accordance with published research. Membrane conductivity was shown to affect the maximum characteristic frequency of the response and supported theoretical descriptions of the phenomenon.

The potential applications of the technique to haematological procedures was investigated using clinical blood samples from leukaemic patients, in association with Leeds General Infirmary. Results suggested that the phenomenon could be exploited to distinguish between different cell populations, B and T lymphocyte sub-sets, and normal and malignant cell types. Recommendations have been made for exploiting the potential of a dielectrophoretic technique in haematological analysis and as a separation method to facilitate autologous bone marrow transplantations in leukaemia treatment.

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DECLARATION

I declare that all the work contained in this thesis is my own except where acknowledged.

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Chapter One

INTRODUCTION

Dielectrophoretic behaviour is exhibited when a neutral particle is induced to undergo translational motion by the application of a non-uniform electric field. It is generated by a specific electro-static charge effect, and was first defined in 1951 by Pohl as;

> *"the relative motion of suspensoid and medium resulting from polarisation forces produced by an inhomogeneous electric field".*

In simpler terms, an uncharged particle can be caused to move by the application of a non-uniform electric field; this phenomenon is known as dielectrophoresis.

The dielectrophoretic response can be demonstrated in organic and inorganic materials. It was first employed as a technique for brake fluid purification and for xerographic image-production (Pohl, 1978). Current interest is concerned with the possible applications of biological dielectrophoresis. These include the detection and separation of mixtures of cells on the basis of species, cell type, stage in life-cycle, disease, viability and nutritional status (Pohl, 1978). As an analytical tool, the technique provides information about the bio-electrical composition of the particle or cell under investigation, which can then be interpreted in terms of biological structure. Dielectrophoresis may also be exploited as a separation method, with many possible clinical applications; advantages include the rapidity of the process and that no prior modification of the cell is required.

The principles of the behaviour of particles in non-uniform electric fields are described in section 1.1; these are elaborated with respect to bio-electrical cell structure in section 1.2. Various methods of measuring the dielectrophoretic response are discussed together with a brief historical background (section 1.3). The justification for investigating the dielectrophoretic behaviour of haematological cells is described and appropriate research evaluated.

1.1 THE PHYSICAL PRINCIPLES OF DIELECTROPHORETIC BEHAVIOUR

Dielectrophoresis is the term used to describe the translational motion of a particle subjected to a non-uniform electric field (Pohl, 1951). Such behaviour is only observed with neutral particles such as biological cells, which possess no net overall charge although there may be local variations in charge sign and density. This phenomenon is distinct from electrophoresis, *i.e.* the movement of a charged particle in response to an external electric field. In contrast, it originates from the asymmetrical distribution of charges induced and displaced in a neutral particle by a non-uniform electric field. These polarisation processes result in motion of the particle. When the strength of this response differs between suspensoid and solvent particles then one particle type is displaced with respect to the other, and dielectrophoresis is said to occur.

The general equation describing the dielectrophoretic force, F, acting to cause the motion of a neutral particle of volume, v, as a result of its position in a non-uniform electric field is;

$$F = pv\,(E.del)\,E$$

(Pohl, 1951)

where p is the effective polarisability of the suspended particle, v is the particle volume, E is the local root-mean-square electric field and del is the del vector operator. Thus, the force is a function of the polarisability and volume of the particle, and the electric field surrounding the particle in all directions.

The polarisation processes induced in a particle subjected to an electric field are characteristic to the particle itself, and are critical in determining its dielectrophoretic response. This property is frequency dependent, therefore the dielectrophoretic force and motion of the particle will vary according to the frequency of current generating the electric field. The dependency between dielectrophoretic response and electric field frequency is highly specific for each particle; this may potentially be exploited as a non-invasive technique for the characterisation of biological cells.

Dielectrophoretic behaviour is common to both organic and inorganic materials, and is exhibited by all neutral matter subjected to non-uniform electric fields. For simplicity the fundamental basis of the phenomenon is described with respect to any neutral particle; where appropriate, specific biological examples such as cells are presented.

1.1.1 Characterisation of Materials by their Response to an Electric Field

A material may be classified on the basis of its response to an external uniform electric field, as either a conductor or an insulator. In the former, charge movement occurs through the material, giving rise to a current. In an insulator, this motion is blocked and the material becomes polarised by the field. An electrical insulator (or near insulator) which can sustain an electric field across it, without incurring thermal damage and breaking down is known as a dielectric (Grant *et al.*, 1978; Bergethon & Simons, 1990).

All biological material may be considered as dielectrical as a result of its capacity to form dipoles upon exposure to an external electric field.

1.1.2 Permanent and Induced Dipoles

A polarized material has a dipole associated with it which may either be permanent or induced by an applied electric field. A dipole is defined as the force due to the separation of two equal and opposite charges, therefore maintaining overall electrical neutrality. Dipoles can be formed in molecules (and consequently in cells) without the application of an external force, due to the differing electro-negativities of their constituent ions. Polar molecules exhibit permanent dipoles - this is a characteristic of all biological molecules. The magnitude of the dipole depends upon the size of the particle and the charge distribution over it (Grant *et al.*, 1978; Bergethon & Simons, 1990).

1.1.3 The Behaviour of Neutral and Charged Particles in Static and Alternating Electric Fields

The dielectrophoretic force is a consequence of the sum dipole occurring in a net uncharged particle, such as a cell, resulting from its permanent dipole (if present), and that due to the application of a non-uniform electric field (Pohl, 1978; Pethig, 1979). For example, consider the effect of simple electrostatic forces acting upon charged and neutral particles in an electric field (fig. 1).

In a uniform electric field, charged particles move to the electrode of opposite polarity. For a neutral particle, polarisation occurs; the induced charges are equal and opposite so that no translational motion results.

In a non-uniform field, charged particles continue to be attracted towards the electrode of opposite polarity. For the polarised neutral particle, the induced charges are unbalanced due to the convergent nature of the field. Where the electric field is strongest, (as indicated by a reduction in spacing between field lines on fig. 1), the charge density upon the particle is greatest. The force per unit area upon the particle increases with field strength, resulting in net movement towards maximal field intensity. In non-uniform fields, the direction of motion of charged particles is dependent upon the direction of the applied field; for the movement of neutral particles, it is always towards increasing field strength independent of electrode polarity.

Reversal of the electrode polarities, and therefore of field direction, causes a charged particle to migrate in the opposite direction to that exhibited previously. Thus an alternating current (a.c.) source will cause the direction of motion to change in phase with the frequency of the current. If the frequency of the applied voltage is increased, then a maximum threshold frequency will be attained where inertia and other forces act to prevent movement, and the particle becomes stationary. This change in direction of motion is not observed with neutral particles. Reversal of current, together with the corresponding alteration of field direction, has the effect of reversing the orientation of the dipole created in the neutral particle. The imbalance of charges is unaffected, so that the particle will still move towards the greatest field intensity. This action is limited by the speed of polarisation of the particle, *i.e.*, if a.c. frequency is increased, a threshold level will be attained where reversal of dipole moment cannot remain in phase with the changing current, and dielectrophoretic motion can not occur.

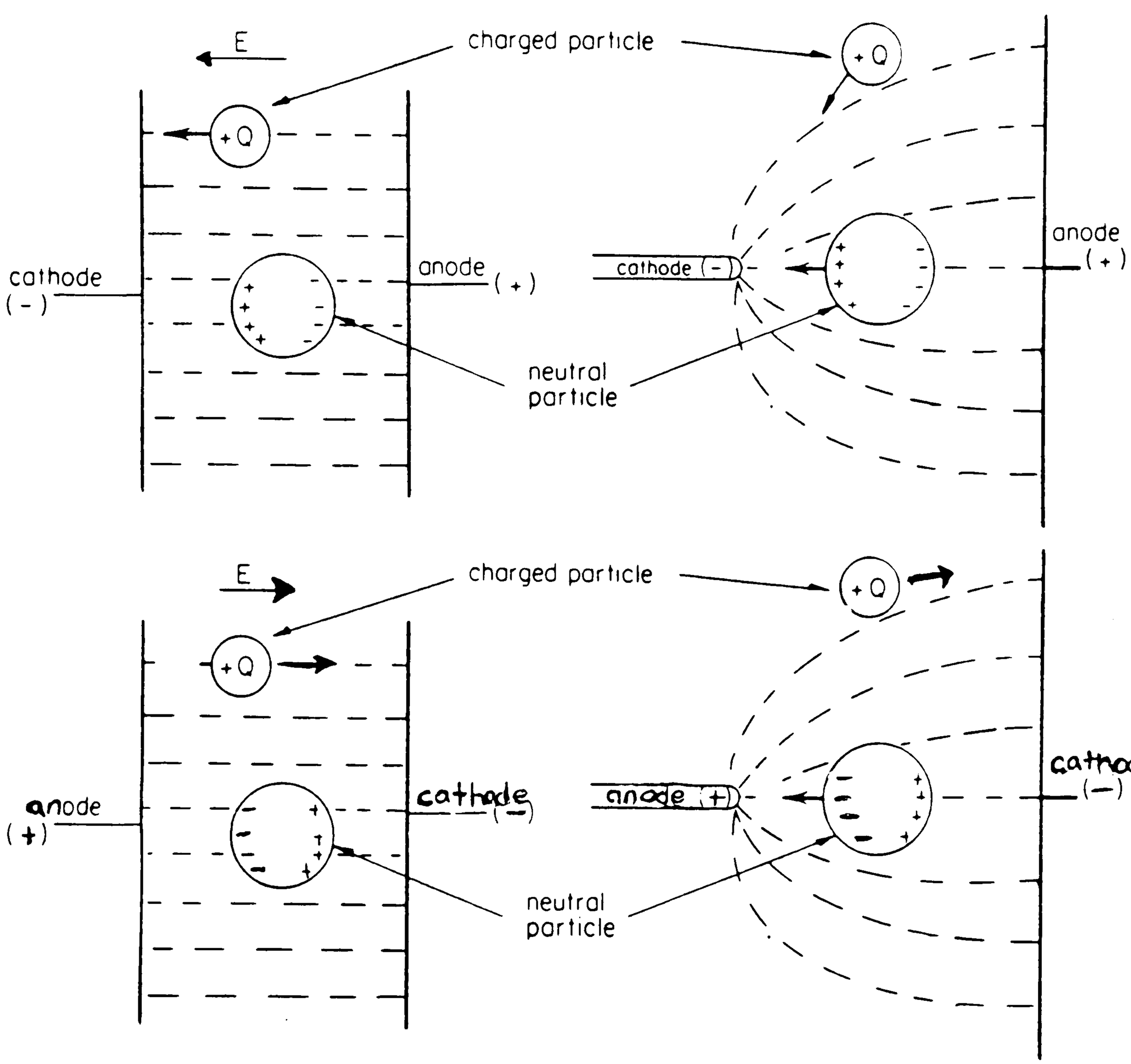


Figure 1: The behaviour of neutral and charged particles in electric fields.

The simple explanation of dielectrophoretic behaviour described so far states that particle movement is always directed towards the region of highest electric field intensity. The real situation is slightly more complex as both suspended particles and suspending medium are effected by the application of a non-uniform electric field. The dielectrophoretic effect observed is due to the net movement of suspended particles relative to that of the medium (Barnaby *et al.*, 1988).

In all circumstances, suspended cells move to the position at which their potential energy is minimum. In non-uniform alternating electric fields the direction of particle movement, *i.e.*, relative to suspension, is determined by the comparative dielectric properties of particle and medium. The cells are described to exhibit positive dielectrophoretic collection if they are caused to move towards the high electric field intensities at electrode edges. Conversely, if the movement of suspension molecules towards the electrodes is promoted, then the cells are, in effect, repelled towards lower field strengths. The movement of cells away from the electrode surface, as determined by the relative properties of the cell and medium, and the frequency of the applied field, is termed negative dielectrophoresis (Huang & Pethig, 1991).

1.1.4 Relaxation of Dipoles

The dipole formed in response to the application of an electric field, re-disperses after the field is removed, due to Brownian motion of charged species within the particle. This process is known as relaxation. The time taken for the sample to change from a state of charge alignment to randomness is known as the relaxation time, τ. When a neutral particle is subjected to an alternating field, the relaxation time is fundamental in dictating whether the dipole can reverse in phase with the changing field, and hence if dielectrophoretic motion is observed. The critical frequency at which dielectrophoretic motion can no longer be sustained is inversely related to the relaxation time of the specific dipole involved;

$$f_R = 1/2\pi\tau$$

where f_R is the frequency of relaxation and τ is the relaxation time (Grant *et al.*, 1978).

There is also a relationship between the relaxation time of the dipole and the constituent charged species involved in the response to the applied electric field. Comparatively long relaxation times are associated with the orientation of large cellular molecules such as proteins. Dipoles derived from charge alignment within smaller water molecules exhibit shorter relaxation times, indicating increased ease of dipole formation and reversal (table 1).

Table 1: Relaxation times and inferences

τ/s	f/Hz	Reason for polarisation
10^{-14}	10^{14}	Induced dipoles of electron wave function
10^{-11}	10^{11}	Orientation of small molecules *e.g.* some ligands
10^{-8}-10^{-6}	10^{8}-10^{6}	Orientation of large polar molecules *e.g.* proteins in water

(Grant *et al.*, 1978)

1.1.5 Dielectric Dispersions And The Implications For Biological Structure

The response of a dielectric material to an alternating electric field may be described by consideration of two parameters - conductivity and permittivity. The conductivity (σ) of a substance is a measure of its ability to transport charge within an electric field; permittivity (ε) conveys its proficiency in storing electrical charge and is proportional to capacitance (Grant *et al.*, 1978; Schwan, 1985a, 1985b; Holzel *et al.*, 1991). Both qualities are particular to the material and exhibit opposing responses to field frequency, *i.e.*, conductivity increases and permittivity decreases as a result of frequency incrementation (Kell & Harris, 1985).

Ease of dipole formation is dependent upon permittivity of the substance. Relative permittivity (ε'), or the dielectric constant, is the ratio of the actual permittivity of the material to that of free space, and is dimensionless. This property provides information about the capacitive nature of the system, *i.e.*, upon how easily it may be distorted by an electric field enabling electricity to be stored (Schwan, 1985b; Holzel *et al.*, 1991).

Electric field strength is inversely proportional to both relative and absolute permittivity. The latter is a fundamental constant describing the permittivity of free space, thus has fixed value. Electric field strength is thereby determined by relative permittivity of the material; the larger the relative permittivity the smaller the maximum field that can be supported, indicating that conduction effects take precedance over polarisation. Typical values for relative permittivity are 80 for water, in comparison with 8-10 for cell membranes, indicating the tendency towards a polarisation response in the latter (Tsong, 1992). Relative permittivity is thus inversely proportional to the extent of dipole formation in the material, which is expressed for unit volume as the dielectric polarisation.

The relative permittivity of a dielectric material decreases with increasing frequency because the motion of the molecular dipoles, as influenced by the relaxation time, can not remain in phase with the changing field, thus polarisation processes are reduced. A decline in permittivity is associated with a corresponding rise in electrical conductance through the material, *i.e.*, conductivity, by means of a short-circuit type process. This in turn indicates the extent to which the electric field is able to penetrate the material.

For example, consider a biological cell subjected to an electrical field of varying frequency. At low frequencies in the kilohertz range, charged species associated with the cell membrane are polarised by the field, creating a large induced dipole moment. The electric field is thus prevented from penetrating the cell by the shielding effect of the membrane. As field frequency is increased, membrane permittivity decreases, and the electric field progressively penetrates the cell interior; at maximum frequencies the field will pass directly through the cell without distortion *i.e.*, it will behave as a conductor (Schwan, 1985a, 1985b).

As ε' falls, energy is absorbed by the field. This results in a characteristic dispersion curve, as shown in figure two, where ε' falls from one plateau to another with increasing frequency of the applied field. The difference in permittivities between the plateaus is proportional to the square of the sum dipole moment of the material (Grant *et al.*, 1978)

Different types of dipole formation are dominant in dictating dielectrophoretic behaviour over different frequency ranges, as described in table one. The dispersion curve of relative permittivity against frequency indicates changeover between predominant dipole type, and characteristically exhibits three distinct regions (fig. 2).

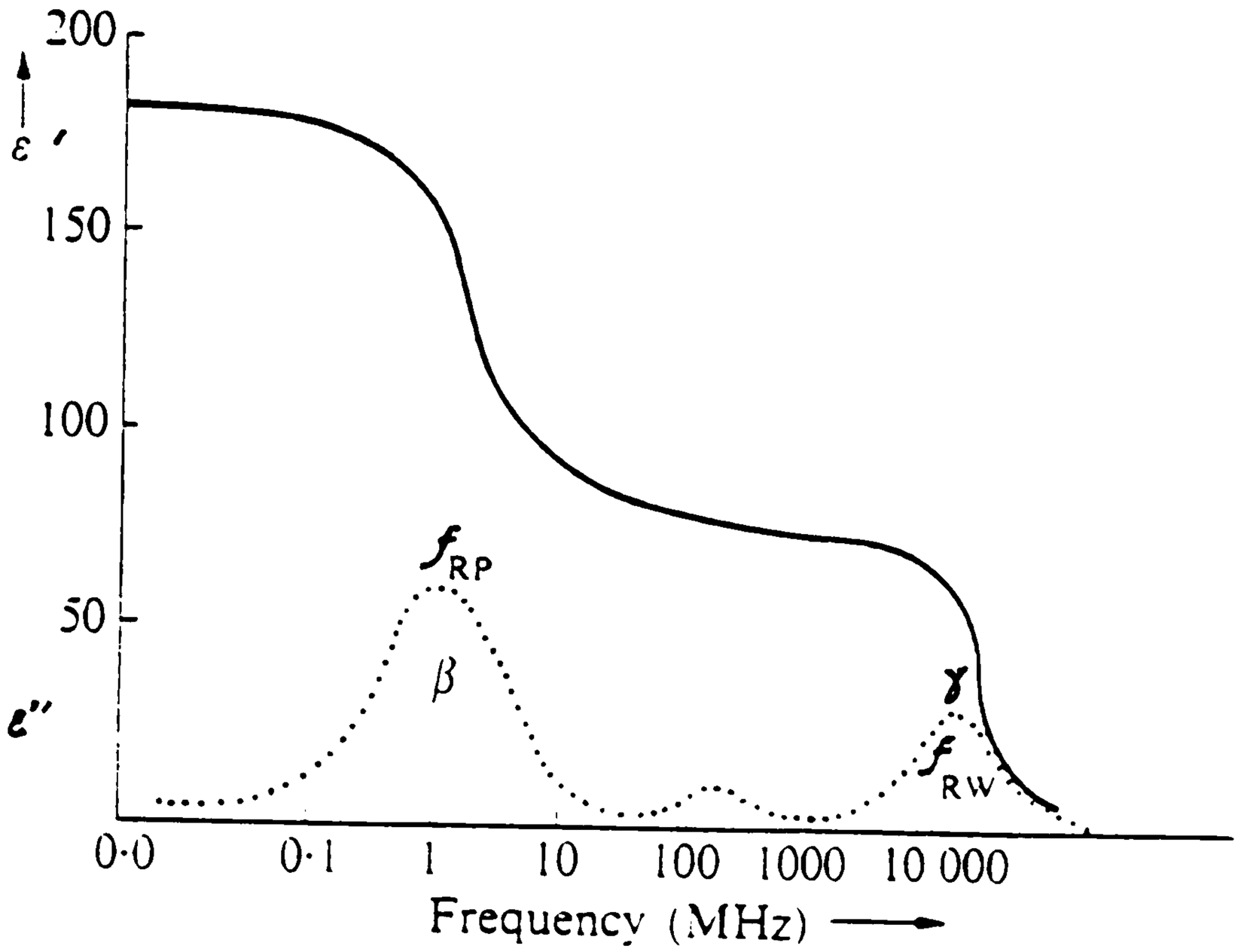


Figure 2: The dielctric dispersion curve for an aqueous solution of haemoglobin. (ε' = relative permittivity, ε" = dielectric loss, f_{RP} = relaxation frequency of protein molecules, f_{RW} = relaxation frequency of water molecules; β, α, identify dispersion regions). (Grant *et al.*, 1978).

In biological dielectrophoresis the dispersion curve may be used to obtain information about biological structure. The various dispersion regions have been correlated with particular processes by regarding the cell as a bio-electronic structure. The dispersion curve exhibited by a cell in aqueous solution, can be described in molecular terms (see Grant *et al.*, 1978). The frequency at which permittivity loss occurs identifies a specific type of dispersion category, which in turn indicates the dielectrophoretically active cellular components:

- α dispersion occurs in the 100 Hz region and is concerned with relaxations and migrations in the ionic layer surrounding the cell membrane (Grant *et al.*, 1978). This is directly dependent upon the metabolic state of the tissue; a characteristic dispersion of this nature only persists for a few hours after cell death (Holzel *et al.*, 1991).
- ß dispersion occurs around 1 MHz (Schwan, 1957, 1985). This is attributed to the inhomogeneity of the material causing the Maxwell-Wagner effect. The latter describes the effect of an electric field upon a heterogeneous medium, causing charge build-up at the various boundaries separating regions of different permittivity and conductivity (Grant *et al.*, 1978; Warren, 1987). A finite time is taken for the charges to reach equilibrium; the effective permittivity and conductivity of the medium will depend upon how this time compares with the periodic time of the field. ß dispersion indicates the structure and width of cell membranes, and reflects the static capacitance of cells (Kell & Harris, 1985). This response typically persists for several days after cell death, indicating its dependence upon more stable structural cell features in comparison to the diffuse ionic layer commanding α dispersion effects (Holzel *et al.*, 1991).
- γ dispersion may be detected at higher frequencies around 10 GHz, and is attributed to the relaxation of water molecules (Schwan, 1965; Grant *et al.*, 1978). It is not affected by cell death for an extended period (Holzel *et al.*, 1991).
- δ dispersion at frequencies in excess of 20 GHz is rarely present, and results from the relaxation of water molecules in the immediate vicinity of the biological macro-molecules (Grant *et al.*, 1978).

A theoretical basis for these processes has been developed by Schwarz (1962).

The ranges of these dispersions and their causes are summarised in figure 3.

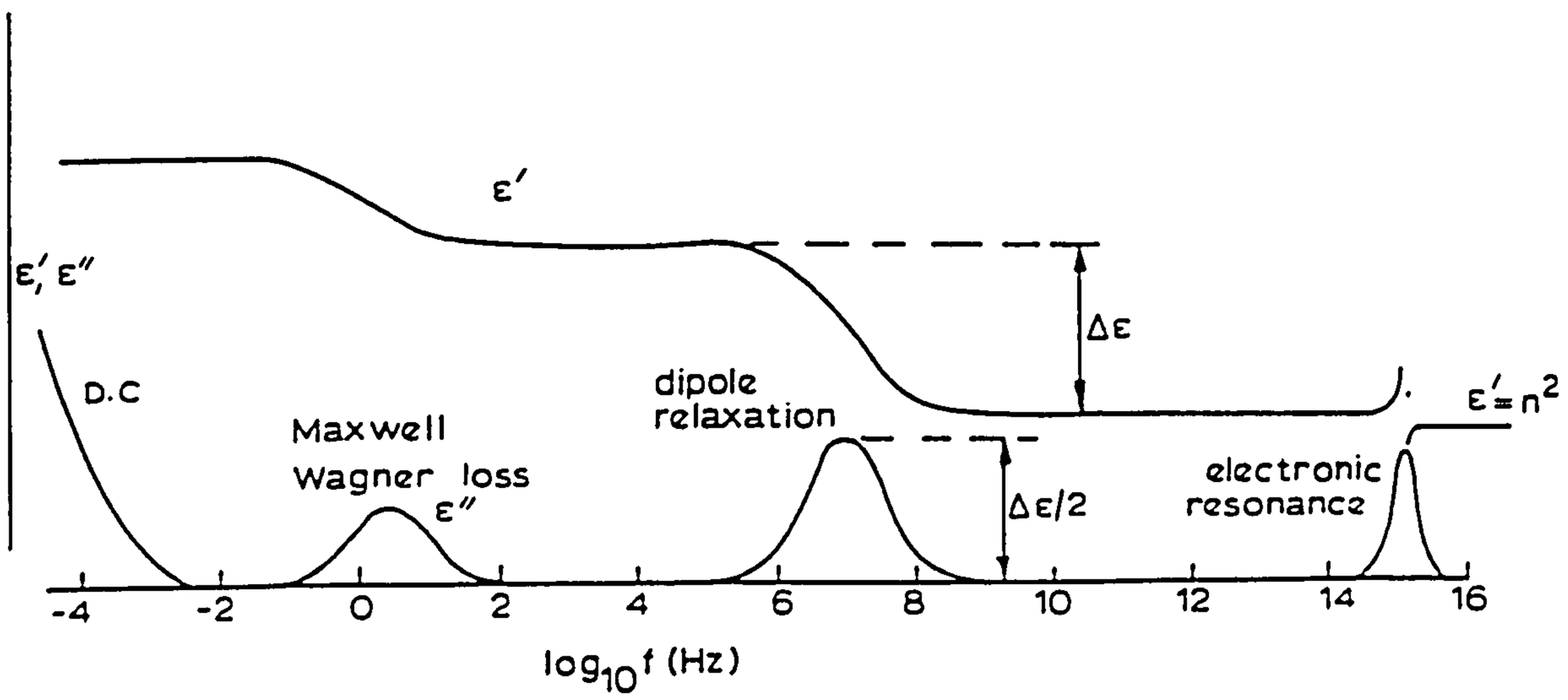


Figure 3: The dependence of relative permittivity and dielectric loss upon electric field frequency. (ε‘ = relative permittivity, ε“ = dielectric loss).

Biological particles typically exhibit a strong positive dielectrophoretic response over a frequency range of 100 kHz to 1 MHz; a weaker response is observed at frequencies below 1 kHz. These two dispersion regions are separated by either a very weak positive response or negative dielectrophoretic behaviour as defined in section 1.1.3 (Lamprecht & Mischel, 1985).

The dielectrophoretic response of a particular cell is, at its highest level of resolution, dependent upon the molecular structure of the cell and therefore is unique. Cells from the same population will theoretically differ in their responses to an applied non-uniform field, but such differences will be minimal and beyond detection by contemporary measurement instruments. This supports the potential use of a dielectrophoretic technique to differentiate between different cell types.

1.1.6 Other Electric Field Induced Phenomena

Dielectrophoretic particle behaviour, as induced by the application of a non-uniform electric field, may be accompanied by associated phenomena. These include; mutual dielectrophoresis (pearl chain formation), cell orientation, deformation (di-electrodeformation), rotation (electro-rotation) and electro-fusion.

a) Mutual dielectrophoresis (pearl chain formation)

The application of a non-uniform electric field to a sample of neutral particles can result in the alignment of particles in the direction of the imposed field, designated "pearl chain" formation, This condition results from the effect of an already polarised particle upon a neutral particle, causing the second also to become polarised (fig. 4), hence the term mutual dielectrophoresis (Pohl, 1978). A chain is built up in this way until the field strength can no longer cause the polarisation of an additional particle.

This property is of particular use as it is exploited as a method of measuring the dielectrophoretic response. The responses of various cell types under differing conditions can be compared on the basis of length of pearl chain formed within a defined time period. This is the dielectrophoretic collection rate (DCR), and has traditionally been measured by manual counting using a microscope. Quantification is as length of chain in graticule units, established over a standard period of time

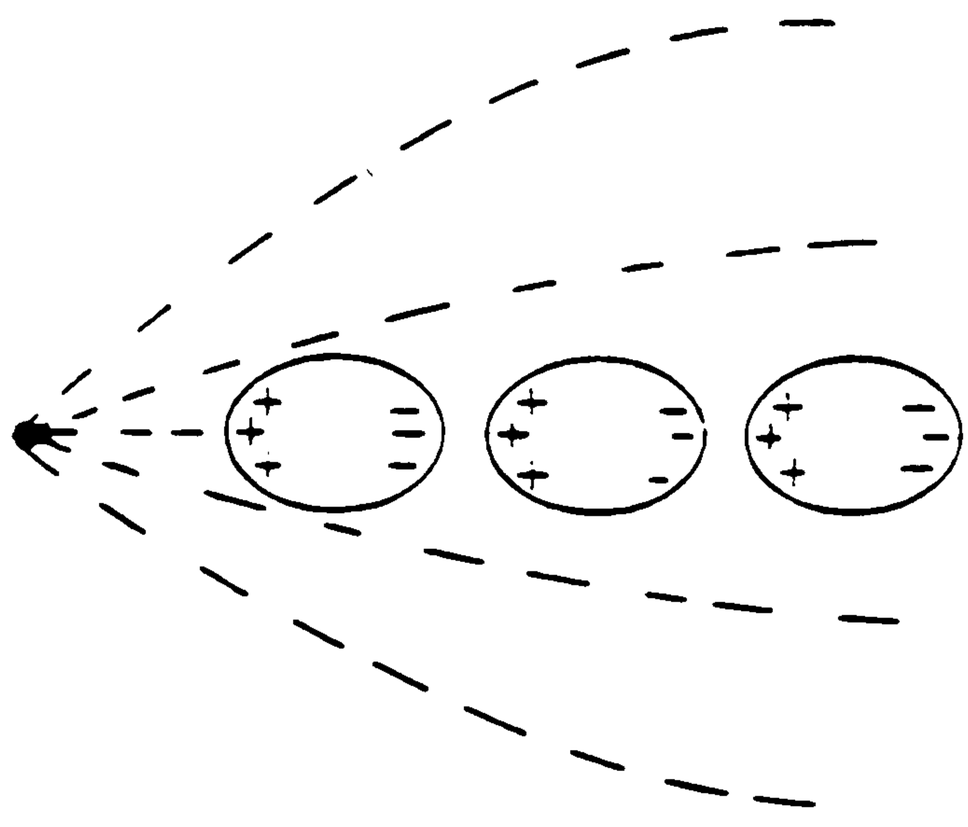


Figure 4: Particle polarisation as a result of mutual dielectrophoresis.

usually specified in minutes (Pohl, 1978). Characteristic spectra can be compiled by measuring the DCR at different applied frequencies and plotting the results.

b) Cell orientation

|In the presence of an electric field cells will move to the position at which their potential energy is minimum (section 1.1.3). Depending upon the field configuration at a particular frequency the cells may collect upon or between electrode bars. This activity may hinder cell enumeration during dielectrophoretic collection.

The frequency of an applied non-uniform electric field also effects the orientation of non-spherical neutral particles (Teixeira-Pinto *et al.*, 1960; Schwan & Sher, 1969). For elongated cells such as the phytoflagellate *Euglena*, low frequency a.c. fields cause the cells to be aligned along the field lines; at high frequencies the cells become orientated perpendicular to the field lines (Teixeira-Pinto *et al.*, 1960). Such orientation is dictated by the particle assuming the configuration of least resistance to the field, as determined by the conduction/polarisation processes induced.

This phenomenon has also been observed during dielectrophoresis of human sickled erythrocytes. At frequencies in excess of 3 MHz these cells orientate perpendicular to the electric field, and to normal erythrocytes (Vienken *et al.*, 1984). This behaviour is attributed to the decrease in membrane fluidity associated with sickle-cell formation, causing greater resistance to electric field-induced charge flow through the cell.

This alignment of non-spherical particles is a characteristic of dielectrophoretic behaviour and is distinct from a similar phenomenon known as electro-rotation.

c) Electro-rotation

The bio-electrical structure of a cell also determines its behaviour in response to a rotating electric field. This phenomenon, known as electro-rotation (or cellular spin resonance - CSR; Pohl *et al.*, 1983), describes the gyrational motion of a particle resulting from a electrically-induced rotating dipole formed within it (Arnold & Zimmermann, 1988; Glaser & Fuhr, 1986). Both direction, and frequency of spin of the particle are dependent upon whether the dipole rotates ahead of or behind the

field in the medium. The angle between these two forces results in a torque on the cell determining frequency of spin, which is much smaller than the frequency of the applied field. The behaviour of cells in rotating electric fields has been described with reference to their surface charges and structure (Fuhr & Kuzmin, 1986). In addition, the frequency at which maximum anti-field rotation is observed is determined by the charging time of the membrane and is correlated with the pathological state of the cell (Arnold & Zimmermann, 1988).

d) Electro-fusion

Cell fusion may be induced by membrane breakdown due to the application of an electric field (Pohl *et al.*, 1984). Such effects may be observed during dielectrophoretic measurements of mammalian cells whereby cells are brought into close contact and are mechanically deformed (Stenger *et al.*, 1991; Kononenko *et al.*, 1991).

This phenomenon has been exploited in biotechnology and genetic engineering (Zimmermann, 1986; Abidor & Sowers, 1992; Berg, 1988). It is of particular advantage for the production of hybridoma cells (Bischoff *et al.*, 1982).

Electrofusion of haematological cells has been investigated by Scheurich *et al.*, (1980) and Chernomordik & Sowers (1991).

1.2 THE BIO-ELECTRICAL STRUCTURE OF THE CELL

The cell is defined as the structural and functional unit of all living organisms. The fundamental property of any cell is the ability to define "self" from "non-self". This is accomplished by the possession of a cell membrane - a physical barrier encompassing the cell, distinguishing between the cell interior and the exterior milieu. This semi-permeable plasma membrane, in addition to defining the outer perimeters of the cell, is also the interface through which the cell interacts with its environment. It is the site for many chemical reactions and for the transport of materials such as metabolites, waste products and ions into and out of the cell. Movement of ions across the cell membrane and their unequal distribution between the cell and its environment stipulates a further role for the membrane in acting as an electrical insulator (Deuticke *et al.*, 1992).

The selective permeability of the plasma membrane enables the cell to maintain a constant internal environment. It also allows the creation of an ionic gradient and thus an electrical potential difference between the inside and the outside of the cell. The latter is known as the membrane potential (ψ) and can be used to indicate the dynamic transport properties of the membrane (Szollosi *et al.*, 1987). In animal cells, the membrane potential difference depends upon the balance between passive and active transport processes controlling ion distribution across the membrane. It is predominantly concerned with differing internal and external concentrations of Na^+, K^+ and Cl^-. This transmembrane potential can be calculated using the Goldman-Hodgkin-Katx constant field equation which considers the intra- and extra-cellular concentrations of different ions, and their associated permeabilities. Although the surface potential can be considered as distinct from the membrane potential the two are inter-related (Tsong & Astumian, 1987).

Plant, fungal and prokaryotic cells often possess an additional structural boundary, outlying the cell membrane, known as the cell wall. This has a significant effect upon the dielectric properties of the cell but is not considered in detail here.

The basic cell membrane consists of a phospholipid bilayer into which proteins may be partially or fully inserted, as described by Singer in 1972 (Hermann *et al.*1990). The lipid component provides the permeability barrier; the proteins are responsible for the characteristic functions of the cell (Cevc *et al.*, 1990). Specific proteins and lipids are associated with particular cell types, functions and species. The ratio of membrane protein to lipid reflects the activity of the cell. For example, myelin sheath cells are composed mainly of lipid, erythrocytes have approximately equal amounts of protein and lipid, whereas the mitochondrial inner membrane predominantly consists of protein, reflecting its high metabolic activity (Stryer, 1988).

The external animal cell surface is coated with carbohydrates, often in the form of glycoproteins and glycolipids. The carbohydrate component of the cell surface is the most variable and accounts for much of its individuality. N-acetylneuraminic acid (NANA) is commonly found as the terminal sugar group on a membrane glycoprotein. This is negatively charged and affects the electrical properties of the cell surface. Other charged glycoproteins and lipids have similar effects causing the cell surface to carry a net negative charge (Fishman, 1985; Warren, 1987; Datta, 1987; Petty, 1993).

1.2.1 Polarisation Processes of Cells in Aqueous Suspensions

Cell polarisation processes in response to an electric field are frequency-dependent, as discussed in section 1.1. The characteristics of this behaviour have been described in detail by Pohl (1978), Pethig (1982), Foster & Schwan (1987) and Glaser & Gingell (1990). A basic summary of these processes of relevance to dielectrophoretic investigations of haematological cells is presented here.

The electrical behaviour of a cell membrane is influenced by its constituent protein and lipid molecules. In aqueous solution the amino acid subunits of various proteins become ionised, depending upon the pH of the medium; this creates dipoles that can interact with an applied electric field. Models of electrostatic interactions as a result of protein structure have been described by Rogers (1986). The amphipathic nature of phospholipid molecules similarly promotes dipole formation.

When in aqueous suspension, the presence of charged groups at the membrane surface will affect the distribution of ions in a boundary layer, the depth of which will depend upon various properties of the membrane and the medium. The net negative charge carried on the cell surface is balanced by equal and opposite charges in the medium directly adjacent to the cell, resulting in the formation of an electrical double layer (fig. 5).

The counter-ion layer surrounding the cell is responsible for the frequency dependent surface conductance. Relaxation frequency of the particle theoretically varies according to the inverse square of particle size, *i.e.*, as particle size increases, characteristic relaxation frequency and termination of dielectrophoretic motion are exhibited at decreasing frequencies (see section 1.1.4). This situation is true for colloidal suspensions but is not necessarily valid for biological cells with their intrinsic high dielectric constants at the low end of the α-dispersion effect (Schwan, 1985a).

Charge distribution within the electrical double layer is asymmetrical and can be considered as two distinct regions. The counter-charge layer directly adjacent to the cell surface is encircled by a more diffuse layer of ionic charge; these are referred to as the Stern and Gouy-Chapman layers respectively. Charge located within the Stern layer is tightly bound to the cell and will move with it through a suspending medium.

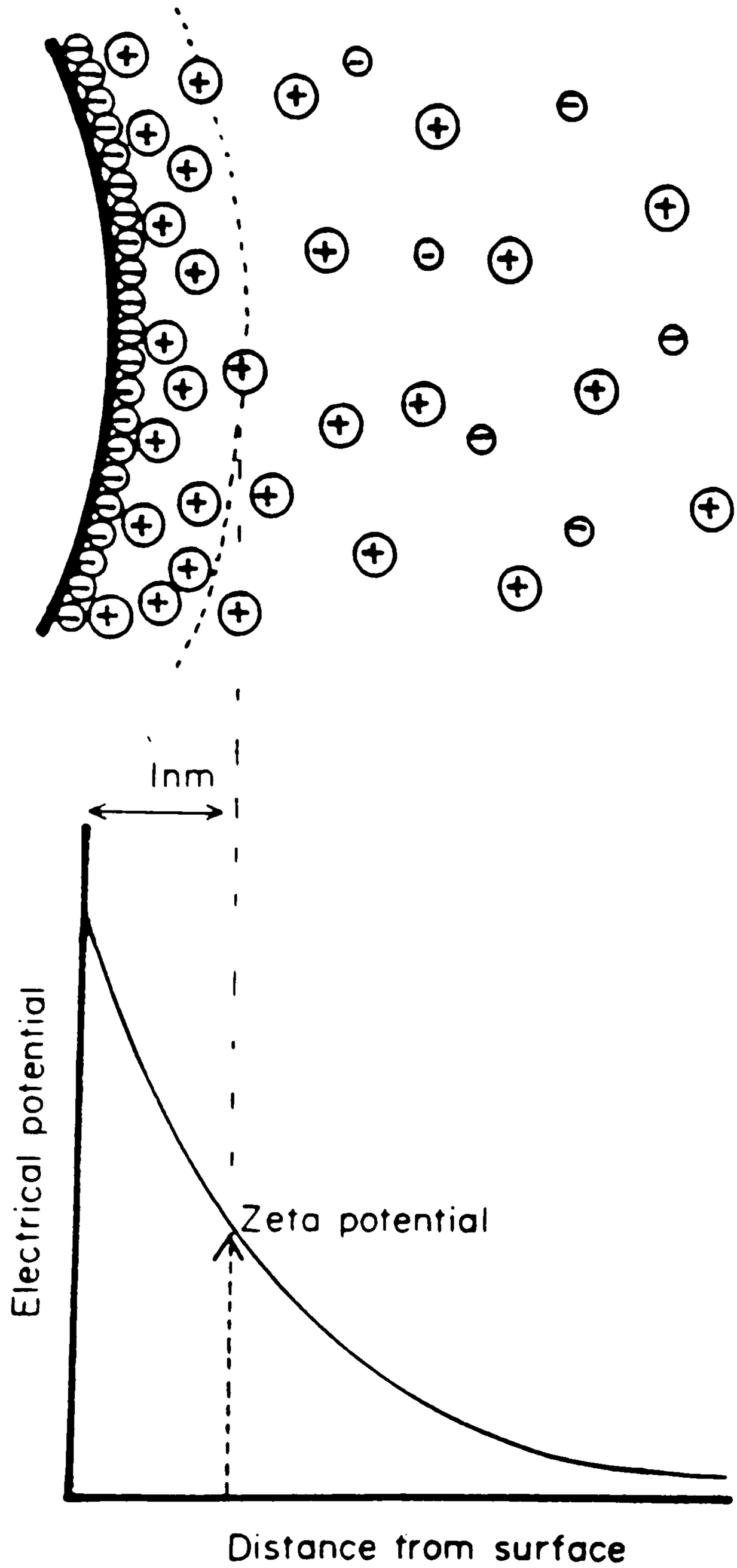


Figure 5: The electrical double layer surrounding a cell in aqueous solution.

The electrical potential at the place of shear is known as the zeta (ζ) potential. This is in the region of 1 nm from the cell surface, and is typically a few millivolts lower than the true surface potential. The magnitude of this potential can be calculated by application of the Stern-Gouy-Chapman model (Bergethon & Simons, 1990). The thickness of the zeta potential layer is determined by the cell surface charge and the properties of the suspending medium (Meryman, 1977).

The dielectrophoretic response is associated with the formation of dipoles and their corresponding relaxation times, as described previously. Exploitation of this behaviour as a non-invasive technique to differentiate between different cell types, involves dipoles formation in the ionic layer surrounding the cell membrane and by the orientation of comparatively large protein molecules. In contrast, dipoles formed by water molecules have shorter relaxation times and the corresponding dielectric dispersion is much more difficult to investigate; the information obtained relates to subcellular structure.

Dielectrophoretic effects of a suspended cell are promoted by a large differential between cell conductivity and that of the medium, to maximise the width of the diffuse ion layer. Alteration of cell or medium dielectric properties will affect dielectrophoretic spectra observed. Information concerning gross cell structure can be obtained from both α and ß dispersions, which are apparent over an alternating electric field frequency range in excess of 100 Hz to 1 MHz (Grant *et al.*, 1978).

1.2.2 Electrical Behaviour of Abnormal Cells

The dependence of the bio-electrical properties of a cell upn its molecular compostition has been described. Differences in surface carbohydrates influence the potential at the membrane surface, therefore altering the electrical double layer formed at the interface between solid and solvent. Abnormal membrane lipid composition may exert similar effects due to its polar groups, and may also affect dielectric behaviour at higher field frequencies where the membrane itself is polarised by the field (Pethig & Kell, 1987; Pethig, 1988; Foster & Schwan, 1989).

The application of cell electrophoresis as an investigative technique has been described by Chaubal (1985). Electrophoretic mobility measurements describe the velocity of particle displacement within an electric field and are directly dependent upon particle charge (Freifelder, 1982). Inferences about the ionic diffuse layer

surrounding the cell can therefore be made by consideration of electrophoretic mobilities, which, unlike zeta potential, can be measured directly (Donath & Pashtuskenko, 1980; Donath & Voigt, 1985). Differences in electrophoretic mobility can potentially be exploited by a dielectrophoretic method to analyse and/or separate cells. Such detectable changes in particle charge have been associated with cell differentiation (Gascoyne & Becker, 1990) and have also been used to characterise leukaemic cells (Holowiecki *et al*, 1985; Knippel.*et al.*, 1991).

The malignant state is associated with higher values for permittivity and conductivity, in comparison to normal tissue (Szent-Gyorgyi, 1957, 1980; Pethig & Kell, 1987; Pethig, 1991). Cancerous cells are reported to contain increased levels of water and sodium ions, thus affecting the electrochemical properties of the membrane. In association with the increased water content the lipid bilayer exhibits increased fluidity. Sialic acid residues on the cell surface are reduced, thus indicating a reduction in surface negativity (Pethig, 1978). Such properties are proposed to initiate differences in dielectrophoretic spectra of normal and malignant cell types (Grant & Spyr, 1985; Pethig, 1988).

1.3 A BRIEF COMMENTARY UPON DIELECTROPHORETIC INVESTIGATIONS OF BIOLOGICAL CELLS

1.3.1 An Historical Overview

The dielectrophoretic phenomenon was first reported in the 1600's by Gilbert who noted a change in the shape of a water drop as electrified amber was brought near (Pohl, 1969). However, it was not until the twentieth century that the processes involved in biological dielectrophoresis were examined in any detail.

Pearl chain formation was first observed by Muth in 1927 (Schwan & Sher, 1969), who investigated the effect of high frequency fields on fat particles. Muth used a uniform electric field which was distorted by the presence of the neutral droplets, resulting in the end-to-end arrangement of the particles. This phenomenon is now known as mutual dielectrophoresis (Pohl, 1978). In 1939 Liebesny studied the effect of a uniform high-frequency field upon erythrocytes and also reported pearl chains formation (Schwan & Sher, 1969; Pohl, 1978).

There was renewed interest in the phenomenon of dielectrophoresis in the late 1950's to early 1960's. In 1959, Heller studied the response of various micro-organisms to high-frequency electric fields and observed pearl chain formation and orientation effects (Schwan & Sher, 1969; Pohl, 1978). The first theoretical treatment of the force was carried out by Schwan in 1960 (Schwan & Sher, 1969), and further developed in later years. In 1963, Sher carried out the first quantitative experiments on pearl chain formation.

The first successful experiments on the use of dielectrophoresis to collect living cells were reported in 1966 by Pohl and Hawk. They used a pin-plate dielectrophoretic chamber and found that live and heat-killed yeast showed different responses to an applied electric field (Pohl, 1978). Dielectrophoresis could thus be used to simultaneously distinguish and separate living and dead cells.

Further investigations by Pohl and Hawk, and others have shown that a particular cell species produces a unique spectrum of collection rate against frequency (fig. 6; Pohl, 1978). Such results are readily reproducible for similar cells under the same physical conditions, but show variation between species, cell status and individual cells. The dielectrophoretic spectrum is thus a characteristic property of the cell type and status.

1.3.2 Measurement of Biological Dielectrophoresis

The basic requirements for inducing and observing dielectrophoretic motion in a suspension of particles are:

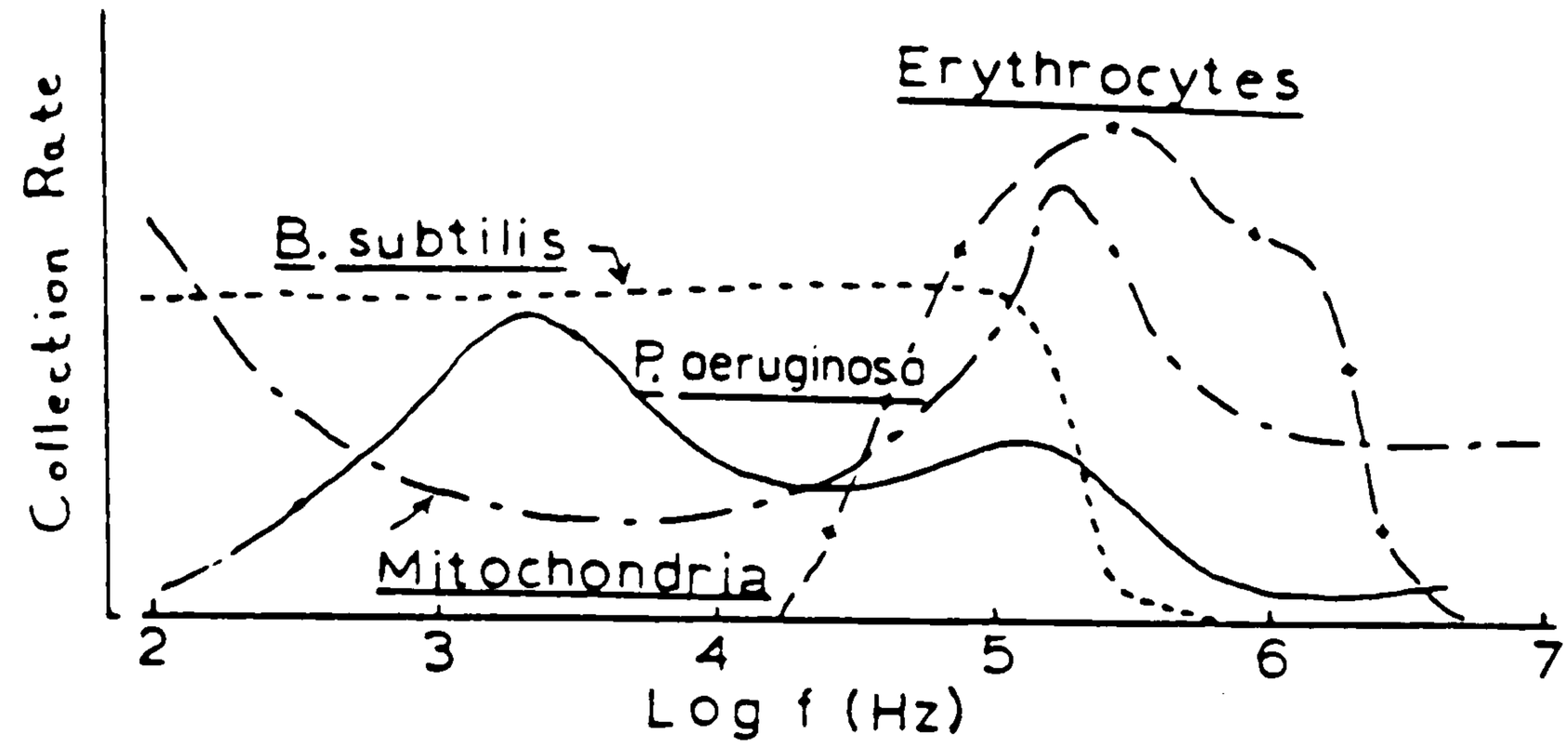
1) an electrode system - this may simply be composed of two wires through which a small electric current can be applied to create a non-uniform field.
2) differing electrical properties of suspensoid and solvent. This is readily accomplished by, for example, the suspension of biological cells in a low ionic strength solution, permitting positive dielectrophoresis of particles with respect to the medium.
3) a method of detecting the response. This may be by direct observation by the unaided eye, or with a microscope, or by monitoring some other property of the suspension affected by the dielectrophoretic activity.

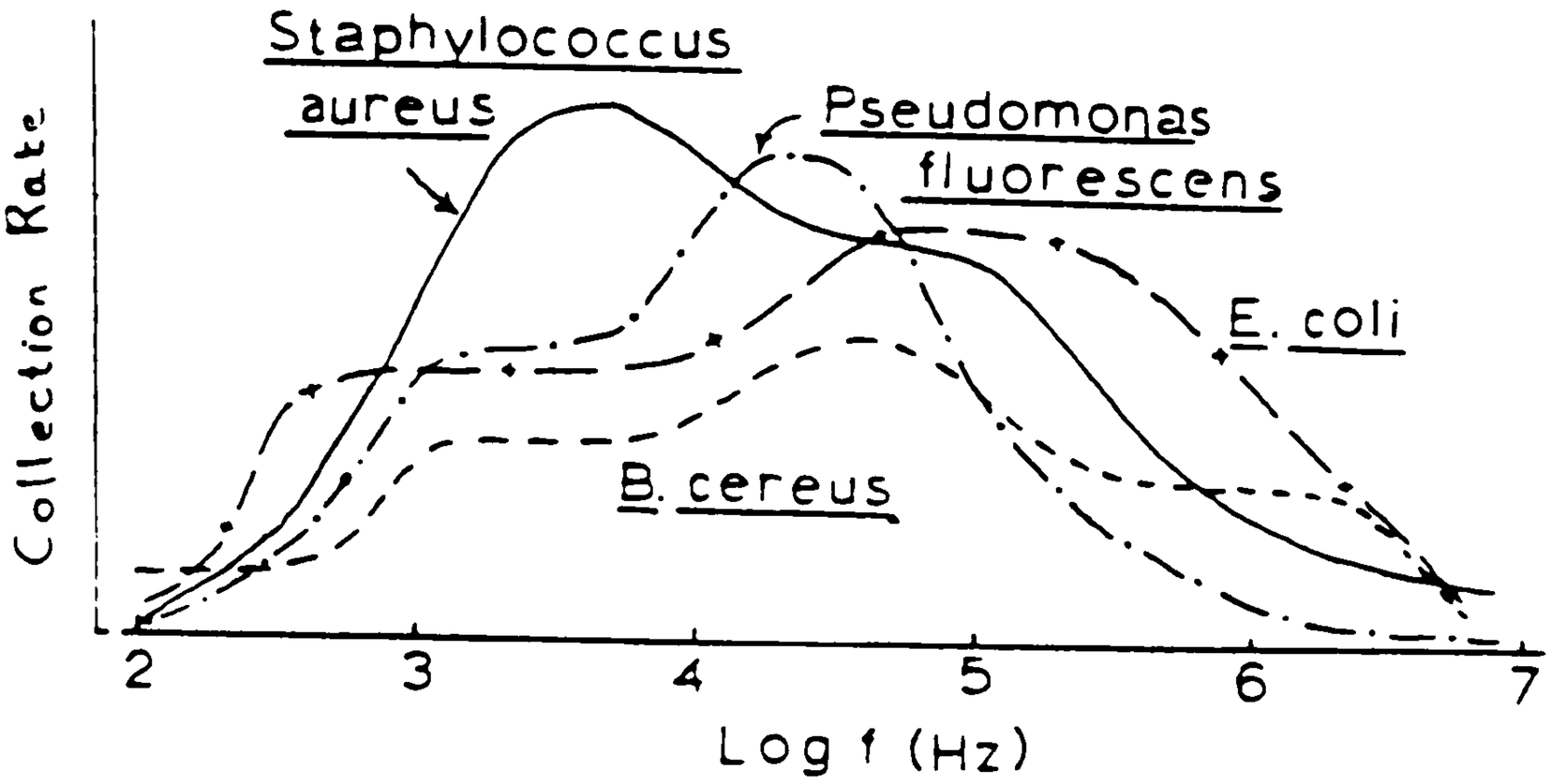
Initial investigations of biological dielectrophoresis were carried out using a crude electrode arrangement known as the pin-plate system; this consisted of a base plate and a wire (used as a point) between which a non-uniform field was created. The force experienced by the sample was considered to be proportional to the length of the pearl chain formed. A graticule microscope was used to determine the average chain length after a set period of pulse application, usually 1 minute, and the measurement was reported as the yield or dielectrophoretic collection rate (DCR; Pohl, 1978). This method of quantification enabled the effect of various field parameters upon collection to be studied. For example, dielectrophoretic spectra of yield against field frequency could be compared for different cell types (fig. 6).

The design and construction of an instrument to measure dielectrophoresis were also important considerations. The pin-plate system used by Pohl and Hawk (1966) consisted of a platinum wire perpendicular to a similar flat plate. This was positioned in a sample chamber supported by a glass slide, then viewed under the microscope. Crane and Pohl (1968) used a pin-pin electrode design also positioned on a glass slide. More recent workers have developed a sealed electrode chamber encasing an inter-digitated array system (*e.g.* Price *et al.*, 1988; Burt *et al.*, 1990).

For dielectrophoresis of suspended particles to occur then the sample must be subjected to an inhomogeneous field. This induces an unequal distribution of charges over a particle and thus an imbalance of forces resulting in net movement. The structure of the electric field itself is determined by the configuration of the electrodes. An inhomogeneous or non-uniform field will be produced by any electrode arrangement except that of parallel plates of infinite extent. However, a distinction must be made between a randomly non-uniform field and one which is regularly non-uniform, allowing the areas of high field intensity to be predicted. An advantage of the pin-plate electrode configuration is that a fair prediction of field pattern could be made (as indicated in fig. 1).

Field geometry varies with electrode configuration (as discussed by Pethig, 1979). Regular non-uniform fields may be generated by spherical, cylindrical or castellated electrodes. In such cases, the dielectrophoretic force on a particle in the field can vary by orders of magnitude according to the position of the particle in relation to the electrodes. This problem has been solved by an electrode system providing isomotive field geometry (Pohl, 1978). This uses one V-shaped and one curved electrode which are shaped according to a specific equation (as deduced by Pethig, 1979). In such a field, the dielectrophoretic force on particles at different distances





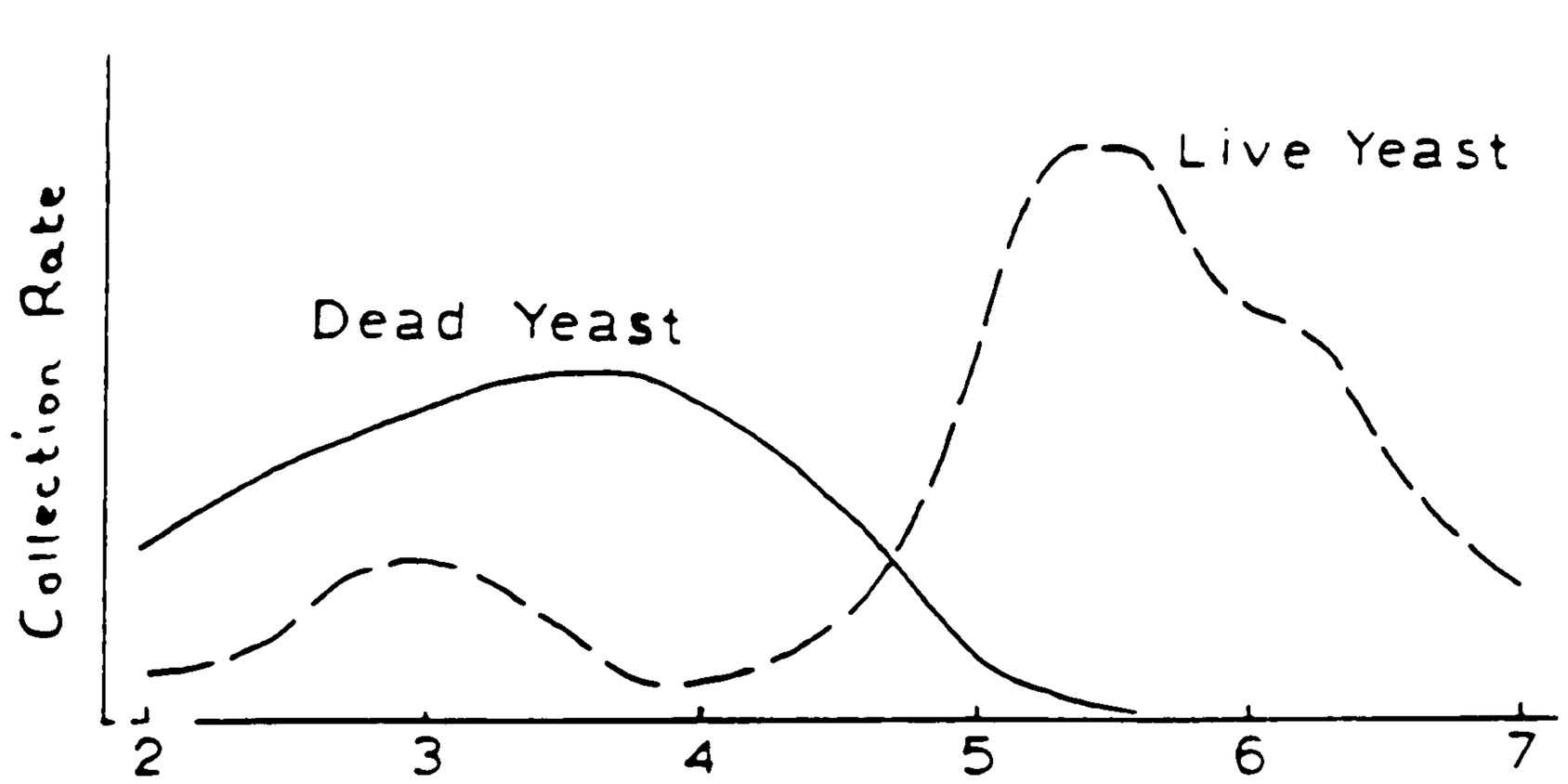


Figure 6: The effect of electric field frequency upon dielectrophoretic collection of various cells, as determined using a manual method (Pohl, 1978).

from the electrodes remains fairly constant. Kaler *et al.*, (1986) used an isomotive dielectrophoresis chamber in their study of yeast cells.

Application of photolithographic techniques to electrode manufacture has enabled micro-electrode arrays to be prepared on glass slides. For the electrode configuration used in these investigations (Chapter Two) the depth of the parallel electrode bars was deemed insignificant with respect to its other dimensions (*i.e.*, 1 μm in comparison with 600 μm by 50 μm). The resulting electric field was considered to be generated by "edge" effects.

Detection of dielectrophoretic collection has been radically changed by the adoption of monitoring procedures which eliminate the requirement for counting cells by direct observation. Spectrophotometric methods have been successfully employed to measure the changes in light absorbance as a result of cellular dielectrophoretic motion (Adamson & Kaler, 1988; Price *et al.*, 1989; Burt *et al*, 1989; Hawkes *et al.*, 1993). Other workers (Kaler *et al.*, 1986) have applied quasi-elastic light scattering techniques for particle detection.

Advances in optical imaging have permitted systems to be re-developed from the basis of direct observation of events occurring at the electrode surface. Kaler and Jones (1990) incorporated a video camera into their system for measuring levitation as a result of the dielectrophoretic force. Using this system, they were able to study the polarisation response of single cells by identifying at what field strength the dielectrophoretic force became equal and opposite to gravity. The experimental chamber was viewed with a microscope and the image recorded on a video system. The latter was used to identify the effect of the conditions imposed and to detect cell position for a feedback control loop.

Recent advances have employed the use of computerized imaging techniques to permit multiple sample measurements and real time analysis (*e.g.* Gascoyne *et al.*, 1992). In the work presented here, such advances have been included in instrumentation development with the aim of applying dielectrophoresis to haematological samples.

1.4 THE DIELECTROPHORETIC RESPONSE OF HAEMATOLOGICAL CELLS

1.4.1 Relevance Of Studies

The purpose of investigating the dielectrophoretic response of haematological cells is bi-functional:

1) The lack of intracellular structures has classically established the mature human erythrocyte as a model system for studying the composition and activities of the plasma membrane (Powers, 1989). As a bio-electrical structure the erythrocyte may be considered as a unit of highly conductive material separated from its surroundings by an insulating boundary. Suspension of erythrocytes in low ionic strength solution permits the electrical activity of the plasma membrane to be examined using the dielectrophoretic system described here. The aim of these studies is to investigate the parameters dictating the dielectrophoretic response and to explain these in terms of biological significance.
2) Differences in the electrical properties of cells have been associated with transformation between normal and malignant cell types (see section 1.2.2). It is suggested that such differences could be detected dielectrophoretically. Information to date regarding human cell surface charge has been centred upon investigation of lymphocytes. Differences in electrophoretic mobility have been detected for normal human B and T lymphocytes (Sherbet, 1978); it is anticipated that similar differences in dielectrophoretic response would be apparent.

Investigations will be carried out to examine the dielectrophoretic response of a range of haematological cell populations, both normal and malignant. This research is directed towards the potential application of a dielectrophoretic technique to clinical procedures, assisting diagnosis and treatment of leukaemias.

1.4.2 Properties And Composition Of Human Blood

Blood is defined as a fluid body tissue that acts as a transport medium within a higher animal (Oxford English Dictionary). It consists of cellular elements suspended in an aqueous solution of salts and proteins, and performs numerous functions, including;

- transportation of oxygen, nutrients, carbon dioxide, chemical messengers, waste and byproducts of metabolism.
- protection against damage from bacteria, fungi, defence against invasion.
- ability to restore the closed vascular system in the case of injury preventing body fluid loss and invasion by foreign material. preservation of a closed vascular system by mounting a coagulation response to injury/impairment.

The blood volume of a typical adult is about 4-6 l and carries in the region of 10^{13} cells (Jandl, 1987). These cellular components may be subdivided into two groups; erythrocytes or red blood cells which account for over 99 % of the circulating cells, and leucocytes or white blood cells (*i.e.*, granulocytes, monocytes and lymphocytes). In addition, non-nucleated cellular fragments known as thrombocytes or platelets also circulate; these are pivotal in the coagulation response. Erythrocytes, leucocytes and thrombocytes are present in the blood system of a normal adult in the ratio of approximately 1000:1:100 cells (Caro *et al.*, 1978). This ratio and total blood cellularity is affected by variables such as age, sex and health. The circulatory life-span of haematological cells varies from hours to months, according to their function, thus production in the bone marrow is continuous. Blood cells may therefore be found throughout the body or localised in particular tissues, at various stages of maturation.

1.4.3 Characteristics Of Haematological Cell Populations

Blood cells are produced in the adult bone marrow by totipotent stem cells which in turn give rise to partially committed lymphoid and myeloid stem cells. The former differentiate further into lymphoblasts which mature to B or T lymphocytes depending upon whether development occurs in the bone marrow alone or partially in the thymus. Myeloid stem cells produce another precursor cell - the colony forming unit granulocyte monocyte (CFU-GM) - in addition to blast cells for erythrocyte and thrombocyte lineages. Monocyte and granulocyte (neutrophil,

basophil and eosinophil) cell lineages arise from monoblasts and myeloblasts respectively, produced from CFU-GM cells. The bone marrow of an average male adult contains roughly 10^{12} haematopoietic cells, of which approximately 0.5×10^{12} are granulocyte precursors, 0.4×10^{12} erythrocyte, and 10^9 mega-karyocytes from which thrombocytes fragment (Powers, 1989).

The general characteristics of the different human blood cell populations are described. Emphasis is given to the erythrocyte (below and section 1.4.4), upon which the majority of these investigations are based, and to various aspects of haematological populations of relevance to dielectrophoretic behaviour (section 1.4.5).

a) Erythrocytes

These cells account for 45 % of the total blood volume and play a major part in the mechanics of the circulation due to their effect upon viscosity (Caro *et al.*, 1978). The primary function of the erythrocyte is as a carrier for the respiratory pigment haemoglobin, enabling oxygen to be transported to the body tissues and the removal of the waste product, carbon dioxide. Cellular sequestration of haemoglobin from the plasma, in contrast to the situation in invertebrates, permits the oxygen demands of mammalian metabolism to be met, and prevents an increase in blood viscosity problematic to the circulation, and water loss from the tissues.

A normal erythrocyte is non-nucleated, and at rest appears as a biconcave disk of diameter 7.82 ± 0.62 μm, with thickness of 0.81 ± 0.35 μm at the central indent and 2.58 ± 0.27 μm at the periphery (Evans & Fung, 1972). This shape is optimally suited to its function in gaseous exchange as it provides a large surface area:volume ratio and permits the cell to deform, enabling passage through capillary beds. Conformation and flexibility are determined by interactions between the cell membrane and an underlying protein skeleton (the cytoskeleton). The erythrocyte is remarkable for its ability to maintain membrane integrity whilst exhibiting extreme deformability under normal physiological circumstances.

Circulating red cells represent over 99% of total blood cell number, amounting to approximately 5×10^9 per ml of blood for a normal adult male (slightly less for females). Over 1% of this cell mass is replaced daily, corresponding to the manufacture of around 2.5×10^6 cells per second (Jandl, 1987; Powers, 1989). Cells

are released into the blood stream as immature nucleated forms (reticulocytes) which then lose all intracellular organelles and have a life-span of approximately 120 days as a respiratory carrier in the circulation. During this period an erythrocyte in the peripheral blood will complete a circuit of the body about every minute; conservative estimates predict that each erythrocyte will travel a distance of over one hundred miles before destruction (Powers, 1989).

The stresses imposed upon the red cell mass as it circulates the body requires that some mechanism acts to limit collision between erythrocytes, resulting in cell damage, or blockage of flow. This is achieved by electrical repulsion between erythrocytes, as a consequence of their strong negative charge conferred by composition and activities of the cell membrane (described in detail in section 1.4.4). Individual erythrocytes in plasma cannot be brought to within 25 nm of each other even when packed by centrifugation, preventing abrasive contact or agglutination by cross-linking of surface antibodies (Jandl, 1987).

Differentiation of erythrocytes from their blast forms is accompanied by specialisation for their vital role, and involves the loss of cytoplasmic organelles. Accordingly, the mature erythrocyte is unable to synthesize new protein or undergo mitosis; in effect it may be considered as a membrane enclosing a solution of protein (95 % haemoglobin) and electrolytes (Lee *et al.*, 1993). There is no provision for storage of metabolites and no mitochondria, thus the erythrocyte is dependent upon anaerobic glycolysis to produce ATP, NADH and 2,3-diphosphoglyerate (2,3-DPG), enabling continued function as an oxygen carrier.

The respiratory pigment carried by the erythrocyte, haemoglobin, consists of a porphyrin nitrogenous ring with a central iron atom (haem) and four polypeptide chains (globin) each with a haem group capable of binding one oxygen molecule. The centre of the haemoglobin molecule may be occupied by a 2,3-DPG molecule; when plasma levels of this substance rise, indicating the prevalence of anaerobic glycolysis, 2,3-DPG is bound by haemoglobin, reducing the affinity of the latter for oxygen and its release to the tissues. Aerobic metabolism is consistent with the production of carbon dioxide waste which diffuses into the erythrocyte and combines with water under the influence of carbonic anhydrase to form carbonic acid. In order to reduce hydrogen ion concentration, non-oxygenated haemoglobin exerts a buffering effect and acts as base, accepting a proton from the acid leaving bicarbonate ions which then diffuse into the plasma down a concentration gradient. To maintain electro-neutrality chloride ions diffuse into the cell; this is known as the

chloride shift. These reactions are reversed in the lungs where haemoglobin is oxygenated reforming carbonic acid, which is then lost as carbon dioxide and water (Powers, 1989; Lee *et al.*, 1993).

The erythrocyte cell surface is the site of a variety of glycolipids and glycoproteins, whose carbohydrate moieties provide the specificity required for their function as receptors and antigenic determinants such as the ABO system. In addition, the external membrane surface under normal physiological conditions must carry a negative charge with respect to its environment. The structure of the erythrocyte membrane is described in section 1.4.4.

b) Granulocytes

The granulocytic cell line accounts for around 75% of peripheral blood leucocytes, *i.e.*, ~ 7 x 10^6 per ml. Granulocytes are approximately 12-14 μm in diameter and are characterised by lobed nuclei, the possession of cytoplasmic granules and their phagocytic activity. They may be categorised on the basis of their staining properties; neutrophils attract both acidic and basic components of Romanowsky stains, eosinophils attract the acidic red eosin component whereas basophils take up the basic methylene blue. Between 60-70 % of all leucocytes are neutrophils, indicating their active role in defence against infections. They are the first cells to respond to bacterial invasion or tissue necrosis. Eosinophils and basophils are typically found at levels of 2-4 % and 0.5-1% respectively, of the total white blood cell population (Tortora & Anagnostakes, 1981). The former is active in allergic reactions and parasitic infections, the latter undergoes de-granulation releasing histamine in response to immunoglobulin E (IgE) stimulated antigen-antibody reactions (Becan-McBride & Ross, 1988). All three types of granulocyte typically remain in the circulation for a period of hours.

c) Lymphocytes

Lymphocytes are the principal component of the body's immune system. Approximately 20-25 % of peripheral blood leucocytes are lymphocytic - this however accounts for only 5 % of the total lymphocyte mass which is in the region of 10^{12} cells (Powers, 1989) and located throughout the lymphoid system and other body tissues.

The lymphocytic lineage is further divided into B or T cells respectively, depending upon whether maturation took place totally in the bone marrow, or if a period was spent in the thymus. B lymphocytes produce antibody in response to the presentation of antigen; T lymphocytes carry out cell-mediated immunity and are categorised as either T helper (T_H) or suppressor (T_S) cells. Both forms are identical in appearance on a blood smear, are of 6-9 μm in diameter and possess a spherical nucleus which is surrounded by a thin layer of non-granular cytoplasm.

Lymphocyte sub-populations include plasma cells (Blymphocytes), macrophages (Blymphocytes), natural killer and K-type cells (non-T, non-Blymphocytes).

d) Monocytes

With a typical cell diameter of 16-20 μm, these are the largest cellular components of normal human blood, comprising up to 10 % of the total leucocyte population (Becan-McBride & Ross, 1988; Powers, 1989). Monocytes have a single kidney-shaped nucleus surrounded by non-granular cytoplasm and exhibit phagocytic activity. They provide the second line of defence against infection by scavenging for and removing foreign material and dead tissue.

e) Thrombocytes

The non-nucleated thrombocytes released from the bone marrow have a seven day life-span in the circulation. Their function is to adhere to breaks in endothelium in presence of coagulation factor VII molecule, forming a primary platelet plug. This is stabilised by sequential activation of serum protein coagulation factors catalysing the formation of a fibrin meshwork. The entrapment of erythrocytes and thrombocytes resulting in the formation of a clot is the end result of the coagulation response.

In a passive unstressed state all leucocytes (granulocytes, lymphocytes and monocytes) are spherical in appearance, with numerous membrane folds permitting a large surface area for interaction with the environment. When activated in response to tissue injury they typically undergo large cytoplasmic deformation, pseudopod

projection and de-granulation, where appropriate. This behaviour is associated with changes in ionic concentrations between the cell and its surroundings, involving alteration of ion transport and distribution across plasma and internal membranes.

1.4.4 The Erythrocyte As A Simple Membrane System

The erythrocyte membrane's composition and structure allow it to perform three basic functions:

- to separate the intracellular fluid environment, containing haemoglobin, from the plasma
- to permit selective transport of nutrients and ions into and out of the cell
- to allow the cell to deform when required allowing passage through capillary beds.

Early investigations of membrane structure focused upon studies of the mature erythrocyte as a model, due to its lack of internal membranes. In 1925, Gorter and Grendel first established from work on erythrocytes, that membranes were based upon lipid bilayers. Also at this time, the electrical properties of the erythrocyte membrane were under investigation by Fricke (1925). Membrane structure was further elaborated in 1972 by Singer and Nicolson, who proposed a fluid mosaic model where proteins are partially or totally inserted in the lipid bilayer. Each leaflet of the phospholipid bilayer is arranged with the phosphate polar head groups of each molecule orientated towards the aqueous environment, whilst the nonpolar fatty acid tails face the hydrophobic leaflet interior. The tails can move laterally within the membrane, hence its fluidity (Lee *et al.*, 1993). This model also allows lateral mobility of inserted proteins, and permits membrane individuality as a result of differing concentrations and locations of protein, lipid and carbohydrate.

The red cell membrane is composed of 41% lipid, 52% protein and 7% carbohydrate by weight (for review see Haest, 1982). As for all membranes, the lipid component provides the permeability barrier, whilst proteins and carbohydrate dictate function and cell specificity. Membrane analysis revealed dissimilarities in lipid distribution between inner and outer bilayer leaflets. The external membrane surface was generally rich in the choline-phospholipids, sphingomyelin (SG) and phosphatidylcholine (PC), whilst the amino-phospholipids, phosphatidyl-ethanolamine (PE) and phosphatidyserine (PS) were preferentially located in the inner leaflet (Bretscher, 1972; Op den Kamp, 1979). This asymmetry participates in

establishing an electrical gradient across the membrane, with the outer surface carrying a negative charge with respect to the membrane interior (Powers, 1989).

The external membrane surface is covered with carbohydrates in the form of glycolipids and glycoproteins which endow the cell with the majority of its specific surface receptors and antigenic determinants. These complex heteroglycans are highly hydrophilic, and in addition to helping maintain the asymmetry of the bilayer, impart negative charge to the surface. Underlying the membrane on its cytoplasmic face there is a flexible protein lattice which maintains the shape of the erythrocyte whilst permitting deformability; this is the cytoskeleton (Bennett, 1985, 1990).

The protein component of the erythrocyte membrane can be analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and the constituent proteins identified by Coomassie Blue and Periodic Acid-Schiff base gel staining techniques. This method also enables the degree of insertion of the protein into the lipid bilayer to be established; proteins easily solubilised indicates partial insertion (peripheral proteins), in contrast, proteins requiring more vigourous extraction due to total insertion are termed integral proteins.

Integral proteins typically act as channels through the lipid bilayer. Band 3 protein, known as the anion channel is important for its antiport mechanism in gaseous exchange; as HCO_3^- ions move out of the cell a Cl^- ion moves inwards to maintain electrical balance (Dalmark, 1976; Lee *et al.*, 1993). In addition this protein carries antigens of the ABO blood group system upon its extracellular portion. Band 4.5 protein is responsible for the facilitated transport of glucose into the cell via a carrier system; this process is insulin independent.

The external erythrocyte surface is covered with the extra-membranous portions of glycoproteins and glycolipids embedded in the lipid bilayer. This region extending 5 - 10 nm from the cell surface is known as the glycocalyx; its main components are glycophorins, band 3 and band 4.5 protein. Glycophorin molecules typically extend 5 nm from the bilayer and carry a large number of sialic acid residues which are negatively charged at physiological pH. These molecules are primarily responsible for the net negative surface charge on the erythrocyte. Polylactosamine chains associated with band 3 and band 4.5 proteins extend approximately 10 nm from the cell surface and also contribute to surface charge (Vitala & Järnefelt, 1985).

1.4.5 Bio-Electrical Properties Of Haematological Cells

The red cell membrane at physiological pH may be considered as a surface layer of approximately 6 nm thick and area of 140 μm^2, containing a distribution of 1.5 x 10^7 electric charges (Voigt & Donath, 1990). When in aqueous solution, the hydrophillic nature of the carbohydrate residues responsible for the surface charge causes a hydration sheet to be formed around the cell. This layer of water molecules hydrogen-bonded to the glycocalyx may extend 50 nm from the cell surface, preventing erythrocyte damage and agglutination by the creation of a zeta potential (Viitala & Järnefelt, 1985). As described in section 1.2, the extent of the zeta layer of charge and counter-charge is dependent upon the magnitude and density of charge at the cell surface and the ionic composition of the medium. In *vivo*, the zeta potential is reduced by the binding of plasma proteins such as albumin to the outer erythrocyte surface; this is concerned with steric hindrance effects as attachment of the large bipolar albumin molecule to the surface limits the number of cations that can bind (Jay, 1975).

For dielectrophoretic effects to be observed, cells must be suspended in solutions of low ionic strength. This has the effect of reducing the density of the charge layer around the cell thus decreasing the minimum intercellular separation, and may result in agglutination. Measurements of the response may be inhibited by shape changes as a result of electro-poration and water influx (Chang *et al.*, 1985).

The biophysical behaviour of erythrocyte suspensions has been reviewed by Chien (1974). A thorough description of the electrokinetic processes together with micro-electrophoresis measurements has been presented by Seaman (1975).

The degree of electrical charge carried by cells of the erythrocyte lineage is correlated with their maturity and helps prevent the release of immature forms from the bone marrow into the bloodstream. The net negative surface charge upon reticulocytes and erythroblasts is less than that of the mature erythrocyte (isoelectric points of 3 versus 1), and acts to impede the progress of immature forms through spleenic channels which are rapidly transversed by erythrocytes (Ponder & Ponder, 1955).

A similar process may be involved with the removal of erythrocytes from the peripheral blood after their 120 day life-span. Research has suggested that as the red cell ages its negative charge diminishes, perhaps promoting capture by the reticulo-

endothelial system (Eylar, 1962; Aminoff, 1985; Clark, 1988). However measurements of surface charge or concentration of sialic acid residues upon cells of different ages have proved inconclusive (Luner *et al.*, , 1977; Walter *et al.*, 1981; Bartosz, 1990).

The phospholipid composition of the membrane also exerts an effect upon the electrical properties of the cell. Amino-phospholipids are preferentially located on the inner leaflet where their anionic groups help to stabilise the cytoskeleton (Lee *et al.*, 1993). The appearance of amino-phospholipids, in particular phosphatidylserine, upon the external face of the lipid bilayer is associated with pro-coagulant activity due to the effects upon cell surface charge (Rosing *et al.*, 1980; Jackson & Nemerson, 1980; Rosing *et al.*, 1988; Herrman *et al.*, 1990). Abnormal amino-phospholipid distributions of the erythrocyte membrane are also associated with certain pathological states; sickle cell anaemia, old red cells, red cells from patients with chronic myeloid leukaemia and tumourigenic cells (as reviewed by Schroit & Zwaal, 1991). It is postulated that differences in cell surface charge as a result of membrane lipid distribution may be detected dielectrophoretically. In addition, abnormal membrane fluidity levels which are observed in particular clinical conditions (Cooper & Meddings, 1991) are proposed to affect dielectrophoretic behaviour.

Cell surface charge differences, as determined by electrophoretic mobilities, are observed between T and B lymphocytes. T cells exhibit a higher mean electrophoretic mobility, as measured in phosphate buffered saline, in comparison to B lymphocytes (in Preece & Sabolovic, 1979). Sabolovic *et al.*, 1985, described a direct relationship between electrophoretic mobility and cell surface phenotype as defined by monoclonal antibodies, and were able to differentiate between populations of T_H, T_S, B, Null and natural killer cells from human blood lymphocytes. Electrophoretic mobility measurements of cell lines and leukaemic cells have indicated cell surface charge differs with respect to cell differentiation and maturity (Babusikova *et al.*, 1985).

Changes in electrical properties of membranes are associated with cell stimulation or activation. Electro-rotation has been used to distinguish between inactive and antibody-secreting lymphocytes (Arnold and Zimmermann, 1985; Hu *et al.*, 1990). The effect of cell stimulation upon ionic membrane processes has been reviewed by Seligmann, (1990).

1.5 RESEARCH AIMS

The fundamental aim of this project is to investigate the dielectrophoretic response of haematological cells and to assess whether there are realisable applications for clinical procedures. This will involve several stages:

- to develop and optimise an experimental system which enables rapid measurement of dielectrophoretic response;
- to define the limits and reliability of this measurement system;
- to establish a protocol for dielectrophoretic investigations of haematological cells;
- to determine the resolution of the technique by considering the effects of cell membrane modification upon dielectrophoretic response;
- to assess the usefulness of a dielectrophoretic technique as a possible haematological procedure for cell identification and separation.

Each phase is now described in more detail.

1.5.1 System Development

The initial research objective is to develop an experimental system allowing rapid measurements of the dielectrophoretic behaviour of haematological cells. Specifications for this system include;

- development of an automated system allowing the controlled application of a non-uniform electric field to a biological sample.
- investigation of the response of a cell population as opposed to that of single cells.
- provision of rapid detection and quantification of the induced response allowing real-time analysis.
- restriction of the dead space volume of system minimising total number of cells required for investigations.
- reduction of experimental time period thus limiting physiological changes occurring in the cell sample.

Preliminary investigations will be concerned with obtaining reproducible dielectrophoretic spectra whilst concentrating upon the optimisation of the experimental system. Restrictions upon experimental procedure concerned with the use of mammalian cell samples will be recognised, but examined in detail after system development.

Prokaryotic cells and latex beads carrying known surface groups (*i.e.* "test particles") will be employed for preliminary investigations due to their ease of manipulation. This allows rapid and inexpensive assessment of system performance to be achieved. Subsequent investigations will utilise formaldehyde stabilised erythrocytes and fresh human blood cells to investigate whether the dielectrophoretic behaviour of haematological cells can be detected.

1.5.2 Reliability of Measurements

Investigations will be undertaken to define the operational parameters of the experimental system. Trials will be carried out with test particles then human erythrocytes to identify possible equipment-based sources of variation in dielectrophoretic measurements. The performance of the system will be assessed and evaluated. Appropriate modifications will be made where necessary.

1.5.3 Experimental Procedures For Haematological Cells

Future application of a dielectrophoretic technique to haematological procedures, such as purification of bone marrow cells for autologous transplantation, requires that cell viability is maintained. In association with this the physiological demands of the cell during the experimental period must be met, and any detrimental consequences of the investigative procedure kept to a minimum.

For dielectrophoretic behaviour of a particle to be observed, in preference to that of its suspending medium, the latter must be ion-deficient with respect to the particle. This enables the polarisation effects induced in the particle by a non-uniform field to dominate the behaviour of the suspension, permitting dielectrophoretic motion. When mammalian cells are placed in hypertonic medium ions and metabolites are lost from the cell. In addition, the absence of an accessory supporting structure such as a cell wall means there is no opposing force restricting volume increase of the

cell. Suspension in hypo-osmotic medium results in movement of water into the cell; this activity affects concentration gradients across the cell membrane and may result in rupture of the cell.

A suspension medium for dielectrophoretic investigations of haematological cells has therefore to satisfy conflicting criteria. Dielectrophoretic effects are promoted by use of a low ionic strength solvent whereas this is not suitable for preservation of cell viability. The osmotic pressure of the medium and its content of nutrients and essential ions must also be considered.

Various solutions will be evaluated for their suitability as an experimental suspension medium. Furthermore, a standard protocol for haematological sample preparation will be defined.

1.5.4 Sensitivity Of Dielectrophoretic Measurements

The effects upon dielectrophoretic spectra as a consequence of various surface active treatments will be explored. All experiments will be carried out using the human erythrocyte as a model cell on account of its simple membrane structure In addition, investigations will be undertaken to assess the sensitivity of the dielectrophoretic technique in distinguishing between different cell samples.

1.5.5 Potential Applications For Haematological Procedures

Trials will be carried out using clinical blood samples to investigate the potential application of dielectrophoretic measurements as a haematological technique. In particular, the possibility of developing this procedure towards bone marrow cell separation in leukaemia therapy will be addressed.

This research will be carried out in association with the Haematological Malignancy Diagnostic Service located at Leeds General Infirmary.

Chapter Two

THE STANDARD AUTOMATED DIELECTROPHORETIC MEASUREMENT SYSTEM

2.1 CRITERIA FOR DEVELOPING A STANDARD SYSTEM TO MEASURE DIELECTROPHORESIS

Application of a dielectrophoretic technique to haematological analysis requires an experimental system of high resolution, capable of recognising subtle differences in cellular response to an electric field. For investigative purposes a sample population of cells should be used, in contrast to analogous methods observing individual cells. Medical diagnosis would potentially involve mixed cell samples, *i.e.* of different lineages and age, which would still be classified as 'normal'. Additional criteria for an experimental system include rapidity of procedure and analysis, preservation of cell viability, and the potential for operation under sterile conditions. The limitations and reproducibility of measurements must also be examined.

System development was carried out using robust particles such as micro-organisms and latex beads, before measurements were made using haematological cells. These test samples were easily prepared and exhibited little interaction with the suspension medium, retaining a detectable dielectrophoretic response for a period of at least two hours.

2.2 BASIC COMPONENTS OF THE EXPERIMENTAL APPARATUS

The standard system for the measurement of the dielectrophoretic response consisted of a micro-electrode chamber (through which a cell suspension was circulated by peristalsis), a signal generator and a computer to co-ordinate the activities of the components. Various optically-based methods to detect the possible effect of the applied field upon the cell sample were evaluated. In addition to its control function, the computer was employed to acquire and analyse data where appropriate, *c.f.* the video technique described in Chapter Three, thus enabling rapid measurements to be made.

2.3 SPECIFICATIONS OF THE MICRO-ELECTRODE ARRAY

2.3.1 Electrode Design

The standard electrode configuration consisted of an interdigitated array of rectangular bars, as indicated in figure seven (Betts & Hawkes, 1991). The original design allowed a choice of either 2 or 16 bar arrays to be selected; the attributes of each are described in section 3.2.3b. Each bar was 4500 µm long, 50 µm wide and less than 1 µm thick, and was continuous with an electrode tab region to which the signal generator could be connected (fig. 7). Spacing between neighbouring bars was set at 50 µm which supported a maximum field strength of 2×10^5 V m^{-1} upon the application of a 10 V signal. The production of such a large active electrode area from a number of individual electrode bars ensured that the entire electrode was not compromised by damage to a single bar.

The template describing the electrode configuration enabled four sets of electrodes to be prepared on one microscope slide.

2.3.2 Electrode Manufacture

Electrodes were produced using photolithographic techniques as detailed in Appendix I. The sequence of events described briefly:

Glass microscope slides were acid-cleaned then vapour-coated with metal. Aluminium was typically used on account of its strong adhesion to glass. The vapour coating process appeared to result in uniform covering of the slide; however, microscopic examination revealed the presence of pin-point holes in the metal layer. Such imperfections could not be avoided due to technical difficulties associated with the vapour-coating procedure.

A layer of photoresist was applied to the slide and a mask aligner employed to imprint the electrode design onto the slide. Unfixed photoresist and the excess underlying metal were removed using washing techniques, leaving the metal electrode configuration upon the slide. A second coating of photoresist was applied. Using a different mask, the photoresist was developed to delineate a channel around the active electrode area but not encasing the electrode tabs. This photoresist was used as a guide

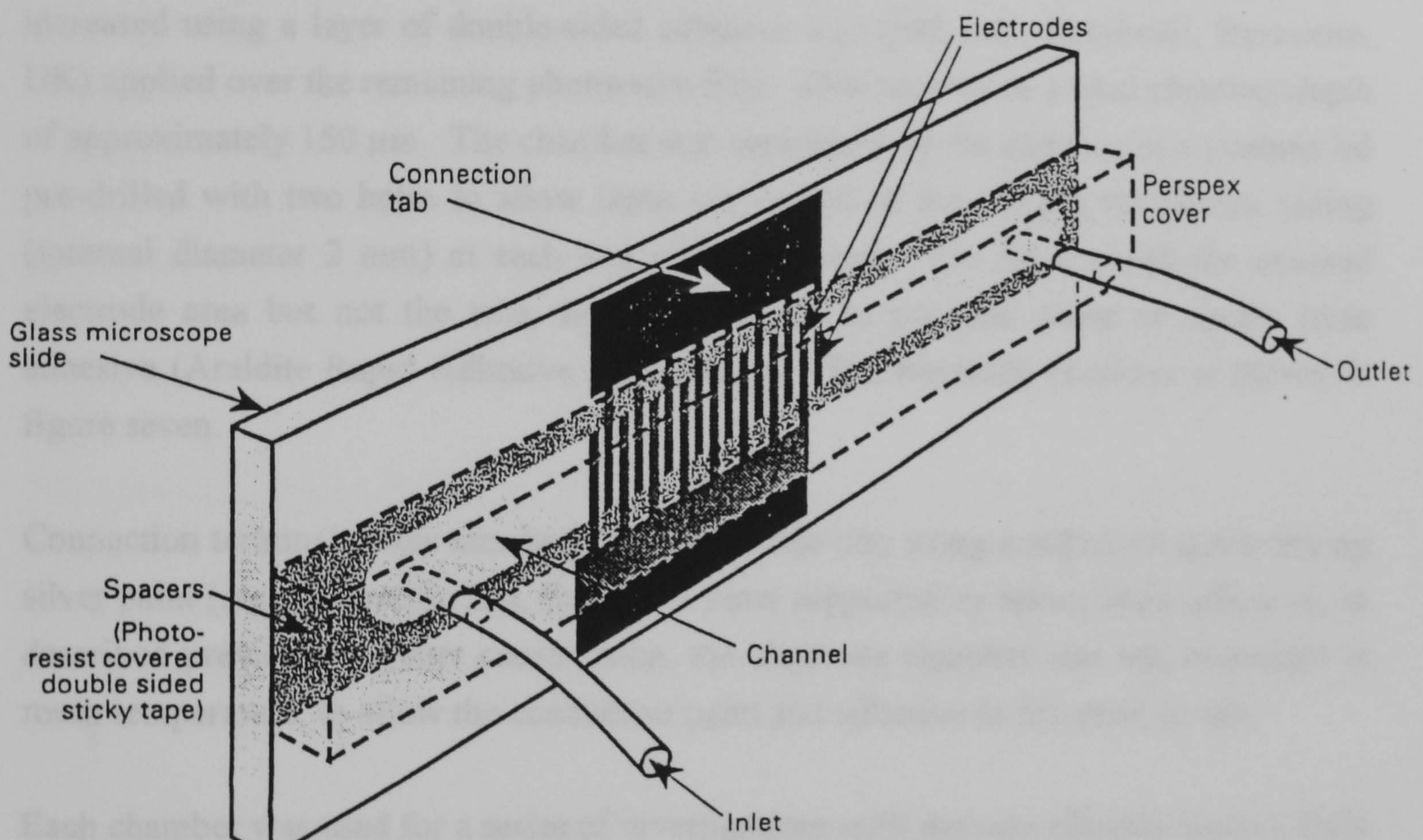


Figure 7: The dielectrophoretic measurement chamber.

for construction of the chamber walls and also made a significant addition (approximately 70 μm) to the chamber depth.

Glass slides bearing the electrode template were prepared in batches of 6-10 and stored at 4° C in the dark until use. When required, a slide was selected and examined microscopically for damage to electrode bars and tabs; if intact this slide was used for chamber construction.

2.4 CONSTRUCTION AND USAGE OF THE ELECTRODE CHAMBER

The electrode chamber walls, delineated by the additional layer of photoresist, were increased using a layer of double-sided adhesive tape (3M Ltd., Bracknell, Berkshire, UK) applied over the remaining photoresist film. This resulted in a total chamber depth of approximately 150 μm. The chamber was completed by the addition of a perspex lid pre-drilled with two holes to allow input and output of the sample via silicon tubing (internal diameter 2 mm) at each end of the channel. The lid covered the encased electrode area but not the tabs, and was secured in position using an epoxy resin adhesive (Araldite Rapid Adhesive, Ciba-Geigy). The electrode chamber is shown in figure seven.

Connection terminals were attached to the electrode tabs using conductive quick-drying silver paint (Agar Scientific Ltd, Essex, UK) and supported by epoxy resin adhesive, as described previously. After construction, the electrode chamber was left overnight at room temperature, to allow the conductive paint and adhesive to dry prior to use.

Each chamber was used for a series of investigations until damage affected electric field production or detection of the dielectrophoretic response. Chambers were rinsed with deionised water between experiments. The adherence of remaining cells to the electrodes was discouraged by the introduction of bubbles into the chamber; surface tension at the air-liquid interface aided the removal of cellular debris, but could also result in electrode damage. After rinsing, the system was drained then the experimental sample re-introduced.

When not in use, the chamber was dried and stored at 4° C in darkness until required. During repeated use of the same chamber, the condition of electrode bars and chamber were assessed, using a microscope, prior to experimentation. If no dielectrophoretic collection response could be elicited by a manual test, as described in section 2.7, the chamber was discarded and a new one prepared.

2.5 SYSTEM DESCRIPTION

The field across the electrodes was produced by a 1 mHz to 50 MHz function generator (Hewlett Packard 8116A, Germany) connected via leads to the electrode tabs. A 50 Ω shunt was attached to the connecting coaxial cable at the junction to the electrodes; its function was to ensure that the output of the signal generator was consistent over the frequency range investigated.

The suspension under investigation was circulated through the electrode chamber from a sample reservoir by a peristaltic pump (Gilson Minipuls, France) (fig. 8). A typical experiment involved the use of two different flow rates, as stated in descriptions for each specific measurement system. A slow flow rate, *e.g.* 1 ml min^{-1}, was in operation prior to, during and after pulse application. Successive field applications were separated by a short rapid flushing step, *e.g.* 4.5 ml min^{-1}, to remove any adherent particles from the electrodes after the response to the field had been measured. Where appropriate, sedimentation of particles in the sample reservoir was discouraged by gentle introduction of filtered air at the reservoir base. A second peristaltic pump was employed for this purpose, permitting a consistent mixing action for the duration of the experiment.

The activities of both the signal generator and the primary peristaltic pump were co-ordinated by a microcomputer (fig. 8; for model details see specific system description) using in-house software. Control from the computer was via an interface card (Hewlett Packard Interface Bus) for the signal generator and a Keithley DAS8-PGA card for the pump.

The microcomputer co-ordinated the activities of the signal generator and the pump using pre-set variables. For a frequency response investigation these variables were as follows:

- minimum frequency limit; the equipment was not operated below 1×10^3 Hz due to the damaging effects of electrolytic activity at lower frequencies.
- maximum frequency limit; this was set at 5×10^7 Hz, the upper operating limit of the Hewlett Packard signal generator employed.
- increment step; expressed as the number of frequencies applied per frequency decade on a logarithmic scale.
- pulse voltage; peak to peak measurement of a sinusoidal waveform.
- base time; the slow flow rate period prior to voltage application.
- pulse time; the length of voltage application

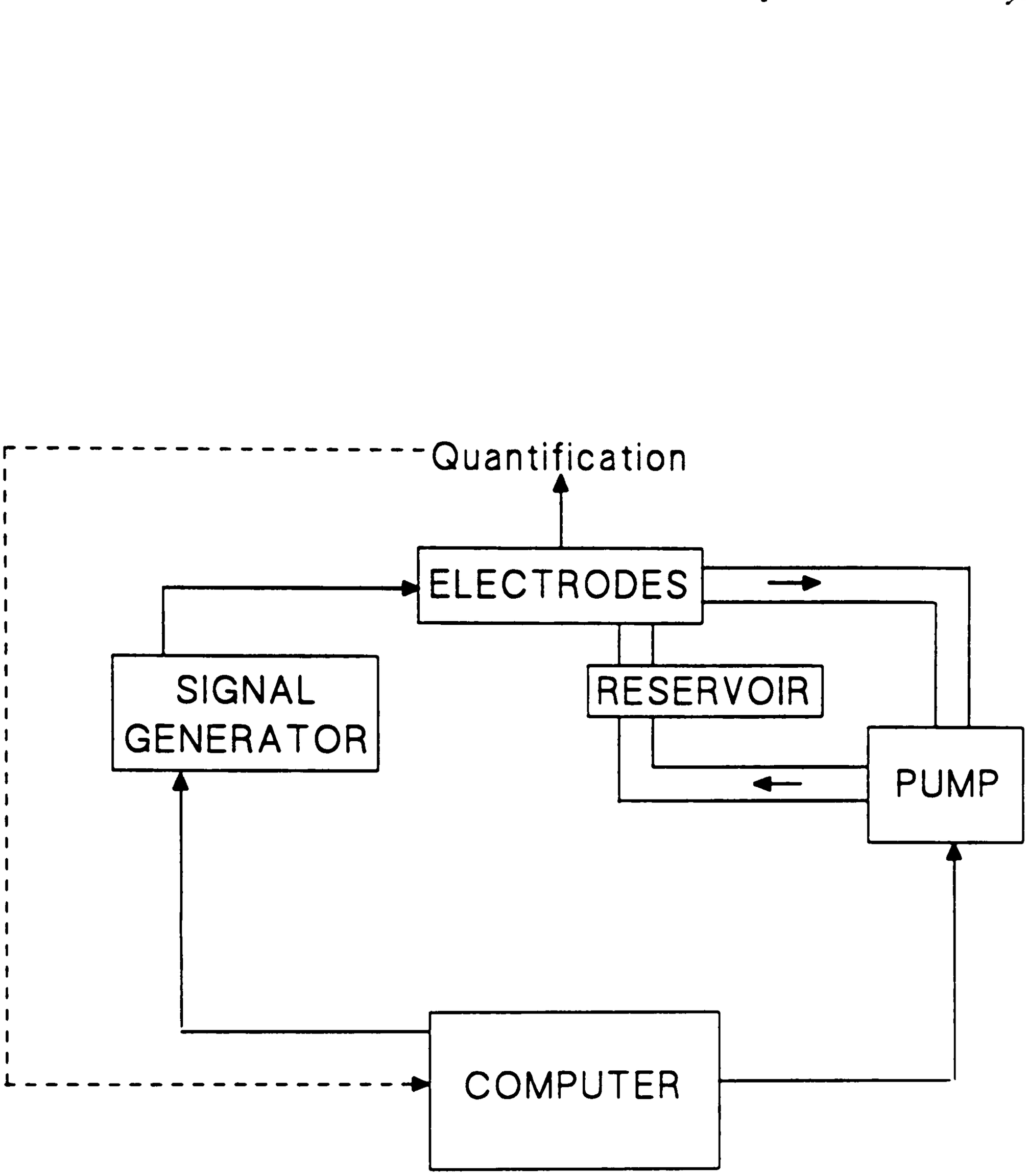


Figure 8: Schematic of the basic experimental apparatus.

- peak time; the period after voltage application.
- pump time; the length of flushing step at increased pump speed.
- experimental pump speed; the rate of flow before, during and after pulse application.
- flush-through pump speed; the value of increased flow rate used to remove adherent particles from electrodes prior to subsequent voltage application.

The experimental sequence is described in a flow diagram (fig. 9).

The in-house software allowed the dielectrophoretic response to be investigated with respect to frequency (as already described), pulse voltage, pulse length or flow rate. In each case, parameters were set on a control screen prior to the initiation of an investigation.

2.6 SUPPLEMENTARY SAMPLE MEASUREMENTS

During an experiment it was necessary to monitor pH and conductivity, both of which have effects upon dielectrophoretic collection. For the investigation of the dielectrophoretic response, the particles to be studied were suspended in low ionic strength solution to prevent dielectrophoresis of the medium occurring in preference to that of the cells (Chapter Six). Non-viable and prokaryotic cells used as test particles displayed little interaction with the suspending medium, as indicated by a rise in conductivity of a few μ Siemens per cm during a typical experimental time period. This increase was attributed to the loss of ions from the boundary layer surrounding the cell into the bulk solution. However when investigating haematological cells, the hostility of the suspension medium induced ion leakage from the cell interior (Chapter Seven). This action had the potential to cause a large enough increase in sample conductivity to influence dielectrophoretic collection. Conductivity was therefore measured as required during the experiment using either a probe immersed in the reservoir (Jencons 4070 hand-held conductivity meter) or an on-line flow-through probe (EDT Instruments RE387 Tx micro-processor conductivity meter). Disadvantages associated with the latter were the tendency for particle sedimentation to occur inside the bulb of the probe during prolonged investigations, and the large dead space volume of the probe, which precluded its use in some of the haematological investigations due to the small size of the sample available (Chapter Seven)

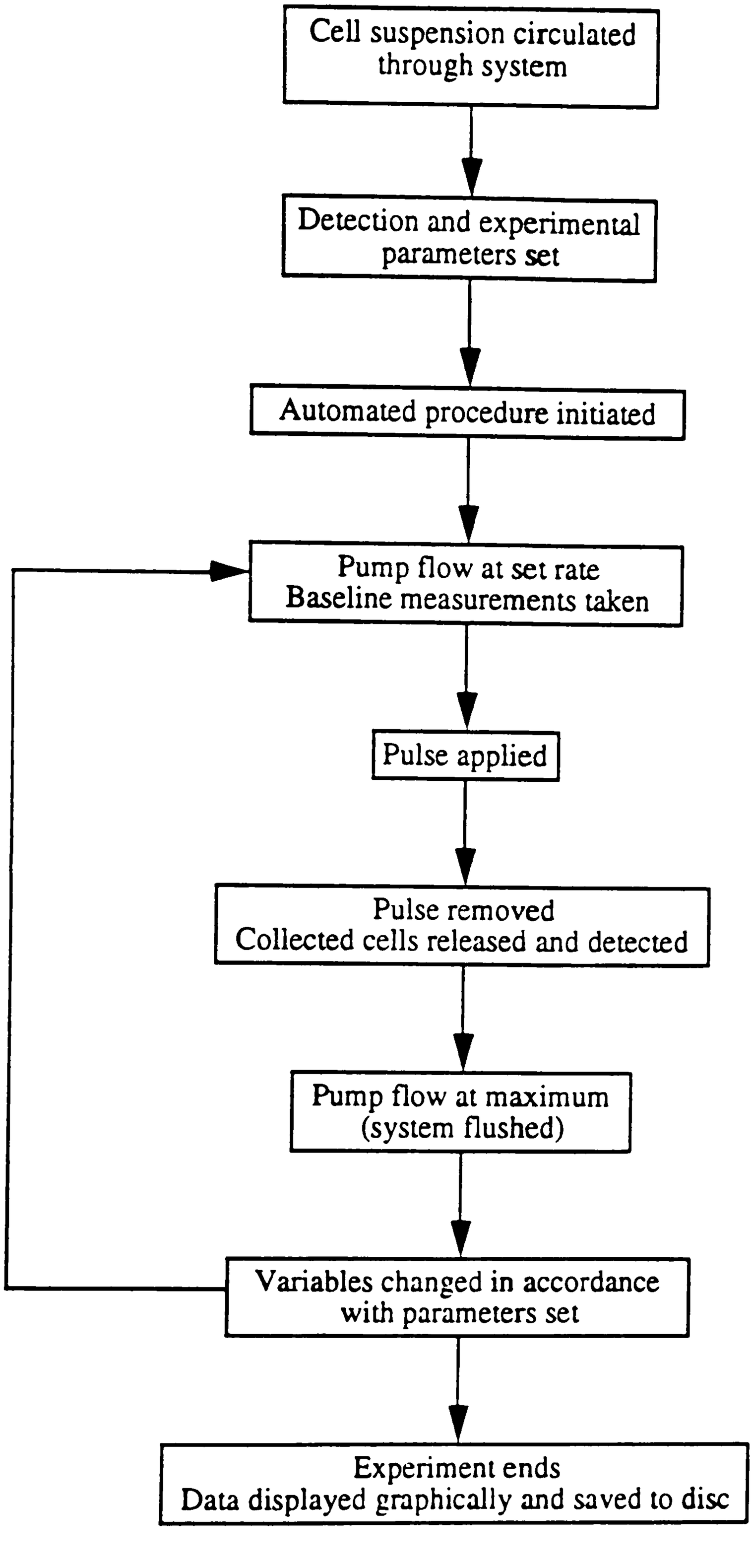


Figure 9: Flow diagram of experimental process including details relating to automated detection and data acquisition.

On-line pH measurements could not be made; electrolyte leakage from the pH electrode caused a rise in conductivity of the sample. Instead, aliquots of 0.5 ml were sampled as required from the system reservoir after thorough mixing, and pH determined using a microprobe adapted for use with small volume, low ionic strength suspensions, connected to a pH meter (model 3220, Jenway). At this stage the cell concentration of the sample could also be determined using a haemocytometer. These measurements were taken at least at the beginning and end of each run to ensure that any differences in collection were not due to changes in pH or sample density.

The dielectrophoretic measurement systems described subsequently were developed from the standard components detailed here. Modifications to the chamber design and the method of detecting the response of particles to a non-uniform electric field are described in Chapters Three to Five.

2.7 SUMMARY OF STANDARD EXPERIMENTAL PROCEDURE

A suspension of particles was prepared in a low conductivity solution, transferred to the system reservoir and circulated continuously through the electrode chamber by peristalsis. Adjustments were made to allow optimum detection of the dielectrophoretic response with the method employed. A manual test was carried out by applying a pulse from the signal generator to establish whether an effect of the field upon the particle could be determined.

After the check to ascertain that there were no faults with the system, the operational parameters were set using the computer, and the experiment initiated.

Chapter Three

A SPECTROPHOTOMETRIC DIELECTROPHORETIC MEASUREMENT SYSTEM

Automated quantification of the dielectrophoretic response was achieved by employing a spectrophotometer to measure the change in absorbance of the cell suspension as an effect of the applied electric field (Hawkes *et al.*, 1993). Dielectrophoretic collection resulted in an alteration of the local cell concentration and thereby affected the absorption properties of the suspension in the immediate vicinity of the electrodes. Absorbance readings were analysed in real-time by the computer and used to construct dielectrophoretic spectra.

3.1 SYSTEM DESCRIPTION

Detection of the dielectrophoretic response by absorbance changes of the sample requires that bulk changes in local cell concentration are apparent. Due to the nature of the response, collection of cells may occur both in the intervals between the electrode bars and upon the bars themselves (section 1.1.6b). Measurement of absorbance between adjacent electrode bars only would result in under-estimation of the total collection. In order to optimise detection of suspension absorbance and its variation with an applied field, measurements were made slightly downstream of the electrode bars after voltage removal. In this manner a gross absorbance change can be recorded, after cell release, as a result of dielectrophoretic collection on and between electrodes. To enable the absorption of the cell suspension to be recorded by a spectrophotometer, modifications to the electrode chamber and an extension to the controlling computer program were required.

The base of the electrode chamber was masked with aluminium foil leaving a sharp-edged rectangular window approximately 8 mm by 2 mm positioned 3 mm from the downstream edge of the electrode bar. The chamber was mounted in the path of the light beam from a LKB Ultrospec II spectrophotometer so that the focussed beam passed through the window downstream of the electrodes (fig. 10).

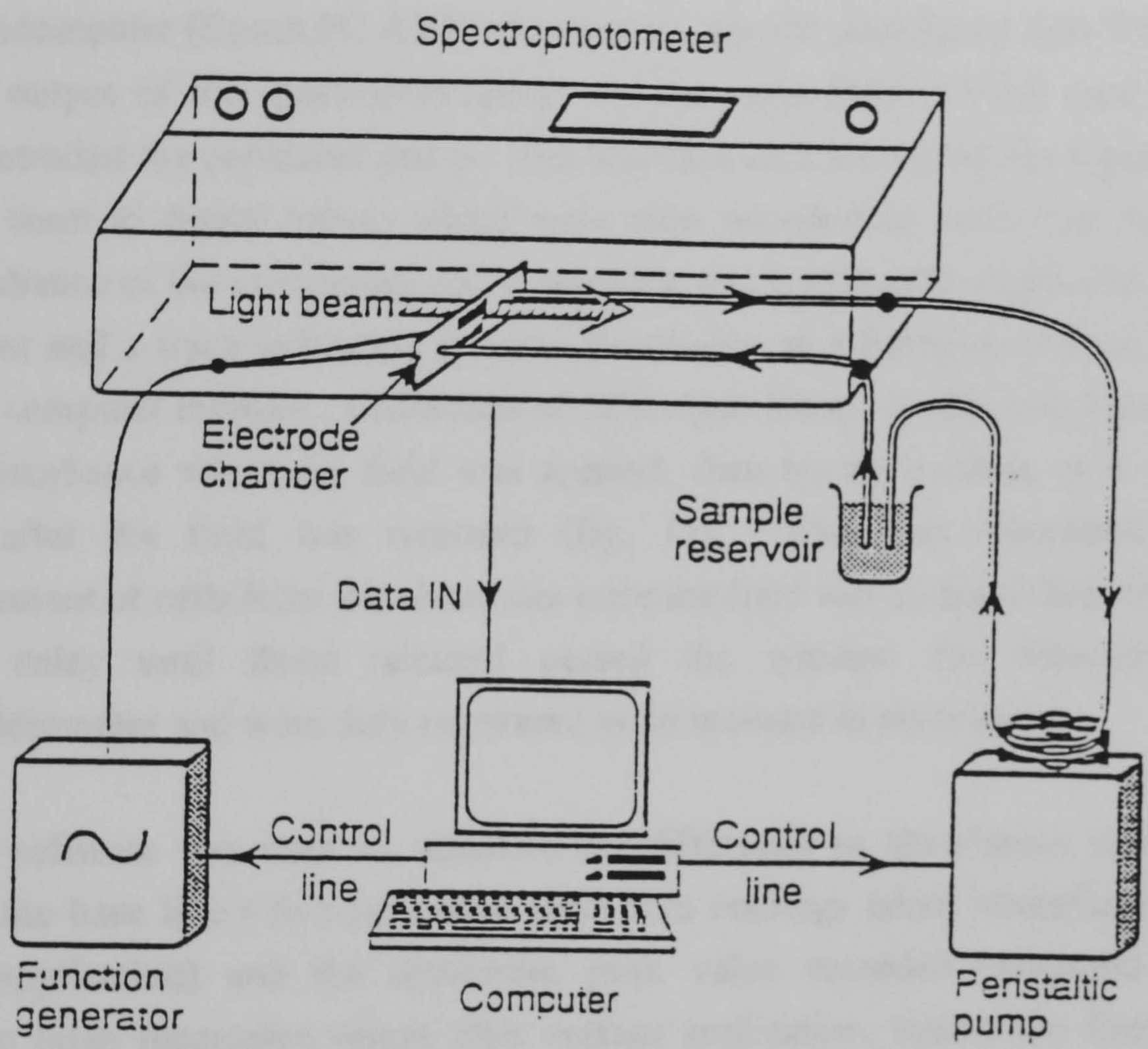


Figure 10: Schematic of the spectrophotometric dielectrophoretic experimental system indicating the position of the light beam with respect to the electrode bars.

A suspension of cells was circulated, by a peristaltic pump, from a reservoir through the electrode chamber, as described previously, (section 2.5). A slow flow rate, *i.e.* up to 1 ml min^{-1}, was used for data collection and a faster rate, *i.e.* up to 4.5 ml min^{-1}, for chamber purging. The wavelength of the light beam from the spectrophotometer selected was that at which absorbance by cells was maximal, and by the suspending medium, minimal. This was determined from a wavelength scan on a Hitachi U2000 spectrophotometer (Hitachi Ltd, Japan). The absorbance reading for the base line flow of cell suspension through the experimental spectrophotometer was set to zero.

The microcomputer (Epson PC AX2) was used to acquire absorbance data from the 1 V analogue output of the spectrophotometer via the same DAS-8 PGA card (Keithley) which controlled the peristaltic pump. The interface card converted the signal from an analogue form to digital values which were then recorded as multi-line ASCII files. The absorbance of the circulating cell suspension was continually monitored during the experiment and a trace indicating relative absorbance as a function of time, displayed upon the computer monitor. Collection of cells upon the electrodes was indicated by a fall in absorbance when the field was applied, then by an increase at a short time interval after the field was removed (fig. 11). This was consistent with the disengagement of cells from the electrodes once the field was extinguished, followed by a short delay until those released passed the window for detection by the spectrophotometer and were duly registered as an increase in absorbance.

In-house software was used to calculate the difference in absorbance measurements between the base line value (averaged from five readings taken immediately prior to voltage application) and the maximum peak value recorded (averaged from the maximum three successive values after voltage application, before the flushing step). This value was calculated for each voltage application as indicated in figure 11a, and was a relative measure of cell collection due to the dielectrophoretic force. At the end of the investigation, a spectrum was compiled by the computer of the difference in absorbance against the experimental parameter investigated, such as field frequency. This was displayed on screen and saved to disk.

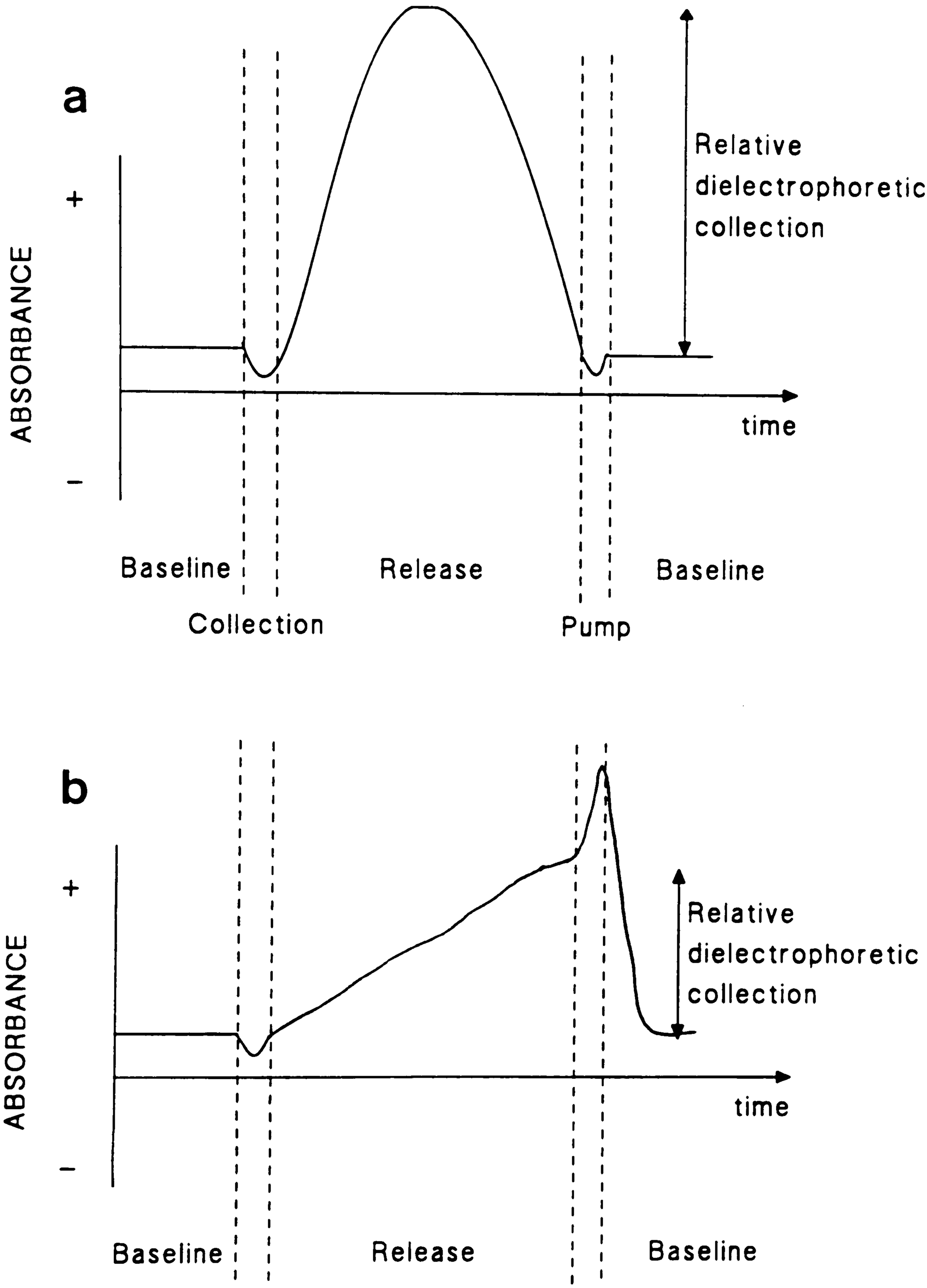


Figure 11: Change in suspension absorbance with time due to a single pulse application. a) Idealised on-line trace indicating quantification of dielectrophoretic response. b) Abnormal trace indicating problems with particle release after voltage removal.

3.2 EVALUATION OF THE SPECTROPHOTOMETRIC METHOD TO MEASURE DIELECTROPHORETIC PHENOMENA

Initial investigations of dielectrophoretic collection and its detection using the spectrophotometric method were carried out using *Saccharomyces cerevisiae* and commercially available polystyrene latex beads. The advantages of these 'test particles' were their availability, robustness, ease of sample preparation and low (zero for latex beads) physiological demands during the experimental period.

3.2.1 Sample Preparation

For the dielectrophoretic collection of suspended particles to occur, in preference to that of the medium, the medium itself must be of low conductivity, as described in Chapter One. For microorganisms and latex particles, this condition was easily satisfied by the use of deionised water as a suspending medium.

Before the electrode chamber was positioned in the spectrophotometer, the movement of particles in response to an applied electric field was visualised using a Nikon Labophot-2 microscope equipped with a long working distance 10x objective lens and a 10x eyepiece. This ensured that the electrodes were operating correctly and that a dielectrophoretic effect could be induced. At this stage it was observed that although test particles suspended in deionised water underwent collection upon the electrode surface, they did not disengage after the field had been removed and remained attached to the electrodes. This occurred with both *Saccharomyces* and latex bead samples thus preventing the composition of dielectrophoretic collection spectra using this method.

a) Use of detergent to promote release of cells from electrodes

Detergents with different types of action were investigated for their ability to promote the disengagement of collected cells after removal of a voltage. Sodium dodecyl sulphate (anionic), hexadecyltrimethyl-ammonium bromide chloride (cationic), N-dodecyl-N, N-dimethyl1-3-ammonio-1-propanesulphate (DDAP; zwitterionic) and Triton X-100 (non-ionic; all were supplied by Sigma) were investigated at a concentration of 0.01 mM. *S. cerevisiae* test samples were prepared from a stock suspension prepared in water following procedures detailed in section 3.2.1b. In each case, the stock suspension was further diluted with an equal volume of 0.02 mM

detergent solution to minimise differences associated with varying cell concentrations. An example of a typical spectrum obtained is shown in figure twelve.

The use of SDS solution as a suspension medium improved the release of yeast cells from electrode bars after removal of an electric field. The detection of increased levels of dielectrophoretic collection, as shown in figure twelve, suggested that the polarising action of the field was enhanced by the use of this anionic detergent; the promotion of cell release was indicated by the on-line trace profile displayed on the monitor (see section 3.2.3). Dielectrophoretic collection was abolished by the use of the cationic detergent investigated. The zwitterionic and non-ionic detergents permitted collection spectra to be obtained, but did not exhibit the increased values or low variability of measurement associated with suspension in SDS solution.

The effect of various agents upon dielectrophoretic collection is examined in detail elsewhere (section 6.4). The action of detergents in enhancing or abolishing the dielectrophoretic effect may reflect its effect upon the zeta charge layer surrounding the cell (section 1.2.1). Particle re-suspension in an anionic detergent solution may promote dielectrophoretic response by increasing the polarisability of the ion layer surrounding the cell. Use of a cationic detergent is postulated to affect the formation of a charge interface between the particle and suspending medium, resulting in a decrease in polarisability. This action is discussed in association with other treatments in Chapter Eight.

On the basis of these investigations, 0.01 mM SDS was employed as a suspending medium for use with the test particles described. This concentration was selected as it was the minimum investigated which supported consistent disengagement of cells after voltage removal, as viewed microscopically. Use of a low concentration of detergent also limited its contributory effects to suspension conductivity and electrode impairment. It is acknowledged that while this solution would not be suitable for use with haematological cells, it enabled the detection of possible dielectrophoretic collection by the spectrophotometer to be assessed.

As a consequence of these investigations, experimental samples were prepared by washing, then suspending the test particles in 0.01 mM Sodium Dodecyl Sulphate (SDS; Sigma) solution.

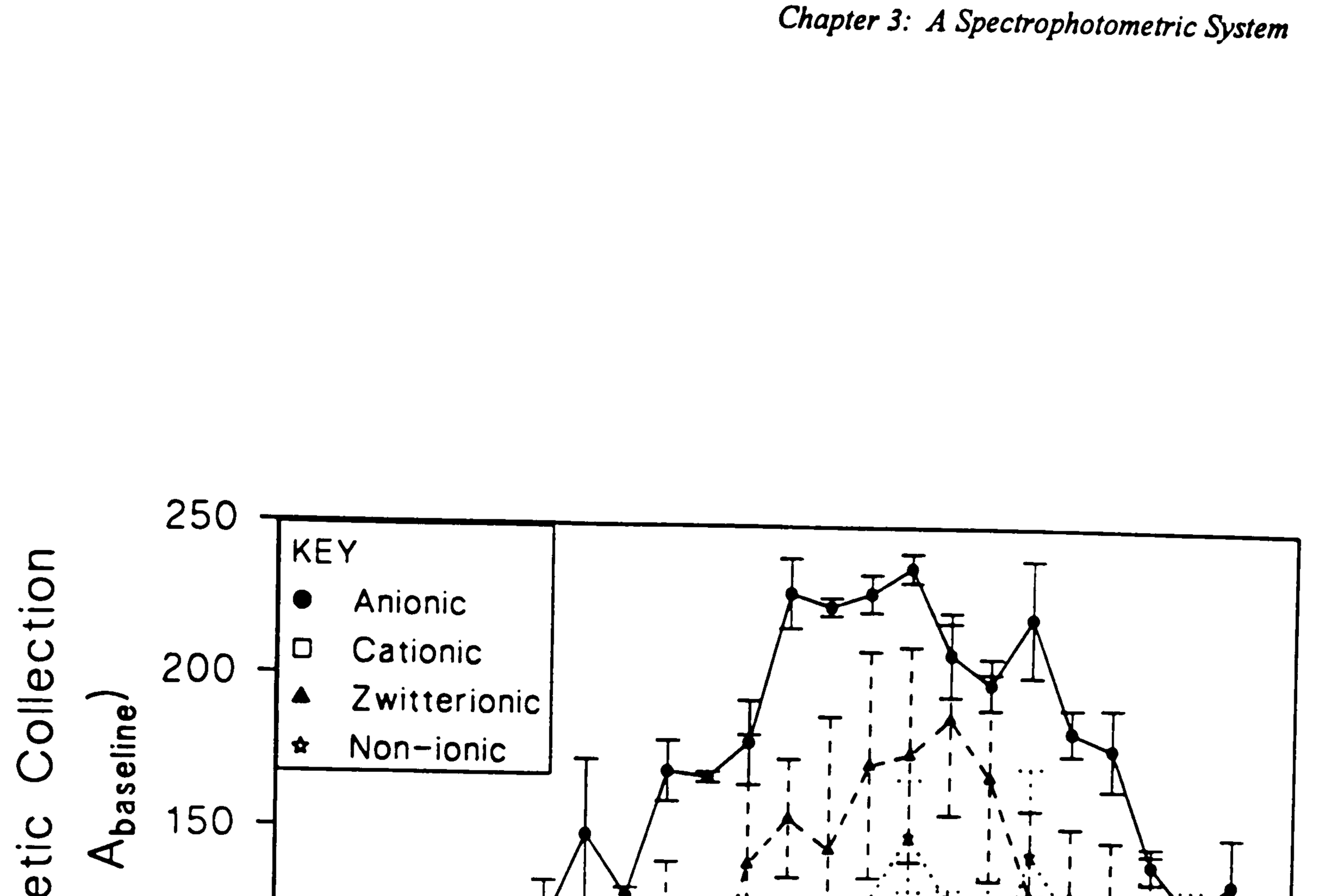


Figure 12: Effect of detergents upon dielectrophoretic collection of *Saccharomyces cerevisiae*. (Detergents used at concentration of 0.01 mM; anionic = sodium dodecyl sulphate, cationic = hexadecyltrimethyl-ammonium bromide chloride, zwitterionic = DDAP, non-ionic = Triton X-100. Pulse of 7 V for 5 s, pH = 6.4-6.7, σ = 1-5 $\mu S\ cm^{-1}$, concentration ~ 1 x 10^8 cells per ml, flow rate = 0.5 ml per min, λ = 550 nm. Each datapoint is the mean ± error bars of SEMs of 3 replicates; points connected through means).

b) Preparation of Saccharomyces cerevisiae samples

A stock culture of *Saccharomyces cerevisiae* cells was incubated at 37° C in glucose-yeast extract broth (GYE; see Appendix II). After a 48 hr period, as established from a growth curve, cells in the exponential phase of growth were obtained by centrifugation at 8000 rpm for 10 min. Harvested cells were washed in the experimental medium (0.01 mM SDS) and similarly re-centrifuged three times to remove high ionic strength culture medium before final re-suspension.

c) Preparation of latex bead samples

Polystyrene latex beads (Interfacial Dynamics Corporation, Portland, Oregon) were also employed as test particles. A range of bead diameters (0.030-2.13 μm), with various charged groups attached, was investigated. The beads are available as a suspension in distilled water containing a trace of EDTA to prevent flocculation.

Samples were prepared by the transfer of a 200 μl aliquot of beads to approximately 5 ml of 0.01 mM SDS solution which was filtered through a 0.2 μm membrane (Sartorius). The beads were then re-suspended into fresh SDS medium. This washing process was repeated three times to ensure removal of bead storage suspension before final re-suspension in 10 ml SDS medium.

Various bead types were tested for dielectrophoretic response using the preparative method and equipment described. No response was elicited using latex beads carrying the following charge groups; zwitter-ionic (bead diameter 0.492 μm), amidine (0.076 and 2.51 μm) and carboxyl (0.516 μm). However beads with sulphate and hydroxyl groups attached, the latter as a result of the manufacturing procedure, were observed to experience dielectrophoretic motion. These observations provide support for the importance of surface groups and their effect upon the electrical double layer (section 1.2.1), in the dielectrophoretic response. It is suggested that beads coated with sulphate and hydroxyl groups effectively increase the extent of their zeta layer due to hydrogen bond formation with water. Manufacturers' information indicated that in aqueous solution, hydrophobic effects were largest for these beads for the selection investigated.

Optimal detection of these sulphated particles was achieved with beads of diameter 2.13 μm, leading to their employment as test particles.

d) Preparation of rapid test samples

Test samples of yeast cells were rapidly prepared from commercially available dried yeast (Rank, Hovis and McDougall) using the syringe filtration washing method as detailed for latex particles. Such samples were used for testing the electrodes and measurement apparatus under controlled conditions prior to investigations.

3.2.2 Comparison Of Dielectrophoretic Collection Spectra Of Test Particles

Test samples of cultured *Saccharomyces cerevisiae*, sulphated latex beads (diameter 2.13 μm) and dried yeast were prepared for dielectrophoretic investigation as required, using procedures already detailed. The same electrode chamber and system parameters were utilised. All samples were prepared in 0.01 mM SDS solution.

The aim of this investigation was to establish the frequency range over which positive dielectrophoretic collection could be detected. Thus, more importance was attached to standardising sample conductivity rather than concentration. The latter approach may be justified by the potential advantages of exploiting frequency differentials for identification and separation purposes. In addition, there is a requirement for a measurable absorbance change to be effected, irrespective of the magnitude of the particles' response to the electric field; *i.e.* weakly responsive particles may be optimally detected at a different concentration than highly responsive particles. For all three samples, concentration was approximately 10^8 particles per ml.

Dielectrophoretic collection as a function of frequency was measured about 20 minutes after sample preparation to allow time for adjustments to be made to the sample if necessary. Typical spectra of dielectrophoretic collection against frequency of applied field obtained from each of the three test particles are shown in figure 13.

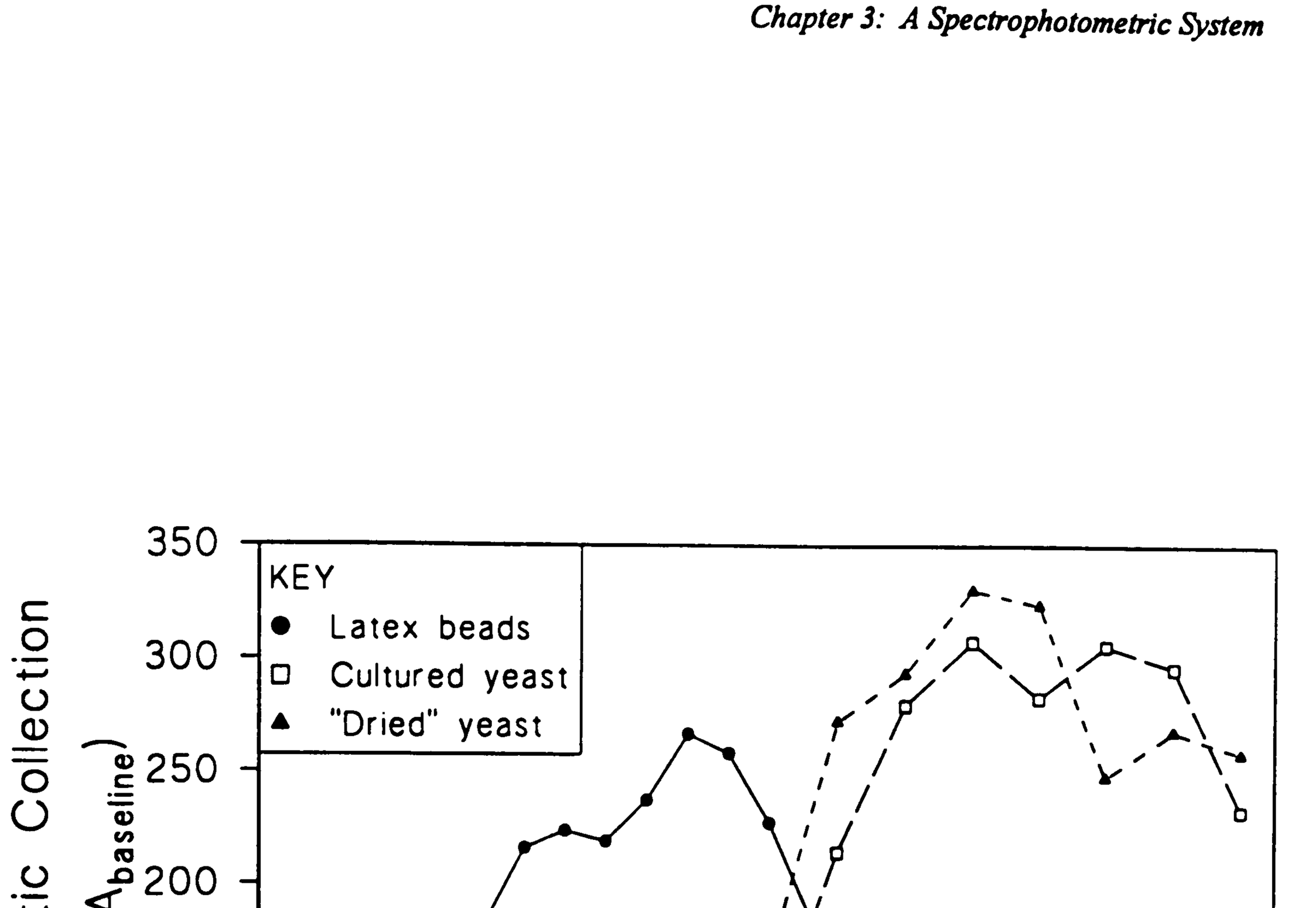


Figure 13: Dielectrophoretic collection of test particles as a function of frequency. (Pulse of 7 V for 3 s, pH = 6.4-6.7, σ = 1-5 μS cm^{-1}, concentration ~ 1 x 10^8 cells per ml, flow rate = 0.5 ml per min, λ = 550 nm. Data from first run only of each sample).

In accordance with dielectrophoretic theory, yeast cells and latex particles exhibit dissimilar collection spectra under similar experimental conditions. Dielectrophoretic behaviour of *Saccharomyces* was shown to be comparable between cultured samples and those obtained from dried yeast, supporting the use of the latter as rapidly-prepared test particles for investigations. Further investigations were undertaken with the primary aim of assessing the measurement system rather than examining the dielectrophoretic response of the test particles.

3.2.3 On-Line Diagnosis Of Collection/Release Abnormalities

The proficiency of the system could be assessed whilst the experimental run was in progress, or upon its completion. In the latter instance, as described in section 3.1, the data obtained from the experiment were displayed on the computer monitor as relative change in absorbance against the experimental variable, and saved to disk.

a) Interpretation of absorbance changes as a function of time in response to a single pulse

It was possible to monitor the performance of the system whilst dielectrophoretic measurements were being made, by consideration of the absorbance values relating to a single voltage application and release. These values from the spectrophotometer were continuously processed by the computer and displayed graphically on the monitor as a function of time. By this method, the gross response of the particles to an applied field could be determined at any point. An ideal trace indicating positive dielectrophoretic collection was typified by a constant base-line value before pulse application, followed by a decrease in absorbance during pulse application, then an increase when the field was removed (fig. 11a).

Measurement of dielectrophoretic response by the automated spectrophotometric system described here depended upon the concurrent release of cells from the electrodes after voltage removal. Due to the analysis method employed, it was essential that the collected cells disengaged from the electrodes simultaneously, to maximise the change in suspension absorbance measured by the spectrophotometer. Such behaviour was subject to the intensity of the force experienced by the cells and the integrity of their membranes; electro-fusion may be induced by the application of a voltage. Competing

forces such as flow and sedimentation rates also act. A collective release of cells was indicated by a well-defined peak on the trace, as described previously.

If inappropriate parameters had been defined, or the integrity of the cell was infringed as a consequence of the suspension medium or the experimental procedure, then the shape of the trace was altered. For example, observation of a maximum absorbance peak during the flush-through step, signifying that discharge of cells from the electrodes was compromised, could indicate electro-fusion preventing cell removal or that the experimental flow rate was too low. A similar trace pattern resulted when particles were suspended in deionised water, and found to remain attached to the electrodes after field extinction, as described in section 3.2.1a. This method of on-line problem diagnosis prompted experimental parameters to be modified, allowing optimum measurements to be obtained by the system.

b) Comparison of collection and release patterns for 2 and 16 bar electrode arrays

Comparison of on-line traces indicating the response to an applied field revealed that collection behaviour varied according to electrode configuration. Traces of absorbance changes obtained from dielectrophoresis of both cultured and dried yeast samples ($n > 20$), using either 2 bar or 16 bar electrode arrays, consistently described one of two distinct qualitative profiles. That from the 2 bar array tended to increase and fall off with time quite rapidly, defining a sharp peak, comparable to the ideal trace form in figure 11a. Conversely, the trace indicating collection on a 16 bar electrode array, was flatter and declined at a slower rate (fig. 11b).

This disparity in trace shape was attributed to the variation in distance between electrode bars and detection window for the two electrode designs. For the 16 bar array, cells released from the upstream bar had further to travel than those from one further down, resulting in a prolonged increase in absorbance. In addition, collection was not equal over the electrode array and was greatest on the first electrode bar, declining along the array, until saturation was attained. This reduction effect could be observed by microscopic examination of the electrodes during pulse application.

When 16 bar electrode arrays were employed, the time course over which the absorbance of the suspension changed in response to dielectrophoretic collection and release was lengthened in comparison to the 2 bar array. Also, the maximum absorbance measured in response to a single pulse was lowered, due to the slower and

prolonged release of particles from the electrodes (*c.f.* fig. 11a & 11b). Dielectrophoretic response to the applied field was quantified by the controlling computer program as the difference between the baseline absorbance reading before, and maximum peak value after, pulse application. This method of calculation resulted in under-estimation of gross change in absorbance and, therefore, dielectrophoretic collection. All further investigations dependent upon cell release utilised 2 bar arrays to promote coherent disengagement of collected cells. The extended electrode array was more suitable for separation applications where a high level of cell collection was desired (see Chapter Eight for further comment).

3.2.4 Assessment Of Variability Between Dielectrophoretic Spectra

The reproducibility of spectra was examined both for variation between consecutive runs of the same *Saccharomyces* sample, and for differences in day-to-day operation of the system.

a) Effect of repeated measurement upon the same sample

Dielectrophoretic collection values for the same sample, as determined using the spectrophotometric system, exhibited some instability with time. Typically, the second consecutive frequency run on the same sample yielded higher collection values than the first taken directly after sample preparation; after this the values decayed with time (see fig. 14).

As figure 14 indicates, both the extent of collection and the frequency range over which dielectrophoretic collection was observed differed between consecutive runs. The data have not been statistically tested; however, this trend of maximum collection values exhibiting an initial increase then a decrease with sample repetition was consistent for all dielectrophoretic investigations undertaken with this measurement system. The phenomenon was further investigated using erythrocytes, as described in Chapter Six. The time elapsed since cell suspension in low ionic strength solution was surmised to affect the dielectrophoretic response; hence rapidity of sample investigation was desirable.

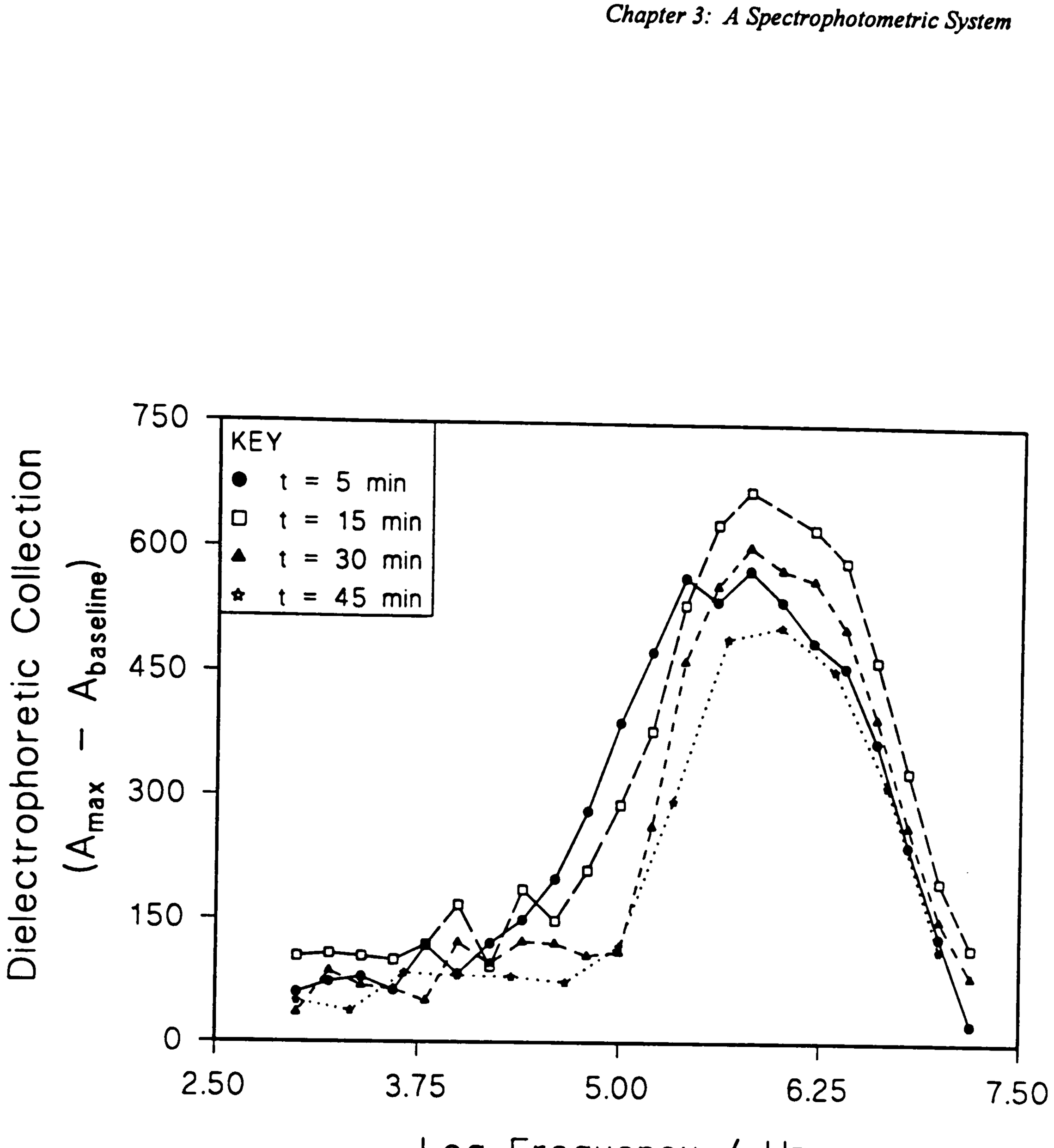


Figure 14: Effect of repeated sample measurement upon dielectrophoretic response of *Saccharomyces cerevisiae*. (Pulse of 5 V for 5 s, pH = 6.4-6.7, σ = 1-5 μS cm^{-1}, concentration = 8.2 ± 0.2 x 10^8 cells per ml, flow rate = 0.5 ml per min, λ = 550 nm. Data from repeated measurement of the same suspension).

The minimum frequency at which dielectrophoretic collection was recorded increased with time elapsed from sample re-suspension. This was attributed to conductivity changes occurring in the cell-dense, low ionic strength solution. For the sample described in figure 14, conductivity rose from 1 $\mu S\ cm^{-1}$ directly after preparation, to 5 $\mu S\ cm^{-1}$ after one hour. This increase could not be avoided given the requirement for a high cell concentration to enable absorbance changes to be monitored by the spectrophotometer. The effect of a rise in conductivity could be modified by suspending the cells in a higher ionic strength medium, but this increased the minimum frequency at which dielectrophoretic collection was observed and reduced the extent of collection. Both of these factors would place the measurement of dielectrophoretic collection beyond the limits of the system described.

Differences in the extent of collection were attributed to the rise in conductivity of the sample and possibly, to sedimentation effects. Measurements of reservoir cell concentration (as determined with a haemocytometer) taken at intervals throughout the experimental time period, showed some variation. The mean cell concentration was $8.2 \pm 0.2 \times 10^8$ cells per ml, n = 9), but did not indicate a trend towards decreased concentration in the bulk sample. Although the suspension was mixed in the reservoir, it may, to some degree, settle out in the system tubing and the electrode chamber. As already stated, the requirement for a relatively concentrated cell suspension is central to the operation of the spectrophotometric system; thus potential cell clumping and subsequent sedimentation could not reasonably be avoided.

b) Variation in dielectrophoretic measurements as a consequence of the experimental system

Day-to-day variation in dielectrophoretic measurements with the same electrode chamber was also observed. Figure 15 shows typical dielectrophoretic collection spectra from similarly prepared dried yeast samples taken over a four day period. Fresh samples were prepared each day prior to experimentation; cell concentration and conductivity were standardised (mean concentration $8.0 \pm 0.2 \times 10^8$ cells per ml, conductivity 1-5 $\mu S\ cm^{-1}$), and samples tested five minutes after suspension.

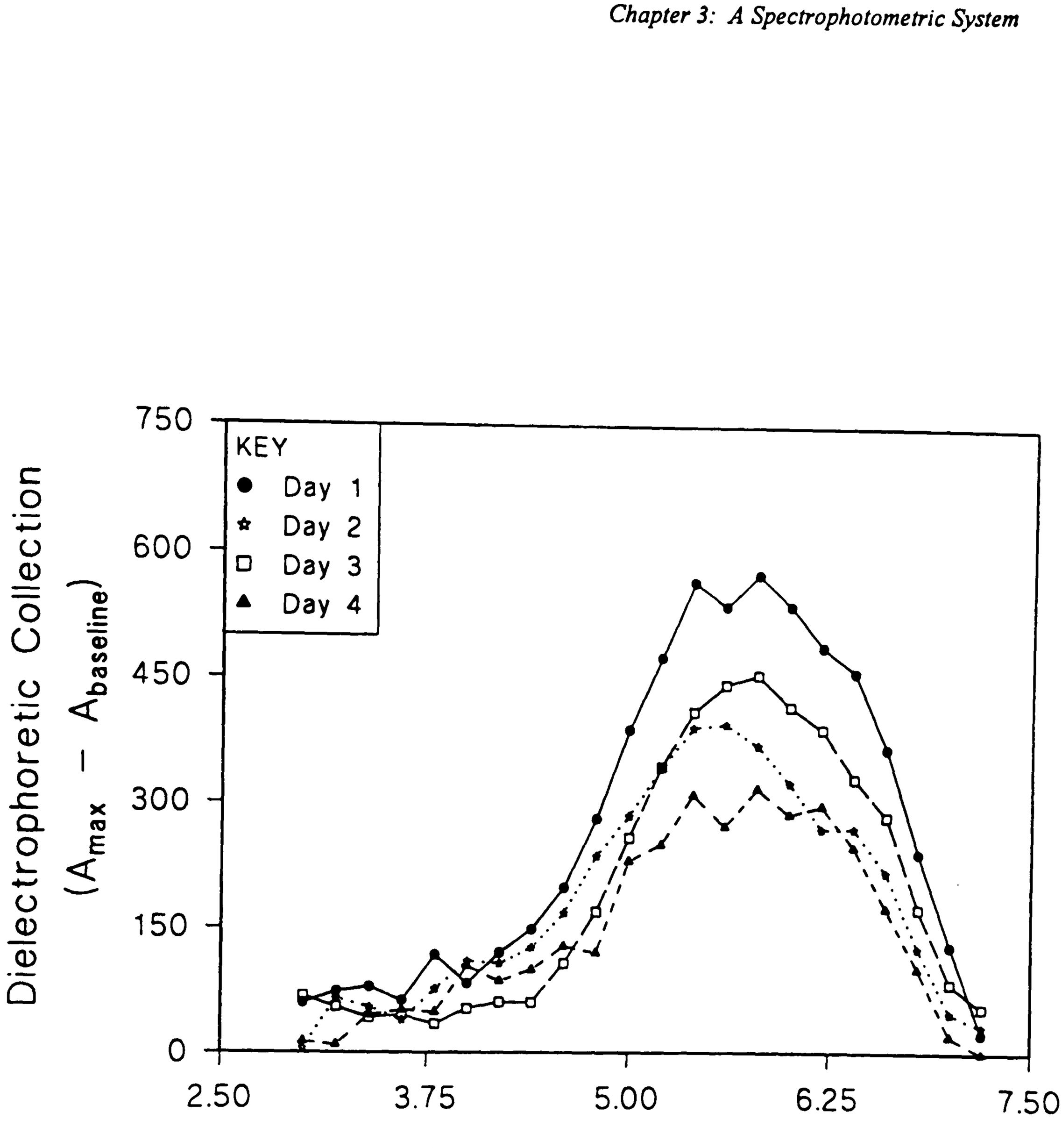


Figure 15: Variability of day-to-day measurements of the dielectrophoretic response of *Saccharomyces cerevisiae*. (Pulse of 5 V for 5 s, pH + 6.4-6.7, σ = 1-5 μS cm^{-1}, concentration = 8.0 ± 0.2 x 10^8 cells per ml, flow rate = 0.5 ml per min, λ = 550 nm. Data from first run only of fresh suspension prepared 5 min prior to experimentation).

Figure 15 suggests that dielectrophoretic collection values attained differed from day to day. In addition, it was noted that the maximum values obtained in an experimental run showed a tendency to decrease with electrode usage (fig. 15). Although this may be due to different sample preparations, observation of the electrode chamber whilst *in situ* over weeks of use revealed visible build-up of debris within the chamber, and thus a decrease in optical quality. Microscopic examination of the chamber on a daily basis showed deterioration of the electrode plates, leading to a reduction in collection efficiency, and the progressive build-up of debris upon the chamber surfaces. The latter event exerts a considerable effect upon experimental data by reducing light transmission through the chamber, thereby increasing all the absorbance measurements determined by the spectrophotometer. This effectively raises the baseline absorbance level, reducing the differential between baseline values and those after cell release, thereby resulting in under-estimation of the dielectrophoretic response.

Problems associated with day-to-day variability of collection values are discussed in more detail with respect to the image-analysis measurement system in section 5.4.

3.2.5 Concluding Remarks From Preliminary Investigations

The utilisation of various test particles to investigate the performance of the dielectrophoretic spectrophotometer has been discussed. Observation of the behaviour of such particles at the electrode surface prior to an experiment was shown to be of great benefit in assessing the quality of cell collection, release and detection. In addition, the function of the on-line trace in diagnosing errors during investigations has been described.

A weak anionic detergent solution was employed as a suspension medium due to its effects in promoting the release of cells from the electrodes after field application. This solution was also found to be beneficial in inhibiting the build-up of debris within the chamber which could otherwise lead to a deterioration in optical quality. Procedures for the preparation of test particles in this suspension medium have been described. Different test particle samples were shown to exhibit dissimilar collection versus frequency spectra.

Various potential sources of inaccuracy when using the dielectrophoretic spectrophotometer have been identified from investigations with test samples. Collection values are affected both by sample age, *i.e.* period of re-suspension in low ionic strength solution, and impairment of the electrode chamber. Both electrode deterioration (altering the electric field induced) and decay in optical quality (effecting cell detection) are sources of inconsistencies in measurements. Such limitations must be taken into account when defining a standard experimental protocol.

3.3 INVESTIGATION OF THE DIELECTROPHORETIC BEHAVIOUR OF HUMAN ERYTHROCYTES USING THE SPECTROPHOTOMETRIC METHOD

3.3.1 Sample Preparation

The dielectrophoretic investigation of eukaryotic cells without cell walls requires that they are suspended in osmotically-balanced medium (section 1.5.3). The criteria specifying a suitable suspension medium for use with human erythrocytes are discussed in detail in section 6.1.2; blood collection and storage procedures are also considered. For these preliminary investigations using the spectrophotometric system, a low ionic strength solution composed of 252 mM glycine 3 mm glucose (GG) (Krishna *et al.*, 1989a, 1989b) was adopted.

Fresh venous blood from one healthy female donor was collected into EDTA-containing vacu-tubes (Sarstedt, Germany), maintained at room temperature and used within 10 hours. A 1 ml aliquot was centrifuged at 1000 rpm for 10 min, the supernatant and buffy coat layer aspirated, and the pellet re-suspended to 10 ml with GG solution. The cells were similarly washed twice prior to final re-suspension in GG solution.

3.3.2 Preliminary Dielectrophoretic Investigations Of Human Erythrocytes

A sample of human erythrocytes was prepared, as already described, to give a concentration of 5 x 10^7 cells per ml. The cell concentration was lower than that adopted for test particles in order to maintain a low conductivity level so that measurements could be made. The sample was introduced to the reservoir, its pH and conductivity recorded, and the experiment initiated. Reservoir cell concentration was determined by removing samples every 5 minutes and counting cells using a haemocytometer. Figure 16 shows a typical dielectrophoretic response spectrum obtained from consecutive investigations of the same sample. Cell concentration remained stable within ten percent of the mean value (n = 5) over the experimental time period.

Figure 16 suggests that although positive dielectrophoretic collection was detected over a consistent frequency range, *i.e.* 300 kHz to 30 MHz, during repeated use of the same sample, the number of affected cells declined rapidly between successive runs. Such a decay was not thought to be due to changes in sample conductivity, as the increase over the experimental period was relatively small (140 to 163 $\mu S\ cm^{-1}$). In addition, the frequency at which dielectrophoretic collection was first observed was consistent, indicating that for these replica spectra, effects due to conductivity differences were minimal.

Further investigations of the trend which suggested that dielectrophoretic collection values decreased with repetition of experimental run, were carried out with a series of similar erythrocyte suspensions. These were prepared in GG solution as required, using blood samples from the same donor, following the procedure already described. In each case, six consecutive dielectrophoretic investigations using constant experimental parameters and electrode chamber were carried out. The first run was undertaken approximately five minutes after final re-suspension of washed erythrocytes; between runs the sample was drained from the chamber and measurement system, mixed well, then re-circulated to help maintain a consistent cell concentration.

Correlating values were obtained from each individual collection spectrum produced for each sample (n = 5), as measured at a frequency of 3.7 MHz which was the mean midpoint frequency value at which collection was observed for the samples investigated. For each sample, collection as a percentage of the first run value was calculated for each subsequent repetition, obtained at approximately ten minute intervals. The mean values and SEM's for percentage maximum dielectrophoretic

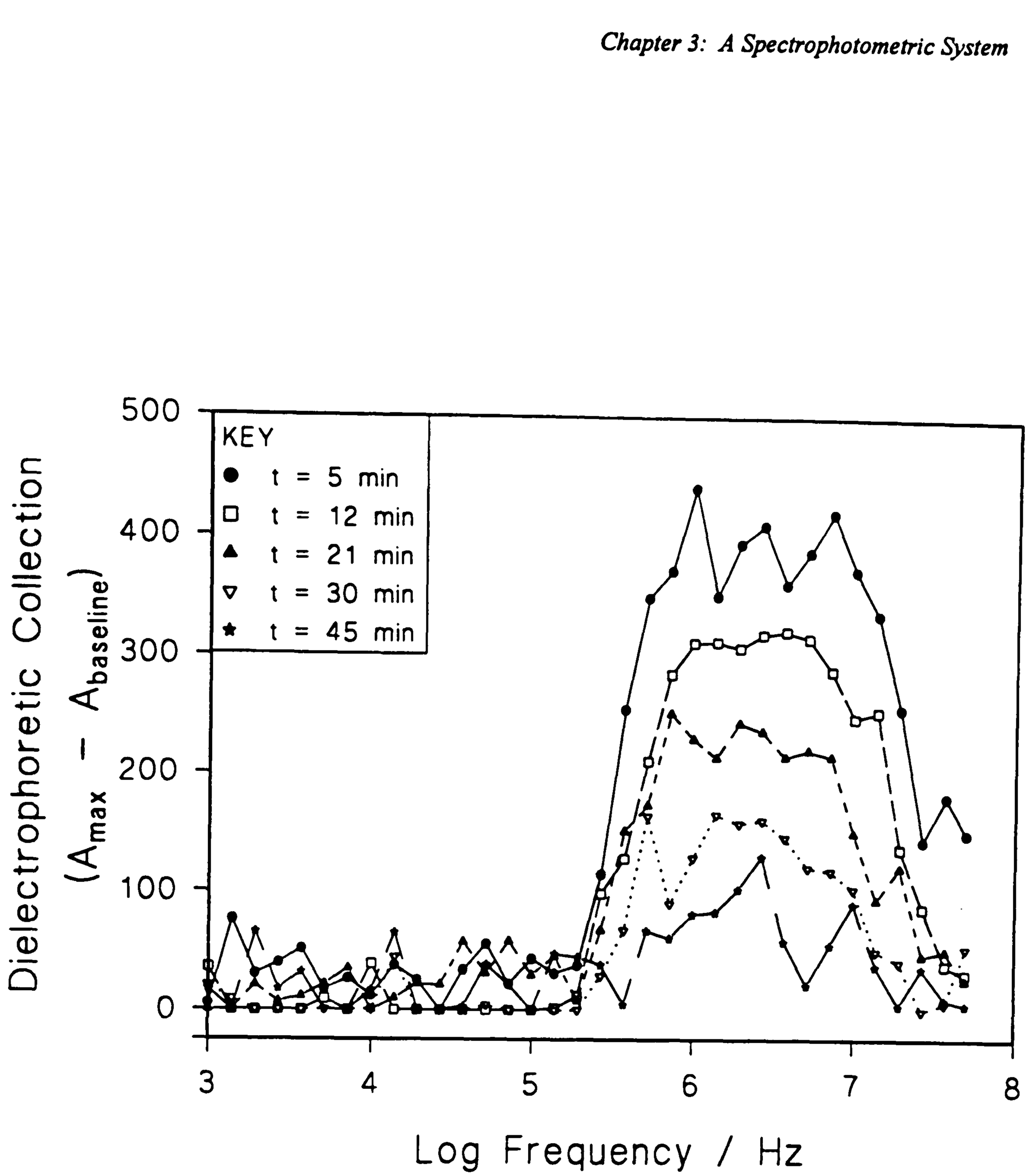


Figure 16: Effect of repeated sample measurement upon dielectrophoretic response of human erythrocytes suspended in glycine-glucose solution. (Pulse of 14 V for 3s, pH = 6.2 ± 0.1, initial σ = 140 μS cm^{-1}, final σ = 163 μS cm^{-1}, concentration = 1.0 ± 0.2 x 10^8 cells per ml, flow rate = 0.5 ml per min, λ = 540 nm. Data from repeated measurements of same suspension).

collection were then calculated for the pooled samples. The time interval between repetitive runs varied slightly due to problems with setting a zero baseline absorbance value on the spectrophotometer between consecutive spectra. In view of this, mean percentage of maximum collection values, with SEM's, were plotted against repetition number (figure 17). A linear regression line was fitted to the data and a correlation value obtained.

Linear regression of percentage decay against repetition number indicated that there was a direct dependence between the two parameters. The hypothesis that dielectrophoretic collection values decreased with use of sample was accepted ($p < 0.01$).

The decay of collection maxima with time was thought to be related more to sample age than variation in conductivity *per se*; mean percentage conductivity rise for data shown in figure 17 was 10.9 ± 5.3. Sample age affects aspects of the measurement/detection system in addition to the dielectrophoretic response. It is possible that cell sedimentation within the system reduces the effective cell concentration. Changes within the cell and its associated boundary layer, resulting from suspension in a low ionic strength medium and indicated by the rise in conductivity, will also affect the dielectrophoretic response. For example, loss of ions from the cell to the suspending medium would have a two-level effect; cell polarisability is decreased whilst that of the medium is increased, thereby reducing the differential between the two. This difference in polarisability, and therefore permittivity, is important in determining whether suspensoid or medium is affected by an applied non-uniform field (see section 1.1.1).

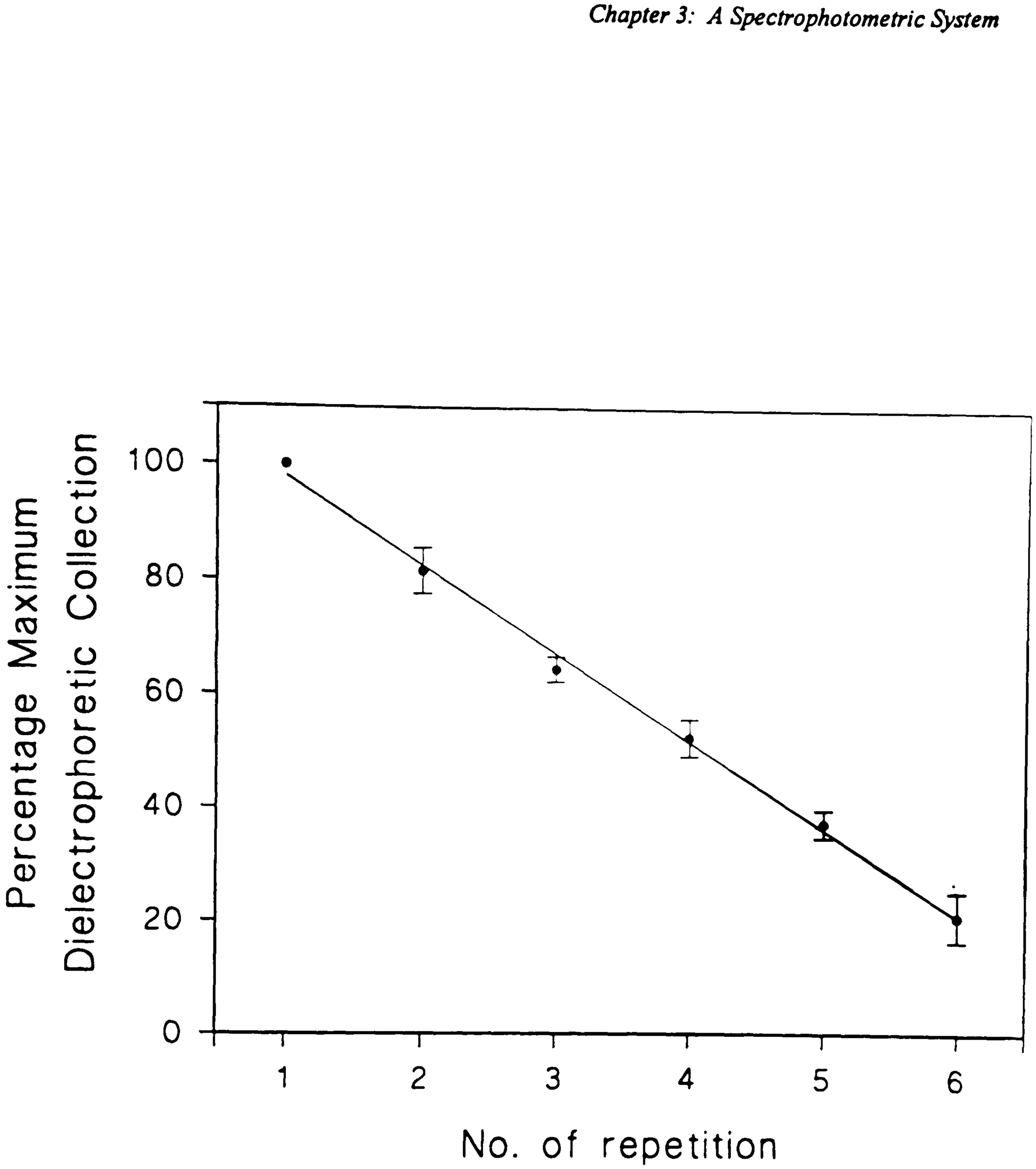


Figure 17: Percentage decay of dielectrophoretic collection as a function of time for human erythrocytes suspended in glycine-glucose solution. (Pulse of 14 V for 3 s, pH = 6.4 ± 0.1, initial σ = 156 ± 18.1 μS cm^{-1}, final σ = 173 ± 8.3 μS cm^{-1}, concentration = 5.0 ± 0.5 x 10^7 cells per ml, flow rate = 0.45 ml per min, λ = 541 nm. Datapoints shown are means ± error bars of SEMs from collection values at 3.7 MHz for 5 replicates).

Linear regression equation: $y = -15.454x + 113.523$, $r^2 = 0.997$, $p = 4 \times 10^{-6}$.

3.3.3 Difficulties Encountered Whilst Attempting To Measure The Dielectrophoretic Response Of Human Erythrocytes

Spectra of the dielectrophoretic response of human erythrocytes may be obtained using the spectrophotometric system described. Collection occurs over the frequency range 300 kHz - 30 MHz as shown in figure 16, under the conditions specified. These preliminary investigations highlighted some of the problems associated with measuring the response of mammalian cells. Such cells are more sensitive to their environment than the test particles employed during system development, and require a more complex suspending medium. Experiments to measure the dielectrophoretic response of erythrocytes using the spectrophotometric system, utilised an osmotically balanced, low conductivity solution described by Krishna *et al.*, (1989). Although this medium preserved erythrocyte integrity and permitted cell dielectrophoresis, it also dissolved the adhesive holding the chamber together, allowing it to enter the chamber interior. This resulted in contamination of the sample, deterioration of optical quality due to adhesive deposition upon inner chamber surfaces, entry of air bubbles with corresponding electrode damage and sample leakage. A variety of adhesives were investigated; a substitute capable of securing glass to perspex which was resistant to attack by GG solution was not found. (The criteria for, and development of, an alternative suspending medium are discussed in section 6.1.2).

The period of re-suspension in low ionic strength solution has been described as a factor affecting the dielectrophoretic response of test particles. This time-span is of more importance when investigating mammalian cells due to the hostility of the suspension medium and its inability to support cell viability over an extended period of hours. The conductivity of the medium increases with time due to ion leakage from the suspended cells. In addition to the physiological implications, this also affects dielectrophoretic behaviour, increasing the minimum field frequency at which collection is induced, and thus, decreasing the magnitude of the response. A technique for rapid, reliable measurement of dielectrophoretic response is therefore desirable.

3.4 CONCLUSION

3.4.1 System Performance And Limitations

A method of detecting dielectrophoretic response by studying the absorbance changes of a cell suspension, downstream of an applied electric field, has been described. Coherent mass cell release from electrodes after positive dielectrophoretic behaviour, was a critical requirement for this process, and was promoted by the use of 2 bar electrode arrays in preference to 16 bar configurations. Disengagement of test particles was further enhanced by the anionic detergent, SDS. Protocols concerning the use of test particle samples were established, and the ability of the system in distinguishing between different particles by their dielectrophoretic spectra has been demonstrated.

Specific advantages of this measurement system include:

- detection of gross dielectrophoretic response relating to a cell population; this was considered preferable for potential applications than measurements of individual cells.
- rapid sample processing; system automation permitted a frequency response spectrum to be compiled within ten minutes of suspension preparation thus limiting sample deterioration, and allowing replica data sets to be obtained in contrast to alternative methods (section 1.3.2).
- synchronisation of components by computer; the activities of the signal generator, peristaltic pump and data acquisition from the spectrophotometer were all controlled by computer.
- on-line fault diagnosis; abnormalities in the relative absorbance trace displayed during the experiment could indicate problems with particle release and detection.

On the basis of these preliminary investigations, some of the inherent problems of dielectrophoretic measurements have been identified.

A major limitation of this system is concerned with its day-to-day variability, as a result of chamber deterioration. Optical quality and electrode condition cannot be assessed without disrupting the investigation as this involves removal of the chamber from the spectrophotometer. The experimental protocol was refined to ensure that microscopic examination of the chamber was carried out before and after an investigation. In addition, comparisons were only made between data obtained from the same experimental set to minimise the effect of chamber deterioration as a variable

parameter. Inconsistencies concerned with the measurement system are discussed further in section 5.4.

Events occurring at the electrode surface could not be observed directly using this system, and were inferred from spectrophotometric readings interpreted by the computer. This method required that particle concentration was at a level where suspension absorbance changes could be detected without the use of a prolonged pulse application. In addition, coherent disengagement of particles from the electrodes was essential, and observations of real field effects (such as electro-rotation and pearl chain formation) could not be made. Improvements to the experimental system permitting visualisation of the electrode bars during an investigation would be beneficial.

Rapidity of measurement has been demonstrated to be of great importance when investigating dielectrophoretic behaviour using this system. The response detected diminished with the time-period for which the particles had been suspended in low ionic strength solution; the frequency range of the response was also altered. It is therefore concluded that the effect of interactions between particles and suspending solution should be taken into account during dielectrophoretic investigations.

3.4.2 Concluding Remarks Regarding The Utilisation Of The Dielectrophoretic Spectrophotometer Method For Measurement From Haematological Cells

The fundamental principle of operation for the dielectrophoretic spectrophotometer concerns the detection of absorbance changes in a particle suspension resulting from the application of an electric field. The optimum sample concentration for each investigation was dependent upon the condition of the electrode chamber and the polarisability of the particles in relation to that of the medium; it typically ranged from 1×10^8 to 1×10^9 particles per ml. The working concentration range was thought excessive when considering haematological samples for two reasons. Firstly, such a high concentration of cells would have a sizeable interaction with a low ionic strength medium, resulting in a conductivity rise. The sample would thereby be unstable with respect to dielectrophoretic behaviour. Also, when investigating sub-populations of haematological cells, a large volume of blood would be required for the isolation of sufficient cells. Such investigations were carried out in association with the regional Haematological Malignancy Diagnostic Service located at Leeds General Infirmary and it was not possible to obtain samples of the required magnitude. Operation of the dielectrophoretic spectrophotometer with a lower sample concentration was possible,

but an extended pulse application was necessary to collect enough cells for subsequent detection. This increased the time to which cells were exposed to deleterious effects of the field.

In addition to disadvantages associated with the requirement for a concentrated sample, the use of a closed experimental system (*i.e.* the chamber interior could not be observed directly) was inconvenient for investigations concerning haematological samples. Such samples are less robust than the test particles employed, and may be damaged during passage through the system, by exposure to electric fields or by prolonged suspension in low ionic strength medium. A dielectrophoretic measurement system permitting direct observation of the chamber interior would be beneficial, allowing cell morphology, electrode and chamber quality, and dielectrophoretic behaviour to be monitored.

Further investigations concentrated upon the development of a system for quantification of the dielectrophoretic behaviour of cells at the electrode surface, as described in Chapters Four and Five. Considerations associated with the measurement of haematological cells are discussed in Chapters Six and Seven.

Chapter Four

A VIDEO TECHNIQUE TO MEASURE DIELECTROPHORESIS

A simple but effective method of investigating dielectrophoretic collection of cells involved video-recording the events occurring at the electrode surface, as viewed microscopically, whilst a succession of electric fields were applied. This procedure, as compared to direct microscopic observation and enumeration, had the advantage of reducing the time course of the experiment, thus minimising the extent of physiological changes in the cell suspension during the investigative period. Subsequent analysis of the recording, exploiting the digital freeze frame facility of the video recorder, allowed total collection at the electrode area observed to be determined at specific time intervals. Additionally, it was possible to describe the motion of the cells and whether any gross morphological changes occurred as a result of the investigation.

4.1 SYSTEM DESCRIPTION

The electrode chamber (section 2.4, fig. 7) was positioned on the stage of a microscope (Nikon Labophot-2) fitted with a solid state colour camera (Hitachi KP-C500) (see figure 18). The chamber was viewed using a 20x objective lens (total magnification 1000x) allowing a section of electrode bar approximately 300 μm in length to be observed. The two bar electrode array and area immediately downstream were positioned in the field of view; the area in the centre of the chamber width was favoured where flow abnormalities were thought to be reduced. The image was viewed on a monitor (Taxan Multivision 775, Kaga Electronics Co., Japan) and simultaneously captured using an S-VHS video cassette recorder (VCR; NEC model DS 6000 K). An audio input to the VCR was used to indicate the period of pulse application for identification purposes during tape play-back and subsequent analysis. Both reflected and transmitted light microscopy were employed, allowing both the upper surfaces of the electrode bars themselves and the intervals between adjacent bars to be observed.

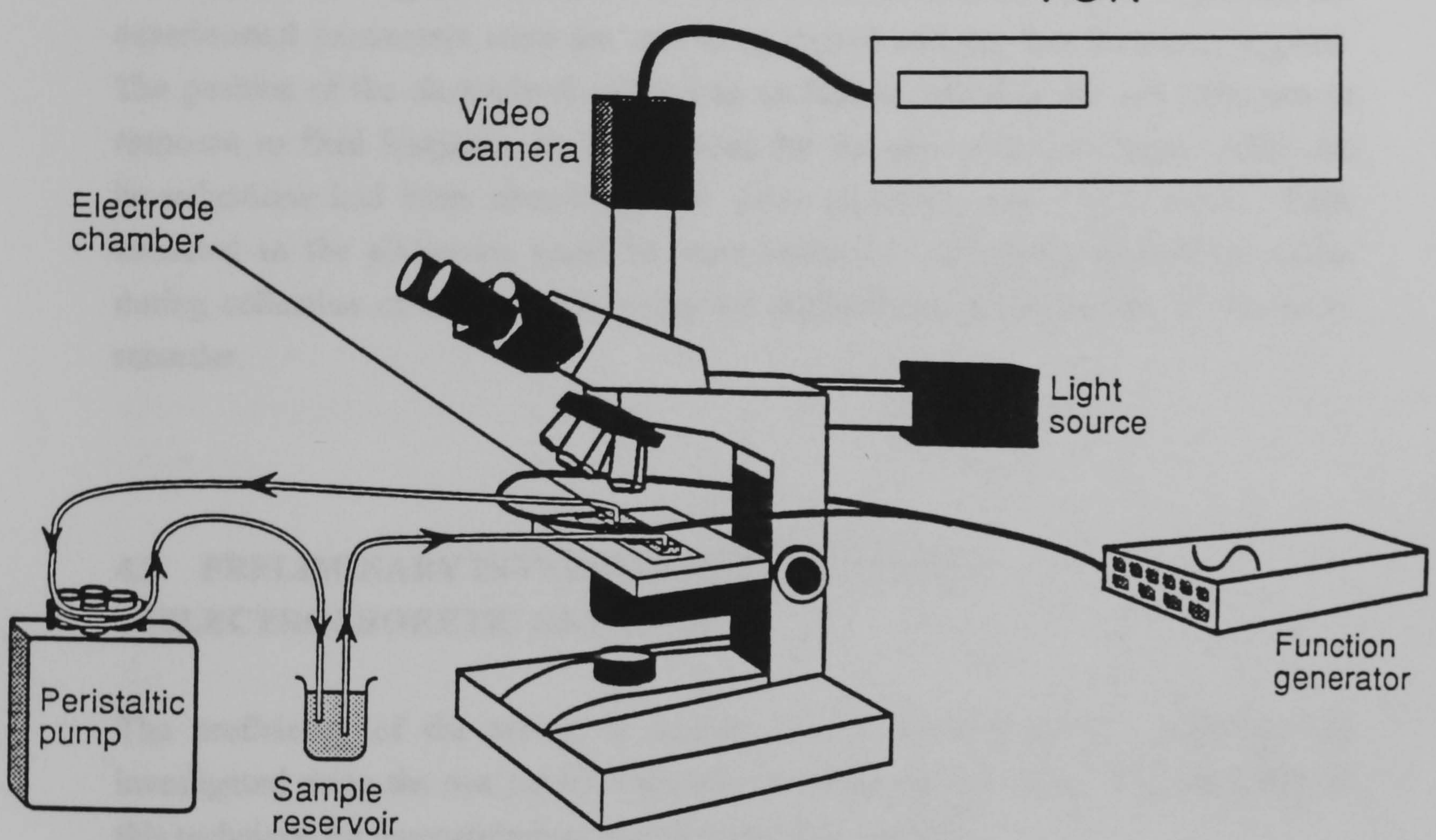


Figure 18: The experimental system for video recording dielectrophoretic behaviour at the electrode surface.

The cell suspension was circulated from the reservoir into the chamber, passing over the electrodes, then from the outlet port to the peristaltic pump returning to the reservoir (fig. 18). The activities of the pump and the signal generator were synchronised by an Epson PC AX2 microcomputer using in-house software as described in Chapter Two.

For a typical investigation of dielectrophoretic response as a function of frequency, the experimental parameters were set, recording started and the first frequency applied. The position of the electrode chamber was unchanged, allowing the cell collection in response to field frequency to be recorded for the same electrode area. After the investigations had been completed, the video recording was played back. Cells attracted to the electrodes could be enumerated for each pulse application, either during collection or after release, using the digital freeze-frame facility of the video recorder.

4.2 PRELIMINARY INVESTIGATIONS TO DETECT DIELECTROPHORETIC RESPONSE

The proficiency of the system in quantifying the dielectrophoretic response was investigated using the test particles already described (section 3.2). The suitability of this technique for haematological samples was also assessed.

4.2.1 Investigations Using Micro-organisms

Test samples of *Saccharomyces cerevisiae* were prepared according to the method detailed in section 3.2.1b and circulated through the experimental system. The response of the cells to a range of applied voltages was observed. Dielectrophoretic effects could be quantified either as cell collection upon the electrode array as induced by an electric field, or cell release after termination of the field. Sample concentration and other system parameters such as flow rate and resolution, determined whether enumeration was performed during or after pulse application. For relatively low cell concentration preparations (*e.g.* 10^3 cells per ml), cells could be quantified at intervals during pulse application as they gathered at the electrodes. At higher concentrations, where a large response was elicited, cell clustering in "pearl chain" type arrangements

(see section 1.1.6a) hindered accurate quantification during collection. In such instances, enumeration of individual cells released from electrodes was more convenient. When analysing video recordings of cell release, cells which had been attracted to the electrodes were easily differentiated from unaffected "baseline" cells by the clarity of their video image. The slower-moving released cells were distinct in appearance whilst the faster unaffected cells presented a more blurred image and were frequently obscure.

Observation of the electrodes during an experiment confirmed that collection of cells could occur both between and upon electrode surfaces (section 1.1.6b), and that destination was dependent upon field frequency. For this reason it was important that the chamber was illuminated by both transmitted and incident light, allowing both intervals between electrodes and the electrode surfaces themselves to be viewed. If such a situation could not be attained then enumeration was confined to cell release after pulse extinction, thus preventing gross under-estimation of the quantity of cells affected.

Preliminary investigations to quantify dielectrophoretic response using the video method were carried out using suspensions of micro-organisms, prepared according to the protocol detailed in section 3.2.1b. Oocysts of the protozoan parasite, *Cryptosporidium parvum*, were also investigated. These oocysts have been identified as a serious contaminant of potable water due to their resistance to water disinfection methods and the low infective dose (as little as ten oocysts) capable of causing severe gastro-enteritis in man. The video technique was successful in distinguishing between autoclaved, ozone-treated and untreated *Cryptosporidium* oocysts, as described by Archer, Betts and Haigh, 1993 - see paper at rear of thesis. Both autoclaved and ozone-treated oocyst samples exhibited maximum dielectrophoretic collection values at a field frequency in the region of 200 kHz; in contrast the collection of untreated oocysts was optimal around 2 MHz. This differential is consistent with the dependence of dielectrophoretic response upon cell viability, as discussed in the Chapter One, and illustrates the potential applications of dielectrophoresis as a separation technique.

4.2.2 Investigations Of The Dielectrophoretic Response Of Human Erythrocytes

Preliminary investigations were carried out using human erythrocytes suspended in GG solution, prepared as described in section 3.3.1. Figure 19 indicates the sequence of events observed at the electrode surface during dielectrophoretic cell collection and release. The effect of the pulse upon the magnitude of the response is clear; collection is inconsistent at the electrode surface, as indicated by pearl chain formation between electrode bars.

Dielectrophoretic electrode chambers used for these investigations varied with respect to electrode bar integrity and optical quality of the chamber. During these trials such equipment-based dissimilarities were unavoidable and required that sample concentration be adjusted for each experimental run, to enable optimum image production. The mean sample erythrocyte concentration at which these investigations were carried out was 10^3 cells per ml (*c.f.* spectrophotometer sample concentrations at $\approx 10^8$ per ml). Investigations were hampered by problems associated with the suspension of mammalian cells in ion-deficient media. The use of GG solution as a suspension medium for erythrocytes was found to be unsatisfactory. However, useful information about the performance and operation of the system could still be obtained and is described subsequently.

Direct observation of the erythrocyte suspension during experimentation indicated changes in cellular morphology, associated with the continued attraction of cells to the electrode surface after pulse removal. These cells were resistant to removal by the system purging step and had an amorphous appearance in that individual cells could not be resolved (suggesting electro-fusion may have occurred). Immobilisation of new cells upon the electrodes appeared to occur to some extent over the entire experimental frequency range from 1 kHz to 10 MHz, but was promoted around 1 MHz in association with gross dielectrophoretic collection. The residual layer upon the electrodes increased in cell density with the application of successive pulses, in particular those eliciting a positive dielectrophoretic response. This amorphous cell mass maintained its response to the applied field as indicated by the movement of cells adhering to the upper electrode surface into the gap between adjacent bars in response to applied fields of particular frequency. The integrity of such cells appeared compromised as indicated by their apparent lack of structure. This impairment was attributed to a combination of ion and metabolite

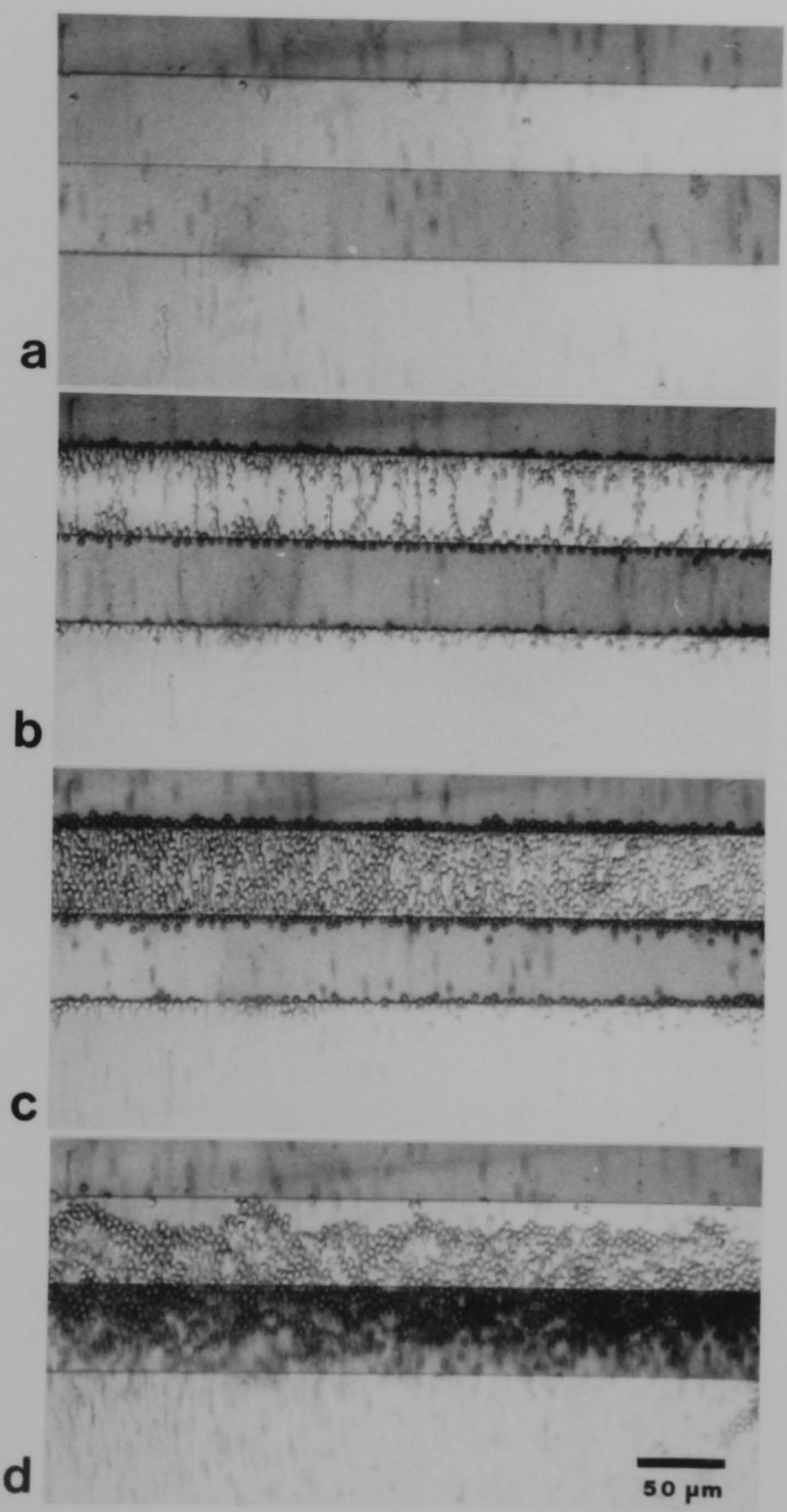


Figure 19: Positive dielectrophoretic collection of human erythrocytes in response to a voltage application: a) Prior to pulse application; b) During application of a 1 MHz 10 V pulse, t = 5 s, indicating pearl chain formation; c) Extensive collection of erythrocytes during application of a 1 MHz 10 V pulse, t = 30 s; d) Release of cells from the electrode surface after removal of the voltage. (Electrode shown as dark bars, suspension flow is from top to bottom).

insufficiencies and to stresses caused by electric field application, possibly promoting electro-fusion events between adjacent cells.

As already discussed, the video analysis technique can be used to enumerate either cells collecting upon the electrode surface during pulse application, or those released after the field is removed. Neither of these methods could be used to quantify erythrocyte dielectrophoretic behaviour under the conditions described. Difficulties associated with monitoring cells during pulse application included the inability to resolve the collection of individual cells upon the electrode surface, and to distinguish between newly recruited erythrocytes and those already present in the boundary layer. In addition, the presence of an increasing build-up of adhered of cells upon the electrode surface affected the field induced by successive voltage applications, and therefore modified the force experienced by passing cells. The alternative quantification procedure involving enumeration of cells after pulse application was also unsuitable due to the persistance of collected cells upon the electrode surface after the field had been removed.

The problem of progressive cell build-up upon the electrode surface has already been shown to affect the force experienced by passing cells. Attempts were made to remove this boundary layer between successive experimental runs by using washing procedures, but these resulted in electrode damage, thereby diminishing potential cell collection in future investigations.

Observation of erythrocyte dielectrophoretic response during these investigations revealed that collection occurred both on and between electrode bars. In addition, the trajectory of motion of collecting cells towards the electrodes was also revealed. It was noted that extremes of the electrode bars were the favoured destination for collecting erythrocytes, and that cells would move along the bars until this position was attained (fig. 20). This tendency was attributed to the increase in electrical field intensity in this region, as indicated on figure 20 by converging field lines. The effect may also have been promoted by the direction of flow in relation to the electrodes. If these are not perpendicularly arranged then collection at the downstream electrode extremity may be enhanced; due to the method of chamber manufacture, alignment is unlikely to be perpendicular.

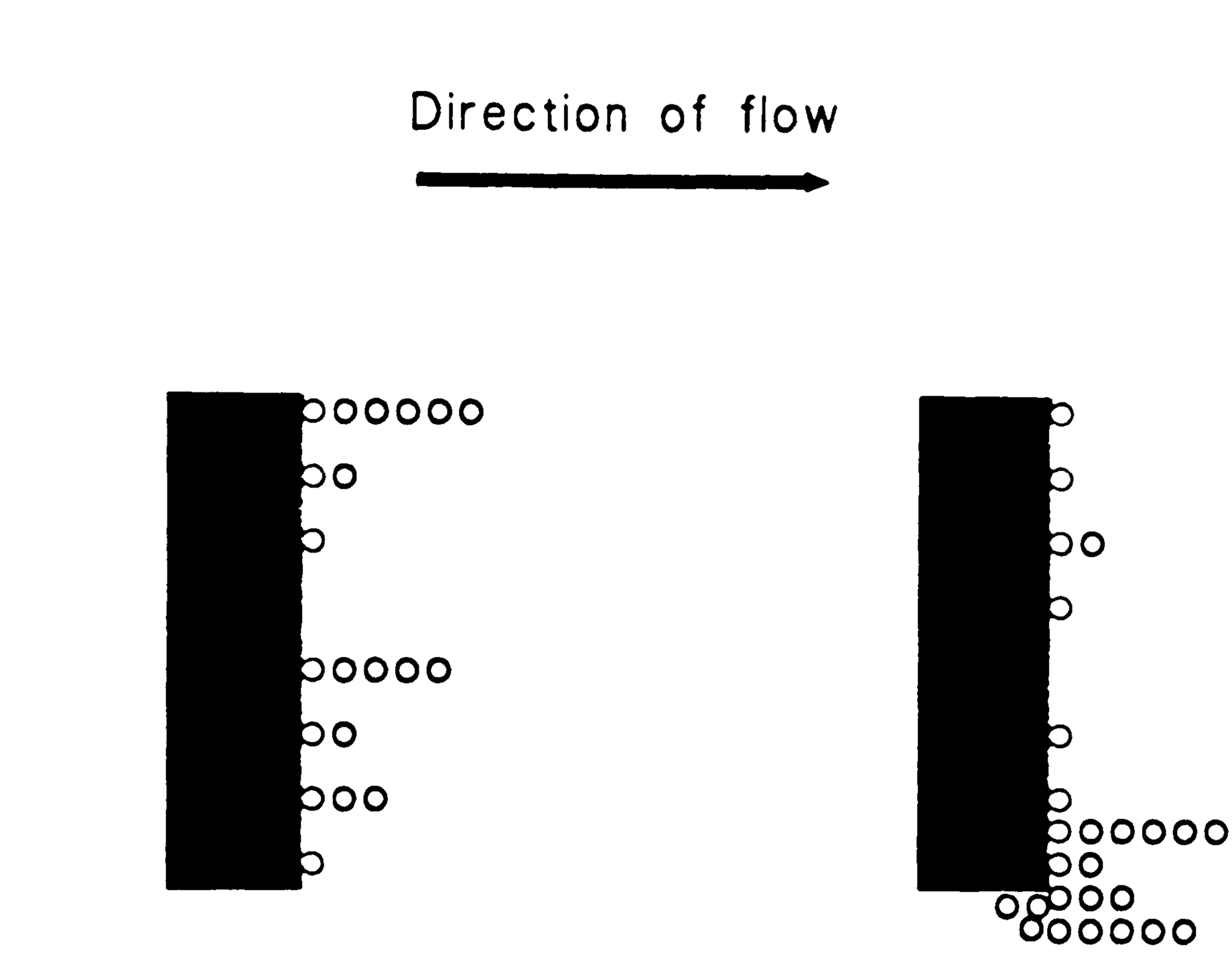


Figure 20: Erythrocyte behaviour at the electrode surface.

Direct examination of erythrocyte behaviour at the electrode surface also revealed that at lower frequencies, prior to dielectrophoretic collection towards the electrode bars, other electro-kinetic phenomena were displayed. The activity of erythrocytes in the presence of a low frequency electric field was suggestive of negative dielectrophoresis and electro-rotation effects. The actual frequency ranges over which such phenomena were apparent depended upon suspension conductivity, amongst other conditions. However, negative dielectrophoretic activity was typically exhibited over the frequency range 1-30 kHz for the erythrocyte investigations carried out ($n > 20$, $\sigma = 107 \pm 5$ $\mu S\ cm^{-1}$); this behaviour was indicated by the removal of cells from the plane of microscope focus during pulse application, *i.e.* electric field induced repulsion from the electrodes. At higher frequencies, for example 150 kHz, this behaviour was reversed and cells were observed to collect upon the electrode surface due to positive dielectrophoretic motion. Between these two extremes of dielectrophoretic behaviour, a third characteristic activity was sometimes apparent. The latter was only displayed over a very narrow frequency range between the two effects already described (*e.g.*, 35-45 kHz), so may not be exhibited at frequencies selected by the controlling computer. When detected, this type of response to an electric field was typified by erythrocytes appearing to "bounce" upon the electrode surface. Their motion was confined to the periphery of the electrode bars and involved the cells moving in and out of the plane of microscope focus; this suggested that they were following a circular trajectory, rising above the electrode surface then falling lower and returning to the electrode bar. Such motion was accompanied by electro-rotation, manifested as cell gyration about its own axis, in addition to the translational movement already described. This third type of behaviour was attributed to a combination of heating and electro-rotation effects. It is proposed that the circular motion of the cells at the electrode surface is due to convection currents implicated at these low frequencies, and is not of significance to measurement of the dielectrophoretic response, except where it results in damage to the cells.

Attempts at enumeration of erythrocyte dielectrophoretic response were hampered by the inability to resolve adjacent collected cells and by their continued attachment to the electrode surface after pulse application. This behaviour was thought to be concerned with the unsuitability of the suspending medium causing impairment of cell integrity. It was decided that future investigations using erythrocytes would be conducted in a superior medium. In addition, due to the prolonged time period required to analyse experimental video-tape recordings, further investigations based

upon direct observation were confined to the development of an automated counting system as discussed in Chapter Five.

4.3 CONCLUSION

4.3.1 System Performance And Limitations

Preliminary investigations using micro-organisms, identified advantages of adopting a direct observation method to quantify the dielectrophoretic response. Such benefits included being able to assess if collections were impaired by either solution or electrode abnormalities. (Checking the electrodes for causes of malfunction when using the spectrophotometric system involved removal of the aluminium foil window from the chamber base and examining the electrode bars using a microscope, before replacing the foil window and re-aligning in the spectrophotometer). Possible electrode decay and the integrity of the suspended cells could be monitored during an experiment.

The advantages of direct observation of the events occurring at the electrode surface were exemplified by initial investigations of erythrocytes suspended in GG solution. Although this had previously not appeared to yield any problems when a spectrophotometric method was used to quantify the dielectrophoretic response, impairment of cell integrity was clearly implicated. It was likely that the latter may have been caused by reduced conductivity of the suspension, associated with the decreased concentration of cells used for these investigations (*i.e.* conductivity tends to increase with cell concentration due to ion leakage). It is suggested that such a gross impairment in cell integrity was not brought about during investigations involving the spectrophotometric determination of dielectrophoretic collection, due to the increased conductivity of the sample. However, in the latter case, the electrodes could not be viewed during the experiment and may have been subject to a build-up of adhered cells thereby decreasing subsequent field strengths and collection values.

Observation of the electrode bars suggested that dielectrophoretic collection was not uniform along their surface. Such non-uniformity was anticipated due to problems associated with attempting to manufacture parallel electrode bars of perfect rectangular area and insignificant thickness; the requirement for such a configuration, necessary to create an electric field of the desired conformation by "edge" effects, was described in section 1.3.2. Unavoidable irregularities introduced during electrode plating and etching, and also through storage and use, would result in some areas of the electrode bars inducing a stronger electric field than other more damaged regions. In an attempt to standardise these inconsistencies concerned with the electrode array, experiments were carried out in which the same electrode area was observed for the duration of the investigation.

Dielectrophoretic cell collection was found to be promoted at the extremes of the electrode bars, as indicated by the observable migration of cells from their first point of collection along the electrode. This tendency was diminished in further system development by decreasing the chamber diameter and limiting the dielectrophoretic effects to continuous regions of the electrode bars (section 5.2).

The video technique proved successful in quantifying the dielectrophoretic response of micro-organisms. The resolution of such cells is aided by the possession of a rigid cell wall structure. They are also resistant to osmotic shock, and may be suspended in de-ionised water. Advantages of this method include direct observation of events at the electrode surface, and that the system may be operated using a relatively low sample concentration. The importance of the former condition can not be over-emphasised. In addition to permitting electrode integrity, chamber optical quality and cell morphology to be monitored, it enables the type of electro-kinetic behaviour induced to be described..

The major disadvantage of this method was the time requirement for analysis of the video recording. The next stage in the development of an apparatus to perform rapid quantification of dielectrophoretic response, was to combine this procedure of direct observation with technology allowing real-time analysis of dielectrophoretic phenomena.

4.3.2 Evaluation Of The Video Technique For Dielectrophoretic Measurement Of Haematological Cells

This method for determining the dielectrophoretic response was particularly useful in resolving some of the problems involved with investigating mammalian cells. Direct observation of suspended erythrocytes exposed to an electric field clearly illustrated the importance of selecting a low-ionic strength medium capable of preserving cell integrity. Previous use of the GG solution had only demonstrated its destructive effect upon the electrode chamber, not the cell sample. It was obvious that an improved suspension medium was required for future investigations.

Another advantage of this dielectrophoretic measurement technique, in comparison with the spectrophotometric method, concerned the cell density requirement for the experimental sample. Use of an absorbance technique required that gross changes were caused in local cell concentration; thus a relatively concentrated sample was imperative. Direct observation greatly reduced the cell concentration requirement. This was of great benefit to subsequent haematological investigations involving leucocytes, which constitute a much smaller fraction of the blood than erythrocytes, thus restricting the volume of blood required from a donor. In addition, cell samples isolated from patients with various haematological disorders were only available in limited volume.

Direct observation of the electro-kinetic behaviour of erythrocytes was far more informative, in comparison to micro-organisms, due to the absence of a rigid cell wall. The morphology of eukaryotic cells may be affected by exposure to an electric field; for erythrocytes this dielectro-deformation is typified by elongation of the normal discoid shape to an ellipsoid (Chang *et al.*, 1985). Forces then operate upon a non-spherical body causing it to orientate with respect to the electric field (section 1.1.6b). For erythrocytes, this response is typically indicated by the relative width of a single pearl chain formation. For example, the deformed cells may be orientated perpendicularly to the field as indicated by the build-up of a comparatively wide pearl chain, or parallel to the field resulting in a spindly pearl chain. This deformation and alignment response is frequency dependent. Erythrocyte deformability is determined by the structure of its membrane and under-lying cytoskeleton and is compromised in certain medical conditions suggesting another potential application for dielectrophoresis technique (Brain, 1982; Brovelli, 1990).

The stress experienced by mammalian cells in response to an electric field can also be monitored by the extent of deformation induced, preventing the application of excessive field strengths, which could otherwise result in electro-fusion (Bryant & Wolfe, 1987).

Video analysis of erythrocytes exhibiting a positive dielectrophoretic response was compromised by problems with release of cells, and resolution during collection. The formation of a residual layer of cells upon the electrode surface also affected the capacity of the electrodes to set up an electric field in response to a voltage application. However, these difficulties were thought to be concerned with the suspending medium used, as opposed to the technique itself.

Future investigations concentrated upon the development of an alternative suspending medium for use with haematological cells. The method of direct observation of events at the electrode surface was retained and developed to allow quantification of the dielectrophoretic response in real time, as described in Chapter Five.

Chapter Five

AN IMAGE-ANALYSIS EXPERIMENTAL SYSTEM TO STUDY THE DIELECTROPHORETIC RESPONSE OF HAEMATOLOGICAL CELLS

This automated system was based upon direct quantification of events occurring at the electrode surface. The number of cells collecting at a particular electrode area (as observed with a microscope), was counted using image-analysis techniques. The advantages of this system were that the cell count was obtained directly, and quantification could be carried out in real-time (*c.f.* the video method, Chapter Four). Additional benefits of this system with reference to haematological investigations were that the concentration of cells required was reduced, and the physiological status of the cells could be monitored during the experiment. Real-time analysis also allowed more measurements to be made of cells subjected to a dielectrophoretic force, thereby increasing confidence in the experimental results.

5.1 SYSTEM DESCRIPTION

The image-analysis system was more complex than the basic system (Chapter Two), and was arranged as shown in figures 21 & 22a. In brief, the electrode chamber was positioned on a microscope stage, the electrode image from the microscope digitised by a computer and visualised on the "secondary" monitor. The "primary" monitor was dedicated to the computing facility, which included control of the experiment, data acquisition and analysis. Conductivity measurements of the suspension were made upon exit from the electrode chamber using a small volume flow-through probe. In addition to the standard peristaltic pump which drove circulation of the sample, a second pump was employed to ensure mixing in the sample reservoir, reducing the sedimentation rate of the erythrocytes.

The electrode chamber was viewed in bright field mode with a charge-coupled device (CCD) camera (Ikegami ICD-42E, Japan) attached to a microscope (Nikon Labophot-2, Japan). A long-working distance objective lens with 10x magnification was employed, which allowed the electrode bars on the lower chamber surface to be studied. The total magnification of the system was 450x, enabling observation of a section of the electrode bar approximately 550 μm in length. The video signal was

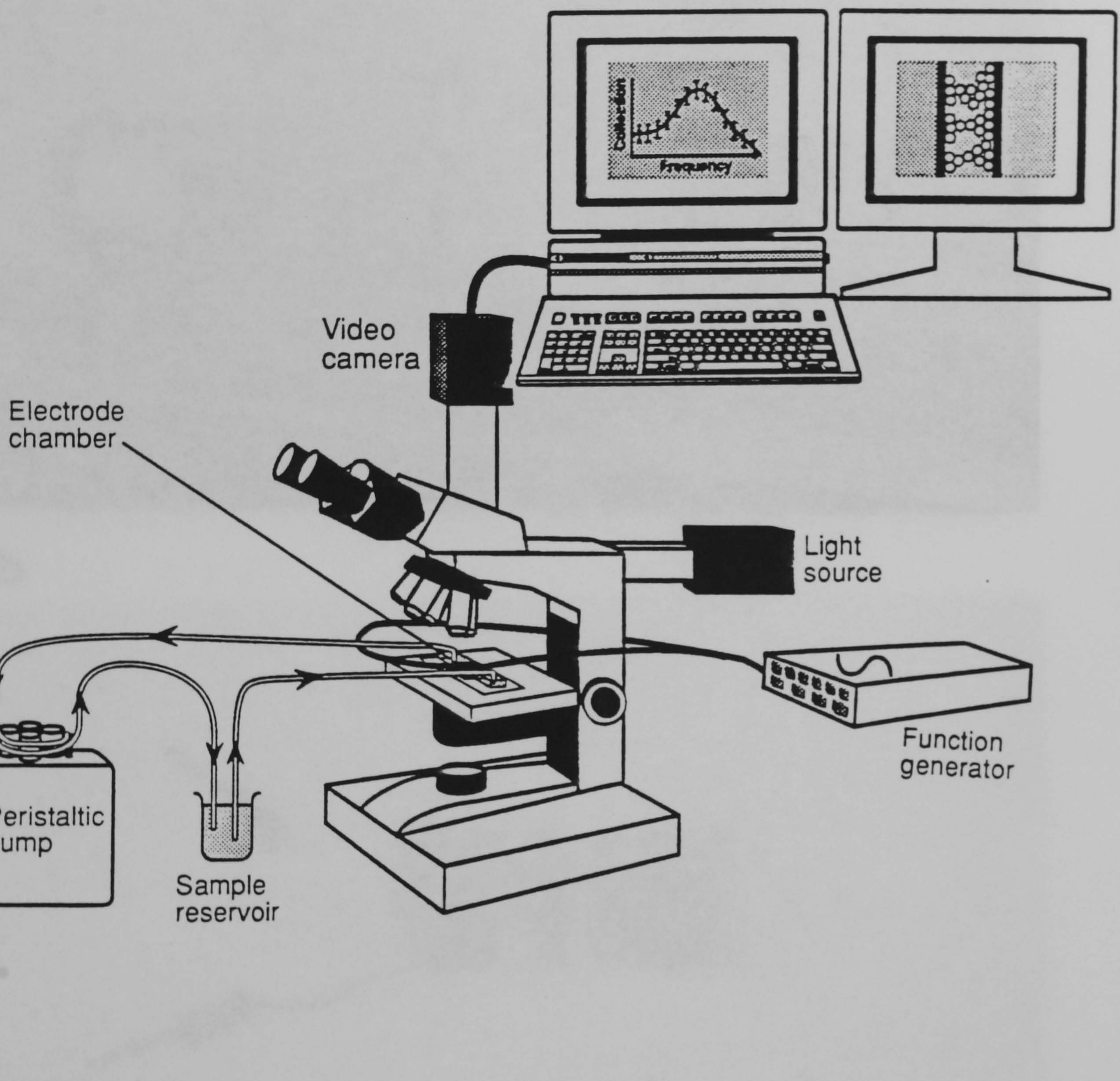


Figure 21: Schematic of the image analysis experimental system

a

b

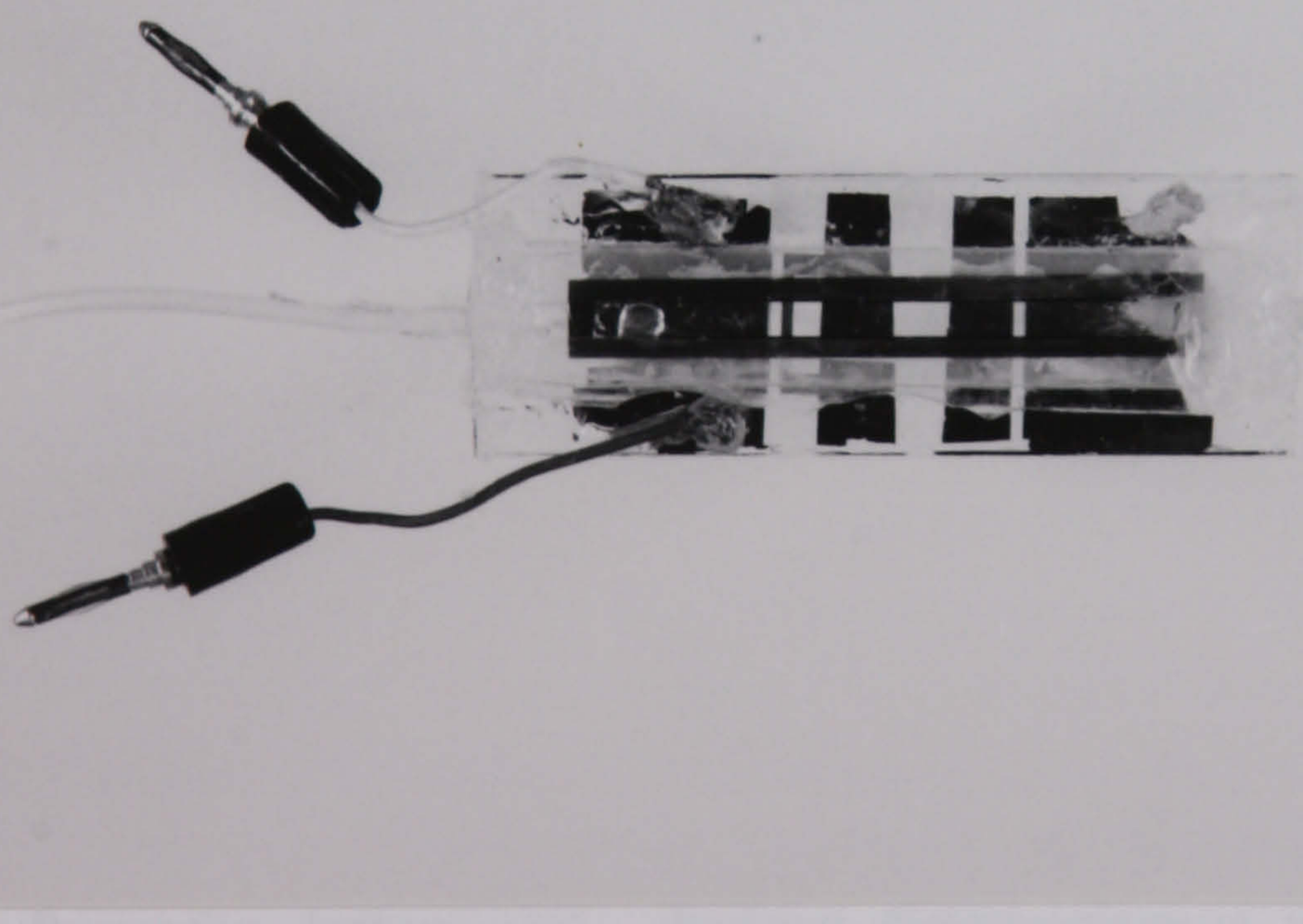


Figure 22: The image analysis experimental system for dielectrophoretic measurements ot haematological cells. a) The laboratory system. b) The modified electrode chamber.

simultaneously processed by a modified image-analysis package (Domino, Perceptive Instruments) and displayed on a monitor (Taxan Multivision 775, Perceptive Instruments) allowing detection parameters to be defined.

Detection after release from the electrodes enabled individual cells to be resolved by the image-analysis system. Attempts at counting during collection upon the electrode surface resulted in adjacent cells being counted as one object. In addition, the counting of released cells eliminated difficulties concerned with cell quantification both on and between the electrode bars (sections 1.1.6b and 4.2.1). The detection frame was positioned slightly downstream of the electrodes to prevent the inclusion of collecting cells in pearl chain formations.

Each image was processed by the image-analysis package then digitised to enable relative collection to be calculated rapidly by the computer. In each case the number of distinct objects within the detection frame was determined. The display on the "secondary" monitor indicated the resolution of detection by visually tagging all counted objects. Quantification in real-time from each successive image frame enabled twelve frames per second to be analysed, giving a relative indication of particle concentration every 0.08 s.

The entire system was co-ordinated by a 80486-based personal computer (AST 486 Bravo), with a Hewlett Packard Interface Bus HP 82335A and Keithley DAS8-PGA card to communicate with the signal generator and peristaltic pump respectively. In-house software was employed to control all parameters including pulse voltage, pulse frequency, pulse length, pump speed and data acquisition. The computer also recorded the conductivity of the suspension, as measured using a flow-through probe with a microprocessor conductivity meter (model RE387 Tx, EDT Instruments), at both the start and finish of each run. (The sequence of events is indicated in figure 9).

During a typical experimental sequence, images were continuously analysed before, during and after, the application of a voltage, as observed on the "secondary" monitor. The data obtained, representing the number of cells detected, was displayed as a trace against time for each pulse application on the "primary" computer monitor (AST SVGA, Perceptive Instruments). Positive dielectrophoresis was indicated by a decrease in counts during voltage application associated with cells collecting upon the electrodes, thereby reducing the number passing through the detection frame for possible enumeration. After the field was removed, the number of detected cells increased to a peak value then decreased to the constant base-line value; this rise in cell

count was consistent with particle release from the electrodes and detection by the image-analysis system. The trace was used diagnostically by monitoring its shape to ascertain whether the appropriate parameters had been selected (section 3.2.3).

The dielectrophoretic effect of the field was quantified for each voltage application using in-house software. This calculated the difference between the average baseline value from the trace (recorded prior to pulse application), and the maximum value after voltage removal (averaged from the highest three successive data points). At the end of the investigation, these values, indicating the effect of electric fields upon the sample, were plotted against the experimental variable, such as field frequency, and the data saved.

5.2 SPECIFIC MODIFICATIONS TO THE STANDARD ELECTRODE AND CHAMBER DESIGN

Investigating cell dielectrophoresis using a direct observation method eliminates the requirement for bulk changes in local suspension concentration to be effected. (The latter was a prerequisite for the spectrophotometric system described in Chapter Three). Instead, accurate quantification is dependent upon the ability of the image-analysis system to resolve individual particles travelling across the detection frame; this is in accordance with synchronised particle release from the electrodes after removal of the field. If these conditions were met, then positive dielectrophoresis would result in a well-defined peak being described by the trace. Synchronised particle release was facilitated by the implementation of a two bar electrode array, the advantages of which were discussed in section 3.2.2b. The optical quality of the electrode system, and therefore the image resolution, was improved by the use of glass in place of perspex for the chamber lid.

Other considerations were taken into account when designing a chamber suitable for use with haematological cells. Difficulties associated with creating apertures through the glass chamber lid were avoided by arranging input and exit portal tubes parallel to the direction of flow through the chamber. To accommodate this, the depth of the chamber was increased using a layer of 1 mm thickness silicon sheet (Bibby Sterilin) as a spacer, allowing the input and exit tubing to be inserted directly between the electrode base slide and lid (fig. 23). This modification improved the uniformity of flow of suspension, and reduced the formation of air locks, within the chamber. These changes were deemed to reduce physical stress experienced by circulating cells and

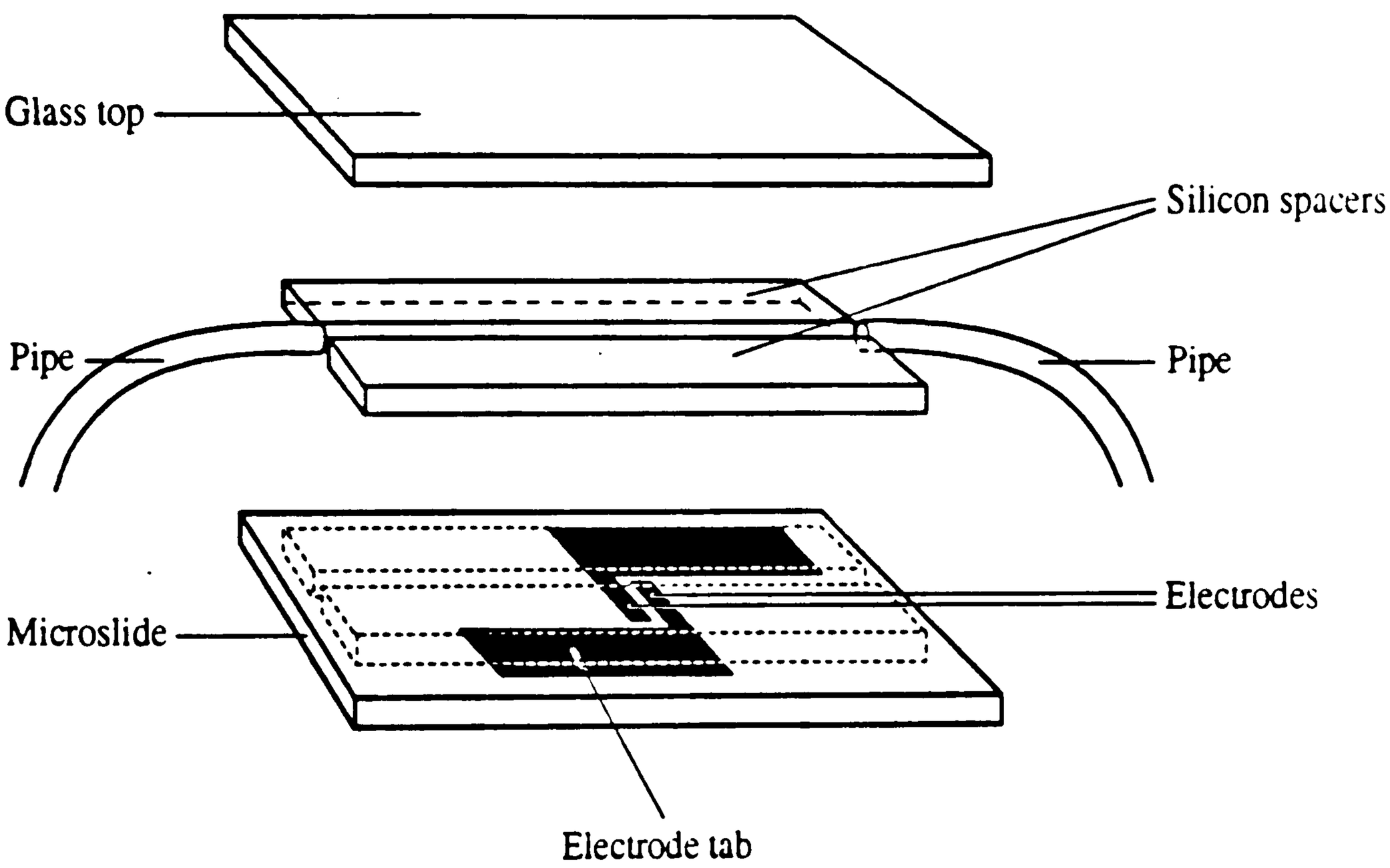


Figure 23: Construction of the modified electrode chamber

only slightly increased the volume of the system to approximately 3 ml, excluding the reservoir..

The silicon spacers were positioned approximately 3000 μm apart, thus defining the chamber width. This decrease in width meant that only the inter-digitated section of the electrodes was exposed to the sample, which diminished aberrant collection patterns resulting from cell build-up at electrode bar extremities (section 4.2.2, fig. 20).

The entire electrode chamber was secured and sealed using silicon based adhesive (Non-corrosive silicone rubber, RS Components, Northants) which also held the portal tubes in position. An additional advantage of this re-designed system was that the layer of adhesive tape in the chamber construction was no longer necessary. This was deemed to be beneficial as it removed the possibility that adhesive could enter the chamber.

5.3 PREPARATION OF THE IMAGE-ANALYSIS SYSTEM FOR AN EXPERIMENTAL INVESTIGATION

The electrode chamber was connected to the signal generator, positioned on the microscope stage, and viewed with the image-analysis ("secondary") monitor. An experimental sample was introduced to the reservoir and circulated through the system by the peristaltic pump at a baseline flow rate of 1.5 ml per min, or 4.5 ml per min for chamber purging. Parameters controlling the actions of the signal generator and the pump were set using the computer.

Prior to an investigation, the image-analysis parameters were defined. The detection box was positioned 25 - 50 μm downstream of the electrode bars as previously described. The frame size was fixed at 700 x 400 pixels (0.17 mm^2), permitting the downstream electrode edge also to be viewed on the image-analysis monitor screen. This allowed the behaviour of the cells subjected to the field to be monitored with respect to their shape, orientation, trajectories of motion towards, and release from, the electrodes.

The optimum image for analysis was produced by slightly de-focussing the microscope, so that detection was centred upon each individual cell. This was especially important during investigations of biconcave erythroid cells; detection of the distended cell rim caused some touching cells to be analysed as one object.

The function of the on-line trace from a single field application in determining whether appropriate parameters had been selected, has already been discussed (section 3.2.3). In this system, the trace could additionally indicate problems with particle detection if a high noise level was expressed. Such noise was usually the result of poor particle resolution; image contrast could have been too high, or too low, causing neighbouring cells to be counted as one object. Both these conditions resulted in under-estimation of particle number. The production of a good image for analysis was affected by changes in light; both ambient, and that dependent upon the concentration of the passing cell suspension. The former condition was relieved by operating the image-analysis equipment under blackout conditions. Fulfillment of the latter criterion to produce an optimum image for analysis is unachievable; this system is based upon affecting changes in density of the cell suspension as a method of quantifying mass dielectrophoretic response. Sample concentrations in the region of 10^7 cells per ml were used in order to satisfy these conditions; determination of this value is discussed further in section 5.4.2a.

During an experiment the proportion of cells detected by the image-analysis package for each pulse application was graphically displayed on the "secondary" monitor. This enabled simple assessment of the performance of the system to be carried out on-line, preventing operation beyond the resolution of the image-analysis package.

5.4 CONSIDERATION OF SYSTEM-BASED VARIABILITY IN MEASUREMENTS

5.4.1 The Requirement For Standardisation

It was important to establish, as with the other systems already described, the dependence of collection data obtained upon the particular electrode chamber used. This was to ensure that spectral differences could be attributed to the sample and not the equipment. The main variable component of the system was the electrode chamber which was susceptible to deterioration and therefore had a finite life-span.

Micro-electrode slides were prepared in batches of 6-10 using photolithographic techniques (Appendix I) and chambers assembled as required. Electrode consistency between batches could not be guaranteed; pin-point holes were created in the metal film during the vacuum plating process and could then act as foci for subsequent deterioration. In addition, oxidation of newly-prepared electrodes in storage prior to

use could not be avoided, generating another potential source of electrode variability within a batch. These sources of variation were not thought to have a sizable effect upon dielectrophoretic collection measurements in this system, but in order to ensure that all electrodes behaved in a similar manner quality control procedures were introduced.

Creation of microscopic holes in the metal layer was discouraged by the use of high quality glass microscope slides and by the vigourous washing of the slide before vacuum plating to promote glass-metal adhesion. In order to minimise the detrimental effects of prolonged storage, electrodes were prepared in batches of 6-10 and were typically used within three months of vacuum coating.

Preliminary investigations with erythrocytes enabled further sources of system-based variability to be identified. As a result of this, additional procedures were adopted and are described in the following sections.

5.4.2 Use Of Fixed Human Erythrocytes As Calibration Particles

Preliminary investigations using the image-analysis dielectrophoretic system were carried out using formaldehyde-fixed human erythrocytes. Such cells were obtained from Interfacial Dynamics Corporation, Portland, Oregon, where they had been prepared by isovolemic fixation. This process abolishes the deformation capacity of the erythrocytes but specific antibody and agglutinin sites retain their reactivity. Cells were obtained as a 10 % suspension in phosphate buffered saline containing 1 % w/w formaldehyde and a trace of EDTA to discourage aggregation.

The inertness of these fixed particles allowed them to be suspended in non-physiological media without undergoing adverse effects. Unlike fresh cells, the fixed erythrocytes were unresponsive to the pH and osmolarity of suspending solution, except where these acted directly to cause a dielectrophoretic change, as opposed to that resulting from a physiological alteration. This meant that the detection and physical limits of the experimental system could be evaluated independently from problems associated with the suspension of viable haematological cells in ion-deficient media. Manufacturer's information regarding cell concentration of the stock was of use as a rough guide to the concentration of test samples prepared, before haemocytometer counts were carried out. For this series of investigations, the fixed cells were employed as test particles to establish the working parameters of the system.

Primary concerns were the investigation of the effect of suspension concentration upon detection resolution, determination of reproducibility between experiments with the same electrode chamber, assessment of variation between electrode batches and performance of different electrode chambers. This allowed a protocol for system operation and treatment of data to be established. For all these investigations, type O+ve fixed cells, as defined by the ABO-Rhesus blood grouping system, were utilised, suspended in sorbitol-glucose solution (SG; Appendix I); the designated suspension solution for haematological investigations, as discussed in section 6.1.2.

a) The effect of sample concentration upon the resolution of detection

Measurement of dielectrophoretic collection after pulse application using the image-analysis system is dependent upon;

- gross collection of cells upon the electrode surface and their unified release to cause a local and transient increase in suspension density;
- that this change in density can be resolved in terms of individual cells and quantified by the image-analysis system.

For dielectrophoretic collection to be measured using this system then differences in cell concentration between baseline flow, prior to pulse application, and release of collected cells after pulse application, must be detected.

To investigate the relationship between cell concentration and system performance, suspensions of varying cell density, as determined using a haemocytometer, were prepared in SG solution using formaldehyde-fixed erythrocytes. The passage of these suspensions through the electrode chamber at typical experimental flow rates (*e.g.* 1.5 ml per min) was observed using the microscope and the image displayed upon the "secondary" monitor. Optical parameters of both the microscope and the computerised analysis system were adjusted to provide optimum resolution of the image. The ability of the image-analysis system to detect and resolve individual cells as they travelled in the plane of the electrode bars was qualitatively assessed for a range of suspension densities.

Trials indicated that fixed erythrocyte concentrations in excess of 10^8 cells per ml did not permit reliable resolution of individual cells by the image-analysis system. At this level image contrast was reduced when pigmented erythrocytes were studied using the chamber design described, resulting in poor resolution of individual cells. At lower

sample concentrations more light can be transmitted through the electrode chamber, generating an improved image and increasing accuracy of computerised cell quantification.

When using low sample concentrations, for example 1 x 10^5 cells per ml, problems associated with resolving individual cells were reduced due to increased spacing of free-flowing cells in the suspension. In such cases the total number of cells per unit time, which could be caused to collect upon the electrode bars in response to a non-uniform electric field was diminished. In order to collect enough cells to cause a detectable change in local concentration of the suspension, a prolonged period of pulse application would be required. Such a situation was undesirable due to the possible detrimental effects upon the cell sample.

For dielectrophoretic measurements of erythrocyte samples using the image-analysis system a working concentration in the region of 10^7 cells per ml was defined. This permitted a sufficient level of dielectrophoretic collection to be exhibited for down-stream quantification without excessive field application, whilst maintaining optical resolution for the analysis system.

b) Establishing a procedure for the use of fixed human erythrocytes as calibration particles

The possibility of using formaldehyde-fixed erythrocyte suspensions prepared by a direct dilution method for rapid calibration of the dielectrophoretic system was investigated. In accordance with this, attempts were made to obtain collection spectra of a fixed erythrocyte suspension prepared directly from the commercial stock, and of a sample washed prior to re-suspension.

A 50 µl aliquot of type 'O' fixed cells mixed with their storage solution was introduced into 10 ml SG solution, resulting in a cell concentration of approximately 3 x 10^6 cells per ml. The suspension was transferred to a reservoir at 25° C and circulated through the system. The effect of a 10 V pulse applied for 10 s over a frequency range 1 kHz-10 MHz was investigated. Measurements of the frequency spectra were repeated four times consecutively in an attempt to determine the consistency of the collection values obtained.

At the beginning and end of each run, a sample was taken from the system reservoir and its pH measured; cell concentration was then determined using a haemocytometer. Conductivity readings were obtained from the on-line probe after the suspension had passed through the electrode chamber at set intervals.

The contaminating effect of the fixed cell storage medium was evaluated by washing the cells prior to suspension in the experimental medium. 100 µl of cell suspension were diluted in 1 ml of SG, then centrifuged at 6500 rpm for 10 s and the supernatant discarded. The washing process was repeated then the pellet re-suspended in SG to a concentration similar to that from direct dilution. As anticipated, the washing process reduced the conductivity of the sample. In order to establish that this difference in conductivity was not the cause of any variation between the two samples, a small volume of 1 mM KCl, typically 10-20 µl, was added to the washed cell sample to increase its conductivity to that of the unwashed sample. The dielectrophoretic response was investigated as before.

Collection data were expressed as the mean collection values, from repetitions of the same sample, against log frequency. Error bars indicating the standard error of the mean were also displayed. Figure 24 shows typical spectra obtained from both washed and unwashed fixed cells using the same electrode chamber and experimental parameters.

The results indicated that dielectrophoretic collection of fixed cells under the same conditions occurred over the same frequency range, and to a similar extent, independently of whether the cells had been washed or not prior to investigation. However, as indicated by the error bars, measurements of the samples of unwashed cells were more variable compared to those of the washed cell suspensions. This variability did not increase with repetition of the experiment indicating that it was not a time-related effect, but was concerned with the sample type.

Contamination of the experimental suspension from the fixed cell storage solution may have had a destabilising effect upon dielectrophoretic collection. It is suggested that this may be attributed to ion chelation by EDTA (the effects of EDTA are examined further in section 6.4.2). For the purposes of electrode calibration it was decided that suspensions of pre-washed formaldehyde-fixed erythrocytes were preferable for use as a standard.

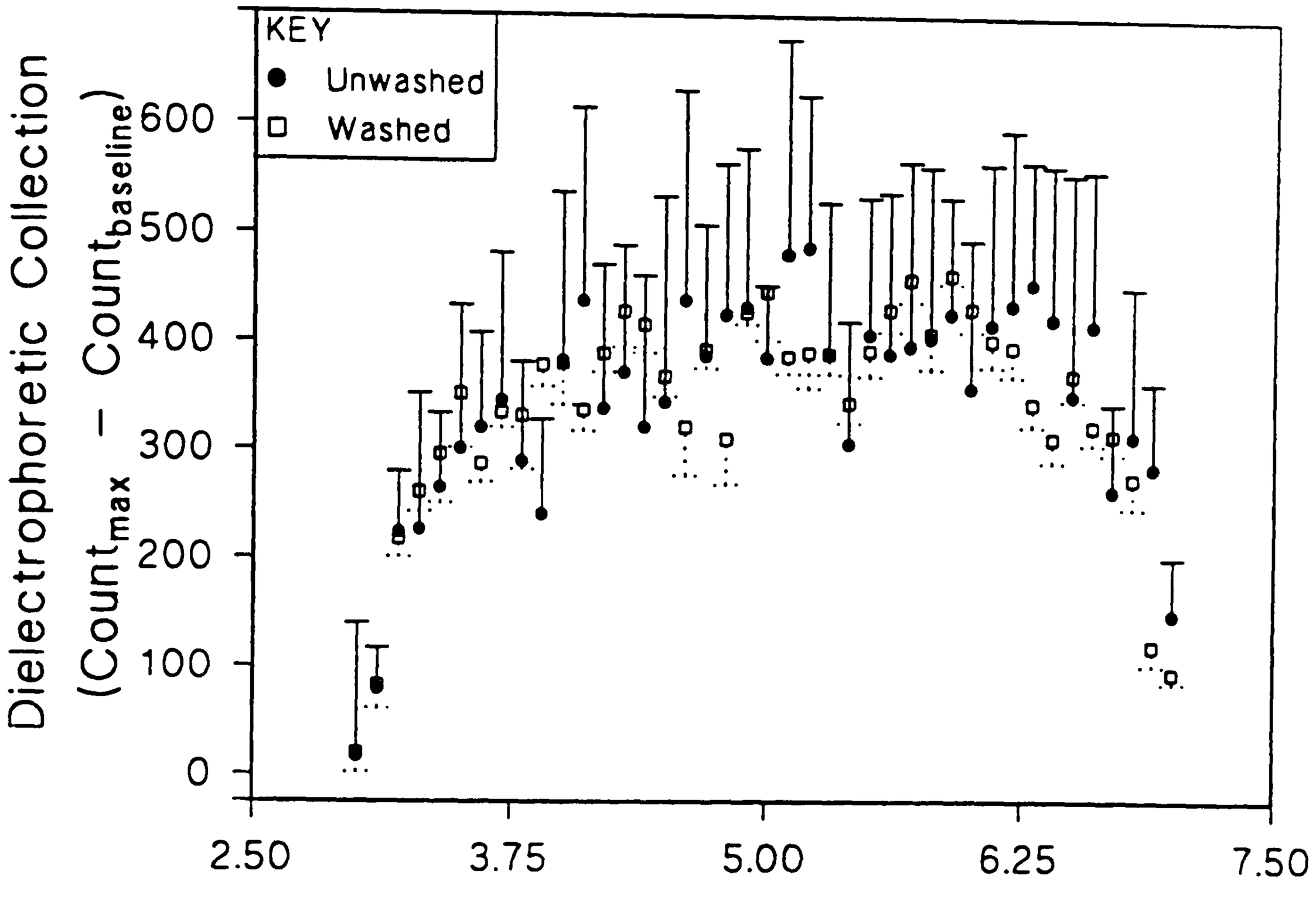


Figure 24: Dielectrophoretic collection of formaldehyde-stabilised human erythrocytes in sorbitol-glucose suspension. (Pulse of 10 V for 10 s, pH = 7.4 ± 0.1, σ = 160 ± 5 μS cm^{-1}, concentration of washed sample = 3.25 x 10^6 cells per ml, concentration of unwashed sample = 3.15 x 10^6 cells per ml. Data represents mean collection values ± error bars of the SEMs for 4 replicates; half error bars are shown for clarity).

5.4.3 Investigation Of Variability Between Unused Electrode Sets

A stock suspension of washed fixed cells in 500 ml SG solution was prepared in accordance with procedures described in section 5.4.2b. This suspension was stored at 4° C in a polypropylene container; use of glassware was avoided where possible, to minimise conductivity increases due to ion leakage from glass, and to prevent premature coagulation when using fresh blood. The stock calibration suspension was then used for dielectrophoretic investigations with each new set of electrodes prior to experimental usage over a ten week trial period.

Experiments were carried out to determine whether a stock suspension of erythrocytes could be used to establish whether new electrodes elicited similar levels of dielectrophoretic activity during test runs prior to experimentation. Use of the same stock suspension, providing it was unaffected by storage, would be advantageous as the cell concentration would be fixed and the testing procedure rapid. The fixed cells were known to be resistant to haemolysis and content leakage for in excess of a 30 day period at 37° C, as established by the manufacturer. It was therefore hypothesised that they would also be stable with respect to dielectrophoretic activity over a prolonged storage period and could be used as a standard to compare the behaviour of different electrode sets.

Prior to experimental investigations each previously unused electrode set was tested, using the standard fixed erythrocyte suspension, to determine whether typical levels of dielectrophoretic collection were obtained. Assuming that the dielectrophoretic activity of the standard suspension remained constant over the storage period, this enabled potential variability in electrode performance arising from production and time-related decay effects to be established. For these measurements, the stock suspension was remixed and allowed to equilibrate to 25° C before use. A 10 ml aliquot was transferred to the system reservoir and circulated through the apparatus. A minimum of four consecutive collection spectra were obtained, investigating the response to a 10 V pulse applied for 10 s over a frequency range of 1 kHz to 10 MHz.

For each new electrode set, the mean dielectrophoretic collection values, with standard errors, were calculated for the frequency range investigated. Collection data for individual electrode sets could then be plotted against frequency. Figure 25 shows results obtained for three new electrode sets used in succession to replace damaged chambers over an eight week period. In this case, the standard suspension had been stored for eight weeks between testing the first and third electrode set but was not

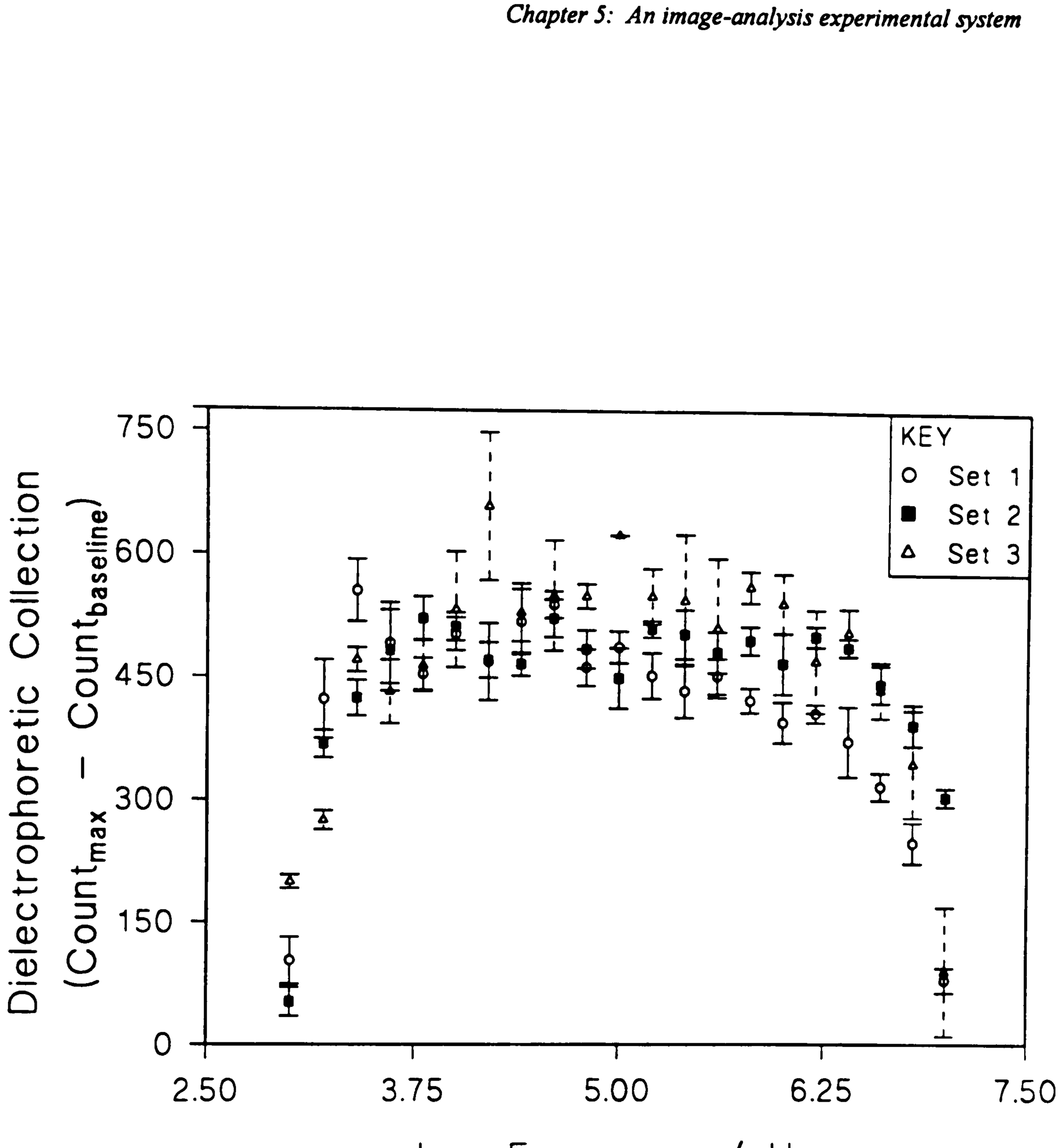


Figure 25: Dielectrophoretic collection of a standard formaldehyde-stabilised erythrocyte suspension as determined by different electrode chambers. (Pulse of 10 V for 10 s, pH = 7.4 ± 0.1, σ = 160 ± 5 μS cm^{-1}, concentration = 3.8 ± 0.1 x 10^6 cells per ml. Data represents mean collection values ± error bars of the SEMs for 5 replicates).

considered to be a source of variation in measurements due to its resistance to decay. Therefore, any inconsistencies in dielectrophoretic response were attributed to system-based variation, in particular that relating to differences between the electrode sets.

Similar dielectrophoretic collection spectra of fixed erythrocytes were produced from investigations using different sets of electrodes, as indicated by the comparison of three sets shown (fig. 25). Collection occurred over the same frequency range although the absolute collection values attained were variable.

Statistical analysis of the data using a Chi-squared test, to determine whether different electrode sets supported the same extent of dielectrophoretic collection, was considered. However, variation in detection levels upon cell enumeration by the image-analysis system, has already been mentioned. The settings for detection levels, light intensity and focus of the microscope were altered on a day-to-day basis where necessary to allow the optimum image to be produced. Accordingly, lighting and detection levels were not the same between test runs of new electrode batches. In view of this it was decided that performing a Chi-squared analysis upon the data would be inappropriate.

Further observations using a stock suspension of fixed erythrocytes for electrode testing over a prolonged period suggested that the washed standard suspension was not entirely unaffected by storage. When not in use, the fixed erythrocytes rapidly sedimented, and appeared more difficult to re-suspend with increased storage time; the removal of flocculation-inhibiting EDTA during washing procedures may be implicated. Communication with the manufacturer of the fixed erythrocytes disclosed that the particles were not totally inert with respect to the zeta layer surrounding the cells, and, upon re-suspension, did undergo some decay before stability was attained. Such changes were insignificant for most purposes, but constitute an additional source of variation when considering the dielectrophoretic response.

It was ultimately decided that a stock suspension of washed fixed erythrocytes, at a standard concentration, would be used to test the performance of new electrodes prior to experimental investigations. Calibration as such was limited to establishing that dielectrophoretic collection occurred to a comparable extent over a similar frequency range for each new electrode set. Some variation in absolute collection values was expected due, for example, to differing light conditions and decay-associated effects of stock suspension as a result of storage time.

5.4.4 Electrode Chamber Maintenance And Durability

For quantification of dielectrophoretic response by actual cell enumeration, a clear image of events occurring at the electrode surface was required. It was, therefore, important that the optical quality of chamber surfaces was maintained. This condition was not as critical when using the spectrophotometric system, as high image resolution was not required. Standard procedure dictated that electrodes were rinsed with de-ionised water between experimental runs; if necessary an air bubble was passed through the chamber to remove adherent cells (section 2.4).

With the image-analysis experimental system the electrodes could be viewed directly at all stages of an investigation. It was noted that with increased use, a build-up of debris occurred upon the glass surfaces of the chamber. These deposits had the effect of fogging the image observed by the detection system, and indicated that the washing procedures adopted were not rigorous enough.

Attempts were made to find a cleaning agent capable of removing inner chamber debris without damaging the electrodes. Various detergents and sterilising agents (e.g. SDS, Tween, Lipsol, Gigasept, ethyl alcohol and hydrogen peroxide), at differing concentrations, were employed. SDS solutions appeared to be most suitable, but were found to remove the metal electrode layer when used at concentrations exceeding 10 mM and bubbled vigorously through the chamber. Other agents exerted similar effects at lower concentrations, or caused debris to remain fixed to the chamber interior. The action of some antibiotics, enzyme solutions and anti-coagulation agents was also investigated; no discernible effect upon debris accumulation or removal was observed, and some anti-coagulants had the adverse effect of dissolving the chamber sealant.

In order to retard the build-up of organic debris upon the inner chamber surfaces, electrodes were rinsed after use with 1 mM SDS solution. The detergent solution helped to remove adherent cells whilst the low concentration prevented gross damage to the electrode metal-plate. Traces of the SDS solution were removed by rinsing with de-ionised water. The chamber was then drained of liquid and stored dry in darkness at 4° C. Such treatment was judged to help preserve the optical quality of the chamber.

During investigations using formaldehyde-fixed erythrocytes, it became apparent that electrode damage was incurred during washing and storage periods, in addition to that due to experimental use. Such damage could be visualised on the image-analysis monitor as electrode erosion, or as the increasing size of holes in the metal layer,

initiated during the vacuum coating stage (section 2.3.2). An obvious consequence of this deterioration was its effect upon the direction and strength of the induced electric field. This in turn affected cell collection in both magnitude and distribution over the electrode bars. Dielectrophoretic collection was non uniform over the entire electrode structure as a result of this deterioration.

The extent of electrode decay was dependent upon the intensity of investigations carried out (for example, low frequency measurements often resulted in impairment), and the vigour of the washing procedures required. Some deterioration was also found to occur during storage prior to use; this was attributed to oxidation reactions of the metal layer, and varied between electrode batches plated at different times. In spite of this variability between batches, only a small number of electrodes were manufactured simultaneously due to production limitations; inter-batch variability was minimal compared with the detrimental effect of storage time.

Longevity of the electrode set was dependent upon the number of investigations carried out and their relative harshness, as already described. When constantly in use, the lifespan could be as little as two weeks, but it should be realised that electrode damage was not the only reason for their replacement - deterioration of optical quality due to debris build-up was also involved. Electrode chambers used in the spectrophotometric system were longer-lasting since detection was less affected by chamber translucence. Electrode longevity increased with system development as the operating limits were established. On average, electrode chambers for the image-analysis system were replaced every three weeks.

An additional source of variability in dielectrophoretic collection measurements using the image-analysis system has been described. Electrode performance can not be considered as constant over a period of usage due to deterioration of both the metal electrode layer and the optical quality of the chamber.

It has therefore been established that electrode performance is not consistent over chamber lifespan, and that detection of dielectrophoretic events may also be impaired.

5.4.5 Summary Of System-Based Variability In Measurements

Several sources of variation in dielectrophoretic measurements using the image-analysis system have been identified in the preceding sections. These include imperfections in the base metal layer generated at the vacuum-coating/etching stage,

deterioration during storage, optical quality of the electrode chamber, and damage to the electrodes as a result of experimental procedures.

As a result of this variability, standard protocols must be refined so that differences between dielectrophoretic collection spectra are associated with the sample investigated, and are not instrument-based.

5.5 MODIFICATIONS TO STANDARD PROCEDURE FOR OPERATION OF THE IMAGE-ANALYSIS SYSTEM

Improvements in the method of characterising dielectrophoretic response from manual techniques described in section 1.3.2 allowed various sources of experimental inaccuracy to be identified. The adoption of a narrower chamber width allowing only the inter-digitated section of the electrode bars to be exposed, prevented the preferential collection of cells at electrode extremities. However, imperfections of the electrode bars, in addition to the deterioration described earlier, illustrated that collection was not uniform over the entire electrode structure.

Measurement of the dielectrophoretic response using the image-analysis system required that an area of the electrode surface was observed. This area comprised a fraction of the total active electrode surface, and was selected by consideration of the flow characteristics of the suspension, visible electrode damage and optical quality of the chamber. Streaming of the suspension was promoted along the channel boundaries, so the central electrode zone was the preferred choice. Areas with visible electrode damage and/or high levels of deposition of debris upon the glass were avoided.

As a consequence of the inconsistency of collection levels attainable with the electrodes, it was established that collection should be quantified in relation to that of a control sample studied over the same electrode locality. In accordance with this, the standard protocol was adjusted; the same electrode area was observed for any series of investigations carried out on a given day, by leaving the chamber *in situ* for the experimental period. Thus, direct comparisons were only made between the same batch of investigations, and differing detection levels resulting from varying light conditions and background debris were therefore insignificant. This allowed the effect of treatments and operational parameters such as voltage, to be compared independently of the day-to-day variability in the dielectrophoretic measurement system.

5.6 EVALUATION OF AN IMAGE-ANALYSIS EXPERIMENTAL SYSTEM TO INVESTIGATE DIELECTROPHORETIC COLLECTION

An experimental system using image-analysis techniques to quantify dielectrophoretic collection has been described. The advantages of this system include:

1) Measurements correspond to a cell sample, not individual cells, so this system is more appropriate for medical applications.
2) Real-time measurements can be achieved limiting errors resulting from physiological changes in the cell sample.
3) Direct observation of the events occurring at the electrode surface allows the behaviour of particles in the vicinity of the applied field to be described (e.g. negative dielectrophoresis, electro-rotation, dielectro-deformation).
4) Electrode condition can be monitored continuously during the experiment - in the event of electrode damage the investigation may be aborted rapidly and restarted after adjustments have been made and/or the electrode replaced.
5) Minimum sample volume and concentration for system operation are both relatively low, permitting dielectrophoretic analysis of small clinical samples.
6) Rapid quantification of the response by the image-analysis system removes the requirement for lengthy analysis after the experiment.
7) Potential sources of error can be recognised allowing preventative steps to be taken.
8) Confidence in experimental data increases since actual electrode performance/dielectrophoretic collection can be observed and some system limitations established.

Problems with detection of particles and day-to-day variation within the system have been described, leading to the adoption of a protocol which permits comparison of relative dielectrophoretic measurements. This procedure allowed treatments to be compared within a series of investigations thus alleviating difficulties associated with decay of collection and detection capacity of the system.

Quantification of relative dielectrophoretic response as a function of collection capacity of a particular electrode area was also of advantage when considering sample cell concentration. Deterioration in optical quality of the electrode chamber obviously affected the clarity of the image perceived by the analysis system. In accordance with this, a standard cell concentration for the sample could not be established. This variation in cell concentration was advantageous when investigating clinical samples of

limited volume; the entire experiment could be carried out at a reduced cell concentration, providing that sufficient measurable dielectrophoretic collection events occurred.

Relative dielectrophoretic response was determined according to the operating capacity of the system for each set of investigations. This method increased confidence in the data by reducing dependency upon potential sources of variation, as already described.

Data were presented graphically as the difference in number of cells detected prior to, and following, a voltage application, against the experimental variable. The possibility of converting the ordinate axis to standard units was considered, but discarded due to variability of light intensity, electrode capacity and suspension concentration. This method reduced the number of sample runs which could be compared together, but limited other sources of inaccuracy which were thought to have a more dominant effect upon dielectrophoretic collection spectra. For each series of investigations, the response of an appropriate control sample was recorded.

5.7 CONCLUSION OF SYSTEM DEVELOPMENT

A rapid system for determination of the dielectrophoretic response has been described. Several constraints of the system have been presented and taken into consideration when defining experimental protocol. Chapters Six and Seven describe investigations carried out using the image-analysis system to examine the dielectrophoretic response of erythrocytes and to assess the potential application of this technique in separating blood cell sub-populations. Evaluation of the system as a method of determining the dielectrophoretic response of haematological cells is discussed in Chapter Eight, with suggestions for future improvements.

Chapter Six

DIELECTROPHORETIC MEASUREMENTS OF HUMAN ERYTHROCYTES USING THE IMAGE-ANALYSIS EXPERIMENTAL SYSTEM

The use of the image-analysis system to investigate the dielectrophoretic response of erythrocytes is described. Considerations for sample preparation are presented with respect to the possible future applications of dielectrophoresis as a clinical tool. The effect of varying physical parameters, such as applied voltage, upon the dielectrophoretic spectra determined by this system is investigated and precise experimental procedure established. The latter part of this chapter is concerned with the relationship between dielectrophoretic behaviour and bioelectronic structure, using the erythrocyte as a simple membrane system.

6.1 PREPARATION OF BLOOD SAMPLES FOR DIELECTROPHORETIC INVESTIGATIONS

6.1.1 Basic Protocol For Experimental Procedure

Blood was obtained by venipuncture of the median cubital vein from a healthy female donor, group A+. Multiple samples were drawn with a 21 gauge needle into evacuated tubes pre-dosed with anti-coagulants (Safety-Monovette, Sarstedt Ltd., Leicester). After venipuncture the whole blood sample was not refrigerated but kept at room temperature and tested within 4 hr, according to the International Committee for Standardisation in Haematology guidelines (ICSH, 1986). In addition to minimising erythrocyte alterations associated with a change in temperature, this protocol was also beneficial in preventing the spontaneous agglutination sometimes observed during sample re-suspension in low ionic strength medium, thought to be triggered by temperature variance.

Aliquots of 0.5 ml of blood in Eppendorff tubes were centrifuged at 1500 rpm for 10 min, then supernatant and buffy coat aspirated. The remaining erythrocyte pellet was re-suspended to 0.5 ml in Hanks Balanced Salt Solution (HBSS; Sigma) and re-centrifuged. This washing procedure was repeated twice. Finally, the cells were re-

suspended in a low ionic strength experimental solution for immediate dielectrophoretic investigations.

Unless otherwise stated, all investigations were carried out using fresh human erythrocytes. If this was not practical due to damaging effects upon cell viability (for example, when establishing the effect of extended pulse application as described in section 6.2.1) then formaldehyde-fixed erythrocytes (section 5.4.2) were used as substitutes. The latter were prepared using the low ionic strength experimental solution for both washing and re-suspension purposes.

6.1.2 A Suspension Medium For Investigating Cellular Dielectrophoresis

a) Specifications for the experimental medium

For a dielectrophoretic response of suspended particles to be elicited then the polarisability of the particles must be greater than that of the suspending medium. For prokaryotic cells, as described during system development (section 3.2.1), suspensions were prepared in de-ionised water. For eukaryotic cells, other considerations must be taken into account, such as osmolarity and pH, to ensure that cells remain viable throughout the experimental period. The low ionic strength environment required for dielectrophoretic motion to be observed is non-physiological and hostile to the cells, and affects their aggregability and deformability properties (Iovtchev *et al.*, 1990). Viability cannot be maintained indefinitely in such solutions due to ion leakage from the cell into the suspending medium and the consequent disturbance of electrochemical gradients.

Preliminary investigations to determine whether the dielectrophoretic response of erythrocytes could be detected by the image-analysis experimental system used suspensions of whole blood in GG solution (Appendix III; Krishna *et al.*, 1989a, 1989b). An experimental sample was produced by direct dilution of whole anti-coagulated blood with an isotonic glycine-glucose solution (GG) in a 1:500 ratio. This method did not prove satisfactory due to the tendency for cell aggregation during blood dilution. Krishna *et al.*, (1989) used GG solution for suspension of washed human erythrocytes to investigate a possible correlation between blood groups and dielectrophoretic collection yield. In the present study, although cell re-suspension was

found to be improved by including a washing step, use of GG solution as an experimental medium was not suitable for investigations using the electrode chamber described in Chapters Two - Five. The glycine component of the solution was found to dissolve the adhesive used to construct the chamber, causing sample leakage and impairment of optical quality by coating inner chamber surfaces (section 3.3.3). In addition, the effect of glycine upon the electrical properties of the erythrocyte surface is unresolved; it is known to exhibit anti-coagulating activity and thus may alter dielectrophoretic response. Observations of erythrocyte dielectrophoretic behaviour in GG solution, using the video method suggested that cell integrity had been impaired as a consequence of the experimental procedure (section 4.2.2). Tsoneva *et al.* (1984) have also noted the destabilising effects of GG solution upon erythrocyte membranes.

Criteria for the selection of a solution suitable for erythrocyte suspension enabling dielectrophoretic measurements to be made using the image-analysis experimental system were established. These included:

- low ionic strength; permitting dielectrophoretic behaviour of the cell to be exhibited.
- iso-osmotic; preventing changes in cell water content, which in addition to the effect upon viability may otherwise alter cell shape and thus the level of detection by image-analysis.
- supporting metabolism; the erythrocyte requires ATP to maintain its physiological functions, the majority of which is obtained from glucose via the Emden-Meyerhof pathway for anaerobic glycolysis.
- minimisation of physiological stress and changes experienced by the cell, where possible.
- maintenance of pH at physiological levels.
- to exhibit minimal detrimental effect upon the electrode array and optical quality of the dielectrophoretic chamber.

To satisfy these conditions a solution of 280 mM sorbitol, 1 mM HEPES supplemented with 5 mg/ml glucose (SG), at pH 7.4 and osmolarity 290 ± 5 mOsm was selected. Sorbitol acts as a stable osmoticum enabling cell volume to be maintained in the short-term (over longer periods ionic losses from the cell result in morphological changes). Glucose is included at a relatively high concentration (plasma levels do not become rate limiting in ATP synthesis until they fall below 10 mg/l, normal plasma levels are approximately 1 mg/ml; Jandl, 1987). This ensures that erythrocyte metabolism, as required for preservation of structural integrity and transport processes, is not limited by substrate availability, even after a period of storage where build-up of ADP is incurred.

Stability of pH was accomplished by use of Goods buffers in preference to those containing phosphate groups (Good *et al.*, 1966; Ferguson *et al.*, 1980). HEPES consists of a large organic molecule which is excluded by cell membranes and has no effect upon mean cell volume or phosphate-requiring energy pathways. In contrast, phosphate groups are able to cross the red cell membrane in exchange for chloride ions, promoting intracellular dehydration (Keidan *et al.*, 1987). The experimental suspension medium described was used for all dielectrophoretic investigations.

b) Effect of suspension medium upon erythrocyte integrity

Erythrocyte survival in SG medium was monitored by measurement of cell count with a haemocytometer, as a function of suspension period. A series of erythrocyte suspensions from the same blood sample were individually prepared in SG solution following standard procedure (Section 6.1.2). Erythrocyte concentration of the five suspensions investigated varied within the range 5×10^6 to 5×10^8 cells per ml; these values were considered to encompass the working range of the image-analysis experimental system, as determined from preliminary investigations (Chapter Five). For each suspension, cell concentration was determined directly after preparation, then at approximately five minute intervals for a period of forty minutes in total. Conductivity of the suspension was recorded using a dip-type probe (Jencons 4070 hand-held conductivity meter), at the beginning and end of this period. The investigation was repeated for a similar series of suspensions prepared in phosphate buffered saline (PBS). To assess the effect of the mechanical action of the experimental system upon circulating cells, suspensions of high and low concentration, within the specified range, prepared in SG and PBS, were circulated through the apparatus at a pump speed of 1.5 ml/min. Cell concentration was determined as described previously. All investigations were carried out at room temperature (23 ± 1 °C).

For each experimental suspension, change in cell concentration was calculated as a percentage of the initial value,in each case. For the series of non-circulated suspensions prepared in SG, the lowest initial density sample exhibited the largest percentage fall in cell concentration over the experimental period (initial concentration = 5.6×10^6 cells per ml, σ = 75 μS, final concentration = 5.3×10^6, σ = 78 μS cm^{-1}; percentage fall in concentration 3 %). During this period, conductivity was fairly constant for all samples, and thus did not indicate that cell lysis had occurred; instead, it was suggested that discrepancies in concentration were primarily associated with cell sedimentation. Similar effects were observed for the non-circulated series of suspensions prepared in

PBS, where concentration appeared to decrease slightly with time (maximum decrease recorded was 4.5 %). Conductivity values for samples suspended in PBS ranged between 1600-1700 mS cm^{-1}. Suspensions circulated through the experimental system also exhibited a slight decay in concentration (maximum percentage decrease for SG- and PBS- suspended samples was 7.3 %) over the experimental period. For all samples, conductivity rose more rapidly for concentrated samples with increased initial levels; however, this increase was below 10 % of the original value for all cases.

Upon the basis of this study, re-suspension of erythrocytes in SG was considered to have little effect upon cell integrity, as determined by concentration measurements. (Similar investigations suggested that minimal changes occurred during an extended observation period of two hours).

It must, however, be noted that these measurements of cell concentration have a certain degree of inaccuracy associated with them. Determination using a haemocytometer involves making multiple counts of cells enclosed in a volume of 0.2 x 0.2 x 0.1 mm, *i.e.* 0.004 mm^3. For each determination, a minimum of six such volumes were counted, as limited by time constraints. At the minimum concentration limit, increasing the mean cell count by one results in a 5 % increase to the calculated cell concentration. Therefore, variability of measurements is comparable in size to the reported decreases in cell concentration.

c) Effect of low ionic strength suspension medium upon resistance of erythrocytes to haemolysis using the osmotic fragility test

The resistance of the erythrocyte to osmotic lysis is a function of membrane integrity, ability to maintain ionic gradients, and the surface area to volume ratio of the cell. The latter may also be used to indicate cell shape; this ratio is smaller for abnormal spherocytic cells than discoid forms, indicating the associated increase in susceptibility to osmotic lysis. The osmotic fragility test employs a simple photometric method to measure cell lysis after incubation of erythrocytes in saline solutions of various dilutions.

Investigations were carried out to establish if suspension in SG solution exerted a detrimental effect upon erythrocyte osmotic fragility, in comparison to similar suspensions prepared in PBS. The effects of circulation through the dielectrophoretic measurement system and of voltage application were also considered.

A 3 ml aliquot of heparinised blood was centrifuged at 1000 rpm for 10 min and the plasma layer carefully aspirated. The erythrocytes were gently re-suspended in SG solution to the original volume, mixed and divided equally between three experimental tubes of 50 ml volume. Two tubes were diluted with SG solution to yield total volumes of 40 ml; the third tube was similarly treated with PBS. This method enabled three suspensions to be prepared at a concentration (mean cell density 1.0-1.2 x 10^8 cells per ml) which compromised between working limits for the image-analysis experimental system and that required for the osmotic fragility test. The initial conductivities of the SG suspensions were 298 and 312 $\mu S\ cm^{-1}$, and thus were low enough for their use in dielectrophoretic investigations. (In comparison, the initial conductivity of the sample suspended in PBS was in excess of 1600 $mS\ cm^{-1}$).

Osmotic fragility was determined by standard protocol which had been slightly adapted in accordance with the lower cell concentrations involved (for procedure, see Appendix IV). Erythrocyte samples prepared in PBS and SG were tested at time zero after re-suspension and after two hours incubation at room temperature (22 ± 1 °C). Effects upon osmotic fragility as a result of dielectrophoretic measurements were assessed by circulating a suspension (1 blood:40 SG) through the system and applying a 10 s, 10 V pulse of frequency 1 MHz every 30 s (base flow rate = 1.5 ml per min, 5 s flush pump at 4.5 ml per min). Saline dilutions were prepared from this experimental sample at t = 5 min and t = 2 hr. Osmotic fragility of whole heparinised blood was also investigated.

Percentage haemolysis with reference to the level observed for the sample in 0.0 % saline solution was plotted as a function of ascending percentage saline concentration. In all cases but one, r^2 values representing the goodness-of-fit between theoretical and real data were in the range 0.94-0.99 (exception - cells circulated through system for 2 hr, $r^2 = 0.81$). This indicated that the data were adequately described by this function, allowing the identification of saline concentrations at which haemolysis was initiated and completed.

At time zero after re-suspension, osmotic fragility values were very similar for erythrocyte samples prepared in SG and PBS, and those obtained from whole blood (data not shown). Control values from the normal blood sample were within normal accepted standards (haemolysis initiated at 0.55 % saline, completed at 0.35 %). For SG and PBS non-circulated samples haemolysis began in 0.6 % saline solutions, and became maximal at 0.4 % saline. After incubation for 2 hr the whole blood sample remained unchanged, although haemolysis was not initiated until the 0.5 % saline dilution for the PBS suspension and 0.4 % for the SG suspension; haemolysis was

complete at 0.3 % saline dilutions. For the experimental samples in SG, osmotic fragility after 5 min and 2 hr displayed no apparent differences from non-circulated samples.

Suspension in SG solution caused no apparent detrimental effect upon osmotic fragility of erythrocytes. Results suggested that resistance to haemolysis was increased - this could be attributed to water loss from the erythrocyte and cell shrinkage, which would affect the surface area:volume ratio.

6.1.3 Effects Of Blood Collection And Storage Procedures Upon Subsequent Dielectrophoretic Investigation.

The erythrocyte is subjected to a series of changes during the period following its removal from the peripheral blood supply to preparation of a low ionic strength sample for dielectrophoretic investigations. These changes include possible damage resulting from phlebotomatic procedures, plasma composition (e.g., effect of anti-coagulant, decline in metabolic substrates), change in temperature and deterioration with storage time. Such effects are rarely considered during dielectrophoretic investigations but were found here to influence spectra obtained.

Common anti-coagulants used in haematological procedures include heparin, citrate and ethylenediamine tetraacetate (EDTA). Heparin acts by preventing the transformation of prothrombin to thrombin; citrate and EDTA act as chelators and prevent the calcium-dependent stages of the coagulation pathway (Becan-McBride & Ross, 1988; Turgeon, 1988). EDTA-treated blood is suitable for most applications and is frequently used for dielectrophoretic investigations. Enhanced cell storage is achieved using a Citrate-Phosphate-Dextrose-Adenine solution (CPDA-1), as used clinically for blood transfusion purposes (Beutler, 1989). In this study, choice of anti-coagulant was initially limited to those used in the Sarstedt Safety-Monovette range (*i.e.*, lithium heparin, potassium EDTA or trisodium citrate), due to University Medical Centre regulations.

Attempts were made to re-suspend erythrocytes (separated from fresh EDTA-treated blood) in SG solution for investigation by the image-analysis dielectrophoretic experimental system following standard procedure (Section 6.1.2). However, difficulties were experienced during the final dilution step due to the tendency for cell aggregation to occur. When re-suspension of EDTA-treated samples was achieved and

the dielectrophoretic response investigated, electric field application typically resulted in the apparent electro-fusion of cells at the electrode surface. Such effects were not noted by Krishna *et al.*, (1989a, 1989b), who measured the dielectrophoretic collection rate of EDTA-treated erythrocytes (1 mg EDTA per ml blood) after washing and re-suspension in GG solution. Burt *et al.*, (1990) described the addition of 1 μM $CaCl_2$ to the low ionic strength suspending solution for use with EDTA-treated cells, to prevent electrofusion effects. In this system a similar addition of calcium chloride, thereby increasing sample conductivity, did not prevent cell electro-fusion.

Successful re-suspension and dielectrophoretic measurement of EDTA-treated erythrocytes appeared to be correlated with sample age and storage conditions. Freshly harvested cells behaved as already described, whereas those incubated at room temperature for a period of hours could be manipulated as required and the dielectrophoretic response investigated. This phenomenon was not exhaustively investigated but it is hypothesised that the initial effect of EDTA is to chelate a large proportion of metal ions, both free in the plasma and in the ion layer surrounding each cell. This action may predispose the cells to agglutination effects, due to depletion of the ionic layer. It is proposed that this boundary layer becomes re-populated with ions over a period of time, as a result of extrusion from the cell, permitting dielectrophoretic behaviour without electro-fusion to be exhibited. Sample age may also account for the successful use of EDTA-treated samples by other workers; in some instances dielectrophoretic measurements are made after a storage period in excess of 48 hr (Burt, 1992). Under such circumstances the possible detrimental effects of EDTA upon cell bioelectrical structure may not be apparent.

The problems experienced during dielectrophoretic investigations of EDTA-treated erythrocytes prompted testing of blood treated with alternative anti-coagulants. Experimental suspensions were prepared from sodium citrate and lithium heparin anti-coagulated blood samples, obtained from the same venipuncture, (procedure outlined in Section 6.1.2). If necessary, adjustments were made so cell concentration and conductivity were consistent between samples. Dielectrophoretic investigations were carried out using the same experimental parameters, upon suspensions prepared within one hour of venipuncture (whole blood maintained at room temperature), and after whole blood storage for 24 hr at 4° C. Characteristic spectra of dielectrophoretic collection against the base 10 logarithm of frequency were compiled from the first experimental run of each sample, initiated within five minutes of erythrocyte re-suspension (fig. 26).

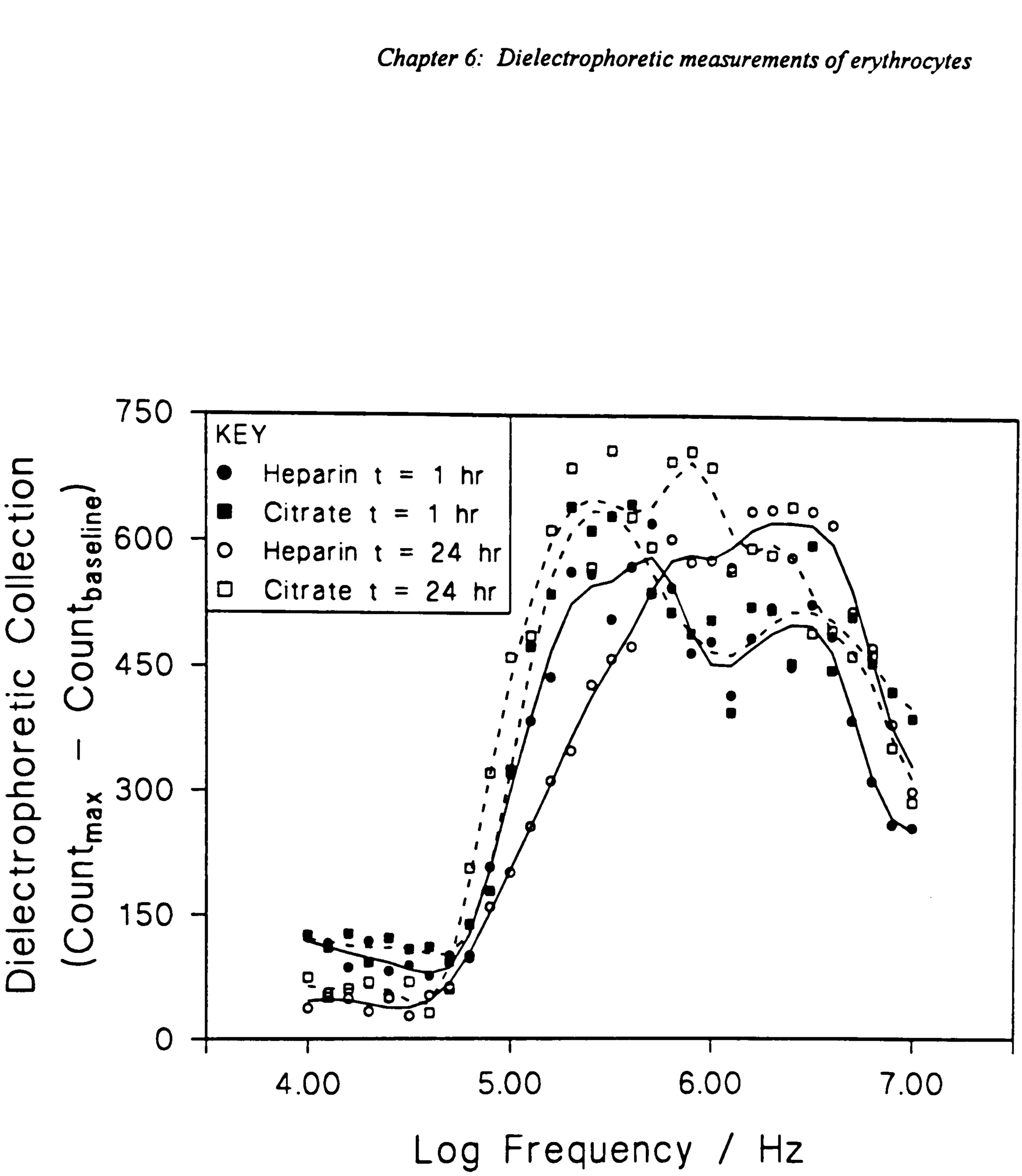


Figure 26: Variation in dielectrophoretic collection of human erythrocytes due to anti-coagulant treatment and storage time. (Pulse of 5 V for 10 s, pH = 7.2 ± 0.1, σ = 80 ± 2.4 μS cm^{-1}, concentration = 1.2 ± 0.15 x 10^{7} cells per ml. Data shown of first run only of 4 different samples. Smoothed curves fitted through data; —— = heparin treated, - - - = citrate treated).

The spectra shown in figure 26 indicate that the dielectrophoretic response of erythrocytes obtained from heparinised blood was affected by age of the whole blood sample, causing a shift towards higher frequencies. The minimum frequency of collection, as determined by suspension conductivity, was consistent for the two anti-coagulants investigated at each time period. The difference observed in collection values from heparinised blood was thought to be associated with the lack of metabolic substrates available during storage and the activity of the anti-coagulant. Standard haematological procedures define that heparinised blood must be used within 48 hours, as compared to slightly longer periods for citrate- and EDTA-treated blood (Powers, 1989). The effect upon dielectrophoretic response as a result of sample age may be attributed to heparin acidity. It is also of interest to note the temporary fall in dielectrophoretic collection values observed around 1 MHz for suspensions prepared from fresh blood samples.

From these investigations it was concluded that both anti-coagulant and blood storage conditions affect erythrocyte dielectrophoretic response. The preferred anti-coagulant, at this stage, for use in accordance with dielectrophoretic investigations was trisodium citrate.

6.2 EFFECTS OF VARYING THE ELECTRIC FIELD APPLICATION ON DIELECTROPHORETIC RESPONSE

6.2.1 Pulse Length

The effect of pulse length upon dielectrophoretic collection was established using similar suspensions of fixed erythrocytes prepared in SG solution (section 5.4.2). A preliminary spectrum of frequency response was used to specify the a.c. frequency at which pulse length was investigated; this was selected from the midpoint of the frequency range generating positive dielectrophoretic collection (400 kHz). Pulse length spectra were obtained using incremented pulse applications at a series of different voltages (fig. 27).

Figure 27 indicates that increased levels of dielectrophoretic collection are observed with extended pulse lengths where field voltage is above a threshold level. For a 2.5 V pulse (fig. 27), increased pulse application has little effect upon dielectrophoretic response, indicating that the field generated is unable to cause collection to a level that may be detected by the image-analysis method.

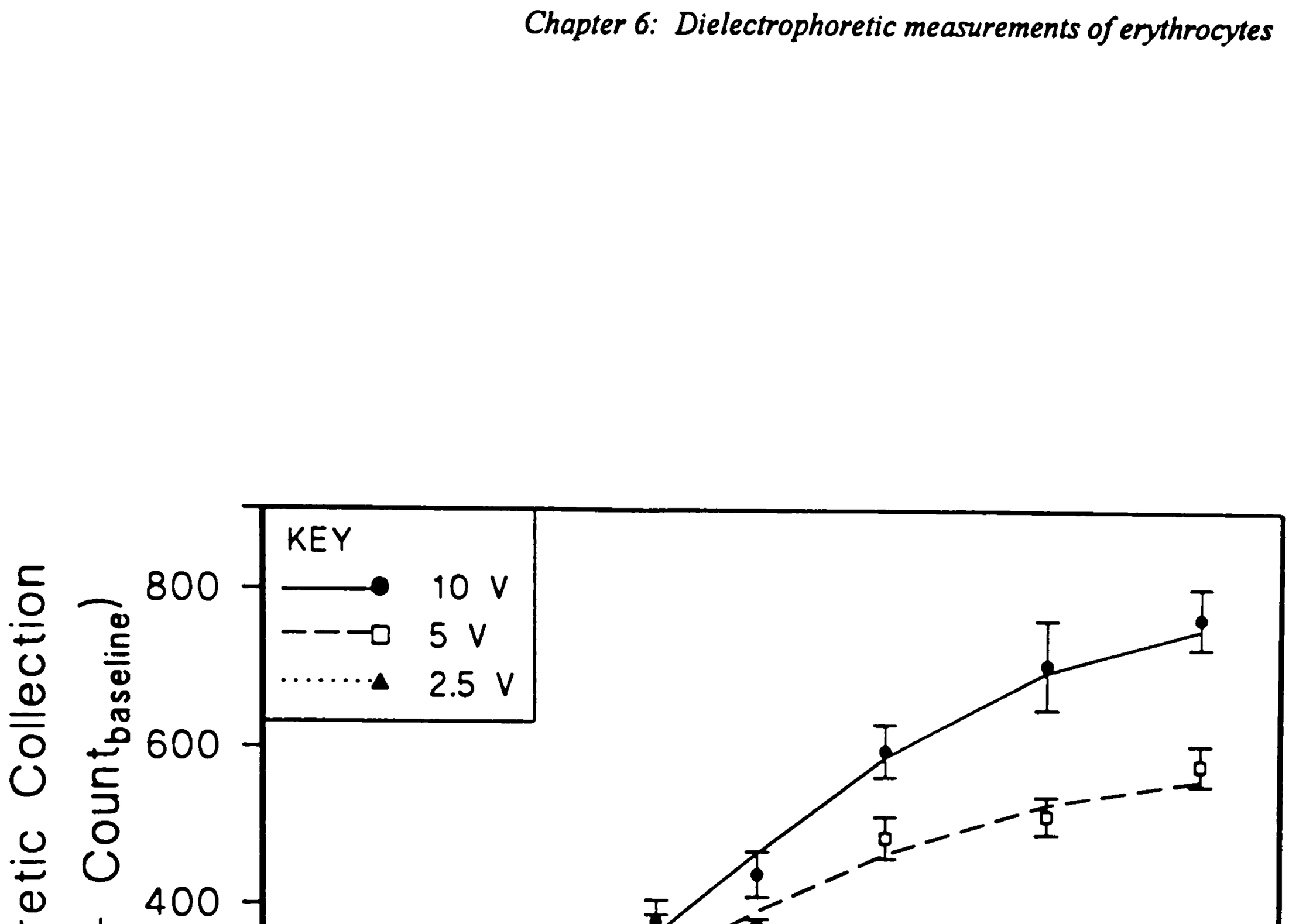


Figure 27: Effect of pulse length upon dielectrophoretic collection of formaldehyde-stabilised erythrocytes. (Frequency = 400 kHz, σ = 116 ± 6 µS cm^{-1}, pH = 7.4, concentration = 1.4 ± 0.1 x 10^7 cells per ml. Datapoints shown are means ± error bars of SEMs of 5 replicates. Linear regressions:

For 2.5 V	$y = 2.7511(x) + 29.2132$	$r^2 = 0.6793$	$p = 4.7 \times 10^{-5}$
For 5 V	$y = 20.1662(x) + 28.0433$	$r^2 = 0.9751$	$p = 1.9 \times 10^{-13}$
For 10 V	$y = 26.7085(x) + 14.9903$	$r^2 = 0.9910$	$p = 8.9 \times 10^{-17}$)

At increased voltages, *i.e.* 5 and 10 V for the data shown in figure 27, and pulse lengths of up to 25 s, dielectrophoretic collection exhibited a linear dependence upon pulse length. The validity of this relationship was indicated by the r^2 and p values obtained from linear regressions (*i.e.*, for 5 V, $r^2 = 0.6793$, $p = 4.7 \times 10^{-5}$; for 10 V, $r^2 = 0.9751$, $p = 1.9 \times 10^{-13}$). However, at increased pulse lengths, figure 27 suggests that dielectrophoretic collection values reach a plateau where collection is independent of pulse length. This effect may represent electrode saturation, *i.e.* that the electric field generated cannot support collection of any more cells, or may indicate the limitations of the image-analysis system in detecting dielectrophoretic response.

6.2.2 Pulse Voltage

The effect of pulse voltage upon dielectrophoretic collection was investigated in a similar manner to that described in the preceding section. Experimental spectra were obtained of collection against pulse voltage for three different pulse lengths, as shown in figure 28; data shown in figures 27 and 28 were obtained in the same experimental session and are directly comparable.

For each pulse length investigated, dielectrophoretic collection was not observed until a threshold voltage level was reached, the magnitude of which decreased with increasing pulse length (figure 28). Above this level dielectrophoretic collection increased with voltage until a plateau was attained. The relationship between dielectrophoretic collection and voltage of the applied pulse was adequately described by an exponential sigmoidal function (see fig. 28), as indicated by p values for this response occurring by chance; $p <= 0.05$ for 10 s and 15 s pulse lengths.

The sigmoidal dependence of dielectrophoretic collection upon voltage indicated that above a threshold level, at a given pulse length, collection values observed were limited by some factor other than pulse voltage. Comparison of data between figures 27 and 28 indicates that pulse length was a predominant limiting parameter. Consequently, dielectrophoretic measurements determined by this method may be constrained by sample concentration, *i.e.*, the number of cells travelling through the region affected by the electric field.

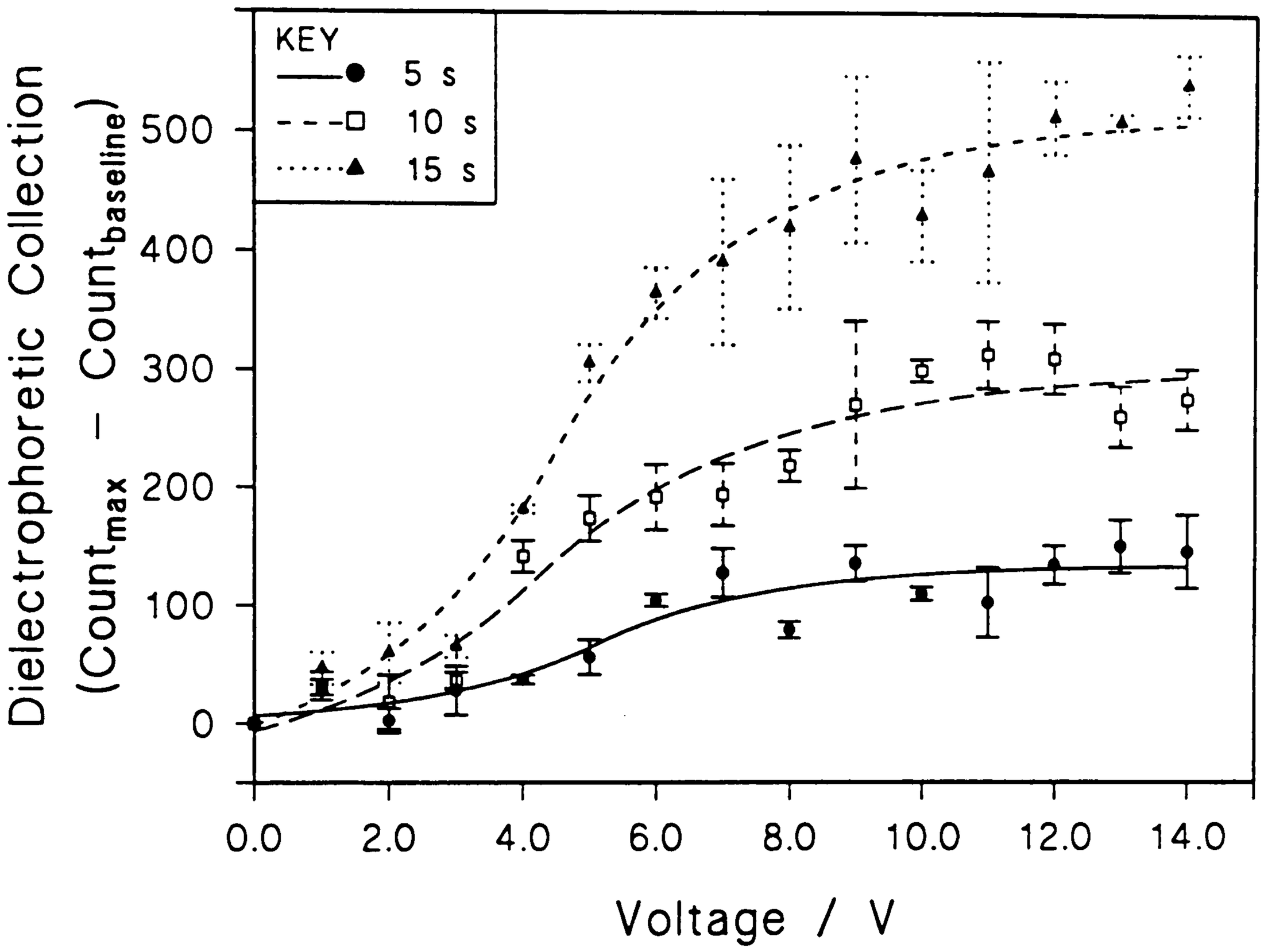


Figure 28: Effect of voltage upon dielectrophoretic collection of formaldehyde-stabilised erythrocytes at constant pulse lengths. (Frequency = 400 kHz, σ = 118 ± 4 $\mu S\ cm^{-1}$, pH = 7.4, concentration = 1.5 ± 0.1 x 10^7 cells per ml. Datapoints shown are means ± error bars of SEMs of 5 replicates. Exponentail sigmoidal curves fitted to the data: For 5 s pulse length, p = 0.88, for 10 s pulse length, p = 0.95, for 15 s pulse length, p = 0.98).

6.2.3 Ascendant And Descendant Frequency Applications

The effect of ascendant and descendant frequency series of electric pulses on dielectrophoretic collection of fresh human erythrocytes was investigated. The results presented in figure 29 indicate that both the range and magnitude of dielectrophoretic collection, as determined by the image-analysis system, are affected by the nature of the frequency series applied. The minimum pulse frequency eliciting positive dielectrophoretic collection is unchanged by the order of experimental frequencies applied (*i.e.*, is approximately 100 kHz for the example shown). However, use of an ascending series of pulse frequencies maintains increased levels of dielectrophoretic collection at frequencies in excess of 1 MHz. This phenomenon is shown in figure 29, where collection values in response to the application of a 10 MHz pulse are approximately 75 % larger during an ascending series in comparison to that observed during a descending series (in each case mean data with SEMs from three replica runs of three individual suspensions are shown).

The variation in collection values observed in the MHz region, as affected by frequency incrementation, was attributed to electrode polarisation. Such effects produce an electrode boundary layer with its own associated dielectric properties which influence the actual field experienced by the cells (Bone, 1985). It is suggested that electrode polarisation creates an additional phase of dissimilar dielectrical properties through which the electric field generated by a voltage application must penetrate. Dipoles formed within additional layer due to high frequency field application are postulated to lag slightly behind the frequency of the alternating current, with which they would then interact to slightly reduce the frequency of the field experienced by the suspended cells. Continuation of high dielectrophoretic collection levels at applied frequencies in excess of 1 MHz may thereby be attributed to dipole persistence at the electrode-medium interphase and are an artifact of the measurement system. .

The results presented here support similar electrode polarisation effects observed during measurement of erythrocyte electro-rotation (Georgiewa *et al.*, 1989). Positively incremented frequency application caused cell electro-rotation to be exhibited at lower frequencies than if a declining frequency series was used. To correlate the effect of electrode polarisation upon electro-rotation with that described here upon dielectrophoretic collection, it is suggested that polarisation effects at low frequencies act to increase the torque experienced by the cell, but have no effect upon translational dielectrophoretic activity.

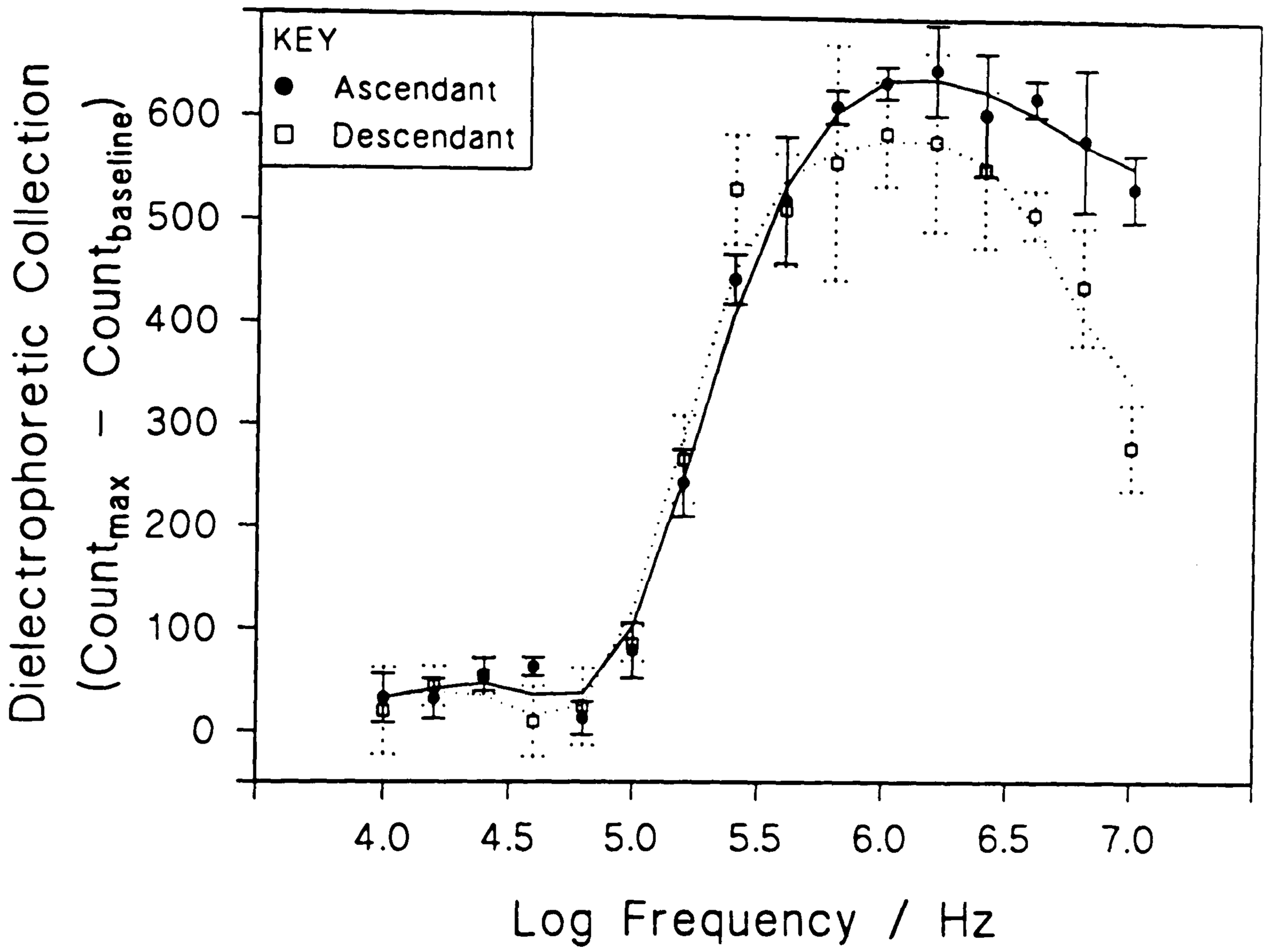


Figure 29: Effect of frequency incrementation series upon dielectrophoretci collection of human erythrocytes. (Pulse = 5 V for 5 s, pH = 7.4 ± 0.1, σ = 120 ± 11 μS cm^{-1}, concentration 8.3 ± 0.2 x 10^6. Data represents means ± error bars of SEMs from 3 replicates of 3 individual samples).

6.3 EFFECT OF EXPERIMENTAL SUSPENSION COMPOSITION UPON DIELECTROPHORETIC RESPONSE

6.3.1 Sample Concentration

The effect of suspension cell concentration with regard to dielectrophoretic measurements using the image-analysis system has been mentioned briefly in section 5.6. Investigations were carried out to qualitate this response and enable additional operational parameters to be defined.

Suspensions of fresh human erythrocytes were prepared in SG, as required, over a range of concentrations from 2.5×10^6 to 4×10^7 cells per ml; all other variables such as conductivity and pH were standardised. For each concentration, dielectrophoretic collection as a function of field frequency was investigated and data displayed as means ± SEMs of five similar trials (fig. 30). The lowest frequency eliciting positive dielectrophoretic collection was found to be independent of sample concentration. The effect of sample concentration upon dielectrophoretic response was determined by plotting mean collection values and SEMs resulting from the application of a 1 MHz pulse, against cell concentration. (A frequency of 1 MHz was selected as this is a convenient value in the middle of the frequency range eliciting a positive dielectrophoretic response for this sample - see figure 30). The sigmoidal relationship between dielectrophoretic collection and cell concentration is shown in figure 31 (r^2 = 0.97). For the concentration range investigated, collection reaches a maximum value after which they are unaffected by further increases in cell concentration.

6.3.2 Suspension Conductivity

The effect of medium conductivity upon dielectrophoretic collection was investigated using suspensions of fresh human erythrocytes, at a standard cell concentration, prepared in SG. The minimum conductivity investigated was that of a control suspension in SG; for subsequent samples aliquots of 0.01 N KCl were added to increase conductivity to a maximum of 400 $\mu S\ cm^{-1}$. For each suspension dielectrophoretic collection as a function of frequency was investigated.

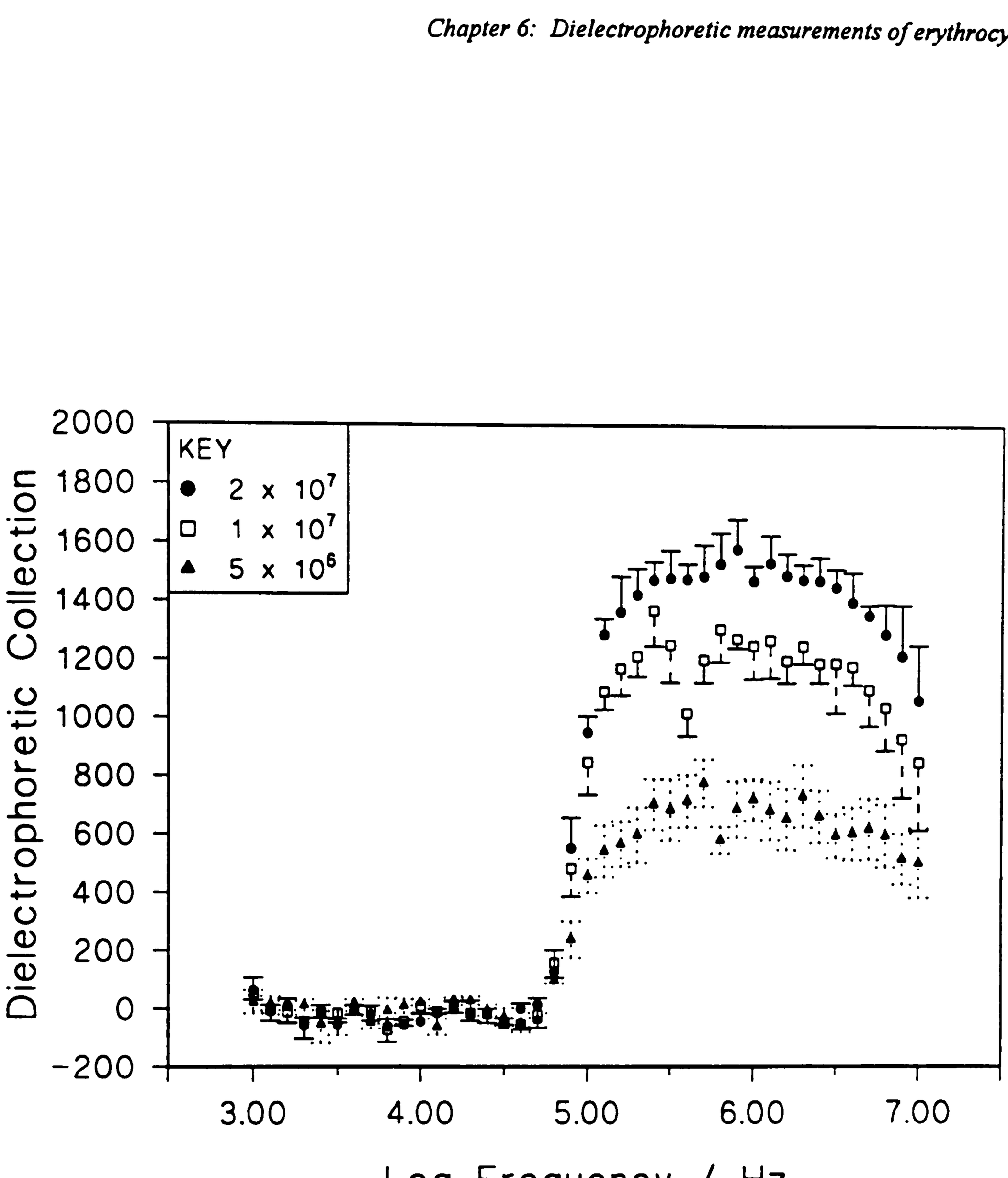


Figure 30: Dielectrophoretic collection of human erythrocytes as a function of frequency for a series of concentrations. (Pulse = 10 V for 10 s, pH = 7.3 ± 0.2, σ = 100 ± 5 $\mu S\ cm^{-1}$. For sample concentrations, measured as cells per ml, see figure insert. Each datapoint is the mean ± SEMs of 5 replicates - for the two larger concentrations half error bars are displayed for clarity).

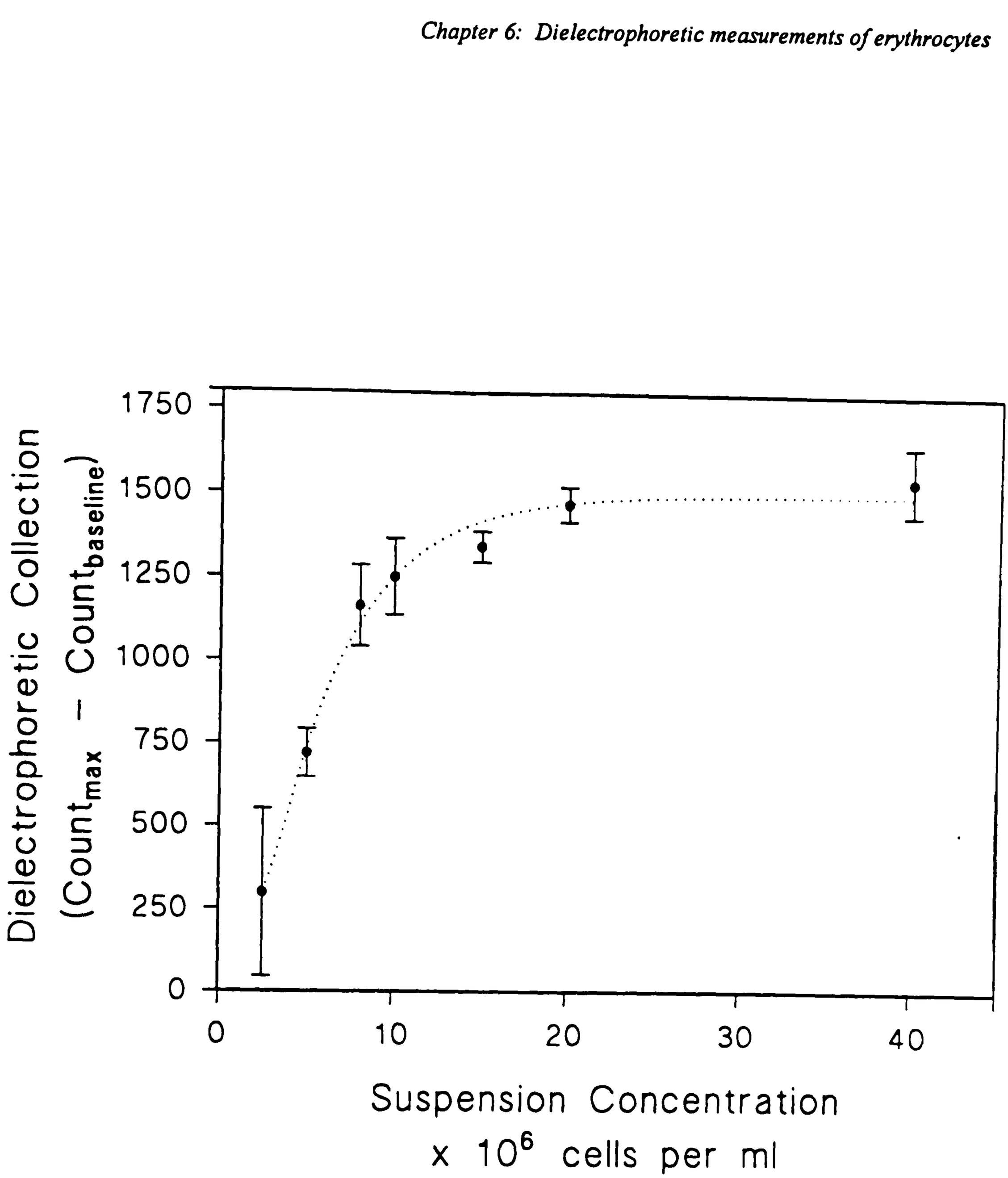


Figure 31: Relationship between dielectrophoretic collection of human erythrocytes and sample cell concentration as determined by an image analysis method. (Frequency = 1 MHz, pulse = 10 V for 10 s, pH = 7.4 ± 0.1, σ = 100 ± 5 $\mu S\ cm^{-1}$. Error bars shown are means ± SEMs of 5 replicates. The data is described by an exponential sigmoid curve with an r^2 value of 0.97).

Figure 32 indicates the effect of suspension conductivity upon both the magnitude and the frequency range of dielectrophoretic collection (minimum and maximum conductivities investigated shown). Increasing suspension conductivity caused a shift of the first collection frequency to higher values and decreased the overall magnitude of the response.

These results are in agreement with established views whereby the onset of dielectrophoretic collection is predominantly dependent upon suspension conductivity. The maximum frequency at which dielectrophoretic collection is expressed is relatively independent of medium conductivity, reflecting its association with dipole formation within the cell (sections 1.1.5 and 1.2.1).

6.4 INVESTIGATION OF THE DIELECTROPHORETIC RESPONSE USING THE ERYTHROCYTE AS A MODEL MEMBRANE SYSTEM

6.4.1 General Method

An aliquot of 0.5 ml of CPDA-1 treated blood (taken from the same donor as for section 6.1.1) was diluted to 1.5 ml with sterile Hanks Balanced Salt Solution (HBSS, Sigma) supplemented with 3 mg/ml glucose . This was centrifuged at 1500 rpm for 5 min, the supernatant aspirated, then the washing procedure repeated twice.

The erythrocytes were then gently re-suspended in 10 ml of HBSS containing the test agent and incubated at 37° C for 1 hr, whilst being gently agitated to promote mixing of the cells with the solution.

After incubation the sample was centrifuged at 1500 rpm for 5 min and the supernatant removed. The cells were gently re-suspended in 10 ml SG solution. The suspension was re-centrifuged and the washing process using SG solution, repeated. If at any stage a red-brown supernatant was obtained indicating cell lysis and a gross impairment of cell integrity, then the sample was discarded.

A 10 µl aliquot of the washed cell pellet was transferred to a fresh tube using a Gilson Microman pipettor and re-suspended in 10 ml of fresh SG solution. The absorbance of the cell suspension was measured at a wavelength of 540 nm using a spectrophotometer

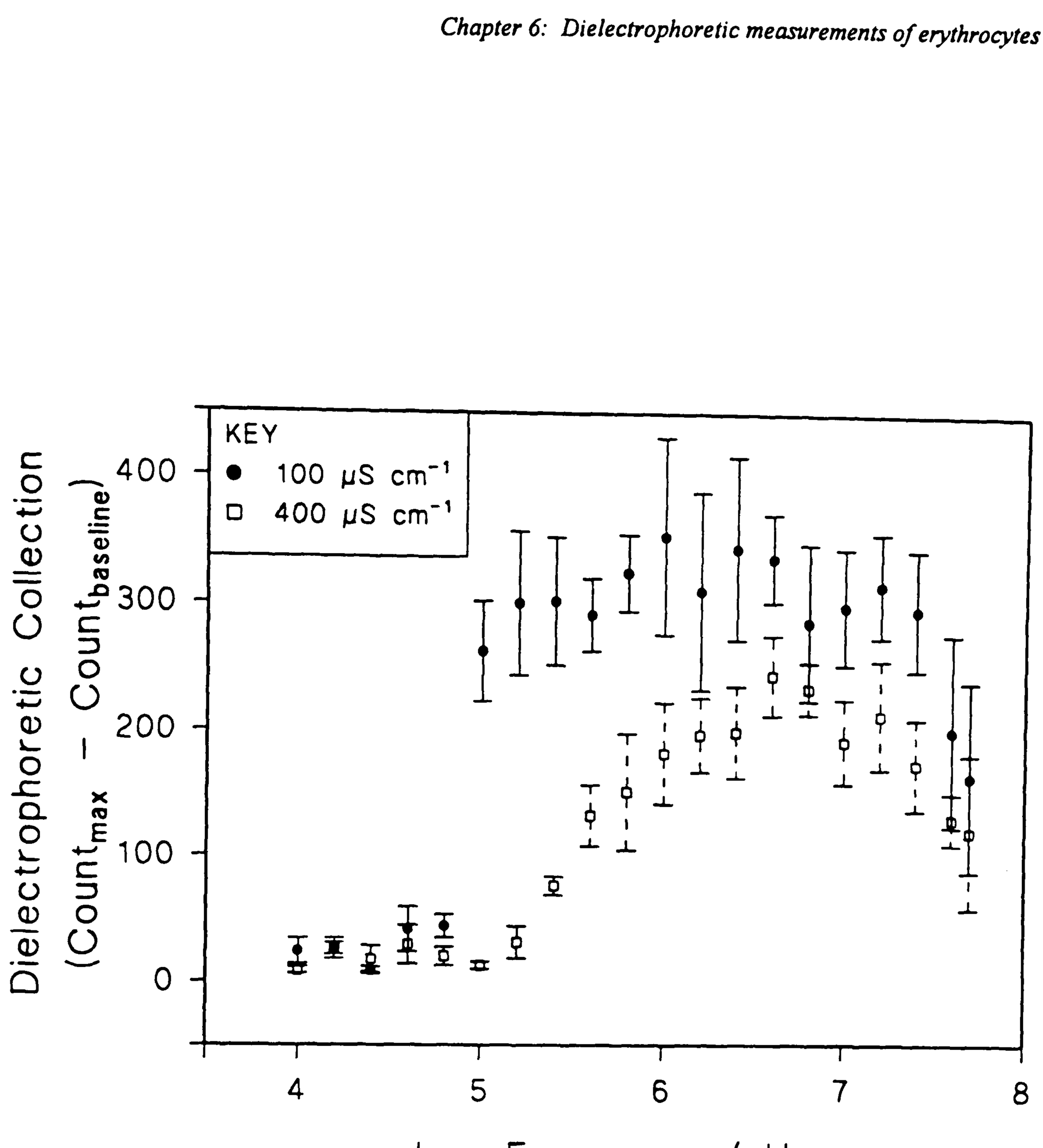


Figure 32: Effect of suspension conductivity upon dielectrophoretic collection of human erythrocytes. (Pulse = 10 V for 10 s, pH = 7.4 ± 0.1, σ = see fig. insert, concentration = 1.2 ± 0.1 x 10^7 cells per ml. Data shown of means ± SEMs form three runs of three replicates at each conductivity).

(LKB Ultrospec II), and compared with a standard curve to give a rapid indication of concentration. The suspension was adjusted as required then absolute cell concentration determined using a haemocytometer. The conductivity of the suspension was measured and adjusted if necessary by the addition of 1mM KCl.

The dielectrophoretic investigation was initiated within 10 min of preparation of the experimental sample to minimise any changes associated with a time delay. Unless stated otherwise, measurements were made to investigate the effect of a 10 V pulse applied for 10 s over a frequency range 1 kHz - 10 MHz. Each test treatment was carried out in triplicate and three consecutive dielectrophoretic collection against frequency spectra obtained for each individual sample. The mean values of collection against frequency were calculated for replica runs of the same suspension, to minimise inconsistencies of measurement, then mean values of the three individual samples calculated for each treatment. Results were plotted as mean collection values with error bars of the SEMs against frequency.

6.4.2 Effect Of EDTA On Dielectrophoretic Collection

The effects of the metal ion chelator EDTA upon dielectrophoretic spectra were investigated. It was hypothesized that the addition of EDTA would diminish the concentration of cations able to participate in the counter-ion layer surrounding the cell, thereby increasing the width of the diffuse ion layer and dielectrophoretic response.

For anti-coagulant activity, EDTA is typically used at a concentration of 1-1.5 mg/ml of blood (approximately 4mM; Turgeon, 1988). In this study, cells were incubated for one hour in HBSS containing 5 mM EDTA, prior to experimentation. The results of these trials are shown in figure 33. It is clear that EDTA has no effect upon the threshold frequency which elicits dielectrophoretic collection, and there are no appreciable differences in spectra over the positive collection frequency range. It would be expected that EDTA chelation of cations in the diffuse double layer surrounding the cell would result in an enhanced dielectrophoretic response, yet this was not observed. The absence of an effect may be attributed to use of HBSS as the incubation medium, thereby supplying an ionic population to surpass the chelation activity of the EDTA concentration used.

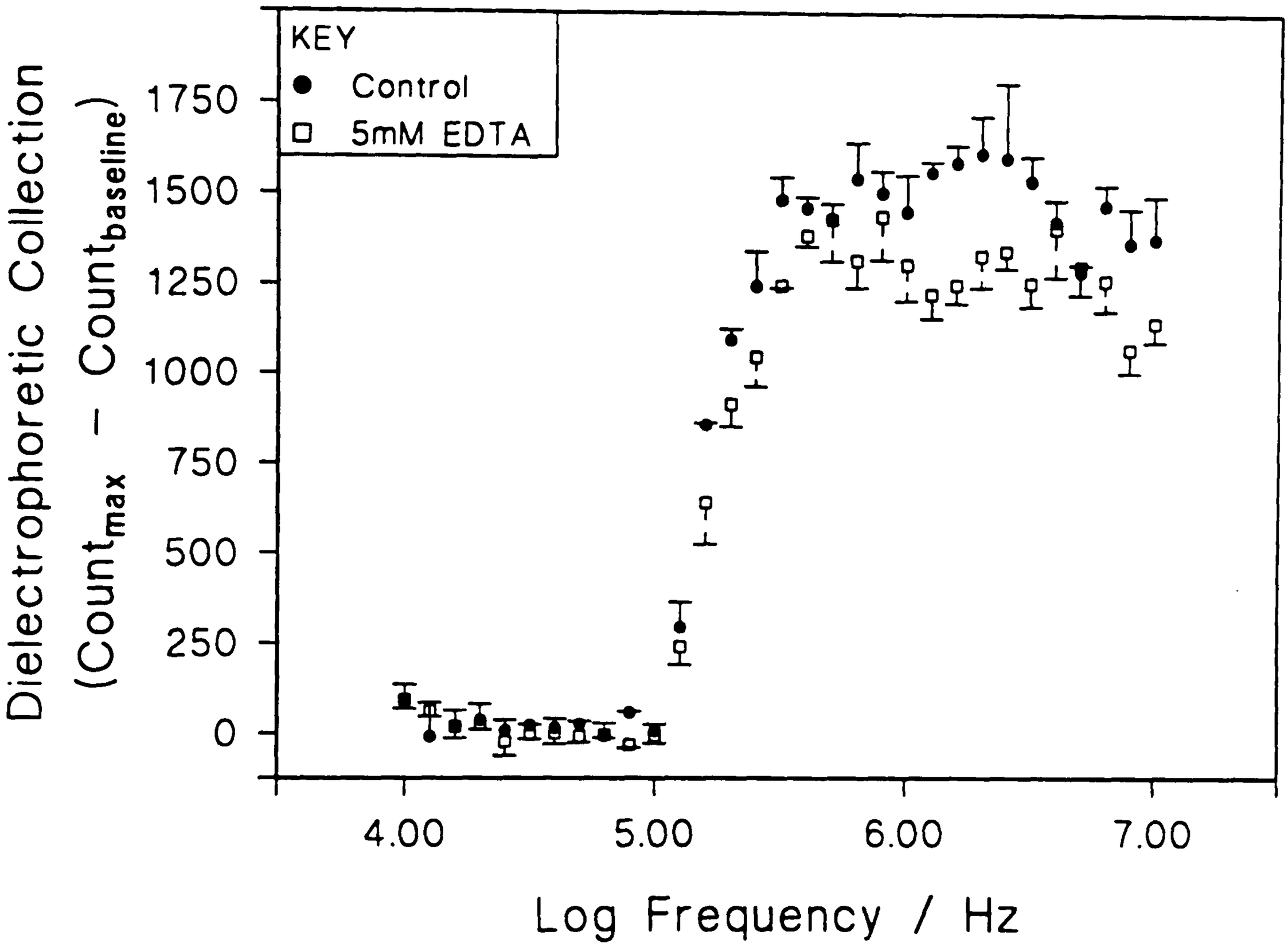


Figure 33: Effect of EDTA treatment upon dielectrophoretic collection spectra of human erythrocytes. (Pulse = 5 V for 10 s, pH = 7.4 ± 0.1, σ = 108 ± 7 μS cm^{-1}, concentration = 7.7 ± 0.2 x 10^6 . Data represents means ± error bars of SEMs from 3 replicates of 3 individual samples; half error bars only shown for clarity).

These experiments demonstrate that the presence of EDTA as an *anti-coagulant* may not prevent a dielectrophoretic response as determined previously (section 6.1.3). However, this activity may be critically dependent upon cell pre-treatment (*i.e.*, ionic and metabolite content of the anti-coagulant used), and storage of cells prior to dielectrophoretic measurement.

6.4.3 Effect Of Neuraminidase Treatment

The dielectrophoretic behaviour exhibited by a cell is dependent upon its surface charge established by charged components of the cell membrane (sections 1.2 and 1.4.4). The effect of enzymatic removal of sialic acid species, the major contributor to erythrocyte surface charge, upon dielectrophoretic spectra was investigated.

Neuraminidase type X from *Clostridium perfringens* (Sigma N2133) (Burt *et al.*, 1990) was suspended in 1 ml HBSS and stored on ice until required. Aliquots of stock suspension were added to the incubation solution prior to the addition of washed erythrocytes. Treatments were carried out at unit activity concentrations of zero (control), 0.10, 0.15, 0.25 and 0.50. Incubation with neuraminidase was for 60 min.

Figure 34 shows the effect of the highest unit concentration of neuraminidase employed. No differences in spectra magnitude or frequency dependency were observed between control and test trials. This also held for the lower unit concentrations. In all cases, values for the standard error of the mean did not exceed 10% of the mean value for the frequency range over which strong dielectrophoretic collection was exhibited (i.e. 100 kHz - 10 MHz).

These results are in accord with those of Burt *et al.* (1990) where similar neuraminidase concentrations were found to have no effect upon erythrocyte dielectrophoresis in response to frequencies above 50 kHz. The influence of cell surface charge upon dielectrophoretic behaviour is predicted to dominate polarisation processes induced by frequencies below 200 Hz (Burt *et al.*, 1989), thus would not be observed using this experimental system.

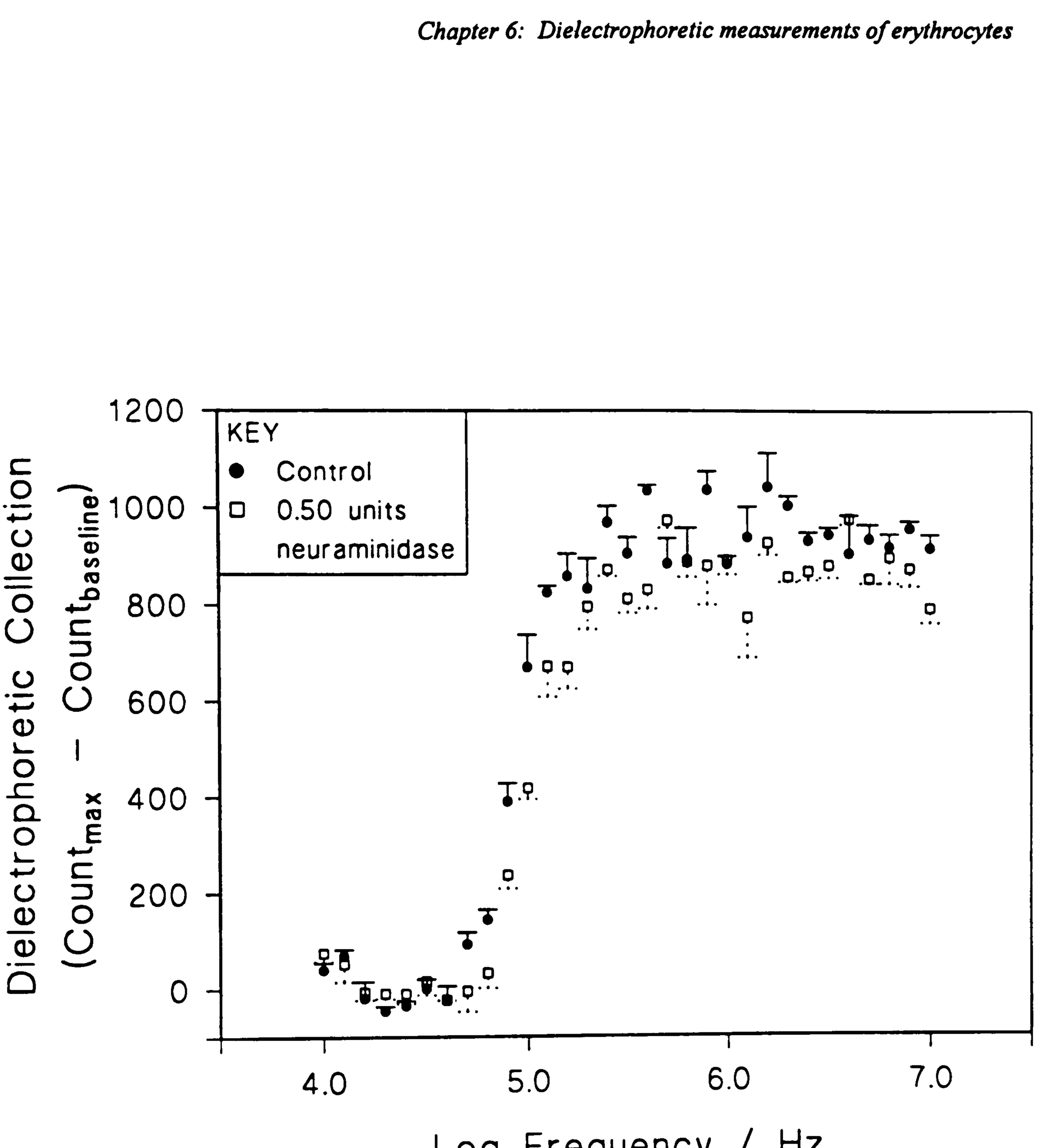


Figure 34: Effect of neuraminidase treatment upon dielectrophoretic collection spectra of human erythrocytes. (Pulse = 10 V for 10s, pH = 7.4 ± 0.1, σ = 100 ± 5 μS cm^{-1}, concentration = 7.2 ± 0.1 x 10^6. Data represents means ± error bars of SEMs from 3 replicates of 3 individual samples; half error bars only shown for clarity).

6.4.4 Effect of DIDS Treatment

The band 3 anion transporter of the erythrocyte membrane can be inhibited by the stilbene derivative DIDS (4,4-diisothiocyanato-stilben-2,2'-disulphonacid-Na-salt; Cabantchik *et al.*, 1978). The effect of preventing anion transport across the cell membrane upon dielectrophoretic spectra was examined. Erythrocytes were incubated with 0.02, 0.04 and 0.06 mM DIDS for 60 min at 37 °C following standard procedure.

DIDS treatment caused a concentration-dependent decrease in dielectrophoretic response although the onset of collection was exhibited at similar frequencies to that of the control (figure 35). At frequencies in excess of 1 MHz DIDS treatment promoted positive dielectrophoretic behaviour. It is postulated that inhibition of an ion-transport system (such as band 3) would allow continued polarisation of the membrane, affording extended dielectrophoretic collection.

Similar investigations to determine the effect of DIDS upon the dielectric properties of erythrocytes have focussed upon electro-rotation (section 1.1.6c) and can be correlated with dielectrophoretic behaviour (Wang *et al.*, 1992). The effect of DIDS upon erythrocyte electro-rotation has been described by Donath *et al.*, (1990) who found that the first characteristic frequency of electro-rotation was decreased by this treatment. They attribute this effect to the decrease in membrane conductivity (due to prevention of band 3 anion exchange) thus increasing membrane capacity. Georgiewa *et al.*, (1989) have indicated that DIDS treatment prevents the efflux of potassium ions through the anion channel which has been proposed to occur in low ionic strength media (Bernhardt *et al.*, 1991). It is postulated that a difference in first dielectrophoretic collection frequency due to DIDS treatment, as observed for electro-rotation data, is not apparent using the current experimental system as measurements are based upon cell populations and not individual cells. For electro-rotation measurements the shift towards lower frequencies associated with DIDS treatment may be concerned with a local decrease in suspension conductivity due to prevention of K^+ efflux. Such variation in conductivity is unlikely to be detected using the image-analysis system to measure gross dielectrophoretic response, thus the lowest field frequency causing collection is constant. Accordingly the effect of DIDS treatment in decreasing ion transport across the membrane and the associated increase in polarisation effects is hypothesised to account for the persistence of dielectrophoretic collection at high frequencies observed here.

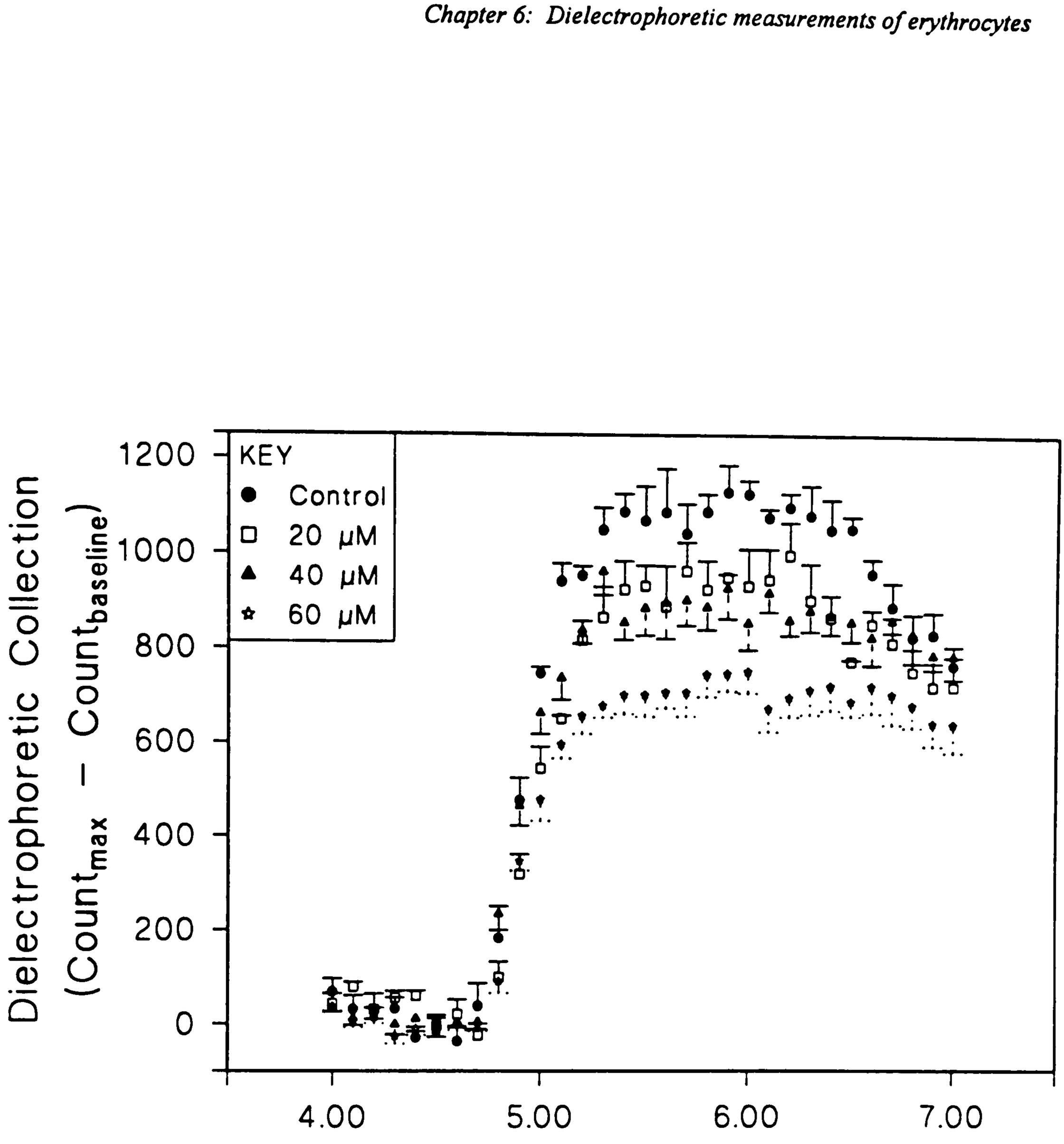


Figure 35: Effect of DIDS treatment upon dielectrophoretic collection spectra of human erythrocytes. (Pulse = 10 V for 10 s, pH = 7.4 ± 0.1, σ = 103 ± 8 μS cm^{-1}, concentration = 7.6 ± 0.1 x 10^6 cells per ml. Data represents means ± error bars of SEMs from 3 replicates of 3 individual samples; half error bars only shown for clarity).

6.4.5 Effect of A23187

The ionophore A23187 was used to alter ion transport across the cell membrane, thereby affecting permeability. In addition, disruption of electrochemical gradients across the membrane induces structural modifications resulting in the efflux of K^+ ions and water; this is known as the Gardos effect (Gardos, 1966). Potassium efflux via the Gardos channel is in addition to that caused by erythrocyte suspension in low ionic strength solution (Georgiewa *et al.*, 1989; Glaser, 1982). For these investigations, ionophore treatment is considered to increase membrane conductivity and decrease intracellular water, with subsequent effects upon cell volume, effective haemoglobin concentration, and cell deformability (Nikinmaa, 1990; Noji *et al.*, 1991).

Erythrocytes were incubated in HBSS containing concentrations of 0, 10, 20 and 40 μM A21387 (Sigma) at 37 °C for 1 hr, (recommended concentration for erythrocyte treatment was 10 μM; Beaven *et al.*, 1990). The frequency dependency of the dielectrophoretic response was investigated and shown in figure 36 (N.B. concentration of 40 μM only displayed).

Treatment with A23187 caused a decrease in dielectrophoretic collection in comparison to that observed using an untreated control sample (fig. 36); this effect was dependent upon A23187 concentration, and was observed for all concentrations investigated. It is possible that decrease in collection is concerned with a reduction in volume of the cell resulting from potassium efflux, as the dielectrophoretic force is directly proportional to particle volume (section 1.1). In addition, increased viscosity of the cell from intracellular dehydration as a result of ionophore treatment, may decrease polarisability over the frequency range investigated, also causing a reduction in the dielectrophoretic force experienced by the cell.

Figure 36 illustrates that ionophore treatment also affected the frequency range over which positive dielectrophoretic behaviour was observed. Onset of collection may be indicated to occur at slightly higher frequencies than for untreated cells, although all samples were maintained at similar conductivities (*c.f.* section 6.3.2). In addition, decline in collection occurred at lower frequencies for A23187-treated erythrocytes than for a control. This result supports the hypothesis that the high frequency dielectrophoretic response, as detected using this system, is indicative of membrane capacity, as discussed in section 6.4.4.

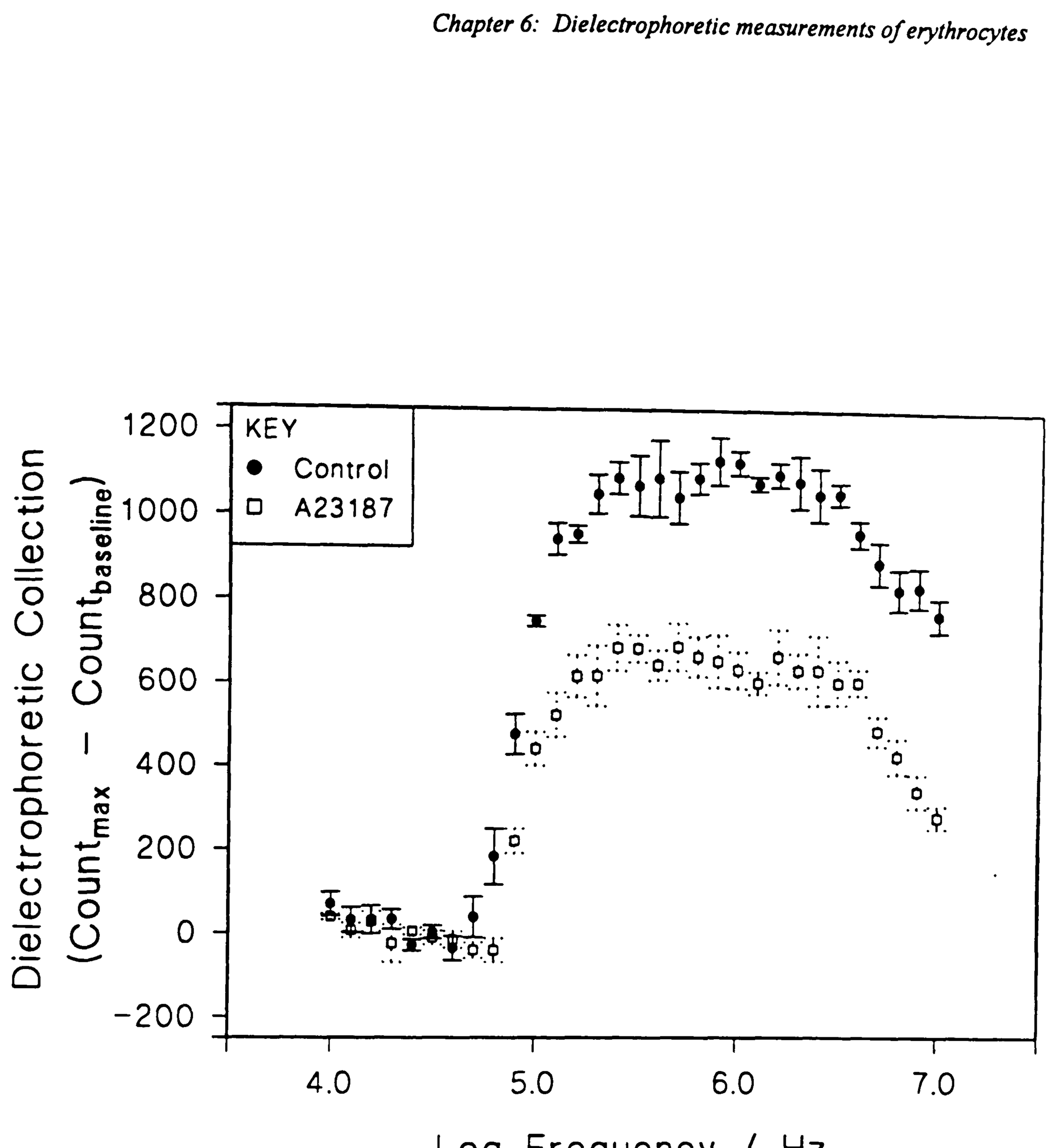


Figure 36: Effect of A23187 treatment upon dielectrophoretic collection spectra of human erythrocytes. (Pulse = 10 V for 10 s, pH = 7.4 ± 0.1, σ = 98 ± 5 μS cm^{-1}, concentration = 7.25 ± 0.1 x 10^6. Data represents means ± error bars of SEMs from 3 replicates of 3 individual samples).

6.5 SUMMARY

In previous studies on dielectrophoretic behaviour of haematological cells, various sugar-based suspensions have been employed to preserved cell integrity whilst allowing the response to a non-uniform electrical field to be measured, *i.e.* they exhibit negligible dispersions over the frequency range of investigation (Arnold *et al.*, 1993). These suspensions include, for example, GG solution (glycine/glucose, pH 6.5; Krishna *et al.*, 1989a, 1989b), D-mannitol/glucose (Kononenko & Kasimova, 1991; Kononenko *et al.*, 1991) and sucrose/glucose (Burt *et al.*, 1990). In this study a sorbitol-glucose (SG) solution was found to have little deleterious effect on either erythrocyte integrity or the measurement system. Some morphological changes were observed when employing SG (*e.g.* echinocyte formation) but these may possibly be attributed to the low ionic strength of the medium rather than its composition (Backman, 1986; Gass *et al.*, 1991). Such changes were typically reversed on transfer to a physiological medium. Osmotic potential of the suspending medium is critical to the detection method used here; swollen cells have a greater probability of being under-estimated as adjacent cells may be indistinguishable.

Washing and re-suspension procedures must be carried out with care to prevent cell agglutination in low ionic strength medium; polypropylene containers were used to prevent cell adherence to vessels. Use of a saline solution to wash cells was inappropriate as this adversely affects the conductivity of the test suspension. Rather, final washing must be performed in the experimental suspension.

Storage and anti-coagulant treatment of cells may also influence responses; compromises must be made between standard clinical procedures and those which enhance the dielectrophoretic response. Prolonged storage of blood samples (*i.e.* 35 days; Moore., 1981) without eradication of dielectrophoretic response may be accomplished using CPDA-1 solution as an anti-coagulant.

Despite initial problems with cell agglutination when EDTA was used as an anti-coagulant, it was possible to observe dielectrophoretic effects; this was critically dependent upon storage period and trauma experienced during phlebotomy procedures (as described in Powers, 1989). Experimental difficulties associated with EDTA-treated blood indicated the possible importance of divalent cation concentration in the cell boundary layer to the manifestation of a dielectrophoretic response. Moreover, as EDTA is used as a standard anti-coagulant in hospital haematological procedures and

has been shown here to permit dielectrophoretic collection, EDTA was the anti-coagulant used in further studies on leucocytes (Chapter Seven).

Key experimental parameters have been defined which allow system operation within saturating levels. It is clear that the mode of electric field application can produce qualitative and quantitative effects on erythrocyte dielectrophoretic collection. Both pulse length and magnitude can become limiting. As yet, the limiting factor at saturating voltage and constant pulse length is unknown. The threshold frequency for positive dielectrophoretic behaviour is independent of suspension concentration, but the latter may limit the extent of collection detected at a given frequency. Further recommendations for system development are discussed in Chapter Eight.

Chapter Seven

PRELIMINARY DIELECTROPHORETIC INVESTIGATIONS OF HUMAN LEUCOCYTES

Dielectrophoretic investigations of human leucocytes were carried out in association with the regional Haematological Malignancy Diagnostic Service (HMDS) based at Leeds General Infirmary. Experiments were conducted to assess the possible use of the image-analysis dielectrophoretic system to differentiate between leukaemic and normal white cell populations.

Preliminary studies were concerned with establishing whether this system could adequately detect and resolve the dielectrophoretic behaviour of leucocytes. Such investigations were facilitated by the use of clinical samples with elevated leucocyte levels. The effects of dielectrophoretic suspension medium and circulation through the experimental system upon leucocyte viability were investigated. The sensitivity of the system, in resolving differences between dielectrophoretic spectra exhibited by cells of varying lineage, phenotype (*i.e.* B and T lymphocytes), and normal versus malignant types was evaluated.

Investigations were restricted both by available samples and by the time period allowed for the study. Their purpose was to identify limitations of the current system and to make recommendations for a more vigorous research programme if initial assessments proved encouraging.

7.1 A BRIEF ACCOUNT OF HAEMATOLOGICAL MALIGNANCIES

In a normal healthy individual relative cell populations in the peripheral blood remain fairly constant, i.e. rate of haematopoesis is comparable with cell destruction. Total leucocyte numbers typically increase in response to infection; this amplification of cell production usually reverts to a normal level after the activity of the immune system has ceased. In certain pathological conditions the balance between blood cell production and destruction is disrupted due to uncontrolled cell proliferation. Such cancers of the blood system are known as leukaemias and are defined as; diseases of the blood-forming organs characterised by an increase of a cell type and its precursors in the blood, together with enlargement and proliferation of lymphoid tissue in the spleen (Becan-McBride & Ross, 1988).

There are many variants of leukaemia, each associated with the proliferation of one or more precursor cell types in the bone marrow, which may lead to abnormal levels of circulating leucocytes and/or the appearance of immature cell types in the blood. Leukaemia can emerge at almost any stage of blood cell development; the level of differentiation at which this occurs is a significant determinant of subsequent clinical behaviour (Lee *et al.*, 1993).

Fundamentally, leukaemias are classified as acute or chronic. The latter group is characterised by the appearance of increased numbers of mature leukocytes on peripheral blood smears (Turgeon, 1988).

Acute leukaemias are distinguished by the presence of blasts and immature leucocytes in the peripheral blood and bone marrow. Anaemia may also develop due to loss of blood and/or the replacement of normal marrow elements. Depending upon the cell lineage affected, acute leukaemias are subdivided as acute lymphoblastic leukaemias (ALL), or the corresponding non-lymphoblastic form (ANLL); these categories are the basis for further classification by the French-American-British (FAB) system (Greaves, 1991). Acute lymphoblastic leukaemia accounts for 90% of childhood leukaemias in the western world. In such cases total leucocyte count is typically elevated and the peripheral blood is usually composed of close to 100 % lymphoblasts, lymphocytes and smudge cells. Remissions and possible cures are more successful in children than in adults where the fatality rate is 70 %, with an average survival time of 3 months if untreated (Turgeon, 1988).

In many cases of leukaemia, treatment consists of correcting the levels of circulating cells and suppressing excessive production in the bone marrow. Leukapheresis is commonly used in the management of patients with extremely high concentrations of circulating immature cells (e.g. blast counts in excess of 100×10^9 per l) where damage to cerebral and pulmonary systems may be implicated (Lee *et al.*, 1993). This method exerts a temporary effect by rapidly reducing the number of circulating blasts.

Long-term treatment consists of preventing abnormal cell proliferation in the bone marrow, involving the destruction or removal of uncontrolled progenitor cells. Chemotherapy uses drugs to attack cells undergoing intensive division; the non-specificity of this mode of action is responsible for some of the extreme side-effects of this treatment. Increasingly, bone marrow transplantation offers the best prospects for recovery. The patient is subjected to radiation and/or chemotherapy at levels which

destroy the existing bone marrow, which is then replaced with that from a donor. The marrow is thus repopulated with non-malignant progenitor cells to restore normal haematopoeitic function (Greaves, 1991; Lee *et al.*, 1993). Success depends upon donor-host compatibility; transplants between siblings offer the greatest chance of success due to the similarity of histocompatability antigens of the individuals. Current interest is concerned with autologous bone marrow transplantation where the question of incompatibility does not arise. Using this method bone marrow is harvested from the patient and treated *in vitro* to remove malignant cells, the patient undergoes chemotherapy and/or radiation treatment to ablate the bone marrow *in vivo*, then is re-infused with the modified marrow. This process circumvents the problems associated with rejection, and is the subject of a recent review (Worthington-White *et al.*, 1992). A similar process still under development involves the purification of haematopoietic stem cells which are found at low concentrations in the peripheral blood. Patient treatment with non-malignant autologous stem cells after marrow ablation had been reported to promote long-term disease-free survival, in some cases where bone marrow transplantation is not appropriate (Zander & Cockerill, 1987; Worthington-White *et al.*, 1992). Other potential methods of treatment including immunotherapy, whereby autologous transplantation of particular leucocyte populations are undertaken to control or eliminate existing neoplasias, have been described (Stevenson *et al.*, 1991). In such procedures there is a requirement for a high degree of purification before transplantation can occur.

The transformation of a normal cell to a malignant state exhibiting uncontrolled proliferation is associated with structural changes which affect the dielectric properties (section 1.2.2). Upon this basis, dielectrophoresis may be used to detect metastatic cells.

It is postulated that dielectrophoresis may find application in the diagnosis of certain haematological disorders, or as a means of cell separation aiding leukaemia treatment. The former condition may involve rapid detection of abnormal individual cells or population levels. This is not restricted to investigations of leucocytes alone, as erythrocytes from patients with leukocyte disorders such as chronic myelogenous leukaemia (CML) have been found to display abnormal cytoskeleton structure in comparison with normal erythrocytes (Kundu *et al.*, 1991). As a separation technique, dielectrophoresis may prove to be a superior method of selecting normal progenitor cells from a marrow aspirate, thus aiding autologous transplantations. In addition, it may also promote the development of other clinical treatments, such as immunotherapy.

7.2 PREPARATION OF WHITE BLOOD CELL SAMPLES

Blood samples and marrow aspirates were obtained approximately six hours after collection from patients. Investigations were carried out on samples surplus to HMDS requirements, thus experimental sample volumes were unpredictable, limiting the scope and reliability of the measurements. In addition, the blood disorder, donor sex and age were variables throughout the study period.

Samples for dielectrophoretic investigations were obtained as either EDTA-treated whole blood samples, or as purified white cell suspensions (preparation methods detailed in Appendix IV). Prior to use, each sample had been analysed by HMDS personnel to identify haematological disorder and relative cell populations. Unless stated otherwise, all samples used in this study had an original blood white cell count in excess of 20×10^9 per litre. Such an elevated level is indicative of a haematological proliferative disorder such as leukaemia. Where possible, specimens exhibiting little heterogeneity were selected to minimise measurements of mixed cell populations. Such conditions were unattainable for marrow samples which consist of highly heterogeneous cell populations at various stages of maturity.

The standard method of experimental sample preparation was adapted to compromise between existing HMDS procedure, requirements for preservation of cell viability, and conditions necessary to generate a dielectrophoretic response. The volume of the sample (*i.e.* gross number of cells) available for experimentation was often a limiting factor.

For these investigations clinical cell suspensions were prepared to a standard concentration and volume. A value of 1×10^6 cells per ml was selected for sample concentration to minimise the number of cells required for dielectrophoretic measurements whilst ensuring that possible cell collection was still detectable. Sample volume was fixed at 10 ml to reduce variance as a result of dissimilarities in the number of times an individual cell was circulated through the system or exposed to the electric field.

Purified white cell populations were obtained from whole blood by sedimentation with an erythrocyte aggregating agent and/or density gradient centrifugation (respectively using Plasmagel, Laboratoire Roger Bellon, Neuilly-sur-Seine, France and Lymphoprep, Nycomed, Oslo, Norway; for protocols see Appendix IV). If required, erythrocyte lysis by ammonium chloride was also employed (Appendix IV). After

separation the cells were washed and re-suspended in phosphate buffered saline, pH 7.4 (PBS). Cell concentration was determined by an electrical impedance method using a Sysmex K-1000 fully-automated haematology analyser (TOA Medical Electronics Co Ltd, Kobe, Japan). Relative cell composition and any abnormalities were noted at this stage.

Difficulties concerned with the measurement of experimental suspension cell concentration, and with the constraints of clinical sample availability, dictated that the following method of suspension preparation was employed. For these investigations, the standard experimental suspension was defined as a 10 ml volume with a concentration of 1 x 10^6 cells per ml, *i.e.* 1 x 10^7 cells in total. The volume of clinical sample in PBS to contain this number of cells was calculated, and the aliquot, typically in the region of 500 µl, transferred to a micro-centrifuge tube. Cells were then separated from their suspending solution by centrifugation at low speed (6500 rpm for 30 s). The supernatant was discarded then the cell pellet re-suspended in 10 ml of sorbitol-glucose (SG) solution, to give a final cell count of 1 x 10^6 cells per ml. If the conductivity of the cell suspension was too high, (*i.e.* greater than 300 µS cm^{-1}), then another centrifugation step was employed and the cells re-suspended in fresh SG solution. Conductivity was increased, if required, by adding a few drops of 1 mM KCl solution. Cell concentration was verified using the haematology analyser (measured cell concentrations ranged from 0.95-1.05 x 10^6 per ml) and the experimental suspension used directly after preparation.

This procedure permitted the rapid preparation of experimental suspensions at a standard concentration. Manipulations made after cells were re-suspended in SG were kept to a minimum; this was especially advantageous for use with leucocyte samples, which were susceptible to morphological change when suspended in the dielectrophoretic experimental medium.

7.3 EFFECTS OF SUSPENDING SOLUTION AND EXPERIMENTAL SYSTEM UPON LEUCOCYTE VIABILITY AND MORPHOLOGY

7.3.1 Effect Of Suspension Medium

As a preliminary to dielectrophoretic investigations, the effects of proposed cell suspension solutions and the experimental apparatus upon leucocyte viability, concentration and morphology were monitored.

Density gradient centrifugation was used to separate B lymphocytes from a peripheral blood sample donated by a patient with a chronic lymphocytic leukaemia of the cellular immune system (B-CLL). The viability of B lymphocytes in 10 ml of Sorbitol-Glucose (SG) or Phosphate Buffered Saline (PBS) solution, (suspension pH 7.4; conductivity 150 μS cm^{-1} and 1400 mS cm^{-1} respectively), was examined using three parameters;

1) Cell density, enumerated by a Sysmex K-1000 haematological analyser.
2) Cell integrity, assessed by fluorescence measurement of propidium iodide uptake (Appendix IV).
3) Cell morphology, examined after May-Grünswald and Wright's staining to allow visualisation of membranes and intracellular structures.

Measurements were taken over a 35 min time period following suspension in the test medium, representing the minimum length required to produce three replica dielectrophoretic spectra using standard experimental variables (*i.e.* response to a 5 s, 10 V pulse over the frequency range 1 kHz - 10 MHz, at intervals of 5 measurements per frequency decade). Samples were maintained at room temperature. Cell density and integrity were measured at 3 min intervals, sacrificing 100 μl aliquots; morphology was examined at zero and 30 min. In accordance with potential applications for cell separation by dielectrophoresis, attempts were made to recover cells from the experimental medium and restore to apparent normal physiological status in a saline solution.

Similar investigations were carried out using suspensions of neutrophils (isolated from the peripheral blood of a patient with a clonal expansion of the neutrophil lineage). These were examined with respect to cell density and morphology over a 35 min time period, as previously.

Initial experiments using lymphocytes indicated that re-suspension in SG solution had little effect upon cell concentration over the time period investigated. In contrast, re-suspension in PBS resulted in an approximate three-fold decline in concentration relative to SG (data not shown). It is possible that cells at low concentration in PBS tend to aggregate, thus excluding them from enumeration by the automatic cell counter. This was supported by qualitative visual examinations of sample preparations. In addition, cells suspended in SG for 30 min exhibited a greater ratio of intact to "smeared" cells (145:16 cells per microscope field for SG, 19:58 for PBS; result from a single determination with erythrocyte contamination present at 6 cells per field).

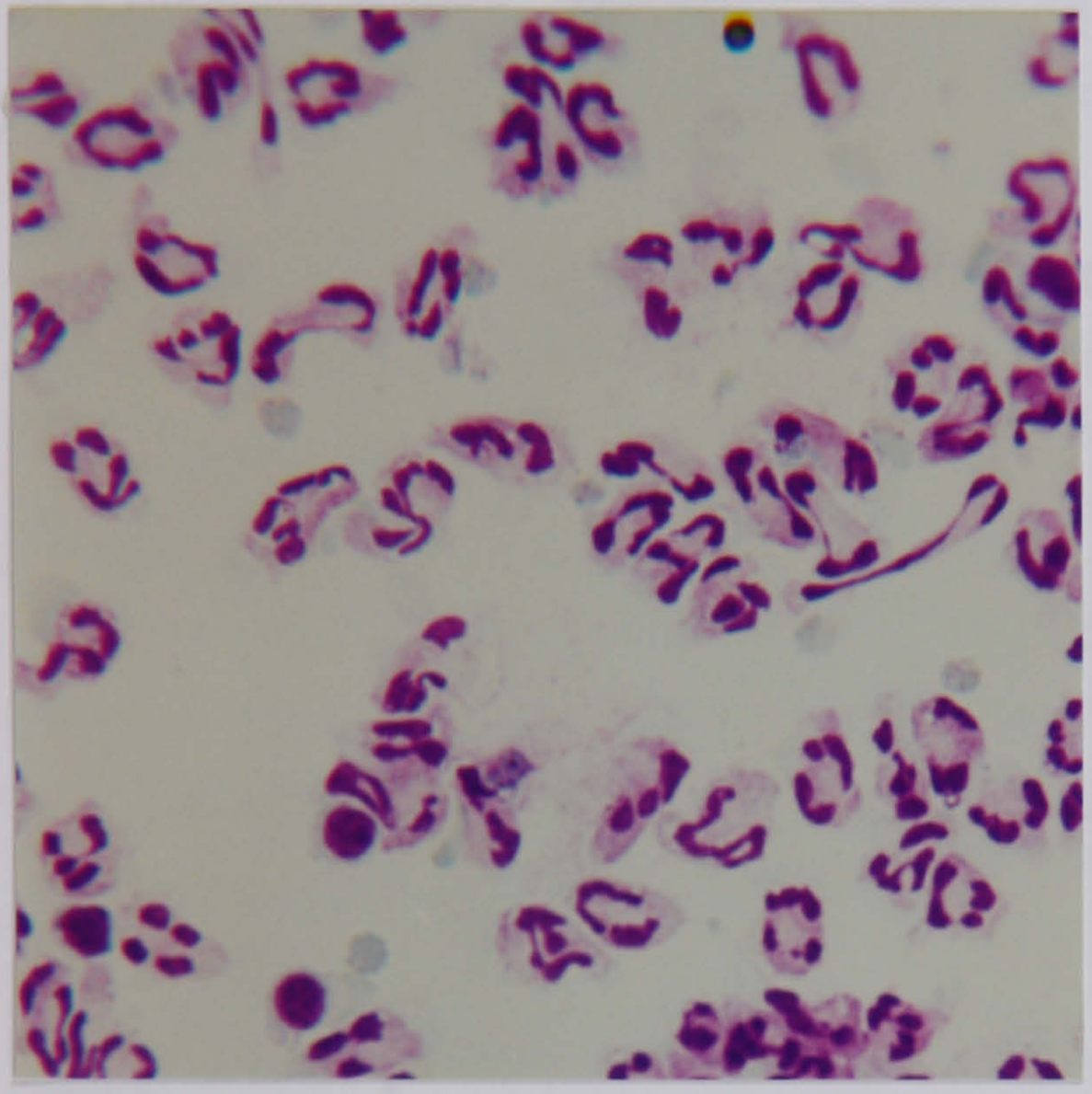
Lymphocyte viability declined by only 5 % over the experimental time period for both test solutions. This indicated that investigations could be carried out using low ionic strength medium without incurring severe apparent damage. Examination of stained cells supported this conclusion.

Neutrophil suspensions prepared in SG and PBS showed little variance in cell concentration over the investigation period, in contrast to lymphocyte suspensions. However, examination of smears prepared at time t = 0 and t = 35 min after re-suspension revealed changes in cellular morphology (fig. 37). At time zero, preparations of both samples exhibited characteristic lobed nuclei. However, after 35 min, smears of cells suspended in SG displayed swollen nuclei, giving a monocytic appearance. The morphology of cells suspended in PBS revealed a similar trend although not as pronounced; nuclei were dilated and appeared less condensed whilst retaining a polymorphonuclear appearance.

Attempts to recover cells by centrifugation followed by re-suspension in fresh medium repeatedly resulted in cell disruption and aggregation. However, this could be overcome by addition of 1% (v/v) foetal calf serum prior to centrifugation.

Initial experiments suggest that the SG solution developed for investigation of erythrocytes did not cause any apparent gross impairment of leucocyte integrity over the time period examined. Moreover, cells could be recovered from the low ionic strength suspension. Although the long-term effects upon the cells was considered beyond the scope of this investigation, re-suspension in SG was judged to be suitable for the purposes of this dielectrophoretic study. In accordance, measurement of cell concentration was deemed to provide a fair indication of cell damage.

a



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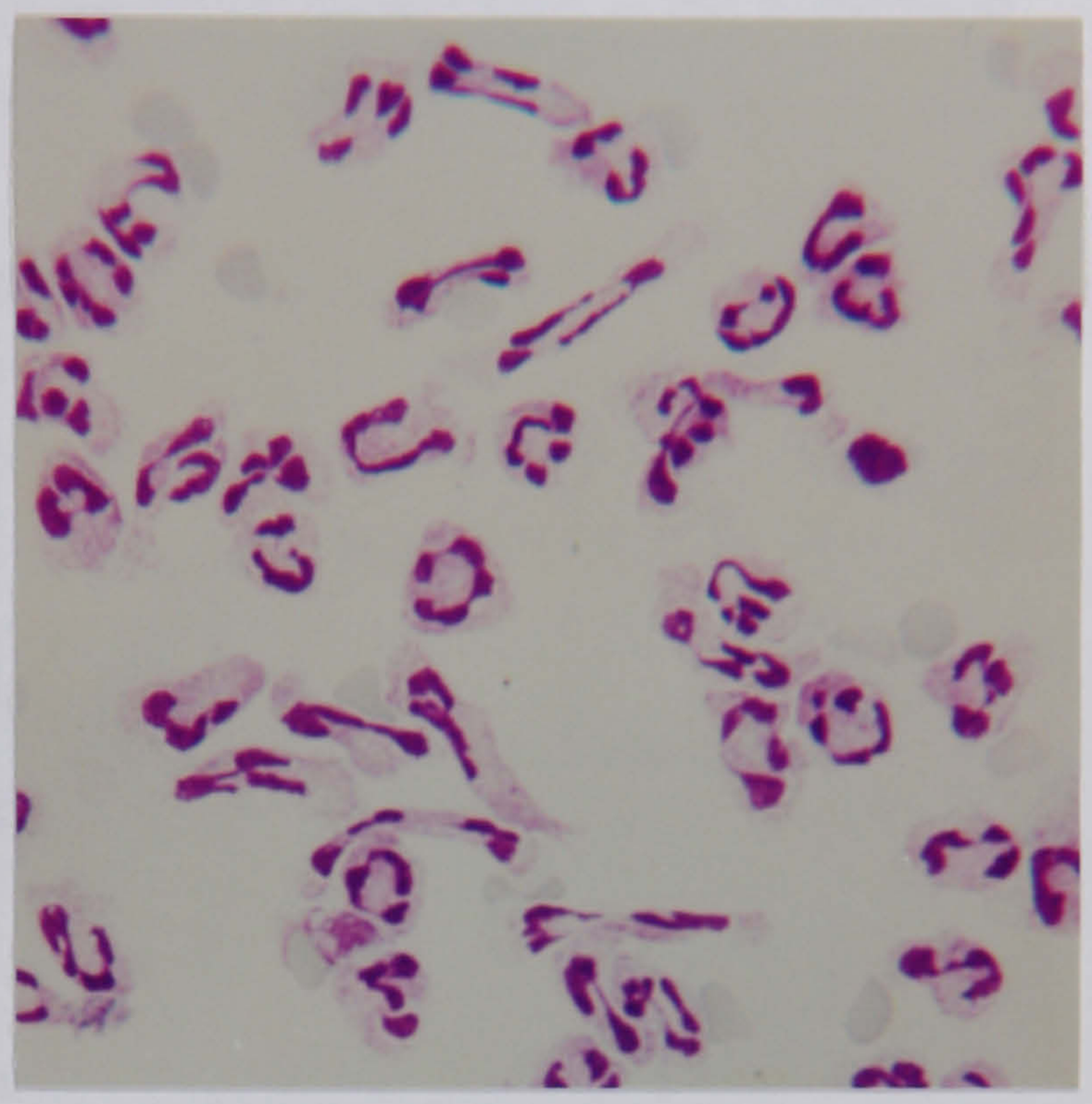

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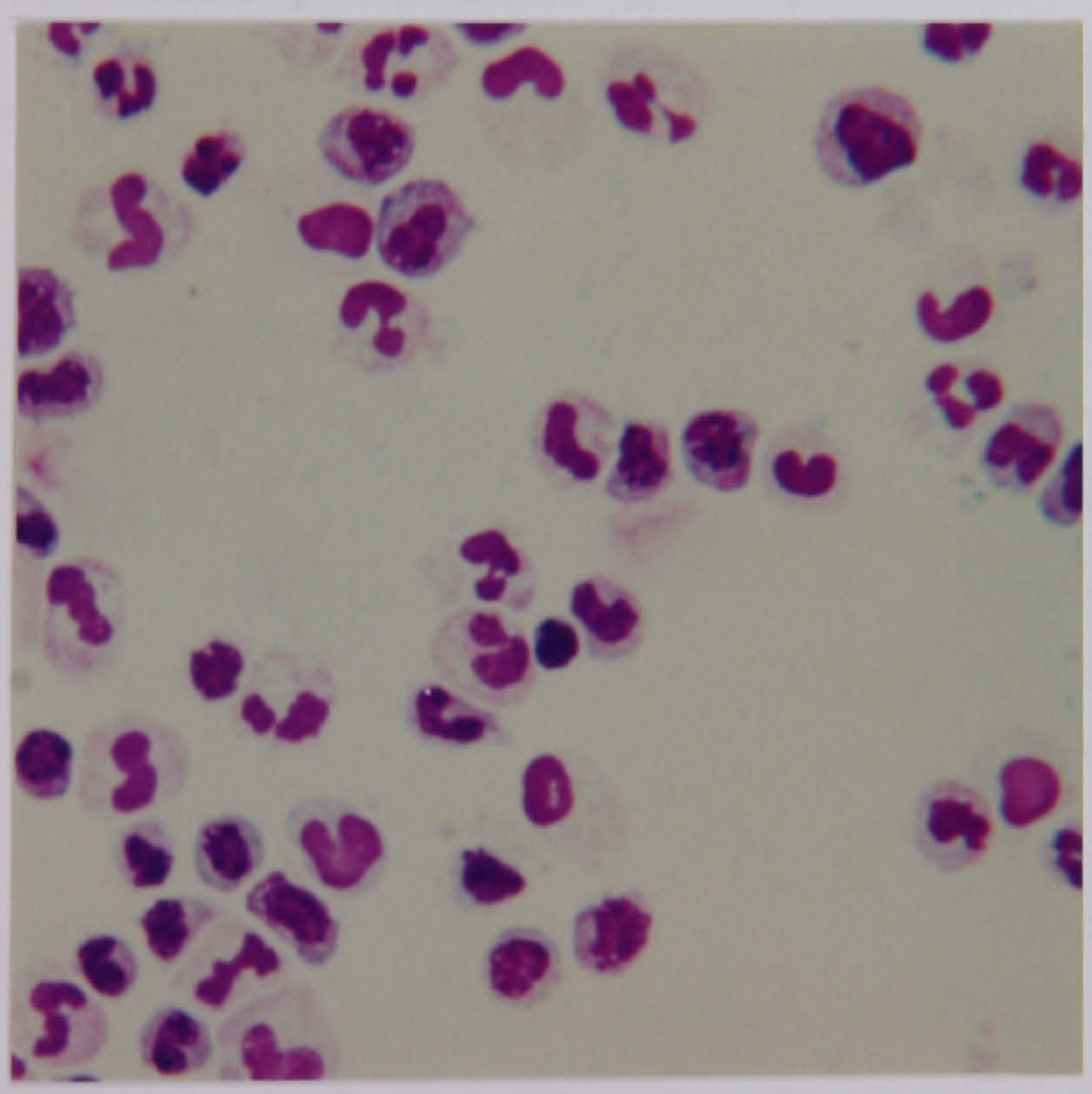

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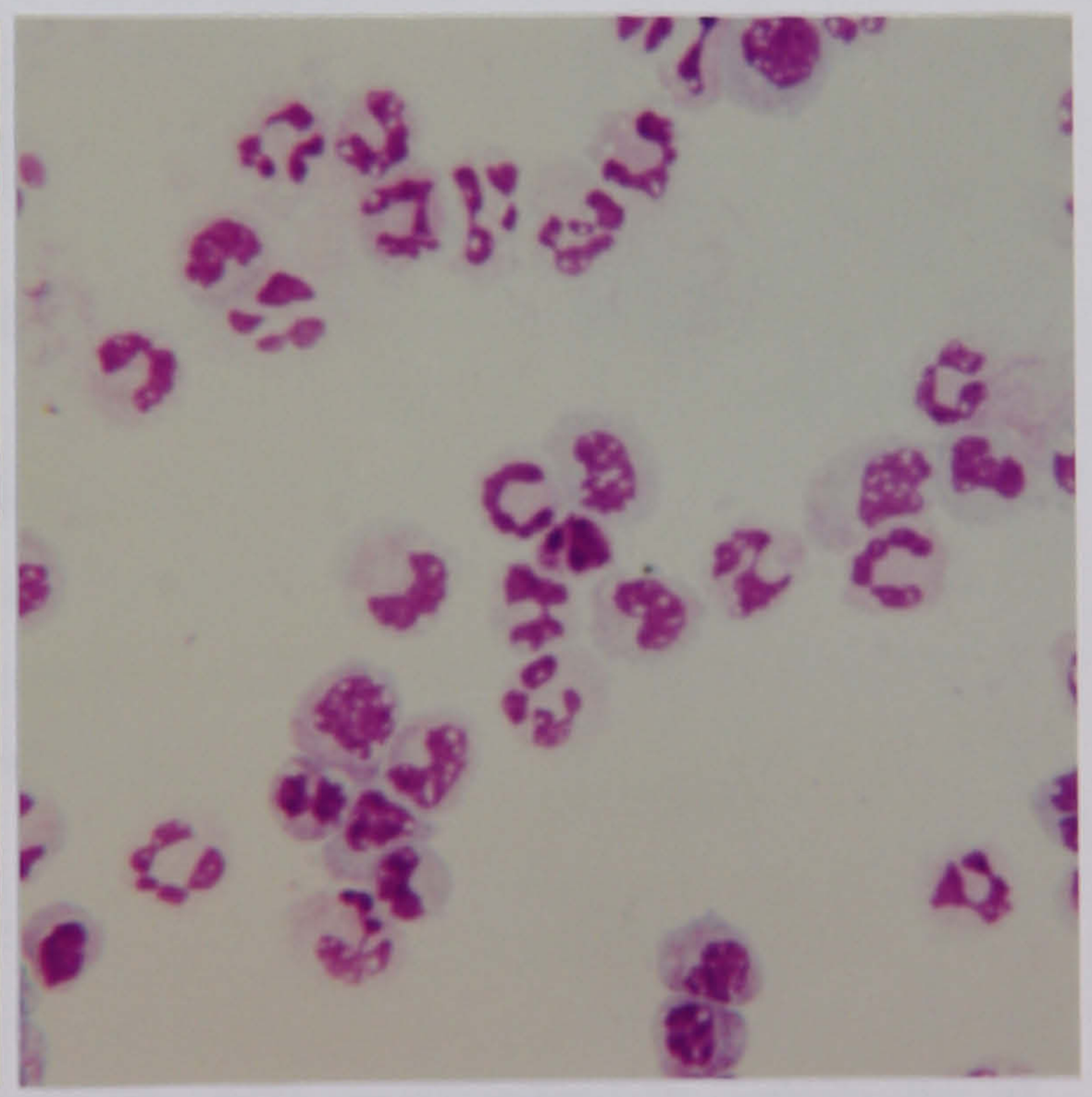

Figure 37: Photographs to indicate the effect of suspension medium upon neutrophil morphology; a) Sorbitol-glucose solution, t = 0 min, b) Phosphate buffered saline solution, t = 0, c) Sorbitol-glucose solution, t = 35 min, d) Phosphate buffered saline solution, t = 35 min.

7.3.2 Effect Of The Experimental System

The mechanical effect of the experimental system upon the cells was evaluated by circulating similarly prepared cell suspensions through the system at different flow rates. Impairment of cell integrity was assessed by measurement of cell concentration as a function of time. The effect of applying a repetitive electric field to the suspension at a frequency likely to cause dielectrophoretic collection was also investigated.

Suspensions of lymphocytes, separated from a B-CLL patient blood sample, were prepared in SG as required and transferred to the reservoir of the experimental system. The suspension was circulated through the electrode chamber and the rest of the system by the peristaltic pump. At five minute intervals a small volume of suspension, typically 500 µl, was removed from the reservoir and the cell count determined using the Sysmex K-1000 haematology analyser. The effect of the rate of flow upon different cell suspensions was investigated. The effect of time upon cell count was also determined for a non-circulated sample.

Experimental parameters were set to define a 5 s baseline period, followed by the application of a 10 V pulse at 1 MHz, a peak time of 5 s and a clearing step of 5 s at maximum pump speed (4.5 ml min^{-1}), before the sequence was repeated. Cell counts were determined at five minute intervals as before. Pulse times of 5 s and 20 s were investigated at a typical experimental flow rate of 1.5 ml min^{-1}.

Data obtained from this investigation using the Sysmex haematology analyser exhibited little variation over the 35 min time period. For each of six flow conditions ranging from 0.1 - 4.5 ml min^{-1}, suspension cell density reading was 0.6 - 0.7 x 10^6 cells per ml at time 0; the range for final readings was 0.6 - 0.8 x 10^6 cells per ml. Analysis of the six data sets individually revealed that in each case, cell concentration was scattered within ± 0.3 x 10^6 cells per ml from the initial value.

Attempts to assess possible damage by the experimental system resulting in a reduced concentration of cells were restricted by the operational parameters of the cell-counting device. Default settings for the Sysmex analyser employed limited its sensitivity of enumeration to units of 0.1 x 10^6 cells per ml. At this level of accuracy only major detrimental effects upon suspension density would be detected.

From these investigations the mechanical effect of the pump upon a circulating cell suspension was judged to have a minimal effect upon cell concentration over the experimental time period. Cell concentration also appeared unaffected by pulse application over a similar time period. Cytological examination revealed no apparent gross changes in cell morphology as a result of sample circulation or pulse application.

7.4 INVESTIGATIONS TO ASSESS WHETHER THE DIELECTROPHORETIC IMAGE ANALYSIS SYSTEM MAY BE USED FOR LEUCOCYTE SEPARATIONS

Experiments were hampered by the quantity and viability status of samples received after HMDS analysis. In many cases the leucocyte population obtained was too small for a sample to be prepared for dielectrophoretic investigation. In addition, if the sample was received as an already purified leucocyte population suspended in PBS then the cell viability tended to be impaired as a result of the separation treatments and of the time elapsed since preparation. Many samples could not be re-suspended in the low ionic strength SG solution and exhibited cell aggregation. Re-suspension was attempted by transferring the cells gently through a series of decreasing ionic strength solutions but similar problems were encountered.

The difficulties associated with the detection of cells after dielectrophoretic collection were heightened by the operation of the system at a lower cell concentration and by the use of cell populations of dissimilar size and morphology. As discussed before (Chapter Five) it was preferable to compare spectra measured during the same experimental period to minimise changes associated with electrode and chamber decay, and variation in resolution of detection. For the purposes of this study, dissimilarities arising from the use of a panel of donors were considered to be negligible in dielectrophoretic terms, in comparison with general differences between haematological cell populations, independent of donor.

7.4.1 The Dielectrophoretic Response Of Different Cell Lineages

Investigations were carried out using lymphocyte and neutrophil suspensions, each prepared from peripheral blood samples of B-CLL and neutrophillia patients respectively (see Appendix IV). Dielectrophoretic responses were compared with that of erythrocytes from a normal donor, under the same experimental conditions.

Suspensions of purified cell populations were prepared in SG as required and conductivity corrected to 120 μS cm^{-1}. The dielectrophoretic response of each sample was investigated over a frequency range of 1 kHz - 10 MHz using a 10 V pulse applied for 10 s. For each spectrum, the frequency at which cells first exhibited attraction towards the electrodes was noted.

Visual observations of onset of collection upon the electrodes varied according to the cell suspension investigated. The mode frequencies of first cell collection for each sample, averaged from six replica runs, were 39.1 kHz for neutrophils, 63 kHz for erythrocytes and 100 kHz for lymphocytes. Figure 38 shows smoothed dielectrophoretic collection values against field frequency from initial runs (data points not shown for clarity). Positive dielectrophoretic collection was detected at lower frequencies for neutrophil suspensions than erythrocytes, which in turn was lower than that of lymphocytes.

Observation of the "secondary" monitor, communicating the events occurring at the electrode surface, in conjunction with the trace display on the "primary" monitor, indicating relative cell enumeration by the analysis package, revealed a discrepancy in detecting the onset of collection. Determination of first collection by the experimental system was typically at higher frequencies than that detected visually.

Spectra obtained from this investigation (fig. 38) suggested that neutrophils were susceptible to dielectrophoretic collection at lower frequencies than lymphocytes or erythrocytes under the same experimental conditions. This was supported by the visual observation of collection upon the electrodes.

Replica runs of the same samples exhibited the same trend in onset of dielectrophoretic collection. However, it was noticeable that collection values for the neutrophil sample decayed dramatically with time. This was thought to be associated with the morphological changes described previously (section 7.3.1, fig. 37). It was therefore decided that spectra obtained from the first run of each sample in addition to the visual observations were sufficient to suggest that there were exploitable differences in dielectrophoretic collection of the cell populations investigated. Attempts to justify this statement statistically were hampered by a lack of suitable samples available within the study period.

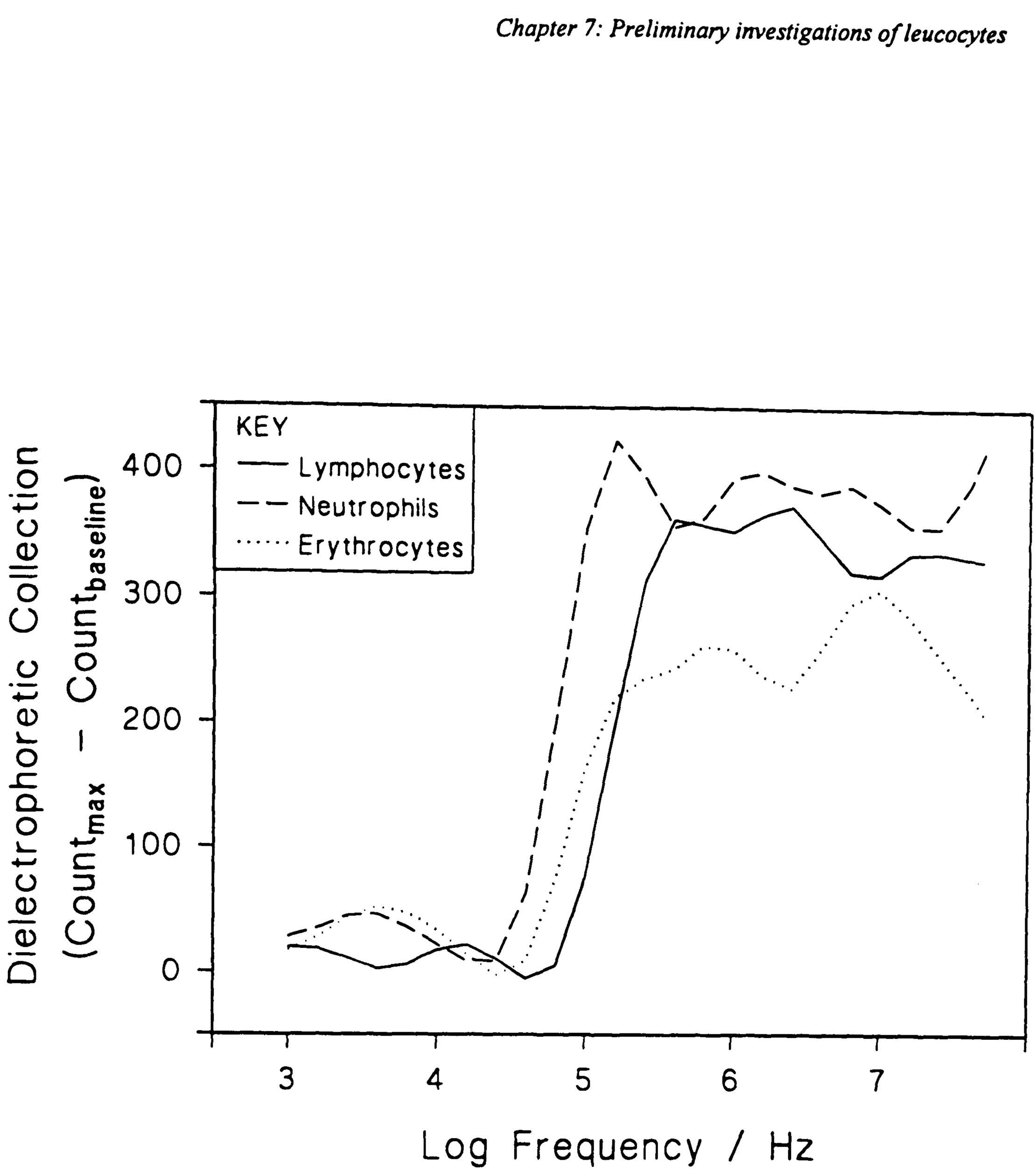

Figure 38: Dielectrophoretic collection of various haematological cell types as described in figure insert. (Pulse = 10 V for 10s, pH = 7.4 ± 0.1, σ = 120 $\mu S\ cm^{-1}$, concentration 1.0 ± 0.1 x 10^6 cells per ml. Data represents first run only for each sample).

7.4.2 Investigation To Differentiate Between High And Low Electrophoretic Mobility Cells Of The Same Population Using A Dielectrophoretic Technique

The resolution of the system was examined by investigating the dielectrophoretic responses of T and B lymphocyte populations isolated from two blood samples exhibiting excessive T or B cell proliferations.

In each case, experimental samples consisted of over 95 % of the relative lymphocyte sub-set, as determined by FACS analysis (Appendix IV). Cell viability was assessed by fluorescence measurement of Propidium Iodide uptake and was established to be greater than 95 % at the beginning of the investigation and remained over 90 % after experimentation.

Cell suspensions were prepared in SG as described in the standard protocol and dielectrophoretic collection investigated as a function of frequency. Mean collection values and SEMs were calculated from three replicates for each sample.

Dielectrophoretic collection spectra displayed dissimilarities in first collection frequency and extent of collection between the T and B cell suspensions investigated (fig. 39). For the three replica B cell suspensions both these parameters were reduced with respect to the T cell suspensions prepared from a different donor.

Spectra obtained from the two samples used in the study suggested that the suspension of predominantly B lymphocytes exhibited dielectrophoretic collection at a lower field frequency than the suspension of T lymphocytes. The T lymphocytes however, demonstrated a much more dramatic onset of collection, as indicated by the steeper gradient, and attained higher collection values than those observed for the B lymphocytes. As both investigations were carried out under the same experimental conditions (*i.e.* at the same conductivity) this difference was deemed a consequence of the bio-electrical character of the two cell types, and not to be an artifact of the system. However the possible effect of dissimilarities between the two donors has not been addressed.

If the *a priori* hypothesis holds true - that the differences between B and T lymphocyte populations are greater than differences between donors - then the investigation suggests that T and B lymphocytes are potentially separable by a dielectrophoretic method. This theory is supported by electrophoretic mobility data; measurements

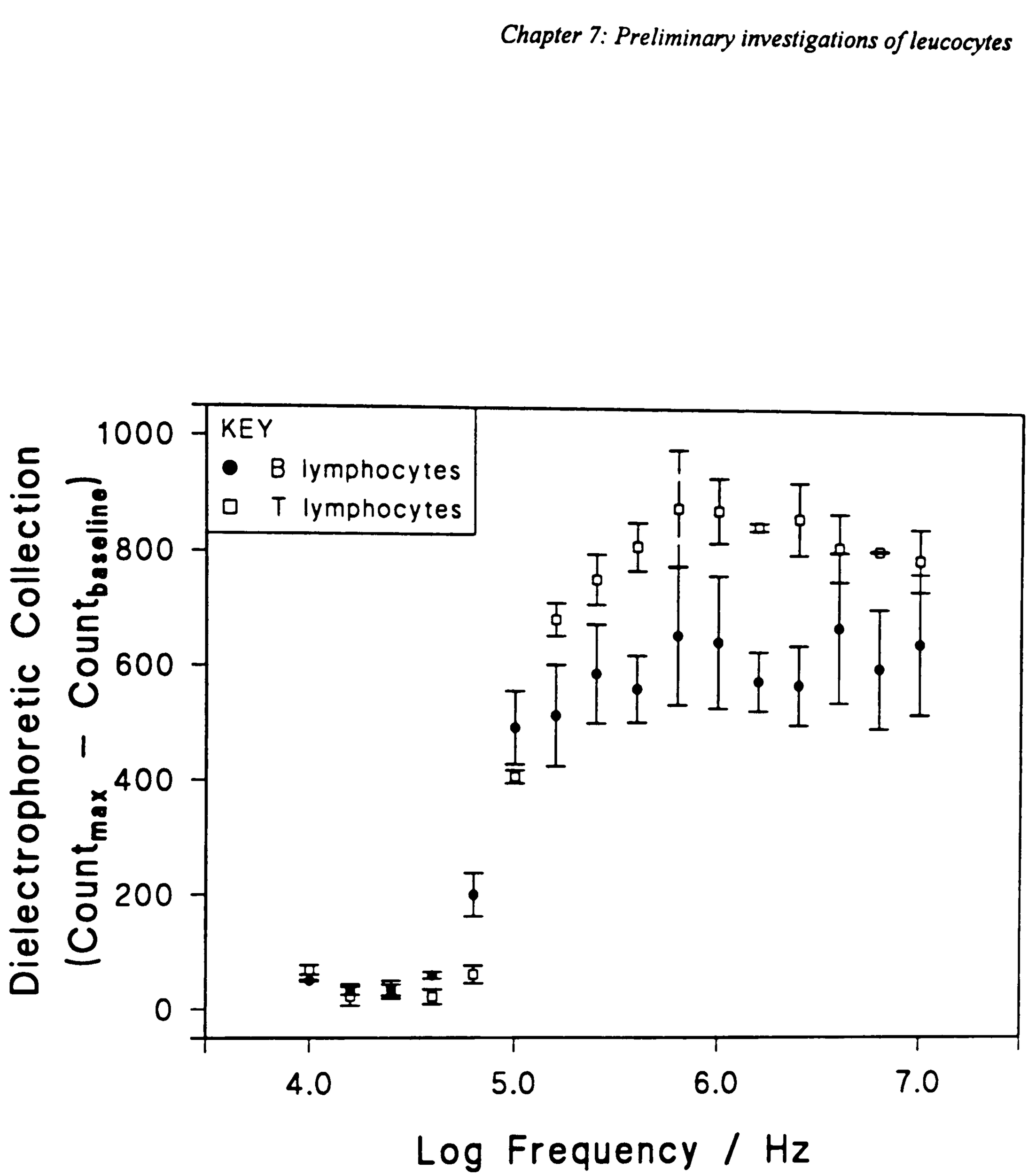

Figure 39: Dielectrophoretic collection spectra of B and T lymphocyte populations. (Pulse = 10 V for 10 s, pH = 7.4 ± 0.1, σ = 80 μS cm^{-1}, concentration = 1.1 ± 0.1 x 10^6 cells per ml. Data shown of mean ± SEMs from 3 replicates).

describing the movement of T lymphocytes in an electric field are greater than the corresponding values for B lymphocytes (Sherbet, 1978).

This suggests that the T lymphocytes will exhibit a higher degree of polarisation with the applied dielectrophoretic field, thus allowing the pearl chain length supported by the field to be longer and result in a greater collection of cells.

Although this investigation of the two different cell populations is preliminary as it involves comparing cells arising from the clonal expansion of one (or a small number) of precursor phenotypes, it does suggest that further investigations are justified. If a difference in dielectrophoretic collection could be detected between normal and cancerous lymphocytes of the same individual then the benefits of potential applications for diagnosis and treatment are considerable.

7.4.3 Manipulations And Dielectrophoretic Investigations Of Bone Marrow Aspirates Using The Image Analysis System

Bone marrow aspirates consist of a mixture of all immature cell types whose properties differ from those of circulating fully differentiated cells (section 1.4.3). For example, reticulocytes carry a smaller negative charge in comparison to mature erythrocytes, as described in section 1.4.5. It has been postulated that bone marrow cells in general have a decreased surface charge associated with them which allows close packing within the bone interior. Such a property might impede re-suspension of the cells in a low ionic strength solution in accordance with the standard protocol adopted for these investigations.

Fresh heparinised marrow aspirates were obtained from a CML donor. Cells were washed in PBS and suspended in SG as described in the standard protocol. The dielectrophoretic response of the sample was investigated and compared with that of lymphocytes separated from peripheral blood of the same donor.

Comparison of dielectrophoretic spectra of bone marrow cells and peripheral blood B lymphocytes indicates a marked difference between frequency of first collection, and in the extent of collection (fig. 40)

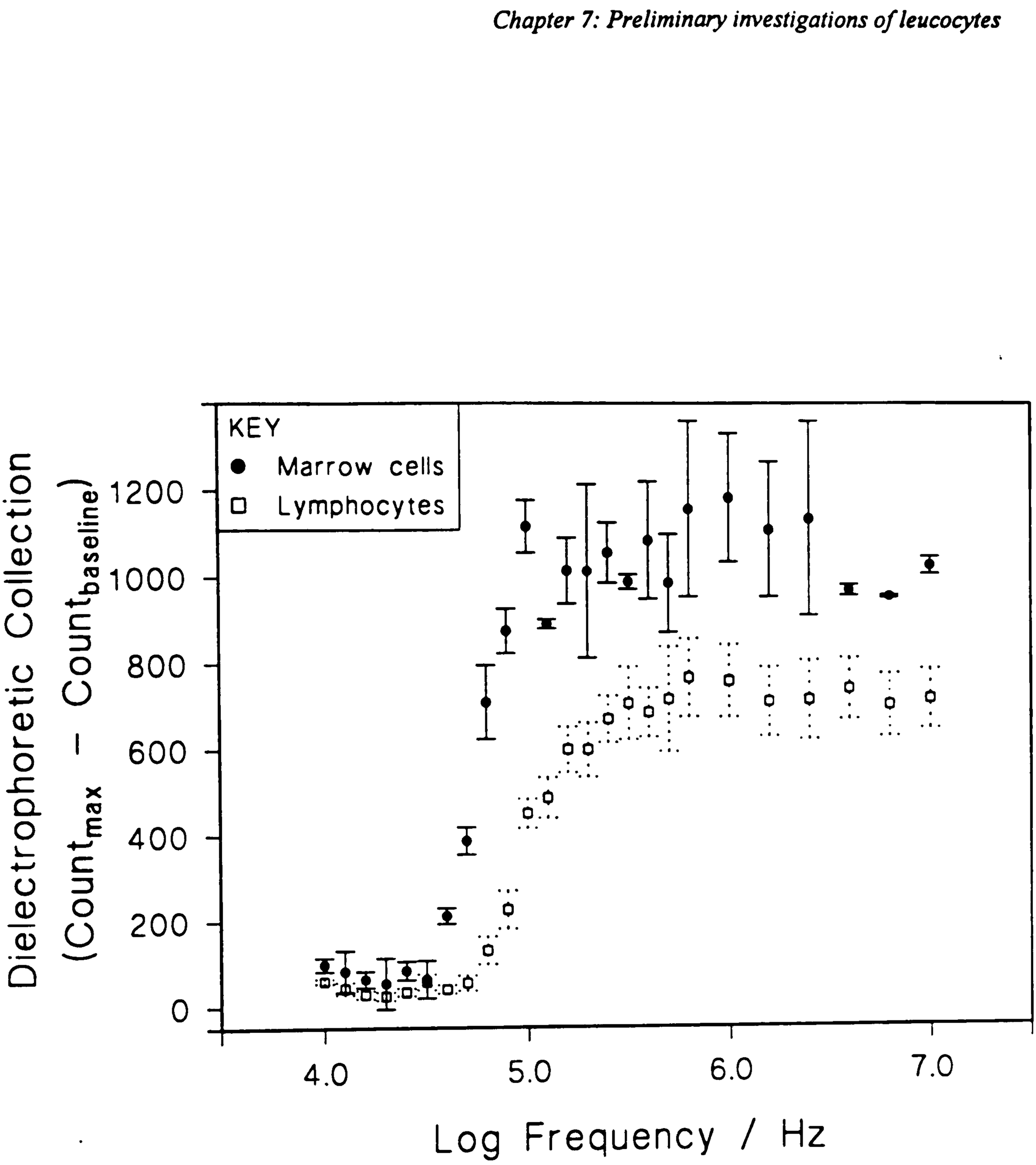

Figure 40: Dielectrophoretic collection of bone marrow cells and peripheral blood lymphocytes. (Pulse = 10 V for 10 s, pH = 7.4 ± 0.1, σ = 80 μS cm^{-1}, concentration = 1.1 ± 0.1 x 10^6 cells per ml. Data shown of mean SEMs from 3 replicates).

Cells harvested from bone marrow were amenable to re-suspension in low ionic strength solution following the protocol as described in section 7.2. Spectra of dielectrophoretic response were obtained where the onset of collection occurred at a lower frequency than that observed using a sample of lymphocytes, prepared from peripheral blood of the same donor, under the same experimental conditions. This difference may reflect the size of the cells under investigation; bone marrow cells tend to be larger than circulating lymphocytes. The spectra also suggest that more bone marrow cells were affected by the non-uniform field than lymphocytes, resulting in higher collection values being observed. As both suspensions were at the same cell concentration and none of the experimental variables had been altered the increased collection values were deemed to be a valid phenomenon and not an artifact. It is suggested that the onset of collection is seen at a lower frequency than for the lymphocytes due to the larger size of the marrow cells (Turgeon, 1988).

7.4.4 Separation Of Cell Populations Using The Present Electrode System.

The improved detection afforded by the image analysis system as a result of slightly de-focussing the image (see section 5.3) proved problematic in this particular study. In such situations although cells undergoing dielectrophoretic collection may be viewed on the monitor, it is very difficult to recognise cells from different haematological populations and thus to identify whether different populations are collecting at different frequencies. In association with this attempts were made to operate the electrode array as a separating device; pulses were applied for an extended time period to cause a substantial amount of dielectrophoretic collection, then the collected cells sampled upon their release from the electrodes.

A fresh suspension of lymphocytes was prepared in SG and introduced into the system reservoir. The experimental apparatus was re-arranged to locate the peristaltic pump between the reservoir and electrode chamber, allowing suspension leaving the chamber to be sampled. A maximum pulse of 14 V at 1 MHz was applied for 10 s resulting in dielectrophoretic collection, as observed microscopically. Investigations were carried out using pulse applications of up to 10 min and collecting sample discharge from the electrode system into the chambers of a 96 well microtitre plate. The plates were then analysed by a Multiskan Plus absorbance analyser.

A variety of time intervals were applied, both for pulse length and for suspension collection into each individual well. Aliquots were collected continuously throughout

the experiment, both prior to, during and after pulse application in an attempt to detect discrete differences in cell concentration of the suspension.

Results obtained from analysis of the multi-well plates did not indicate any differences in the absorbance, and therefore the concentration, of the suspension at any stage. This was attributed to several possibilities:

- The maximum volume of cells collected by an extended pulse application is still negligible in contrast with the cell concentration of the bulk suspension;
- Cell collection upon the electrodes does not increase steadily with pulse application but will attain a steady state where no additional collection occurs;
- The dead space of the system is massive in relation to cell size, thus it is unlikely that cells released from dielectrophoretic collection will travel to the collection point as a discrete package;
- The nature of the peristaltic pump also causes some disturbances in the flow.

It was established that the electrode design used in this study for the investigation of dielectrophoretic response was incompatible with requirements for analysis of cells collected upon the electrodes. Accordingly, this design was unsuitable to determine whether components of a mixed cell suspension would collect at different frequencies upon the electrodes, promoting dielectrophoretic separation of different cell types. Possible improvements to this electrode chamber design towards cell separation are suggested in Chapter Eight.

7.5 CONCLUSIONS AND RECOMMENDATIONS

The preliminary investigations described here have indicated that there are potential applications of dielectrophoresis to haematological research. Findings from this short research programme have suggested that, in accordance with dielectrophoretic theory, blood cells exhibit different responses to the application of a non-uniform electric field, which may be exploited for cell separation.

Various modifications to the experimental design are required before a fuller, more comprehensive research programme can be initiated.

Upon the basis of these preliminary investigations a grant was made available from the Hospital Trust Fund for the continuation of this research.

Chapter Eight

GENERAL DISCUSSION

8.1 PRACTICAL CONSIDERATIONS FOR EXPERIMENTAL DETERMINATION OF THE DIELECTROPHORETIC RESPONSE

Use of a dielectrophoretic technique for cell analysis and separation has many potential advantages, notably that it is non-invasive and requires no pre-modification of the cell. Major drawbacks concerned with exploitation of this phenomenon are concerned with quantification of the response and assessment of confidence in the values obtained. However, automated control of both electric field application and detection of induced particle behaviour have revolutionised this procedure, realising its applicability to clinical investigations.

The current study has identified some of the parameters affecting the dielectrophoretic response, which are of relevance to the anticipated use of this technique in medical research. Other factors concerning the mode of measurement are also discussed.

The development of an automated dielectrophoretic experimental system for the rapid measurement of haematological cells has been described. Using the image analysis system the mean positive dielectrophoretic response induced in a suspension of cells by the application of a non-uniform electric field was quantified and standard errors of the means calculated to indicate the variability of data values. Deterioration in the response as an effect of time was also established; this was difficult to detect by non-automated techniques due to longevity of the procedure.

Both spectrophotometric and image analysis experimental systems (Chapters Three and Five) quantify dielectrophoretic response in relative terms. A discrepancy between these values and the actual events at the electrode surface was introduced by the method of calculating relative collection. In these systems "relative" was defined as the difference between baseline and maximum absorbance or count values - the characteristics of cell disengagement from the electrodes was ignored. Improved quantification would result from calculation of the summation of individual measurements recorded after pulse application. Other parameters to be considered for standardisation of relative collection values include; electrode area observed (where

applicable), proportion of total dielectrophoretic response detected and variation of shear stresses within the flow chamber. Such variables were constant for measurements described here.

When dielectrophoretic analysis of a cell population, as opposed to a single cell, is carried out, care must be taken to ensure that the resultant effect is not beyond the detection parameters of the system. Various investigations using the current experimental system (section 6.2) have demonstrated that saturation effects may readily be observed if the collection capacity of the electrodes, or resolution of detection, are surpassed. In such circumstances, results obtained from quantification of cell collection will not be proportional to the dielectrophoretic force induced in response to the application of an electric field.

The effects of frequency incrementation (section 6.2.3), particle concentration (section 6.3.1), suspension composition and conductivity (sections 6.1.2 and 6.3.2) and collection and storage of haematological samples (section 6.1.3) upon the observed dielectrophoretic response have been described. For most cases optimal values for these parameters can be defined according to the detection capacity of the system. To prevent anomalous results due to electrode polarisation effects it is suggested that a biphasic method of pulse application is adopted, whereby alternate high and low frequency pulses are applied. Dielectrophoretic frequency spectra obtained using this method may exhibit less variation from that predicted by mathematical models, enabling further analysis of the response.

Dielectrophoretic spectra obtained here by determination of gross cell collection in response to an applied non-uniform electric field are a consequence of a number of effects, and not directly attributable to simple dielectrophoretic behaviour. Measurements of the response of a cell suspension, in contrast to that of an individual cell, means that in addition to the effect of the field, each cell is also influenced by the behaviour of others in the vicinity and may be subject to mutual dielectrophoresis (see Chapter One). The contribution of these effects to the dielectrophoretic collection values determined during these investigations is suggested to be frequency-dependent. Observation of cell collection at the electrode surface reveals that cell build-up is not uniform over the electrode length, but consists of pearl chains of varying magnitude interspersed with gaps where minimal collection is apparent. In such instances it is possible that mutual dielectrophoretic effects due to cell-cell interactions take precedence over simple dielectrophoretic motion towards the electrodes. If this were the case over the entire frequency range investigated, then positive dielectrophoretic

effects only would result, *i.e.*, two interacting cells have the same phase shifts of their induced dipole moments, thus the second cannot be repelled from the first (Gimsa, 1994). However, at low frequencies (typically below 100 kHz - see section 4.2.2), negative dielectrophoretic collection was implicated; this situation may only be generated by simple dielectrophoretic action between the cell and the electrode.

Mathematical models defining the dielectrophoretic response in terms of various membrane parameters have been described (Gimsa *et al.*, 1991a, 1991b; Marszalek *et al.*, 1991). A cell surrounded by aqueous solution is considered as two regions of differing electrical properties, separated by a non-conducting barrier. The basic one-shell model describes a spherical cell in terms of three discrete regions; the internal cell composition, the membrane shell, and the external solution. This does not take into account the double layer nature of a biological membrane, nor the non-uniformity existing at region boundaries due to ion distribution. Also, the cell exerts an effect upon its immediate environment causing the formation of an ionic diffuse layer which effectively shields the cell. More complex multi-shell models attempt to deal with such behaviour.

Collection spectra predicted from mathematical models displayed dissimilarities from typical spectra obtained using the measurement system described in earlier chapters. Negative dielectrophoretic behaviour could not be quantified by this system and positive collection spectra were skewed towards high frequencies (section 6.2.3). Gimsa *et al.*, (1994) noted that simple spherical models did not adequately describe the dielectric behaviour of erythrocytes, and that ellipsoidal models were more appropriate. In addition, models have concentrated upon predicting the response of a single cell (Crane & Pohl, 1973; Barnes, 1985; Dimitrov & Zhelev, 1987; Benguigui & Lin, 1988; Chizmadzhev *et al.*, 1989). The limitations of modelling electrical behaviour have been described by Drago *et al.*, 1984). Accordingly, data obtained using this experimental system could not be compared with that predicted mathematically, as current models were not considered to be suitable to describe the observed response of a cell suspension.

Dielectrophoretic theory predicts that collection as a function of frequency rises from a minimum negative plateau (*i.e.*, repulsive, negative collection), until a maximum positive plateau is attained, then falls to a steady negative value. Dispersion of the membrane potential is responsible for the low frequency change between negative and positive dielectrophoretic behaviour; subsequent decline in collection is attributed to the prevalence of conduction over polarisation in the cell cytoplasm (Gimsa *et al.*, 1991). This model allows two characteristic frequencies (f_{ct1} and f_{ct2}) to be defined

at which dielectrophoretic force is zero, during the transitions between positive and negative behaviour.

Attempts were made to obtain values for the two characteristic frequencies at which dielectrophoretic response was zero by fitting curves to the experimental data. However, the curve-fit function could not be justified due to the complexity of collection spectra obtained (see Appendix V).

Although definitive values of field frequencies at which the dielectrophoretic force was zero could not be calculated, the effects of various treatments upon the onset and fall of collection values, as determined using this experimental system, could be investigated. Deductions could thereby be made concerning the electrical properties of the cell from collection spectra generated by this system.

8.2 PHYSICAL PARAMETERS AFFECTING DIELECTROPHORETIC RESPONSE

The erythrocyte may be described as a membranous bag containing a saturated solution of haemoglobin. It is interesting to note that the ß dispersion due to protein relaxation for an aqueous solution of haemoglobin is observed at a field frequency of 1 MHz (Grant *et al.*, 1978). All investigations of erythrocyte dielectrophoretic spectra exhibited strongly positive dielectrophoretic collection at this frequency, possibly indicating that polarisation of cellular haemoglobin may participate in the response. Such an activity is not predicted by current dielectric theory whereby dispersions in the kHz - MHz range are attributed to interfacial polarisations occurring within the cell membrane and external ion layer; the cell interior is shielded from the field by these effects. However, lack of internal membrane structures and the association between haemoglobin and iron (II) molecules may enable intracellular polarisation to be exhibited over the frequency range investigated. It would be of interest to investigate whether haemoglobin dispersion contributed to the dielectrophoretic response of the erythrocyte; this could be carried out using membrane ghosts. The effects of the cell content upon dielectrophoretic activity could be evaluated; the response of ghost membranes re-loaded with haemoglobin could then be compared with that of fresh erythrocytes. It should be noted that although a link between dispersion of native haemoglobin and that of an erythrocyte is suggested, the relaxation of this protein is not the predominant determinant of the erythrocyte response, as many biological cells

exhibit positive dielectrophoretic behaviour over this region (for example see figure 13).

The dependence of the dielectrophoretic response on the dielectrical properties of the cell, in particular the cell membrane, and those of the interphase region between the cell and its suspension medium has been described. Investigations were carried out to assess the effect of altering some of these parameters upon the dielectrophoretic collection behaviour of the cell (section 6.4). These results were in accordance with mathematical descriptions of the diffuse layer in terms of the interfacial β dispersions (Garcia *et al.*, 1985).

During development of an experimental system various detergents were used to promote cell disengagement from the electrode surface after dielectrophoretic collection (section 3.2.1.a). The detergents investigated exerted dissimilar effects upon dielectrophoretic behaviour; significantly, use of an anionic detergent increased collection whereas a cationic detergent inhibited the response. It is postulated that negatively charged anionic detergent molecules orientate within the diffuse layer surrounding the cell with their electro-negative groups directed towards the medium, away from the cell. In such circumstances the ionic layer around the cell is extended as counter-ions must be attracted to polar groups on the detergent molecule to maintain overall electro-neutrality. In contrast, the width of the diffuse layer is proposed to be decreased by cationic detergents whereby the polar group faces the cell, diminishing the extent of the surface charge.

The influence of cell surface charge on the magnitude of dielectrophoretic response was also demonstrated by the increased levels of collection reported for T lymphocytes (high electrophoretic mobility) in comparison to B lymphocytes (low electrophoretic mobility; section 7.4.2). However this observation was not supported by investigations to reduce the cell surface charge using neuraminidase (section 6.4.3).

The effect of membrane conductance upon the dielectrophoretic response of erythrocytes was examined using DIDS to inhibit anion exchange (decreasing conductivity) and A23187 to promote ion transport across the membrane. Both treatments affected the collection behaviour exhibited at high field frequencies and were therefore presumed to alter the second critical frequency (f_{ct2}) at which the dielectrophoretic force is zero. Increased membrane conductivity resulting from DIDS treatment appeared to increase this frequency whereas ionophore treatment caused a decrease. The f_{ct2} parameter is determined by polarisation processes occurring within

the cell, as opposed to those induced within the diffuse layer (Gimsa *et al.*, 1991). Increasing membrane conductivity is therefore postulated to increase the probability that the response to a high-frequency pulse application will be predominantly conductive rather than dispersive in nature. Correspondingly the prevalence for conduction of the electric field at high frequencies will reduce the frequency range over which a dielectrophoretic response is apparent.

Reduction in collection values observed for both DIDS- and A23187- treated samples is hypothesised to result from cell shape changes induced by the disruption of electrochemical gradients. In addition to affecting cell volume (which is known to influence dielectrophoretic behaviour; section 1.1)), shrinkage (exemplified by echinocyte formation) would cause some previously exposed membrane areas to be hidden within invaginations, and therefore alter the properties of the boundary layer surrounding the cell. The protrusion of surface groups into the diffuse layer is thought to be responsible for ß dispersions of the cell becoming apparent at higher frequencies than can be predicted mathematically (Kell & Harris, 1989).

In conclusion, the investigations described in this study indicate that the nature of the dielectrophoretic response is complex due to the interdependency of electrical parameters.

8.3 APPLICATIONS OF DIELECTROPHORESIS FOR HAEMATOLOGICAL PROCEDURES

Opposing criteria may be defined for the utilisation of a dielectrophoretic technique as either an analytical tool or a separation device. For the latter, exploitation of a collection frequency differential enables one cell type to be collected preferentially with respect to others in a mixed cell population. In such circumstances, the device must enable physical isolation of those cells preferentially collected. Use of the present electrode system for separation was hampered by difficulties associated with collection of cell aliquots after dielectrophoretic activity (section 7.4.4). Markx *et al.*, (1994) have been able to achieve selective isolation of micro-organisms using inter-digitated and castellated electrodes. This was accomplished by flushing the electrode chamber with fresh medium whilst pulse application was continued, thus allowing cells exhibiting a strong positive dielectrophoretic response to be retained upon the electrode surface, whilst those less affected were removed by the flow. Collected cells were then released by removal of the voltage and flushed from the chamber. It is anticipated that

a similar effect could be observed by manipulation of the electrode system described here, although prolonged voltage application may result in mammalian cell damage (Mahaworasilda *et al.*, 1994).

Alternatively it may be possible to attain continuous cell separation by deflection of collected cells towards a specified flow exit port. The behaviour of cells at the electrode surface has been reported to be affected by the direction of the electric field in opposition to the suspension flow rate (section 4.2.2). Where the electrode bars were not perpendicular to the direction of flow, cells were forced to collect preferentially at the downstream bar terminus. Manipulation of this effect may enable deflection of cells exhibiting positive dielectrophoretic activity to a chamber exit incidental to the bulk suspension flow.

Cells of differing electrophoretic mobilities are likely candidates for potential separation by dielectrophoretic means. In the present study the magnitude of dielectrophoretic collection was greater for T lymphocytes, which have an higher electrophoretic mobility (*i.e.*, increased negative charge), in comparison to B lymphocytes. Mature thymocytes are known to have a higher electrophoretic mobility than the younger cells (Rychly & Ziska, 1985), thus potentially enabling the separation of fully differentiated cells from their precursors; this type of discrimination may be of assistance in therapeutic cell apheresis of leukaemia sufferers where elevated levels of immature cells are found in the peripheral blood. Burt *et al.*, (1990) have shown using cell lines that dielectrophoretic response is altered as a result of induced differentiation. In addition, the electrophoretic mobility of human peripheral B lymphocytes that are actively secreting antibody is above that of resting cells (Bauer *et al.*, 1988; Grinstein & Dixon, 1989), which may have clinical applications.

Application of a dielectrophoretic technique to facilitate autologous bone marrow transplants would have great potential impact. Ideally this would enable the total removal of all malignant cells prior to re-infusion, but enhancement of the ratio of normal:malignant cells could also aid remission of the disease. Investigations presented here have indicated that human haematological cells may be resolved on the basis of cell lineage thus dictating that further development of this technique is of paramount importance.

8.4 RECOMMENDATIONS FOR FUTURE DEVELOPMENT OF A DIELECTROPHORETIC TECHNIQUE FOR HAEMATOLOGICAL APPLICATIONS

1) Re-design of the electrode chamber specifically for either analysis or separation procedures, and to enable quantification of both positive and negative dielectrophoretic behaviour.

2) Minimisation of other conflicting sources operating within the system, for example sedimentation may be reduced by vertical orientation of the chamber, use of a syringe pump for sample circulation would improve the uniformity of flow.

3) Standardisation of measurements concerned with system variability and correlation with an established technique of surface charge determination.

4) Carry out more extensive studies involving control donors and clinical blood samples to confirm that differences in dielectrophoretic behaviour between different cell lineages is greater than that between different donors.

5) Possible use of the technique in association with dialysis to reduce the numbers of circulating abnormal cells

APPENDIX I

PROTOCOL FOR MANUFACTURE OF ALUMINIUM MICROELECTRODES

All operations were carried out using the clean room facilities of the University of York Electronics Department.

1) Clean slides

Glass slides (BDH Super Premium microslides, cat. no. 406/0180/02) were individually cleaned with mechanical abrasive and stored in de-ionised water. These were then immersed in boiling acetone and dried using a pressurised nitrogen gun.

2) Metal-coat slides

One side of the cleaned microslide was coated with aluminium using a vacuum evaporator.

3) Apply first covering of photoresist

The metal-coated surface of each slide was covered with a layer of syringe-filtered photoresist (Shipley, cat. no. 1400-27) and placed on a slide-spinner at 1500 rpm for 30 s, resulting in a uniformly thin coating. The slides were then transferred to a hotplate for a pre-exposure bake at 95 °C for 1 min.

4) Expose slide to electrode template

The photoresist-coated slide and mask describing the electrode configuration were positioned using a mask aligner (Micro Controle/ Sulzer Electro Technique, model number MA750). The slide was exposed using ultra-violet illumination for 7 s.

5) Development of photoresist

The electrode design was carried upon the microslide as described by the exposed photoresist. Development of this motif rendered the exposed photoresist and underlying layer of aluminium resistant to acid-etching allowing excess metal to be removed, leaving the electrode design. The slide was gently agitated in developer (MF 319), removing unexposed photoresist, until the electrode image was apparent (approximately 40 s), then baked at 95 °C for 15 min.

6) Etching

Acid etching was carried out using a solution of < 1 part nitric acid : 1 part acetic acid : 1 part orthophosphoric acid, in turn diluted for use as 6 parts etch solution: 1 part water. Aluminium not protected by exposed photoresist was removed by immersion in this mixture until the glass slide was observed.

7) Removal of excess photoresist

Exposed photoresist was removed using boiling acetone, taking care not to rub the metal.

8) Addition of adhesion promoter

Adhesion promoter was freshly prepared and consisted of 1 part deionised water : 1 part Selectiplast HTR AP3 (Ciba Geigy) : 98 parts propan-2-ol. The slides were covered with promoter and left for 15 s before being spun at 1500 rpm for 15 s. The slides were then baked at 95 °C for 1 min.

9) Application of second coat of photoresist to delineate chamber boundaries

The aluminium coated surface of the slides was covered with a second photoresist (Selectilux HTR3-200, Ciba-Geigy) and spun at 550 rpm for 15s, then the speed increased to 1500 rpm for an additional 3 s. The slides were baked prior to exposure at 95 °C for 35 min. The combination of this photoresist and conditions resulted in a durable layer approximately 70 μm thick.

10) Exposure of slide to channel template

The slide and channel template were positioned using the mask aligner as before. The photoresist was exposed using ultra-violet illumination for 1 min 45 s.

11) Development of photoresist

Excess photoresist was removed by gentle rubbing of the surface with a cotton bud moistened with Selectiplast HTR D2 (Ciba-Geigy) until the channel motif was revealed (between 60-90 s). The slide was then rinsed in a 50/50 solution of HTR D2 and IPA for 15 s, then in IPA for 15s before being dried with a pressurised nitrogen gun. The slide was then inspected to evaluate the success of the development process which could be repeated if necessary. When satisfactory the slides were baked at 150 °C for 80 min and were then ready for use.

APPENDIX II
CULTURE MEDIA

Glucose-yeast extract broth

	g/l de-ionised water
Glucose	20
Yeast extract	5

To pH 5.6 with 1 M NaOH

Sterilisation by autoclaving at 115°C , 15 psi for 20 min.

APPENDIX III
SOLUTIONS FOR BLOOD CELL SUSPENSION

1) Citrate phosphate dextrose adenine formula 1 anticoagulant solution (CPDA-1)

	g/100 ml de-ionised water
Sodium citrate	2.63
Anhydrous glucose	2.90
Citric acid monohydrate	0.327
Sodium acid phosphate	0.251
Adenine	0.0275

Used at 63 ml: 450 ml blood

2) Glycine-glucose (GG) experimental medium

(Pohl, 1978; Krishna *et al.*, 1989a, 1989b)

9 parts 2.1 % (v/v) glycine
1 part 5.5 % (v/v) glucose

3) Sorbitol-glucose (SG) experimental medium

280 mM sorbitol
1 mM HEPES
5 mg/ml glucose
pH 7.4 with NaOH

Osmolarity 290 ± 5 mOsm
N.B. pH adjustment must be carried out at experimental temperature.
Sterile filtered with 0.2 μm pore.

4) Phosphate-buffered saline (PBS)

	g/l de-ionised water
NaCl	8.5
KH2PO4	1.36

pH 7.2 with NaOH

APPENDIX IV
HAEMATOLOGICAL METHODOLOGY

1) Erythrocyte lysis using ammonium chloride solution

Ammonium chloride at 0.75% (w/v) (Sigma Chemical Co. Ltd., Poole, UK) was dissolved in a 20 mM solution of HEPES buffer (ICN Flow Labs. Ltd), and the pH was adjusted to pH 7.4. On the day of use, 0.25% (v/v) autologous plasma was added to the ammonium chloride solution, which was filtered through a 0.2 um pore, sterile filter before use.

Excess ammonium chloride solution was added to the tube containing the cell sample contaminated with erythrocytes. This was incubated at 37°C for 10 min to allow lysis to occur then washed in PBS, centrifuged at 1500 rpm for 10 min and the debris-rich supernatant discarded. The pelleted cells were then re-suspended in PBS.

(Bøyum A, Løhaug D and Tresland L, 1991 Density-dependent separation of white blood cells. pp 217-239 in Blood Separation and Plasma Fractionation, ed Harris JR, Wiley-Liss, Inc)

2) Trypan Blue Exclusion Test

2 volumes of neutrophil suspension were added to 3 volumes of PBS and 5 volumes of 0.4% (w/v) Trypan Blue Solution (Sigma Chemical Co. Ltd) and mixed. The cells were incubated for 15 minutes at room temperature and a small aliquot examined by light microscopy. The number of cells which remained colourless were counted as a proportion of the total number of cells. Live (viable) neutrophils exclude the dye and remain colourless, whereas non-viable cells (with permeable membranes) take up the dye and stain blue.

3) Neutrophil separation by sedimentation with the erythrocyte-aggregating agent, Plasmagel

An equal volume of Plasmagel (Laboratoire Roger Bellon, Neuilly-sur-Seine, France) was layered onto a clinical blood sample using aseptic technique. The suspension was gently mixed with a pipette, then left to stand for approximately 20 min until erythrocyte sedimentation had occurred leaving a clear supernatant. The supernatant was aspirated, centrifuged at 1000 rpm for 20 min, washed in PBS and re-centrifuged. The final suspension in PBS was analysed by FACScan - if erythrocyte concentration was still greater than that of the leucocytes then an ammonium chloride lysis step was employed.

4) Lymphoprep separation

10 ml of whole blood from a clinical sample was gently layered onto 10 ml of Lymphoprep (Sigma). This was centrifuged at 1400 rpm for 20 min. The upper layer of the leucocyte band was aspirated and transferred to a second tube for washing.

5) The Becton Dickinson FACScan

The fluorescence-activated cell analyser (FACScan) is a flow cytometer system permitting the analysis of all cells, or only those with defined characteristics, as determined by the experimental parameters selected. Detailed accounts of principles of operation and standard protocols are given in manufacturers' information and Current Protocols in Immunology; a brief guide is presented here.

Cells for analysis are suspended in saline and aspirated into the analyser. A stream of cells is caused to pass through the path of a laser beam (488 nm excitation) resulting in light scatter in all directions. This scatter is detected in the forward (FSc) and side (SSc) directions only, and correlates with cell size and cellular complexity respectively. Fluorescence emission may also be detected by photomultiplier tubes - the bandwidth of detection is specified by optical filters in front of the tubes, and is referred to as a channel. Typically, the FL1 channel detects emission at a wavelength of 530 nm $\pm$ 30 nm, FL2 at 585 nm $\pm$ 26 nm and FL3 at >630 nm; this enables comparisons to be made between different fluorochromes.

Electronic gates may be set to select parameters for data acquisition. For example, gates can be applied to a simple plot of SSc against FSc, *i.e* granularity against size, to specify mononuclear cells only from a normal peripheral blood sample. Fluorescence data can then be acquired for monocyte and lymphocyte populations alone, allowing differential fluorescence emissions of mononuclear cells to be detected.

For these investigations the FACScan flow cytometer (Becton Dickinson) was utilised in association with the Consort 30 four parameter analysis program. The instrument was prepared and standardised in accordance with manufacturer's protocol. For standard use typical cell concentrations are approximately 5 x 10^6 cells per ml at a sample flow rate of approximately 1000 events per second. For this study, dielectrophoretic sample cell concentrations were around one fifth of the standard value; for each sample 3000 events were analysed.

A) Determination of viability of a cell sample using the fluorescence activated cell analyser (FACScan)

The percentage viability of a cell sample was determined by fluorescence analysis after treatment with a fluorophore, propidium iodide (PI). This red fluorescent dye is taken up by dead cells and binds to nucleic acids, but is excluded from intact cells. Excitation by the FACScan light source results in red spectrum emissions by PI and subsequent detection by the FL2 photo-multiplier tube. Therefore, a contour plot of red

fluorescence intensity (FL2) against green fluorescence intensity (FL1) should reveal two cell populations:
- FL1-/FL2+ (red but not green) indicating non-viable cells
- FL1-/FL2- (not red and not green) indicating viable cells

Electronic gating can be employed to establish the relative populations of the two phenotypes.

Cell samples at a standard concentration of 1 x 10^6 cells per ml were labelled by adding 100 µl of 50 µg/ml PI in PBS to an equal volume of cell sample. The suspension was mixed by gentle agitation then allowed to stand for 30 s. If required, extra isotonic saline solution was added to increase the volume, then the sample analysed using the FACScan. Percentage viability was established from the proportion of cells that did not fluoresce.

B) Lymphocyte characterisation using the FACScan

B and T lymphocytes may be differentiated by labelling with fluorochome-conjugated monoclonal antibodies to their specific antigens and analysing the results using the FACScan.

Becton-Dickinson cluster determinant (CD) membrane markers (i.e. monoclonal antibodies) 19 and 21 were used to label B lymphocytes; CD 2 and 3 labelled T lymphocytes. By use of a combination of antibodies with opposing coloured fluorochromes the relative populations of B and T lymphocytes may be assessed. In the antibody panel used in these experiments fluorescent markers attached to CD19 and CD21 antibodies are excited by red light whilst those to CD2 and CD3 are excited by green. The FACScan was used to compare red fluorescence intensity with green for a lymphocyte population and the data analysed using Quadrant Statistics. Relative populations with red fluorescence intensity and low green, and high green and low red, may then be determined using electronic gating techniques. The former corresponds to B lymphocytes and the latter to the T lymphocyte population.

A 100 µl aliquot of blood was added to a tube containing 20 µl of the antibody panel CD 19/21 vs 2/3, and incubated at room temperature in the dark for 10 min. Cell lysis was induced by the addition of 2 ml of Facslyse solution (Becton Dickinson) and the suspension incubated for a further 5 min. To remove excess reagents the suspension was transferred to a micro-centrifuge and spun at 6500 rpm for 30 s, the supernatant discarded and the pellet resuspended in PBS. The centrifugation step was repeated and the pellet re-suspended in Isoton for FACScan analysis.

The total composition of the sample was analysed first by data collection of side scatter of laser light (SSc) against forward scatter (FSc). This is in effect a measure of granularity against size. The population of cells to be examined is chosen using an electronic gate *i.e.*, non-lymphocytes can be excluded. A contour map was produced with the Facscan using data acquired on parameters FL2 against FL1. Electronic gating was applied to divide the map into quadrants enabling the relative populations to be quantified.

6) Osmotic fragility test

The effect of low ionic strength suspension medium upon erythrocytes was assessed using osmotic fragility to determine the resistance of cells to hypotonic saline solution. Resistance is a function of membrane integrity, cell shape and volume, and the capacity to maintain an ionic gradient.

Standard procedure required that blood samples were diluted 100 x into saline - this was adapted to allow for lower cell concentrations comparable with those used in the dielectrophoretic investigation to be utilised.

All saline dilutions were incubated for 24 hr at 37 C to create hypoglycemic conditions and increase the sensitivity of the test. After incubation, tubes were re-mixed then centrifuged at 1500 g for 5 min. The absorbance of the supernatant was measured at 540 nm against (?) that of an unlysed whole blood sample (0.85% saline) and haemolysis calculated:

% Haemolysis = (absorbance of supernatant/absorbance of 0.00% tube) x 100

The test is based upon a simple photometric method to measure cell lysis after incubation of erythrocytes in saline solutions of various dilutions. The standard period of incubation is for 30 min at room temperature, but a more sensitive investigation also puts the cells under hypoglycemic stress by incubation for 24 hr at 37° C.

A series of saline solutions at varying concentrations are prepared, i.e. 1, 0.85, 0.75, 0.65, 0.55, 0.5, 0.45, 0.4, 0.35, 0.3, 0.2, 0.1, and 0 % saline. Dilutions of 1:100 blood to saline are prepared and mixed by inversion. Samples are incubated at room temperature for 30 min, re-mixed, then centrifuged at 1200 g for 5 min. The absorbance of the supernatant was read at 540 nm with a spectrophotometer, against the 0.85% saline sample. The highest density reading should be given by the 0 % tube where complete haemolysis should have occurred.

Results were plotted as % haemolysis vs % saline soln. Typically for normal samples one hundred percent haemolysis occurs over a very small concentration range around 0.52% saline.

APPENDIX V
MATHEMATICAL MODELLING OF THE DIELECTROPHORETIC RESPONSE

The curve fit function applied to dielectrophoretic spectra was

$$y = a * \exp ((1-\exp(b1*(c1-w1))) * (1-\exp(b2*(c2-w)))$$

where:

a = a scale parameter corresponding to the maximum collection level divided by e (the base of natural logs)

b1 = the slope of the rise

c1 = the position of the rise where collection is 1/e of maximum

b2 = the slope of the fall

c2 = the position of the fall where collection is 1/e of maximum

w = omega

(Byrne & Robinson, 1994)

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Rapid dielectrophoretic analysis of erythrocytes

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Abstract

An advanced, computer operated dielectrophoretic system was developed for the analysis of human erythrocytes. The response of cell suspensions to applied non-uniform electrical fields was rapidly determined using automated procedures and real-time imaging. Reproducible and characteristic spectra consisting of dielectrophoretic response plotted against field frequency were generated.

Introduction

Dielectrophoresis can be defined as the relative movement of particles suspended in a medium resulting from their polarization induced by an inhomogeneous electric field (Pohl, 1951). The magnitude of the response, but not the direction, is dependent upon the frequency of the applied electric field and can be visualized as a dielectrophoretic collection of particles in formations known as pearl chains. For the motion towards the electrodes to persist, the dipole formed as a result of the polarization must be able to reverse in phase with the applied field. The properties of the induced dipole are determined by the structure of the particle. For every particle type a unique profile of response against electric field frequency can be constructed and exploited for characterization or separation purposes if internal and external conditions of the system are controlled.

Biophysical characteristics of cells are affected by membrane irregularities such as those found in pathological conditions and these could influence the dielectrophoretic response. Routine haematological investigations to detect abnormal cells are often laborious and require skilled personnel. Many of these procedures could potentially be replaced with an efficient dielectrophoretic technique.

Materials and methods

Two aluminium electrodes (50 μm wide separated by a 50 μm gap) were laid down onto a glass microscope slide using photolithographic techniques. The electrode bars were enclosed by silicon rubber spacers (thickness 1 mm; Bibby Sterilin) and by a glass cover (Figure 1). Input and output tubes were

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incorporated into the chamber at each end resulting in a flow-through system adapted from that previously described (Betts and Hawkes, 1994; Hawkes *et al.*, 1993). A cell suspension supplied from a temperature controlled reservoir was then circulated (Figure 2) throughout the chamber in a continuous manner by a peristaltic pump (Gilson Minpuls 3, France).

The field across the electrodes was produced using a signal generator (Hewlett Packard 8116A, Germany) connected by leads to the electrode tabs. The electrodes were viewed using a charge coupled device (CCD) camera (Ikegami ICD-42E, Japan) attached to a microscope (Nikon Labophot-2, Japan) at a magnification of x450. The video signal was simultaneously processed by a modified image-analysis package (Domino, Perceptive Instruments, England) and viewed on a monitor, allowing operating and detection parameters to be defined (Quinn *et al.*, 1994). The area analysed was 25—50 μm downstream of the electrode bars and was used to detect the package of cells released after removal of the field. The processing facility conditioned and then digitized the image enabling it to be accessed by special digital processing logic. This allowed quantification in real-time from each successive image frame.

The entire system was co-ordinated by a 80486-based personal computer (AST 486 Bravo) using in-house software to control all parameters including pulse voltage, pulse frequency, pulse length, pump speed and data acquisition. A typical sequence of events is shown in Figure 3.

The effects of the dielectrophoretic force were interpreted from image analysis data by the in-house software for each applied pulse. Average measurements were calculated from baseline readings for each sequence of events, and from the three consecutive maximum values recorded after pulse removal. The effect of the field was estimated by calculating the difference between these two values in each case. A large positive value indicated cell collection and that positive dielectrophoresis had occurred.

Blood samples (O+ve) from healthy volunteers were citrated and maintained at 4°C until their use, within 4 h. Sample volumes of 0.5 ml were centrifuged at 1,500 rpm for 5 min and the plasma plus buffy coat removed. The cells were then washed with 10 ml of suspending solution (280 mM sorbitol; 5 mg/ml glucose; 1 mM Hepes; pH 7.4) at 37°C, recentrifuged and the supernatant aspirated. The sample was prepared by diluting 10 μl of the cell pellet with 10 ml of suspending solution. This method was found to give reproducible cell concentrations.

The sample was introduced into the reservoir (37°C) and circulated throughout the system. The experimental parameters were set to study the effect of a 5 V pulse applied for 5 s over the frequency range 10 kHz to 10 MHz. Conductivity and pH were monitored throughout the investigation and were found to be relatively consistent over the experimental period.

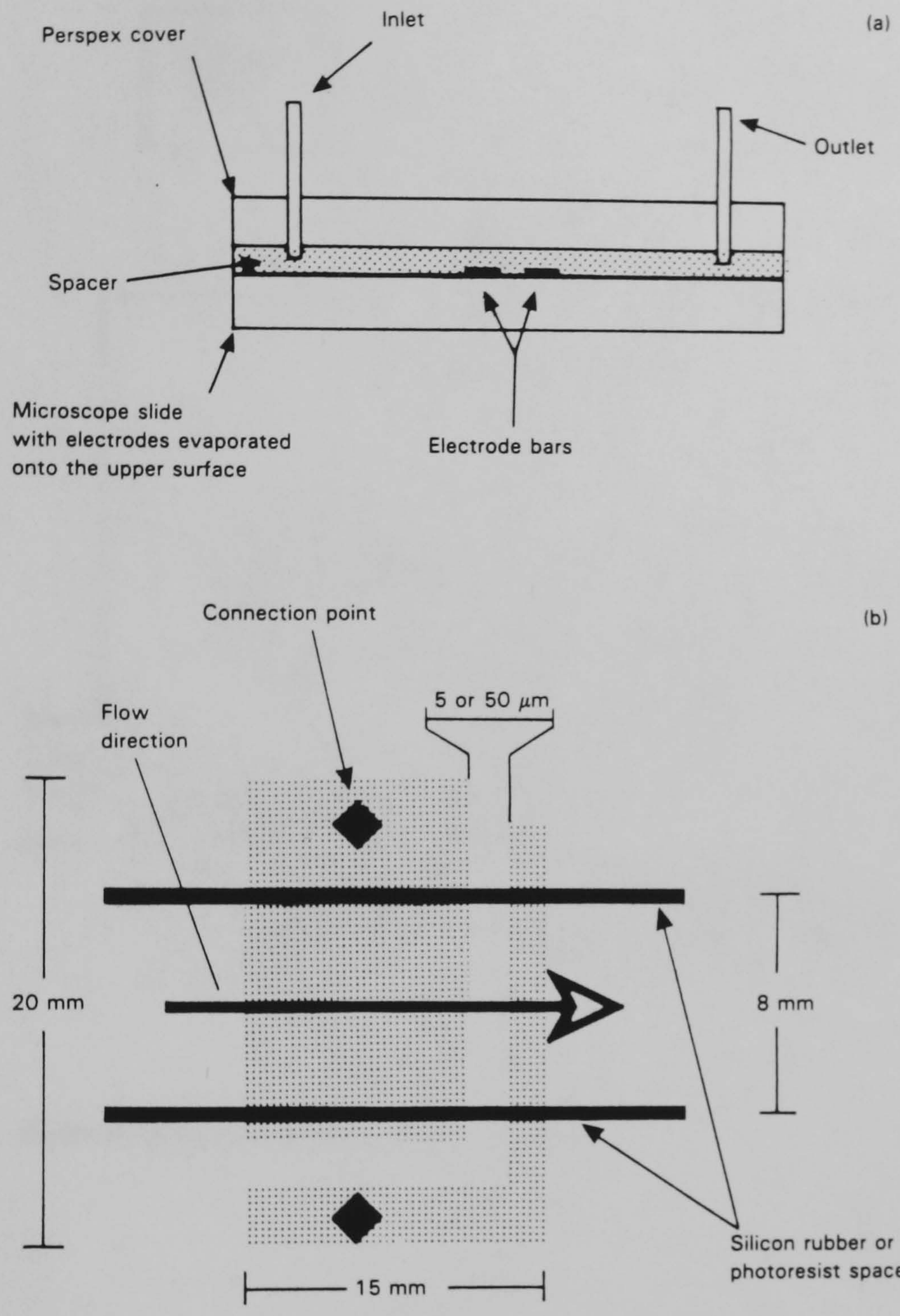

Figure 1 The dielectrophoresis electrode chamber arrangement. (Figure 1a) Side elevation showing electrodes and suspension inlet and outlet. (Figure 1b) Plan view showing electrodes, electrical connection points, electrode gap and spacer position.

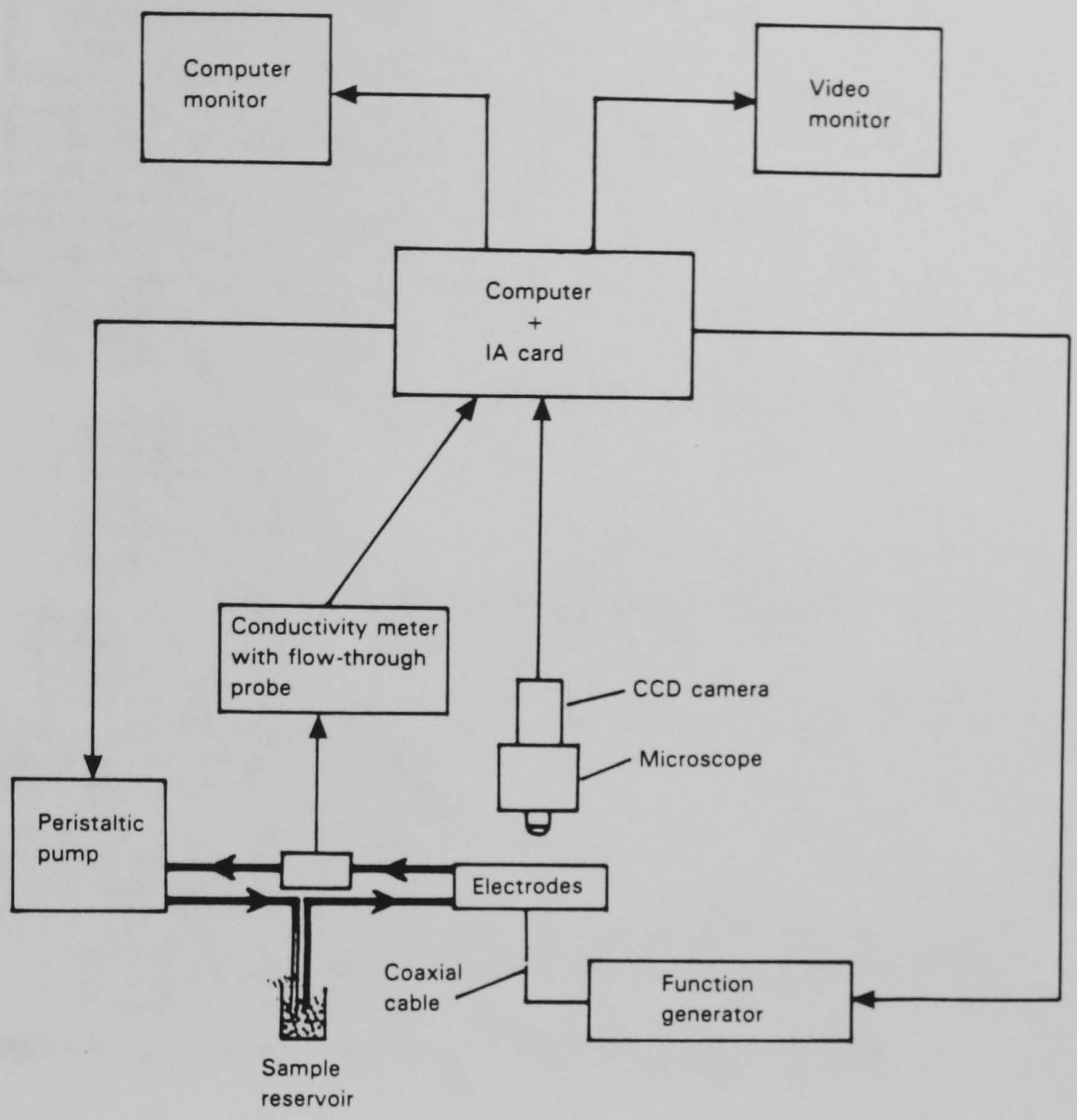

Figure 2 Configuration of experimental system.

Results and discussion

A typical dielectrophoretic spectrum generated during experiments on nine blood samples is shown in Figure 4. The frequencies at which positive dielectrophoresis was first determined by the system and at which collection declined were dependent upon the electrical parameters of the cells (*e.g.* cell surface chemical groups present), their suspending solution (*e.g.* conductivity, pH) and the limitations of this system (*e.g.* frequency range available). This was also true of the general shape of the characteristic collection spectrum for erythrocytes.

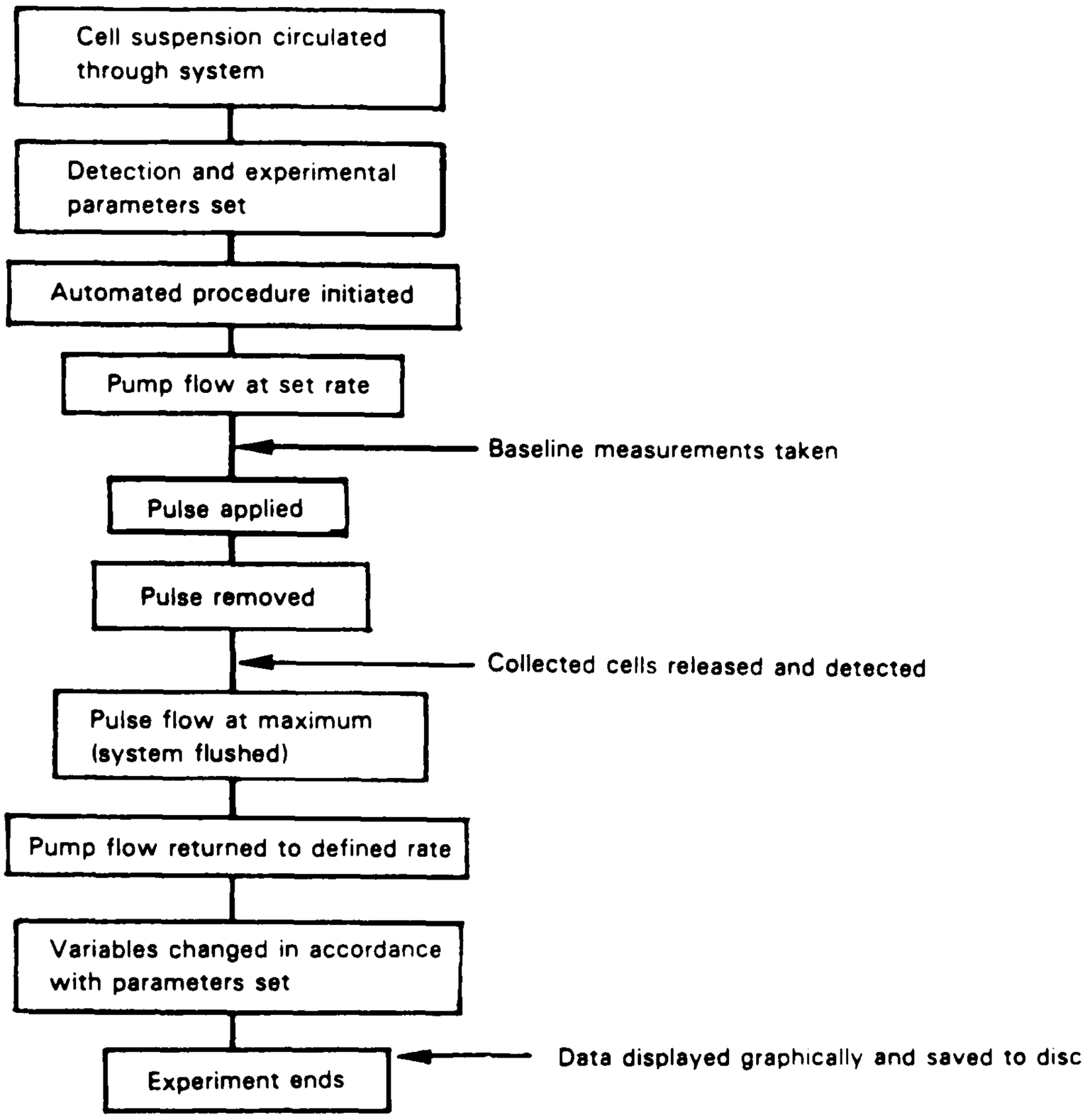

Figure 3 Flow diagram summarizing automated experimental sequence.

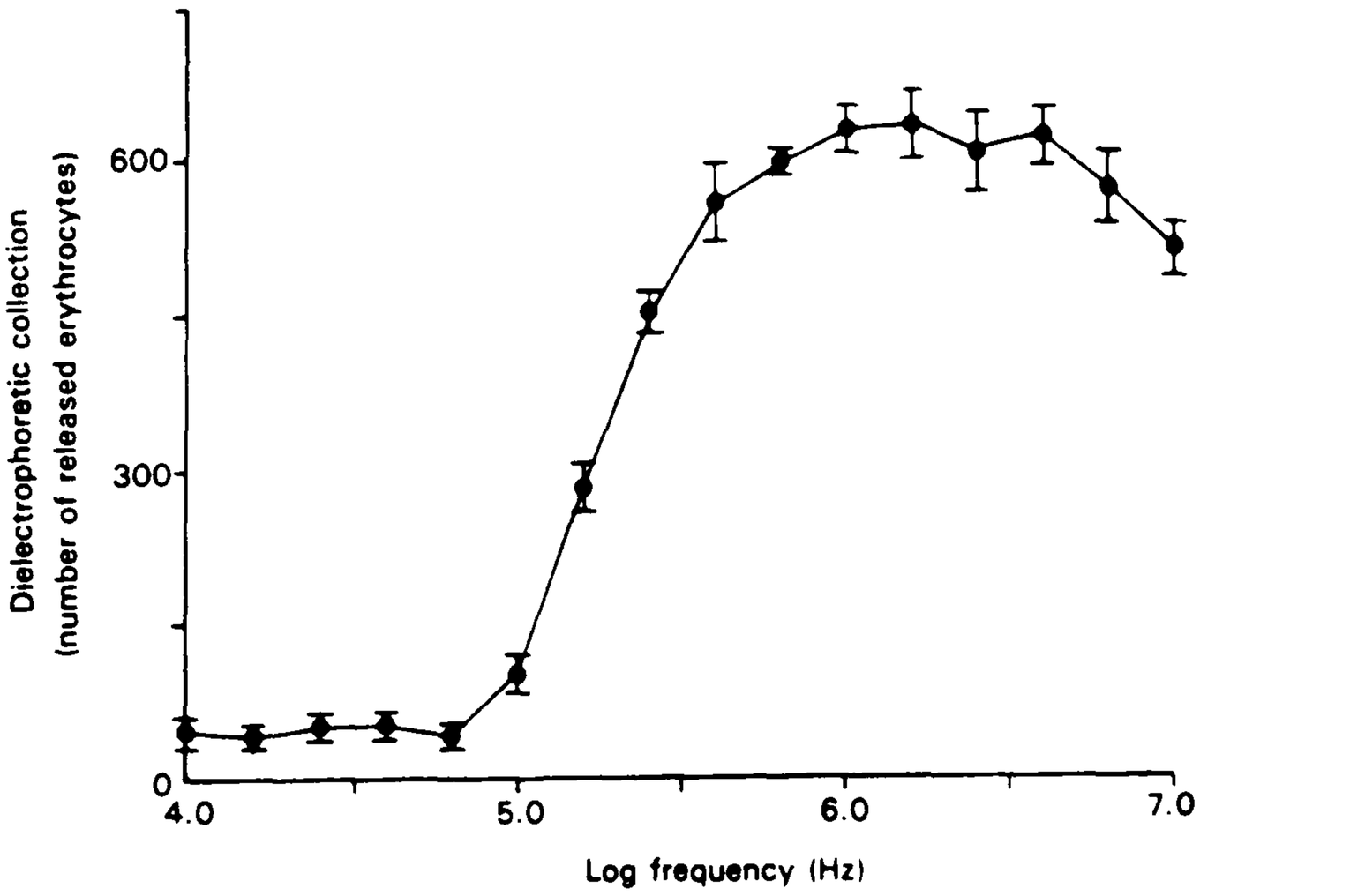

Figure 4 A typical dielectrophoretic collection spectrum generated from nine blood samples and showing standard errors of the mean (σ = 126–135 μs; cell concentration = 7.5 x 10 ; pH 7.4).

The bioelectrical characteristics of normal and neoplastic cells have been reported to differ (Pethig, 1984; Burt *et al.*, 1990) and there are indications that the ABO classification system can also be differentiated (Krishna *et al.*, 1989). Alterations in the electrical composition of cells with abnormal pathology result in spectral differences as determined by this method. It is anticipated that developments of the personal computer-based system described in this paper will find future use as a diagnostic tool, providing a rapid assessment of pathological conditions. Further refinements could enable a similar system to be utilized for cell separation assisting clinical treatments.

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Rapid differentiation of untreated, autoclaved and ozone-treated *Cryptosporidium parvum* oocysts using dielectrophoresis

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Abstract

A novel automated dielectrophoretic electrode apparatus and procedure was used to differentiate between untreated, autoclaved and ozone treated *Cryptosporidium parvum* oocysts recovered from water. A freeze-frame video technique enabled images of oocysts to be captured when they collected at sites above and between the electrodes upon application of an electric field. The number of oocysts collecting could then be conveniently counted. Varying the frequency of the applied electric field allowed the construction of characteristic spectra for each sample.

Introduction

The protozoan parasite *Cryptosporidium parvum* has a complex life cycle including sexual and asexual phases together with an oocyst stage which is capable of environmental transmission (Fayer and Ungar, 1986; Janoff and Reller, 1987). It is the only species of *Cryptosporidium* which infects humans (Crawford and Vermund, 1988) and water is an important vehicle in this respect (Anon, 1990; Barer and Wright, 1990). Cryptosporidiosis is now recognised as an important, and previously overlooked, cause of severe gastroenteritis in man and mortality in immunocompromised patients. The infective potential of *Cryptosporidium* will probably lead to a requirement for analysis of potable water for viable oocysts.

The most commonly used water disinfection methods such as chlorination are unreliable for this organism and the infective dose in humans is very low (as few as ten oocysts) and so research is currently very active in the areas of oocyst inactivation and detection (Vesey *et al.*, 1991). At present the detection of *Cryptosporidium* oocysts in water is time-consuming and inefficient. The principal technique involves the staining of concentrated samples for assay using immunofluorescence (Rose *et al.*, 1989) but this can suffer from inadequate antigen recognition by the antibodies used and is of little value in determining viability. *In vitro* excystation of the parasite or *in vivo* infectivity studies are currently used to assess viability (Fayer *et al.*, 1990) but these are slow, laborious and expensive, and interpretation is difficult. Flow cytometry, supplemented with fluorescein isothiocyanate (FITC) antibody staining of oocysts, has been assessed as a detection method (Howes, 1991). Although improvements in efficiency, sensitivity and speed have been made using this

method, without specific staining of viable oocysts the determination of oocyst infectivity potential cannot be achieved (Woolf, 1992). There is thus a need for a technique capable of monitoring the effectiveness of water disinfection treatments. Ideally this technique should be rapid, automated and capable of being installed *in situ* at water treatment plants.

Dielectrophoresis is a term used to describe the movement of particles in non-uniform electric fields (Pohl, 1951) and the possible exploitation of this phenomenon for the separation and characterisation of micro-organisms and other particles has been recognised, although largely unrealised in terms of practical applications. The theoretical aspects of dielectrophoresis have been described elsewhere (Pohl, 1973).

The dielectrophoretic collection of any particle can be measured across a wide frequency range and plotted as a characteristic spectrum which can be compared with reference spectra. It is the aim of this paper to illustrate that rapid microbiological analysis is one of the many potential practical applications of dielectrophoresis. A technique is presented utilising a novel dielectrophoretic electrode chamber and a digital freeze-frame video detection system which is capable of obtaining spectra for suspensions containing relatively low concentrations of particles.

Materials and methods

Micro-organism

Oocysts of *Cryptosporidium parvum* were supplied by Yorkshire Water plc, Bradford, England, as suspensions in distilled water. The ozone treated samples were prepared at Elvington Water Treatment Plant, York, England.

Preparation of oocyst suspensions

Untreated *Cryptosporidium* oocysts (Ui)

A 1 ml volume of an untreated stock sample (1×10^8 oocysts/ml) was suspended in 20 ml of 0.5 mM sodium dodecyl sulphate (SDS) solution (Sigma Chemical Company) and centrifuged for 10 min at 12,000—13,000 rpm. The supernatant was drawn off and the pellet resuspended in 4 ml of 0.5 mM SDS solution.

Autoclaved *Cryptosporidium* oocysts (Ti)

A small volume of an untreated stock sample (1×10^8 oocysts ml^{-1}) was autoclaved at 121°C for 10 min and then prepared as for the untreated oocysts (Ui).

Untreated *Cryptosporidium* oocysts (Uii)

A 7.5 ml volume of an untreated stock sample (1×10^6 oocysts ml^{-1}) was suspended in 20 ml of distilled water and centrifuged for 10 min at 12,000—13,000 rpm. The supernatant was drawn off and the pellet resuspended in 4 ml of 0.02 mM SDS solution.

***Cryptosporidium* oocysts treated with 1.15 mg l^{-1} (Tii) or 3.3 mg l^{-1} (Tiii) ozone**

A 10 ml volume of a stock sample treated with 1.15 mg l^{-1} or 3.3 mg l^{-1} of ozone (both samples 7.5×10^5 oocysts ml^{-1}) was suspended in 20 ml of distilled water and centrifuged for 10 min at 12,000—13,000 rpm. The supernatant was drawn off and the pellet resuspended in 4 ml of 0.02 mM SDS solution.

Design and manufacture of the electrodes and electrode chamber

The electrodes used consist of an array of sixteen parallel gold-coated chromium bars each 50 μm wide and separated by a distance of 50 μm. These were deposited upon a glass microscope slide using a photolithographic technique similar to that described by Price *et al.* (1987). The thickness of the bars was estimated to be less than 1 μm.

The electrode chamber (Figure 1) allows a suspension to flow over the electrodes in a channel approximately 150 μm high. This channel was formed by creating a space between two slides using double-sided sticky-tape. A perspex slide, predrilled with two holes, was then placed onto the electrode slide so that the holes were located in the centre of the channel on either side of the electrode array. A tube was inserted into each hole and secured by a small block of perspex containing a hole. Once in position the two slides were sealed together using an epoxy resin adhesive.

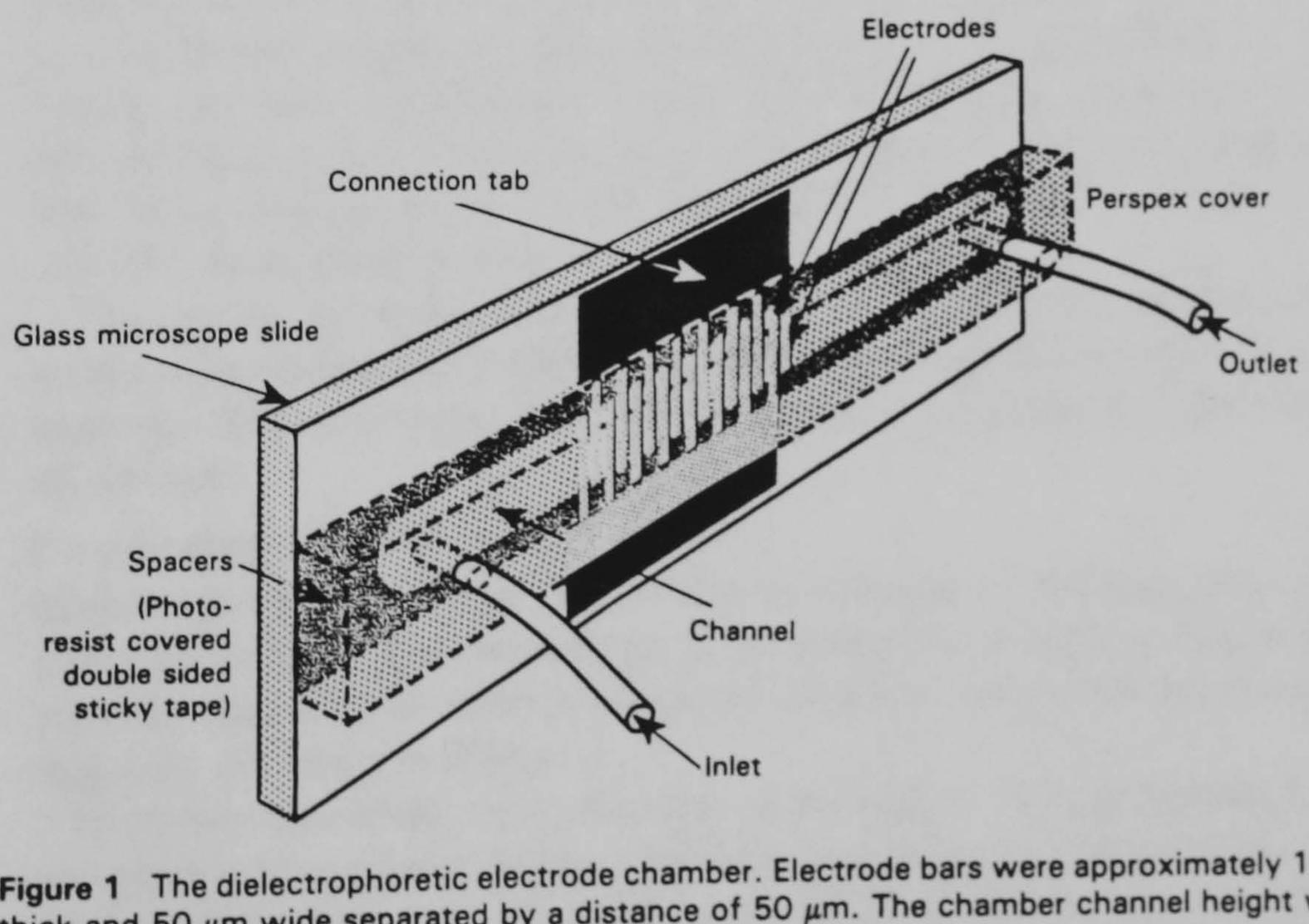

Figure 1 The dielectrophoretic electrode chamber. Electrode bars were approximately 1 μm thick and 50 μm wide separated by a distance of 50 μm. The chamber channel height was approximately 150 μm.

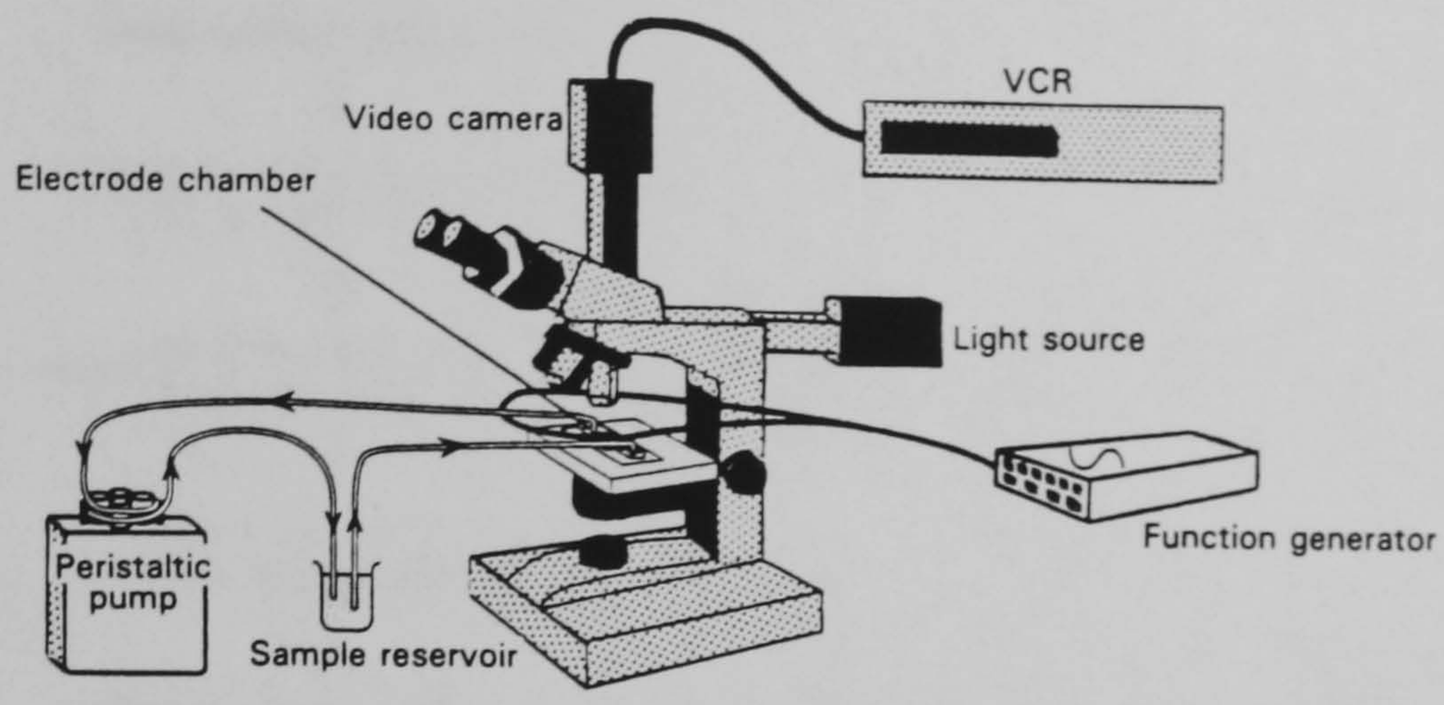

Figure 2 The dielectrophoretic apparatus system. The electrode chamber was located on the microscope stage and was supplied with bacterial suspension through tubes from and to the sample reservoir by a peristaltic pump. A function generator was connected to the electrodes and supplied a pulsed voltage with a frequency incremented through a chosen range for each experiment. The pump and function generator were controlled by a computer (not shown). A video camera and video cassette recorder (VCR) were used to observe and record cell collection.

Experimental equipment and connections

The apparatus was connected as shown in Figure 2. The peristaltic pump (Gilson Minipuls 3) was used to draw the sample suspension through the electrode channel and back into the reservoir. A microscope with the facility to observe both transmitted and reflected light (Nikon Labphot-2) was fitted with a solid state colour camera (Hitachi KP-C500) to monitor the first few bars of the electrode array and enable the image to be captured using an S-VHS video cassette recorder (NEC DS 6000 K). Throughout the experiment 100-fold magnification was employed.

The system was controlled using an Epson PC AX2 microcomputer which set the pulse voltage and frequency applied to the electrodes by a pulse/function generator (Hewlett Packard 8116A), controlled the pump and determined all timings.

Dielectrophoretic collection measurements

Measurements were made at increments throughout the frequency range 1 kHz to 50 MHz. The pulse length and voltage were chosen so as to provide suitable maxima and minima of oocyst collection. A typical experimental sequence is shown in Figure 3.

Replicate runs of the entire experiment were made for each sample. Upon completion of the experiments, the video recording was played back and a section of the first three electrode bars, counting from the up-stream end of the electrode array, was outlined on the monitor screen. Using the digital

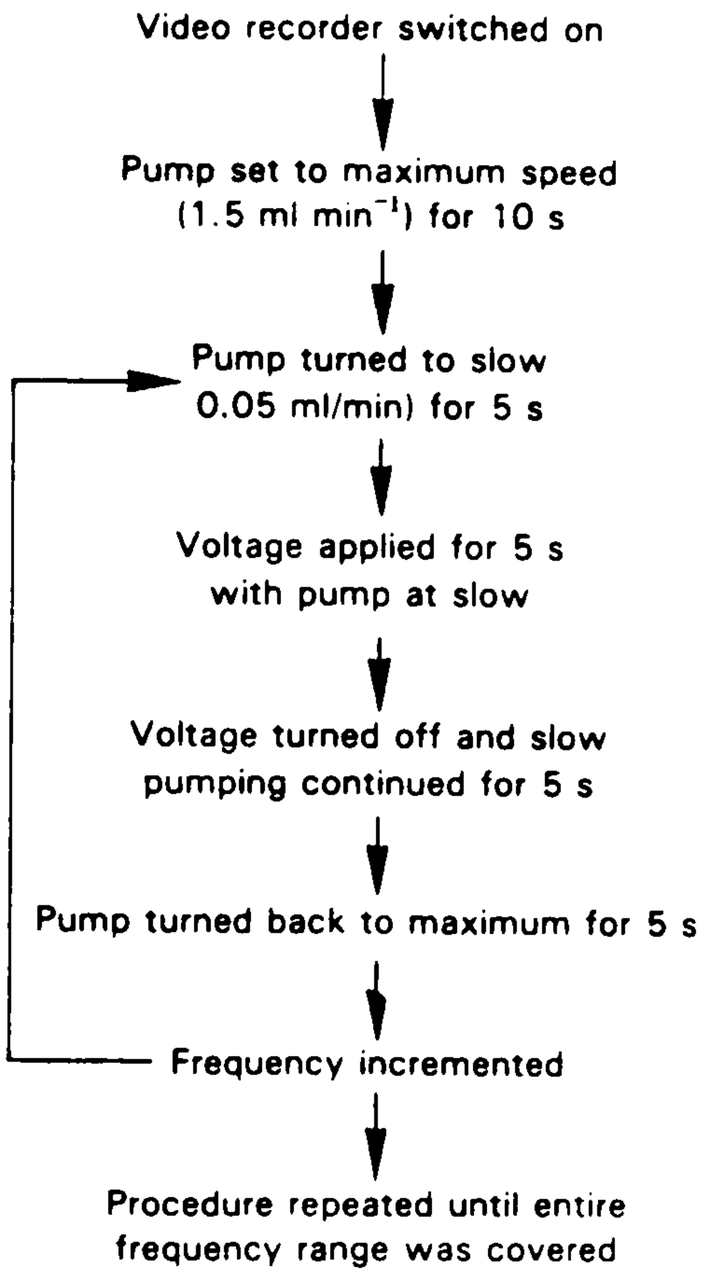

Figure 3 Typical experimental sequence to obtain dielectrophoretic collection spectra.

freeze-frame facility of the video recorder, cells which had collected both on and between the electrodes within this area were counted. Counting was undertaken on frames immediately prior to the release of the applied pulse at each frequency.

Results

Significant differences were observed between the spectra obtained from the untreated sample (Ui) and those from the autoclaved sample (Ti) (Figure 4). The autoclaved sample exhibited very little collection above 5 MHz in contrast to the untreated sample which continued to collect up to and beyond 10 MHz. The spectra show the collection observed between 1 kHz and 10 MHz. Untreated oocysts, and to a lesser extent ozone treated oocysts, were observed to undergo positive dielectrophoresis at frequencies as high as 50 MHz. However, transmission line effects may make present quantitative measurements unreliable above 10 MHz. The lower numbers of oocysts collecting in the case of the autoclaved sample was due to the unavoidable use of a different (and less efficient) set of electrodes.

The results of experiments using ozone treated samples are shown in Figure 5. The major difference between the spectra obtained from the treated samples (Tii) and (Tiii) and the untreated sample (Uii) was pulse frequency

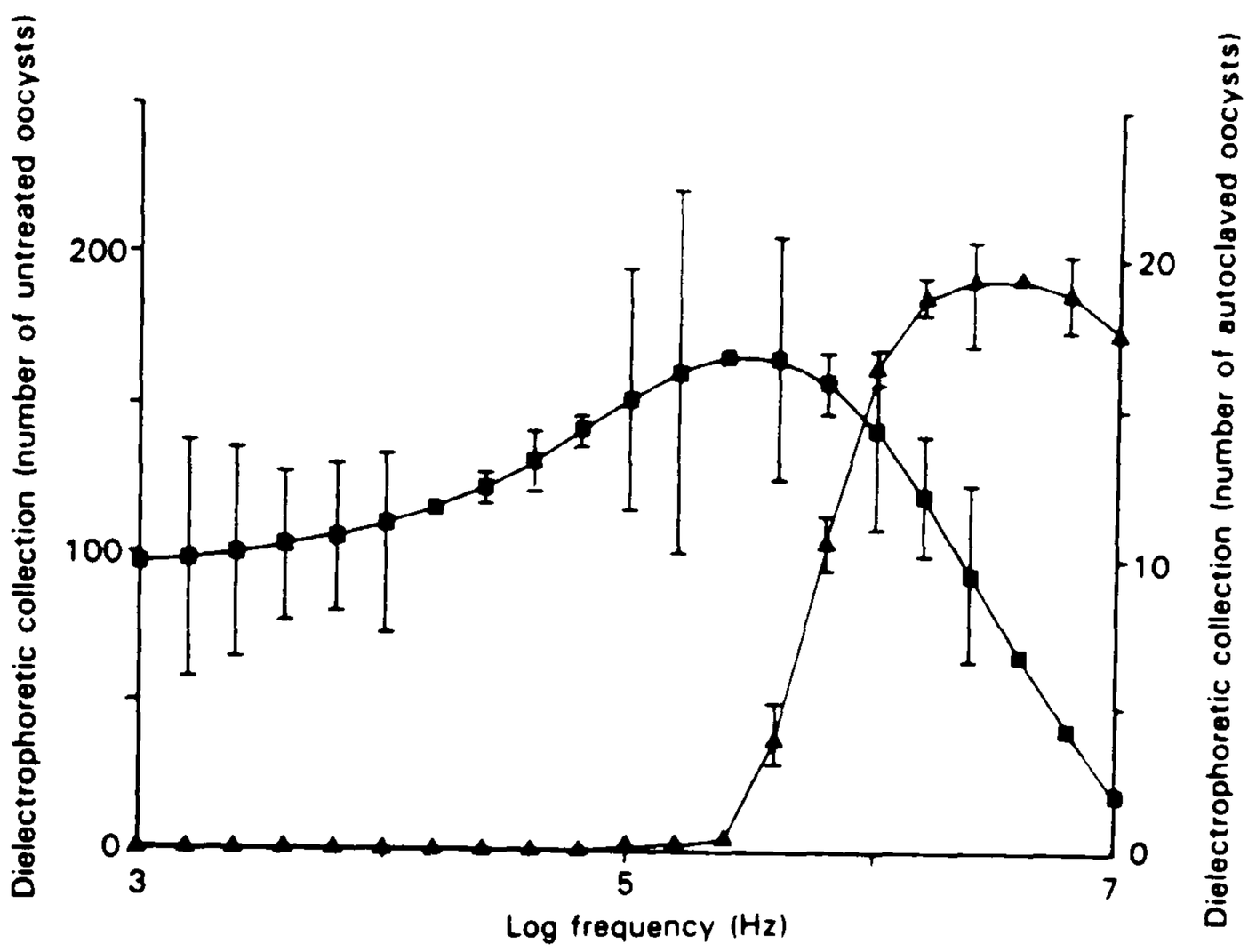

Figure 4 Dielectrophoretic collection spectra comparing untreated (▲ , Ui) and autoclaved (■ , Ti) samples of *Cryptosporidium* oocysts.

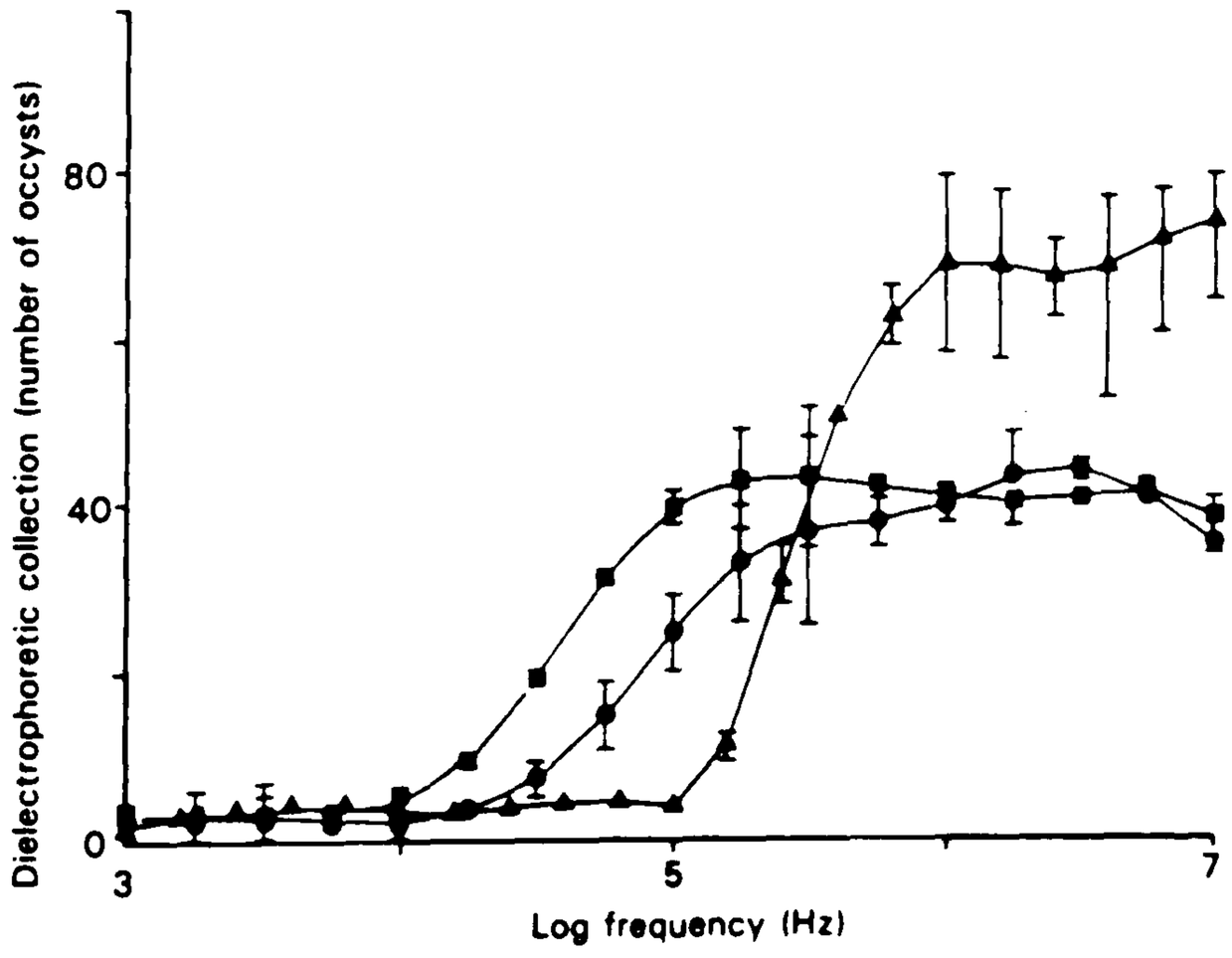

Figure 5 Dielectrophoretic collection spectra comparing untreated (▲ , Uii) and ozone treated (●, Tii 1.5 mg^{-1}; ■ , Tiii 3.3 mg l^{-1}) *Cryptosporidium* oocysts.

at which cell collection began to rise sharply. For the untreated sample this onset occurred at around 200 kHz which was an order of magnitude higher than that shown by the sample treated with 3.3 mg l^{-1} ozone. The sample treated with 1.15 mg l^{-1} ozone yielded a collection spectrum which started to increase at a point between the onsets of collection shown by the untreated and 3.3 mg l^{-1} ozone treated samples. These three samples (Uii, Tii and Tiii) were dielectrophoresed using a single set of electrodes.

Discussion

The technique described is capable of distinguishing between untreated *Cryptosporidium parvum* oocysts and those which have undergone three different disinfection processes. The ability of dielectrophoretic measurements to distinguish between viable and non-viable micro-organisms has also been demonstrated using yeast cells (Crane and Pohl, 1969) and *Escherichia coli*.

Another important observation during the course of these experiments concerns the nature of the collection upon the electrodes, which can occur in addition to collection between the electrodes. At frequencies lower than about 30 kHz collection was predominantly along the centre of the electrode bars, whereas above this frequency collection between the electrodes became increasingly important and by 100 kHz accounted for almost all the collection seen. This observation has important implications for any detection or observation system. For example, a microscope unable to gather reflected light would be of no use in observing collection along the centre of the electrode bars. Moreover, a turbidimetric system measuring the density of cells through the electrodes would not detect those cells collected upon, and therefore screened by, the electrodes.

Whilst the results presented were obtained from samples containing up to 10^8 oocysts ml^{-1}, spectra were easily constructed from samples containing as few as 10^3 oocysts ml^{-1}. The apparatus could be readily adapted to operate with computer controlled image analysis which would provide automatic construction and comparison of spectra for very rapid sample throughput.

Further applications of this system are numerous but it is particularly useful for those involving the detection and characterisation of very low concentrations of particles and cells. This has previously been a major problem when utilising spectrophotometric measurements of collected particles, although this type of system would still be useful in many applications (Betts and Hawkes, 1991). Incorporation of the described apparatus into a system including concentration of oocysts, by conventional or preferably dielectrophoretic means, could provide a complete and rapid analytical tool capable of screening large volumes of water.

Acknowledgements

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